Doctoral Thesis

Stability and aggregation kinetics of colloidal systems application to polymer colloids, proteins and peptides

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Stability and aggregation kinetics of colloidal systems: Application to polymer colloids, proteins and peptides

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for the degree of
Doctor of Sciences
presented by
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Chiudo con un ricordo e una dedica, alle mie nonne Maria.
Abstract

The stability behavior of several colloidal dispersions involved in a wide range of relevant technological applications and biological systems is analyzed by combining experimental characterization to modeling analysis. Aggregation kinetics and aggregate morphology are investigated by light scattering, chromatography, microscopy and a series of biophysical techniques, and the obtained results are modeled by means of population balance equations.

In the first part of the work, this approach is applied to the stability of polymer particle dispersions and to polymerization kinetics in heterogeneous systems.

In the second part of the work, the analysis is extended to protein systems exhibiting different aggregation mechanisms: reversible oligomerization, precipitation of amorphous aggregates, and secondary fibril-fibril aggregation reactions. Different proteins with increasing level of size and structure complexity are considered, ranging from short peptide to globular multi-domain proteins.

For all the investigated systems, the combination of mathematical models with experimental data provides mechanistic insights into the aggregation process as well as quantitative analysis of the effect of key parameters on aggregation kinetics and aggregate morphology. The results indicate that mechanistic approaches, commonly applied in polymer and colloid engineering, represent powerful tool to describe the behavior of more complex systems such as proteins.
Sommario

La stabilità di dispersioni colloidali coinvolte in un ampio campo di sistemi biologici e importanti applicazioni tecnologiche è stata analizzata combinando caratterizzazione sperimentale e analisi modellistica. Le cinetiche di aggregazione e la morfologia degli aggregati sono stati studiati tramite “light scattering”, cromatografia, microscopia e una serie di tecniche biofisiche, e i risultati ottenuti sono stati modellati tramite le equazioni di bilancio di popolazione.

Nella prima parte del lavoro, questo approccio è stato applicato allo studio di stabilità di dispersioni di particelle polimeriche e di cinetiche di polimerizzazione in sistemi eterogenei.

Nella seconda parte del lavoro, l’analisi è stata estesa alla stabilità di proteine in soluzione. Diversi meccanismi di aggregazione sono stati analizzati: formazione reversibile di oligomeri, precipitazione di depositi amorfi e reazioni secondarie di aggregazione tra fibrille. Sono state considerate diverse proteine con un livello crescita di dimensione e complessità di struttura, estendendo il campo di ricerca da un corto peptide a complesse proteine globulari a struttura modulare.

In tutti i sistemi studiati, la combinazione di modelli matematici e dati sperimentali ha evidenziato nuovi aspetti meccanistici del processo di aggregazione oltre a permettere un’analisi quantitativa dell’effetto di parametri chiave sulle cinetiche di aggregazione e sulla morfologia degli aggregati.

I risultati indicano che l’approccio meccanisticamente, tradizionalmente applicato all’ingegneria di polimeri e colloidii, e’ un efficace strumento anche per descrivere il comportamento di sistemi più complessi, quali la stabilità di proteine in soluzione.
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Chapter 1

Introduction

1.1 Colloidal dispersions

Colloids are subdivided bulk substances with size in the range from few nm to about 1 micron uniformly distributed in a dispersion medium.¹ Such subdivided matters (colloidal particles) could include gas, liquid or solid. Aerosols, emulsions and foams are examples of colloidal dispersions.² Solid particles dispersed in a liquid medium represent typical sol colloids. In most cases, the dispersed colloidal particles are thermodynamically unstable, referred to as lyophobic (“solvent-fearing”),³ and therefore they tend to aggregate in time. In order to avoid aggregation, colloidal particles are typically stabilized kinetically by electrostatic repulsions. In the energy landscape the contribution of the electrostatic repulsion superimposes the Van der Waals attraction and generates an energy barrier that reduces the aggregation rate. This behavior is quantitatively described by the DLVO (Derjaguin-Landau-Vervey-Overbeek) theory. Depending on the specific system, steric repulsion and other types of intermolecular forces can also contribute to the interaction potential.⁴,⁵

Colloidal systems are present in many industrial processes including painting, ceramics, papermaking, soaps, cosmetics, and pharmaceutics. Relevant examples of sol colloids are found in various polymerization processes. Emulsion polymerization is the most common polymerization process producing millions of tons of polymer per year in the form of latexes (polymer particles dispersed typically in water). Dispersion and precipitation polymerizations are alternative processes which differ in the level of stabilization and in the size of the final particles.⁶ The stability of the polymer particles during reaction affects the polymerization kinetics and the final product properties.
In many practical applications, controlled aggregation of the colloidal particles is necessary, such as in the separation of colloidal particles from the disperse medium or in the production of materials with well-defined morphology starting from colloidal particles. In other applications, instead, one has to keep the particles uniformly dispersed in the medium and to strictly avoid their aggregation, such as in paintings or film forming dispersions. In all cases, a fundamental and quantitative understanding of aggregation processes is desirable. Many works in the literature have investigated both experimentally and theoretically the aggregation kinetics of colloidal dispersions.\(^7\) From the experimental point of view, aggregate (or cluster) size and morphology are monitored by light scattering and microscopy techniques. In parallel with the experimental characterization, modeling activities have been developed based on Monte Carlo simulations and population balance equations. These methods describe the properties of a colloidal system accounting for the interaction potential between two approaching particles. The extensive studies showed universal behaviors of colloidal aggregation induced by Brownian motion,\(^8,9,10\) with the aggregation kinetics and fractal morphology of the clusters following two limiting regimes: power-law kinetics and fractal dimension of around 1.8 for rapid, diffusion-limited cluster aggregation (DLCA),\(^11\) and exponential kinetics and fractal dimension of around 2.1 for slow, reaction-limited cluster aggregation (RLCA).\(^12\)

### 1.2 Protein Aggregation

Many solutions of polymers and biomacromolecules exhibit features of colloidal dispersions, although they represent true solutions. Such colloids are thermodynamically stable, defined as lyophilic (“solvent-loving”).\(^3\) Aqueous solutions of biomacromolecules, such as nucleic acids and proteins, are ubiquitous in biological systems. Proteins represent a unique class of lyophilic colloids with heterogeneous structures, the amphoteric polyelectrolyte nature and the simultaneous presence of hydrophobic and hydrophilic patches.\(^13\) Evolution has optimized protein folding and regulatory mechanisms in living organisms to avoid aggregation.\(^14,15\) Globular proteins show stable native structure in which hydrophilic groups are solvent-exposed, while hydrophobic patches and backbone
hydrogen bonds are wrapped intramolecularly. In some cases, the protein stability requires binding partnerships or complexation. However, not all proteins appear to be equally optimized versus aggregation.

Aberrant aggregation of proteins in physiological conditions is involved in several human disorders including Alzheimer’s, Parkinson’s, prion disease and systemic amyloidoses. Deposits of regular, filamentous aggregates, so-called amyloids, are observed in the intra- and extra-cellular compartments of damaged organs. In certain diseases, protein amorphous deposits are detected as well. The relationship between the pathogenicity and the aggregates is still unclear: aggregates could be the cause of the dysfunction but, on the other hand, they could also be a consequence of the diseases. According to recent studies, small oligomers formed in the early stages of aggregation represent the most toxic species, but the exact mechanism of action is still not understood.

Protein stability is a crucial problem also in biotechnology. In recent years, several therapeutic proteins have been developed against diseases such as multiple sclerosis, rheumatoid arthritis, tumors, hemophilia. The industrial production of these drugs involves a series of steps (expression, purification, virus inactivation, shipping and storage) which expose the proteins to different types of stresses: acidic, thermal, mechanical. Such stresses induce protein unfolding and, consequently, aggregation. Aggregation must be strictly avoided, not only because it leads to loss of valuable product, but, most importantly, because aggregates are potentially immunogenic. The stability during manufacturing and administration is therefore crucial for the successful application of the drugs.

Our current understanding and control of protein aggregation is still unsatisfactory, mainly due to the complexity of the problem, which involves contributions from several disciplines, including medicine, molecular biology, biochemistry, biophysics, and bioengineering. However, the level of knowledge of the aggregation mechanism has increased significantly in the last twenty years. Thanks to these advances, recently, protein aggregation has also been exploited in bio-nanotechnology for the production of novel, functionalized biomaterials by self-assembling of small peptides.
The protein aggregation mechanism is much more complex with respect to those of typical colloidal dispersions. Protein structure and intermolecular interactions are extremely sensitive to small changes of environmental parameters and even single-point mutations of the primary sequence. Depending on the specific protein and the experimental conditions, several aggregation pathways and a very broad range of aggregates have been observed. Establishing universal behaviors is more challenging in comparison with colloidal dispersions. Moreover, in order to comprehensively describe aggregation in living organisms, we have to take into account the complex composition of the cell environment.\textsuperscript{26}

However, accumulated evidences from \textit{in silico}, \textit{in vitro} and \textit{in vivo} studies show some generalities in protein behavior. In particular, the fibril state appears to be a generic property of the polypeptide chain; aggregation is an alternative pathway of the folding process, which, on the other hand, is strongly affected by the primary sequence.\textsuperscript{16} Indeed, almost all proteins are able to form amyloid fibrils if incubated in suitable conditions.\textsuperscript{27}

The aggregation propensity has been correlated to three general physicochemical properties of the protein: 1) hydrophobicity, 2) tendency to form backbone hydrogen-bonds and \(\beta\)-sheet structure, and 3) charges.\textsuperscript{28} In their native state, proteins show properties corresponding to low aggregation tendency, at least during their life spans. Mutation in protein primary sequence, change in environmental parameters or presence of infectious seeds may modify the delicate balance of non-covalent interactions involved in protein stability and form an alternative fold state. In many protein systems, aggregation is connected to the formation of a partially folded structure. Such intermediate structure shows flexibility, combining with the mentioned physicochemical properties suitable to promote aggregation. The formation of a partially folded structure appears to be fundamental also in the aggregation of naturally unfolded proteins.\textsuperscript{29}

Many biophysical studies of protein aggregation \textit{in vitro} indicate some generalities in protein aggregation behavior also from the mechanistic point of view.\textsuperscript{30} In most of the cases, monomeric proteins aggregate via a multi-steps process involving nucleation and growth.\textsuperscript{31} Growth may occur either by monomer addition (as in amyloid fibril formation)
or by further aggregation between formed aggregates (more often observed for amorphous deposits).\textsuperscript{32}

When protein aggregation is dominated by monomer addition, the self-assembling mechanism resembles polymerization reactions, where the reactive monomeric unit is substituted by the single macromolecule. The stability of protein aggregates shares similarities also with the stability of sol colloids. Significant differences arise clearly from the complex and non-homogeneous structure of the protein, which complicates the physical description of the interaction potential between two aggregating units. Protein conformational stability is strongly interconnected with its colloidal stability, and discriminating the two contributions is often extremely difficult.\textsuperscript{33} Despite such differences, colloidal approaches based on DLVO theory and population balance equations represent useful tools to approach the aggregation mechanism and the intermolecular forces responsible for protein stability.\textsuperscript{31}

\subsection*{1.3 Outline of the work}

This work attempts the investigation of differences and analogies between the stability of polymer colloids and protein solutions. On this purpose, the stability of several dispersed systems covering a wide range of applications has been considered: these include dispersions of polymer particles in water, reacting polymer macromolecules in a non-solvent medium, and proteins aqueous solutions. In the case of protein solutions, different systems with increasing size and protein structure complexity were analyzed in parallel: a synthetic amphiphillic peptide with applications in bio-nanotechnology, an immunoglobulin light chain fragment involved in biomedical research, and complete immunoglobulins developed for therapeutic applications.

For each study, the stability behavior is characterized experimentally by light scattering, chromatography, microscopy and a series of biophysical techniques. In protein studies, information on protein structure are assessed by several spectroscopic and fluorescence techniques. The attention is focused on the effect of several parameters on aggregation kinetics and aggregate morphology. For polymer colloids, these include primary particle
morphology, surfactant surface coverage, copolymer composition and shear rate. For all the investigated protein systems, the protein structure stability and aggregation is analyzed as a function of temperature, pH, type and concentration of co-solutes. The experimental activity is combined to population balance equation models to get mechanistic insights into the investigated processes. This is applied to describe both the polymerization kinetics in heterogeneous system and the protein aggregation mechanism. The investigated systems can be divided into two parts:

The first part covers Chapters 2 and 3 and is dedicated to polymer colloids:

Chapter 2 describes the aggregation/gelation under intense-shear of strawberry-like particle dispersions produced by emulsion polymerization. The particles consist of a rubber core surrounded by plastic patches. We show how the particle morphology and the surfactant surface coverage affect the extent of coalescence of particles during aggregation. Because polymer clusters with more compact structure contain less residual dispersing medium, the structural properties of the clusters significantly affect the properties of the final polymer products and the ability to handle the product during post-treatment.

In Chapter 3 an example of precipitation polymerization is investigated, i.e., the copolymerization of vinyl-imidazole and vinyl-pyrrolidone in organic solvent. We characterize the reaction kinetics and the size distribution of the precipitating polymer particles, and we analyze the influence of particle stability on the reaction kinetics. Moreover, a detailed kinetic model is developed in order to elucidate the reaction locus and the relevance of mass transport of the active chains between the continuous and the dispersed phase.

The second part includes Chapters 4 through 7 and deals with protein systems:

In Chapter 4 we present an example of exploitation of protein aggregation in nanotechnology, namely a synthetic peptide designed to self-assemble in a controlled way to fibrils and higher ordered structures. These biomaterials have several applications in medicine and biotechnology for cell culture, tissue repairing and engineering. However, the industrial production is still limited by the poor control on the peptide stability. In this work, the stability of the amphiphilic peptide and the fibril dispersions is analyzed as a function of several operating parameters. We found that fibrils elongate via an end-to-end
aggregation mechanism and we applied population balance equations to simulate the aggregation process.

In Chapter 5 the stability of an immunoglobulin light chain fragment is considered. Excessive production of monoclonal light chains due to multiple myeloma can induce aggregation-related disorders, such as light chain amyloidosis and light chain deposition diseases. In this work, we produce a non-amyloidogenic IgE $\lambda$ light chain dimer from human mammalian cells U266, which originated from a patient suffering from multiple myeloma in which neither amyloids nor amorphous deposits were detected. We investigate the effect of several physicochemical parameters on the in vitro stability of this protein. The features of the non-amyloidogenic fragment considered in this work will be compared to the behavior of various amyloidogenic variants which are going to be analyzed in a following study. The comparative analysis of fragments with different aggregation propensity and clinical consequences could potentially lead to identify the key factors involved in amyloids formation and organ disfunction. This project is conducted in collaboration with the group of Prof. Merlini at Policlinico San Matteo (University of Pavia, Italy).

In Chapter 6 the aggregation stability of complete immunoglobulins G (IgG) is investigated. We focus on the aggregation during the protein production process and specifically on the downstream, i.e., the purification portion of it. In particular, we explore a wide range of operating conditions (i.e., pH, buffer nature, type and concentration of salt, temperature, IgG subclass, protein concentration) in order to identify the possible aggregation scenarios and find the key parameters determining the protein stability.

In Chapter 7 we show that the IgG aggregates considered in the previous chapter exhibit effective fractal morphology and we model the aggregation kinetics by means of population balance equations (PBEs). This study offers an example of the potentiality of the PBE approach in protein aggregation kinetics, providing important quantitative information on the effective protein-protein interactions and their dependence on operating parameters, such as temperature, pH and type of anion.

Finally, conclusive remarks and outlook of the present work are summarized in Chapter 8.
Chapter 2

Effect of primary particle morphology and surfactants on structure of gels formed in intense turbulent shear

2.1 Introduction

Polymer gels are encountered in many industrial processes related to the production of ceramics, food, medicine, controlled porous materials, and so forth.\textsuperscript{34} Emulsion polymerization process produces polymers in the form of latexes; after the polymerization reaction, through a proper coagulation process, the submicrometer polymer particles can be separated from the dispersing medium forming clusters or gels. Gelation can be obtained by destabilizing the colloidal system using electrolytes or applying external energy to overcome the repulsive interaction energy barrier, such as imposing a high shear rate through turbulent flow. For the latter case, the liquid-like particle dispersion is first expected to be transferred into the liquid-like cluster dispersion under shear and then to solid-like state through interconnection among clusters when the imposed shear is stopped. Because polymer clusters with more compact structure contain less residual dispersing medium, the structural properties of the clusters significantly affect the properties of the final polymer products and the ability to handle the product during post-treatment.

The aggregation/gelation of charge-stabilized \textit{rigid} colloidal particles induced by electrolytes has been well studied in the literature under both stagnant conditions\textsuperscript{35-43} and shearing flow.\textsuperscript{44-46} In general, the added electrolytes after the aggregation cannot be completely eliminated from the polymers and lead to changes in electronic and mechanical properties of the final polymer products as well as their color. The aggregation/gelation of \textit{rigid} polymer particles without using electrolytes can be achieved by applying a sufficiently strong shear rate so as to overcome the repulsive interaction energy barrier.\textsuperscript{47-50}
It is found that when a colloidal system is stabilized by the typical DLVO (Derjaguin-Landau-Verwey-Overbeek) interactions, such a shear-induced aggregation/gelation does in fact take place, as has been recently explained theoretically by Zaccone et al.\textsuperscript{51} It is also found that the fractal dimension of the clusters constituting the gel is 2.4\(\pm\)0.04, independent of the original primary particle size and concentration and the imposed shear rate.\textsuperscript{50} However, it has been observed that when ionic surfactants are used and the density of the adsorbed surfactant on the particle surface reaches a certain value, no gelation or even aggregation is possible, even at extremely large shear rates.\textsuperscript{49,51} This somewhat unexpected behavior was attributed to the additional non-DLVO interaction, the strongly repulsive, short-range hydration force generated by the adsorbed surfactant.\textsuperscript{47-49} This is based on the fact that as the ionic surfactant progressively adsorbs on the particle surface, the hydrophilicity of the particle surface increases, eventually leading to some ordering of the water molecules, which has been confirmed by various experiments to be the source of the hydration force.\textsuperscript{52-55}

With respect to rigid particles, the aggregation of soft particles has been less frequently studied in the literature.\textsuperscript{56-60} Recently, Gauer et al.\textsuperscript{61-65} systematically investigated the aggregation of elastomer particles under stagnant conditions and demonstrated that upon physical contact elastomer particles may coalesce through intermolecular diffusion or viscous flow during aggregation as they do during the film formation of latex paints and coatings.\textsuperscript{66-68} Coalescence during aggregation results in more compact clusters, and the extent of coalescence depends not only on the material properties of the soft particles\textsuperscript{63} but also on surface properties\textsuperscript{62,64} and temperature.\textsuperscript{64}

In this work, we studied the dependence of the structure properties of the gel on the primary particle morphology. The primary particles consist of a rubber core surrounded by a plastic shell. Depending on the shell-to-core mass ratio, the latex particles can be strawberry-like,\textsuperscript{69} with the core partially covered by the shell, and thus able to coalesce when they aggregate. Previous studies on the aggregation of particles of this type under stagnant conditions found that coalescence among particles in the clusters is negligible at room temperature and indeed occurs when the temperature is greater than a certain value. In this work, we study such coalescence behaviour under intense shear-induced
aggregation. We have prepared two sets of primary particles: the first set contains particles with the same mass of the rubber core but different masses of the shell, thus leading to different core surface coverage by the shell. The particles in the second set vary in their morphology somewhat randomly with respect to size, the shell-to-core mass ratio and the shell-to-core volume ratio, which are used to verify the results obtained from the first set. All particles are stabilized by both fixed charges originating from polymer chain-end groups and mobile charges from the adsorption of a sulfonate surfactant. This strongly repulsive system is fully stable under stagnant conditions but can be destabilized under turbulent conditions.

In the second part of the chapter, we study the role played by surfactants in the shear-induced aggregation/gelation of the soft strawberry-like particles. The main objective is twofold: first, we would like to determine if the observed partial coalescence during intense shear-induced gelation can be altered by adsorbing an ionic or a non-ionic (steric) surfactant on the particle surface. Second, as mentioned above, previous studies demonstrated that when a sufficient amount of ionic surfactant is adsorbed on the particle surface, short-range hydration forces can be generated, which protect the particles from aggregating even in the presence of very intense shear. Now, we would like to explore whether similar behavior can also be obtained with non-ionic (steric) surfactants.

2.2 Materials and Methods

The colloidal system

The system used for this study is a colloidal dispersion of nanosized particles supplied by BASF SE (Ludwigshafen, Germany), produced by emulsion polymerization. The particles exhibit a core-shell structure with the core of acrylic elastomer \((T_g<253\, K)\) and the shell of polystyrene. Two different sets of particles were considered, one with fixed core and a varying shell (indicated as \(FC\)) and the other with a varying core (\(VC\)). For the latter, both the particle radius and the shell-to-core mass ratio are varied. The mean radius of the particles was determined by both dynamic (DLS) and static (SLS) light scattering measurements. The form factor of the primary particles, \(P(q)\) measured by SLS, has been
fitted using the Rayleigh-Debye-Ganz (RDG) scattering theory which is valid for small spherical particles.\(^\text{70}\)

\[ P(q) = 9 \left[ \frac{\sin(qR_p) - qR_p \cos(qR_p)}{(qR_p)^3} \right]^2 \]  

(2.1)

where \( R_p \) is the radius of the primary particles and \( q \) is the magnitude of the wave vector defined as

\[ q = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \]  

(2.2)

with \( n_0 \) being the refractive index of the disperse medium, \( \lambda_0 \) being the laser wavelength and \( \theta \) being the scattering angle. The radius of each type of particle and the corresponding name that will use in the following text are reported in Table 2.1.

The particles are stabilized by a commercial surfactant system, Emulgator K30/95 (Bayer, Germany). The adsorption isotherm of this surfactant on the surface of polystyrene primary particles has already been obtained in a previous work.\(^\text{48}\) On the basis of the obtained adsorption isotherm, the surfactant density on the particle surface in this work can be estimated to be equal to \( \Gamma_s = 2.85 \times 10^{-7} \text{ mol/m}^2 \). By considering the molecular area to be around 25 Å\(^2\),\(^\text{48}\) the surfactant surface coverage can be estimated to be rather small, about 2.5%. Because the mass percentage of surfactant added during emulsion polymerization is the same for all types of particles and their sizes do not change significantly, it is reasonable to consider that the surfactant surface coverage is small in all cases.

In the experiments with added surfactants, all the surfactants and the residual initiator used during the polymerization, as well as the other possible electrolytes, have been previously removed completely by mixing the dispersions with a mixture of cationic and anionic ion-exchange resins (Dowex MR-3, Sigma-Aldrich), according to the procedure described elsewhere.\(^\text{47}\) After the dispersions were cleaned, the surface tension of the mother liquor was very close to that of the Milli-Q (Millipore) deionized water, indicating that the colloidal dispersions are free of surfactants. Note that the dispersions after cleaning are still very stable under quiescent conditions because of the presence of polymer chain-end charge groups (sulfate) on the surface, which originate from the initiator. In fact, the
measured zeta-potential at $\phi=5.0 \times 10^{-4}$ is -58 mV, indicating a stable colloidal system.

The surfactants

To investigate the role played by the surfactants in shear-induced aggregation/gelation, we have considered two of them: Emulgator K30/95 (Bayer) and Tween 20 (polyoxyethylene (20) sorbitan monolaurate) (AppliChem). The former is a multicomponent sulfonate (ionic) surfactant system with mean molecular formula: $C_{15.05}H_{30.78}(SO_3Na)_{1.32}$, whose detailed information can be found elsewhere,\(^4\) while the latter is a nonionic surfactant. The adsorption (binding) of both surfactants on the particle surface mainly relies on their linear hydrophobic hydrocarbon chain, while the stability mechanism is related to charges from sulfonate groups for the former and to steric effect of the complex polyoxyethylene group for the latter. Moreover, to investigate the effect of the hydrophobic part of the steric surfactant, we have also investigated Triton X-100 ($p$-(1,1,3,3-tetramethylbutyl)-phenyl)poly(oxyethylene) (Merck), whose hydrophobic binding to the particle surface is related to the tetramethylbutyl-phenyl groups.

Table 2.1  Morphology parameters of the two sets of core-shell particles used in this work.

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<th>Set</th>
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<td>Particle name</td>
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<td>FC2</td>
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<tr>
<td>$R_p$ (nm)</td>
<td>52</td>
<td>53.3</td>
</tr>
<tr>
<td>$R_C$ (nm)</td>
<td>42.5</td>
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<tr>
<td>$MR_{sc}$</td>
<td>0.67</td>
<td>1.0</td>
</tr>
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</table>
Gelation through a microchannel

To explore the effect of the particle morphology on the shear-induced gel structure, we have performed shear-induced gelation on all of the latexes in Table 2.1 in the range of the particle volume fraction $\phi=0.2-0.35$ without using any screening electrolytes. To explore the effect of surfactant type and surface density on shear-induced gelation, for each surfactant we have performed the shear-induced aggregation (gelation) experiments at the same volume fraction, $\phi=0.15$, and Peclet number, $Pe=220$, but with different amounts of surfactants.

It has been found that without added screening electrolytes, all the colloidal systems are very stable, and their aggregation cannot be realized by the common flow devices such as stirred tanks, Couette devices, and so forth, where the local shear rate is generally within $2\times10^3$ 1/s. Thus, we have applied a commercially available device, Homogenizer HC-5000 (Microfluidics, USA), with a z-shape microchannel with a rectangular cross-section, referred to as z-MC in the following, whose fluid dynamic behaviour has been characterized elsewhere.\(^7^1\) The high-shear turbulent flow in the z-MC is generated by the pressure at the inlet through a piston pump driven by compressed air. The relation between the shear rate $G$ in the z-MC and the inlet pressure $P$, reported by the device supplier using water, is $G \ [1/s]=1.02\times10^4 \ P \ [\text{bar}]$, where $P \in [20,150]$ bar. Thus, we can obtain a shear rate in the range $G \in [2\times10^5, 1.5\times10^6] \ 1/s$ or a Peclet number, $Pe = \frac{3\pi R_p^3 \mu G}{k_B T}$, where $\mu$ the medium viscosity, $k_B$ is the Boltzmann constant) in the range $[55, 275]$ (based on water at $T=298$K). The pressure at the inlet of the capillary has been measured by means of an electronic pressure transducer and used to compute the $Pe$ in the z-MC from the above correlation. It should be pointed out that such computed shear rates have to be taken as indicative, because when aggregation or gelation occurs within the z-MC, the fluid properties change substantially along the microchannel, and the real shear rate in the z-MC can be substantially different from the one calculated by the above correlation.

It has been confirmed that the shear rate generated in the z-MC is high enough to realize the gelation of all the colloidal systems in Table 2.1. It should be noted that at such high shear rates cavitation within the z-MC may occur and the additional hydrodynamic...
stress generated by cavitation bubble collapse may contribute to the colloidal aggregation.\textsuperscript{72}

**Sample characterization**

When the shear-induced gelation occurs for any of the latexes in Table 2.1, it is found that there is always some residual liquid separated from the gel in the given range of $\phi = 0.2-0.35$. When this liquid is properly diluted and characterized by light scattering, we observed two distinct classes of clusters, one composed mainly of primary particles plus small numbers of doublets and triplets and another composed of clusters with sizes that are at least 2 orders of magnitude larger than the former. This phenomenon is typical of the shear-induced gelation of repulsive colloidal systems and has been illustrated in details previously.\textsuperscript{49} To characterize such systems, we introduce the conversion of the primary particles to the gel, $x$. In particular, we quantify the concentration of primary particles in the residual liquid of the gel based on the light-scattering intensity at $90^\circ$. For each kind of particle, a calibration curve relating the particle concentration to the intensity was previously measured (data not shown). Note that the intensity of the residual liquids is measured after filtration with a filter of 5 $\mu$m openings because clusters larger than 5 $\mu$m are considered to be part of the gel phase. The remaining liquid contains mainly the primary particles with small numbers of doublets and triplets, and corrections are also made for different scattered intensities of the doublets and triplets in order to estimate the conversion correctly.\textsuperscript{49} A BI-200SM goniometer (Brookhaven) light scattering device with a Ventus LP532 (Laser Quantum, UK) solid-state laser with a wavelength $\lambda_0 = 532$ nm is used.

To obtain the fractal dimension of the clusters from which the gels are constructed, we have first redispersed the gel pieces in water under gentle agitation with a magnetic stirring bar until reaching an $\sim 100$ $\mu$m cluster size, and then measured the average structure factor of the obtained clusters, $\langle S(q) \rangle$, using a small-angle light scattering (SALS) instrument,

$$
\langle S(q) \rangle = \frac{I(q)}{I(0)P(q)} \quad (2.3)
$$
where $I(q)$ is the angle-dependent scattered intensity, $I(0)$ is the intensity at zero angle, and $P(q)$ is the form factor of the primary particles measured using the same instrument. For very large clusters, the power-law regime of the average structure factor leads to an estimate of the fractal dimension, $D_f$:  

$$S(q) \sim q^{-D_f} \quad \text{for} \quad 1/\langle R_g \rangle << q << 1/\langle R_p \rangle$$  

(2.4)

The SALS instrument used in this work is a Mastersizer 2000 (Malvern, UK). Details of the measurements and characteristics of the instrument may be found elsewhere.

It is worth noting that because all the latexes are stable under stagnant conditions, the clusters generated in the z-MC under the extremely high shear are also very stable under stagnant conditions. Thus, any shear rate that may be involved during the sample preparation for all of the analyses does not significantly alter the size and structure of the formed clusters, because it is at least a few orders of magnitude smaller than that generated in the z-MC. In fact, reproducibility of the SALS measurements is always excellent.

Standard SEM pictures were also taken after drying the gel at room temperature. Because coalescence may occur after drying if the rubbery core is not fully covered by the plastic shell, the SEM pictures allow us to observe the extent of coalescence, thus the extent of the core coverage by the shell.

### 2.3 Effect of particle morphology

#### 2.3.1. Particles with fixed core mass.

Let us first consider the particles of Set A in Table 2.1 with the same rubber core but a different shell mass, thus a different shell thickness. We have performed the shear-induced gelation at fixed values of the initial particle volume fraction, $\phi_{\text{ini}}=0.20$, and the shear rate, $G=8.0 \times 10^5$ 1/s, and found that the gel is indeed formed in all cases. Then, the average structure factors $\langle S(q) \rangle$ of the re-disperse clusters from all the gels are determined by SALS. Figure 2.1 shows the $\langle S(q) \rangle$ curves for clusters generated from particles FC1, FC3 and FC5, respectively.
Figure 2.1 Average structure factor of the clusters constructing the gel, \(\langle S(q) \rangle\), where the power-law regime leads to an estimate of the fractal dimension \(D_f\): FC1 (○), FC3 (◊), FC5 (□).

Figure 2.2 Fractal dimension \(D_f\) of the clusters constructing the gel (error bars indicate experimental errors) (a) and conversion \(x\) of the primary particles to gel (b) as a function of the shell-to-core mass ratio, \(MR_{sc}\), for particles in Set A of Table 2.1. \(G = 8.0 \times 10^5\) 1/s.

The slope of the power-law regime of the \(\langle S(q) \rangle\) curve gives the value of the fractal dimension, \(D_f\). For particles in Set A, because the core is the same for all the particles, changes in the shell mass correspond to variations in the shell-to-core mass ratio, \(MR_{sc}\). Figure 2.2a reports the \(D_f\) value as a function of the shell-to-core mass ratio, \(MR_{sc}\). It is
seen that the $D_f$ value decreases as $MR_{sc}$ (i.e., the shell mass) increases. This means that a more open structure is obtained as the shell mass increases.

To understand the variations in the structure of the gels better, we have made the SEM pictures of the gels after drying at room temperature; these are shown in Figure 2.3. It is seen that starting from the gel of the FC1 particles with the smallest shell mass to that of the FC5 particles with the largest shell mass, one can see a clear trend in the structure variations. In particular, for the gel of the FC1 particles in Figure 2.3a it is practically impossible to identify the primary particles. Although small white pieces can be identified on the surface by enlarging the image further, their estimated average diameter is only in the range of 40-50 nm, which is too small to be primary particles. Thus, Figure 2.3a clearly indicates that the core of the FC1 particles is not fully covered by the plastic shell and coalescence (fusion) among the rubber cores occurs during the shear-induced gelation. The white spots are mostly the pieces of the plastic shells surrounding the rubber cores.

Starting from Figure 2.3b corresponding to the gel of the FC2 particles, the identities of the primary particles become more and more evident as the shell mass increases. This indicates that the particle coalescence progressively reduces with the shell mass. In fact, in Figure 2.3e the gel produced from the FC5 particles is identical to those formed from typical rigid particles, indicating non-coalescence of the particles. Based on the observations from the SEM pictures in Figure 2.3, one can conclude that the value of the fractal dimension reported in Figure 2.2a reflects the extent of coalescence during shear-induced gelation, i.e., the larger the fractal dimension, the larger the extent of coalescence.
This result, as also observed in aggregation under stagnant conditions,\textsuperscript{69} indicates that for coalescence systems the fractal dimension estimated from the average structure factor of the clusters can well reflect the extent of coalescence. Note that when coalescence occurs in a cluster, the cluster has been modeled as a fractal object rather than a real fractal object. In fact, many porous materials are often modeled as fractal objects in the literature,\textsuperscript{75-77} even though they are unnecessarily true fractal objects.

It should be pointed out that drying the gel would alter the extent of coalescence, and
the extent of coalescence shown in the SEM pictures in Figure 2.3 is only indicative. One should not consider that these pictures correspond correctly to the gels formed during the shear-induced gelation. In fact, the $D_f$ value of about 2.65 for the FC1 gel in Figure 2.2a does not correspond to a so compact, basically homogeneous, gel state as shown in Figure 2.3a.

An important quantity that is connected to the extent of coalescence is the conversion of the primary particles $x$ to the gel network during gelation, which is shown in Figure 2.2b as a function of $MR_{sc}$. Note that the $x$ values reported in Figure 2.2b refer to experiments with primary particle volume fraction, $\phi_{ini}=0.35$. Repetition of experiments at different $\phi_{ini}$ values allows us to check reliability of the small but systematic differences in conversion (less than 1%). It is seen that in contrast to the fractal dimension, the conversion increases as $MR_{sc}$ increases. This is due to the cubic dependence of the shear-induced aggregation kinetic constant, $\beta_{i,j}^{shear}$, on the radii of the aggregating clusters, $R_i$ and $R_j$:44

$$\beta_{i,j}^{shear} = \frac{4}{3} G (R_i + R_j)^3$$  \hspace{1cm} (2.5)

For given $i$ and $j$ primary particles in the clusters, the less the extent of coalescence is, the larger their radii; it follows that the faster the aggregation proceeds. Thus, at fixed values of the shear rate and residence time in the z-MC, with the assumption of coalescence faster than aggregation, the conversion must increase as the extent of coalescence decreases. This explains the results in Figure 2.2b when one considers that Figures 2.2a and 2.3 demonstrate that the extent of coalescence decreases as the shell mass increases.

The aggregation and coalescence behaviors of the FC1 particles were investigated under stagnant conditions.69 It was found that when the diffusion-limited cluster aggregation (DLCA) was carried out at $T=298$ K, the fractal clusters with typical $D_f=1.87$ were obtained. This means that under stagnant conditions, coalescence of the FC1 particles is insignificant at room temperature, even though one would expect that the uncovered soft core with $T_g<253$ K could “flow out” through the open areas of the shell and coalesce. This is mostly related to the fact that although the rubber core is not fully covered by the plastic shell, the polymer chains of the shell are grafted on the rubber core and generate certain
resistance for the contacting and inter-diffusion of the rubber cores. This indicates that the coalescence observed above during the shear-induced aggregation of the FC1 particles is mainly due to shear stress, which overcomes such resistance, probably related to two mechanisms: (1) shear stress increases mobility of the grafted plastic patches, leading to better exposure of the rubber core for coalescence; (2) shear stress induces strong cluster restructuring, which results in more probability for contacting between uncovered parts of rubbery cores of different particles.

When the DLCA clusters formed at room temperature were heated to \( T \geq 333 \text{ K} \), the \( D_f \) value increases as \( T \) increases.\(^6\)\(^9\) Therefore, similar to shear stress, thermal energy can also improve coalescence of particles, but for the latter the coalescence is mostly due to the improved flow properties and softness of the core and shell materials at higher temperatures. From this viewpoint, because cavitation bubbles may be generated at so high turbulence during the processing of the latex through the \( z\)-MC, their collapse may lead to local hot-spots, thus improving coalescence.

In Figure 2.2, as \( MR_{sc} \) increases, both the conversion and the fractal dimension reach a plateau, approximately starting at \( MR_{sc}=1.6 \), corresponding to the FC4 particles. This suggests that at this point the core surface coverage by the shell is large enough to prevent coalescence of the particles. The negligible coalescence in the case of the FC4 particles is confirmed by the SEM picture in Figure 2.3d. Then, we can assume that starting at the FC4 particles, the core is fully covered by the shell. To verify this, let us perform a simple mass balance calculation based on the particle size information from the light scattering measurements. The radius of the FC4 particles determined through DLS is \( R_p=58.5 \text{ nm} \). When the core is fully covered by the shell, the total volume of the particle can be expressed as:

\[
V_{TOT} = \frac{M_C}{\rho_C} + \frac{M_S}{\rho_S} = \frac{4}{3} \pi R_p^3 \tag{2.6}
\]

where \( M_C \) and \( \rho_C \) (=1.04 kg/l), and \( M_S \) and \( \rho_S \) (=1.06 kg/l), are the mass and density of the core and shell, respectively. Since \( R_p \) is known and \( M_S=1.67M_C \) given in Table 2.1, from eq 2.6 one can estimate the mass of the core, \( M_C \). Then, with \( M_C=(4/3)\pi R_c^3 \rho_C \), we can predict the radius of the core, \( R_c=42.3 \text{ nm} \). This is very consistent with \( R_c=42.5 \text{ nm} \)
determined before grafting the plastic shell, as given in Table 2.1, thus confirming the full coverage of the core by the shell starting at the FC4 particles. Note that eq 2.6 is valid only for a sphere. The \( R_p \) values in Table 2.1 were determined by DLS experiments, i.e., we have assumed that hydrodynamic radius equals particle radius. This is true only for a sphere. In the cases with the core not fully covered by the shell, the hydrodynamic radius determined by DLS must be smaller than the real particle radius. Therefore, eq 2.6 is invalid in those cases.

It is expected that when the core is only partially covered, the particle surface is rather irregular, mostly strawberry-like, as demonstrated by the SEM picture of the FC1 particles.\(^{69}\) In this case, it could be very useful to estimate the coverage of the core by the shell. To this aim, we assume that the shell is composed of a few pieces of cylindrical spots, whose radius and height are the same for all types of particles before the core is getting fully covered. In this way, the difference in the shell for different particles is only the number of the spots. From the SEM pictures, we have observed that the average radius of the spots is \( R_{sp}=25 \) nm. Since the FC4 particle has been considered to be the critical one with the core just fully covered by the shell, the difference between its radius and the radius of the core should represent the height of the spots, \( H_{sp} \); it follows that \( H_{sp}=R_p-R_c=58.5-42.5=16 \) nm.

With the known value of the shell-to-core mass ratio, \( MR_{SC} \), the number of the shell spots can be computed by

\[
N_{sp} = \frac{V_S}{V_{sp}} = \frac{4}{3} \frac{\rho_c}{\rho_s} \frac{R_c^3 \cdot MR_{SC}}{R_{sp}^3 \cdot H_{sp}} \tag{2.7}
\]

The corresponding core surface coverage by the shell, \( \Gamma \), is given by

\[
\Gamma = \frac{N_{sp} \cdot R_{sp}^2}{4 \cdot (R_C + H_{sp}/2)^2} \tag{2.8}
\]

Note that the surface area of the core has been evaluated at the radius of the core, \( R_C \), plus half of the height of the shell spot, \( H_{sp}/2 \). The number of shell pieces and the core surface coverage computed from eqs 2.7 and 2.6 for the primary particles with fixed core (i.e., Set A in Table 2.1) are reported as a function of the shell-to-core mass ratio in Figure 2.4. As expected both quantities increase as \( MR_{SC} \) increases. Note that for large \( MR_{SC} \) values, the
computed $\Gamma$ values become larger than unity. This arises because all the $\Gamma$ values are estimated using a fixed height of the shell spot, $H_{SP}$, as defined above. In reality, when the core is fully covered, further increase in the shell-to-core mass ratio would lead to increase in $H_{SP}$, but not in $\Gamma$. Under such a situation, if one keeps $H_{SP}$ fixed, the $\Gamma$ value will become larger than unity.

**Figure 2.4** Number of shell pieces $N_{SP}$ (●) and rubber core surface coverage $\Gamma$ (◊) as a function of the shell-to-core mass ratio $MR_{SC}$ for the particles in Set A of Table 2.1.

**2.3.2. Particles with a Varying Core.** The results from the subsection above clearly reveal that the key parameter controlling the coalescence (thus gel structure and particle conversion) is the core surface coverage, $\Gamma$. In order to further verify this, we have prepared another set of particles, Set B in Table 2.1, where the radius of the core $R_C$ is varying and the value of the shell-to-core mass ratio $MR_{SC}$ is somewhat random. Then, the shear-induced gelation for the Set B particles is carried out under the same conditions as for the Set A particles, and the corresponding fractal dimension of the clusters $D_f$ and the particle conversion to the gels $x$ are measured. The obtained results together with those from the Set A particles are shown as a function of the core surface coverage $\Gamma$ in Figure 2.5. It is seen that both the $D_f$ and $x$ values are well correlated to the $\Gamma$ values, thus
supporting that $\Gamma$ is indeed the key parameter controlling the extent of coalescence. Moreover, the $D_f$ values in Figure 2.5 can be well correlated through the following linear expression with $\Gamma$:

$$D_f = -0.66 \cdot \Gamma + 2.97$$

(2.9)

When this correlation is extrapolated to $\Gamma=0$, i.e., in the absence of the plastic shell, we have $D_f=2.97$, very close to 3, corresponding to complete coalescence of the particles. This is expected because without the plastic shell, aggregated rubber cores would coalesce to form spheres.

To further support that the core surface coverage $\Gamma$ is the key parameter determining the cluster structure and the particle conversion, we have plotted the data in Figure 2.5 as a function of the other two important parameters, the shell-to-core mass ratio $MR_{SC}$ and the particle radius $R_P$, as shown in Figures 2.7 and 2.8, respectively. As can be seen, both the $D_f$ and $x$ data do not collapse to a single curve when they are correlated with $MR_{SC}$ or $R_P$.

![Figure 2.5](image)

**Figure 2.5** The $D_f$ values of the clusters for the gels formed from the particles in both Set A and Set B of Table 2.1 (a), as well as the corresponding conversion $x$ of the particles to gels (b), as a function of the core surface coverage $\Gamma$. The solid line in (a) is given by eq 2.9.
2.4 Surfactant Effect

2.4.1 Surfactant adsorption behavior

The adsorption isotherms of Tween 20 and Emulgator K30/95 on the particles have been determined using a standard surface tension technique at the particle volume fraction of $\phi=0.005$. In the case of strawberry-like core-shell particles with the rubbery core partially covered by a plastic shell, there are two types of surface for surfactant adsorption. In order to assess if selective adsorption on different surfaces occurs, for each surfactant we have measured the adsorption isotherm also for particles of similar sizes but made of the pure rubbery core and the pure plastic shell materials, which were also supplied by BASF SE. The obtained results are shown in Figures 2.8a and 2.8b for Tween
20 and Emulgator K30/95, respectively, in terms of the surfactant density on the particle surface ($\Gamma$) as a function of the surfactant concentration in the aqueous phase ($C_{s,eq}$) at equilibrium. Note that in the case of strawberry-like particles, to calculate the particle surface area in order to compute $\Gamma$, we have assumed that the particles are spherical and defined by the nominal radius, $R_p=51.4$ nm, as discussed above.

![Figure 2.8](image)

**Figure 2.8** Adsorption isotherms of surfactants on three different particles: (●) strawberry-like core-shell particles, (●) particles made of the rubbery core material and (▲) of the plastic shell material. (a) Tween 20 and (b) Emulgator K30/95. $\phi=0.005$; $T=25^\circ C$. The solid curves serve to guide the eye.

It is seen that for both surfactants the adsorption isotherm for the particles made of the pure rubbery core material lies slightly above that corresponding to the pure plastic shell material, while the one for the strawberry-like particles is in between. This may indicate a
certain preference for the surfactants adsorption on the rubber with respect to the plastic material.

All the adsorption isotherms are of the S-shape, indicating changes in the adsorption mechanism as the surfactant concentration increases,\textsuperscript{78-80} \textit{i.e.}, at low surfactant concentrations the adsorbed surfactant forms a homogeneous dilute gaseous-like layer of individual molecules, while above a certain value they self-organize to form inhomogeneous hemi-micelles (aggregates) on the surface, leading to a substantial increment of the surfactant surface density.

The transition from homogeneous to inhomogeneous state in Figure 2.8a occurs at \( C_{s,eq} \approx 2.4 \times 10^{-5} \text{ mol/L} \) with the surfactant surface density, \( \Gamma \approx 2.6 \times 10^{-6} \text{ mol/m}^2 \), while it occurs at a much low surfactant surface density in Figure 2.8b. The saturation adsorption cannot be defined in both cases because the CMC occurs earlier. On the other hand, from the experimental point at the largest surfactant concentration before the CMC, even though not at saturation, the estimated surface area, \( A_m \) occupied by each surfactant molecule, is only \( 17.5 \text{ Å}^2 \) for Tween 20 and \( 22.2 \text{ Å}^2 \) for Emulgator K30/95, while from the literature it should be \( 60.3 \text{ Å}^2 \) for a Tween 20 molecule\textsuperscript{81} and \( >25 \text{ Å}^2 \) for a sulfonate molecule.\textsuperscript{82} Such difference confirms that the adsorbed surfactants are in the inhomogeneous hemi-micelles form, and the obtained \( A_m \) values are only their projection area on the surface.

2.4.2 Shear-induced gelation in the absence of surfactant

Let us first consider the case without adding any surfactant. As mentioned above, in this case the colloidal system with the strawberry-like particle morphology (particles FC1) is still very stable under quiescent conditions, due to the presence of the polymer chain-end charged groups (sulfate) on the surface, originating from the initiator. Such a stable colloidal system has been forced to pass through the z-MC at the particle volume fraction, \( \phi = 0.15 \), and the Peclet number, \( Pe = 220 \ (G = 8.0 \times 10^5 \text{ } 1/\text{s}) \). It was found that shear-induced gelation occurred and a solid-like gel was obtained at the outlet of the z-MC. The measured conversion of primary particles to the Class-2 clusters forming the gel is \( x = 89.4\% \), and the
estimated fractal dimension of the clusters is $D_f=2.76\pm0.04$, indicating a rather compact structure of the clusters.

We have also performed the shear-induced gelation for the particles made of the pure plastic shell material at the same values of $\phi$ and $Pe$. The conversion of primary particles to gel is $x=96\%$, significantly larger than that in the case of the strawberry-like particles. Moreover, from the average structure factor, $\langle S(q) \rangle$, the estimated fractal dimension of the Class-2 clusters is only $D_f=2.4\pm0.02$, indicating a much more open structure than in the previous case. Note that the $D_f$ value of 2.4 is typical for the Class-2 clusters formed from rigid (plastic) primary particles. Thus, the larger $D_f$ value of the Class-2 clusters of the strawberry-like core-shell particles reveals that partial coalescence among the particles occurs during the shear-induced aggregation (gelation).

It should be mentioned that we did not perform the shear-induced aggregation of the particles made of the pure rubbery core material, because when these particles are forced to pass through the z-MC, they and their clusters stick on the z-MC wall, leading to clogging of the channel.

### 2.4.3 Shear-induced aggregation in the presence of ionic surfactant (Emulgator K30/95)

A set of latexes of the strawberry-like particles FC1 have been prepared at the same particle volume fraction ($\phi=0.15$) but with different amount of the ionic surfactant, Emulgator K30/95. These latexes have been forced to pass through the z-MC to perform the shear-induced aggregation at the fixed $Pe=220$. Similarly to the above cases in the absence of surfactant, for each latex after passing through the z-MC, we have determined two quantities, the conversion of the particles to the Class-2 clusters, $x$, and the fractal dimension of the Class-2 clusters forming the gel, $D_f$. The results are shown in Figure 2.9a as a function of the surfactant surface density. It is seen that the $x$ value decreases monotonically as the surfactant surface density increases. Moreover, we have observed three distinct regimes as a function of the surfactant surface density, as indicated in the figure: For $\Gamma<6.07\times10^{-7}$ mol/m² ($A_m>274$ Å²), the samples after passing through the z-MC
are in solid-like gel state, while for $\Gamma > 6.07 \times 10^{-7}$ mol/m$^2$ ($A_m < 274$ Å$^2$), they are in liquid-like state, which means that the amount of the Class-2 clusters formed within the z-MC are not enough to interconnect forming a standing gel network. Thus, $\Gamma = 6.07 \times 10^{-7}$ mol/m$^2$ ($A_m = 274$ Å$^2$) is the critical surfactant surface density for the transition from a solid-like gel to a liquid-like state. When the surfactant surface density further increases to have $\Gamma \geq 1.12 \times 10^{-6}$ mol/m$^2$ ($A_m \leq 148$ Å$^2$) the conversion of the particles to the Class-2 clusters becomes $x = 0$, i.e., there is no change in the latex after passing through the z-MC.

The above effect of the surfactant surface density on the shear-induced aggregation has already been observed previously for the same surfactant but on different (plastic) colloidal particles. As explained in that work, this behavior has been attributed to the non-DLVO, very strong, short-range repulsive hydration force generated by the adsorbed surfactant on the particles. Such hydration force, at very low ionic strength (without adding any salt), increases as the $\Gamma$ value increases, and when the $\Gamma$ value reaches a certain value, the repulsive hydration force becomes extremely high such that aggregation and corresponding gelation become unachievable even at the highest collision energy that the adopted device can generate. Therefore, $\Gamma = 1.12 \times 10^{-6}$ mol/m$^2$ represents the critical $\Gamma$ value above which the latex becomes extremely stable against shear and no sheared-induced aggregation/gelation occurs.

The fractal dimension of the Class-2 clusters shown in Figure 2.9a is constant, independent of the surfactant surface density, and equal to that ($D_f = 2.76$) in the absence of surfactant. This means that the adsorbed surfactant does not play any role in protecting the particles from coalescence. Gauer et al.,$^{61,62}$ when studying aggregation of elastomer colloids under stagnant conditions, also observed that ionic surfactant cannot protect particles from coalescence. These results suggest that, under the shearing force or van der Waals attraction, relocation of the adsorbed surfactant molecules occurs when two particles approach each other, leading to exposure of the rubbery core surface to intermolecular diffusion (coalescence).
Figure 2.9 Fractal dimension $D_f$ of the clusters constituting the gel (♦) and conversion $x$ of the primary particles to gel (●) as a function of the surface density of three different surfactants: (a) Emulgator K30/95 (ionic), (b) Tween 20 (nonionic), and (c) Triton X-100 (nonionic). $Pe=220$ and $\phi=0.15$. 
2.4.4 Shear-induced aggregation in the presence of nonionic surfactant

For the nonionic surfactant, Tween 20, we have also prepared a set of latexes with the strawberry-like particles by adding different amounts of the surfactant at $\phi=0.15$. Each of the latexes has been forced to pass through the z-MC at $Pe=220$. The conversion of the particles to the Class-2 clusters, $x$, and the fractal dimension of the Class-2 clusters forming the gel, $D_f$, for the latexes after passing through the z-MC are shown in Figure 2.9b as a function of the surfactant surface density. When the conversion curve is compared to that in Figure 2.9a, the difference between ionic and nonionic surfactants is not substantial, i.e., in both cases the conversion decreases as the surfactant surface density increases. In particular, we observe that the stability of the particles in the intense shear flow is similar for the ionic and non-ionic surfactant, at least when the conversion is made for the same surface surfactant density, which, as seen in the contest of the adsorption isotherms in Figure 2.8, corresponds to rather different values of the surfactant aqueous solution. Such stabilization due to the steric effect is known, to be related to two mechanisms: 1) osmotic repulsion due to the difference in the osmotic pressure of the solvent in the overlapping zone with respect to that in solution and 2) elastic repulsion corresponding to compression-induced net loss in configurational entropy of the chains. Romero-Cano et al. have experimentally investigated the steric effect of nonionic surfactant, Triton X-100, on the colloidal aggregation under stagnant conditions, and found that above a certain value of the adsorbed surfactant surface density, no Brownian-motion-induced aggregation occurs even at very high ionic strengths. Similarly, in this work if the surface density of the adsorbed Tween 20 is larger than a certain value ($\Gamma=1.17\times10^{-6}$ mol/m$^2$ or $A_m=142$ Å$^2$) no shear-induced aggregation occurs even at extremely high shear rate. Therefore, similarly to ionic surfactant (with hydration), properly adsorbed nonionic surfactant can also protect colloidal particles from intense shear-induced aggregation. Somehow surprisingly, the critical value of $\Gamma=5.94\times10^{-7}$ mol/m$^2$ ($A_m=280$ Å$^2$) at which the transition from solid-like gels to liquid-like states occurs is very similar for Tween 20 and for Emulgator K30/95.

On the other hand, when the fractal dimension curve in Figure 2.9b is compared to that in Figure 2.9a, there are clear differences. Unlike the ionic surfactant, adsorption of Tween
20 on the strawberry-like particles does reduce the fractal dimension of the Class-2 clusters forming the gel. In particular, when the surfactant surface density is low, there is a sharp decrease in the fractal dimension as the surfactant surface density increases. This may be related to interactions of the adsorbed polyoxyethylene (POE)-type surfactants that lead to bridging, thus lowering the interfacial free energy. As proposed by Zhao and Brown, such bridging may involve interactions between the adsorbed surfactant molecules and the neighboring polymer chains and occurs both through the surfactant hydrocarbon tail and its POE chain hydrogen-bonding with the fixed surface charge (sulfate) groups. Cabane et al. studied the interactions between SDS (sodium dodecyl sulfate) and POE and found that POE can interact with the sulfate groups at the interface of SDS micelles to form POE-SDS aggregates. Zana et al. also suggested, based on fluorescence measurements, that POE-SDS interactions mostly occur at the SDS micelle surface. Such bridging may be a general phenomenon on a “hairy” surface if the adsorbed surfactant molecule has a suitable length, and reinforces the affinity between the surfactant molecule and the particle surface.

Based on the above observations, bridging can explain the $D_f$ curve in Figure 2.9b. Initially, since the adsorbed surfactant molecules are very few, each of the POE chains on the Tween 20 molecule would interact with a sulfate group, leading to very strong affinity between the Tween 20 molecule and the surface. The Tween 20 molecules adsorbed in this way are difficult to be relocated when two particles approach during the intense shear-induced aggregation, thus reducing the particle coalescence. It follows that, initially, the $D_f$ value decreases as the Tween 20 surface density increases in Figure 2.9b. Then, as the Tween 20 surface density progressively increases, the $D_f$ value reaches a plateau. This arises because the availability of the sulfate groups is limited, and now it becomes impossible for each of the POE chains to get a sulfate group for bridging. In this case, the adsorption strength of the Tween 20 molecules is substantially reduced, and consequently, some of the adsorbed Tween 20 molecules can be relocated during aggregation and cannot contribute to the protection of the rubbery core from coalescence. When the Tween 20 surface density reaches the critical value ($\Gamma=5.94\times10^{-7}$ mol/m$^2$ or $A_m=280$ Å$^2$) for the solid-like to liquid-like transition, the $D_f$ value decreases sharply as the Tween 20 surface density increases. This may be explained by the two facts: 1) the particle surface is
composed of both plastic and rubbery materials and the coalescence occurs only at the rubbery parts and 2) considering that each Tween 20 molecule requires 60.3 Å² at the criticality the area occupied by each molecule is only 4-5 times of this value. Then, even if the surfactant molecules can be partially relocated during the particle approaching, the exposed rubbery surfaces become smaller and smaller with increasing the surfactant density, and eventually not only coalescence but also aggregation becomes impossible.

To further verify the above shear-induced aggregation behaviour of the strawberry-like particles covered by a nonionic surfactant, we have also carried out the same experiments using another non-ionic surfactant, Triton X-100. With respect to Tween 20, it has fewer POE groups (thus less bridging with sulfate groups), but stronger hydrophobic interactions with the surface, due to the tetramethylbutyl-phenyl groups. The results are shown in Figure 2.9c as a function of the surfactant surface density, which is estimated from the adsorption isotherm on a similar surface measured by Jodar-Reyes et al. When it is compared to Figure 2.9b, it is seen that both the \( x \) and \( D_f \) curves are very similar. Thus, the adsorption of Triton X-100 molecules can also reduce the particle coalescence, and, at substantially large surface densities, it can also protect the particles from the intense shear-induced aggregation and gelation. However, both the critical \( \Gamma \) values for the solid-like to liquid-like transition and for zero conversion are larger for Triton X-100 than for Tween 20. Thus, with respect to Tween 20, Triton X-100 is less effective in protecting the particle coalescence and aggregation, mostly due to fewer hydrophilic POE groups.

2.5 Concluding remarks

In this work, we investigated the intense shear-induced gelation of core-shell particles. The core is composed of an acrylic elastomer (\( T_g < 253 \) K) grafted with the shell of polystyrene. Depending on the shell-to-core mass ratio, the core can be either partially or fully covered by the shell. The former leads to strawberry-like morphology, with potential coalescence among particles when they aggregate. The main objective of this work is to understand the effect of such core-shell morphology and of surfactants on the gel structure and conversion of the particles to gels.
It is found that when the core mass is fixed and the shell mass is varying, the fractal dimension $D_f$ of the clusters from which the gel is constructed decreases as the shell mass increases (i.e., a more open gel structure is obtained), and after the shell mass reaches a certain value, the $D_f$ value reaches a plateau. The SEM pictures of the gels reveal that the structure variations arise because of coalescence among particles, and the extent of coalescence decreases as the shell mass increases. More interestingly, in the region where $D_f$ reaches a plateau, the coalescence is also negligible. These results indicate that for coalescing systems the fractal dimension estimated from the average structure factor of the clusters can well reflect the extent of coalescence, even though in this case the clusters have been modelled as fractal rather than real objects.

The conversion $x$ of the particles to the gels instead increases as the shell mass increases, (i.e., as the extent of coalescence decreases.) This is related to the cubic dependence of the shear-induced aggregation rate on the cluster size: the smaller the extent of coalescence, the larger the cluster size, thus the larger the aggregation rate, leading to a larger conversion.

At this point, it is clear that the key parameter controlling the gel structure and the particle conversion is the core surface coverage by the shell. To verify this conclusion further, we have carried out the shear-induced gelation of another set of particles with varying core mass. It is found that the only parameter that can well correlate the $D_f$ and $x$ values from the two sets of particles is the core surface coverage, thus confirming the conclusion of this work.

Next, the role of the surfactant type on the shear-induced aggregation/gelation of strawberry-like particles has been investigated. An ionic (sulfonate) surfactant, Emulgator K30/95, and a nonionic (steric) surfactant, Tween 20, have been added to the system, and the same shear-induced aggregation/gelation has been carried out at $Pe=220$ and $\phi=0.15$. It is found that for both surfactants the conversion of the primary particles to the clusters constituting the gel decreases as the surfactant surface density increases. As the surfactant surface density increases above certain critical values, first gelation and then even aggregation cannot occur any longer. In the case of ionic surfactants, this behavior was already reported in the literature,\textsuperscript{47-49} and is attributed to the occurrence of the non-DLVO,
short-range repulsive hydration forces, protecting the particles from aggregation. In this work, we have demonstrated that it is also possible to protect particles from aggregation under intense shear using nonionic surfactants, by taking advantage of steric repulsion.

Another important observation is that although the ionic sulfonate surfactant can protect the particles from aggregation, it cannot protect the strawberry-like particles from coalescence once the clusters are formed, while the nonionic steric surfactant can do it. This is demonstrated by a reduction in the fractal dimension from 2.76 to 2.45. These results indicate that the adsorption of the ionic surfactant on the surface is not very strong and relocation of the adsorbed surfactant might occur as the particles approach each other during aggregation, thus leading to exposure of the rubbery core to intermolecular diffusion, and then coalescence. However, the adsorption of the nonionic surfactant, mostly due to bridging between the surface sulfate groups and the groups able to give hydrogen-bonding, such as for example the POE groups in Tween 20, is very strong, and its relocation during shear-induced aggregation is very difficult. In this case, the presence of the surfactant molecules between the particle surfaces can protect the rubbery cores from coalescence.

Nomenclature

- $D_f$: fractal dimension
- FC: particles with fixed core (from FC1 to FC5)
- $G$: shear rate [1/s]
- $H_{SP}$: height of plastic spots [nm]
- $I(0)$: intensity at zero angle [kcps]
- $I(q)$: angle dependent scattered intensity [kcps]
- $M_C$: core mass [kg]
- $MR_{SC}$: core to shell mass ratio
- $M_S$: shell mass [kg]
- $n_\theta$: refractive index of the solution
- $N_{SP}$: number of shell spots
- $P$: inlet pressure
$P(q)$  form factor of primary particles
$Pe$  Peclet number
$q$  magnitude of the wave vector [nm$^{-1}$]
$R_C$  core radius [nm]
$R_g$  gyration radius [nm]
$R_i$  radius of the aggregating cluster $i$ [nm]
$R_p$  radius of primary particles [nm]
$R_{SP}$  average plastic spot radius [nm]
$S(q)$  structure factor of obtained clusters
$VC$  particles with varying core (from VC1 to VC5)
$V_S$  volume of the plastic shell [nm$^3$]
$V_{SP}$  volume of one single plastic spot [nm$^3$]
$x$  conversion of primary particles to gel
$z$-MC  z-shape microchannel

**Greek letters**

$\beta_{i,j}^{\text{shear}}$  shear-induced aggregation kinetic constant [m$^3$/s]
$\Gamma$  core surface coverage
$\Gamma_S$  surfactant surface density [mol/m$^2$]
$\theta$  scattering angle [rad]
$\lambda_0$  laser wavelength [nm]
$\rho_C$  core density [kg/l]
$\rho_S$  shell density [kg/l]
$\phi$  particle volume fraction
Chapter 3

Precipitation polymerization reactions:
the case of vinyl-imidazole and vinyl-pyrrolidone
copolymerization in organic solvent

3.1 Introduction

Copolymers containing vinylimidazole (VI) and vinylpyrrolidone (VP) are highly functionalized polymers exhibiting attractive combinations of the different properties of the two corresponding homopolymers. Polyvinylpyrrolidone (PVP) is the technically most important polymer belonging to the family of poly-N-vinylamides. This material exhibits interesting properties since it contains both a non-polar substructure and a polar amide group which allow the pyrrolidone unit to favorably interact with hydrophobic as well as hydrogen-bonded and dipolar compounds. On the other hand, the imidazole structure of VI possesses a marked tendency to complex formation both with organic substrates (e.g. proteins, enzymes and dyes) and with metal ions, such as iron, copper, nickel and silver. Due to this ability to form complexes with various low molecular weight compounds, VI copolymers are used in several industrial applications, such as photographic industry, dyeing of textile fibers and plastic surfaces, removal of heavy metals in wines, printing of special papers, and coupling agents in corrosion prevention. Finally, the presence of the imidazole ring (contained in L-histidine) in the active site of several hydrolytic enzymes has suggested the use of PVI and its copolymers as catalysts of activated ester hydrolysis.

VI/VP copolymers are water-soluble and industrially produced by solution polymerization in water. This process exhibits distinct limitations; in particular high
molecular weight polymers cannot be produced. On the other hand, this is possible in precipitation polymerization in a non-solvent medium of the polymer where bimolecular terminations are significantly lowered by diffusion limitations.

The aim of this work is to develop a better understanding of the precipitation polymerization process which can be regarded as a promising alternative to the conventional solution polymerization for many industrial applications. For this we take as a model system the VI/VP copolymerization in an organic solvent, i.e. butyl acetate (BA), which on the other hand has also a significant industrial relevance.

It is worth noting that the PVP homopolymerization\textsuperscript{90,95-98} and copolymerization with several monomers\textsuperscript{99,100} have been widely investigated in the literature. On the other hand, despite the large number of industrial applications, relatively few data are available in the literature for polyvinylimidazole (PVI) and the VI/VP copolymers. Furthermore, the published studies both on PVP and on PVI focus on bulk and solution polymerization,\textsuperscript{89,91-93,95-97,99-105} while to the best of our knowledge the precipitation process has never been considered in the literature so far.

Precipitation polymerization is generically defined as a heterogeneous polymerization in which the produced polymer is insoluble in the reaction medium.\textsuperscript{106} Both initiator and monomer are soluble in the solvent and the reaction starts as a homogeneous polymerization in the continuous phase. With the proceeding of the reaction, the polymer chains grow and precipitate out of the continuous phase, thus nucleating a second, dispersed phase. Since monomer and initiator are soluble also in the newly formed, polymer-rich phase, from this point on, i.e. after a few percent of conversion, the reaction occurs in both phases. Since the difference in reactivity between the two phases can be significant, the properties of the polymer chains can be largely different depending upon the locus where they were produced. Therefore, the identification of the contribution of each reaction locus is crucial for proper understanding and design of the process.

Several papers appeared recently aimed to determine the role of competing reaction loci in heterogeneous processes combining experimental data and mathematical models.\textsuperscript{107-112} Müller et al. developed a kinetic model for two types of heterogeneous polymerization in supercritical carbon dioxide, i.e. dispersion polymerization of methyl methacrylate\textsuperscript{107,108}
and precipitation polymerization of vinylidene fluoride.\textsuperscript{109} in both systems the interphase transport of active chains was found to be crucial in determining the reaction locus. The relevant parameter is the ratio of the macro-radical diffusion rate to the termination rate, referred to as parameter $\Omega_j$ in [107-108], where $j$ is the index specifying the phase, continuous or dispersed. In general, two limiting conditions apply: $\Omega_j \gg 1$, where the polymer chains initiated in the $j$-th phase are able to transfer into another phase before terminating, and $\Omega_j << 1$ where all polymer chains terminate in the same phase where they are initiated.

In the system considered in this work since the polymer chains are strongly insoluble in the continuous phase the transfer of active growing radicals from the polymer particles to the continuous phase can be neglected. We need then to consider the $\Omega$ parameter in the continuous phase only, and we can have two limiting conditions: $\Omega \gg 1$, where the radicals produced in the continuous phase are transferred to the polymer particles before terminating, which implies that the polymer particles are the only reaction locus; $\Omega << 1$, where the radicals generated in the continuous phase grow and terminate in the same phase, which implies that two reactions loci are operative. Only after termination the radicals produced in the continuous phase precipitate and mix with the chains produced in the other reaction locus, i.e. the polymer particles.

In order to identify the major mechanisms determining the product quality for the heterogeneous precipitation copolymerization process, several reactions have been performed in a well stirred, isothermal, glass reactor while changing operating parameters such as monomer mixture composition (from VI homopolymer to the 50/50 copolymer), monomer mixture holdup, initiator amount and stirring rate. The kinetics of the system was monitored by sampling at suitable interval time intervals the reacting mixture and measuring conversion through gravimetry and gas chromatography (GC), and molecular weight distributions (MWD) by gel permeation chromatography (GPC). Moreover, since the final product of the precipitation process is a particulate polymer dispersed in the solvent, the particle size distribution (PSD) and the structure of the formed aggregates have been characterized by scanning electron microscopy (SEM) and small angle light scattering (SALS).
After the experimental characterization of the system, a kinetic model accounting for the polymerization reaction in both phases is developed. A kinetic scheme of free-radical polymerization is applied to each phase and the diffusion of active chains from the continuous to the dispersed phase is accounted for. In the approach used by Müller et al.,\textsuperscript{107} the mass transfer rate was described in detail, specifically accounting for the dependence of the diffusion coefficient on chain length. This approach becomes too complex in terms of number of adjustable parameters in the case of copolymerization reactions and therefore a simpler approach has been considered. In particular, the transport rate is expressed as a fraction $\alpha$ of the overall radical production rate by initiation. It is clear that as $\alpha$ increases from zero to one the model predicts all possible situations ranging from complete segregation, i.e. all radicals grow and terminate in the same phase where they have been generated ($Q << 1$), to complete transport to the polymer particles ($Q >> 1$).

First, a reduced version of the model has been used to predict the reaction kinetics as a function of several operating parameters, such as copolymer composition, initiator concentration and monomer concentration. A good comparison with experimental results has been obtained. Moreover, it is found that the system operates in a situation close to complete phase segregation, i.e. with an $\alpha$ value close to zero.

Finally, the complete version of the model has been considered. This includes the detailed population balances of active and dead polymer chains in each phase, and therefore it allows computing the complete distribution of the molecular weight. The model predictions compare favorably to the experimental data, suggesting the presence of a crosslinking reaction taking place in the dispersed phase and confirming the presence of two significant reaction loci in the system.
3.2 Experimental analysis
3.2.1 Materials and Methods

Chemicals
VI and VP monomers and the initiator, i.e. tert-Butyl peroxy-2-ethylhexanoate (T21S), were supplied by BASF (Ludwigshafen, DE). The organic solvent, i.e. n-Butyl acetate (BA), was purchased from Acros (Geel, BE). The alcoholic solvent, i.e. 1-Butanol, was obtained from Aldrich (Buchs, CH). All the chemicals were used as received without any further purification.

Reactor and procedures
Batch reactions were carried out in an automated lab reactor (Labmax©, Mettler-Toledo, CH), which ensures accurate control of the temperature inside the reactor and of the rotational speed of the mechanical stirrer. A glass, round-bottom, jacketed reactor of 0.5 liter volume has been used. A glass reactor was selected to prevent any interaction between the radicals and the reactor walls and to allow the visualization of macroscopic changes in the reaction system. To achieve good mixing inside the reactor, an anchor-type stirrer has been used. The reactor was equipped with a condenser to avoid monomer and solvent loss by evaporation.

The mixture of monomers and solvent was prepared and then charged to the reactor. Before starting the reaction, stripping flushing nitrogen inside the reactor was applied in order to remove oxygen from the reactants, which could inhibit the radical reactions. To obtain better stripping efficiency, nitrogen was flushed trough a needle dipped inside the liquid mixture. Such stripping was applied for about 40 minutes at pressure of about 1.2 bars. Once this step was completed, the condenser was closed with a gummy balloon and the pressure set to a value slightly larger than the atmospheric one to prevent oxygen to enter the reactor.

As soon as the reactor had reached the set-point temperature (90°C), three samples of the reacting mixture were taken using a Pasteur pipette. Since the sampling requires the opening of the reactor cap, nitrogen flow was maintained during this step to prevent the
entrance of oxygen. Such samples were stored into glass vials and later analyzed by gas chromatographic analysis. This same sampling procedure was applied for all samples taken during the reaction.

After those preliminary operations, the initiator was injected by a syringe through the rubber septum. The introduction of the initiator corresponds to the time zero of the reaction. To measure the conversion, samples were taken during the reaction and immediately quenched by ice bath to avoid any polymerization inside the sampling vial.

### Gas Chromatography

Gas Chromatography (GC) was applied to measure conversion and cumulative composition by evaluating the residual amount of monomer. A Hewlett Packard gas chromatograph HP6890, equipped with autosampler, thermal conductivity detector and fused silica column (CP-WAX 52 CB, 30m × 0.32mm × 0.5 um, from Varian (Palo Alto, USA)) has been used. To avoid plugging of the column by the polymer particles, every sample was solubilized in a 50-50 vol.-% water-ethanol solution before injection into the GC. Calibration curves were evaluated for both monomers. A linear calibration equation such as \( A = C \cdot x \) (where \( A \) represents the peak area and \( x \) the moles of monomer) has been used, being the constant \( C = 1.2 \cdot 10^{10} \) and \( 9.7 \cdot 10^{9} \) for VI and VP, respectively.

Given the proportionality between the area of the peaks in the chromatogram and the concentration of the monomers, conversion can be directly evaluated as follows:

\[
Conv(t) = \frac{A_0 - A(t)}{A_0}
\]  

(3.1)

where \( A_0 \) is the area of the monomer peak in the mixture at time zero and \( A(t) \) is the area of the monomer peak at time \( t \). The consistency between the conversion evaluated in this way and by gravimetry (cf. next subsection) was checked.

As a further check of the accuracy of the chromatographic measurements, the ratio between the areas of solvent and ethanol peaks was also evaluated: since these species do not react, such ratio should remain constant all along the reaction, as it was the case in all our experiments.
Thermogravimetry analysis (TGA)

The total conversion was also measured by the Halogen Moisture Analyzer HG53 from Mettler Toledo (CH). Such instrument measures the moisture content of a sample by weighting the sample before and after its heating by an integral halogen dryer unit. On completion of drying, the dry content of the sample is displayed and the conversion of the reaction is evaluated knowing the expected dry content at complete conversion. The conversions measured by this technique were in good agreement with the values obtained by GC. Only in a few reactions, a slight disagreement was found at low reaction times. Since the thermogravimetric technique has a small accuracy at low conversion, in these cases only the data obtained by the more reliable GC technique were considered.

Gel Permeation Chromatography

Molecular weight distributions were analyzed by Gel Permeation Chromatography (GPC). The samples taken during the reaction were dried overnight in a vacuum oven at 20°C and 50 mbar. The dried polymer was then analyzed in BASF laboratories using the two columns Suprema Max linear XL and Suprema Max linear M from PSS (Mainz, DE). A water solution of 0.02 mol/l formic acid and 0.2 mol/l potassium chloride was used as eluent. Samples at concentration of 1.5 mg/ml were injected into the column after filtration with a 0.2 μm filter. Refractive index signal was detected by the differential refractometer Agilent 1200 (Santa Clara, CA, USA) and the UV signal by the Gilson UV-Detector UV/VIS 155 (Middleton, WI, USA). The calibration curve was obtained with Pullulan standards from PSS (Mainz, DE) in the range 5.6-1,660 kDa.

Small Angle Light Scattering (SALS)

To evaluate the size distribution of the polymer particles, Small Angle Static Light Scattering (SALS) measurements were carried out. The SALS instrument used in this work is a Mastersizer 2000 (Malvern, UK). Characteristics of the instrument may be found elsewhere. Since it was necessary to stir the suspension during the measurements to avoid sedimentation of the particles, measurements at different stirring rates were performed in order to exclude possible influence of the stirring rate on the measured size distribution.
Scanning Electron Microscopy (SEM)
Polymer samples were diluted in butyl-acetate, transferred on a silica specimen holder using a micropipette, and left drying at room temperature. The dilution was adjusted in order to avoid aggregation of particles during the drying process: therefore, the obtained images can be considered representative of the real structure of the aggregates in the suspension. Finally, the dried samples were imaged on a Gemini 1530 microscope (Zeiss, FEG) at low voltage (1 kV) to minimize charging effects.

3.2.2 Reaction Kinetics
A first, “reference” copolymerization reaction was carried out at 90°C, at stirring rate of 200 rpm, 10% of monomer content, composition VI/VP = 75/25 w/w and initiator amount equal to 0.6% of monomer content. Then, to investigate independently the effect of the operating parameters on the polymerization kinetics, several reactions were performed changing the value of one single parameter at a time with respect to the reference case. The effects of total monomer concentration, monomer feed composition (from VI homopolymer to copolymer VI/VP 50/50), initiator concentration and stirring rate effect were examined. A summary of all the experimental runs is reported in Table 3.1.
Different monomer mixture concentrations were investigated in the range 4-15% for VI homopolymerization. At the larger monomer concentration values, a clear precipitation of polymer on the walls of the reactor was observed. Such instability of the polymer suspension was affecting the sampling reproducibility and the sample homogeneity under stirring. A macroscopic phase separation of a yoghurt-like phase surrounding a liquid-stirred phase was observed. Therefore, the results collected at monomer concentration larger than 15% were discarded. The conversion-time curves for the homopolymerization reactions are shown in Figure 3.1: an increasing rate of reaction is found at increasing monomer concentration.
Table 3.1 Summary of all the experimental runs.

<table>
<thead>
<tr>
<th>Total monomer concentration (w% of mixture)</th>
<th>Monomer feed composition VI/VP</th>
<th>Initiator (w% of monomer)</th>
<th>Stirring rate (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>75/25</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>75/25</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>75/25</td>
<td>0.6</td>
<td>300</td>
</tr>
<tr>
<td>10</td>
<td>50/50</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>90/10</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>80/20</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>60/40</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>75/25</td>
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<td>200</td>
</tr>
<tr>
<td>10</td>
<td>75/25</td>
<td>0.3</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
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<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>7.5</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
</tr>
<tr>
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<td>100/0</td>
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<td>200</td>
</tr>
<tr>
<td>10</td>
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<td>1.8</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>100/0</td>
<td>0.2</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>90/10</td>
<td>0.6</td>
<td>300</td>
</tr>
<tr>
<td>10</td>
<td>90/10</td>
<td>0.6</td>
<td>100</td>
</tr>
</tbody>
</table>

The effect of the initiator was investigated by changing its initial concentration. Reactions with 0.3%, 0.6% and 1.2% of initiator with respect to the monomer content were carried out for the copolymer VI/VP 75/25. The corresponding curves of conversion versus time are shown in Figure 3.2. It is worth noting that at 0.3% of initiator content complete conversion was not reached even after 3 h, in contrast to the reactions performed with larger initiator amounts. This behavior is not related to initiator depletion; in fact, about 20% of the initial charge is still present after 3 h, being the half-time of this initiator at the operating temperature equal to 1.17 h.
Figure 3.1. Monomer conversion as a function of time for VI homopolymerization at various monomer concentrations: 4% (□), 7.5% (◊), 10% (△), 15% (○).

Figure 3.2. Monomer conversion as a function of time for VI/VP 75/25 copolymerization at various initiator concentrations: 0.3% (○), 0.6% (△), 1.2% (□) with respect to monomer.

Figure 3.3. Monomer conversion as a function of time for copolymerizations at various VI/VP feed compositions: 50/50 (□), 60/40 (◊), 75/25 (○), 90/10 (△), VI homopolymer (●).
The effect of the feed composition was studied ranging from 100% VI homopolymer to composition VI/VP = 50/50. The corresponding curves of conversion vs. time are compared in Figure 3.3. At increasing VP content in the monomer feed, an increase of the reaction rate is observed, thus indicating a larger reactivity of VP with respect to VI.

Figure 3.4. Cumulative polymer composition as a function of conversion for VI/VP 75/25 copolymerization (dashed line: feed composition).

Figure 3.5. Cumulative polymer composition as a function of conversion for VI/VP copolymerization at various monomer mixture composition: 50/50 (□), 60/40 (◊), 75/25 (○), 90/10 (△) (dashed lines: feed compositions).

In Figure 3.4, the cumulative copolymer composition as a function of conversion as measured by GC is shown for feed composition VI/VP = 75/25 (reference reaction).
beginning of the reaction the composition of the polymer is richer in VI with respect to the feed composition. Considering that VP was found to be more reactive than VI (Figure 3.3), this suggests that the cross propagation reaction between an active polymer chain terminating with a VP unit and VI is more favored compared to the direct propagation reactions VP-VP, and of course they are both faster than the propagation of radical chains terminating with a VI unit. During the reaction, a compositional drift is found, approaching the feed composition at complete conversion. Such behavior was observed at all feed compositions, as shown in Figure 3.5.

The Molecular Weight Distributions (MWD) of various copolymers at complete conversion are shown together with that of the VI homopolymer in Figure 3.6. It can be seen that all the distributions are quite broad, covering about three orders of magnitude of molecular weight. Moreover, a shoulder appears at about 10 times the peak molecular weight, and its location shifts towards larger molecular weights at increasing VP content in the feed. The presence of such considerable amount of polymer at large molecular weight is most probably due to the formation of nonlinear chains by some branching or crosslinking reactions. Indeed, while performing the GPC measurements, difficulties in filtering the samples were encountered, thus indicating the presence of some micro-gel polymer.

Figure 3.6. Final molecular weight distributions. VI/VP copolymers: 60/40 (→), 75/25 (···), 90/10 (--; VI homopolymer (→).
Figure 3.7. Normalized number average molecular weight versus dimensionless time for different VI/VP copolymer compositions: 60/40 (◊), 75/25 (○), VI homopolymer (□).

The broad MWDs shown in Figure 3.6 actually require some further comments since in fact the shoulders are an artifact due to the exclusion of the largest macromolecules produced by branching or crosslinking. In other words, such huge polymer chains leave the column all together in the initial part of the eluted chromatogram leading to a shoulder in the corresponding MWD. Most likely the “real” MWD exhibits some kind of tailing, corresponding to the formation of larger and larger macromolecules.

In order to further support the conclusion above, the final sample of the copolymer VI/VP 75/25 was re-analyzed using another bench of columns and a different eluent. Namely, a Suprema Max 10 000Å 10 μm (8×300 mm) column from PSS (Mainz, DE) and water solution of 0.3 M formic acid as eluent were used. It was found that the shoulder in the high-MW region in Figure 3.6 is indeed replaced by a smoother, more regular tail in the distributions (data not shown).

The presence of this fictitious shoulder affects also the reliability of the average MW values estimated from such distributions. Being the inaccuracies concentrated in the high molecular weight region of the chromatogram, we expect the weight average molecular weight, $\bar{M}_w$, strongly affected and the number average, $\bar{M}_n$, less affected. Accordingly, all estimated $\bar{M}_w$ (and polydispersity) values have been discarded and only
values will be presumed reliable in the rest of this work. The measured values of three experiments are shown in Figure 3.7 as a function of time. In all cases, after an initial increase, the average molecular weight decreases in time. This behavior is expected in free-radical polymerization, where the number average $\overline{MW}_n$ is proportional to the ratio of monomer and initiator, i.e. $\overline{MW}_n = k_p M \sqrt{k_i R}$, being $M$ the monomer concentration, $k_p$ and $k_i$ the propagation and termination rate constants, respectively, and $R_i$ the initiation rate. Depending on the consumption rates of monomer and initiator the number average $\overline{MW}_n$ can increase or decrease. Moreover, after precipitation of polymer particles the reaction can occur in two phases, i.e. the solvent-rich phase and the dispersed phase. Since the two phases exhibit different reactivity, they are expected to produce polymers with different molecular weights. Therefore, the time evolution of the total molecular weight depends on the time evolution of the molecular weight inside a single phase and of the relative extent of the polymerization rate in the two phases. In order to quantify these effects a detailed kinetic model has been developed and will be discussed in the second part of this work.

Given the broadening of the molecular weight distributions, the possible reaction mechanisms underlying the formation of branched/crosslinked large chains need to be discussed. Our experimental results (microgel in the samples, MWD tailing in the large MW region) are consistent with a mechanism previously reported in the literature involving a crosslinking reaction of the imidazole group of VI. First evidences of such a reaction were reported by Bamford and Schofield, who studied the polymerization of VI in bulk and in different solvents. In particular, the kinetic study of VI polymerization in ethanol at different concentrations showed that the reaction order with respect to monomer approaches 1 in dilute solution and 0 at high monomer concentration. It was also observed that the reaction order with respect to the initiator approaches 0.5 in dilute solutions and 1 at high monomer concentrations. These results were interpreted by considering that the propagating radical could add the monomer onto an internal double bond of the imidazole ring, thus generating an unreactive species with low tendency to continue propagation.
Later on, Chapiro\textsuperscript{92} proposed a second mechanism: according to his considerations the degradative addition to monomer proposed by Bamford and Schofield should induce a gradual decrease of the overall rate of polymerization, while in all the investigated systems an auto-accelerated behavior was observed. Furthermore, if the internal double bonds of the monomer can participate significantly to the reaction, VI should always behave as a di-functional monomer leading to crosslinked polymer. On the contrary, gel formation was observed only under specific conditions, i.e. when high concentrations of PVI are present in the monomer. Such conditions are achieved at high conversion values when the polymer is formed in a concentrated monomer solution or when the polymer is insoluble in the reacting medium and precipitates as a separate phase swollen by the monomer. Notably, the latter case corresponds to the situation encountered in the precipitation process analyzed here. In order to explain this peculiar behavior of VI polymerization, Chapiro proposed a chain transfer to polymer reaction between the growing chains and the internal double bonds of PVI. The reaction is slow in comparison to propagation; therefore gel formation takes place only when the polymer concentration in the monomer solution becomes large enough. The different reactivity of the double bonds in the monomer and in the polymer can be explained by the conjugation of the vinyl group with the imidazole ring.
that enhances monomer stability. This mechanism, schematically represented in Figure 3.8, is consistent with all kinetics data reported in literature so far as well as with our own data, and therefore it will be adopted in developing the kinetic scheme of this system in the following part of this work. However, more experimental evidences are required to discriminate ultimately between the degradative addition proposed by Bamford and the chain transfer to polymer reaction proposed by Chapiro.

3.2.3 Particle Size Distribution (PSD)

Before starting the reaction, the mixture is a transparent, homogeneous solution. As soon as the reaction starts, polymer chains are formed, become insoluble in the solvent and nucleate polymer particles, thus forming the new, dispersed phase. Since no stabilizer is used these so-called “primary” polymer particles grow, aggregate to clusters and the system becomes a viscous, slurry-like suspension. The dimension and structure of such aggregates have a great impact on the product quality with respect to its final application, and therefore their size characterization is of interest. A typical SEM picture of a sample at the end of the reaction is shown in Figure 3.9. The clusters appear as compact structures in which the primary particles are still recognizable, indicating that they keep their identity during growth and aggregation. The final radius of the primary particles can be estimated around 55 nm.

Figure 3.9. SEM picture of polymer aggregates: dried final sample of the reference reaction VI/VP 75/25.
The particle size distribution (PSD) of the polymer aggregates has been characterized off-line by small angle light scattering (SALS). Static light scattering measures the intensity of the scattered radiation $I$ as a function of the wave vector $q$:

$$q = \frac{4\pi n}{\lambda_0} \sin \left( \frac{\theta}{2} \right)$$

where $\theta$ is the scattering angle, $n$ is the refractive index of the solvent and $\lambda_0$ is the wavelength of the radiation. From the measured intensity of the scattered radiation at different angles the corresponding PSD can be evaluated. PSDs reconstructed directly by the software of the light scattering device were considered. In order to avoid multiple scattering effects, the sampled polymer suspensions in BA were diluted by the same solvent until a polymer concentration of about 0.1% was reached.

The PSDs of the final samples are shown in Figure 3.10 for two different copolymers (VI/VP=75/25 and VI/VP=90/10) produced at various stirring rates. All PSDs are bimodal, with a major peak around 80-100 $\mu$m and a second, smaller peak around 10 $\mu$m. Both the particle sizes corresponding to such two peaks as well as the ratio between the two peak heights are affected by the stirring rate.
Figure 3.10. Final PSD for a VI/VP 75/25 copolymer (a) and a VI/VP 90/10 copolymer (b) at various stirring rates: 100 rpm (---), 200 rpm (--), 300 rpm (—).

Figure 3.11. Monomer conversion versus time for a VI/VP 75/25 copolymer at various stirring rates: 100 rpm (Δ), 200 rpm (○), 300 rpm (▽).
On the other hand, the stirring rate has no effect on the kinetics of the process, as it is clearly shown in Figure 3.11, where the conversion of the reference reaction VI/VP 75/25 carried out at different stirring rates is reported as a function of time. This result is particularly relevant, since it indicates that the polymerization kinetics is not affected by the PSD and thus the polymerization reaction and the particle precipitation can be described as two independent processes. Accordingly, the particle interphase area which is obviously affected by aggregation does not affect the polymerization kinetics. This means that the diffusional transports of the different species between the two phases do not affect the polymerization rate. Such behavior is found when the mass transport rate from one phase to another is so large to establish instantaneous equilibrium, or when the same transport rate is so small to be negligible. Based on the SEM pictures the primary particles exhibit a radius of about 55 nm which corresponds obviously to a very large interphase area. On the other hand, the primary particles are aggregated in quite compact clusters, where only a small fraction of the total “geometric” area may be accessible. To discriminate between such two opposite cases (very small or very large mass transport rates), a quantitative model has been developed and it will be discussed in the second part of this work.

It is worth noting that the bimodality of the PSD is affected not only by the stirring rate, but also by the copolymer composition. In Figure 3.12 the final PSDs for copolymers with various compositions are compared. The bimodality is more pronounced for the VI homopolymer, while the PSD gradually evolves towards a single, broader distribution at increasing VP content in the monomer feed.

In order to investigate the mechanisms determining the bimodality of the PSD, it is convenient to monitor its evolution in time. In Figures 3.13 and 3.14, the PSDs for two different systems, VI/VP copolymer 75/25 at 200 rpm (Figure 3.13) and VI/VP copolymer 90/10 at 100 rpm (Figure 3.14), are shown as a function of time. In both cases the PSD is monomodal during the early stages of the reaction. As the reaction proceeds, the precipitation of polymer chains and the subsequent growth and aggregation of the primary particles shift the peak to larger sizes. At some point a maximum size seems to be reached, while a second mode at smaller aggregate size appears. Note that this behavior is very
similar to the one observed in the precipitation polymerization of vinyl chloride (PVC):\textsuperscript{114} at few % of conversion particle nuclei of around 100 nm are formed by flocculation of microdomains of precipitated macromolecules. The primary particles later stick together in clusters still remaining well separate (similar clusters are formed in our system as shown in the SEM picture of Figure 3.9). Even after aggregation, the primary particles continue to grow because of reaction, thus increasing their size and decreasing the void space inside the clusters. At larger conversion such PVC clusters reach a maximum size of about 2-10 μm and further aggregate into larger structure of about 50-300 μm (below indicated as “grains”). Such an upper limit corresponds to the critical size above which the breakage process becomes dominant and prevents further growth by balancing the aggregation process. In the case of the system investigated in this work, the time evolution of the PSD can be explained through the same arguments. The value of the upper mode, i.e. 80-100 μm, is slightly different, as expected being different the mixing conditions. Breakage occurs through the energy introduced by the shear which apparently is sufficient to break the grains into clusters of around 2-10 μm, thus leading to the formation of the second mode in the PSD. On the other hand, such hydrodynamic energy is not sufficient to overcome the strength of the primary particle bonds inside a cluster and therefore the clusters cannot be further broken into smaller fragments.

The effect of shear rate and copolymer composition on both the maximum size and the evolution kinetics of the PSD is related to the aggregation/breakage processes. It is well known in fact that the shear rate in the reactor affects directly the aggregation and breakage rate coefficients.\textsuperscript{115} The particle material (i.e., copolymer composition) affects the aggregate strength\textsuperscript{116} since it affects the strength of the bounds among primary particles, particularly in the presence of partial interfusion or coalescence, and therefore affects breakage and consequently the PSD. In the system under examination, we observe that with increasing the VP content the bimodality in the PSD is less pronounced. Most likely, increasing the VP content the coalescence among the clusters inside a grain increases. Therefore, the clusters loose partially their identity and the fragment size distribution resulting from a breakage event is more uniform, as shown in Figure 3.13.
Figure 3.12. Final PSD for different VI/VP copolymers at 200 rpm: 50/50 (○), 60/40 (⋅⋅⋅), 80/20 (→), 90/10 (⋯), homopolymer VI (―).

Figure 3.13. PSD for a VI/VP copolymer 75/25 at various times: 10 min (―), 20 min (⋅⋅⋅), 30 min (→), 50 min (⋯), 180 min (○), 610 min (◊).

Figure 3.14. PSD for a VI/VP copolymer 90/10 at 100 rpm at various times: 20 min (―), 50 min (⋅⋅⋅), 180 min (⋯).
Some information about the structure of the precipitated aggregates can be obtained from the so-called average structure factor $\langle S(q) \rangle$ which can be computed from the measured intensity of the light scattered at different angles $I(q)$, as follows:73

$$\langle S(q) \rangle = \frac{I(q)}{I(0)P(q)}$$

(3.3)

where $I(q)$ is the angle-dependent scattered intensity, $I(0)$ is the intensity at zero angle and $P(q)$ is the form factor of the primary particles, which depends on the shape and the size of the particle. In the case of spherical particles, the form factor $P(q)$ is given by:

$$P(q) = \left[ \frac{3\left( \sin(qR_p) - qR_p \cos(qR_p) \right)}{(qR_p)^3} \right]^2$$

(3.4)

where $R_p$ is the primary particle radius, estimated by SEM pictures.

The estimated $\langle S(q) \rangle$ for the final sample of the reference reaction VI/VP 75/25 is shown in Figure 3.15. All the other structure factors evaluated for different conditions show very similar behaviour. The average radius of gyration of the clusters, $\langle R_g \rangle$, can be evaluated according to the Guinier equation:73

$$\langle S(q) \rangle = \exp\left(-q^2\langle R_g \rangle^2 / 3 \right) \quad \text{for} \quad q < 1/\langle R_g \rangle$$

(3.5)

The structure factor contains information about the correlations among the particles in a cluster. The typical measured $\langle S(q) \rangle$ shows two bendings, the first at $q \sim 2\times10^{-5}$ nm$^{-1}$ and the second at $q \sim 2\times10^{-4}$ nm$^{-1}$. The inverse of the $q$ vector represents the length scale of the scattering aggregates. Therefore the two bendings correspond to two different classes of aggregates with length scales of 50 μm and 5 μm, respectively. Indeed by applying the Guinier equation in these two regions the $\langle R_g \rangle$ values were evaluated as $\sim 70$ μm and $\sim 8$ μm for the first and second bending, respectively. Hence the analysis of the $\langle S(q) \rangle$ confirms the presence of two classes of aggregates with different size, thus confirming the bimodal PSDs previously discussed.
Figure 3.15. Structure factor as a function of the wave vector for the final sample of the reference copolymerization reaction VI/VP 75/25. The two Guinier regimes and the power-law regime are highlighted.

In addition, the analysis of the structure factor can provide information about the structure of the aggregates. In particular, for sufficiently large fractal clusters, the structure factor exhibits a power law behaviour, whose slope in the plot shown in Figure 15 represents the fractal dimension ($D_f$) of the aggregates:

$$\left\langle S(q) \right\rangle \sim q^{-D_f} \quad \text{for} \quad \frac{1}{\langle R_g \rangle} \ll q \ll \frac{1}{R_p} \quad (3.6)$$

It can be seen that the measured values of $\left\langle S(q) \right\rangle$ as a function of $q$ shown in Figure 3.15 follows indeed a power law for $q > 2 \times 10^{-4}$ nm$^{-1}$. Therefore, we can conclude that in the 5 μm scale the polymer clusters exhibit fractal geometry, with fractal dimension $D_f$ around 2.5. For larger aggregates, no conclusions can be drawn, since the possible power law decay is hidden by the superposition of the scattering given by the smaller clusters. In order to put the obtained values in a perspective we should consider that the diffusion limited cluster aggregation, which occurs in absence of stabilizer, forms very open aggregates with $D_f \approx 1.8$, while a full, compact sphere has $D_f = 3$. Therefore, the estimated value of $D_f \approx 2.5$ corresponds to quite compact clusters, in agreement with the SEM picture in Figure 3.9. This further confirms that the mode about 10 μm in the PSD corresponds to the fused clusters inside which the primary particles grow during the reaction, thus reducing the void space. It is worth noting that the clusters obtained in this work in a stirred tank reactor
exhibit a $D_f$ value which is in close agreement with the $D_f$ value of clusters obtained by the shear-induced aggregation of polymer primary particles in other devices, i.e. in a microchannel ($D_f \sim 2.4$)$^{50}$ and in a rheometer ($D_f \sim 2.7$)$^{117}$. This suggests that shear-induced aggregation leads to a fractal scaling weakly dependent on system conditions, as for example applied shear stress and primary particles.
3.3 Kinetic model

3.3.1 Reduced model: reaction locus, mass transport and kinetic

Model assumptions

Considering the results of the previous experimental investigation, the model has been developed based on the following assumptions.

1. The possible presence of two reaction loci is accounted for: the solvent-rich, continuous phase and the polymer-rich, dispersed phase. The latter one is made of polymer particles, nucleated at the beginning and then growing all along the process.

2. As confirmed by experiments, the separability between reaction kinetics and particle precipitation/aggregation processes applies. Therefore, the actual morphology of the produced polymer particles is neglected and the kinetic polymerization model does not require any information about the particle size distribution.

3. The nucleation of the particles is very fast compared to the total reaction duration. Therefore, the number of particles can be considered constant and equal to its final value since the beginning of the copolymerization. This number has been evaluated given the final polymer volume and the size of the primary particles measured by SEM.

4. Low molecular weight species, such as initiator, monomers and solvent, are considered at thermodynamic equilibrium and their partitioning between the two phases is described in terms of partition coefficients.

5. The dead chains formed in the solvent-rich phase are considered instantaneously transported to the polymer-rich phase, since the kinetics of their diffusion is not relevant to the model predictions. On the other hand, the mass transport rate of the active chains is accounted for as a function of the total rate of radical production by initiation: given the large affinity of the polymer chains for the dispersed phase, such transport is considered irreversible, from the solvent to the particles.

6. The volumes of the two phases are evaluated applying the volume additivity rule as the sum of the molar volumes of all species times the corresponding number of moles. In terms of concentrations, the following two equations apply:
\[
\frac{[A^1] \cdot MW_A}{\rho_A} + \frac{[B^1] \cdot MW_B}{\rho_B} + \frac{[Sol^1] \cdot MW_{Sol}}{\rho_{Sol}} + \frac{[I^1] \cdot MW_I}{\rho_I} = 1
\]  
(3.7)

\[
\frac{[P^2] \cdot MW_p}{\rho_p} + \frac{[A^2] \cdot MW_A}{\rho_A} + \frac{[B^2] \cdot MW_B}{\rho_B} + \frac{[Sol^2] \cdot MW_{Sol}}{\rho_{Sol}} + \frac{[I^2] \cdot MW_I}{\rho_I} = 1
\]  
(3.8)

where the meaning of all variables is provided in the section Symbols.

7. The free-radical kinetic scheme shown in Table 3.2 has been considered in both phases. Note that three indices are used for the generic polymer chain: the superscript \( j \) indicates the phase (\( j = 1 \) for the continuous phase and \( j = 2 \) for the dispersed one) and the two subscripts \( m \) and \( n \) the number of monomer units of type A and B, respectively. According to the experimental evidences reported above, a cross-linking reaction is likely to occur in the dispersed phase. This reaction allows the rebirth of dead polymer chains, which restart propagating and enlarging their size, without changing the total number of active chains in the system. Accordingly, the crosslinking reaction does not affect the reaction kinetics but only the molecular weight distribution of the final product. Given the complexity of the system only termination by combination and disproportionation mechanisms have been arbitrarily considered, while chain transfer to monomer has been neglected. However, such termination mechanism could be easily introduced in the model as soon as experimental evidences become available.
Table 3.2. Kinetic scheme for radical copolymerization of VI (A) and VP (B) as applied to both the continuous (j=1) and the dispersed phase (j=2).

<table>
<thead>
<tr>
<th>Type of Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator decomposition</td>
</tr>
<tr>
<td>( I^j \xrightarrow{k_{i,j}} 2I^{*j} )</td>
</tr>
<tr>
<td>Chain initiation</td>
</tr>
<tr>
<td>( I^{*j} + A^j \xrightarrow{k_{i,j}} A^{*j} )</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + A^j \xrightarrow{k</em>{ii,j}} A^{*j}_{m+1,n} )</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + B^j \xrightarrow{k</em>{ii,j}} B^{*j}_{m,n+1} )</td>
</tr>
<tr>
<td>Propagation</td>
</tr>
<tr>
<td>( B^{*j}<em>{m,n} + A^j \xrightarrow{k</em>{ii,j}} A^{*j}_{m+1,n} )</td>
</tr>
<tr>
<td>( B^{*j}<em>{m,n} + B^j \xrightarrow{k</em>{ii,j}} B^{*j}_{m,n+1} )</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + A^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m+r,n+q}_{j} )</td>
</tr>
<tr>
<td>Termination by combination</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + B^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m+r,n+q}_{j} )</td>
</tr>
<tr>
<td>( B^{*j}<em>{m,n} + B^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m+r,n+q}_{j} )</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + A^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m,n}<em>{j} + P^{p,q}</em>{j} )</td>
</tr>
<tr>
<td>Termination by disproportionation</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + B^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m,n}<em>{j} + P^{p,q}</em>{j} )</td>
</tr>
<tr>
<td>( B^{*j}<em>{m,n} + B^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} P^{m,n}<em>{j} + P^{p,q}</em>{j} )</td>
</tr>
<tr>
<td>( A^{*j}<em>{m,n} + P^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} A^{*j}_{m+r,n+q} )</td>
</tr>
<tr>
<td>Cross-linking</td>
</tr>
<tr>
<td>( B^{*j}<em>{2m,n} + P^{*j}</em>{r,q} \xrightarrow{k_{ct,j}} A^{*j}_{2m+r,n+q} )</td>
</tr>
</tbody>
</table>

Mass balance equations

By applying the long chain approximation,\textsuperscript{118,119} the mass balances can be expressed with the same formalism of a homopolymerization by introducing appropriate pseudo-kinetic rate constants designated by an asterisk in the following. The latter involve the kinetic parameters for the two homopolymers, the cross propagation and termination rate...
constants and the probability of having a radical with terminal unit VI \( (p_A = \frac{[A^*][R^*]}{[R^*]} ) \)

or VP \( (p_B = \frac{[B^*][R^*]}{[R^*]} ) \).

\[
k_{pA}^j = k_{pA}^j \cdot p_A^j + k_{pBA}^j \cdot p_B^j
\]

\[
k_{pB}^j = k_{pB}^j \cdot p_B^j + k_{pAB}^j \cdot p_A^j
\]

\[
\frac{k_p^j \cdot [M^j]}{k_p^j \cdot [A^j] + k_{pA}^j \cdot [B^j]} = k_{pA}^j \cdot p_A^j + k_{pBA}^j \cdot p_B^j
\]

\[
(k_{id}^j + k_{ic}^j) = (k_{idAA}^j + k_{icAA}^j) \cdot (p_A^j)^2 + 2 \cdot (k_{idAB}^j + k_{icAB}^j) \cdot p_B^j \cdot p_A^j + (k_{idB}^j + k_{icB}^j) \cdot (p_B^j)^2
\]

The probabilities of each radical terminal unit are given by:

\[
p_A = \frac{k_{pA} [A]}{k_{pA} [B] + k_{pBA} [A]}
\]

\[
p_B = \frac{k_{pB} [B]}{k_{pB} [B] + k_{pBA} [A]}
\]

The variation of the total radical concentration in time \((R^*)\) is related to the initiation and termination reactions and to the mass transfer term. The latter has been expressed in lumped form as the fraction \(\alpha \) of the total radicals produced by initiation, thus summarizing the relevance of the radical mass transfer in the system into a single, adjustable parameter.

In case of \(\alpha = 1\), all radicals produced by initiation in the continuous phase are transferred to the dispersed phase before termination. Vice versa, \(\alpha = 0\) corresponds to the case of complete phase segregation. Since the life-time of an active chain is only a very small fraction of the characteristic time of the process, the approximation of quasi-steady state (QSSA) can be applied. The resulting mass balances for the total active chain concentration in the two phases are given by:

\[
\frac{d}{dt} \left( v^1 \cdot [R^*] \right) = R_i^1 - \left( k_{id}^1 + k_{ic}^1 \right) \cdot [R^*] \cdot \alpha \cdot R_i^1 = 0
\]

\[
\frac{d}{dt} \left( v^2 \cdot [R^*] \right) = R_i^2 - \left( k_{id}^2 + k_{ic}^2 \right) \cdot [R^*] \cdot \alpha \cdot R_i^2 = 0
\]

On the other hand, the concentration of monomers, initiator and solvent is evaluated coupling the corresponding total mass balances to the equilibrium condition in terms of
partition coefficients \((K_i)\). Thus, the following equations apply to VI, VP, initiator, solvent and polymer:

\[
\begin{align*}
\frac{dA}{dt} &= \frac{d}{dt}(V^1 \cdot [A^1] + V^2 \cdot [A^2]) = -\sum_{j=1}^{2} \left( (k^c_{pA} \cdot [R^i_j] + k_{dA} \cdot [I^i_j]) \cdot [A^j] \right) \cdot V^j \\
\left(\frac{[A^j]}{[A^2]}\right) &= K_A 
\end{align*}
\tag{3.17}
\]

\[
\begin{align*}
\frac{dB}{dt} &= \frac{d}{dt}(V^1 \cdot [B^1] + V^2 \cdot [B^2]) = -\sum_{j=1}^{2} \left( (k^c_{pB} \cdot [R^i_j] + k_{dB} \cdot [I^i_j]) \cdot [B^j] \right) \cdot V^j \\
\left(\frac{[B^j]}{[B^2]}\right) &= K_B 
\end{align*}
\tag{3.18}
\]

\[
\begin{align*}
\frac{dI}{dt} &= \frac{d}{dt}(V^1 \cdot [I^1] + V^2 \cdot [I^2]) = -\sum_{j=1}^{2} R^i_j \cdot V^j = -\sum_{j=1}^{2} 2 \cdot f \cdot k^d_{d} \cdot [I^j] \cdot V^j \\
\left(\frac{[I^j]}{[I^2]}\right) &= K_I 
\end{align*}
\tag{3.19}
\]

\[
\begin{align*}
\frac{dS}{dt} &= \frac{d}{dt}(V^1 \cdot [S^1] + V^2 \cdot [S^2]) = 0 \\
\left(\frac{[S^j]}{[S^2]}\right) &= K_S 
\end{align*}
\tag{3.20}
\]

\[
\begin{align*}
\frac{dP^2}{dt} &= \frac{d}{dt}(V^2 \cdot [P^2]) = \sum_{j=1}^{2} k^p_{F} \cdot [M^j] \cdot [R^i_j] \cdot V^j 
\end{align*}
\tag{3.21}
\]

Thus summarizing, the reduced model is made of 13 equations ((7), (8), (16)-(21)), with 13 unknowns \((V^1, V^2, [A^1], [A^2], [B^1], [B^2], [I^1], [I^2], [S^1], [S^2], [R^i], [R^s], [P^2])\). The resulting mixed system of ordinary differential and algebraic equations has been implemented and solved in Matlab©, using an iterative method for the algebraic part and the solver ODE23s for the differential part.
Parameter evaluation

The model presented above includes several physics-chemical parameters: kinetic parameters (i.e., propagation, termination and initiation rate coefficients, monomer reactivity ratios) for both phases, thermodynamic parameters (i.e., partition coefficients of all the species) and the empirical parameter $\alpha$, describing the interphase transport rate of growing radical chains. Due to the lack of literature studies on the copolymerization of VI and VP, a significant effort was needed to get reliable and a priori estimates of such parameters.

According to the study of Chapiro et al.\textsuperscript{92,93,101} on VI polymerization in bulk and in different solvents, the VI reactivity changes in different solvents following the ranking: alcoholic solvent $>$ organic solvent $\geq$ bulk. However, in all cases the differences of reaction rate are quite small and never exceed a factor two. Therefore, the kinetic parameters of VI were estimated from ad-hoc solution polymerization experiments and then applied to precipitation polymerization. According to the experimental evidences, the propagation rate constant in organic solvent was set slightly smaller than the value in alcoholic solvent ($1.4 \times 10^6$ cm$^3$/mol/s versus $2 \times 10^6$ cm$^3$/mol/s), and the same value was assumed for both the continuous and the polymer phase.

The termination rate constant was assumed independent upon conversion and the reported value for the continuous phase (see Table 3.3) was estimated by direct fitting to the experimental data of number average molecular weight measured in the solution polymerization experiments mentioned above. The termination rate constant in the dispersed phase is expected to be strongly affected by diffusion limitations, which reduce the probability of collision between two active chains. Therefore, larger molecular weights are obtained. The termination rate constant for the dispersed phase (reported in Table 3.3) has been evaluated by fitting to the experimental data of number average molecular weight obtained in precipitation polymerization, assuming most of the polymer produced in the dispersed phase. It is worth noticing that according to this procedure the estimated rate constants are affected by the calibration considered in the experimental measurements of the molecular weights.
### Table 3.3: Model parameter values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator decomposition, $k_d^1, k_d^2$</td>
<td>$5.28 \times 10^{-4}$</td>
<td>1/s</td>
<td>Akzo Nobel, NE</td>
</tr>
<tr>
<td>Efficiency factor, $f^1, f^2$</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI propagation rate constant for the continuous phase, $k_{pAA}^1$</td>
<td>$1.4 \times 10^6$</td>
<td>cm$^3$/mol/s</td>
<td>Solution polymerization</td>
</tr>
<tr>
<td>VI disproportionation termination rate constant for the continuous phase, $k_{tdAA}^1$</td>
<td>$7.0 \times 10^9$</td>
<td>cm$^3$/mol/s</td>
<td>Solution polymerization</td>
</tr>
<tr>
<td>VI combination termination rate constant for the continuous phase, $k_{tcAA}^1$</td>
<td>$1.0 \times 10^{11}$</td>
<td>cm$^3$/mol/s</td>
<td>Solution polymerization</td>
</tr>
<tr>
<td>VI partition coefficient, $K_A$</td>
<td>0.30</td>
<td>-</td>
<td>BASF, DE</td>
</tr>
<tr>
<td>VP partition coefficient, $K_B$</td>
<td>0.19</td>
<td>-</td>
<td>BASF, DE</td>
</tr>
<tr>
<td>Solvent partition coefficient, $K_S$</td>
<td>1000</td>
<td>-</td>
<td>Fitting</td>
</tr>
<tr>
<td>VI propagation rate constant for the dispersed phase, $k_{pAA}^2$</td>
<td>$1.4 \times 10^6$</td>
<td>cm$^3$/mol/s</td>
<td>Same as continuous phase</td>
</tr>
<tr>
<td>VI termination rate constant for the dispersed phase, $k_{tcAA}^2$</td>
<td>$1 \times 10^9$</td>
<td>cm$^3$/mol/s</td>
<td>Fitting (MW data)</td>
</tr>
<tr>
<td>VP propagation rate constant for the dispersed phase, $k_{pBB}^2$</td>
<td>$4.4 \times 10^6$</td>
<td>cm$^3$/mol/s</td>
<td>Literature$^{97}$</td>
</tr>
<tr>
<td>VP propagation rate constant for the continuous phase, $k_{pBB}^1$</td>
<td>$4.4 \times 10^6$</td>
<td>cm$^3$/mol/s</td>
<td>Same as dispersed phase</td>
</tr>
<tr>
<td>VP termination rate constant for the continuous phase, $k_{tBB}^1$</td>
<td>$1 \times 10^{11}$</td>
<td>cm$^3$/mol/s</td>
<td>Same as VI value</td>
</tr>
<tr>
<td>VP termination rate constant for the dispersed phase, $k_{tBB}^2$</td>
<td>$1 \times 10^9$</td>
<td>cm$^3$/mol/s</td>
<td>Same as VI value</td>
</tr>
<tr>
<td>VI reactivity ratio for continuous and dispersed phase, $r_{VI}$</td>
<td>1.85</td>
<td>-</td>
<td>Fitting</td>
</tr>
<tr>
<td>VP reactivity ratio for continuous and dispersed phase, $r_{VP}$</td>
<td>0.9</td>
<td>-</td>
<td>Fitting</td>
</tr>
</tbody>
</table>
Compared to VI, more data are available in the literature for VP homopolymerization. Several authors investigated the effect of the solvent on VP polymerization,\textsuperscript{90,97} and reported the reactivity in water to be larger than in organic solvents. The propagation rate constants have been reported in the literature for bulk and solution polymerization in water by Stach et al.\textsuperscript{97} The propagation rate constants both for the dispersed and the continuous phase were assumed equal to the values in bulk (see Table 3.3). Moreover, the same values of termination rate constants used for VI were applied considering that they both are strongly diffusion controlled.

Few data of monomer reactivity ratios are reported in the literature for bulk copolymerization: Petrak et al.\textsuperscript{104} reported $r_{VI} = 0.95$ and $r_{VP} = 0.17$, while Martinez-Piña et al.\textsuperscript{89} $r_{VI} = 0.07$ and $r_{VP} = 1$. These values are definitely too different. Therefore, as an alternative, the prediction of such parameters was attempted using the Q-e theory.\textsuperscript{120} Accordingly, the monomer reactivity ratios of two reacting monomers 1 and 2 are evaluated as:

\begin{align}
    r_{12} &= \left( \frac{Q_1}{Q_2} \right) \exp \left[ -e_1 \left( e_2 - e_1 \right) \right] \quad (3.22) \\
    r_{21} &= \left( \frac{Q_2}{Q_1} \right) \exp \left[ -e_2 \left( e_2 - e_1 \right) \right] \quad (3.23)
\end{align}

where the parameter $Q$ is related to the monomer reactivity and the parameter $e$ to the permanent electric charge on the monomer. Values of $Q$ and $e$ are published in literature for many systems. The Q-e values for VI were obtained from Petrak et al.\textsuperscript{104} while the Q-e values for VP from the Polymer Handbook.\textsuperscript{121} The estimated values $r_{VI} = 2.2$ and $r_{VP} = 0.19$ are significantly different from both sets of data reported in the literature.

Therefore, to achieve a more reliable parameter evaluation, the monomer reactivity ratios were estimated from our own experimental data as described in the following. In the precipitation process the experimental curve of copolymer composition versus time exhibits a composition drift at all feed compositions. Since at the beginning of the process most of the polymer is produced in the solvent-rich phase, the value of the copolymer composition at time zero represents the composition of the copolymer instantaneously produced in the continuous phase. Therefore, the copolymer compositions at time-zero ($F_{VI}$) have been reported as a function of the feed composition in the Mayo-Lewis plot in
Figure 3.16 and the monomer reactivity ratios for the continuous phase were evaluated by fitting such experimental data. The estimated values are $r_{VI} = 1.85$ and $r_{VP} = 0.9$, respectively.

![Figure 3.16](image1.png)

**Figure 3.16.** Instantaneous copolymer composition measured at time zero ($F_{vi}$) as a function of the monomer feed composition ($X_{vi}$). Experimental data (○); calculated curve (—).

![Figure 3.17](image2.png)

**Figure 3.17.** Mayo-Lewis plot obtained with monomer reactivity ratio values from different sources: Petrak et al.\textsuperscript{104} (—•—); Martinez-Piña et al.\textsuperscript{89} (····); Q-e theory (○○); current work (—).

Furthermore, it is found that in the composition range investigated in Figure 3.16 (VI fraction from 0.5 to 1) the estimated $F_{vi}$ values are very close to the ones evaluated with
the Q-e theory and not far from those reported by Petrak et al.,\textsuperscript{104} as shown in Figure 3.17. Therefore, the parameter values estimated using our own experimental results have been used in the rest of the work. Since for both monomers we assumed equal reactivity in the two phases, it’s reasonable to apply the same assumption to the monomer reactivity ratios and the same values estimated in solution have been applied to both phases – continuous and dispersed.

The decomposition rate constant of the initiator was provided by Akzo Nobel (Amsterdam, NE) and the efficiency factor was kept equal to the one estimated in solution polymerization.

The monomer partition coefficients were evaluated from independent experiments measuring the monomer amount at equilibrium between the two phases, solvent-rich and polymer-rich. After the system equilibration, the mixture of polymer, solvent and monomer was filtrated to separate the two phases, the polymer phase was washed with the solvent and the filtrate collected. The monomer amount in the two filtrates was measured by GC and the monomer amount in the dispersed phase was estimated by subtracting this value to the known initial amount of monomer. In order to cross-check the estimated value, a second type of experiments was carried out, in which samples were analyzed by GC without separating the two phases. Therefore, different assumptions were applied in the two cases: in the first case it was assumed that all the monomer not in polymer phase was collected in the filtrate, while in the second it was assumed that solvent and polymer are fully incompatible, thus neglecting the solubility of the solvent in the polymer phase. Both methods gave similar results, thus confirming the general reliability of the obtained values. The estimated partition coefficients are reported in Table 3.4: even though some composition effect is present, average constant values have been used at all compositions as reported in Table 3.3.

The solvent partition coefficient was assumed in large favor of the continuous phase in order to reproduce its limited solubility into the polymer.
Table 3.4. Experimental values of monomer partition coefficients. \( K \) is expressed as the ratio of the mass fraction in the continuous phase to the mass fraction in the disperse phase.

\[
\begin{align*}
\text{VI in PVI} \\
K_d &= 0.3
\end{align*}
\]

\[
\begin{align*}
\text{VI in 50-50 VI-co-VP} \\
\text{In absence of VP} & \quad \text{In presence of VP} \\
K_d &= 0.25 & K_d &= 0.31
\end{align*}
\]

\[
\begin{align*}
\text{VP in 50-50 VI-co-VP} \\
\text{In absence of VI} & \quad \text{In presence of VI} \\
K_b &= 0.38 & K_b &= 0.19
\end{align*}
\]

Table 3.5. Summary of all considered experimental runs. Bth=Butanol, BA=Butyl-acetate.

<table>
<thead>
<tr>
<th>Run</th>
<th>Total monomer concentration (%)</th>
<th>Monomer feed composition VI/VP</th>
<th>Initiator (% referred to the monomer)</th>
<th>Stirring rate (rpm)</th>
<th>Temperature (ºC)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
<td>100</td>
<td>Bth</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>75/25</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>50/50</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>90/10</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>75/25</td>
<td>1.2</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>75/25</td>
<td>0.3</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>7</td>
<td>7.5</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>100/0</td>
<td>0.6</td>
<td>200</td>
<td>90</td>
<td>BA</td>
</tr>
</tbody>
</table>

A summary of all model parameter values is reported in Table 3.3. Note that the fraction of initiated radicals transported to the dispersed phase (\( \alpha \)) and the initiator partition coefficient are the only two parameters which have been fitted so as to reproduce the
experimental data. It is worth noting that these cover a quite large range of operating conditions as it can be seen in Table 3.5 where all the experiments used for such a comparison are listed. This is to support the reliability of the model and of the corresponding parameter values. A detailed discussion of the comparison between experimental data and model simulations is reported in the sequel.

VI Solution homopolymerization

The model was initially validated in the case of VI homopolymerization in butanol, in order to test the corresponding model parameter values for the continuous phase. The model equations described above were solved for one single phase, thus setting to zero all terms related to radical mass transport. The comparison between model simulations and experimental data of conversion is shown in Figure 3.18. The simulated residual amounts of monomer and initiator are also reported. The model reproduces the reaction kinetics with accuracy, predicting also a limiting conversion below 80% due to the initiator depletion.

Figure 3.18. VI homopolymerization in butanol at 20% VI concentration (run 1 in Table 3.5). % conversion: experimental data (○), model simulation (—); % residual VI (—); % residual initiator (····).
Interphase transport of radical species

In order to understand the relevance of the radical interphase transport in the system and to identify the contribution of each reaction locus, we performed a parametric analysis of the model with respect to the parameter $\alpha$ and the initiator partition coefficient $K_I$ in terms of conversion and copolymer composition. Since the two monomers have the same reactivity ratios in the two phases but different partition coefficients, the composition drift reflects the shift of the reaction locus during the reaction. Such shift can be explained in two different ways: 1) because of mass transport of active chains from the continuous to the dispersed phase; 2) because of large solubility of the initiator in the dispersed phase, thus shifting the reaction locus even in absence of mass transport. In order to identify which one of the two cases applies, the model behavior was investigated in four cases, corresponding to limiting values of the two adjustable parameters: complete segregation ($\alpha = 0$) and extremely fast inter-phase transport ($\alpha = 1$) of the radicals with initiator partitioning largely in favor of continuous ($K_I = 1000$) or dispersed phase ($K_I = 0.01$). In Figure 3.19, the comparisons between experimental data and model simulations in terms of conversion and cumulative composition are shown for the reference copolymerization reaction VI/VP 75/25. The model predicts satisfactorily the copolymer composition drift only in the case $\alpha = 0$ and $K_I = 0.01$, i.e. complete segregation and initiator partitioning largely in favor of the dispersed phase.

In Figure 3.20 the predicted amount of polymer produced in the dispersed phase with $K_I = 0.01$ is shown for the two limiting $\alpha$ values. In the case of very fast radical transport ($\alpha = 1$), all chains are growing in the dispersed phase from the very beginning of the reaction. On the contrary, the reaction starts in the continuous phase at $\alpha = 0$ (complete segregation) and progressively shifts to the dispersed phase. Such shift of the reaction locus is explained by the partitioning of monomers and initiator which is largely in favor of the polymer-rich phase. At about 20% of conversion the reaction locus is mainly represented by the dispersed phase, but at least 10% of the polymer is still formed in the solvent-rich phase at the end of the process. It is apparent that both phases contribute significantly to the polymerization process although to different extent which changes in time.
Figure 3.19. Conversion versus time for $K_f=0.01$ (a) and $K_f=100$ (c), cumulative composition versus conversion for $K_f=0.01$ (b) and $K_f=100$ (d). Experimental data (○), model simulations in the two limiting cases $α=1$ (─) and $α=0$ (--) . Reaction VI/VP 75/25 (run 2 in Table 3.5).

Figure 3.20. Wt.-% of polymer produced in the dispersed phase as a function of conversion. Model simulations in the two limiting cases $α=1$ (─) and $α=0$ (--) . Reaction VI/VP 75/25 (run 2 in Table 3.5).
As final check of the reliability of such conclusion, the value of the mass transfer coefficient has been estimated so as to verify that the corresponding rate of radical mass transport is negligible with respect to that of termination.

In general, the irreversible rate of interphase transfer of the active chains can be expressed as follows:

\[
    r_{\text{transport}} = k_m \cdot \frac{A_{\text{int}}}{V_i} \left[ R^{*i} \right] \tag{3.24}
\]

where \( k_m \) is the overall mass transport coefficient, \( A_{\text{int}} \) the interphase area, \( V_i \) the volume of phase \( i \) and \( R^{*i} \) the total radical concentration in the continuous phase. The overall transport coefficient is related to the diffusion coefficient \( (D_m) \) as follows:

\[
    k_m = \frac{D_m}{\delta} \tag{3.25}
\]

where \( \delta \) is the characteristic length of the corresponding transport process.

Clusters of primary particles of about 10 \( \mu \)m have been observed already after few percent of conversion by light scattering measurements; such clusters form larger aggregates of about 100 \( \mu \)m (so called “grains”) at larger conversion values. From preliminary computations it is easy to see that the rate determining step for the radical transport in the particle phase is the mass transfer in the clusters. Considering the characteristic length of diffusion, \( \delta \), equal to the radius of the particle cluster and the diffusion coefficient in the dispersed phase, \( D_m \approx 10^{-8} \text{ cm}^2\text{s}^{-1} \), Equation (3.25) leads to \( k_m = 2 \cdot 10^{-5} \text{ cm/s} \). This leads to a characteristic time for radical transport, \( t_d = \left( k_m N_p s_p \right)^{-1} \approx 50 \text{ s} \), where \( N_p \) is the number of clusters per unit of volume and \( s_p \) is the cluster surface. Such value can be compared to the characteristic time for termination, \( t_t = (k_t R)^{-1} \approx 0.3 \text{ s} \). The ratio between the two yields on \( \Omega \approx 5 \cdot 10^{-3} \), which is significantly smaller than one. This implies that the aggregation process occurring since the beginning of the reaction reduces significantly the interphase area and, consequently, the chain interphase transport from the continuous to the dispersed phase. Therefore, the radicals formed in the solution terminate in the same phase, i.e. the two phases are separated (with respect to radical exchange). This conclusion is
agreement with the experimental evidences reported above: by changing the stirring rate in the reactor, different particle (or cluster) size distributions are obtained but the reaction rate in each phase stays constant. Therefore, even though different values of interfacial areas are established, the interphase transport is anyway so small that radicals are not exchanged between the phases and thus the reaction rate in each phase is not affected. This corresponds to the limiting case of complete phase segregation, i.e. $\alpha = 0$.

Following the previous arguments, a set of predictive simulations was carried out assuming complete segregation and the parameter values evaluated for the copolymer reaction 75/25, without any further adjustment of the model parameter values. In the following, the comparisons between model predictions and experimental data are reported at different copolymer compositions (Figure 3.21), initiator amounts (Figure 3.22) and monomer concentrations (Figure 3.23). The agreement is satisfactory in all cases thus strongly supporting the reliability of the developed model. However, it is worth noticing that the analysis of the transport rate was based mainly on the transition of the copolymer composition. More experimental evidences would be helpful to further validate the mechanistic picture here proposed.

**Figure 3.21.** Conversion versus time for several VI/VP feed composition. Simulations: 90/10 (···), 75/25 (--), 50/50 (—). Experimental data: 90/10 (Δ), 75/25 (○), 50/50 (□) (run 4, 2 and 3 in Table 3.5).
3.3.2 Comprehensive model: molecular weight distribution

In order to simulate the molecular weight distribution (MWD) of the product, the reduced model developed in the previous sections was augmented by considering the population balance equations (PBEs) of active and terminated chains in both phases. Once more, the QSSA is applied to all active species. It is to be noted that since the crosslinking reaction does not affect the polymerization reaction but only the MWD, its corresponding rate
constant $k_{cr^*}$ appears only in this augmented version of the kinetic model and its value will have to be fitted by comparison with MWD experimental data. The PBEs for the generic chain of length $n$ in each phase are as follows:

$$\frac{d}{dt} \left( R_{n}^{\ast} \right) = -k_{p} \left[ M^{1} \right] \left[ R_{n}^{\ast} \right] + k_{p} \left[ M^{2} \right] \left[ R_{n-1}^{\ast} \right] - \left( k_{d} + k_{c} \right) \left[ R_{n}^{\ast} \right]\sum_{j=1}^{n} \left[ R_{j}^{\ast} \right] = 0 \quad (3.26)$$

$$\frac{d}{dt} \left( P_{n}^{\ast} \right) = \frac{1}{2} k_{p} \left[ M^{1} \right] \left[ R_{n}^{\ast} \right] + k_{p} \left[ M^{2} \right] \left[ R_{n-1}^{\ast} \right] - \left( k_{d} + k_{c} \right) \left[ R_{n}^{\ast} \right]\sum_{j=1}^{n} \left[ R_{j}^{\ast} \right] \quad (3.27)$$

$$\frac{d}{dt} \left( R_{n}^{*2} \right) = -k_{p} \left[ M^{1} \right] \left[ R_{n}^{*2} \right] + k_{p} \left[ M^{2} \right] \left[ R_{n-1}^{*2} \right] - \left( k_{d} + k_{c} \right) \left[ R_{n}^{*2} \right]\sum_{j=1}^{n} \left[ R_{j}^{*2} \right]$$

$$\frac{d}{dt} \left( P_{n}^{*2} \right) = \frac{1}{2} k_{p} \left[ M^{1} \right] \left[ R_{n}^{*2} \right] + k_{p} \left[ M^{2} \right] \left[ R_{n-1}^{*2} \right] - \left( k_{d} + k_{c} \right) \left[ R_{n}^{*2} \right]\sum_{j=1}^{n} \left[ R_{j}^{*2} \right] \quad (3.28)$$

$$\frac{d}{dt} \left( D_{n}^{*2} \right) = \frac{1}{2} k_{p} \left[ M^{1} \right] \left[ R_{n}^{*2} \right] + k_{p} \left[ M^{2} \right] \left[ R_{n-1}^{*2} \right] - \left( k_{d} + k_{c} \right) \left[ R_{n}^{*2} \right]\sum_{j=1}^{n} \left[ R_{j}^{*2} \right] - k_{cr^*} n \left[ P_{n}^{*2} \right]\sum_{j=1}^{n} \left[ R_{j}^{*2} \right] \quad (3.29)$$

Such equations have been solved for all chain lengths, from $n = 1$ to a large enough maximum value ($n=3\times10^5$). This way, the complete cumulated distribution of chain length is calculated at any time and in each phase. From such distributions the first three order moments are calculated and from these the relevant cumulative average properties have been evaluated for each phase:

$$\bar{n}_{N} = \frac{\mu_{1}}{\mu_{0}} \quad (3.30)$$

$$\bar{n}_{W} = \frac{\mu_{2}}{\mu_{1}} \quad (3.31)$$

$$PD = \frac{\mu_{2} \cdot \mu_{0}}{\left( \mu_{1} \right)^{2}} \quad (3.32)$$

To calculate the so-called number and weight average molecular weights ($\overline{MW_{N}}$ and $\overline{MW_{W}}$), an average molecular weight of the repeating unit is needed. For a copolymer, the molecular weight of the repeating unit is a function of the chain composition and, therefore, of time. This was computed for each phase using the corresponding current copolymer composition as follows:
\[
\overline{MW}_{\text{MON}} = MW_{i_1} \cdot y_{i_1} + MW_{i_P} \cdot y_{i_P}
\]  
(3.33)

\[
\overline{MW}_N = n_N \cdot \overline{MW}_{\text{MON}}
\]  
(3.34)

\[
\overline{MW}_w = n_w \cdot \overline{MW}_{\text{MON}}
\]  
(3.35)

The selected numerical method for solving the PBE (direct integration of the mass balances for each individual chain length) asks for a large computational effort, and it becomes quickly unfeasible if long chains and fast kinetics are present in the system. In order to reduce the computational effort, the integration of a limited number of differential equations, corresponding to a finite number of moments, has been performed. The equations for the moments of zero, first, and second order for both phases and for both active and dead polymer chains are reported in the Appendix A. The complete chain length distribution is finally estimated from such moments assuming a suitable model distribution, such as the perturbed Gamma distribution:\textsuperscript{122}

\[
f(n) = \frac{\lambda}{(\lambda - 1)!} \cdot \frac{\mu_0}{a} \cdot \left(\frac{\lambda n}{a}\right)^{\lambda-1} \cdot \exp\left(-\frac{\lambda n}{a}\right)
\]  
(3.36)

The parameters of the selected model distribution, \(\lambda\) and \(a\), are expressed as a function of the calculated moments as follows:

\[
a = \frac{\mu_0}{\mu_0}
\]  
(3.37)

\[
\lambda = \frac{a^2}{\mu_2 / \mu_0 - a^2}
\]  
(3.38)

Such reconstruction of the molecular weight distribution is reliable provided that the real distribution conforms to the selected model distribution and, therefore, it is reasonably estimated using the first three moments only. The reliability of the reconstructed distributions was checked by comparing them to the distributions evaluated by solving the complete set of differential equations. The agreement was always very good and the latter approach was therefore adopted.

In order to better understand the system behavior we can, using the model, estimate separately the polymer produced in the continuous phase and the one produced in the
dispersed phase. Of course, the overall molecular weight distribution is the sum of the two distributions:

\[ f_n(n, t) = \sum_{j=1}^{2} f_n^j(n, t) \]  

(3.39)

where, as usual, subscript \( j \) indicates the phase.

The rate of the crosslinking reaction is proportional to the total mass of dead chains, and therefore, the large polymer concentration in the dispersed-phase favors the occurrence of such reaction. Through crosslinking, dead polymer chains are re-activated with a correspondingly large increase of their size until gel formation. As a consequence, the resulting MWD becomes very broad, with a significant fraction of high MW polymer. In order to evaluate the heterogeneous MWD the equations of the first three order moments of the dispersed phase have been solved applying the numerical procedure described in Appendix A.

**Figure 3.24.** Molecular weight distribution (MWD) of the final polymer of the reference reaction VI/VP 75/25 (run 2 in Table 3.5): simulated continuous phase (---), simulated dispersed phase (---), simulated overall (---), experimental data (···).

The crosslinking rate constant \( k_{cr}^* \), which is at this point the only still unknown model parameter, has been evaluated by fitting the model predictions to the experimental MWD data (\( k_{cr}^* = 30 \text{ cm}^3/\text{mol/s} \)). An example of such a comparison is shown in Figure 3.24. The experimental data correspond to the final copolymer of reaction VI/VP 75/25. With regard
to the simulated distribution, the contribution of the two phases has been distinguished: polymer chains at high MW are mainly produced in the dispersed phase, while a smaller fraction of polymer at lower MW is formed in the continuous phase, which however significantly contributes to the broadening of the overall MWD. As discussed in the experimental part, the experimentally observed shoulder at high MW is induced by a cut-off of the GPC column and, therefore, is not reliable. The “true” distribution should be characterized by a more regular tailing at high MW or, at least, by a slighter shoulder. Keeping in mind this experimental limitation, the agreement in Figure 9 and the corresponding $k_{cr}*$ values were accepted. The broad MWD corresponds to quite large values of the polydispersity, PD, and final values as large as 3.5 are computed by the model as shown in Figure 3.25. It is seen that the major contribution is given by the dispersed phase, where the crosslinking reaction occurs. This reaction is proportional to the concentration of total polymer, and the crosslinking rate increases at increasing conversion. As a consequence, also the fraction of polymer with large MW and PD values increases in time. The evolution of the MWD in time is shown in Figure 3.26, where the comparison between simulated and experimental MWD for the reaction at different conversions is shown. Very similar behavior was observed both experimentally and through model calculations for reactions at different feed composition (homopolymer VI, VI/VP 60/40).

![Figure 3.25](image-url)

**Figure 3.25.** PD versus time for the reference reaction VI/VP 75/25 (run 2 in Table 3.5): simulated continuous phase (···), simulated dispersed phase (--), simulated overall (−).
Figure 3.26. Simulated (a) and experimental (b) molecular weight distribution (MWD) of the reference reaction VI/VP 75/25 (run 2 in Table 3.5) at different conversion values: 60% (⋯), 77% (−), 90% (−).

Unlikely weight average molecular weights ($\overline{MW_w}$) and PD values, the measured number average molecular weights ($\overline{MW_N}$) are only slightly affected by the experimental limitation in the high MW region mentioned above. Accordingly, these experimental values can be considered for a quantitative comparison to model simulations. An example of such a comparison for two different copolymer compositions is shown in Figure 3.27. The model correctly predicts the effect of the copolymer composition on the evolution of $\overline{MW_N}$ in time: larger $\overline{MW_N}$ are obtained by increasing the VP feed fraction.

Figure 3.27. Number average molecular weight ($\overline{MW_N}$) versus time. VI homopolymerization (run 8 in Table 3.5): experimental data (□) simulated curve (−); VI/VP 75/25 co-polymerization (run 2 in Table 3.5): experimental data (○) simulated curve (−).
3.4 Concluding remarks

In this work the precipitation copolymerization of vinylimidazole (VI) and vinylpyrrolidone (VP) in organic solvent, i.e. butyl acetate, was investigated. The effect of different operating conditions on the polymerization kinetics was analyzed. Copolymerizations with different initiator concentrations, different monomer hold ups, and different monomer feed compositions and different stirring rates were carried out. Conversion was measured by gas chromatography while molecular weight distributions (MWD) and number average molecular weight were obtained by gel permeation chromatography. The latter analysis showed broad MWD and large MW polymer chains formed by a crosslinking reaction. Evidences of formation of micro-gel were also reported.

It is found that the kinetics of the copolymerization reaction depends upon the monomer feed composition, and in particular the reaction rate increases at increasing VP content. Faster kinetics is obtained also increasing the initiator concentration and the monomer concentration. A composition drift was observed in all reactions, being the produced polymer richer in VI with respect to the feed composition at low conversion. Since VP is more reactive, such composition drift indicates that the cross propagation reaction between a polymer chain terminating with a VP unit and VI monomer is favored respect to all the other possible propagation reactions.

In parallel with the kinetic characterizations, the structure of the precipitated aggregates was analyzed by scanning electron microscopy and static light scattering (SLS). Pictures reveal quite compact clusters in which primary particles keep their identity during the entire process and even allow an estimate of their final size. Particle size distributions (PSD) of the polymer aggregates were measured by SLS. Bimodal PSDs were obtained, with one smaller peak at around 10 μm, corresponding to clusters of primary particles, and a second major peak in the 80 μm range, corresponding to grains containing several clusters. It has also been shown by SLS that at least the smaller clusters can be considered fractal objects with fractal dimension around 2.5, thus corresponding to compact aggregates in agreement with SEM pictures. The maximum peak, the ratio between the two modes and the kinetics of evolution of the PSD are related to aggregation and breakage.
processes occurring inside the reactor. The PSD depends on operative parameters like copolymer composition and stirring rate, which affect aggregate strength and aggregation/breakage coefficients, respectively. In particular, increasing the VP content the clusters of primary particles inside a grain partially coalesce and lose their identity. Therefore, the fragment size distribution resulting from a breakage event is more homogeneous and the bimodality in the final PSD is less marked.

It has been observed that the stirring rate influences the PSD but doesn’t affect the polymerization reaction rate. Therefore the kinetics of the polymerization reaction can be investigated independently of the stability behavior (i.e. aggregation/breakage processes) inside the examined range of reaction conditions. This implies that the polymerization rate is not affected by any mass transport process from one phase to another. This is in fact the case when interphase mass transport is either extremely fast, so as to guarantee interphase equilibrium conditions at all times, or completely absent. In order to discriminate between these two limiting situations, as well as to develop a detailed quantitative understanding of this system, a detailed kinetic model is developed using a two steps procedure.

First, a reduced version of the model was applied in order to elucidate the reaction locus and the relevance of mass transport of the active chains between the continuous and the dispersed phase. The latter was taken into account as a fraction $\alpha$ of the radical initiation rate in the continuous phase. The lumped parameter $\alpha$ was estimated performing a parametric analysis of the model against experimental data relative to the compositional drift as a function of conversion. Such analysis allows concluding that the system is composed by two fully segregated phases, being the mass transport of active chains negligible. The reaction starts in the continuous phase but since both monomers and initiator are much more soluble in the dispersed-phase, during growing of polymer particles the reaction locus progressively shifts to the dispersed phase.

It is concluded that the behavior of the system is dictated mainly by the dispersed phase (according to the simulations around 90% of the polymer is produced in the precipitated polymer particles) but the contribution of the continuous phase is significant, especially in the early stages of the reaction, and cannot be neglected for a reliable simulation of the process performance.
In a second step, the model was augmented by introducing the population balance equations for active and terminated polymer chains so as to describe molecular weight distributions (MWD) and average molecular weights. In this model a crosslinking reaction has been introduced in the kinetic scheme. Since most polymer chains are produced in the dispersed phase, the average MW is close to the MW characteristic of the dispersed phase. However, the final polymer contains about 10% of polymer produced in the continuous phase which is characterized by a significantly lower MW. The crosslinking reaction produces chains at large MW and can eventually induce gel formation. As a consequence of the heterogeneity of the system and of the crosslinking reaction, the resulting MWD is broad and the corresponding PD values are large. Even though experimental limitations were encountered in the analysis of the MWD in the large MW range, the model is shown to be able to describe the time evolution of the number average MW and of the complete MWD rather satisfactorily.

In conclusion, the developed comprehensive model represents a reliable tool for the simulation of the VI-VP precipitation polymerization process. The model reliability is based on the very wide range of operating conditions investigated, the very small number of fitted parameter values and the fact that the time evolutions of conversion, composition and MWD have been considered in the model validation procedure. The developed model and parameter estimation procedure is general and can be extended to other precipitation polymerization processes.

**Symbols**

Roman symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Total amount of VI in the system [mol]</td>
</tr>
<tr>
<td>$[A^+]$</td>
<td>Concentration of VI in phase $j$ [mol·cm$^{-3}$]</td>
</tr>
<tr>
<td>$A_{\text{int}}$</td>
<td>Inter-phase surface area between continuous and dispersed phase [cm$^2$]</td>
</tr>
<tr>
<td>$B$</td>
<td>Total amount of VP in the system [mol]</td>
</tr>
<tr>
<td>$[B^+]$</td>
<td>Concentration of VP in phase $j$ [mol·cm$^{-3}$]</td>
</tr>
<tr>
<td>$D_{\text{Diff},x}$</td>
<td>Diffusion coefficient of an active polymer chain of length $x$ [cm$^2$·s$^{-1}$]</td>
</tr>
</tbody>
</table>
\( f \)  
Efficiency factor in initiation rate [-]

\( I \)  
Total amount of initiator in the system [mol]

\([I']\)  
Concentration of initiator in phase \( j \) [mol·cm\(^{-3}\)]

\([I'^*]\)  
Concentration of initiator radical in phase \( j \) [mol·cm\(^{-3}\)]

\( K_i \)  
Thermodynamic partitioning coefficient of specie \( i \) [-]

\( k_{Al/j} \)  
VI chain initiation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{Bl/j} \)  
VP chain initiation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k^{*}_{cr} \)  
Cross-linking reaction rate constant [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_d^j \)  
Initiator decomposition rate constant in phase \( j \) [s\(^{-1}\)]

\( k_m \)  
Macro-radical transport rate coefficient [cm·s\(^{-1}\)]

\( k_{pAI}^{*j} \)  
VI pseudo-homogeneous propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pAI}^j \)  
VI propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pAB}^j \)  
VI cross propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pBI}^{*j} \)  
VP pseudo-homogeneous propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pBI}^j \)  
VP propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pBB}^j \)  
VP cross propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{pAI}^j \)  
VP propagation rate constant in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{c}^{*} \)  
Pseudo-homogeneous rate constant for termination by combination in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{c,AA}^j \)  
Rate constant for termination by combination between two radicals VI in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{c,AB}^j \)  
Rate constant for termination by combination between one radical VI and one radical VP in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{c,BB}^j \)  
Rate constant for termination by combination between two radicals VP in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{c,d}^{*} \)  
Pseudo-homogeneous rate constant for termination by disproportionation in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]

\( k_{d,AA}^j \)  
Rate constant for termination by disproportionation between two radicals VI in phase \( j \) [cm\(^3\)·mol\(^{-1}\)·s\(^{-1}\)]
$k_{AB}^{J}$ Rate constant for termination by disproportionation between one radical VI and one radical VP in phase $j$ [cm$^3$·mol$^{-1}$·s$^{-1}$]  

$k_{BB}^{J}$ Rate constant for termination by disproportionation between two radicals VP in phase $j$ [cm$^3$·mol$^{-1}$·s$^{-1}$]  

$m$ Monomer thermodynamic partitioning coefficient [-]  

$[M_i^J]$ Concentration of total monomer in phase $j$ [mol·cm$^{-3}$]  

$MW_i$ Molecular weight of specie $i$ [g·mol$^{-1}$]  

$MW_{MON}$ Average molecular weight of adding monomer unit [g·mol$^{-1}$]  

$MW_N$ Number average molecular weight [g·mol$^{-1}$]  

$MW_w$ Weight average molecular weight [g·mol$^{-1}$]  

$n_N$ Number average chain length [-]  

$n_w$ Weight average chain length [-]  

$[P^J]$ Total concentration of terminated chains in phase $j$ [mol·cm$^{-3}$]  

$[P_n^J]$ Concentration of terminated chains with length $n$ in phase $j$ [mol·cm$^{-3}$]  

$p_A^J$ Probability of finding a chain terminating with a VI unit in phase $j$ [-]  

$p_B^J$ Probability of finding a chain terminating with a VP unit in phase $j$ [-]  

$[R^J]$ Total concentration of active chains in phase $j$ [mol·cm$^{-3}$]  

$[R_n^J]$ Concentration of active chains with length $n$ in phase $j$ [mol·cm$^{-3}$]  

$r_A^J$ VI reactivity ratio [-]  

$r_B^J$ VP reactivity ratio [-]  

$R_i^J$ Initiator decomposition rate in phase $j$ [mol·cm$^{-3}$·s$^{-1}$]  

$r_{transport}$ Macro-radical transport rate from continuous to dispersed phase [mol·cm$^{-3}$·s$^{-1}$]  

$T$ System temperature [K]  

$V_i^J$ Volume of phase $j$ [cm$^3$]  

$y_{VI}$ Cumulative VI fraction in terminated polymer [-]  

$y_{VP}$ Cumulative VP fraction in terminated polymer [-]
Greek symbols

$\alpha$ Fraction of macro-radicals initiated in the continuous phase which are transferred into the dispersed phase before terminating

$\delta$ Characteristic length for macro-radical mass transfer [cm]

$\lambda^j_i$ Moment of order $i$ in phase $j$ for active polymer chains [mol·cm$^{-3}$]

$\mu^j_i$ Moment of order $i$ in phase $j$ for terminated polymer chains [mol·cm$^{-3}$]

$\rho_i$ Density of specie $i$ [g·cm$^{-3}$]

$\Phi$ Objective function defined for optimization procedure for parameter evaluation [mol·cm$^{-3}$]
Chapter 4

Stability of a model amphiphilic peptide in aqueous solutions

4.1 Introduction

In nanotechnology several functional materials are designed based on the spontaneous self-assembling of single building blocks into highly organized structures.\textsuperscript{123-125} This “bottom-up” approach involves a reduction of the total free energy of the system through a combination of weak and strong intra and inter-molecule interactions such as hydrogen forces, ionic bonds and hydrophobic interactions.\textsuperscript{126}

Ionic complementary peptides represent a family of monomers able to spontaneously self-assemble in aqueous solutions.\textsuperscript{127-131} These short, synthetic oligopeptides of 8-16 amino acid residues are either inspired by fragments of naturally occurring proteins or are \textit{de novo} designed. The peculiar structure of the macromolecules displays on one side completely hydrophobic residues and on the other side positively and negatively charged residues arranged alternatively. Based on charge distributions, one can classify the ionic-complementary peptides into different types: type I: -+, type II: --++, type III: ----+++ and type IV: ----++++.\textsuperscript{128} Depending on the intrinsic properties of the structure (e.g., amino acid periodicity, charge distribution, intrinsic chirality, etc.), different peptides have different propensity to form inter- and/or intramolecular hydrogen bonds\textsuperscript{128,132}, thus leading to different secondary structures (\textit{\alpha}-helix, \textit{\beta}-sheet or random coil)\textsuperscript{133,134} and aggregation states.\textsuperscript{135} These characteristics make the self-assembly of amphiphillic peptides particularly attractive. For example, one can sensitively tune the charge properties of the peptides, by modifying either their intrinsic structure or the environmental conditions (pH, ionic strength, etc.), to produce in a controlled way various stable and resistant nanomaterials. These include fibrils,\textsuperscript{126} various types of fibril networks,\textsuperscript{128} gels (i.e.,
hydrogels with extremely high water content) and membranes. Potential applications have been found for such biocompatible materials in medicine, biotechnology, nanotechnology and biology. Examples include 3D cell culture, tissue repairing and engineering, cosmetic industry, drug release and drug delivery, biological surface engineering, separation matrices and membrane protein stabilization. Moreover, self-complementary peptides represent a useful model system to investigate the in vitro formation of amyloid fibrils involved in several neurodegenerative diseases.

RADARADARADARADA (RADA 16-I) is a peptide of 16 amino acid residues, belonging to the family of self-complementary peptides. It consists of repeated segments of hydrophobic (alanine) and hydrophilic (arginine and aspartic acid) amino groups. RADA 16-I has a high propensity to self-assemble to form very stable β-sheet structures, leading to a series of organized structures. For example, under acidic pH conditions, nanofibrils are observed, while higher order nanofibril scaffold are obtained by increasing pH or adding salt. RADA 16-I hydrogels with extremely high water content are ideal materials for making 3D scaffolds for cell culture and tissue engineering. Although significant attention has been given in the literature to the application of RADA 16-I hydrogels, only few fundamental studies can be found focusing on the aggregation (self-assembling) mechanism.

The biophysical analysis of RADA 16-I self-assembling mechanism is interesting not only academically but also in practical applications. An example involves the stability of the dispersion of assembled RADA 16-I fibrils, which still limits their industrial productions and handling. Due to the large aggregation propensity, avoiding RADA 16-I assembling into fibrils is extremely difficult. A completely molecular solution of RADA 16-I has in fact never been observed in the literature, and its fibril dispersions represent the starting materials for obtaining scaffolds and gels in practical applications. Moreover, such applications require well controlled stability of the fibril dispersion in order to assemble them into desired higher ordered structures.

In this work, we identify the existence of stable fibril units of RADA 16-I at low pH. During their incubation, such fibrils elongate to longer fibrils through end-to-end
fibril-fibril association. Several biophysical techniques have been applied to characterize the system. Fibrils size and morphology were characterized by Atomic Force Microscopy, Cryo-Scanning Electron Microscopy, Transmission Electron Microscopy, Dynamic Light Scattering and Static Light Scattering. The fraction of RADA 16-I monomers in the system was quantified by Size Exclusion Chromatography, while protein secondary structure was analyzed by Circular Dichroism and Fourier Transform Infrared Spectroscopy. Using such a comprehensive characterizations, the stability behavior of the fibril dispersions is investigated as a function of several parameters, such as pH, anion type and temperature.

4.2 Material and Methods

Material

The RADA 16-I (Ac-R-A-D-A-R-A-D-A-R-A-D-A-R-A-D-A-NHCOCH3) peptide was provided by Lonza Ltd (Visp, Switzerland) as lyophilized powder in the form of chloride and trifluoroacetic salt. The material was used as received without further purification. To prepare the starting material for our investigations, we dispersed the peptide powder into Milli-Q (Milli-pore) deionized water under mild shaking for 10 minutes for homogenization. The peptide concentration in the dispersion was measured by UV absorbance at 217, 230 and 240 nm. It is worth noticing that for all the experiments shown in this work very similar results have been obtained from three different production batches, with the peptide in the form of both chloride and trifluoroacetic salts.

Atomic Force Microscopy (AFM)

10 µL of 150 fold diluted samples were spotted on a freshly cleaved mica surface for 30 seconds before washing with Milli-Q deionized water to remove unattached materials and gently drying under nitrogen flux. Samples were imaged at room temperature by a Nanoscope IIIa (Digital Instrument, USA) operating in tapping mode. Scan rate of 0.8 Hz and antimony doped silicon cantilevers with resonance frequency in the range 325-382 kHz and tip radius of 8 nm (Veeco, Plainview, NY, USA) were used.
Electron Microscopy (EM)

Samples for cryogenic scanning electron microscopy (cryo-SEM) were frozen at about -160 °C and transferred via the BAL-TEC airlock shuttle system VCT 100 to the freeze-etching unit BAF 060 from BAL-TEC (Liechtenstein), where the continuous phase was sublimated under high vacuum at -80 °C (within ca. 120min) followed by coating with tungsten (2 nm, at 10° angle). Images were recorded on a Gemini 1530 FEG scanning electron microscope (Zeiss, Germany) equipped with a cold stage.

Samples for transmission electron microscopy (TEM) were loaded on a carbon grid (Quantifoil, Germany) and negative stained with a 2% uranyl acetate aqueous solution. Pictures were recorded on a FEI Morgagni 268.

Light Scattering (LS)

Dynamic light scattering (DLS) and static light scattering (SLS) measurements were performed on-line using a goniometer, BI-200SM (Brookhaven Instruments, Holtsville, NY, USA) covering angles from $\theta = 16$ to 150°. A solid-state laser, Ventus LP532 (Laser Quantum, Manchester, UK) with a wavelength $\lambda_0 = 532$ nm was used as the light source.

DLS was also measured at a fixed angle of $\theta = 173^\circ$ using a Zetasizer Nano (Malvern, UK) with laser beam of wavelength $\lambda_0 = 633$ nm. Micro UV-Cuvettes with dimension 12.5×12.5×45 mm (70 µL) and light path 1 cm (Brand GmbH, Wertheim, Germany) were used.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was performed with a Superdex Peptide 10/300 GL, 10 mm×300 mm size-exclusion column (GE Healthcare, Upssala, Sweden) mounted on a Agilent 1100 series HPLC unit (Santa Clara, CA, USA) consisting of a quaternary pump with degasser, an autosampler, a column oven, and a DAD detector. Each sample was eluted for 70 min at a constant flow rate of 0.4 mL/min using as mobile phase the same buffer of the analyzed sample. The UV absorbance peaks were detected at 217
Circular Dichroism (CD)

Circular dichroism (CD) spectra were collected using a Jasco-815 CD spectrophotometer (Jasco, Easton, MD, USA). Far-UV CD spectra were recorded from 260 to 190 nm with the temperature of the cell holder controlled at 25 °C. A quartz cuvette with 0.1 cm path length was used. Spectra obtained after buffer subtraction were corrected for protein concentration and smoothed using the Savitsky-Golay function.

Fourier Transform Infrared Spectroscopy (FTIR)

Hydrated thin film attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) measurements were performed on a Nicolet Nexus 870 FTIR ESP instrument equipped with an ATR Nicolet Omni-Sampler device (Nicolet, Madison, WI, USA). Aliquots of 10 µL were spotted on the crystal surface and let drying under nitrogen flux. The spectra were collected in the wavelength range from 1700 to 1600 cm\(^{-1}\) at 1 cm\(^{-1}\) resolution and smoothed using the Savitsky-Golay function after buffer subtraction.

4.3 Characterization of fibril size and morphology

After the peptide powder is dissolved in water at a concentration in the range of 0.5-1 g/L, a transparent homogeneous solution is obtained (see Figure 4.1a). The AFM analysis in Figure 4.1b shows the presence of a significant amount of nano-scale fibrils with length in the range of about 200-400 nm, which is consistent with previous findings in the literature.\(^{149}\) Notably, the obtained fibrils cannot be further broken by either sonication or shaking or stirring, indicating that these short fibrils exhibit high resistance against shearing forces.
Figure 4.1 (a) typical transparent dispersion of the RADA 16-I fibrils in aqueous solutions at pH 2.0-4.5 and peptide concentration of 0.5-1 g/L, and (b) a typical AFM picture showing the presence of nanofibrils in the dispersions.

Figure 4.2 Fibril length distribution obtained from AFM pictures, in the freshly prepared peptide dispersion in several aqueous solutions.

Three different solvents were used to dissolve the peptide: Milli-Q deionized water (pH was automatically shifted to around 4.5 due to the counterions of the lyophilized peptide), 0.013 M trifluoroacetic acid (TFA) solution at pH 2.0 and 0.01M hydrochloric acid (HCl) solution at pH 2.0. In each solution, the fibril length distribution was evaluated
from the AFM pictures by manually counting and measuring the length of the fibrils. Under each condition at least three independent samples and 350 fibrils were considered to ensure statistical reliability. Figure 4.2 shows the fibril length distributions in the three solutions at the peptide concentration of 1 g/L. All the distributions are rather similar in shape but the average length is slightly dependent on pH. The mean length and the standard deviation of the fibril distributions are, respectively, 296 and ±131 nm in water at pH 4.5, 370 and ±134 nm in TFA solution at pH 2.0, 332 and ±138 nm in HCl solutions at pH 2.0. In order to get more details on the fibril structure, we have analyzed the samples using both cryo-SEM and TEM. As can be seen in Figure 4.3, both techniques confirm the length distribution obtained from AFM. The fibril diameter in all the considered solutions is about 14 nm according to AFM and cryo-SEM pictures and about 10 nm according to TEM pictures. It is well known that due to the excluded volume of the AFM tip during the tipping of the surface, the fibril diameter measured by AFM cannot be considered reliable. Moreover, the drying procedure necessary during sample preparation for AFM and cryo-SEM analysis could induce flattening of the fibril structure on the surface. Since TEM requires very mild drying conditions, the fibril diameter given by TEM can be considered the most reliable. However, also in this case the staining technique could affect the results. Therefore, a detailed model of the peptide fibrils cannot be obtained with any of the applied techniques.

It is known that the length and the height of a RADA 16-I peptide in a stretched conformation are equal to 5 and 1.3 nm, respectively. Based on the mechanism proposed in the literature, the peptides form the fibrils by aggregation along their stretched backbone via hydrogen bonding. In this way, the obtained fibrils would have a width or diameter of about 5 nm, which is only half of the value determined from TEM in Figure 4.3b. On the other hand, the fibrils could also further assemble by lateral association into thicker fibrils. Then, the observed width or diameter of 10 nm would indicate that the observed fibril is composed of two fibrils joined together by lateral association. No twisting of the fibrils occurs, according to TEM picture in Figure 4.3b. The height of the fibrils has been evaluated from the AFM pictures equal to 1.16±0.22 nm, rather consistent with the height of the RADA-I peptide.
Figure 4.3 Cryo-SEM (a) and TEM (b) pictures of the fibrils in the aqueous dispersions of the RADA 16-I lyophilized powder at pH 4.5 and concentration of 0.5 g/L.

In parallel with microscopy analysis, the fibrils size was analyzed by light scattering techniques. Dynamic light scattering measures the intensity fluctuation due to the Brownian motion of the particles as a function of the delay time, $\tau$, from which the intensity autocorrelation function $g^2(\tau)$ and the field autocorrelation function $g^f(\tau)$ can be defined:

$$g^2(\tau) = \frac{\langle I(q,0)I(q,\tau) \rangle}{\langle I(q) \rangle^2}$$  \hspace{1cm} (4.1)
\[ g^2(\tau) = 1 + \sigma \left( g^1(\tau) \right)^2 \]  

(4.2)

where \( q \) is the scattering wavevector, \( q = (4\pi n / \lambda_0) \sin(\theta / 2) \), with \( \theta \) the scattering angle, \( n \) the refractive index of the solvent and \( \lambda_0 \) the wavelength of the laser beam. The field autocorrelation function decays exponentially with \( \tau \) :\(^{150}\)

\[ g^1(\tau) = \exp(-\Gamma \tau) \]  

(4.3)

where the decay rate coefficient, \( \Gamma \), is a function of the particle diffusivity. For an asymmetric particle, \( \Gamma \) is composed of two terms: one related to the translational diffusion \( (D) \) and another to the rotational motion of the particle along its axis \( (\Theta) \):\(^{151}\)

\[ \Gamma = D \cdot q^2 + \Theta \]  

(4.4)

The decay rate coefficient \( \Gamma \) has been evaluated with two different instruments at different angles and plotted in Figure 4.4a as a function of \( q^2 \). It is seen that all the data can be well represented by a straight line with a negligible intercept (i.e., \( \Theta \approx 0 \)), indicating that for the given fibril system, the contribution of the rotational diffusion is negligible. The estimated translational diffusion coefficient is \( D = 6.6 \ \mu \text{m}^2/\text{s} \).

Let us assume that the short RADA 16-I fibrils are rigid and rod-like. The fibril dimension can then be estimated from the translational diffusion coefficient according to the Doi-Edward relationship:\(^{152}\)

\[ D = \frac{kT}{3\pi \eta L} \ln \left( \frac{L}{b} \right) \]  

(4.5)

where \( k \) is the Boltzmann constant, \( T \) the temperature, \( \eta \) the solvent viscosity, \( L \) and \( b \) the length and the diameter of the fibril, respectively. For a polydisperse system, the measured diffusion coefficient is a z-average value, \( \langle D \rangle_z \), which can be computed from the length distribution of individual fibrils:\(^{153}\)

\[ \langle D \rangle_z(q) = \frac{\sum_{i=1}^{N} N_i \cdot D_i \cdot P_i(q) \cdot L_i^2}{\sum_{i=1}^{N} N_i \cdot P_i(q) \cdot L_i^2} \]  

(4.6)

where \( N_i \) is the number concentration of fibrils with length \( L_i \) and \( P_i(q) \) is the form factor for a rigid rod with length \( L_i \) at the wavevector \( q \), given by \(^{154} \)
\[ P_l(q) = \frac{2}{L_i} \int_0^{qL_i} \frac{\sin(x)}{x} dx - \left[ \frac{2}{qL_i} \sin\left(\frac{qL_i}{2}\right) \right]^2 \]  \hspace{1cm} (4.7)

Figure 4.4 (a) the decay coefficient of the DLS measurements, \( \Gamma \), as a function of the wavevector, \( q^2 \), measured using BI-200SM (Brookhaven) (◊) and Nanosizer (Malvern) (○). The continuous line is the best fit of \( \Gamma = D \cdot q^2 \) with \( D = 6.6 \, \mu \text{m}^2/\text{s} \). (b) Measured autocorrelation functions at the angles, 60° (○), 90° (□), 120° (Δ) and 150° (◊), compared with the computed ones using eq 4.3 (continuous line).

From the fibril length distributions determined from AFM in Figure 1b and the \( D_i \) values of individual fibrils computed from eq 5 considering \( b = 10 \, \text{nm} \), we have calculated the \( \langle D \rangle_z \) value from eq 6. The obtained \( \langle D \rangle_z \) values are only slightly dependent on the scattering
angle: 7.9 μm²/s at 173° and 7.7 μm²/s at 60°, and are reasonably in agreement with the value (6.6 μm²/s) from the DLS measurement. As a consequence, from the calculated \( \langle D \rangle_z \) values we can predict the autocorrelation function at different angles, and the obtained results are compared to the measured ones in Figure 4.4b. As can be seen, the agreement is rather satisfactory. The difference at large delay times occurs because we have used a single \( \Gamma \) value in the autocorrelation function simulations, without accounting for the polidispersity of the samples.

Given the low peptide concentration (0.5-1 g/L), we can consider the fibril dispersions as dilute systems. Then, the form factor of the fibrils, \( P(q) \), can be determined from the static light scattering experiments, i.e., from the measured intensity at different angles, \( I(q) \).\(^{155}\)

\[
P(q) = \frac{I(q)}{I(0)}
\]  

(4.8)

where \( I(0) \) is the intensity at zero angle.

Then, for an ensemble of polydisperse rods, the average form factor, \( \langle P(q) \rangle \), can be expressed using eq 4.7 as follows\(^{73}\)

\[
\langle P(q) \rangle = \frac{\sum_{i=1}^{N} N_i \cdot P_i(q) \cdot L_i^2}{\sum_{i=1}^{N} N_i \cdot L_i^2}
\]  

(4.9)

Using the fibril length distribution in Figure 4.2, we have computed the average form factor, \( \langle P(q) \rangle \), from eqs 4.7 and 4.9, and the result is compared with the measured \( \langle P(q) \rangle \) in Figure 4.5. It can be seen that the calculated \( \langle P(q) \rangle \) represents well the experimental data.

The radius of gyration of a rigid rod can be calculated from its length, as \( R_g^2 = L^2/12 \).\(^{155}\) Thus, the average radius of gyration of our fibrils, \( \langle R_g \rangle \), can also be computed from the known fibril length distribution in Figure 4.2, using the following expression:\(^{7}\)

\[
\langle R_g \rangle^2 = \frac{\sum_{i=1}^{N} N_i \cdot R_{g,i}^2 \cdot L_i^2}{\sum_{i=1}^{N} N_i \cdot L_i^2}
\]  

(4.10)
The obtained value is $\langle R_g \rangle = 125$ nm, which is in good agreement with that estimated from the form factor based on the Guinier plot (114 nm). Therefore, the \textit{in situ} light scattering measurements correlate well with microscopy characterizations and also confirm the reliability of the measured fibril length distributions in Figure 4.2.

![Graph](image)

**Figure 4.5** The average form factor, $\langle P(q) \rangle$, of the fibrils, measured by SLS (symbols), compared with the calculated one (solid curve) according to eq 4.8.

It is difficult to determine whether or not the monomeric form of the peptide is present in the system using light scattering techniques, because of the substantial difference in size between the monomers and the fibrils. The size of the monomers is also below the lower limit of the used microscopy techniques. Thus, we have applied SEC to quantify the monomers. Different cut-off (20 nm and 220 nm) filters were applied to prepare the samples in order to verify that no artifacts were induced by the filtering procedure. It is found that in the aqueous solution at pH 4.5 no monomer can be detected, while at pH 2.0, apart from the fibrils, we have observed $60\pm25$ % monomers both in the TFA as well as in the HCl solution. It is worth noticing that the amount of monomers determined by SEC at low pH varies significantly even though the same preparative procedure was rigorously followed for several repetitions. This is likely due to the difference in the fibril length.
distributions of different resuspensions of the same powder even at the same concentration. In fact, the distributions reported in Figure 1b are the average of different resuspensions. At a given peptide concentration, samples with a shorter average size contain more fibrils than those with a larger average size, thus possessing more fibril ends from which the monomers can detach. Undetectable amount of monomers at pH 4.5 in the aqueous solution was confirmed by several repetitions. Undetectable amount of monomers at pH 4.5 in the aqueous solution was confirmed by several repetitions.

From the above results we can then conclude that all the peptides in the original powder are practically in the form of fibrils, and the presence of monomers at low pH is likely due to the dissolution of the fibrils. It is in fact well known that TFA is able to dissolve protein aggregates into monomers by disrupting the hydrogen bonds.\textsuperscript{156} From our present results, we see that HCl can also be used for the same purpose.

To better understand the state of the peptides under different conditions, we have investigated their secondary structure both in the solution and in the original powder, using FTIR and CD. Due to the well-known interference between TFA and protein in I-amide region, the peptide dispersion in the TFA solution was not analyzed by FTIR. In Figure 4.6a the FTIR spectra of the peptide in water at pH 4.5 and in the original powder are shown. They are rather similar and show a maximum at 1627 cm\textsuperscript{-1}, characteristic of the β-sheet structure. A significant shoulder in the range of 1650-1670 cm\textsuperscript{-1} indicates presence of β-sheet turn structures, as observed in other amphiphilic peptide systems.\textsuperscript{157} These results suggest that the peptides in water at pH 4.5, as well as in the original powder, possess an ordered structure, i.e., fibrils, thus confirming that the monomers at pH 2.0 come from dissolution of the fibrils.

Figure 4.6b shows the CD spectra of the peptide dispersion in water at pH 4.5 and in the 0.013 M TFA solution at pH 2.0. For the former, the minimum at 215 nm clearly indicates the β-sheet structure, thus consistent with the FTIR data. For the latter, instead, the minimum is located at 200 nm, characteristic of random coil, more disordered structures. Since in this case the monomers are present, this result indicates that the monomers exhibit a random coil structure in the aqueous solution. The shoulder around 215 nm corresponds to the remaining fibrils. According to CDpro analysis
(http://lamar.colostate.edu/~sreeram/CDPro/main.html), the amount of β-strand is 65.5% at pH 4.5 and 13.2% at pH 2.0, while the unordered structure content is 10% at pH 4.5 and 66.1% at pH 2.0.

Figure 4.6 (a) FTIR spectra of the lyophilized RADA 16-I powder (■) and its dispersion in water at pH 4.5 (○), and (b) CD spectra of the RADA 16-I dispersion in the 0.013 M TFA solution at pH 2.0 (---) and in water at pH 4.5 (―).
4.4 Stability and aggregation kinetics

We have monitored the stability of the fibril dispersions at room temperature in three aqueous solutions: water at pH 4.5, 0.15 M TFA solution at pH 2.0, and 0.01 M HCl solution at pH 2.0, respectively, at the peptide concentration of 1 g/L, using DLS. In Figure 4.7 the measured average hydrodynamic (or equivalent-sphere) radii, $\langle R_h \rangle$, are shown as a function of the incubation time.

![Figure 4.7](image_url)

**Figure 4.7** Time evolution of the average hydrodynamic radius, $\langle R_h \rangle$, of the fibrils in the 0.13 M TFA solution at pH 2.0 (■), in the 0.01 M HCl solution at pH 2.0 (▲) and in distilled water at pH 4.5 (♦), measured at 25 °C and peptide concentration of 1 g/L.

In all the three solutions, the $\langle R_h \rangle$ value of the fibrils increases with time slowly, indicating the occurrence of slow aggregation of the fibrils. The aggregation rate in the three solutions follows the order, 0.013 M TFA at pH 2.0 > 0.01 M HCl at pH 2.0 > water at pH 4.5. Since the isoelectric point of RADA 16-I is pI=7.2, at both pH 4.5 and 2.0 the net charge on the surface is positive. The net charges of RADA16-I monomer calculated from the pK values of the amino-acid side chains are about +0.75 at pH 4.5 and +4 at pH 2.0, and, in fact, the zeta potential values are 36.2 mV in water at pH 4.5 and 40.8 mV in 0.013 M TFA at pH 2. Therefore, the better stability of the fibril dispersion in water at pH 4.5 cannot be explained by the charging effect. We believe that it results from the hydrophobic effect. In
particular, at pH 2.0 more aspartic residues are protonated, leading to an increase in the hydrophobicity of the peptide, thus in the propensity to aggregation in water. TFA is a strong counterion-pairing agent and has a larger propensity to bind charged groups with respect to Cl. Then, the larger aggregation extent in the 0.013 M TFA solution than in the 0.01 M HCl solution at pH 2.0 is consistent with the more efficient reduction of the surface charges, thus promoting the aggregation, in this case due to a charging effect.

For the fibril dispersion in water at pH 4.5, since negligible monomers are present, the observed aggregation is clearly due to fibril-fibril aggregation. In the 0.013 M TFA and 0.01 M HCl solutions at pH 2.0, however, the characterization results shown in the previous section indicate that the system contains a substantial amount of monomers. It would then be reasonable to consider the participation of the monomers in the aggregation, particularly considering that they would be favored with respect to fibril-fibril aggregation due to their higher diffusivity. However, after a detailed SEC analysis of samples taken along the incubation time, we found that although the amount of the monomers decreases with time, several peaks at longer eluting times, representing molecules smaller than RADA 16-I, appear and increase with the incubation time, as clearly shown in Figure 4.8a. Mass spectrometry analysis confirmed the fragmentation of the monomeric RADA16-I into smaller molecules (data not shown). More importantly, as shown in Figure 4.8b, the area of the SEC chromatogram for the RADA 16-I peak decreases and the total area for the smaller molecules peaks increases as the incubation time increases, while the area sum of all the peaks remains practically constant. These results indicate that the decrease in the amount of monomers along the incubation time is not due to aggregation to fibrils. It is instead related to some chemical degradation of the peptides (e.g., hydrolyzation) at low pH. On the other hand, this result reveals that, similar to the case at pH 4.5, the increase in the fibril size is also due to fibril-fibril aggregation.
Figure 4.8 For the peptide distribution in the 0.01 M HCl solution at pH 2.0: (a) SEC chromatograms at several incubation times, where the peak at the eluting time of 34 min represents the monomer, RADA 16-I, and (b) percentage of the SEC chromatogram area corresponding to the remaining monomers (●) and the fragments eluting at larger times (■).
Figure 4.9 AFM (a) and TEM (b) pictures of the fibrils in the RADA 16-I dispersion in the 0.013 M TFA solution at pH 2.0 and at the peptide concentration of 1 g/L after 25 days incubation, and (c) fibril length distributions obtained from the AFM pictures at time 0 (black) and after 25 days incubation (white).
Samples at the incubation time of 25 days have been analyzed by AFM and TEM, and the corresponding pictures are shown in Figures 4.9a and 4.9b, respectively. The most important observations from these pictures, as well as from the fibril length distributions shown in Figure 4.9c, are that the fibrils become longer but their diameter remains the same as before the incubation. The same result has been found for fibrils incubated in water at pH 4.5 for 5 months (data not shown). This suggests that the main mechanism responsible for the fibril aggregation is the end-to-end fibril elongation and that lateral aggregation is negligible. Such mechanism is consistent with the sliding diffusion dynamic re-assembly proposed by Yokoi and coworkers for the RADA 16-I fibril aggregation. They showed that after sonication and breakage of longer fibrils, the fragmented fibrils re-assembly into the original fibrils, due to the unpaired hydrophobic and hydrophilic patches at the end of the fibril created by the breakage.

The above results have been confirmed by incubation at higher temperatures to accelerate the aggregation. As can be seen from Figure 4.10, increasing temperature induces both faster aggregation kinetics and faster peptide degradation.

Let us now apply the Smoluchowski kinetic approach, i.e., the population balance equations (PBE), to simulate the growth of the fibrils, based on the end-to-end fibril-fibril aggregation mechanism. The PBE in this case can be written as:

$$\frac{dN_i}{dt} = \frac{1}{2} \sum_{j=1}^{\infty} k_{ij} N_j N_{i-j} - N_i \sum_{j=1}^{\infty} k_{ij} N_j$$

(4.11)

where $N_i$ is the number concentration of fibrils with length $i$, and $k_{ij}$ the rate constant for the aggregation between two fibrils with length $i$ and $j$, respectively. The first and the second terms on the right hand side of eq 4.11 represent the birth and death of the fibril of a given length, respectively. The aggregation rate constant can be expressed as:

$$k_{ij} = \alpha \cdot k_{ij}^{\text{diff}}$$

(4.12)

where $k_{ij}^{\text{diff}}$ is the rate constant for diffusion-limited aggregation and $\alpha (\in [0,1])$ is a parameter representing the efficiency of the aggregation with respect to the diffusion-limited aggregation. Following Hill and Pallitto and Murphy, $k_{ij}^{\text{diff}}$ for the end-to-end fibril aggregation can be expressed as:
\[ k_{ij}^{\text{Diff}} = \frac{k_B T}{3\eta} \frac{N_A}{1000} (\delta \cdot \omega)^2 \left( \ln \left( \frac{L_i / b}{L_i + L_j} \right) + \frac{\ln \left( L_j / b \right)}{L_j} + \frac{\ln \left( L_j / b \right)}{L_i} \right) \]  
\[ \nu_j = 0.312 + 0.565 \left( \frac{L_j}{b} \right)^{-1} - 0.100 \left( \frac{L_j}{b} \right)^{-2} \]  

where \( T \) is temperature, \( k_B \) the Boltzmann constant, \( N_A \) the Avogadro number, \( \eta \) the viscosity of the solvent, \( \omega \) the minimum allowed angle between two fibrils, and \( \delta \) the minimum allowed distance between two fibrils. A reference value from literature was used for \( \delta \cdot \omega \left( =4.5 \times 10^{-10} \text{ cm} \cdot \text{rad} \right) \) and it was assumed temperature independent. The parameter \( \alpha \) in eq 4.12 was used as an adjustable parameter to reproduce the experimental fibril length distributions.

By numerical integration of the population balance equations, we obtain the time evolution of the fibril distribution, from which the corresponding average hydrodynamic radius, \( \langle R_h \rangle \), can be evaluated from the average diffusion coefficient, \( \langle D \rangle_z \), calculated according to eq 4.6. The fibril distribution estimated from the AFM pictures in Figure 4.2 has been considered as the initial condition. The simulated curves are compared with the experimental data at different aggregation temperatures in Figure 4.10a.
Figure 4.10 (a) The measured time evolutions of the average hydrodynamic radius, $\langle R_h \rangle$, of the fibrils in the 0.013 M TFA solution at pH 2.0 at $T=4 \, ^\circ\mathrm{C}$ (□), 25 $^\circ\mathrm{C}$ (▲), 37 $^\circ\mathrm{C}$ (○) and 50 $^\circ\mathrm{C}$ (♦), compared with the PBE simulations (solid curves) based on eq 4.11, and (b) area percentage of the remaining monomers (open symbols) and the corresponding fragments (filled symbols) in the size exclusion chromatograms for incubation at 37 $^\circ\mathrm{C}$ (○ and ♦) and 50 $^\circ\mathrm{C}$ (◊ and ♣).
It is seen that the model simulation results are in good agreement with the experimental data when the fibrils are small. As the fibril length increases, due to the fibril flexibility, bending, curvature, etc., the fibrils cannot be modeled as rod-like objects. As a consequence, the expressions of the $R_h$ and of the aggregation rate considered in the model do not apply anymore, and the simulation results deviate significantly from the experimental data. The $\alpha$ values obtained from the simulations at different conditions are summarized in Table 4.1: all of them are substantially smaller than unity, indicating that all the investigated aggregation processes are substantially slower than diffusion limited aggregation. Thus, the aggregation rate is not controlled by diffusion but it is rather an activated process which requires the overcoming of some activation energy barrier. This can be estimated to be in the order of 20 kcal/mol by considering the Arrhenius-type behavior of $k_{11}$ (eq 4.12): $k_{11} = k_0 \exp\left(-E_{act}/RT\right)$.

**Table 4.1.** The $\alpha$ values in eq 4.12 estimated by fitting the experimental aggregation kinetics data

<table>
<thead>
<tr>
<th>Solution type</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 °C</td>
</tr>
<tr>
<td>0.013 M TFA at pH 2.0</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.01 M HCl at pH 2.0</td>
<td></td>
</tr>
<tr>
<td>water at pH 4.5</td>
<td></td>
</tr>
</tbody>
</table>

**4.5 Concluding remarks**

In this work we have investigated the dispersion behavior of the RADA 16-I peptide powder in various aqueous solutions. First of all, using several microscopy and light scattering techniques, we have identified the existence of stable RADA 16-I nanofibrils in the pH range of 2.0-4.5. Such fibrils show a $\beta$-sheet structure and have length of around 200-300 nm and diameter of 10 nm. The latter would indicate that each fibril is constituted of two filaments of stretched monomer aligned laterally. The small fibrils
cannot be broken by the mechanical stress generated from sonication.

When the peptide powder is dispersed in water at pH 4.5 at a concentration of 1 g/L, all the peptides are in the form of fibrils with undetectable amount of monomers. When it is dispersed in the 0.013 M TFA or 0.01 M HCl solution at pH 2.0, however, the dispersion contains substantial amount of monomers. FTIR data indicate that the peptides in the powder are already in the form of fibrils, and the monomers at pH 2.0 arise from dissolution of the fibrils induced by the presence of TFA or Cl.

The stability of the fibril dispersions in water with respect to their incubation time has been investigated at the peptide concentration of 1 g/L. It is found that very slow end-to-end aggregation of the fibrils occurs, leading to elongation of the fibrils without changing their diameter. It has been observed that the monomers do not participate in the fibril elongation. The elongation rate is faster for the peptide dispersion in the presence of TFA or Cl at pH 2.0 than in pure water at pH 4.5, indicating the effect of pH and nature of the anions. The aggregation can be accelerated by temperature in the range of 4-50 °C.

The aggregation kinetics under different conditions has been simulated by the Smoluchowski kinetic model, population balance equations, considering the fibrils to be rod-like. Good agreement between simulations and experiments has been obtained when the incubation time is short, but significant deviation occurs at long incubation time, where the fibrils most probably grow too long to be considered as rod-like. It is also found that the aggregation process is not limited by diffusion but rather is an activated process with energy barrier in the order of 20 kcal/mol.

The characterization of the fibril dispersions and of their stability developed in this work represents the starting point for possible future studies addressing the kinetics of gel formation and the morphology of the fibrillar gels induced by the destabilization of such dispersions.
Chapter 5

Production, characterization and in vitro aggregation behavior of a non-amyloidogenic \( \lambda \) light chain fragment originating from multiple myeloma

5.1 Introduction

The aggregation stability of the immunoglobulin light chain fragments is involved in several disorders related to the abnormal proliferation of bone marrow monoclonal plasma B cells and the subsequent excessive production of monoclonal light chains. 10-15\% of the patients affected by multiple myeloma and with large light chain serum concentration are subsequently affected by the aggregation-related diseases,\textsuperscript{161} such as light chain amyloidosis (AL),\textsuperscript{162} light chain deposition diseases (LCDD),\textsuperscript{163} and cast nephropathy.\textsuperscript{164} Light chain amyloidosis (AL) is the most common form of sporadic systemic amyloidosis,\textsuperscript{18,165} characterized by deposition of insoluble amyloid fibrils in organs such as kidney, heart and liver, which leads to organ failure; it can act also on peripheral nerve, gastrointestinal track and lungs.\textsuperscript{166} In opposition to the fibrillar structures encountered in AL, in LCDD the aggregates are amorphous and granular. The basement membrane of kidney is the main target organ, although also heart and liver may be affected.\textsuperscript{167} Typically, only one of the two forms of disease occurs in patients.\textsuperscript{168} A great challenge in understanding the cause and mechanism of aggregation is given by the extremely large number of the possible mutated variant sequences involved. In fact, each patient produces antibodies which have undergone antigen-driven selection. Therefore, the heterogeneity of possible diseases and related protein aggregates are due both to fragment primary sequence and to environmental factors. Several works show that the \( \kappa \) and \( \lambda \) types of light chain dominate in LCDD and AL, respectively.\textsuperscript{169} Despite such tentative of
classification based on clinical analysis, the factors determining whether or not some variants are pathological or lead to fibrillar aggregates or amorphous deposits are far from being understood.

In addition to protein structure, protein amount affects propensity to deposit: circulating disulfide-bound light chain dimers can interfere with normal clearance and metabolism, increasing their serum level. Therefore, it is important to clarify the stability of the dimers and the relationship between dimer/monomer equilibrium and aggregation mechanism.

In this work, we investigate the effect of several physicochemical parameters on the \textit{in vitro} aggregation propensity of a $\lambda$ light chain IgE dimer. This protein was obtained from a human myeloma cell line U266, coming from a patient suffering from multiple myeloma in which neither amyloids nor amorphous deposits were detected. The production of protein in a reasonable amount and in a reproducible way is often a challenge in protein aggregation studies. Commonly, light chain variants are produced recombinantly from bacteria or extracted from urine. Despite the larger effort demanded compared to the commonly employed bacteria systems, the use of eukaryotic cells allows the production of proteins with a glycosylation pattern close to the physiological one. Although the production of light chain fragment and complete immunoglobulin by U266 cells has been already studied, to our knowledge this is the first aggregation study on proteins obtained by such cell line.

We applied several biophysical techniques to investigate the protein secondary structure stability and aggregation behavior. The protein secondary structure was characterized by spectroscopic techniques such as circular dichroism (CD), intrinsic tryptophan fluorescence and 8-anilino-1-naphthalenesulfonic acid (ANS) binding, while the aggregation was monitored by dynamic light scattering (DLS), thioflavin T (ThT) assay, size exclusion chromatography (SEC) and asymmetrical field flow fractionation (FFF). Aggregates morphology was investigated by Congo Red binding, thin film attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) and atomic force microscopy (AFM). The study shows the relationship between secondary structure and
aggregation for the investigated $\lambda$ light chain dimer and underlines the importance of environmental factors, particularly the kind and concentration of salt, on protein stability. Moreover, we have successfully determined the original full-length sequence of the monoclonal light chain produced by the human U266 cell clone. This observation will enable us to make comparative analysis with other studies describing the features of immunoglobulin light chains with a known sequence.

5.2 Materials and Methods

Cell growth, protein purification and characterization

The IgE $\lambda$ light chain was produced from the human mammalian cell line U266 obtained from a 53-years-old patient suffering from multiple myeloma.$^{173}$ Cells were received from the institute “Mario Negri” (Milan, Italy) in RPMI-1640 medium (Sigma-Aldrich, Steinheim, DE) with addition of 10% fetal bovine serum (FBS) (PAN Biotech, München, DE) and 0.1% Penicillin-Streptomycin Solution (Sigma-Aldrich, Steinheim, DE) to avoid bacterial contaminations. The cells were adapted from the 10% FBS medium to a 1% FBS medium to simplify the purification step.

The collected supernatant containing the desired product was concentrated 10-fold using a Sartoflow® Slice 200 Benchtop Crossflow system with 10kDa cut-off membrane (Sartorius GmbH, Göttingen, DE). The concentrated cell culture supernatant was then prepared for cation exchange chromatography by three-fold dilution with deionized water for decreasing the ionic strength and by adjusting the pH to a value in the range 5.0-5.5 using glacial acetic acid (Carbo Erba Reagents, Rodano, Italy). The solution was then filtered using the Sartoflow® Slice 200 Benchtop Crossflow system with 200 nm cut-off membrane.

The clarified cell culture supernatant was then purified by combining cation exchange chromatography with size exclusion chromatography (SEC). In principle, pure protein can be obtained using a SEC column alone. However, since the SEC technique can operate only at a relatively low flow rate and only a limited amount of material can be loaded, it has extremely low productivity. Therefore, we have developed a cation-exchange process
to purify and simultaneously concentrate the IgE \( \lambda \) light chain fragment. All preparative chromatography steps were operated on AKTA equipment (GE Healthcare, Uppsala, SE). The cation-exchange step was carried out using Poros HS50 (Applied Biosystems, Hercules, CA, USA), packed into a KronLab TAC 15/125G0-SR column with 1.5 cm inner diameter and 7.6 cm length. The chromatography method included a loading step at 10 mL/min (340 cm/h) and a linear gradient elution step from 0-100% buffer B in 10 min at 9 mL/min (305 cm/h). As mobile phases, 25 mM acetate buffer (pH 5.0, buffer A) and 25 mM acetate buffer containing 1 M NaCl (pH 5.0, buffer B) were used. The gradient elution was fractionated and the purity of the fractions was determined by analytical SEC on a HP Agilent Series 1100 equipped with a Superdex 75 10/300 GL column. The fractions with the largest content of \( \lambda \) light chains were pooled, concentrated and injected into a HiLoad™ 26/60 Superdex™ 75 prep grade column (GE Healthcare, Uppsala, SE). The fractions with the largest content of \( \lambda \) light chain were washed four times with 25 mM PBS (pH 7.4, 1g/L NaN\(_3\)) and concentrated to reach a final concentration of 1 mg/mL and stored in refrigeration. With this process, from 6 litres of supernatant we can obtain 15 mg of \( \lambda \) chain with a purity of 97%, where bovine serum albumin is the main impurity. Three batch productions indicated good reproducibility of the developed purification process.

The protein concentration was evaluated using a Pierce® BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA) and UV absorbance at 280nm.

The protein purity was assessed by SDS-PAGE with silver staining and Western blot analysis. For the latter, the monoclonal anti-human lambda light chain (bound and free) antibody produced in mouse (Sigma-Aldrich, Steinheim, DE) and the HRP-linked anti-alpha microglobulin antibody were used as primary and secondary antibody, respectively.

To measure protein charge properties, a protein stock solution sample was loaded into an IEF gel (pH 3-10) and run in a PhastSystem™ instrument (GE Healthcare, Buckinghamshire, UK).

**Mass Spectroscopy (MS)**

The mass spectrometric analysis was performed on Bruker's ESI-Qq-TOF (Bruker, Billerica, MA, USA). The protein samples at pH 2.7 and 7.4 were ionized by a nano-spray
Characterization of the IgE $\lambda$ light chain in U266 cells.

Total RNA was extracted from $10^7$ U266 cells. The IgE $\lambda$ light chain nucleotide sequence was cloned by a universal inverse-PCR strategy. Briefly, primers specific for the 5’ ($\lambda$-C$_{\text{LA}}$) and 3’ ($\lambda$-C$_{\text{LB}}$) regions of the constant region of the $\lambda$ light chain were used to deduce the V$_\lambda$ light chain sequence for specific variable region primer design. The PCR primers were characterized by the following sequences: $\lambda$-C$_{\text{LA}}$: 5’-AGTGTGGCCTTTGTTGGCTTG-3’ (from codon +132 to +126), $\lambda$-C$_{\text{LB}}$: 5’-GTCACGCATGAAGGGAGC-3’ (from codon +196 to +204). In order to obtain the original full-length light chain sequence (variable region + constant region, from codon +1 to +216), standard RT-PCR was employed using the same RNA, 5’ clone-specific primer (5’-CAGTCTGCCTCTAGCTAGCTG-3’, from codon +1 to +7 of variable region) and a 3’ universal C$\lambda$ carboxy-terminal primer, corresponding to the last amino acids of the constant region (5’-TGAACATCTCTGAGGGGCAAC-3’, from codon +210 to +216). The PCR fragment was ligated into a cloning vector and amplified. After recombinant plasmid purification, insert was sequenced (Figure 5.1).

Figure 5.1. U266 derived IgE $\lambda$ light chain nucleotide sequence. Black: variable region; Gray: junction region; underscore: constant region.
To determine the IgE λ light chain presumed germline, sequence alignment was made with the current releases of EMBL-GenBank, V-BASE (V BASE Sequence Directory, MRC Centre for Protein Engineering, Cambridge, UK) and IMGT sequence directories. The sequence showed the highest homology with the published IGVL2-8 (Figure 5.2).

**Figure 5.2.** Deduced amino acid sequence of the IGVL2-8 derived U266 λ light chain variable region. Amino acid changes from the germ line donor, IGVL2-8, are highlighted. FR: framework region; CDR: complementarity determining region.

**Circular dichroism (CD)**

Circular dichroism (CD) spectra were measured using a Jasco-815 CD spectrophotometer (Jasco, Easton, MD, USA). Far-UV CD spectra were recorded from 260 to 190 nm with the temperature of the cell holder controlled at 25 °C. A quartz cuvette with 0.1 cm path length was used. Spectra obtained after buffer subtraction were corrected for protein concentration and smoothed using the Savitsky-Golay function.

Protein thermal stability was evaluated by recording the spectra at several temperatures and monitoring the change in molar ellipticity at 196 nm. The temperature was increased from 25 °C to 80 °C in 5 °C steps. The solution was equilibrated at each temperature for 10 min before measuring.

The fraction of unfolded conformation ($F_u$) was obtained assuming a two-state folding mechanism according to Equation 5.1:

$$F_u = \left( \theta_f - \theta \right) / \left( \theta_f - \theta_u \right) \quad (5.1)$$

where $\theta_f$ and $\theta_u$ represent the ellipticity values characteristic of folded and fully unfolded protein. From the sigmoidal curve-fit to experimental data the unfolding temperature ($T_u$) was evaluated.
**Intrinsic Tryptophan Fluorescence (Trp) and 8-anilino-1-naphthalenesulfonic acid (ANS) binding**

The measurements were performed on a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA, USA). Intrinsic tryptophan fluorescence analysis was performed exciting the sample at 295 nm and collecting emission spectra between 305 and 450 nm. The effect of denaturant on conformational stability was investigated by incubating 0.8 g/L light-chain solutions in 25 mM PBS buffer at pH 7.4 with 0.15 M NaCl and with guanidine hydrochloride (GuHCl) in the concentration range from 0 to 5 M at 25°C for 40 min. Changes in protein structure were estimated by fluorescence intensity values assuming a two-state folding mechanism according to Equation 1. From the sigmoidal curve-fit to experimental data the midpoint of the unfolding transition was evaluated ($C_m$).

For 8-anilino-1-naphthalenesulfonic acid (ANS) binding, emission spectra of 0.3 g/L light chain solution in several buffers with 25 µM ANS were collected at 20°C between 420 and 600 nm using 380 nm as excitation wavelength.

**Dynamic Light Scattering (DLS)**

A 10 g/L light chain stock solution was diluted to the desired concentration (0.3-1 g/L) by the desired investigated buffer solutions. DLS measurements were performed on-line using a Zetasizer Nano (Malvern, Malvern Worsc, UK). All samples were filtered with a 0.02 µm cut-off, Anotop 10 syringe filter (Whatman, Kent, UK) immediately before the experiment.

**Size Exclusion Chromatography (SEC)**

The size exclusion chromatography (SEC) technique was performed with a Superdex 75 10/300 GL, 10 mm×300 mm size-exclusion column (GE Healthcare, Uppsala, SE) on a Agilent 1200 series HPLC unit (Santa Clara, CA, USA). Each sample was eluted for 60 min at a constant flow rate of 0.5 mL/min using as mobile phase a 100 mM Na₂SO₄, 25 mM Na₂HPO₄ solution at pH 7.4, filtered with a 0.45 µm cut-off, Durapore membrane.
filter (Millipore, Billerica, MA, USA). The UV absorbance peaks were detected at 280 nm and 220 nm.

**Field Flow Fractionation (FFF)**

The asymmetrical Field Flow Fractionation (FFF) assay was performed using a AF4 Eclipse 3+ (Wyatt, Dernbach, DE), coupled with a 1200 Series isocratic pump from Agilent (Santa Clara, CA, USA). A 275 mm LC channel for aqueous solvents was used for Eclipse 3, with a trapezoidal spacer (350 μm thick, 26.5 cm long) and a Nadir reg. cellulose membrane with 1 kDa cut-off at the bottom (Wyatt, Dernbach, DE). The detector flow was set constant at 1 mL/min, and a step gradient of cross flow from 5 mL/min to 0 mL/min was applied after 30 min. 20 mM HCl buffer solution at pH 2.0 was used as mobile phase, after filtration through a 0.1 μm cur-off, Durapore membrane filter (Millipore, Billerica, MA, USA). 25 μL of sample were injected at desired time interval.

**Atomic Force Microscope (AFM)**

10 μL of 30 fold diluted samples were spotted on a freshly cleaved mica surface for 30 seconds before washing with millipore water to remove unattached material and gently drying under nitrogen flux. Samples were imaged at room temperature by a Nanoscope IIIa (Digital Instrument, USA) operating in tapping mode. Scan rate of 0.8 Hz and antimony doped silicon cantilevers with resonance frequency in the range 325-382 kHz and tip radius of 8 nm (Veeco, Plainview, NY, USA) were used.

**Fourier Transform Infrared Spectroscopy (FTIR)**

Hydrated thin film attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) spectra were acquired on a Nicolet Nexus 870 FTIR ESP instrument equipped with a ATR Nicolet Omni-Sampler device (Nicolet, Madison, WI, USA). Aliquots of 10 μL were spotted on the crystal surface and let drying under nitrogen flux. The spectra were collected in the wavelength range from 1700 to 1600 cm⁻¹ at 1 cm⁻¹ resolution and smoothed using the Savitsky-Golay function after buffer subtraction.
**Thioflavin T assay (ThT) and Congo Red binding**

Off-line Thioflavin T assays were measured during aggregation on a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA, USA). Samples of 10 μL were diluted into 990 μL 25 mM PBS buffer at pH 7.4 with 10 μM Thioflavin T (Acros Organics, Geel, Belgium). The measurements were performed at 25°C with excitation at 450 nm and emission at 485 nm.

Congo Red assay was performed recording the UV spectrum between 400 and 700 nm on a Varian Cary 300 Scan UV-Visible Spectrophotometer (Varian, Palo Alto, CA, USA). 7.5 μL of protein aggregate solution after 10 h of incubation were added to a blank solution of 5 μM Congo Red (Fisher Scientific, Loughborough, UK) in 25 mM phosphate buffer solution at pH 7.4 and left incubated for 30 minutes at room temperature. In presence of amyloid fibrils the spectrum obtained by the difference between the sample and the blank solution should present a maximum at 540 nm.179

**5.3 Protein characterization**

Many domains (both single variable and complete constant-variable domains) have been found to form dimers in physiological and stable conditions.175 Particularly, amyloidogenic light chains are commonly found to be in equilibrium between the dimer and the monomeric form in patients’ urine.175 After the production and purification step, the protein has been characterized in terms of monomer-dimer equilibrium at physiological and low pH. The SEC chromatograms reported in Figure 5.3a show a single, symmetric peak under both conditions. According to the column calibration curve, the elution volume of such a peak corresponds to a molecular weight of 42 kDa, indicating the presence of a dimer consisting of both variable and constant domain.161 The linearity between the elution volume and the natural logarithm of the molecular weight applies well only for globular proteins and non-spherical macromolecules can deviate from such relationship.180 Therefore, to get a more accurate evaluation of the molecular weight, mass spectroscopy (MS) analysis was performed.
Figure 5.3 Protein characterization: a) SEC chromatogram of 1 g/L light chain solution in 25 mM PBS buffer at pH 7.4 (―) and in 20 mM HCl at pH 2.0 (--). Insertion: calibration curve obtained with bovine serum albumin (60 kDa), chymotrypsinogen A (25 kDa) and lysozyme (14.5 kDa); b) and c) Mass spectroscopy analysis in 25 mM PBS buffer at pH 7.4 (b) and in 20 mM HCl at pH 2.0 (c).

The results in Figure 5.3b and 5.3c show one single peak with molecular weight equal to 45.7 kDa for both conditions, in agreement with SEC analysis. It is worth noting that the results of SEC and MS are inconsistent with the Western Blot and SDS-Page analysis (data not shown), which revealed two bands, at around 50 kDa and 25 kDa. This may arise from
the denaturating conditions used in the electrophoresis analysis, which can induce breakage of the dimer disulphide-bond. Indeed, when SDS-Page was performed in the presence of a reducing agent, only the 25 kDa monomer band was detected. We can conclude that the produced light chains are composed of both constant and variable part and associate into dimers through covalent disulphide bonds, which can be broken under reducing conditions.

The nucleotide sequence of U266 derived IgE λ has been characterized as described in the Materials and Methods section.

In addition, the pI of the protein was measured to be 8.5-9.0 by isoelectric focusing (data not shown), in agreement with the theoretical pI (8.25), calculated using a dedicated tool available on the Expasy proteomic server website (www.expasy.org).

5.4 Effect of environmental factors on structure stability and aggregation behavior

The effect of pH, denaturant and temperature on the structure stability of the light chain was investigated by spectroscopic techniques.

In Figure 5.4a the far-UV CD spectra of the protein at pH 7.4 and pH 2.0 are reported. The spectrum at pH 7.4 shows a minimum at 220 nm characteristic of the Greek key β-barrel folding of the immunoglobulin fragment.181,182 The β-sheet structure content increases as the pH value decreases from 7.4 to 2.0 and the minimum shifts from 220 to 218 nm.

The unfolded protein fraction as a function of guanidinium hydrochloride (GuHCl) has been evaluated by intrinsic tryptophan fluorescence, while the temperature stability of the light chain was investigated by CD temperature-step measurements. The GuHCl concentration required to unfold half of the protein ($F_u=0.5$) is equal to about 1.5 M (Figure 5.4b), while the unfolding temperature ($T_u$) at physiological pH was estimated to be 55 ºC (Figure 5.4c).
Figure 5.4 Light chain structural changes induced by pH, denaturant addition and temperature. a) CD spectra for the 0.3 g/L light chain solution in 25 mM phosphate buffer solution at pH 7.4 (—) and in 20 mM HCl at pH 2.0 (—); b) Fraction of unfolded protein as a function of guanidinium hydrochloride (GuHCl) concentration at pH 7.4 evaluated by intrinsic tryptophan fluorescence measurements (see Materials and Methods); c) Fraction of unfolded protein as a function of temperature evaluated by CD measurements (see Materials and Methods). The continuous lines correspond to the interpolation of experimental data according to Eq.5.1.
Table 5.1 Summary of all investigated conditions. Protein concentration is 1 g/L in all cases.

<table>
<thead>
<tr>
<th>Run</th>
<th>Buffer</th>
<th>pH</th>
<th>Salt</th>
<th>T</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 mM PBS</td>
<td>7.4</td>
<td>0.15 M NaCl</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>-</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.15 M NaCl</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.45 M NaCl</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.15 M NaH₂PO₄</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.49 M NaH₂PO₄</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.064 M Na₂SO₄</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>8</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.15 M Na₂SO₄</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.5 M Na₂SO₄</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>20 mM HCl</td>
<td>2.0</td>
<td>0.45 M KCl</td>
<td>37°C</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
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<td>7.4</td>
<td>-</td>
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<td>yes</td>
</tr>
<tr>
<td>12</td>
<td>25 mM PBS</td>
<td>7.4</td>
<td>0.15M NaCl + 1.3 to 1.5M GnHCl</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>13</td>
<td>25 mM PBS</td>
<td>7.4</td>
<td>0.15M Na₂SO₄ + 1.5M GnHCl</td>
<td>37°C</td>
<td>no</td>
</tr>
<tr>
<td>14</td>
<td>25 mM PBS</td>
<td>7.4</td>
<td>0.15M Na₂SO₄ + seeds</td>
<td>37°C</td>
<td>no</td>
</tr>
</tbody>
</table>

After characterizing the structure stability of the protein, the aggregation propensity of the light chain in several conditions was assessed by DLS. A summary of all the experimental runs is reported in Table 5.1. For the condition at low pH, the effect of biologically relevant salts, i.e. sodium chloride (NaCl), sodium phosphate (NaH₂PO₄) and sodium sulphate (Na₂SO₄), was considered.

In physiological conditions, i.e., 25 mM phosphate buffer solution (PBS) with 0.15 M NaCl at pH 7.4 and 37 °C (run 1 in Table 5.1), the protein solutions were stable for over five months. Also in 20 mM HCl solution at pH 2.0 with or without 0.15 M NaCl or 0.15 M NaH₂PO₄ no aggregation could be detected after several days of incubation (run 2, 3 and 5 in Table 5.1).
Figure 5.5 a) Light scattering intensities measured on-line by in situ DLS, for the 1 g/L light chain solution in 20 mM HCl buffer at pH 2.0 without salt (Δ), with 0.15 M NaCl (—), NaH₂PO₄ (···), Na₂SO₄ (—) (runs 3, 4, 6 and 9 in Table 5.1); b) The same as a) but salts are compared at similar ionic strength (see text): 0.45 M NaCl (—), 0.49 M NaH₂PO₄ (···), 0.15 M Na₂SO₄ (—) (runs 4, 6 and 8 in Table 5.1); c) The same as a) but for a solution in 20 mM HCl buffer at pH 2.0, with 0.45 M NaCl (—) and KCl (···) (runs 4 and 10 in Table 5.1).
The situation changed when the concentrations of NaCl and NaH$_2$PO$_4$ were increased to 0.45 M and 0.49 M, respectively (run 4 and 6 in Table 5.1) or when Na$_2$SO$_4$ was added (runs 7 to 9 in Table 5.1). The stability behavior for the different salts and different salt concentrations, followed by DLS, is shown in Figure 5.5. In Figure 5.5a, the results obtained using different salts but at the same constant concentration of 0.15 M are compared, while in Figure 5.5b salts are compared at similar ionic strength $I$, being $I$ defined as $I = \frac{1}{2} \sum_{i=1}^{n} c_i z_i^2$, where $c_i$ is the concentration of ionic species and $z_i$ the corresponding valence ($I$ is equal to 0.45 M for a 0.45 M NaCl and 0.49 M NaH$_2$PO$_4$ solution, and equal to 0.35 M for a 0.15 M Na$_2$SO$_4$ solution). In all cases where instability occurred, the scattered intensity increased almost linearly from the beginning of the incubation, without the lag phase typically encountered in amyloidogenic systems, indicating that the aggregate formation started immediately after incubation without a lag-phase. NaH$_2$PO$_4$ followed a behavior similar to NaCl, i.e., aggregation was observed only at sufficiently large concentration values, but aggregation was slower with NaH$_2$PO$_4$ than with NaCl. Instead, Na$_2$SO$_4$ showed a peculiar effect in accelerating the aggregation at low pH, which occurred even at low salt concentration (64 mM, corresponding to an ionic strength of 0.15 M).

To investigate such a peculiar effect, the initial light chain secondary structure in the presence of the sulfate and the chloride anion was characterized by CD spectroscopy and ANS binding, a method probing the solvent accessibility of hydrophobic patches. As shown in Figure 5.6a, with respect to the case in the absence of salt, in the presence of NaCl the minimum in the far-UV CD spectrum is decreased, indicating an increase of β-sheet structure; moreover, the minimum shifts from 218 to 217 nm. Such structural rearrangement is accompanied by a burying of hydrophobic patches, as indicated by the decrease of the maximum ANS fluorescence (Figure 5.6b) as a consequence of the reduced binding of the hydrophobic dye. In the presence of the sulfate anions, the light chain structure changes significantly: the far-UV CD spectrum shows a minimum at 204.6 nm, corresponding to a disordered structure (Figure 5.6a). In such a more open, disordered structure, with respect to the β-sheet, the hydrophobic patches expose more in the solvent.
Indeed, the ANS binding in this case is larger than that in the presence of the chloride anion, as indicated by the increase in maximum ANS fluorescence and decrease in $\lambda_{\text{max}}$ (blue shift) from 481 to 477 nm (Figure 5.6b). It is likely that such significant structural change forms an intermediate more prone to aggregate.

**Figure 5.6** Light chain structural changes induced by salt addition monitored by CD (a) and ANS binding (b). Experiments were performed at 20 °C for a 0.3 g/L protein solution in 25 mM PBS at pH 7.4 (···) and in 20 mM HCl buffer at pH 2.0 without salt (Δ), with 0.15 M NaCl (--) and 0.15 M Na$_2$SO$_4$ (—).
Since at low pH the protein is positively charged (pI=8.5-9), only anions are expected to affect intra and intermolecular interactions. To verify the absence of the cation effect on aggregation, an additional experiment in 20 mM HCl at pH 2.0 with 0.45 M KCl was performed (run 11 in Table 5.1). The result is compared with that with 0.45 NaCl in Figure 5.5c, confirming the absence of the cation effect for the investigated salts.

After considering aggregation at low pH, we have also investigated temperature- and denaturant-induced aggregation at physiological pH. Incubation at the unfolding temperature $T_u=55 \, ^\circ C$ (run 11 in Table 5.1) caused aggregate formation after few hours. Instead, incubation in 25 mM PBS at pH=7.4 with GuHCl in the concentration range from 1.3 to 1.5 M (run 12 and 13 in Table 5.1) could not induce any aggregation in the presence of either 0.15 M NaCl or 0.15 M Na$_2$SO$_4$. This result suggests that a generic partial protein unfolding is insufficient to promote aggregation, but specific unfolded configurations are necessary to induce aggregation.

### 5.5 Aggregation pathway and aggregate morphology

The aggregation pathway was investigated by several techniques taking as a reference condition run 8 in Table 5.1 (20 mM HCl buffer at pH 2.0 with 0.15 M Na$_2$SO$_4$, 37 $^\circ C$). In Figure 5.7a the time evolution of the hydrodynamic radius ($<R_h>$) followed on-line by DLS is shown. As mentioned above, the aggregation induced by the sulfate addition occurs without lag-phase. The dimer conversion was measured by taking samples at different times during the aggregation and analyzing them off-line by SEC and FFF. The results obtained by the two techniques were consistent, showing a significant decrease of the dimer peak in the chromatograms over time (data not shown). From the value of the area under the peak, the amount of the residual massive dimer fraction has been evaluated and shown in Figure 5.7b. It is seen that the dimer conversion is almost completed already after 6 hours while aggregation is still on-going (Figure 5.7a). This implies that aggregation does not occur via dimer addition only but also among larger aggregates. However, larger aggregates could not be detected with neither of the two techniques mentioned above, probably because of interactions with the stationary phases of the two instruments.
The change of secondary structure during aggregation was followed by spectroscopic techniques (intrinsic tryptophan, CD and ThT fluorescence). The intrinsic tryptophan spectra showed an increase in the maximum fluorescence value over time (Figure 5.7c), indicating that, as a consequence of structural rearrangements, the tryptophan residues exposed during denaturation at low pH become less solvent-exposed during the aggregation. The change in the protein secondary structure along the aggregation is confirmed by CD spectroscopy, as shown in Figure 5.7d. The far-UV CD spectra show a progressive significant shift from a random-coil structure to a more ordered β-sheet structure, with the minimum shifted from 205 to 216 nm.

Figure 5.7 Aggregation kinetics of light chain solution in 20 mM HCl at pH 2.0, T=37 °C, with 0.15 M Na₂SO₄ (run 8 in Table 5.1). Time evolutions of a) the average hydrodynamic radius <R_h>, measured on-line by in situ DLS; b) residual massive dimer fraction (m) evaluated by SEC (■) and FFF (○); c) Intrinsic tryptophan spectra; d) CD spectra. Experiments were performed at a protein concentration of 1 g/L for a) and b) and 0.3 g/L for c) and d).
Figure 5.8: Time evolution of ThT fluorescence values (●) and DLS intensity (♦) under the conditions of Run 8 in Table 5.1. Insert represents ThT values at longer incubation times.

Figure 5.9: Congo Red spectrum obtained by the difference between the samples and the blank solution: light chain aggregates after 10 h incubation under the conditions of Run 8 in Table 5.1 (---); stable light chain solution at pH 7.4 (…); insulin fibrils (—).

Despite the formation of more ordered β-sheet structure during aggregation, the aggregates showed low ThT signal increment with respect to the starting value, indicating lower ThT binding. The time evolution of ThT fluorescence as well as DLS intensity values is shown in Figure 5.8: despite the large increase of the light scattering intensity in the first 10 hours, the increase of ThT absolute fluorescence values is small even after one month of incubation, indicating a low increase in the β-sheet content. After such period, aggregates were analyzed also with Congo Red test, the most common and efficient test applied to
detect the presence of fibrils of light chain.\textsuperscript{179} The result of the test was negative, confirming the absence of fibrillar structure: in Figure 5.9 it can be seen how the spectra of the light chain aggregates and of the stable light chain solution are similar and show no maximum; on the other hand, the spectrum of insulin fibrils shows a clear maximum at 541 nm. The results confirm the absence of fibrillar, amyloidogenic structures in the light chain aggregates.

The aggregate morphology was further studied by AFM microscopy and hydrated thin film ATR-FTIR. In Figure 5.10 the pictures of samples taken after one month incubation in the conditions of runs 4, 6, 8 and 11 in Table 5.1 are shown. It can be seen that only spherical aggregates are visible for all the investigated conditions.

![AFM pictures](image)

**Figure 5.10** AFM pictures of light chain aggregates obtained after one month incubation in 20mM HCl at pH 2.0 with 0.45 M NaCl (a), with 0.15 M Na$_2$SO$_4$ (b) or with 0.49 M NaHPO$_4$ (c) and after two hours incubation in 25 mM PBS at pH 7.4, T=55 °C (d) (runs 4, 8, 6 and 11 in Table 5.1).
FTIR spectra of native state dimer and protein aggregates are reported in Figure 5.11, where the spectrum of the native dimer shows a maximum at 1641 cm⁻¹, characteristic of the β-sheet structure of the immunoglobulin fragment and corresponding to the minimum at 220 nm in CD spectrum in Figure 5.4a. The spectrum shows also a significant presence of disordered, α-helix and turns/loops structure, corresponding to the area between 1647 and 1695 cm⁻¹, in analogy to other spectra of variable domain reported in literature. In the aggregate spectra the percentage of the area between wavenumbers 1615 and 1640 cm⁻¹ with respect to the total area increases and the maximum shifts to 1635.4 cm⁻¹. Such changes indicate rearrangement of the secondary structure inside the aggregates and, particularly, an increase of the β-sheet structure content with respect to the native state, in agreement with the intrinsic tryptophan fluorescence and the CD data shown in Figure 5.7c and 5.7d, respectively. Nevertheless, the disordered, α-helix and turns/loops structures are still significantly present, and the absence of the predominance of the β-sheet structure confirms again the non-amyloidogenic nature of the formed aggregates. The shift of the maximum is less pronounced in the case of NaH₂PO₄, due to the slower aggregation kinetics.

![Figure 5.11 Thin film ATR-FTIR spectra of native light chain dimer (--) and of light chain aggregates obtained after one month incubation in 20 mM HCl at pH 2.0 with 0.45 M NaCl (---), with 0.15 M Na₂SO₄ (•••), or with 0.49 M NaH₂PO₄ (−−−) (runs 4, 8, and 6 in Table 5.1).](image-url)
The ability of the obtained aggregates to seed aggregation was investigated by adding fragments of aggregates obtained in 20 mM HCl buffer at pH 2.0 with 0.5 M Na₂SO₄ to a 1 g/L light chain solution. The aggregates were broken by sonication as reported by Kim et al.¹⁸⁶ using two different sonication times of 0.5 and 5 minutes. The seeds were added at a concentration of 0.1 and 0.2 g/L to a solution at physiological pH with Na₂SO₄ (runs 14 in Table 5.1): no aggregation was observed after several months. The results indicate that amorphous aggregates alone are unable to induce aggregation under the conditions where the peculiar, partially unfolded, configuration necessary to trigger aggregation is absent.

5.6 Discussion

In many studies related to protein aggregation it has been shown that the secondary structure thermodynamic stability and in vitro fibril formation propensity are strongly related.¹⁷²,¹⁸⁶-¹⁸⁹ Temperature, pH and denaturants, such as urea and guanidinium hydrochloride, at suitable concentrations may promote protein conformation changes, particularly total or partial protein unfolding, leading to aggregation.¹⁸¹,¹⁹⁰-¹⁹² The aggregation mechanism and kinetics as well as the morphology of the final products have been shown to depend on the specific unfolded intermediate structure.¹⁹³-¹⁹⁶ For instance, Khurana et al.¹⁸⁸ studied the aggregation of a recombinant non-amyloidogenic light chain variant (stable in physiological conditions) induced by pH, and found that amorphous aggregates were obtained at pH between 3 and 4, while at pH 2 fibrillar structures were formed. In another study,¹⁶⁸ the morphology of the aggregates obtained in vitro by a single variant was significantly affected by the chemical composition of biologically relevant lipid-derived aldehydes added into the system. Also in this case, aggregation occurred via protein secondary structure changes. These examples clearly underlined the important role played by the protein secondary structure in the delicate balance between electrostatic and hydrophobic interactions responsible for the protein stability.¹⁹⁷

Another physical factor significantly affecting the stability behavior of the light chain is the protein native oligomeric state, i.e., monomer, dimer, and their equilibrium state. In
the case of an amyloidogenic variant a single amino acid mutation located at the dimer interface could cause a significant difference in the dimer stability, shifting the equilibrium versus the monomer and promoting aggregation.\textsuperscript{198} This finding is supported by the fact that fibril formation kinetics was accelerated when monomer-dimer equilibrium was shifted versus monomer by decreasing the total protein concentration or by denaturant addition.\textsuperscript{172,181,199} In the proposed aggregation schemes, fibrillation proceeded only via monomer addition, while dimers were responsible for the off-pathway amorphous oligomers observed together with amyloid fibrils.

The results shown in this work indicate that the light chain dimer is very stable in physiological conditions and dimer denaturation is a necessary but not a sufficient condition to induce aggregation. This follows from our observation that protein denaturation either by shifting the pH from 7 to 2 or by adding GuHCl at physiological pH was not resulting in any instability. Instead, aggregation was observed at low pH when salt was added at large concentrations or at physiological pH when the solution was heated to the protein $T_m$. The results indicate that a specific configuration change is necessary to induce aggregation in globular proteins.

From a kinetic point of view, we observed that when aggregation occurs it starts immediately after incubation without any lag-phase, as indicated by DLS data (Figures 5.5 and 5.7a). The aggregation is accompanied by structural rearrangements which decrease the solvent exposure of tryptophan residues (Figure 5.7c) and increase the protein β-sheet content (CD data in Figure 5.7d). Such increase in the β-sheet content in the aggregates compared to the native state is confirmed by FTIR analysis (Figure 5.11). Despite such increase, the aggregates retain a large amount of random coil, α-helix and loops structures and do not bind ThT or Congo Red dye as amyloid fibrils do. AFM confirms the absence of fibrils and shows the presence of amorphous spherical aggregates (Figure 5.10). Moreover, the addition of such amorphous aggregates in native proteins is unable to induce aggregation in physiological conditions, most probably because they cannot induce the necessary conformational change in the native fold protein. At least for the reference reaction (run 9 of Table 5.1) in FFF and SEC chromatograms, the decrease in the dimer peak was not accompanied by the increase in the monomer peak, indicating that
aggregation was not occurring via monomer formation. The lack of monomer formation and the absence of fibrils, together with the observations reported in the literature, suggest that the monomer is likely the only repetitive unit present in the path-way to amyloid fibrils, while dimers are responsible for amorphous aggregates.

Considering the experimental evidences previously discussed, we proposed the reaction scheme shown in Figure 5.12: under suitable conditions the native dimer is partially denatured into a reactive species $D^*$, which may be in equilibrium with the native dimer $D$. $D^*$ has suitable conformational characteristics to aggregate further irreversibly into larger amorphous aggregates.

$$D ⇌ D^* \rightarrow \text{Aggr.}$$

**Figure 5.12** Scheme of aggregation mechanism. $D^*$ represents the salt-induced or temperature-induced intermediate dimer prone to aggregate.

Of particular interest is the fact that one of the few conditions where aggregation was observed is the addition of salt at low pH. The effect of the salt on the stability of the light chain dimer can be explained by a combination of several factors. First of all, salt ions at low salt concentration screen the repulsive electrostatic interactions between proteins, as described by the DLVO (Derjaguin-Landau-Vervey-Overbeek) theory (see Introduction section). Second, at the large concentrations considered in this work (0.15-0.45 M), additional, anion binding effects must be considered, which may change the protein surface chemistry and therefore the protein secondary structure, inducing a conformation more prone to aggregate. Further, salt-protein preferential exclusion may lead to salting-out effect, resulting in protein precipitation. Unlike unspecific salt screening effects, such effects are strongly ion specific. In this work only anions have been found to affect aggregation kinetics, while the investigated monovalent cations (Na and K) do not have any effect. The specific anion effect on several biological phenomena has been widely reported in the literature and often compared to the so-called Hofmeister series based on
anion capacity to affect protein stability, protein precipitation and water structure. Despite the large use of the Hofmeister series, its molecular origin is still under debate.\textsuperscript{200} When compared at the same concentration and similar ionic strength, the order of the anions in accelerating \textit{in vitro} light chain aggregation found in this work is: $\text{SO}_4^{2-} > \text{Cl}^- > \text{H}_2\text{PO}_4^-$, somehow in disagreement with the Hofmeister order: $\text{SO}_4^{2-} > \text{H}_2\text{PO}_4^- > \text{Cl}^-$.\textsuperscript{200}

The peculiar effect of the sulfate anion in promoting aggregation has also an \textit{in vivo} biologically relevance due to the involvement of sulfonated glycosaminoglycans (GAGs) in amyloid fibril formation of several proteins (ex. light-chain,\textsuperscript{182,203-205} $\beta_2$-microglobulin,\textsuperscript{206} transthyretin,\textsuperscript{207} human muscle acylphosphatase,\textsuperscript{208} gelsolin\textsuperscript{209}). Despite the large number of reported evidences, a clear explanation of the mechanism by which GAGs and sulfate anion promote aggregation is still lacking. In this work, we show that not only fibrillation but also aggregation into amorphous aggregates is accelerated by the sulfate anion. The peculiar effect of the sulfate may be explained considering several properties of bivalent anions: 1) at a given concentration, the screening of electrostatic repulsion of a bivalent anion is larger with respect to monovalent anions; 2) a bivalent anion can act as a bridge between two positively charged proteins favoring their aggregation;\textsuperscript{210} 3) according to the Hofmeister series, sulfate is the most kosmotropic anion with the largest propensity to induce salting-out effect without changing the native structure; 4) the bivalency promotes a more effective anion binding, which may significantly changes intramolecular interactions, stabilizing an unfolded intermediate with respect to the native state. The experimental results indicate that, at least for the dimer investigated in this work, at low pH the last one of the above effects is predominant: sulfate anion binding induces a disruption of the $\beta$–sheet structure of the immunoglobulin variant into a more disordered structure, which apparently exhibits larger propensity to aggregate. It is worth noting that at physiological pH the protein has a lower net positive charge and anion binding is significantly reduced. Indeed, no aggregation was observed at physiological pH, even in presence of Na$_2$SO$_4$ or GuHCl.
5.7 Concluding remarks

In this work, a non-amyloidogenic light chain dimer has been expressed in human myeloma cell line U266 adapted to low serum media, and its *in vitro* aggregation behavior has been investigated. It is found that the dimer is very stable in physiological conditions and aggregation is observed only when specific denaturing conditions are applied.

Aggregation starts immediately without any lag phase or nucleation process and forms spherical, amorphous aggregates. The impossibility to obtain fibrils *in vitro* from a light chain dimer has a significant relevance also for the *in vivo* systems: the results of this study together with data reported in literature suggest that the presence of light chain monomer is fundamental for the formation of amyloid fibrils, while dimers are responsible for oligomers or amorphous deposits.

It is found that at low pH the salt anion has a significant specific effect on protein aggregation kinetics according to the following order: $\text{SO}_4^{2-} >> \text{Cl}^\cdot > \text{H}_2\text{PO}_4^\cdot$. In particular, the sulfate anion accelerates aggregation by inducing protein secondary structure change.

The light chain sequence has been characterized in order to allow comparative analysis with following studies investigating the features of amyloidogenic light chains with a known sequence. Such variants will be analyzed in the same way as the non-amyloidogenic fragment considered in this work. The comparison among variants with different aggregation propensity and biological consequences would potentially shed insights into the correlation between protein physicochemical properties, protein aggregation and organ disfunction.
Chapter 6

Aggregation stability of monoclonal antibodies during downstream processing

6.1 Introduction

Thanks to increased understanding of the biological background of various human diseases, protein drugs have been increasingly developed and now occupy a significant part of the whole pharmaceutical market. Several therapeutic proteins versus cancer, rheumatoid arthritis and other immunological disorders are currently approved or in clinical trials. Particularly, monoclonal antibodies represent the most rapidly growing product inside the family of recombinant therapeutic proteins. The successful commercialization of protein drug is often limited by instability problems, which can be both chemical (e.g., deamidation, oxidation, hydrolysis) and physical (e.g., conformational changes, adsorption to surfaces, aggregation). Protein aggregation represents the most common form of all possible deteriorations and can basically be encountered in each step of the production process. The spectrum of the possible products resulting from aggregation is wide. The several types of aggregates may be classified in covalent/non-covalent, reversible/irreversible, native/denatured, soluble/non-soluble. Obviously, protein aggregation in pharmaceutical products must be avoided since it usually leads to inactivation of the drug, and can even trigger an immunogenic reaction of the organism. Since the debate on the possible immunoresponse created by aggregates is still open, strict specification limits are imposed by the drug manufacturers as a precaution.

Several external factors may induce protein instability during production, storage, transportation and handling of the pharmaceutical component: temperature, buffer formulation (pH, presence of solutes, co-solvents, surfactants, salts), mechanical...
stress\textsuperscript{218,219} and surface interaction\textsuperscript{220}. Such environmental factors affect at a molecular level the protein-protein interactions\textsuperscript{201} with consequences on the two main aspects involved in the aggregation process: the colloidal stability of the protein solution and the thermodynamic folding of the protein.\textsuperscript{33} The two processes are strictly connected one to another and are often inseparable.

Salt has a particular effect on protein properties, including protein solubility and aggregation stability. “Salting-out” of proteins is commonly applied in protein crystallization and bioseparations.\textsuperscript{221} Since the discovery of Franz Hofmeister in 1888 it is known that the propensity of salts to induce protein precipitation is ion specific.\textsuperscript{222} Hofmeister determined an ion series, known as the Hofmeister series or lyotropic series. Originally, the specific ions effect was related to capacity of ions in breaking or preserving the structure of water. In the Hofmeister series the ions are ranked from the smaller, tightly hydrated (kosmotropic) ions to the larger, less hydrated (chaotropic) ions:
\[
\begin{align*}
\text{SO}_4^{2-} & > \text{HPO}_4^{2-} > \text{Cl}^- > \text{NO}_3^- > \text{ClO}_4^- \\
\text{NH}_4^+ & > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ca}^{2+}
\end{align*}
\]
The Hofmeister series was later observed in many chemical-physical phenomena\textsuperscript{223} ranging from polymers and colloids\textsuperscript{224} to biological systems,\textsuperscript{202,225} with only slight modifications in the ranking.\textsuperscript{217,225,226} When pH is larger than the protein isoelectric point (pI), the anion effect on salting-out follows the direct Hofmeister series, while if pH is below the pI the reversed series is observed.

Although great attention is given by the physics community to salt-protein and salt mediated protein-protein interactions,\textsuperscript{13,227-229} the molecular origin of the Hofmeister series and of the reverse behavior below protein pI is still unclear.\textsuperscript{226} Moreover, many other puzzling experimental observations of protein-salt solutions, including re-stabilization of protein colloids at high ionic strength,\textsuperscript{230} are poorly understood.

Salt-induced protein aggregation and gelation can be seen as special cases of salting-out where the protein is assembling mainly in a non-native configurational state. The possible final products cover a wide range of sizes and can show either ordered structures or amorphous morphology. Important examples are found not only in pharmaceutical science but also in biomedical research and food technology. A specific anion effect has been
reported for the in vitro aggregation of several amyloidogenic proteins (e.g., A-β,\textsuperscript{231} α-synuclein,\textsuperscript{232} prion protein\textsuperscript{233}), an important class of proteins involved in human neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Huntington’s and prion diseases.

The aggregation propensity of a given protein depends not only on the environment but also on the intrinsic primary sequence and structure of the protein. Antibodies (mAbs) are multi-domain proteins consisting of a constant domain (Fc) and two identical domains containing the variant region (Fab). IgG is one class of immunoglobulins showing several subclasses in humans (IgG1, IgG2, IgG3 and IgG4),\textsuperscript{234} which share the constant Fc domain and differ in the variable Fab domain. In order to correlate aggregation propensity and structure properties, several recent works compare different IgG subclasses.\textsuperscript{235-237} Particular attention is given in elucidating the role of the single domains (Fc and Fab) in protein self-assembling. In parallel with the experimental approach, in silico studies try to identify aggregating-prone regions or “hot” spots in order to optimize protein sequence engineering against aggregation.\textsuperscript{238,239} Protein glycosilation is also found to have an important role in preventing aggregation.\textsuperscript{240}

The large number of intrinsic and extrinsic factors involved in protein stability complicates the rationalization of the problem. On the one hand, different proteins share common features in aggregation behavior: aggregation propensity is correlated to general physicochemical properties of the protein\textsuperscript{28} and the different observed aggregation mechanisms have been classified in a limited number of classes.\textsuperscript{32,194} On the other hand, it is still not possible to predict a priori the stability of a given protein, and the optimization of formulations and processing conditions requires ultimately specific studies. Investigation of the aggregation mechanism and of analogies among different proteins under different operating conditions may allow us reaching general conclusions about the environmental or structure protein characteristics which determine its aggregation.

In this work, we focus on the aggregation of an IgG immunoglobulin during its production process and specifically on the downstream, i.e. the purification portion of it. During this step the conditions of pH and buffer composition are changed in order to favor chromatographic adsorption and desorption. These conditions favor also the formation of
aggregates which have to be removed, thus leading to significant loss in the process yield.\textsuperscript{241} In particular, we investigate the conditions relevant for virus inactivation and Protein A chromatography, which represents the typical capture process in antibodies purification.\textsuperscript{242} The effect of several operating parameters (i.e., pH, protein concentration, buffer nature, concentration of salts) and of antibody subclass on the protein solution stability is investigated.

In the first part of the chapter, the stability of an IgG2 under conditions leading to formation of reversible oligomers is analysed by a combination of several orthogonal techniques. The oligomer formation and distribution have been continuously followed on-line by dynamic light scattering (DLS) and off-line by size exclusion chromatography (SEC) and asymmetric field flow fractionation (FFF). Circular dichroism (CD) studies were also performed to correlate aggregation tendency with change in the protein secondary structure. Moreover, on-line and off-line data have been compared to give important information about the reversibility of the aggregation. The obtained oligomer information during aggregation is then modelled in the frame of a modified Lumry-Eyring model,\textsuperscript{30,32,243} which considers the native monomers to reversibly unfold into a partially unfolded, aggregation-prone state.\textsuperscript{188} This part represents also a general procedure for analyzing protein oligomers, which, produced in the early stages of the aggregation, are considered to be crucial, toxic species in many diseases.\textsuperscript{244} An example is represented by the amyloidosis diseases related to aggregation of light chains of antibodies discussed in Chapter 5.\textsuperscript{166} Since the size of the light chain fragments is not much different from that of the complete antibody and the two proteins share common characteristics, the approach described in this work may be used to detect light chain oligomers during aggregation.

In the second part of the chapter, we deepen the investigation of the salt effect in triggering the aggregation. On this purpose, we quantify the aggregation kinetics for different salts and salt concentration in the range 0-200 mM at different pH values, using size exclusion chromatography (SEC). We show how the salt effect is strongly ion specific and pH dependent, and we identify the presence of a maximum of aggregation extent as a function of salt concentration. Moreover, we discuss the salt effect by correlating the aggregation data to the experimental investigation of protein structure and solution surface
tensions. The work provides general considerations on the interactions between ions and macromolecules, which can be applied to other systems including amyloidogenic proteins\textsuperscript{232} and polymers.\textsuperscript{224}

In the third part of the chapter, we identify operative conditions under which oligomers are not stable and we study the kinetics and mechanism of the aggregation/precipitation process.

Finally, we investigate two more IgG1 proteins and explore a sufficiently large range of operating conditions in order to compare the aggregation mechanism and the stability characteristics of different antibodies. The aggregation data have been correlated to protein structure, thermal stability, hydrophobicity and charge.

6.2 Materials and Methods

Antibodies and chemicals
IgG2 (pI between 7.35 and 8.15), IgG1.1 (pI about 8.5), and IgG1.2 (pI between 8.5 and 9) with MW ~ 150 kDa were stored as 45 g/L solutions in 25 mM sodium phosphate at pH 6.0 with 100 mM NaCl at 4 °C.

All buffers for aggregation studies were filtered using 0.22 μm cut-off sterile syringe filters Pall\textregistered Acrodisc\textregistered 32 mm (Pall Life Sciences, NY, USA). 0.5 g/L of sodium azide was added to all solutions to prevent formation and proliferation of bacterial growth. All chemicals were supplied by Sigma Aldrich (Buchs, CH).

Dynamic Light Scattering (DLS)
The average size of the aggregates or oligomers was measured on-line by the \textit{in-situ} dynamic light scattering (DLS) technique, using a Zetasizer Nano (Malvern, UK). All samples were filtered with a 0.02 μm cut-off, Anotop 10 syringe filter (Whatman, Kent, UK) before starting the aggregation. The solutions for aggregation studies were prepared by manually diluting the mother solution to 1 g/L by selected buffer solutions. For each condition at least three independent samples were prepared and analyzed and average values were considered.
Size Exclusion Chromatography (SEC)

Samples were analyzed by size exclusion chromatography (SEC) technique with a Superdex 200 10/300 GL, 10 mm x 300 mm size-exclusion column (GE Healthcare, Uppsala, Sweden) assembled on a Agilent 1200 series HPLC unit (Santa Clara, CA, USA) consisting of a quaternary pump with degasser, an autosampler with a cooling unit, a column oven, and a DAD detector. Each sample was eluted for 60 min at a constant flow rate of 0.5 mL/min using as mobile phase a 100 mM sodium sulphate, 25 mM Na₂HPO₄ solution at pH 7.0, filtered with a 0.45 μm cut-off, Durapore membrane filter (Millipore, Billerica, MA, USA). The UV absorbance peaks were detected at 280 nm and 220 nm.

Field Flow Fractionations (FFF)

Paralleling with the SEC technique, the asymmetrical flow field flow fractionations (AF4) technique was also used to analyze the oligomer composition. The applied instrument was an AF4 Eclipse 3+ (Wyatt, Santa Barbara, CA, USA), coupled with a 1200 Series isocratic pump from Agilent (Santa Clara, CA, USA). A 275 mm LC channel for aqueous solvents was used for Eclipse 3, with a trapezoidal spacer (350 μm thick, 26.5 cm long) and a Nadir reg. cellulose membrane with 10 kDa cut-off at the bottom (Wyatt, Dernbach, Germany). The detector flow was set constant at 1 mL/min, and a step gradient of cross flow from 5 ml/min to 0 ml/min was applied after 30 min. 25 mM citric acid solution at pH 3.0 was used as mobile phase, after filtration through a 0.1 μm cur-off, Durapore membrane filter (Millipore, Billerica, MA, USA).

Circular Dichroism (CD)

For the variations in the secondary structure of the antibody during aggregation, we have monitored the Far-UV circular dichroism (CD) spectra of the same antibody solutions using a Jasco-815 CD spectrophotometer (Mary’s Court, Easton, MD) with the temperature of the cell holder controlled at 25 °C. A quartz cuvette with 0.1 cm path length was used. Spectra obtained after buffer subtraction were corrected for protein concentration and smoothed using the Savitsky-Golay function. Thermal stability of 0.3 g/L protein solutions in 25 mM citric acid buffer at pH 3.0 without salt was evaluated by
recording the spectra at several temperatures heating from 15 °C to 90 °C with a step of 1 °C. The protein unfolding was monitored at 210 nm.

**Intrinsic Tryptophan Fluorescence (Trp)**

The fluorescence analysis were performed on a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA, USA) by exciting 0.3 g/L protein solutions at 295 nm and collecting emission spectra between 305 and 450 nm.

**Thioflavin-T (ThT) and 8-Anilinonaphthalene-1-sulfonate (ANS) binding**

ThT and ANS fluorescence were measured using an EnSpire 2300 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) with a standard 96 wells black plate with a transparent bottom (ProxiPlate-96, Perkin Helmer, USA). For kinetics studies, 25 μM ThT or ANS were added to the investigated solutions: ThT emission at 485 nm was recorded after excitation at a wavelength of 450 nm, while ANS emission at 490 nm was recorded after excitation at a wavelength of 403 nm. To avoid heterogeneity in the measurements, fluorescence values were averaged over 9 different points inside each well (grid 3×3). Three repetitions were performed for each sample.

Emission spectra of 0.3 g/L light chain solution in several buffers with 15 μM ANS were collected at 20°C between 420 and 600 nm using 380 nm as excitation wavelength.

**Zeta Potential**

Zeta potential values of proteins (z) have been evaluated by a Zetasizer Nano (Malvern, Worcestershire, UK) measuring the electrophoretic mobility (μ) via laser Doppler effect. From the electrophoretic mobility the zeta potential is calculated according to Henry equation: \[ \mu = \frac{2 \cdot \varepsilon \cdot z}{3 \cdot \eta} \], where \( \varepsilon \) and \( \eta \) are the dielectric constant and the viscosity of the medium, respectively. 1 g/L protein solutions in 25 mM citric acid buffer in the pH range from 3.0 to 6.0 and in 25 mM phosphate buffer in the pH range from 7.0 to 9.0 have been measured at 25 °C. Five repetitions of three independent samples were recorded for each condition and average values have been considered.
Surface Tension measurements

Surface tension values were measured using a DCAT 21 tensiometer with a PT11 Platinum-Iridium Wilhelmy Plate (dataphysics, Filderstadt, Germany). The sample temperature was fixed at 25°C. 0.1 g/L lysozyme (Sigma Aldrich, Buchs, Switzerland) solutions were filtered using 0.1 µm cut-off sterile syringe filters PALL® Acrodisc® 32 mm (PALL Life Sciences, Port Washington, NY, USA). For each condition, 3 repetitions of 5 independent samples were performed in order to ensure experimental reproducibility.

6.3 Reversible Oligomer Formation

6.3.1 Experimental observation: pH effect

As already mentioned in the Introduction, protein A affinity chromatography is commonly used as the capture step for purification of monoclonal antibodies and Fc fusion proteins in drug industry. The recovery of the product bound to the stationary phase requires the elution with a low pH mobile phase (usually between pH 3.0 and 4.0), which is well known to potentially induce aggregation. Particularly, low pH values can lead to changes in the structure of the Fc domain, while Fab fragments have been shown to be more sensitive to temperature variations. Moreover, highly concentrated antibody solutions, which are desired for the economy of large scale purification processes, further increase the risk of inducing aggregation under low pH conditions. The effect of pH was first investigated for a solution of 1 g/L IgG in 25 mM citric acid buffer with 0.15 M NaCl (a buffer commonly used in protein A chromatography) at several pH values. The stability of such solutions was followed on-line by in-situ DLS. The time evolution of the average hydrodynamic radius $\langle R_h \rangle$ of the distribution of IgG monomers and oligomers is shown in Figure 6.1. A typical example of the reproducibility of the data is shown in Figure 6.1a, where several independent samples incubated under the same conditions are compared. It can be noticed the very good reproducibility of the data. Similar reproducibility has been found for all the other conditions and the small error bars will not be reported in the following.
Figure 6.1 a) Reproducibility of the time evolution of the average hydrodynamic radius $\langle R_h \rangle$, measured online by *in-situ* DLS, for the 1 g/L IgG solution in 25 mM citric acid buffer with 0.15 M NaCl at pH 3.0; b) the same as a) but at pH 3.0 (●), 3.5 (□), 4.0 (◊), 4.5 (■) and pH 6 (○); c) the same as b) but for the 1 g/L IgG solution in 25 mM phosphate buffer (○), 25 mM citric acid buffer (◊) and 25 mM acetic acid buffer (□) with 0.15 M NaCl at pH 3.0.
In Figure 6.1b it is seen that for pH values between 4.5 and 6.0 the solutions are very stable ($\langle R_h \rangle = 6$ nm is in fact the value corresponding to the monomer IgG under examination), while aggregation occurs when pH drops below a critical value of about 4.0.

Moreover, for all cases where aggregation occurs, the aggregation starts immediately after incubation without any lag-time, which is commonly encountered in other protein aggregating systems. From the average $\langle R_h \rangle$ values shown in Figure 6.1b, only small oligomers are produced (probably no more than 2 or 3 monomer units) and basically stop growing after about 1.5 h. No macroscopic changes in the solutions can in fact be observed even after 1 month of incubation.

**Buffer and salt concentration effect**

To observe the effect of the ionic strength and the nature of the buffer on the IgG aggregation, several experiments at different salt concentrations and in different buffers (phosphate, citric acid and acetic acid buffer), but at a constant pH 3.0, were performed. The conditions for all the experimental runs are described in Table 6.1.

The stability of the IgG solutions in the concentration range from 1 to 5 g/L was first checked in various buffers, typically used in protein A chromatography, without adding additional salts (runs 1 to 4 in Table 6.1). All antibody solutions were stable after 4 weeks, with an average hydrodynamic radius of around 6 nm, which depends slightly on the type of buffer.

The addition of sodium chloride to different buffers leads to different consequences. Figure 6.1c shows the time evolution of $\langle R_h \rangle$ for 1 g/L IgG solutions at 0.15 M sodium chloride in three different buffers. In the case of phosphate buffer the antibody solution remains stable for more than 3 weeks, while in acetate and citric acid buffers aggregation occurs with very similar kinetics and extent. Next, for the acetic and citric acid buffers, the effect of the NaCl concentration on IgG aggregation has been investigated in a large range of NaCl concentrations between 0 and 0.5 M. Figure 6.2 shows the time evolutions of $\langle R_h \rangle$ for the IgG solutions in the citric acid buffer at different salt concentrations (runs 4, 5, 6, 9 and 10 in Table 6.1), and the corresponding CD spectra after 24 h of incubation are shown.
in Figure 6.3b, while in Figure 6.3a a typical example of reproducibility of CD data is reported. It can be seen that as the salt concentration increases, the aggregation extent increases, and the minimum (at 217 nm) in the CD spectra, corresponding to the β-sheet structure of the immunoglobulin, moves downwards more and more, indicating that the aggregation is accompanied by an increase in the more ordered β-sheet structure content.

Table 6.1 Summary of experimental runs. All experiments were performed at pH 3.0.

<table>
<thead>
<tr>
<th>Run</th>
<th>IgG Conc. (g/L)</th>
<th>Buffer</th>
<th>NaCl Conc. Mol/L</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 to 5</td>
<td>25 mM Phosphate</td>
<td>0</td>
<td>Stable</td>
</tr>
<tr>
<td>2</td>
<td>1 to 5</td>
<td>0.1 M Glycine-HCl</td>
<td>0</td>
<td>Stable</td>
</tr>
<tr>
<td>3</td>
<td>1 to 5</td>
<td>25 mM-1 M Acetic Acid</td>
<td>0</td>
<td>Stable</td>
</tr>
<tr>
<td>4</td>
<td>1 to 5</td>
<td>25 mM Citric Acid</td>
<td>0</td>
<td>Stable</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>25 mM Citric Acid</td>
<td>0.1</td>
<td>Unstable</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>25 mM Citric Acid</td>
<td>0.15</td>
<td>Unstable</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>25 mM Citric Acid</td>
<td>0.15</td>
<td>Unstable</td>
</tr>
<tr>
<td>8</td>
<td>0.1</td>
<td>25 mM Citric Acid</td>
<td>0.15</td>
<td>Unstable</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>25 mM Citric Acid</td>
<td>0.3</td>
<td>Unstable</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>25 mM Citric Acid</td>
<td>0.5</td>
<td>Unstable</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>25 mM Acetic Acid</td>
<td>0.1</td>
<td>Unstable</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>25 mM Acetic Acid</td>
<td>0.15</td>
<td>Unstable</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>25 mM Acetic Acid</td>
<td>0.3</td>
<td>Unstable</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>25 mM Acetic Acid</td>
<td>0.5</td>
<td>Unstable</td>
</tr>
</tbody>
</table>
In the case of 0.3 M NaCl in Figure 6.2 (run 6 in Table 6.1), the corresponding time evolution of the CD spectra has also been measured and shown in Figure 6.3c. It is evident that the minimum in the CD spectra moves progressively downwards with time. However, after a few hours, the change in the spectra for different times (e.g., at 22h and 48h) becomes insignificant. This is consistent with the aggregation extent measured by DLS in Figure 6.2, indicating that antibody aggregation and change in secondary structure occur simultaneously.

**Figure 6.2** Time evolutions of the average hydrodynamic radius, measured on-line by the *in-situ* DLS, for the 1 g/L IgG solution in 25 mM citric acid buffer at pH 3.0 and different salt concentrations: 0 M (○), 0.1 M (■), 0.15 M (▲), 0.3 M (●), 0.5 M (◊) (Runs 4, 5, 6, 9 and 10 in Table 6.1).

In the case of the acetic acid buffer, the observed aggregation behavior and changes in the secondary structure for the experimental runs 11 to 14 in Table 6.1 are very similar to those shown above in the case of the citric acid buffer. Thus, for brevity, the data are not shown, but the conclusions reached above are also valid for the acetic acid buffer. On the other hand, this is not the case for the phosphate buffer where, as shown in Figure 6.1c no aggregation occurs.
Figure 6.3 a) Reproducibility of CD spectrum for the 1 g/L IgG solution in 25 mM citric buffer at pH 3.0; b) CD spectra of the same solution as in a) after 1 day incubation at different salt concentrations: 0 M (1), 0.1 M (2), 0.15 M (3), 0.3 M (4), 0.5 M (5); b) CD spectra for Case 4 in a), measured at different aggregation times: 0 min (1), 10 min (2), 30 min (3), 50 min (4), 90 min (5), 22 h (6), 48 h (7).
Oligomer distribution

Samples at different incubation times were taken and analyzed by SEC and FFF. Examples of the obtained oligomer distribution are shown in Figure 6.4, corresponding to run 6 in Table 6.1. The distributions obtained from the two techniques are consistent (note that in FFF larger species elute after smaller ones, while in SEC they elute in opposite order). At time=0, only the monomers and a small (negligible) fraction of dimers are present. After 3 h incubation, a sharp decrease in the monomer concentration and formation of a significant quantity of dimers can be observed. However, after the first few hours, both the FFF and SEC chromatograms show that the concentrations of both monomers and dimers vary very slowly, which is in good agreement with the DLS data in Fig. 6.1 and the CD spectra in Figure 6.3c.

In the FFF chromatograms in Figure 6.4a the dimer peaks are asymmetrical and not well separated from the peaks of trimers and larger species while the separation is much more clear in the SEC chromatograms. Nevertheless, a clear shoulder around the elution time of 15 min can be observed, representing proper separation between dimers and trimers. From both the SEC and FFF chromatograms, the mass fractions of each species can be evaluated based on the ratio of the area under the corresponding peak and the total area. The underlying assumption of such approach is that the absorbance capacity of the oligomers depends linearly on their size, i.e., a dimer absorbs twice as a monomer, a trimer three times, and so forth. As a confirmation of this assumption, in all the experimental runs the total area remained constant along the aggregation time, in both SEC and FFF techniques.

In Figure 6.4c a typical example of the reproducibility of SEC data is shown. It can be seen how, in analogy with DLS and CD data, also the SEC data are very well reproducible.

Moreover, the assignment of the peaks in the chromatograms to monomer, dimer and trimer is confirmed by the molecular weight measurements of the fractionated samples, obtained by static light scattering, as shown in Figure 6.4d.
Figure 6.4 a) FFF chromatograms at different times along the aggregation for run 6 in Table 6.1; b) SEC chromatograms at different times for the same system in a); c) Reproducibility of residual monomer evaluated from SEC chromatograms for Run 6 in Table 6.1; d) Molecular weight measurements of the fractionated sample obtained by static light scattering. The oligomerization was accelerated by incubating the sample for 1 h at 37 °C.

It is known that both SEC and FFF can be affected by several parameters during the analysis such as interactions between proteins and the stationary phase, dilution effect of the injected sample, shear-induced aggregation during the focus mode of FFF, etc. Thus, it is important to check the reliability of the obtained distributions. Moreover, comparing the results from different techniques can give information about the strength of the formed aggregates and their reversibility. For example, weakly aggregated oligomers would be destroyed easily upon dilution during the SEC or FFF analysis, and the obtained distributions would be inconsistent with the on-line DLS measurements. To verify this, we have coupled an on-line DLS system (Wyatt, Dernbach, DE) with the SEC column, so as
to estimate the hydrodynamic radius, $R_h$ of dimer and trimer, which were found to be equal to 7.3 and 9.7 nm, respectively. These values are then used to calculate the time evolution of the $Z$-average hydrodynamic radius of the same system based on the following well-known relationship:\(^{247}\)

$$\langle R_h \rangle = \frac{\sum_i n_i(t) \cdot i^2}{\sum_i n_i(t) \cdot i^2 \cdot R_{h,i}} \quad (6.1)$$

where $i$ is mass of the oligomer (i.e., the number of the monomeric IgGs forming the oligomer), while $n_i$ and $R_{h,i}$ are the number concentration and the hydrodynamic radius of the oligomer with mass $i$, respectively. In particular, in the $\langle R_h \rangle$ computations using Eq. 1 for each corresponding system, we have applied the oligomer distributions $n_i$ that are determined from the analyses of the SEC and FFF techniques, respectively.

**Figure 6.5** Time evolutions of the average hydrodynamic radius measured on-line by the *in-situ* DLS for runs 6 and 9 in Table 6.1, compared with those reconstructed from the oligomer distribution data obtained from FFF and SEC techniques. Run 6: DLS (■), SEC (○), FFF (△); Run 9: DLS (●), SEC (□), FFF (◊).

In Figure 6.5, we have compared such computed time evolutions of $\langle R_h \rangle$ to those measured directly on-line by the *in-situ* DLS technique, in the cases of run 6 and run 9 in Table 6.1. It can be seen that the agreement between the three techniques is very good. Therefore, the obtained oligomer distributions from the SEC and FFF techniques are reliable and reflect
the true oligomer distributions in the system. Moreover, these results indicate that the oligomers formed during aggregation are strong enough to survive during the SEC and FFF fractionation processes.

![Figure 6.6](image)

**Figure 6.6** Distributions of the oligomers determined from the SEC technique for (a) sample from run 7 in Table 6.1 after 4 hours incubation, (b) the same as (a) but diluted to 1 g/L IgG concentration in 25 mM citric acid buffer at pH 3.0 without adding salt and the analysis performed after 1 hour, and (c) the same as (b) but the analysis performed after 48 hours.

Furthermore, we have also investigated the long-term reversibility of the aggregation process. To this aim, we have collected two samples after 4 hours incubation in the case of run 7 in Table 6.1: one sample was immediately analyzed by SEC, while another was diluted tenfold in a salt-free citric acid buffer, so as to alter the equilibrium, and analyzed by SEC after one hour and after 48 hours. The results, shown in Figure 6.6, indicate that aggregation is indeed reversible, and the monomers can be recovered by dilution in a salt-free buffer. It should be pointed out, however, that further investigations are required to understand whether the recovered monomers have the original secondary structure or keep the same structure as in the oligomers.
6.3.2 Kinetic Modelling of the Oligomer Formation

Protein aggregation induced by conformational changes is commonly described by the Lumry-Eyring model, developed in 1954. According to this model, a native protein unfolds in a reversible way into an unfolded state which then aggregates irreversibly. Several recent studies published in the literature showed that not only the completely unfolded species but also different types of intermediates (near native or partly folded) can be the aggregation-prone form of the monomeric species.

\[
N \xrightleftharpoons[K_{eq}]{k_{eq}} I
\]

\[
K_{eq} = \left( \frac{[I]}{[N]} \right)_{eq} = \frac{k_{un}}{k_{fold}}
\]

\[
I + I \xrightarrow[k_{-1}]{k_{1}} D
\]

\[
I + D \xrightarrow[k_{-2}]{k_{2}} T
\]

**Figure 6.7** Scheme of the aggregation process based on a modified Lumry-Eyring model.

In the present work, following the approach of Roberts, we modify the Lumry-Eyring model to describe the IgG aggregation process, by taking into account the reversibility of each step. The main assumptions involved in the model are:

- A unique reactive intermediate prone to aggregation is present, indicated as \( I \). With respect to the aggregation process, the intermediate (\( I \)) and native (\( N \)) species can reach thermodynamic equilibrium instantaneously, which can be described through the equilibrium constant \( K_{eq} = \frac{k_{un}}{k_{fold}} \).
- Two \( I \) species can aggregate to form a dimer (\( D \)). Larger oligomers are formed only by subsequent addiction of an \( I \) unit, while aggregation between two oligomers has been neglected.
- Since the observed oligomers larger than trimer are negligible, trimers (\( T \)) have been considered as the largest species present in the system.
All the aggregation steps have been considered reversible and characterized by intrinsic forward and backward rate constants. Note that reversibility was not taken into account for in the original Lumry-Eyring model, but it has been observed in several studies.\textsuperscript{30,252}

The resulting reaction scheme is shown in Figure 6.7, based on which the following mass balances can be derived:

\begin{align*}
\frac{d[N]}{dt} &= -k_{\text{unf}} [N] + k_{\text{fold}} [I] \\
\frac{d[I]}{dt} &= k_{\text{unf}} [N] - k_{\text{fold}} [I] - 2k_1 [I]^2 + 2k_{-1} [D] - k_2 [I][D] + k_{-2} [T] \\
\frac{d[D]}{dt} &= k_1 [I]^2 - k_{-1} [D] - k_2 [I][D] + k_{-2} [T] \\
\frac{d[T]}{dt} &= k_2 [I][D] - k_{-2} [T]
\end{align*}

where $[N]$, $[I]$, $[D]$ and $[T]$ are the molar concentrations of native IgG, intermediate species prone to aggregation, dimers and trimers, respectively; $k_1$ and $k_2$ are the rate constants for the dimer and trimer formation, respectively, while $k_{-1}$ and $k_{-2}$ are the rate constants for the corresponding backward reactions.

For the experimental techniques applied in this work, it is impossible to distinguish between the $N$ and $I$ species. The experimentally measurable quantity is in fact only the total monomer concentration $[M] = [N] + [I]$, whose mass balance is given by the sum of Eqs 2 and 3. Since the $N$ and $I$ species are at equilibrium, the $I$ concentration can be expressed as a fraction of the total monomer concentration $f$, $[I] = f \times [M]$, where $f = K_{eq} / (1 + K_{eq})$. Then, Eqs 6.2 to 6.5 can be re-written using only experimentally measurable quantities as follows:

\begin{align*}
\frac{d[M]}{dt} &= -2k_{1,\text{app}} [M]^2 + 2k_{-1} [D] - k_{2,\text{app}} [M][D] + k_{-2} [T] \\
\frac{d[D]}{dt} &= k_{1,\text{app}} [M]^2 - k_{-1} [D] - k_{2,\text{app}} [M][D] + k_{-2} [T] \\
\frac{d[T]}{dt} &= k_{2,\text{app}} [M][D] - k_{-2} [T]
\end{align*}
where the apparent rate constants, $k_{1,\text{app}} = f^2 \times k_1$ and $k_{2,\text{app}} = f \times k_2$, have been introduced. It is worth noting that such apparent rate constants are composed of two terms describing the two main aspects of the process: the conformational stability behavior of the macromolecule (represented by $f$) and the kinetic colloidal stability of the solution (represented by the intrinsic kinetic constants $k_1$ and $k_2$). The two aspects are intrinsically interconnected, and their individual contribution to the aggregation process cannot be distinguished.

The $k_{1,\text{app}}$ value can be evaluated experimentally from the early stage of the aggregation where the only species present in the system are monomers and dimers while trimers are negligible. Then, at time $t \rightarrow 0$, Eq. 6.6 reduces to

$$\frac{d[M]}{dt} = -2k_{1,\text{app}} \cdot [M]^2,$$

which can be easily integrated giving:

$$\frac{1}{[M]} - \frac{1}{[M_0]} = 2k_{1,\text{app}} \cdot t,$$

where $[M_0]$ is the given concentration of total monomers at $t=0$. Thus, the plot of $1/[M]$-$1/[M_0]$ versus $t$ should be a straight line whose slope gives $k_{1,\text{app}}$. Indeed, this was verified, as shown in Figure 6.8 for experimental runs 6, 9 and 10 in Table 6.1.

The other three unknown kinetic parameters, $k_{2,\text{app}}$, $k_1^{-1}$ and $k_2^{-1}$, have been estimated by solving Eqs 6.6 to 6.8 and fitting the experimental data, minimizing an error function. Applying such procedure it is possible to estimate with uniqueness the kinetic constant $k_1^{-1}$ and the equilibrium constants $K_{2,eq} = k_{2,\text{app}} / k_2^{-1}$, for the formation of trimers. The summary of the estimated parameter values is reported in Table 6.2.
Figure 6.8 Monomer conversion, plotted in the form of $1/[M] - 1/[M_0]$ (according to Eq. 6.10), versus time for the 1 g/L IgG solution in 25 mM citric acid buffer at pH 3.0 and different salt concentrations: 0.15 M (▲), 0.3 M (●), 0.5 M (◊) (Runs 6, 9 and 10 in Table 6.1). Continuous lines represent fitting to experimental data.

Table 6.2 Values of the rate constants corresponding to the kinetic scheme in Figure 6.7 estimated at different salt concentrations.

<table>
<thead>
<tr>
<th>NaCl Conc [mol/L]</th>
<th>$k_{1,\text{app}}$ [L·mol$^{-1}$·s$^{-1}$]</th>
<th>$k_1$ [1·s$^{-1}$]</th>
<th>$K_{2,\text{eq}} = k_{2,\text{app}}/k_2$ [L·mol$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5.3</td>
<td>1.50·10$^{-4}$</td>
<td>4.29·10$^4$</td>
</tr>
<tr>
<td>0.15</td>
<td>15.5</td>
<td>1.37·10$^{-4}$</td>
<td>1.05·10$^5$</td>
</tr>
<tr>
<td>0.3</td>
<td>30</td>
<td>3.59·10$^{-5}$</td>
<td>1.54·10$^7$</td>
</tr>
<tr>
<td>0.5</td>
<td>32.5</td>
<td>3.02·10$^{-5}$</td>
<td>5.49·10$^7$</td>
</tr>
</tbody>
</table>

It is worth noting that based on the physicochemical meaning of the kinetic rate constants they are not expected to depend on the IgG concentration but to do so for NaCl. Therefore, we fit run 6 in Figure 6.9a and then, with the same kinetic parameters, we predict runs 7 and 8 in Figure 6.9b (for sake of simplicity, only time evolutions of the residual monomers are reported).
Figure 6.9 Comparison between model predictions (continuous curves) and experimental data (symbols). a) Time evolutions of monomer (○), dimer (□) and trimer (◊) mass fractions for run 6 in Table 6.1; b) effect of the IgG concentration on the kinetics: 0.1 g/L (◊), 1 g/L (○), 10 g/L (□) (i.e., Runs 7, 6 and 8 in Table 6.1, respectively).
6.4 Salt effect

6.4.1 Salt effect on aggregation kinetics

In order to better understand the salt effect on the oligomer formation, the aggregation kinetics has been investigated using different types of salts, at a fixed concentration of 150 mM in 25 mM citric acid buffer solution, and at pH 3.0, 4.0 and 4.5, respectively. Note that for divalent anion (or cation), the salt concentration was fixed at 75 mM to allow comparison with equal number of cation (or anion), respectively. All antibody solutions were incubated at room temperature at a protein concentration of 1 g/L or 0.5 g/L.

The effect of anion type on the aggregation kinetics is shown in Figures 6.10a and 6.10b for pH 3.0 and 4.0, respectively. In all the cases, the corresponding cation was sodium. In Figure 6.10a the error bars are reported as example of the good reproducibility of the data. When not visible, error bars are smaller than the symbols. The rate of monomer conversion in both Figures 6.10a and 6.10b follows the ascending order:

\[
\text{HPO}_4^{2-} > \text{Cl}^- > \text{SO}_4^{2-} > \text{NO}_3^- > \text{ClO}_4^-
\]

This agrees well with the Hofmeister series, with the only important exception of sulfate, which behaves similarly to nitrate at pH 3.0 and to chloride at pH 4.0.

To better view the effect of anion type and pH on the extent of oligomerization, we have plotted the monomer conversion at a fixed incubation time, 6 hours, for different anions as a function of pH, as shown in Figure 6.11. It is seen that for chloride, nitrate and phosphate, the protein is more prone to aggregate at pH 4.0 than at pH 3.0 or 4.5, indicating presence of a maximum aggregation propensity in the pH range of 3.0-4.5; on the other hand, for perchlorate and sulfate a monotonic behavior is observed. Moreover, without adding salts, although aggregation does not occur at pH 3.0 and 4.5, it does occur at pH 4.0, as shown in Figure 6.11, which also indicates the presence of a maximum aggregation propensity in between.

From a colloidal interaction point of view, in the given pH range below the pI, lowering pH leads to increase in the net charge, thus in the particle stability, as typically observed for colloidal dispersions. However, what we observed here is that lowering the pH can either increase or decrease the stability, depending on the type of salt. This clearly
indicates that the effect of pH on the aggregation is not purely due to colloidal interactions and mostly connected to the pH dependent unfolding of the protein and the formation of an aggregating-prone structure (intermediate).

**Figure 6.10.** Anion effect on protein monomer conversion for 1 g/L protein solution in 25 mM citric acid buffer and 150 mM of several Na salts at pH 3.0 (a) and pH 4.0 (b).
Figure 6.11. pH effect on monomer conversion at 6 hours of incubation of 0.5 g/L protein solution in 25 mM citric acid buffer with 150 mM of different sodium salts.

Figures 6.12a and 6.12b show the effect of cation type on the monomer conversion to aggregates at pH 3.0 and 4.0, respectively, where the corresponding anion is either chloride or sulfate. No data are reported in the case of pH 4.5, because no aggregation was observed for all the salts. In Figure 6.12a at pH 3.0 and protein concentration of 1 g/L, the effect of cation type on the aggregation kinetics is insignificant. However, in Figure 6.12b at pH 4.0 and protein concentration of 0.5 g/L, substantial effect of cation type has been observed, and the aggregation rate follows the order:

\[\text{Li}^+ > \text{K}^+ > \text{Ca}^{2+} > \text{Na}^+\]

Such an order is not in agreement with neither the Hofmeister series nor the hydration radius series\(^{253}\) nor the electroselectivity series.\(^{254}\) To explain the difference in the effect of cation type between Figure 6.12a and 6.12b, we may consider what has changed when the pH changes from 3.0 to 4.0. Since the pI of the protein is between 7.35 and 8.15, positive charges are dominating on the protein surface at pH 3.0 and 4.0. However, in this case the amount of negative charges on the surface becomes very sensitive to the changes in pH, and in particular, it is much larger at pH 4.0 than at pH 3.0. Then, this indicates that the difference between the results in Figures 6.12a and 6.12b and the cation-specific effect in
Figure 6.12b are mostly related to complex (chemical, electrostatic, polarization, etc.) interactions between cations and negative charges on the protein surface.

Figure 6.12. Cation effect on protein monomer conversion in 25 mM citric acid buffer and 150 mM of several Cl and SO₄ salts at pH 3.0 (a) and of several Cl at pH 4.0 (b). Protein concentration was 1 g/L in (a) and 0.5 g/L in (b).

By applying the model developed in Paragraph 6.3.2, we estimated the dimerization equilibrium constant $K_1 = k_{1,app} / k_{-1}$ as a function of salt concentration by fitting the model
simulations to SEC experimental data. We focus on the anion effect, and in particular on two anions: sulfate and chloride, for comparative studies. Sulfate is a peculiar anion involved in the aggregation of proteins in human diseases\textsuperscript{205,206,209} and in the form of ammonium salt is the most common agent used for salting-out of biomacromolecules in industrial purification processes.\textsuperscript{255} Figures 6.13a and 6.13b show the dimerization equilibrium constant values, $K_1$, as a function of salt concentration, which have been obtained by fitting the SEC data, at pH 3.0 and 4.0, respectively. The obtained values are in the range 100-500 m$^3$/mol\textsuperscript{-1}, corresponding to a free energy of dimerization of about 2.7-4 kJ mol\textsuperscript{-1}.

Let us first consider the case of sulfate. In both Figures 6.13a and 6.13b in the range of low salt concentrations, the $K_1$ value increases as the sulfate concentration increases, till reaching a maximum value of about 500 m$^3$/mol. Then, the $K_1$ value starts to decrease with the sulfate concentration, a kind of re-stabilization effect. The main difference between pH 3.0 and pH 4.0 is that the entire curve moves towards lower sulfate concentration at pH 4.0. In the other words, at low sulfate concentrations, the $K_1$ value or the monomer conversion is larger at pH 4.0 than at pH 3.0, while at large sulfate concentrations, the aggregation rate is larger at pH 3.0.

In the case of chloride in Figure 6.13, the $K_1$ value is always larger at pH 4.0 than at pH 3.0 in the given range of the salt concentration. Moreover, at pH 4.0, as the chloride concentration increases, the $K_1$ value also increases to reach a maximum of around 300 m$^3$/mol and then decreases. At pH 3.0, however, the $K_1$ value increases almost linearly with the chloride concentration without reaching a maximum.

When comparing the behavior of the two anions it is seen that in general, the $K_1$ value is substantially larger for sulfate than for chloride, except for salt concentrations larger than 150 mM at pH 4.0, where it is comparable for the two anions. The electrostatic contribution in DLVO interactions\textsuperscript{256} could be used to explain these results at low salt concentrations, i.e., the sulfate anion with its divalent charge is more efficient than the monovalent chloride in screening the electrostatic repulsion between two positively
charged protein monomers, therefore promoting faster aggregation. However, at larger salt concentrations other explanations are obviously needed.

**Figure 6.13.** Dimerization equilibrium constant as a function of sodium chloride (□) and sodium sulfate (●) concentration at pH 3.0 (a) and pH 4.0 (b).

Thus summarizing, from the experimental data shown in Figures 6.10-6.13, we can draw two main conclusions about the protein aggregation behavior: a) specific effect of salt type and b) a maximum of the aggregation propensity as a function of salt concentration.

Peculiar behaviors of this type in salt-protein systems have been often reported in the literature\(^{257}\) and their origin is currently still under debate. Ions specific effects are traditionally related to the effect of the ion on the bulk water structure.\(^{258}\) However, such hypothesis was recently disproved by experiments, showing that the structuring effect of
ions is restricted only to the first solvation shell and therefore cannot influence the interaction of the ion with proteins.\textsuperscript{226,259,260} More recent studies explain the ion specific effect considering the different polarizability of the different ions, and therefore different ionic dispersion forces acting between each type of ions and the protein.\textsuperscript{202,261,262} According to this interpretation, the specific ion effects are related to direct ion-macromolecule interactions more than to bulk properties.\textsuperscript{226,263} Specific interactions at the protein interface are also considered to be the reason for the stabilizing effect of cosolutes commonly added in protein formulations, such as arginine.\textsuperscript{264}

An important aspect involved in ion-protein interactions is the ion binding to the protein charged groups. Different ions have different propensity to bind to the surface charged groups according to the electroselectivity series: \textsuperscript{265,266}

\[ \text{H}_3\text{PO}_4^- < \text{Cl}^- < \text{NO}_3^- < \text{ClO}_4^- < \text{SO}_4^{2-} \]

Such a series is opposite to the Hofmeister series, with the only exception of the sulfate anion, which due to its divalent charge has the largest tendency to interact with charged components. The sulfate anion has therefore two opposite limiting positions in the Hofmeister and in electroselectivity series and, consequently, two very pronounced and conflicting properties: the largest salting-out activity, which promotes aggregation, and the strongest counterion binding, which increases protein surface dipoles and generates hydration forces with stabilizing effect.\textsuperscript{229,263} The competition between these two opposite effects is the source of the peculiar behavior of such anion observed in this work as well as in other systems.\textsuperscript{267,268} For divalent ions, the re-stabilizing effect has been explained also by considering charge inversion induced by their binding.\textsuperscript{269} However, in this work re-stabilization at large salt concentration has been observed also for chloride anion at pH 4.0 (Figure 13b), which is obviously not related to charge inversion. Moreover, at large ionic strength, where re-stabilization effects are usually observed, electrostatic repulsion forces would be almost completely screened.

Another aspect to be accounted for is that anions can bind not only to charged groups but also to polar and apolar groups, as well as to the peptide bonds. The less hydrated chaotropic ions will interact more favorably with low solvated and apolar regions of the protein according to the reversed Hofmeister series. We tested the hydrophobicity affinity
of the anions by injecting pulses of anions in a reverse phase column (Kromasil® 100-10-C18, Eka Chemicals Bohus, Sweden) and measuring the retention time with a conductivity detector (CDD-10AVP, Shimadzu, Reinach, Switzerland) using a solution of 10 mM HCl with 5.6% w/w acetonitrile at pH = 2.0 as running buffer. As it can be seen from the data shown in Figure 6.14, the reversed Hofmeister series was confirmed, with the retention time increasing according to the order:

\[ \text{HSO}_4^- / \text{SO}_4^{2-} \sim \text{HPO}_4^{2-} \sim \text{Cl}^- < \text{NO}_3^- < \text{ClO}_4^- \]

![Figure 6.14](image-url)

**Figure 6.14** Retention time of different anions in a reverse phase column. The largest the retention time the largest the affinity of the anion to the hydrophobic surface.

The global effect arising from the superimposition of the specific interactions of ions with charged groups, peptide bonds, polar and apolar patches of the protein surface can be described by a single specific (preferential) interaction parameter, introduced by Arakawa and Timasheff to explain the specific salt effect in protein salting-out\(^{255,270}\) and the stabilizing effects of arginine\(^{271}\) and sugars.\(^{272}\) The preferential interaction parameter for a system composed of protein (p), salt (s) and water (w) is defined as \( \frac{\partial m_s}{\partial m_p} \), where \( m_i \) and \( \mu_i \) are the molality and the chemical potential of component \( i \), respectively.\(^{270}\) This parameter is related to the change in the protein free energy induced by the addition of salt (or a generic cosolute): \( \frac{\partial \mu_p}{\partial m_s} \).
and represents the type of interaction between the salt and the protein: a negative preferential binding parameter indicates depletion of salt in the proximity of the protein (preferential exclusion) while a positive preferential binding parameter corresponds to preferential binding of the salt. When the ion is preferentially excluded, the protein surface energy increases, and therefore the protein solubility decreases. An increase in the protein surface energy would favor more compact native structure of the protein, but at the same time it would promote also aggregation, since the surface of a dimer is smaller than the total surface of two monomers. These considerations explain the non-intuitive observation that the ions that are the best at stabilizing the protein secondary structure are also the ones that most promote aggregation.

It is worth noticing that the mentioned preferential interactions affect and are also affected by the protein conformational structure and surface charge. Aggregation of globular proteins is commonly triggered by a change in the native protein structure. Since several structural conformations of a protein may be present at the same time, the added ions would favor one or another conformation, depending on the specific system, thus promoting or not aggregation. Moreover, protein surface is notoriously inhomogeneous and several binding mechanisms may occur at localized sites. The same ion can preferentially bind or be excluded in different regions of the same protein.

Considering all the above mentioned aspects, it is not surprising that the protein aggregation stability exhibits a rather complex dependence on pH, salt type and salt concentration. Although the various phenomena are strongly interconnected and therefore their independent study is challenging, in the following we investigate separately the salt effect on protein conformational stability and on surface energy.

6.4.2 Salt effect on protein structure and surface energy

The effect of salt on protein structure was assessed by a series of spectroscopic techniques at low protein concentration (0.3 g/L) to reduce aggregation during the measurements. Circular dichroism (CD) gives information about the total amount of secondary structures present in a protein. In Figure 6.15a the CD spectra of several protein solutions are shown. In physiological conditions (25 mM phosphate buffer solution at pH 7.0), the spectrum
shows the characteristic minimum at about 216 nm, representative of the ordered β-sheet structure of immunoglobulins. When pH is reduced to 3.0 (25 mM citric acid buffer without addition of salt), the shape of the CD spectrum remains similar to that in physiological conditions but the intensity is significantly reduced, indicating a loss in the ordered β-sheet content. Such a decrease is due to the intra-molecular repulsion forces generated by the positive charges induced by reducing pH. The addition of 150 mM of different salts at pH 3.0 leads to the CD spectra intermediate between physiological conditions and pH 3.0 without salt. This indicates that the presence of the salts re-generates partially the original β-sheet structure. The re-stabilization of the protein structure with addition of anions has already been observed in the literature and explained by anion-binding, which reduces the intramolecular charge repulsion. Moreover, as stated above, the salt-induced increase of protein surface energy would favor a more compact, stable structure. A peculiar CD spectrum is observed for the very chaotropic perchlorate anion (Figure 6.15a), indicating a significant change in the antibody secondary structure. The effect of nitrate anion could not be analyzed due to interference of the anion with CD measurements.

The CD spectra are consistent with the fluorescence spectra of the 1-anilino-8-naphtalene sulfonate (ANS) binding studies shown in Figure 6.15b. ANS is a hydrophobic dye whose fluorescence increases upon binding to protein. Thus, ANS binding is considered representative of the global hydrophobicity of protein surfaces. As expected, under physiological conditions the fluorescence value is very low, because folding of globular proteins normally minimizes the exposure of the hydrophobic patches and exposes the more hydrophilic parts to water. Reducing pH to 3.0 leads to a loss in the β-sheet content, as indicated by the CD spectra, and to an increase in hydrophobicity due to exposure of buried patches, in agreement with the increase of the ANS fluorescence signal shown in Figure 6.15b. The restoration of the ordered structure by introduction of the anions corresponds to a reduction in hydrophobicity. Thus, the fluorescence spectra in the presence of anions shown in Figure 6.15b are again intermediate between physiological conditions and pH 3.0 without salt. In order to quantify the effect of the anion on the protein hydrophobicity, we have plotted the peak of the ANS fluorescence spectra as a
function of the sulfate and chloride concentration in Figure 6.15c, which clearly shows reduction in hydrophobicity as the anion concentration increases.

![Circular dichroism spectra](image)

Figure 6.15. Circular dichroism spectra (a), ANS fluorescence spectra (b) and intrinsic tryptophan fluorescence spectra (d) of 0.3 g/L antibody solution in 25 citric acid buffer at pH 7.0 and pH 3.0 without salt and at pH 3.0 with 150 mM of different salts. c) The same as in b) but with different concentrations of sodium sulfate (white bars) and sodium chloride (grey bars).

Protein structure changes were analyzed also by intrinsic tryptophan fluorescence measurements. Tryptophan fluorescence depends on the polarity of the environment around the residue: a change of the tertiary structure leading, for example, to more solvent-exposure is reflected by a change in the fluorescence spectrum. As it can be seen in Figure 6.15d, the tryptophan fluorescence in physiological condition is very low, indicating burying of the residue. The largest value of fluorescence is observed at pH 3.0 in the presence of perchlorate, thus confirming the denaturation effect of such anion observed by
CD analysis (Figure 6.15a). At pH 3.0 without salt and with 150 mM of sulfate, chloride, phosphate the spectra are similar, indicating no dramatic change in the tertiary structure of the protein, although the secondary structure content and the general hydrophobicity of the protein is different with and without salt, as discussed earlier based on the CD and ANS results. It is worth noting that the spectrum in the presence of 150 mM of nitrate is significantly different with respect to those of chloride, phosphate and sulfate, indicating that this chaotropic anion, in analogy with perchlorate, affects the antibody structure.

By connecting the results of the structure analyses above to the aggregation kinetics data discussed in the previous section we can conclude that aggregation correlates not only to the hydrophobicity content of the protein but rather to a suitable aggregation-prone intermediate state. A clear evidence of this is that the antibody structure at pH 3.0 without salt has the largest hydrophobicity but its solution is stable, mostly due to its disordered structure and increased number of surface charges. Adding salt should screen progressively the surface charges and trigger aggregation. On the other hand, salt addition promotes the formation of a less hydrophobic and more compact structure, less prone to aggregation. The increase of surface dipoles due to counterion binding also contributes to the reduction of hydrophobicity. The competition among structure re-organization, hydrophobicity reduction and charge screening induced by salt explain the maximum of the aggregation propensity as a function of salt concentration, as observed in Figure 6.13.

The behavior of aggregation propensity as a function of pH without salt addition (Figure 6.11) is most likely related to the competition among the same effects. In particular, the antibody at pH 4.5 retains ordered structures with low surface hydrophobicity. The opposite situation occurs at pH 3.0, where the hydrophobicity is the highest, but the antibody structure is more disordered and charged. In both situations, no aggregation is observed. Then, the observed aggregation at pH 4.0 is most probably promoted by two factors: reduced charges (with respect to pH 3.0) and less ordered structures (with respect to pH 4.5).

It is worth noticing that only for the chaotropic perchlorate and nitrate anions the increase in aggregation can be connected with changes of the protein structure. For all the other ions, at least in the resolution limit of the applied techniques, no significant effect of
salt type on protein structure was detected. This suggests that, for a given salt concentration, ion specific effects are not connected to changes in protein structure properties, but most likely to salt-mediated protein-protein interactions, in particular solvation forces. These include hydrophobic forces due to salt exclusion, hydration forces related to counterion binding and dispersion forces between ion and macromolecule.\textsuperscript{202}

Hydrophobic forces lead to an increase of the protein surface energy due to the salt exclusion from the apolar portions of the protein surface. This increment is similar to the increase of water surface tension upon addition of salts due to ion exclusion from the air-water interface. Indeed, the increase in the protein surface energy in the presence of a cosolute can be directly linked to the bulk surface tension.\textsuperscript{221,276} We checked the capability of the different anions to affect the protein surface energy by measuring the surface tension of 0.1 g/L protein solutions in the presence of sulfate and chloride at different concentrations. Due to the large amount of protein required for the measurements, a globular model protein, lysozyme, was used instead of the antibody and the results are shown in Figure 6.16. It is seen that the sulfate anion can increase significantly the surface tension while chloride has a negligible impact. These results are consistent with the larger values of surface tension increment of aqueous solutions reported in the literature for sulfate with respect to chloride\textsuperscript{225,265,277} and confirm the impact of the type of salt on protein surface energy and, consequently, on protein aggregation stability.

![Figure 6.16](image.png)

**Figure 6.16.** Surface tension of 0.1 g/L lysozyme solutions in 25 mM citric acid buffer at pH 3.0 in the absence or presence of sulfate and chloride anions.
6.5 Irreversible precipitation

6.5.1 Effect of operating conditions on IgG2 stability

After characterizing the IgG2 stability at room temperature (Paragraph 6.3 and 6.4), the IgG2 aggregation behavior was investigated in the temperature range from 4 °C to 37 °C and in the pH range from 3.0 to 4.5 at different ionic strengths (25 mM citric acid buffer with and without 150 mM NaCl or Na₂SO₄). The aggregation kinetics is followed in situ by dynamic light scattering (DLS) and samples taken at different incubation times are analyzed by size exclusion chromatography (SEC). To describe the aggregation state of the IgG2 under different conditions, we show in Figure 6.17 the size exclusion chromatograms of 1 g/L protein solutions after a characteristic incubation time of 6 hours in 25 mM citric acid buffer with 0.15 M Na₂SO₄ for several pH and temperature values. The peak at 24 min elution time corresponds to monomeric antibody, while the peaks at shorter elution times represent aggregates. Aggregates larger than about six monomeric units are excluded from the column volume and elute all simultaneously in the void peak at 18 min (Figure 6.17I). At the considered ionic strength, three types of behavior can be identified in the pH-temperature plane investigated in Figure 6.17: 1) stable solutions; 2) formation of oligomers with small aggregation number and 3) formation of high molecular weight (HMW) aggregates. Aggregation is promoted either decreasing pH or increasing temperature. In particular, by increasing temperature from 4 °C to 25 °C and from 25 °C to 37 °C the critical pH value for oligomer formation shifts from 3.0 to 4.0 and from 4.0 to 4.5, respectively. Moreover, at pH 3.0 and 37 °C the formed oligomers are not stable and grow to larger aggregates. The SEC data are in agreement with the time evolution of average sizes measured by dynamic light scattering (DLS) and discussed later.

To investigate the effect of ionic strength, the same study was repeated in the absence and in the presence of 0.15 M NaCl, as shown in Figure 6.18. For sake of simplicity, only the pH effect at 37 °C is shown. The dependence of aggregation rate on pH in the presence of NaCl is analogous to the one described above for the sulfate anion (Figures 6.17C, 6.17F and 6.17I), with the formation of large aggregates when decreasing pH from 4.0 to 3.0 (Figures 6.18D, 6.18E and 6.18F). On the other hand, the aggregation
rate in the presence of NaCl is rather slower than in the presence of sulfate anion, as indicated by the SEC chromatograms shown in Figures 6.17I and 6.17F and by the DLS measurements (data not shown).

<table>
<thead>
<tr>
<th>pH</th>
<th>4°C</th>
<th>25°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>Stable</td>
<td>Stable</td>
<td>Soluble oligomers</td>
</tr>
<tr>
<td>4</td>
<td>Stable</td>
<td>Soluble oligomers</td>
<td>Soluble oligomers</td>
</tr>
<tr>
<td>3</td>
<td>Soluble oligomers</td>
<td>Soluble oligomers</td>
<td>HMW precipitates</td>
</tr>
</tbody>
</table>

**Figure 6.17** SEC chromatograms of 1 g/L protein solution after 6 hours of incubation in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at several pH and temperature values.

A different behavior is observed in the absence of salt. In Paragraph 6.4.1 no aggregation was observed at pH 3.0 and 4.5, and only a small amount of oligomers was detected at pH 4.0. On the other hand, in this work, due to the thermal stress at 37 °C, a significant amount of oligomers is formed even in the absence of salt at all pH values (Figure 6.18A,
6.18B and 6.18C). The extent of oligomerization depends on the pH value and exhibits the same maximum observed at room temperature, with the system more stable at pH 4.5 and pH 3.0 than at pH 4.0. The maximum aggregation propensity as a function of pH, as well as the specific salt effect, confirm therefore the findings discussed above.

**Figure 6.18** SEC chromatograms of 1 g/L protein solution after 6 hours of incubation in 25 mM citric acid buffer at 37 °C and several pH values with and without 0.15 M NaCl.

The oligomers observed in Figure 6.17 can be either intermediate species in the system evolution to the larger precipitates or the product of an alternative aggregation mechanism. In order to better investigate these two cases, we followed the oligomer stability for longer times and in different conditions.

In Figure 6.19a the three situations which appeared less stable for the incubation time of 6 h (Figures 6.17I, 6.17F and 6.17H) are now monitored for 15 hours by showing the average hydrodynamic radius, \(<R_h>\), measured by DLS as a function of time. It is seen that the oligomers in Figure 6.17I are not stable and undergo massive aggregation after about 12 h, while the other two remains substantially stable in the 15 hours. The DLS
intensity signal for the oligomers in Figure 6.17I increases up to about 12 h due to aggregation, and later decreases, indicating precipitation of the aggregates (data not shown). According to small angle light scattering measurements these aggregates reach sizes of tens-hundreds µm (data not shown). At the end of the aggregation process the aggregates are visually insoluble, and precipitate on the bottom of the cuvette.

Figure 6.19. Time evolution of the average hydrodynamic radius, $<R_h>$, measured by dynamic light scattering for a 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na$_2$SO$_4$. a) at 37 °C and pH 3.0 (17I) (○), at 25 °C and pH 3.0 (17H) (▲), and at 37 °C and pH 4.0 (17F) (□); b) at 25 °C and pH 3.0 (17H) (▲), and at 37 °C and pH 4.0 (17F) (□).
By prolonging the experiment duration to 45 days we see that also oligomers in Figure 6.17H and 6.17F aggregate but in a longer time scale involving tens of days. Oligomers in Figure 6.17H undergo massive aggregation after about 30 days, while precipitation of oligomers in Figure 6.17F was observed after more than two months. It is worth noting that the profile of the time evolution of the average size is similar in all the investigated conditions, as seen by comparing the slower kinetics in Figure 6.19b to the faster kinetics at 37 °C and pH 3 in Figure 6.19a. A slow, almost linear increase of the average size during the initial times is followed by a fast acceleration leading to the formation of insoluble aggregates which, eventually, precipitate. From the data shown in Figure 6.19 we can conclude that the oligomers observed in Figure 6.17 are intermediates in the coagulation process leading to larger precipitates. The kinetics of oligomer-oligomer aggregation and, therefore, the oligomers life-span depend on the operating conditions, and particularly on pH and temperature, i.e., on the denaturing conditions.

These results evidence the multi-variable nature of this stability problem, which involves a combined effect of salt, pH and temperature on the aggregation kinetics. The latter is most likely related to unfolding of the native structure, induced with different combinations of operating parameters. On the other hand, the aggregation mechanism seems to be the same in all conditions as discussed in the next section.

6.5.2 IgG2 aggregation/precipitation mechanism

The mechanism of coagulation was investigated in more detail for the following reference conditions: 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na₂SO₄ and 37 °C. In Figure 6.20 the first 3 hours of the time evolution of the SEC chromatograms and the residual monomer amount in the reference conditions and different pH values are compared. In particular, we consider in the sequel three pH values: 3.0, 4.0 and 4.5, which correspond to the operating conditions I, F and C in Figure 6.17, and therefore are referred to 17I, 17F and 17C in the sequel, respectively. It is seen that the time evolution of the chromatograms at pH 4.0 (Figure 6.20a) and pH 4.5 (Figure 6.20b) is similar, with a kinetics slightly faster at pH 4.0. On the other hand, at pH 3.0 already after 3 h species
larger than trimer are observed (Figure 6.20c). Despite this, after 3 hours of incubation the residual monomer at all pH values is comparable, being about 10% at pH 3.0 and about 30% at pH 4.0 and 4.5 (Figure 6.20d).

**Figure 6.20.** Time evolution of normalized size exclusion chromatograms of 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at 37 °C and several pH values: a) pH 4.5 (17C); b) pH 4.0 (17F); c) pH 3.0 (17I). At pH 4.0 and 4.5 only oligomers are visible, while at pH 3.0 aggregation proceeds to HMW aggregates; d) residual monomer fraction versus time evaluated from the SEC chromatograms.

The difference in aggregate size at the different pH values increases further at 6 h of incubation. The corresponding SEC chromatograms are shown in Figure 6.17 (i.e., 17C, 17F and 17I), while the DLS data are shown in Figure 6.19a, indicating an average radius, $<R_d>$, of 30±1 nm at pH 3.0 and of 10±1 nm at pH 4.0. Despite such a difference in size, the residual monomer is rather similar at both pH values (Figure 6.20d). Nevertheless, at longer times (about 10-12 hours), for the case pH=3.0 (Figure 6.19a) a strong acceleration in aggregation is observed through the strong increase of the average
hydrodynamic radius measured by DLS. Since this occurs when the monomer consumption is almost complete, we can conclude that most likely the formation of HMW aggregates is not due to monomer addition but to cluster-cluster aggregation.

From the experimental evidences collected so far we can describe the aggregation process as a two steps process. In the first one a slow, possibly reversible oligomerization (cluster-monomer) occurs, while in the second a fast and auto-accelerating cluster-cluster aggregation takes place. The transition between the two can occur at different times and it is indeed accelerated at pH 3.0 with respect to pH 4.0 and 4.5. On the other hand, it is worth noting that as long as only the monomer conversion is concerned the pH plays a minor role as shown in Figure 6.20d.

We investigated the reversibility of the aggregates formed during the second stage by diluting samples to lower concentrations in 25 mM phosphate buffer at pH 7.0 without salt: decrease in average size and formation of monomer was not observed after 15 days, indicating that the formed aggregates are mostly irreversible.

Changes in protein secondary structure during aggregation were assessed by Thioflavin-T (ThT) and 8-Anilinonaphthalene-1-sulfonate (ANS) binding studies, which give information about the β-sheet content and the hydrophobicity of the protein, respectively. In Figure 6.21a the ThT fluorescence values versus time are shown for the three pH values investigated, corresponding to conditions 17F, 17C and 117. It is seen that the fluorescence signal increases with an extent proportional to aggregation, indicating that the latter is accompanied by β-sheet formation: at pH 3.0 a continuous increase of fluorescence is observed, while at pH 4.0 and 4.5 only an initial increase related to oligomer formation is detected. A similar behavior was observed for ANS binding (data not shown).
Figure 6.21. a) ThT fluorescence values for 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at 37 °C at pH 3.0 (17I) (□), pH 4.0 (17F) (■) and pH 4.5 (17C) (○); b) ThT fluorescence values (Δ) and average hydrodynamic radius (●) versus monomer conversion for the reference reaction at pH 3.0 (17F); c) ThT (○) and ANS (■) fluorescence values versus average hydrodynamic radius for the reference reaction at pH 3.0 (17F).
In Figure 6.21b the correlation between aggregation and structure changes is evidenced for the more unstable condition, 6.17I, by plotting the ThT fluorescence values and $<R_h>$ versus monomer conversion: both quantities increase non-linearly with respect to monomer conversion and follow a similar profile, thus proving the strong correlation between the two quantities. This is confirmed by plotting ThT and ANS values versus $<R_h>$, as reported in Figure 6.21c: the fluorescence values grow rapidly with the average size indicating the structure re-arrangement of the formed oligomers. With increasing average size, the change in fluorescence values reduces until reaching a plateau: this corresponds to the fast acceleration in aggregation kinetics observed in Figure 6.19b. During this step, the aggregates grow too fast to allow internal re-arrangements.

Re-organization of microdomains inside the clusters and formation of distorted intermolecular $\beta$-sheet during aggregation have been observed also for antibodies. However, the features of such re-structuring are still unclear. We compared the increase of ThT fluorescence during IgG coagulation observed above with the increase found during aggregation of an equivalent amount of insulin in similar conditions of pH and ionic strength. Under such conditions insulin forms fibrillar aggregates with a large amount of amyloid $\beta$-sheet structures. The increase in ThT fluorescence value during IgG coalescence is about 4% of the increase during insulin aggregation, indicating that the $\beta$-sheet formation in antibody aggregates is quite limited with respect to the extremely regular architecture of amyloid fibrils.

Based on the experimental evidences collected so far we can propose the coagulation mechanism schematically depicted in Figure 6.22. The aggregation is initiated by a reversible oligomerization involving mainly monomers, dimers and trimers. In suitable conditions, for example of pH and temperature, such oligomers are not stable and grow via irreversible cluster-cluster aggregation. The formed large aggregates become progressively less soluble and, eventually, precipitate. In all the investigated conditions the aggregation follows a unique mechanism whose kinetics depends on the operating condition. The aggregation is associated to a change of secondary structure inside the cluster, as detected by ThT and ANS binding.
6.6 Correlation between aggregation stability and IgG properties

The previous analysis was extended to other two model immunoglobulins belonging to the IgG1 subclass, referred to in the following as IgG1.1 and IgG1.2. The stability of the three antibodies is compared in Table 6.3, where the stability character observed by SEC and DLS after a characteristic time of 6 hours at different pH values and temperatures is reported. It can be seen how the aggregation propensity is different for the different antibodies. At pH 4.5 IgG2 is more unstable than the two IgG1s, while at lower pH the situation is more complex: at pH 4.0 and 37 °C, IgG1.1 is stable, while IgG1.2 and IgG2 form unstable oligomers which aggregate during time. Precipitation is observed after some weeks for IgG1.2 and after months for IgG2. A similar behavior is observed at 25 °C and pH 3.0: in this case, IgG1.2 is the less stable and significant precipitation is observed within 6 hours of incubation. Finally, at 37 °C and pH 3.0 all antibodies precipitate.

The coagulation kinetics at 37 °C and pH 3.0 was followed by DLS, ThT and ANS binding, and monomer conversion was measured by SEC. The DLS data in Figure 6.23a show that the aggregation kinetics follows the order: IgG1.2 > IgG1.1 > IgG2. It is worth noticing the different behavior between IgG2 and IgG1.1: the latter is characterized by a nucleation step similar to IgG2, but significantly slower, as indicated by the smaller size of
the aggregates in this period of time in Figure 6.23a and the lower monomer conversion values in Figure 6.23b. Once a critical concentration of IgG1.1 nuclei has been reached (at about 5 h), coagulation occurs with a much faster kinetics with respect to IgG2 (Figure 6.23a). On the other hand, IgG1.2 shows in Figure 6.23a to exhibit the fastest aggregation kinetics, with basically no nucleation time.

Table 6.3. Type of aggregates observed by DLS and SEC for a 1 g/L solution of the three different IgGs after 6 hours of incubation in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at several pH and temperature values.

<table>
<thead>
<tr>
<th>pH</th>
<th>T (°C)</th>
<th>IgG2</th>
<th>IgG1.1</th>
<th>IgG1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>37</td>
<td>Soluble oligomers</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>4.0</td>
<td>37</td>
<td>Soluble oligomers</td>
<td>Stable</td>
<td>Soluble oligomers</td>
</tr>
<tr>
<td>3.0</td>
<td>25</td>
<td>Soluble oligomers</td>
<td>Stable</td>
<td>Insoluble aggregates</td>
</tr>
<tr>
<td>3.0</td>
<td>37</td>
<td>Micron aggregates</td>
<td>Micron aggregates</td>
<td>Insoluble aggregates</td>
</tr>
</tbody>
</table>

By looking at the ThT values in Figure 6.23c, it appears that the three antibodies exhibit different initial values, indicating a different structure of the non-native monomers. In particular we see that the aggregation of all antibodies is accompanied by structure changes, particularly during oligomer formation, and the fluorescence values of the final aggregates correlate with that of the initial monomers and not with aggregation rate. The slower IgG1.1 oligomer formation is in fact accompanied by a larger increase of order β-sheet structure with respect to the faster aggregating IgG2. On the other hand, IgG1.2 shows the lowest ThT binding, although its aggregation kinetics is the fastest of the three antibodies. Likely, because of the fast aggregation kinetics, the internal domains do not have time to re-organize. ANS fluorescence values are rather similar for all antibodies.
(data not shown) and clear conclusions about monomers and aggregates hydrophobicity are difficult to reach.

**Figure 6.23.** Time evolution of the average hydrodynamic radius, $<R_h>$, (a), and of residual monomer (b) for a 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na$_2$SO$_4$ at 37 °C and pH 3.0; c) ThT fluorescence values versus average hydrodynamic radius, $<R_h>$, at the same conditions as a): IgG2 ($\circ$), IgG1.1 (■) and IgG1.2 (Δ). Continuous lines are a guide to the eyes.
In order to elucidate which property of the antibody is crucial for the aggregation propensity, the antibodies were characterized in terms of thermal stability, secondary structure, hydrophobicity and surface charge.

The thermal stability at pH 3.0 was investigated by circular dichroism (CD) in absence of salt and at low protein concentration to reduce aggregation during the measurements. In Figure 6.24a the normalized CD mean residue ellipticity (MRE) at 210 nm as a function of temperature is reported for the different antibodies. Given the multi-domain nature of the protein, a two-state unfolding model would likely not apply for the considered antibodies. A simple sigmoidal function was used to fit the data. The thermal stability follows the order: IgG1.1 (most stable) > IgG2 > IgG1.2 (less stable), which is the same order observed for the aggregation stability in the first 3 hours of incubation at pH 3.0 (Figure 6.23a).

The far-UV CD spectra at 20 ºC and pH 3.0 without salt are shown in Figure 6.24b: the three antibodies show different secondary structures, although all the spectra have a minimum at about 216 nm, characteristic of the large β-sheet content of immunoglobulins. In the IgG1.1 spectrum the minimum is shifted from 216 to 218 nm, but the β-sheet content is comparable to IgG2. The IgG1.2, characterized by a large propensity to aggregate, has a significant lower secondary structure amount compared to the other IgGs, in agreement with the low ThT binding reported in Figure 6.23c.

The global hydrophobicity of the three antibodies was assessed by ANS measurements. The spectra were recorded at 20 ºC for 0.3 g/L protein solutions in 25 mM citric acid buffer with 25 µM ANS. In Figure 6.25a the maximum fluorescence values of the ANS emission spectra for the three antibodies solutions at different pH values are compared. According to ANS analysis at all pH values the protein hydrophobicity follows the order: IgG2 > IgG1.1 > IgG1.2. The IgG1.2 is the less hydrophobic, despite the largest reactivity at pH 3.0. In addition, at pH 4.0 IgG1.1 is more hydrophobic and more stable than IgG1.2. The ANS fluorescence values increase by decreasing the pH value, confirming denaturation and exposure of hydrophobic patches at lower pH.
Figure 6.24. a) Thermal stability of the three investigated antibodies: normalized CD mean residue ellipticity (MRE) at 210 nm versus temperature for a 0.3 g/L protein solution in 25 mM citric acid buffer at pH 3.0: IgG2 (○), IgG1.1 (□) and IgG1.2 (△). Lines represent sigmoidal fitting to experimental data. b) Far-UV CD spectra at 20 °C for 0.3 g/L solutions of the three investigated antibodies in 25 mM citric acid buffer at pH 3.0: IgG2 (—), IgG1.1 ( - - ) and IgG1.2 ( — • ).

Finally, the zeta potential values of the three antibodies were measured as a function of pH (Figure 6.25b): at pH 3.0 the surface charge follows the order IgG1.1 > IgG2 > IgG1.2, which is in agreement with the ranking of aggregation propensity in the first three hours (Figure 6.23a). On the other hand, at pH 4.0 IgG1.1 has the lowest surface charge although
it is the most stable of the three antibodies. Moreover, at pH 4.5 the surface charge of IgG1.1 and IgG2 is similar, despite their different aggregation tendency.

Figure 6.26. a) ANS fluorescence emission values at 485 nm for 0.3 g/L solutions of the three investigated antibodies at 20 °C in 25 mM citric acid buffer with 25 μM ANS at different pH values. b) Zeta potential values as a function of pH for 0.3 g/L solutions of the three investigated antibodies in 25 mM citric acid buffer in the pH range from pH 3.0 to 4.5 and in 25 mM phosphate buffer in the pH range from 6.0 to 9.5: IgG2 (○), IgG1.1 (□) and IgG1.2 (∆).
For all the three considered antibodies the aggregation propensity increases with decreasing pH at a fixed temperature or with increasing temperature at a fixed pH. In both cases, the change of conditions (pH or temperature) promotes unfolding of the native protein. Only when no salt is added to the buffer solution the IgG2 oligomerization is slower at pH 3.0 than at pH 4.0, as indicated by the SEC data (Figure 2). In this case, the electrostatic repulsions are not screened, and stability is promoted by decreasing the pH value below the protein pI, i.e., by increasing the positive net surface charge.

At pH 3.0 the order in thermal stability (Figure 9a) correlates with the order in aggregation stability in the first 3 hours (Figure 8a), confirming the important role of structure stability in determining aggregation propensity in acidic conditions. In general, antibody thermal stability is significantly reduced at low pH, particularly due to the sensitivity of the CH2 domain in the Fc fragment to acidic conditions. IgG2 antibodies are more prone to aggregate than IgG1 antibodies in destabilizing conditions, i.e. physiological pH and elevated temperature, or acidic conditions at room temperature. This behavior is generally related to the larger stability of the CH2 domain in IgG1 antibodies with respect to IgG2 antibodies. However, a general conclusion on IgG subclasses cannot be achieved. In this work, the investigated IgG2 is in fact more unstable than the considered IgG1s at pH 4.5, while IgG1.2 (but not IgG1.1) is more unstable than IgG2 in the pH range from 3.0 to 4.0. These results would indicate that also the Fab fragments contribute to the structure and aggregation stability, in agreement with previous studies reported in the literature.

The analysis of the two-steps aggregation mechanism gives information about the relationship between the stability of non-native monomers and the stability of aggregates. On one hand, interactions between non-native monomers should reflect interactions between non-native aggregates. On the other hand, aggregation reduces the number of hydrophobic patches exposed during unfolding, and aggregates could therefore interact more similarly to native proteins. In this work, exposure of the model IgG2 to mildly destabilizing conditions was sufficient to promote reversible-oligomerization: during the self-assembling the available hydrophobic patches are covered, and oligomers are rather stable. When exposed to stronger destabilizing conditions (pH 3.0 and larger temperature),
the level of unfolding of the monomeric protein increases, exposing a larger number of hydrophobic patches. Under these conditions the oligomerization is not enough to cover the aggregating-prone patches, and the formed oligomers aggregate to larger ones and eventually precipitate. We conclude that, for the IgG2 under investigation, the extent of monomer unfolding is reflected in the stability of the formed aggregates.

On the other hand, the non-native IgG1.1 monomer has a lower tendency to aggregate with respect to IgG2, likely due to the lower unfolding extent. However, the formed oligomers aggregate much faster than the IgG2 aggregates. This behavior could be explained considering the larger ordered structure of IgG1.1 oligomers, as indicate by ThT binding in Figure 8c: during the slow oligomerization step, oligomers have more time to re-arrange in a more organized structure, which is more prone to aggregate. However, IgG1.2 shows the largest aggregation kinetics (Figure 8a) and the lowest amount of ordered secondary structure (Figure 8c and 9b).

In the case of amyloidogenic proteins, i.e., a class of proteins self-assembling in regular fibrillar structures known as amyloids, the aggregation propensity was found to correlate with general physicochemical properties of the proteins, namely hydrophobicity, surface charge and amount of ordered structure. For the three antibodies investigated in this work, the change in zeta potential and in hydrophobicity due to different protein structure or different pH does not correlate with the antibody aggregation propensity. The results suggest that for multi-domain proteins such as antibodies it is difficult to associate the monomer and oligomer reactivity to generic protein physicochemical properties, such as hydrophobicity or charges. Aggregation kinetics in acidic conditions appears therefore related to specific features of the non-native monomer and oligomers, which are still unclear. Aggregation may occur in specific binding sites, as recently shown for antibody dimer formation at low pH.

It is worth noticing that the oligomerization and the structure change occurring during aggregation are the less understood steps also in the kinetics of amyloid fibril formation. Investigation of IgG aggregation could therefore give mechanistic insights also in the aggregation of amyloidogenic proteins.
6.7 Concluding remarks

In the present work, the aggregation behavior of monoclonal antibodies (mAb) solutions has been investigated under industrial relevant conditions, typically encountered during purification through protein A chromatography, i.e., acidic conditions and presence of salt in the concentration range of 0.1-0.5 M. An IgG2 and two IgG1s were considered as model proteins. The aggregation was followed on-line by dynamic light scattering (DLS) and the oligomer distributions were analyzed off-line through both FFF (field flow fractionations) and SEC (size exclusion chromatography) separation techniques. The aggregation data were correlated to protein structure properties investigated by circular dichroism (CD) and fluorescence techniques.

Two distinct steps are identified for the IgG2 aggregation: 1) reversible oligomerization involving mainly monomers, dimers and trimers, and 2) irreversible cluster-cluster aggregation leading to high molecular weight aggregates, which, eventually, precipitate. Temperature, pH and ionic strength determine both nucleation and growth rate by affecting the protein conformational state. In particular, mild destabilizing conditions promote formation of stable oligomers; on the other hand, stronger denaturing conditions induce oligomer-oligomer aggregation.

Both the FFF and SEC techniques have been successfully applied to measure the oligomer distributions (i.e., monomer, dimer and trimer), and the results between the two techniques are in good agreement. To support the reliability of the obtained oligomer distributions, we have applied them to reconstruct the time evolution of the average hydrodynamic radius and compared the obtained values to the on-line DLS measurements, and good agreement has been obtained. The approach applied in this work represents an example of oligomer determination, which may be applied in other protein-aggregation systems to obtain important information about oligomer distribution and reversibility.

The aggregation kinetic data have been interpreted using a modified Lumry-Eyring model, where reversibility has been introduced into the reaction scheme. The key assumption of the model is an initial reversible conformational change resulting in an
aggregation-prone intermediate $I$. It is found that with properly estimated parameters, the proposed kinetic model can well predict the time evolution of all the oligomers determined from the FFF and SEC techniques and properly account for the effect of the IgG and salt concentrations on the aggregation kinetics.

The experimental investigation of the salt effect showed the following interesting features. a) The extent of aggregation depends on the salt type and the pH value. b) The salt effect is strongly ion specific: particularly, at pH 4.0 both the cation type and anion type affect aggregation, while at pH 3.0 the cation type does not play any role. The ranking of the anion effect follows the Hofmeister series, with the only exception of the sulfate, while the cation effect does not. c) A maximum of the aggregation propensity (i.e. presence of re-stabilization) as a function of salt concentration is observed.

By correlating the aggregation data to experimental information on protein structures and surface energy, it is found that changes in pH and addition of salt induce aggregation not only by altering colloidal interactions but also by promoting formation of a structure intermediate characterized by a certain degree of hydrophobicity and partially ordered secondary structure. The effect of salt on the colloidal interactions between two protein monomers is multifold. Apart from the typical charge screening effect, preferential ion exclusion can induce hydrophobic forces which increase protein surface energy and promote aggregation. On the other hand, counterion binding generates the hydration forces, stabilizing the protein. The complex interactions between the protein structure properties and the various solvation forces explain the presence of the maximum of dimerization extent as a function of salt concentration, as well as ion specificity and the peculiar behavior of sulfate anion.

In analogy with IgG2, the stability of the two IgG1s decreases with increasing denaturing conditions, i.e. lowering pH and increasing temperature. However, significantly different aggregation rates are observed for the three antibodies in different operating conditions. In particular, the two IgG1s show lower nucleation rate, but faster cluster-cluster aggregation rate with respect to IgG2. Moreover, IgG2 forms oligomers with long life-spans in a broad range of operative conditions. For all the three antibodies, the oligomerization step is accompanied by conformational changes with an increase in the
ordered, β-sheet structures.

The aggregation propensity of the investigated antibodies in acidic conditions does not correlate with antibody subclass, surface net charge and hydrophobicity of the non-native state. The results indicate that the aggregation tendency is dictated by specific features of the non-native structure, which are still to a good extent unclear.
Chapter 7

Population balance equations modeling of antibody aggregation kinetics

7.1 Introduction

As mentioned in Chapter 4, 5 and 6, protein self-assembling is widely investigated for its implications in a wide range of fields, such as biomedical research, nanotechnology, food and pharmaceutical industry. A large number of experimental investigations have been reported aimed at the fundamental description of the aggregation process and the individuation of the key factors responsible for protein stability. The experimental analyses described in Chapter 4, 5 and 6 are examples of this approach. In the different protein systems reported in the literature many different types of aggregates, ranging from oligomers to large precipitates, have been observed. Such aggregates could represent the final or intermediate products of several possible aggregation mechanisms and pathways.

Mathematical models of aggregation kinetics represent a useful tool for the mechanistic description of the aggregation process from soluble monomers to larger aggregates. From the comparison between model simulations and experimental data meaningful lumped, kinetic parameters can be quantitatively estimated and important information about the process can be derived. This includes identification of key steps of the process, presence of alternative paths, presence of a critical concentration, size of the critical nucleus. In addition, mathematical models find relevant application in the prediction of therapeutic proteins shelf-life, which represents a great challenge in pharmaceutical industry.
Different approaches have been followed in the literature to model protein aggregation. Most of the kinetic models are based on mass action laws. Roberts and coworkers identified the most common mechanisms observed in protein aggregation and applied the developed models to describe the aggregation behavior of several model therapeutic proteins, including $\alpha$-chymotrypsinogen A, stimulating factors and monoclonal antibodies.

Despite their large heterogeneity, most aggregation behaviors can be included in an universal multi-step Lurny-Eyring modified model. The aggregation scheme involves the formation of an aggregating-prone intermediate in a non-native state, reversible oligomer formation, nucleation of reversible or irreversible aggregates and growth to larger aggregates. The latter step can occur either by cluster-cluster coagulation or by monomer addition to pre-formed nuclei. The specific protein and the operating conditions under consideration determine the features of the individual steps and, consequently, the overall mechanism. Moreover, different pathways are not mutually exclusive and could also occur simultaneously.

Recently, models based on population balance equations (PBEs), which are commonly used to describe the kinetics of sol colloidal dispersions, have been applied to the kinetics of formation of amyloid fibrils. The application of PBEs to protein aggregation kinetics has several motivations: 1) it provides quantitative mechanistic insights into the single steps constituting the overall process; 2) from the evaluated aggregation rate coefficients (kernels), quantitative information on average protein-protein interactions can be obtained. Ideally, the time evolution of the aggregate size distribution can be described a priori by means of PBE simulations provided that protein-protein interaction potential is properly characterized. For lyophobic colloids, quantification of potential interactions is successfully achieved by mean filed approaches such as the DLVO theory and its modifications. For complex, lyophilic macromolecules, such as proteins, the situation is more challenging. Protein surfaces exhibit anisotropic distribution of charges and simultaneous presence of hydrophobic and hydrophilic patches. Donnan effects and additional interactions, including depletion, hydrophobic and solvation forces, must be
accounted for in protein interaction potential.\textsuperscript{221} Moreover, strong intermolecular forces are often confined to specific patches of the protein surface.\textsuperscript{239} As a consequence, structure stability is strongly interconnected to colloidal stability, and the two contributions superimpose in determining aggregation propensity.\textsuperscript{33} Given such considerations, the detailed description of the protein structure appears fundamental to properly catch protein-protein interactions. However, despite the mentioned limitations, mean field models are often used to describe average protein-protein interactions responsible for aggregation, particularly when electrostatic interactions dominate.\textsuperscript{307} Globular proteins are particularly suitable for this approach: Randolph, Carpenter and co-authors applied the colloidal formalism to describe several model proteins including Fc fusion protein,\textsuperscript{308} human stimulating factor,\textsuperscript{309} human interleukin-1 receptor antagonist\textsuperscript{310} and \textit{Bacillus halmapalus} alpha-amylase.\textsuperscript{311} Additionally, also short peptides and amyloidogenic proteins have been modeled along these lines.\textsuperscript{312,313}

In this chapter, we investigate the aggregates morphology and the aggregation kinetics of the model monoclonal antibody IgG2 whose stability behavior was characterized previously in Chapter 6. Under the considered conditions, the IgG aggregates via a two-step process: reversible nucleation of oligomers followed by irreversible cluster-cluster coagulation. We develop a comprehensive population balance model including both steps of aggregation. By fitting the model simulations to experimental data, we quantify the effective monomer-monomer and aggregate-aggregate interaction potentials as a function of several operating conditions. Moreover, the analysis is extended to data reported in the literature for other systems where however growth by cluster-cluster coagulation dominates over chain polymerization, similarly to the one considered in this work.

\section*{7.2 Experimental Methods}

\textbf{Light Scattering}

Dynamic light scattering (DLS) was measured at a fixed angle of $\theta = 173^\circ$ using a Zetasizer Nano (Malvern, UK) with laser beam of wavelength $\lambda_0 = 633$ nm.
Static light scattering (SLS) measurements were performed using a goniometer, BI-200SM (Brookhaven Instruments, Holtsville, NY, USA) covering angles from $\theta = 16$ to $150^\circ$. A solid-state laser, Ventus LP532 (Laser Quantum, Manchester, UK) with a wavelength $\lambda_0 = 532$ nm was used as the light source. With the same instrument, DLS was measured at a fixed angle of $\theta = 145^\circ$.

Small angle light scattering (SALS) was performed on a Mastersizer 2000 (Malvern, UK) covering angles from $\theta = 0.02$ to $40^\circ$ and with a light source with wavelength $\lambda_0 = 633$ nm.

**Atomic Force microscopy**

10 µL of 150 fold diluted samples were spotted on a freshly cleaved mica surface for 30 seconds before washing with Milli-Q (Milli-pore) deionized water to remove unattached material and gently drying under nitrogen flux. Samples were imaged at room temperature by a Nanoscope IIIa (Digital Instrument, USA) operating in tapping mode. Scan rate of 0.8 Hz and antimony doped silicon cantilevers with resonance frequency in the range 325-382 kHz and tip radius of 8 nm (Veeco, Plainview, NY, USA) were used.

**Transmission Electron Microscopy**

Samples for transmission electron microscopy (TEM) were loaded on a carbon grid (Quantifoil, DE) and negative stained with a 2% uranyl acetate aqueous solution. Pictures were recorded on a FEI Morgagni 268.

### 7.3 Aggregation kinetics and structure morphology

The aggregation kinetics of the model IgG2 at various operating conditions has been measured by in situ dynamic light scattering (DLS) at 173°. The pH effect was investigated selecting as reference system 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na$_2$SO$_4$ at 37 °C. The time evolution of the average hydrodynamic radius, $<R_h>$, for various pH values is shown in Figure 1.1a.
Figure 7.1. a) pH effect on the time evolution of the average hydrodynamic radius, $<R_h>$, measured by dynamic light scattering for a 1 g/L mAb solution in 25 mM citric acid buffer with 0.15 M $\text{Na}_2\text{SO}_4$ at several pH values and 37 °C; b) same as a) but at pH 3.0 at several temperatures; c) same as a) at pH 3.0 and 37 °C with 0.15 M of several counterions.
It is seen that decreasing the pH from 4.5 to 3.25 the oligomers become progressively more unstable, but limited aggregation is observed. At the critical pH value of 3.0, the aggregation kinetics increases dramatically: aggregates increase their size until they become insoluble and, eventually, precipitate. Due to aggregate sedimentation, the light scattering signal decreases when the average $<R_h>$ reaches a value of about 200 nm. Therefore, we only considered smaller size values, corresponding to homogeneous samples. The aggregation kinetics at pH 3.0 is very sensitive to temperature (Figure 7.1b) and type of salt (Figure 7.1c). The anion effect on the coagulation rate at 37 °C follows the order: NO$_3$ > SO$_4$ > Cl > H$_2$PO$_4$. Notably, the same ranking was previously observed for the oligomerization of the same antibody in Chapter 6 and for the aggregation of the IgE light chain fragment in Chapter 5.

The morphology of the formed aggregates during incubation at several temperatures and with different salts has been investigated by transmission electron microscopy (TEM) and by atomic force microscopy (AFM), which offered a better resolution for small species (few tens of nm) with respect to TEM. 1g/L protein solution in 25 mM citric acid buffer at pH 3.0 was selected as reference condition. In Figure 7.2a the AFM picture of a sample taken after 6 hours incubation at 37 °C with 0.15 M Na$_2$SO$_4$ is shown, indicating the presence of globular aggregates with radius of about 25 nm, consistent with DLS measurements (Figure 7.1a), and similar in shape to intermediate aggregates reported in the literature.$^{314}$ When aggregation proceeds further, irregular, amorphous aggregates are formed, as seen in the TEM picture in Figure 7.2b, which corresponds to a sample after 1 month incubation at 25 °C with 0.5 M NaCl. Also in this case, the sizes shown by TEM are somehow consistent with the hydrodynamic radius of about 60 nm given by DLS, although the irregular shape and the heterogeneity of the aggregates complicates the comparison. Moreover, the drying procedure required by the microscopy technique could affect the aggregate size, and therefore the comparison between the sizes obtained by microscopy and DLS must be taken only as indicative. Finally, TEM pictures of samples after 24 hours incubation at 37 °C with 0.15 M of different salts are reported in Figure 7.3, where fractal like clusters appear with relatively large compactness.
Figure 7.2. a) AFM picture of 1 g/L mAb solution in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at 37 °C and pH 3.0 after 6 h; b) TEM picture of 1 g/L mAb solution in 25 mM citric acid buffer with 0.5 M NaCl at 25 °C and pH 3.0 after 1 month.
Figure 7.3. TEM pictures of 1 g/L mAb solution after 24 hours incubation in 25 mM citric acid buffer at 37 °C and pH 3.0, with 0.15 M NaCl (a), 0.15 M Na₂SO₄ (b) and 0.15 M NaNO₃ (c)
The fractal behavior of the clusters was proved by small angle light scattering (SALS), which gives information about the structure factor, \( S(q) \), of the clusters:

\[
S(q) = \frac{I(q)}{I(0)P(q)}
\]  \hspace{1cm} (7.1)

where \( I(q) \) is the angle-dependent scattered intensity, \( I(0) \) is the intensity at zero angle, \( P(q) \) is the form factor of the primary particles and \( q \) is the scattering vector defined as:

\[
q = \frac{4\pi n}{\lambda_0} \sin \left( \frac{\theta}{2} \right)
\]  \hspace{1cm} (7.2)

where \( \theta \) is the scattering angle, \( n \) is the refractive index of the solvent and \( \lambda_0 \) is the wavelength of the laser beam. The light scattering has been measured in the small angle region since this angle interval is suitable for the analysis of aggregates with size in the range of several microns, which is the characteristic size of the IgG aggregates obtained at the end of the process. For such larger clusters, the power-law regime of the average structure factor leads to an estimate of the fractal dimension, \( D_f \):

\[
S(q) \sim q^{-D_f} \quad \text{for} \quad 1/\langle R_g \rangle << q << 1/\langle R_p \rangle
\]  \hspace{1cm} (7.3)

where \( R_p \) is the radius of the primary particle inside the cluster and \( \langle R_g \rangle \) is the average radius of gyration of the cluster distribution. In Figure 7.4 the structure factor of a sample after 12 hours of incubation at 37 °C with 0.15 M Na\(_2\)SO\(_4\) is reported. From the fitting in the power-law regime a fractal dimension equal to 2.6 can be evaluated. Such value is well in agreement with the value of \( D_f = 2.56 \) reported in the literature for antibody aggregates.\(^{315} \) Moreover, \( D_f = 2.6 \) corresponds to quite compact structures, which is consistent with the TEM pictures shown in Figure 7.3.

From the fractal dimension and \( \langle R_b \rangle \) measured by DLS, the aggregate average molecular weight can be evaluated according to the following scaling:

\[
\langle MW \rangle = MW_0 \left( \frac{\langle R_b \rangle}{R_0} \right)^{D_f}
\]  \hspace{1cm} (7.4)

Considering \( D_f = 2.6 \), we compare the values evaluated with Equation 7.4 to the molecular weights directly measured by static light scattering. In Figure 7.5 the comparison is reported for a 1 g/L mAb solution in 25 mM citric acid buffer with 0.15 M Na\(_2\)SO\(_4\) at 25
°C (Figure 7.5a), and with 0.15 M NaCl at 37 °C (Figure 7.5b): the values measured by
SLS are reasonably in agreement with the ones estimated from Equation 7.4.

![Figure 7.4](image)

**Figure 7.4.** Structure factor of a 1 g/L protein sample after 24 hours incubation in 25 mM citric acid buffer at
37 °C and pH 3.0, with 0.15 M Na$_2$SO$_4$.

![Figure 7.5](image)

**Figure 7.5.** Comparison between normalized average number molecular weight as a function of time measured directly by static light scattering (▲) and estimated from DLS data using Eq. 7.4 (○) for a 1 g/L mAb solution in 25 citric acid buffer at pH 3.0 with a) 0.15 M NaSO$_4$ at 25 °C and b) with 0.15 M NaCl at 37 °C.
The time evolution of the structure factor during aggregation has been analyzed by static light scattering, as reported in Figure 7.6. The signal has been recorded in the wide-angle interval, 16°-150°, which is suitable for the analysis of the aggregates with radius of tens-hundreds nm produced during aggregation. Simultaneously, DLS was acquired on the same instrument at 145°. 1 g/L protein solution in 25 mM citric acid buffer with 0.15 M Na₂SO₄ at pH 3.0 and 37 °C was selected as reference conditions. Using the Fisher-Burford formula for the structure factor of fractal aggregates:

\[
S(q) = \left(1 + \frac{2}{3D_f}q^2 \left(\langle R_g \rangle\right)^2 \right)^{-D_f/2} \tag{7.5}
\]

the average gyration radius, \(\langle R_g \rangle\), has been fitted to the experimental data using \(D_f = 2.6\). This leads to the estimation of two independent moments of the cluster distribution, namely the average \(\langle R_g \rangle\):

\[
\langle R_g \rangle^2 = \sum_{i=1}^{N_i} \frac{N_i m_i^2 R_{g,i}^2}{\sum_{i=1}^{N_i} N_i m_i^2} \tag{7.6}
\]

and the average \(\langle R_h \rangle\):

\[
\langle R_h \rangle = \frac{\sum_{i=1}^{N_i} N_i m_i^2 S_i(q)}{\sum_{i=1}^{N_i} N_i m_i^2 S_i(q) R_{h,i}} \tag{7.7}
\]

where \(N_i, m_i\) and \(S_i(q)\) are the number, mass and structure factor at the scattering vector \(q\) of aggregates containing \(i\) units, respectively.

The time evolution of \(\langle R_g \rangle\) and \(\langle R_h \rangle\) are reported in Figure 7.7. As expected, the two quantities are initially very similar but as aggregation proceeds \(\langle R_g \rangle\) increases more rapidly than \(\langle R_h \rangle\), being \(\langle R_g \rangle\) a larger order moment of the cluster distribution.
Figure 7.6. Time evolution of the structure factor of a 1 g/L protein sample incubated in 25 mM citric acid buffer at 37 °C and pH 3.0, with 0.15 M Na$_2$SO$_4$. Continuous lines are fitted curves according to Eq. 7.5.

Figure 7.7. Values of the average radius of gyration $<R_g>$ (●) and the average hydrodynamic radius $<R_h>$ (▲), measured experimentally as a function of the aggregation time using the RLCA kernel (Eq. 7.12). Continuous curve and dashed curve represent PBE model simulation results of $<R_h>$ and $<R_g>$, respectively.
7.4 Aggregation model and Population Balance Equations

The antibody reversible oligomerization in acidic conditions was modeled in Chapter 6, where the attention was focused on the early stages of the aggregation. The aggregation was well described by a modified Lumry-Eyring model, including the denaturation of monomer followed by reversible formation of dimers and trimers.

In this chapter, we consider the aggregation of oligomers with monomer and other oligomers to larger aggregates. In the following, we will define both oligomers and larger aggregates as clusters. While, as mentioned above, the aggregation steps involving monomer are reversible, the aggregation between two clusters is irreversible. Thus, summarizing, the aggregation process can be described with the reaction scheme shown in Figure 7.8. The aggregation is initiated by the formation of a reactive intermediate with a metastable conformation, $N_1^*$, from the antibody in the native form, $N$. Such intermediate can aggregate reversibly both with other monomeric intermediates ($N_1^*$) and with oligomers ($N_2^*, N_3^*, ...$). We distinguish the reactivity between two monomers and between a monomer and an oligomer by introducing two different sets of rate constants: $k_1$ and $k_{-1}$, and $k_2$ and $k_{-2}$, describing the forward and the backward aggregation rate constants for monomer-monomer and monomer-oligomer aggregation, respectively. It is worth noticing that $k_1$ and $k_2$ are rate constants of reactions which lump together the equilibrium between native ($N$) and intermediate ($N_1^*$) state of the protein, and the following propagation step. The irreversible aggregation between two generic clusters containing $i$ and $j$ units is described by the aggregation rate constant $k_{ij}$.
\begin{align*}
N & \xrightleftharpoons[k_{eq}]{K_{eq}} N_1^* \\
K_{eq} &= \left( \frac{[N_1^*]}{[N]} \right)_{eq} = \frac{k_{un}}{k_{fold}} \\
N_1^* + N_1^* & \xrightleftharpoons[k_{1}]{k_{1}} N_2^* \\
N_1^* + N_2^* & \xrightleftharpoons[k_{2}]{k_{2}} N_3^* \\
3 < j < n & : \\
N_1^* + N_j^* & \xrightleftharpoons[k_{2}]{k_{2}} N_{j+1}^* \\
2 < i, j < n & : \\
N_i^* + N_j^* & \rightarrow N_{i+j}^* \\
\text{Figure 7.8. Scheme of the aggregation process based on a modified Lumry-Eyring model.}
\end{align*}

From this kinetic scheme, the following population balance equations have been derived for intermediate monomer, \(N_1^*\), dimer, \(N_2^*\), trimer, \(N_3^*\), and the generic cluster containing \(i\) units (\(N_i^*\)):

\begin{align*}
\frac{dN_1^*}{dt} &= -2k_1N_1^{**} + 2k_{-1}N_1^* - k_2N_1^* \left( \sum_{j=2}^{i} (N_j^*) \right) + k_2 \sum_{j=3}^{i} (N_j^*) \\
\text{(7.8)} \\
\frac{dN_2^*}{dt} &= k_1N_1^{**} - k_{-1}N_2^* - k_2N_1^*N_2^* + k_2N_3^* - N_2^* \left( \sum_{j=2}^{i} k_{2,j}N_j^* \right) \\
\text{(7.9)} \\
\frac{dN_3^*}{dt} &= k_2N_1^*N_2^* - k_{-2}N_3^* - k_2N_1^*N_3^* + k_2N_4^* - N_3^* \left( \sum_{j=2}^{i} k_{3,j}N_j^* \right) \\
\text{(7.10)} \\
\text{i > 3} \\
\frac{dN_i^*}{dt} &= \frac{1}{2} \sum_{j=2}^{i+1} k_{i,j}N_i^*N_{i-j}^* - N_i^* \sum_{j=2}^{i} k_{i,j}N_j^* - k_2N_i^* + k_2N_{i+1}^* \\
\text{(7.11)}
\end{align*}
With reference to a given population of clusters, \(N_i^*\), the terms on the right side of eq.7.11 represent on one hand its increase by aggregation of smaller clusters and on the other its decrease due to reaction with other aggregates. It is worth noticing that eq. 7.8-7.11 represent essentially a generalization of the Smoluchowski’s population balance equations\(^{316}\) to account for the reversibility of oligomer formation and the different values of the kinetic rate constants describing monomer-monomer, monomer-cluster, and cluster-cluster aggregation.

Let us consider the coagulation kinetics at pH 3.0 and 37 °C with 0.15 M Na\(_2\)SO\(_4\) (Figure 7.1a): as described in Chapter 6, under these conditions the reversible nucleation of oligomers is confined to the very early stages of the process. Accordingly, in this case we can neglect this step and assume that the coagulation starts from a homogeneous population of oligomers with \(<R_h>= 9\) nm, corresponding to trimers. Which implies, in eq. 7.8-7.11, to set \(N_1\) and \(N_2\) equal to zero. Then, the PBEs reduce to:

\[
\frac{dN_i^*}{dt} = \frac{1}{2} \sum_{j=2}^{i-1} k_{i,j} N_i^* N_j^* - N_i^* \sum_{j=2}^{\infty} k_{i,j} N_j^* \quad i \geq 3 \tag{7.12}
\]

The solution of the PBEs involves the choice of the aggregation kernel, \(k_{ij}\). When no repulsive barrier exists among the proteins, the process is controlled by Brownian motions and aggregation occurs under fast, diffusion limited conditions (DLCA). On the other hand, when an energetic barrier stabilizes two approaching particles, the aggregation efficiency is reduced, the system is under reactive limited conditions (RLCA) and shows lower coagulation rates. The general kernel for RLCA aggregation of fractal objects is:

\[
k_{i,j} = \frac{k_B}{W} B_{i,j} P_{i,j} \tag{7.13}
\]

where \(k_B\) is the Smoluchowski rate constant for diffusion limited aggregation, defined as:

\[
k_B = \frac{8kT}{3\eta}, \quad \text{where} \quad k \text{ is Boltzmann constant, } T \text{ is temperature and } \eta \text{ the viscosity. } W \text{ is the so-called stability ratio, accounting for the energetic barrier between two approaching primary particles, } B_{i,j} \text{ is the correction for the Brownian term that accounts for the fractal nature of the aggregates and for the aggregation between unequal size clusters:}
\]
\[ B_{i,j} = \frac{\left( i^{1/D_f} + j^{1/D_f} \right) \left( 1/i^{1/D_f} + 1/j^{1/D_f} \right)}{4} \]  

(7.14)

where the experimentally measured fractal dimension of \( D_f = 2.6 \) has been used. Finally, \( P_{i,j} \) is the correction term for RLCA conditions with respect to DLCA conditions. Several expressions of \( P_{i,j} \) have been proposed in the literature.\(^7\) In the absence of precise information about the relationship between aggregates structure and reactivity, we selected the product kernel: \(^{317}\)

\[ P_{i,j} = (ij) \lambda \]  

(7.15)

which includes the entire size dependence of the RLCA aggregation rate in a semiempirical parameter \( \lambda \), which based on purely fractal scaling arguments is expected to be in the order of \( 1 - 1/D_f \) and therefore, with \( D_f = 2.6, \lambda = 0.61. \(^{318}\)

The system of ODEs (eqs 7.8-7.11) has been solved numerically using the algorithm proposed by Kumar and Ramkrishna.\(^7,319,320\) Both DLCA and RLCA kernels were tested by comparing model simulations to the experimental data of the time evolution of \( <R_h> \) and \( <R_g> \). While the DLCA predicted an aggregation time in the order of milliseconds and therefore far away from the experimental data, a satisfactory agreement was obtained using the RLCA kernel with fitting parameters \( \lambda = 0.6 \pm 0.01 \) and \( W = 3.3 \pm 0.1 \cdot 10^8 \), as shown in Figure 7.7. The mean squared error, defined as \( E = \sum_{i=1}^{N} \left( y_i - y_{est} \right)^2 / N \), where \( y \) represents the measured normalized average radius of hydration and gyration, \( y_{est} \) the variable estimated by the model and \( N \) the number of experimental points, is \( 2.5 \pm 0.2 \cdot 10^{-3} \). It is worth noticing that the fitted value \( \lambda = 0.6 \) is very close to the expected value of 0.61 estimated above through fractal scaling arguments. The model simulations are in reasonable agreement with the experimental data, although the time evolution of \( <R_h> \) and \( <R_g> \) are slightly over- and underestimated, respectively. Since the difference between these two average moments is related to the polydispersity of the distribution, this indicates that the experimental distributions are broader than the simulated ones.
It is worth noticing that the estimated value of $W = 3.3 \cdot 10^8$ is close to the value $W = 9.1 \cdot 10^8$ previously reported in the literature for antibody aggregation induced at neutral pH and large temperature.\textsuperscript{315} These large $W$ values indicate that protein aggregates are significantly stabilized, even in non-native conditions.

Eq. 7.12 can be conveniently expressed in dimensionless form:

$$\frac{dX_i}{d\tau} = \frac{1}{2} \sum_{j=2}^{i-1} \beta_{i,j} X_j X_{i-j} - X_i \sum_{j=2}^{\infty} \beta_{i,j} X_j$$

(7.16)

where $X_i$ is the dimensionless cluster number concentration $X_i = N_i / N_{i,0}$, with $N_{i,0}$ the initial number concentration of protein particles, $\beta_{i,j} = B_{i,j} P_{i,j}$ and $\tau = t / t_c$ is the dimensionless time normalized with respect to the characteristic aggregation time, $t_c$, defined as:

$$\frac{1}{t_c} = \frac{1}{W} k_g \cdot N_{1,0}$$

(7.17)

From the dimensionless form of the PBE (eq. 7.16) it can be seen that the time evolution of the cluster distribution depends only on the two parameters $D_f$ and $\lambda$, included in $B_{i,j}$ and $P_{i,j}$, respectively. This implies that, if the aggregation process produces clusters with the same structure ($D_f$ and $\lambda$), the aggregation kinetics (e.g., the time evolution of the average hydrodynamic radius) follows a single master curve when plotted versus the dimensionless time, $\tau$, independently on the particle material, particle concentration and conditions of aggregation. This has been shown in the past for polymeric colloidal systems in RLCA and DLCA conditions.\textsuperscript{7,321,322}

Let us consider now the aggregation kinetics under various different conditions reported in Figure 7.1. We see that the aggregation time scales vary quite a bit, ranging from a few hours (Figure 7.1a and 7.1b) to several days (Figure 7.1c). However, when introducing the dimensionless time, $\tau$, and using $W$ as a fitting parameter, all the different kinetic profiles collapse on a single master curve, as shown in Figure 7.9. This important result indicates that, at least in the conditions investigated in this work, aggregation follows a unique mechanism well represented by the adopted kinetic scheme of aggregation and kernel model. It is worth noticing that this conclusion applies for various conditions at
acidic pH: several temperatures at fixed pH (Figure 7.9a), several counterions at fixed temperature and pH (Figure 7.9b) and different salt concentrations at different pH (Figure 7.9b). The analysis was extended to two studies reported in the literature, which describe a coagulation mechanism similar the one observed in this work: a 10 g/L IgG1 solution at physiological pH 6.5 at 60 °C, and 1 g/L IgG1 solution at pH 4.5 and 58 °C. In Figure 7.9c it can be seen that these antibodies behave similarly to the IgG investigated in this work and in fact collapse on the same master curve. The results shown in Figure 7.9 indicate a certain level of generality in the antibody aggregation behavior, at least when condensation mechanism dominates chain polymerization.

The $W$ values estimated for all the different conditions investigated in Figure 7.9 are reported in Table 7.1. The quantification of $W$ allows some important considerations: the large $W$ values in all conditions indicate that even in non-native conditions aggregation of immunoglobulins is not under diffusive control, but rather it is an activated process with some energy barrier to be overcome for aggregation to occur. In Figure 7.10 the effective cluster kinetic rate coefficients, $k_c = \frac{k_v}{W}$, is shown as a function of the reciprocal temperatures: it is seen that in the investigated temperature range, the kinetics follows Arrhenius law, with an effective activation energy of 60 kcal/mol.
Figure 7.9. a) Time evolution of the normalized average hydrodynamic radius versus dimensionless time for a 1 g/L mAb solution in 25 citric acid buffer at pH 3.0 and with 0.15 M Na₂SO₄ at: 25 °C (Δ), 30 °C (●), 35 °C (×), 37 °C (○), 39 °C (◦), 45 °C (□); b) same as a) but at pH 3.0 and 37 °C with 0.15 M NaH₂PO₄ (×), NaCl (●), Na₂SO₄ (○), NaNO₃ (Δ), at pH 4.0 and 55 °C with 0.15 M Na₂SO₄ (○), and at pH 3.0 and 25 °C with 0.5 M NaCl (◦); c) Kinetics data of model IgG of this work (○) are compared to data of other antibodies taken from ref. [323] (○) and ref. [281] (□) (see main text for details).
Table 7.1. Effective stability ratio values ($W$) obtained by scaling the dimensionless time evolution of $<R_g>$ in various conditions and with different antibodies on a single master curve, as shown in Figure 7.9. The average uncertainty of the fitted parameters is ±5% of the value.

<table>
<thead>
<tr>
<th></th>
<th>25 °C</th>
<th>30 °C</th>
<th>35 °C</th>
<th>37 °C</th>
<th>39 °C</th>
<th>45 °C</th>
</tr>
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<tbody>
<tr>
<td>pH 3.0 and 0.15 M Na$_2$SO$_4$ at various temperatures</td>
<td>$1.7\cdot10^{10}$</td>
<td>$2.5\cdot10^9$</td>
<td>$5.1\cdot10^8$</td>
<td>$3.3\cdot10^8$</td>
<td>$1.7\cdot10^8$</td>
<td>$4.3\cdot10^7$</td>
</tr>
<tr>
<td>pH 3.0 and 37 °C, with 0.15 M solutions of various salts</td>
<td>NaH$_2$PO$_4$</td>
<td>NaCl</td>
<td>Na$_2$SO$_4$</td>
<td>NaNO$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$6.3\cdot10^9$</td>
<td>$2.5\cdot10^9$</td>
<td>$3.3\cdot10^8$</td>
<td>$2.2\cdot10^8$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pH 4.0 and 55 °C, with 0.15 M Na$_2$SO$_4$  
1.8·10$^{10}$

pH 3.0 and 25 °C, with 0.5 M NaCl  
$1.8\cdot10^{10}$

Ref. [323] IgG1, pH 6.5 and 60 °C  
$2.5\cdot10^9$

Ref. [281] IgG1, pH 4.5 and 58 °C  
$7.2\cdot10^8$

Figure 7.10. Arrhenius plot of the effective kinetic rate constants, $k_C = \frac{k_s}{W}$, at pH 3.0 with 0.15 M Na$_2$SO$_4$ evaluated from the $W$ values reported in Table 7.1. The fitting provides an activation energy of 60 kcal/mol.
Another interesting comparison can be made by considering that in the kernel expression of eq. 7.13 the effect of the aggregate size $i$ and $j$ is confined in the terms $B_{ij}$ and $P_{ij}$, while the Fuchs stability ratio $W$ refers to primary particles, i.e. to the aggregation between two oligomers containing one monomer unit. Therefore, this in principle should correspond to the kernel $k_i$. However, this cannot be the case because eq. 7.13 does not account for structure changes which are particularly strong when going from $N_1^*$ to $N_2^*$, $N_3^*$ and then larger oligomers. This is in fact the rational why in the kinetic scheme in Figure 7.9 the aggregation kernel of the first three oligomers have been treated separately from that of larger oligomers. It is now interesting to compare such $W$, representing the stability of “oligomer of size one”, with $W_1 = k_2/k_1$, which represents the stability of the non-native monomer species. This should reflect the different stability character of oligomers and monomers depurated from the size effect. For this, the oligomerization rate constant, $k_i$, at 25 °C and low protein concentration (0.1-0.5 g/L) for chloride was used as reported in Chapter 6, while for the other anions it was measured using the same procedure. The obtained $W$ and $W_1$ values are compared in Figure 7.11. It is seen that the anion effect on the interactions between two non-native monomers is reflected in the interactions between two non-native aggregates. Moreover, the stability ratios are smaller for monomers at 25 °C than for aggregates at 37 °C, indicating a significantly larger reactivity for the monomers. For the aggregation at pH 3.0 with 0.15 M Na$_2$SO$_4$ at 25 °C $W_1$ and $W$ were in fact found equal to $W_1 = 1.5 \cdot 10^8$ (Figure 7.11) and $W = 1.7 \cdot 10^{10}$ (Table 7.1), i.e. a difference of two orders of magnitude.

We conclude that the incubation of antibody at pH 3.0 induces formation of a reactive non-native structure which exposes aggregating-prone (likely hydrophobic) patches; during aggregation, the number of these patches reduces and, consequently, the reactivity of aggregates is lower than that of monomers. The transition in reactivity occurs sharply within few aggregating units, together with the change in secondary structure of the oligomers.
7.5 Concluding remarks

We investigated the aggregates morphology and aggregation kinetics of a model IgG2 in acidic conditions. It was found that antibody aggregates exhibit an effective fractal morphology, with fractal dimension equal to 2.6, in agreement with TEM pictures.

Two average moments of the cluster distribution, namely the average radius of gyration, $<R_g>$, and the average hydrodynamic radius, $<R_h>$, have been measured in situ by light scattering. A comprehensive population balance model based on modified Smoluchowski equations has been developed to describe the two-step aggregation process: reversible oligomerization followed by cluster-cluster aggregation. The model simulations are in good agreement with experimental data, and indicate that non-native antibody aggregation does not occur under diffusive control, i.e. it is an activated process.

Introducing a dimensionless time scaled on the Fuchs stability ratio ($W$), the time evolutions of $<R_h>$ measured under a great variety of operating conditions (temperature, pH, type and concentration of salt) collapse on a single master curve. This applies also to...
data reported in the literature when growth by cluster-cluster coagulation dominates, indicating a certain level of generality in the antibodies aggregation behavior.

The described analysis gives important quantitative information on the effective protein-protein interactions and their dependence on operating parameters, such as temperature, pH and type of anion. In particular, it is found that the aggregation rate increases with increasing temperature according to an Arrhenius law. Moreover, the reactivity of the aggregates is lower than the one of the intermediate monomers. This is likely due to the reduction during aggregation of the number of available, aggregating-prone (hydrophobic) patches induced by monomer unfolding at pH 3.0.
Chapter 8

Concluding remarks and Outlook

In the first part of the work, we analyzed the stability of polymer particle dispersions and the polymerization kinetics in heterogeneous systems by combining experimental characterizations to population balance equation (PBE) modeling. In the second part, the same methodology was applied to investigate the stability of several protein solutions. For all the investigated systems, the combination of mathematical models to experimental data provided mechanistic insights of the aggregation process as well as quantitative analysis of the effect of key parameters on aggregation rate and aggregate morphology. The most meaningful results are reported in the following. Taken together, the results indicate that mechanistic approaches, commonly applied in polymer and colloid engineering, represent reliable tools to describe the behavior of more complex aggregation systems such as proteins.

Polymer particle dispersions
The shear-induced gelation of soft, strawberry-like particle dispersions is considered (Chapter 2). It is found that the gel structure and the conversion of the primary particles to gel can be controlled by tuning surface properties of the primary particles, in particular the rubber core surface coverage by the plastic shell and the surfactant surface coverage. Above a critical surface coverage, ionic and steric surfactants can protect particles from aggregation through short-range hydration and steric repulsive forces, respectively. Moreover, nonionic steric surfactants can also protect particles from coalescence, while ionic surfactant cannot.
Precipitation polymerization

A novel precipitation polymerization system, namely the copolymerization of vinyl-imidazole and vinyl-pyrrolidone in organic solvent, has been investigated (Chapter 3). Experimental data have been combined to a comprehensive kinetic model to elucidate key features of the process, such as the reaction locus and the role of mass transport from the continuous phase to the precipitated one. It is concluded that the dispersed phase dictates the behavior of the system and that the transport of active chains from the continuous phase to the dispersed one is negligible. As a consequence, for the system under consideration, the size distribution of the precipitating particles (PSD) does not affect the reaction rate. On the other hand, the developed model is sufficiently general to account for the effect of PSD on reaction rate and can be easily applied to other systems where mass transport does occur. In addition, this analysis allows the quantification of the effects of operative parameters, such as copolymer composition and stirring rate, on the PSD, which impacts the properties of the final product.

Protein solutions

The stability behavior of different proteins with increasing size has been investigated experimentally in parallel as a function of several environmental parameters (Chapters 4, 5 and 6). Different aggregate morphologies are observed: the amphiphilic peptide RADA self-assembles into fibrils, while both the light chain dimer and the entire antibody form amorphous aggregates.

The detailed characterization of the RADA fibril dispersions and of their stability (Chapter 4) provide relevant information about the state of the peptide and the end-to-end fibril-fibril aggregation mechanism in acidic conditions. Moreover, this work represents the starting point for possible future studies addressing the morphology and the properties of the fibrillar gels obtained by destabilizing the fibril dispersions (e.g. by salt addition or pH shift).

The impossibility to obtain fibrils from the considered light chain dimer (Chapter 5), together with data reported in the literature, suggests that the occurrence of amyloidosis in patients requires the presence of the light chain fragment in the monomer form, while
dimer can form only amorphous oligomers or amorphous deposits. This is going to be verified by on-going studies on several amyloidogenic variants which showed different aggregation behavior in patients. Such variants will be analyzed in the same way as the non-amyloidogenic fragment considered in this work. The comparative analysis of fragments with different aggregation propensity and clinical consequences could improve our knowledge on the relationship between aggregation behavior and \textit{in vivo} pathogenicity. 

This project is conducted in collaboration with the group of Prof. Merlini at the Policlinico San Matteo (University of Pavia, Italy).

The investigated light chain dimer and complete antibodies (Chapter 5 and 6) show similar stability behaviors. Changes in pH and salt addition induce aggregation not only by altering the typical charge screening and various stabilizing and destabilizing solvation forces, but also by promoting formation of structural metastable states characterized by a partially ordered structure and certain degree of hydrophobicity. The complex interactions between the solvation forces and the protein secondary structure, induced by salts, result in the observed re-stabilization at large salt concentrations, as well as ion specificity and the peculiar behavior of sulfate anion.

Two distinct steps are identified for the full length antibodies aggregation (Chapter 6): 1) reversible oligomerization involving mainly monomers, dimers and trimers, and 2) irreversible cluster-cluster aggregation leading to larger aggregates in the micron range, which, eventually, precipitate. Temperature, pH and ionic strength determine both oligomerization and growth rate by affecting the protein conformational state. In particular, mild destabilizing conditions promote formation of stable oligomers; on the other hand, stronger denaturing conditions induce oligomer-oligomer aggregation. For the different antibodies under investigation, the aggregation propensity in acidic conditions does not correlate with antibody subclass, hydrophobicity and surface charge of the non-native state.

The reversible antibody oligomer formation has been interpreted using a modified Lumry-Eyring model. By combining model simulations with experimental data, we quantified the effect of type and concentration of salt on the dimerization equilibrium constant, which is directly related to the protein-protein interaction potential. It would be of interest to
compare the obtained values with the potentials estimated by alternative techniques, such as by measurements of the second virial coefficient. Moreover, despite the limitations described in the Introduction section, it would be worth attempting the theoretical evaluation of the interaction potentials by means of coarse-grained (DLVO-like) approaches. This requires additional studies on the role of the anion on the single interaction forces between two approaching units. The results of these studies on the specific anion effect, in particular sulfate, would have also an in vivo biologically relevance due to the involvement of sulfonated glycosaminoglycans (GAGs) in amyloid fibril formation of several proteins.

In the last part of this thesis, PBE modeling is applied to describe the antibodies aggregation kinetics to high molecular weight aggregates (Chapter 7): it is found that, by introducing a dimensionless time scaled on the Fuchs stability ratio ($W$), the time evolutions of the average hydrodynamic radius, $<R_h>$, measured under a great variety of operating conditions (temperature, pH, type and concentration of salt) collapse on a single master curve. The described analysis gives important quantitative information on the effective protein-protein interactions and their dependence on operating parameters, such as temperature, pH and type of anion. These results find relevant potential applications in shelf-life prediction and in optimization of formulation for therapeutic proteins. The PBE approach applied in Chapter 7 is sufficiently general to describe different aggregation mechanisms, including the nucleation polymerization mechanism involved in amyloid fibril formation and secondary fibril-fibril aggregation reactions. An example has been provided in Chapter 4, where the peptide fibril elongation by end-to-end fibril-fibril aggregation mechanism is well described by PBE simulations.
Appendix A

Comprehensive Model for precipitation polymerization kinetics
(Chapter 3)

Given the kinetic scheme shown in Table 3.2 the following Population Balance Equations (PBEs) for active and terminated chains state. The superscript indicates the phase, with index=1 corresponding to the continuous phase and index = 2 to the dispersed phase. For active chains QSSA has been applied.

\[
\frac{d}{dt} \left[ \frac{1}{V^1} \left[ R_{n_1}^{*1} \right] \right] = -k_{p1} \left[ M^1 \right] \left[ R_{n_1}^{*1} \right] + k_{p1} \left[ M^1 \right] \left[ R_{n_1-1}^{*1} \right] - \left( k_{id} + k_{i} \right) \left[ R_{n_1}^{*1} \right] \sum_{j=1}^{n} \left[ R_{j}^{*1} \right] + \delta_{n,0} R_{1}^{*1} = 0
\]

\[
\frac{d}{dt} \left[ \frac{1}{V^1} \left[ P_{n_1}^{1} \right] \right] = \frac{1}{2} k_{c} \sum_{j=1}^{n} \left[ R_{j}^{*} \right] \left[ R_{j}^{*1} \right] \left[ R_{n_1-j}^{*1} \right] - k_{id} \left[ R_{n_1}^{*1} \right] \left[ R_{j}^{*1} \right] \sum_{j=1}^{n} \left[ R_{j}^{*1} \right] \sum_{j=1}^{n} \left[ R_{j}^{*1} \right]
\]

\[
\frac{d}{dt} \left[ \frac{1}{V^2} \left[ R_{n_2}^{*2} \right] \right] = -k_{p2} \left[ M^2 \right] \left[ R_{n_2}^{*2} \right] + k_{p2} \left[ M^2 \right] \left[ R_{n_2-1}^{*2} \right] - \left( k_{id} + k_{i} \right) \left[ R_{n_2}^{*2} \right] \sum_{j=1}^{n} \left[ R_{j}^{*2} \right] - k_{cr} \left[ R_{n_2}^{*2} \right] \sum_{j=1}^{n} \left[ P_{j}^{*} \right] \sum_{j=1}^{n} \left[ R_{j}^{*2} \right] + \delta_{n,0} R_{2}^{*2} = 0
\]

\[
\frac{d}{dt} \left[ \frac{1}{V^2} \left[ P_{n_2}^{2} \right] \right] = \frac{1}{2} k_{c} \sum_{j=1}^{n} \left[ R_{j}^{*2} \right] \left[ R_{j}^{*2} \right] \left[ R_{n_2-j}^{*2} \right] - k_{id} \left[ R_{n_2}^{*2} \right] \sum_{j=1}^{n} \left[ R_{j}^{*2} \right] - k_{cr} n \left[ P_{n_2}^{2} \right] \sum_{j=1}^{n} \left[ R_{j}^{*2} \right]
\]

where \( \delta_{n,0} \) is the Kronecker index and \( R_{j} \) is the initiation rate (\( R_{j} = 2 \eta \cdot k_{j} \cdot [I] \)).

Introducing the moments for active (\( \lambda_{n} = \sum_{n=1}^{\infty} n^{i} \left[ R_{n}^{*} \right] \)) and terminated chains (\( \mu_{n} = \sum_{n=1}^{\infty} n^{i} \left[ P_{n}^{*} \right] \)) the following equations were derived for the moments of the first, second and third order in the two phases:

\[
\frac{d}{dt} \lambda_{n}^{1} = R_{j} \left( k_{id} + k_{i} \right) \left( \lambda_{n}^{1} \right)^{2}
\]
\[
\frac{d\lambda_1^1}{dt} = R_i^1 + k_p^1 \left[ M^1 \right] \lambda_0^1 - \left( k_{sd}^1 + k_{sc}^1 \right) \lambda_0^1 \lambda_1^1 
\]
(A6)

\[
\frac{d\lambda_1^2}{dt} = R_i^1 + k_p^1 \left[ M^1 \right] \left( \lambda_0^1 + 2 \lambda_1^1 \right) - \left( k_{sd}^1 + k_{sc}^1 \right) \lambda_0^1 \lambda_2^1 
\]
(A7)

\[
\frac{d\lambda_2^2}{dt} = R_i^2 - \left( k_{sd}^2 + k_{sc}^2 \right) \left( \lambda_0^2 \right)^2 
\]
(A8)

\[
\frac{d\lambda_2^3}{dt} = R_i^2 + k_p^2 \left[ M^2 \right] \lambda_0^2 - \left( k_{sd}^2 + k_{sc}^2 \right) \lambda_0^2 \lambda_3^2 + k_{cr} \mu_2^2 \lambda_0^2 
\]
(A9)

\[
\frac{d\lambda_2^4}{dt} = R_i^2 + k_p^2 \left[ M^2 \right] \left( \lambda_0^2 + 2 \lambda_2^2 \right) - \left( k_{sd}^2 + k_{sc}^2 \right) \lambda_0^2 \lambda_4^2 + k_{cr} \left( \lambda_0^2 \mu_2^2 + 2 \mu_2^2 \lambda_4^2 \right) 
\]
(A10)

\[
\frac{d\mu_0^1}{dt} = \left( \frac{k_{sc}^1}{2} + k_{sd}^1 \right) \left( \lambda_0^1 \right)^2 
\]
(A11)

\[
\frac{d\mu_1^1}{dt} = \left( k_{sd}^1 + k_{sc}^1 \right) \lambda_0^1 \lambda_1^1 
\]
(A12)

\[
\frac{d\mu_2^1}{dt} = k_{sc}^1 \left[ \lambda_0^1 \lambda_1^1 + \left( \lambda_1^1 \right)^2 \right] + k_{sd}^1 \lambda_0^1 \lambda_2^1 
\]
(A13)

\[
\frac{d\mu_2^2}{dt} = \left( \frac{k_{sc}^2}{2} + k_{sd}^2 \right) \left( \lambda_0^2 \right)^2 - k_{cr} \mu_2^2 \lambda_0^2 
\]
(A14)

\[
\frac{d\mu_2^3}{dt} = \left( k_{sd}^2 + k_{sc}^2 \right) \lambda_0^2 \lambda_1^2 - k_{cr} \mu_2^2 \lambda_0^2 
\]
(A15)

\[
\frac{d\mu_2^4}{dt} = k_{sc}^2 \left[ \lambda_0^2 \lambda_2^2 + \left( \lambda_2^2 \right)^2 \right] + k_{sd}^2 \lambda_0^2 \lambda_4^2 - k_{cr} \mu_2^2 \lambda_0^2 
\]
(A16)

Closure equation
\[
\mu_3 = \frac{\mu_2 \left( 2 \mu_2 \mu_0 - \mu_4^2 \right)}{\mu_1 \mu_0} 
\]
(A17)

In order to apply the method of moments in a reliable way, Numerical Fractionation\textsuperscript{324} has been applied to the PBEs for the dispersed phase. Namely, the active and dead chain populations are divided in a finite number of classes or generations, \( NG \), based on their size according to the following fractionation rules:
- all linear chains are in generation 1;
- a chain in the first generation is transferred to the second one by a crosslinking event;
- a chain is transferred from generation $g$ to $g+1$ (for $g \geq 2$) by crosslinking or termination by combination of two chains in $g$;
- a chain formed by the combination termination of a chain in $g$ and a chain belonging to any of the previous generations, remains in $g$.

The polymer contained in the last generation has such a large crosslinking extent to be considered gel and the ratio of the polymer in such generation to the overall amount of polymer is the gel fraction. To set a proper number of classes, the value of $NG$ is increased until convergence in the predicted gel point, the conversion value corresponding to the onset of gelation.\textsuperscript{325} This way, 6 polymer generations have been found enough for reliable estimation of the gel point. The overall MWD can be evaluated by summing the narrower MWDs of all generations. The fractionated PBEs are reported in the following. Note that the superscript has a different meaning respect to the previous equations and refers now to the number of the generation, running from the first ($G=1$) to the last ($G=NG$) class. Since these equations apply only to one phase, it’s implicit that all concentrations, moments and kinetic constants refer to the dispersed phase. All moments with no superscript are the overall moments of the entire dispersed phase, i.e. the summation of the moments of the single generations. The model equations have been implemented in a code in Fortran 90 using the solver DLSODA from the library ODEPACK.\textsuperscript{326}

Active chains

\[
G = 1
\]

\[
\frac{d\left[R_n^1\right]}{dt} = -k_p \cdot [M] \cdot \left[R_n^1\right] + k_p \cdot [M] \cdot \left[R_{n-1}^1\right] - (k_{id} + k_{ic}) \cdot \left[R_n^1\right] \cdot \lambda_0 - k_{cr} \cdot \left[R_n^1\right] \cdot \mu_i + \delta_{n,i} \cdot R_i
\]

(A18)
\[ G = g \]
\[ \frac{d[R^g_n]}{dt} = -k_{p_1}^* [M] [R^g_n] + k_{p_1}^* [M] [R^{g-1}_{n-1}] - (k_{id} + k_{ic})^* [R^g_n] \lambda_0 - k_{cr}^* [R^g_n] \mu_t \]
\[ + k_{cr}^* \sum_{j=1}^{n-1} [R_j^{g-1}] (n - j) [P_{n-j}^{g-1}] \]
\[ + k_{cr}^* \sum_{j=1}^{n-1} [\sum_{j=1}^{n-1} [R_j^h] (n - j) [P_{n-j}^h] + \sum_{j=1}^{n-1} [R_j^g] (n - j) [P_{n-j}^h] \]
\[ G = NG \]
\[ \frac{d[R_{n-1}^{NG}]}{dt} = -k_{p_1}^* [M] [R_{n-1}^{NG}] + k_{p_1}^* [M] [R_{n-1}^{NG}] - (k_{id} + k_{ic})^* [R_{n-1}^{NG}] \lambda_0 \]
\[ - k_{cr}^* [R_{n-1}^{NG}] \mu_t + k_{cr}^* \sum_{j=1}^{n-1} [R_j^{NG-1}] (n - j) [P_{n-j}^{NG-1}] \]
\[ + k_{cr}^* \sum_{j=1}^{n-1} [R_j^{NG}] (n - j) [P_{n-j}^{NG}] \]
\[ + k_{cr}^* \sum_{j=1}^{n-1} [R_j^{NG}] (n - j) [P_{n-j}^{NG}] + \sum_{j=1}^{n-1} [R_j^{NG}] (n - j) [P_{n-j}^{h}] \]

Terminated chains

\[ G = 1 \]
\[ \frac{d[P_n^1]}{dt} = \frac{1}{2} k_{ic}^* \sum_{j=1}^{n-1} [R_j^1] [R_{n-j}^1] + k_{ic}^* \lambda_0 [R_n^1] - k_{cr}^* n [P_n^1] \lambda_0 \]
\[ G = 2 \]
\[ \frac{d[P_n^2]}{dt} = k_{ic}^* \sum_{j=1}^{n-1} [R_j^2] [R_{n-j}^2] + k_{ic}^* \lambda_0 [R_n^2] - k_{cr}^* n [P_n^2] \lambda_0 \]
\[ G = g \]
\[ \frac{d[P_n^g]}{dt} = k_{ic}^* \sum_{j=1}^{n-1} [R_j^g] [R_{n-j}^g] + k_{ic}^* \sum_{j=1}^{n-1} [R_j^{g-1}] [R_{n-j}^{g-1}] \]
\[ + k_{id} \lambda_0 [R_n^g] - k_{cr}^* \lambda_0 [P_n^g] \]
\[ G = NG \]

\[
\frac{d\left[ P_n^{NG} \right]}{dt} = k_{tc} * \sum_{j=1}^{n-1} \left[ R^j \right] \left[ R_{n-j}^{NG} \right] + \frac{k_{tc}}{2} \sum_{j=1}^{n-1} \left[ R_j \right] \left[ R_{n-j}^{NG} \right] \]

\[
+ k_{tc} * \sum_{j=1}^{n-1} \left[ R_j \right] \left[ R_{n-j}^{NG} \right] + k_{id} \lambda_0 \left[ R_n^{NG} \right] - k_{cr} \lambda_0 n \left[ P_n^{NG} \right] \]

\[ (A24) \]

The equations of the moments of the first, second and third order for active and terminated chains in the different generations are the following:

Active chains

\[ \lambda_0 \]

\[
\frac{d\lambda_0^1}{dt} = R_1 - \left( k_{id} + k_{tc} \right) \lambda_0^1 - k_{cr} \mu_1 \lambda_0^1 \]

\[ (A25) \]

\[
\frac{d\lambda_0^2}{dt} = -\left( k_{id} + k_{tc} \right) \lambda_0^2 - k_{cr} \mu_1 \lambda_0^2 + k_{cr} \mu_1^2 \lambda_0^1 + \mu_1^2 \lambda_0^2 \]

\[ (A26) \]

\[
\frac{d\lambda_0^g}{dt} = -\left( k_{id} + k_{tc} \right) \lambda_0^g - k_{cr} \mu_1 \lambda_0^g + k_{cr} \mu_1^{g-1} \lambda_0^{g-1} + k_{cr} \sum_{h=1}^{g-1} \left( \mu_1^h \lambda_0^h + \mu_1^h \lambda_0^g \right) \]

\[ (A27) \]

\[
\frac{d\lambda_0^{NG}}{dt} = -\left( k_{id} + k_{tc} \right) \lambda_0^{NG} - k_{cr} \mu_1 \lambda_0^{NG} + k_{cr} \mu_1^{NG-1} \lambda_0^{NG-1} + k_{cr} \mu_1^{NG} \lambda_0^{NG} \]

\[ (A28) \]

\[ \lambda_1 \]

\[
\frac{d\lambda_1^1}{dt} = R_1 + k_p \cdot [M] \lambda_0^1 - \left( k_{id} + k_{tc} \right) \lambda_0^1 - k_{cr} \mu_1 \lambda_1^1 \]

\[ (A29) \]

\[
\frac{d\lambda_1^2}{dt} = k_p \cdot [M] \lambda_0^2 - \left( k_{id} + k_{tc} \right) \lambda_0^2 - k_{cr} \mu_1 \lambda_2^2 + \mu_1^2 \lambda_0^1 + \lambda_0^1 \lambda_1^2 + \lambda_0^2 \lambda_1^2 \]

\[ (A30) \]
\[
\frac{d\lambda_i^g}{dt} = k_p \cdot [M]^\ast \lambda_i^g - (k_{td} + k_{tc}) \lambda_i^g - k_{cr}^* \mu_i \lambda_i^g + k_{cr}^* \left( \mu_{g-1} \lambda_{g-1}^g + \lambda_{0}^{g-1} \mu_{2}^{g-1} \right) \\
+ k_{cr}^* \sum_{h=1}^{g-1} \left( \mu_i^h \lambda_i^h + \lambda_0^h \mu_2^h + \mu_i^h \lambda_3^h + \lambda_0^h \mu_2^h \right) 
\]  
\text{(A31)}

\[
\frac{d\lambda_i^{NG}}{dt} = k_p \cdot [M]^\ast \lambda_i^{NG} - (k_{td} + k_{tc}) \lambda_i^{NG} \\
- k_{cr}^* \mu_i \lambda_i^{NG} + k_{cr}^* \left( \mu_i^{NG-1} \lambda_i^{NG-1} + \lambda_0^{NG-1} \mu_2^{NG-1} \right) + k_{cr}^* \left( \mu_i^{NG} \lambda_i^{NG} + \lambda_0^{NG} \mu_2^{NG} \right) \\
+ k_{cr}^* \sum_{h=1}^{NG-1} \left( \mu_i^h \lambda_i^h + \lambda_0^h \mu_2^h + \mu_i^h \lambda_3^h + \lambda_0^h \mu_2^h \right) 
\]  
\text{(A32)}

\[
\lambda_2 
\]  
\[
\frac{d\lambda_2^1}{dt} = R_i + k_p \cdot [M]^\ast \left( 2\lambda_1^1 + \lambda_0^1 \right) - (k_{td} + k_{tc}) \lambda_2^1 - k_{cr} \mu_i \lambda_2^1 
\]  
\text{(A33)}

\[
\frac{d\lambda_2^2}{dt} = k_p \cdot [M]^\ast \left( 2\lambda_2^2 + \lambda_0^2 \right) - (k_{td} + k_{tc}) \lambda_2^2 - k_{cr} \mu_i \lambda_2^2 \\
+ k_{cr}^* \left( \lambda_0^1 \mu_1^1 + 2\lambda_2^1 \mu_1^1 + 2\lambda_2^1 \mu_1^1 + 2\lambda_2^1 \mu_1^1 + 2\lambda_2^1 \mu_1^1 \\
+ 2\lambda_2^1 \mu_1^1 + \mu_i^1 \mu_2^1 + 2\lambda_2^1 \mu_2^1 + 2\lambda_2^1 \mu_2^1 \right) 
\]  
\text{(A34)}

\[
\frac{d\lambda_2^g}{dt} = k_p \cdot [M]^\ast \left( 2\lambda_2^g + \lambda_0^g \right) - (k_{td} + k_{tc}) \lambda_2^g - k_{cr}^* \mu_i \lambda_2^g \\
+ k_{cr}^* \left( \lambda_0^{g-1} \mu_3^{g-1} + 2\lambda_2^{g-1} \mu_5^{g-1} + 2\lambda_2^{g-1} \mu_5^{g-1} \right) \\
+ k_{cr}^* \sum_{h=1}^{g-1} \left( \lambda_0^h \mu_2^h + 2\lambda_2^h \mu_2^h + 2\lambda_2^h \mu_2^h + \lambda_0^h \mu_3^h + 2\lambda_2^h \mu_3^h + 2\lambda_2^h \mu_3^h \right) 
\]  
\text{(A35)}

\[
\frac{d\lambda_2^{NG}}{dt} = k_p \cdot [M]^\ast \left( 2\lambda_2^{NG} + \lambda_0^{NG} \right) - (k_{td} + k_{tc}) \lambda_2^{NG} \\
- k_{cr}^* \mu_i \lambda_2^{NG} + k_{cr}^* \left( \lambda_2^{NG-1} \mu_3^{NG-1} + 2\lambda_2^{NG-1} \mu_5^{NG-1} + 2\lambda_2^{NG-1} \mu_5^{NG-1} \right) \\
+ k_{cr}^* \left( \lambda_0^{NG} \mu_3^{NG} + 2\lambda_2^{NG} \mu_5^{NG} + 2\lambda_2^{NG} \mu_5^{NG} \right) \\
+ k_{cr}^* \sum_{h=1}^{NG-1} \left( \lambda_0^h \mu_2^h + 2\lambda_2^h \mu_2^h + 2\lambda_2^h \mu_2^h + \lambda_0^h \mu_3^h + 2\lambda_2^h \mu_3^h + 2\lambda_2^h \mu_3^h \right) 
\]  
\text{(A36)}
Terminated chains

\[ \mu_0 \]

\[
\frac{d \mu_0^1}{dt} = \frac{k_{ic}}{2} \lambda_0^1 \lambda_0^1 + k_{id}^* \lambda_0^1 \lambda_0^2 - k_{cr}^* \lambda_0^1 \mu_1^1
\] (A37)

\[
\frac{d \mu_0^2}{dt} = \frac{k_{ic}}{2} \lambda_0^2 \lambda_0^2 + k_{id}^* \lambda_0^2 \lambda_0^2 - k_{cr}^* \lambda_0^2 \mu_1^2
\] (A38)

\[
\frac{d \mu_0^g}{dt} = \frac{k_{ic}}{2} \lambda_0^g \lambda_0^g + \sum_{h=1}^{g-1} \lambda_0^h \lambda_0^g + k_{id}^* \lambda_0^g \lambda_0^g - k_{cr}^* \lambda_0^g \mu_1^g
\] (A39)

\[
\frac{d \mu_0^{NG}}{dt} = \frac{k_{ic}}{2} \lambda_0^{NG-1} \lambda_0^{NG-1} + k_{ic}^* \lambda_0^{NG} \lambda_0^{NG} + \sum_{h=1}^{NG-1} \lambda_0^h \lambda_0^{NG} + k_{id}^* \lambda_0^{NG} \lambda_0^{NG} - k_{cr}^* \lambda_0^{NG} \mu_1^{NG}
\] (A40)

\[ \mu_1 \]

\[
\frac{d \mu_1^1}{dt} = k_{ic}^* \lambda_1^1 \lambda_1^1 + k_{id} \lambda_1^1 \lambda_1^2 - k_{cr}^* \lambda_0^1 \mu_1^1
\] (A41)

\[
\frac{d \mu_1^2}{dt} = k_{ic}^* \left( \lambda_1^2 \lambda_1^2 + \lambda_1^1 \lambda_1^2 \right) + k_{id}^* \lambda_1^2 \lambda_1^2 - k_{cr}^* \lambda_0^1 \mu_2^2
\] (A42)

\[
\frac{d \mu_1^g}{dt} = k_{ic}^* \lambda_1^g \lambda_1^g + \frac{k_{id}}{2} \lambda_1^g \lambda_1^g + \sum_{h=1}^{g-1} \lambda_1^h \lambda_1^g + k_{id}^* \lambda_1^g \lambda_1^g - k_{cr}^* \lambda_0^g \mu_1^g
\] (A43)

\[
\frac{d \mu_1^{NG}}{dt} = \frac{k_{ic}}{2} \lambda_1^{NG-1} \lambda_1^{NG-1} + k_{ic}^* \lambda_1^{NG} \lambda_1^{NG} + \sum_{h=1}^{NG-1} \lambda_1^h \lambda_1^{NG} + k_{id}^* \lambda_1^{NG} \lambda_1^{NG} - k_{cr}^* \lambda_0^{NG} \mu_1^{NG}
\] (A44)

\[ \mu_2 \]

\[
\frac{d \mu_2^1}{dt} = k_{ic}^* \left( \lambda_2^1 \lambda_2^1 + \left( \lambda_1^1 \right)^2 \right) + k_{id}^* \lambda_2^1 \lambda_2^1 - k_{cr}^* \lambda_0^1 \mu_1^1
\] (A45)

\[
\frac{d \mu_2^2}{dt} = k_{ic}^* \left( \lambda_2^2 \lambda_2^2 + 2 \lambda_1^1 \lambda_2^2 + \lambda_1^1 \lambda_2^2 \right) + k_{id}^* \lambda_2^2 \lambda_2^2 - k_{cr}^* \lambda_0^2 \mu_2^2
\] (A46)
\[ \frac{d \mu_2^g}{dt} = k_{tc} \left( \lambda_2^g \lambda_0^g + \left( \lambda_1^g \right)^2 \right) + k_{tc} \sum_{h=1}^{g-1} \left( \lambda_2^h \lambda_0^g + 2 \lambda_1^h \lambda_1^g + \lambda_0^h \lambda_2^g \right) \]
\[ + k_{id} \lambda_0^g \lambda_2^g - k_{cr} \lambda_0^g \mu_3^g \]  
(A47)

\[ \frac{d \mu_2^{NG}}{dt} = k_{tc} \left( \lambda_2^{NG-1} \lambda_0^{NG-1} + \left( \lambda_1^{NG-1} \right)^2 \right) + k_{tc} \left( \lambda_2^{NG} \lambda_0^{NG} + \left( \lambda_1^{NG} \right)^2 \right) \]
\[ + k_{id} \lambda_0^{NG} \lambda_2^{NG} - k_{cr} \lambda_0^{NG} \mu_3^{NG} \]  
(A48)

In the derivation of the moment equations the Equation (A17) was again considered as closure equation. The double summations, which appear while applying the moment operator \( \sum_{n=0}^{\infty} n^k \) to Equation (A19)-(A24), were solved using the relations reported by Goldstein and Amudson\textsuperscript{327} and Butte\textsuperscript{e} et al.\textsuperscript{328}
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9/2005-7/2007: MSc graduation with full marks and honors (110/110 cum laude) in Chemical Engineering, from Politecnico di Milano, Milano, Italy.


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Refereed Journal Publications


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