Intermolecular interactions underlying the mechanisms 
and kinetics of protein aggregation

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II. Abstract

The thermodynamic and kinetic stability of colloidal systems is governed by the intermolecular forces acting between two interacting particles. These interactions determine the time scale and the mechanism of the aggregation process, as well as the morphology of the produced aggregates. In this work, we investigate the role of intermolecular interactions in the self-assembly of peptides and proteins, which currently represents a challenging problem underlying a large number of systems of both fundamental and practical importance in biology and biotechnology.

In particular, we study the role of electrostatic interactions in the aggregation mechanism of human insulin and a model amphiphilic peptide (RADA 16-I) at different self-assembly levels by changing the buffer composition in terms of pH value, ionic strength and co-solute composition. The complex aggregation behavior has been characterized by applying a variety of experimental biophysical techniques coupled with modelling activities based on concepts developed in the frame of colloid science, such as Smoluchowski kinetic approach (Population Balance Equations), DLVO theory and fractal gel model. We found that electrostatic interactions play a major role in the self-assembly of monomeric proteins and peptides into amyloid fibrils, in the end-to-end fibril-fibril aggregation into longer filaments as well as in the sol-gel transition of fibril dispersions. However, specific ion effects are also observed to affect dramatically aggregation kinetics and mechanisms.

In the last part of this thesis we discuss the effect of shear stress on protein stability. An elongational flow and simple shear were used to study the self-assembly of proteins differing in the average hydrodynamic size as well as in the properties of protein surface. We show that the properties of protein surface (i.e., the increase of the attractive hydrophobic interactions) rather than its size, together with the stretching properties of the elongational flow, are responsible for the shear-induced protein aggregation.


Im letzten Teil dieser Arbeit wird der Effekt von Scherkräften auf die Proteininstabilität untersucht. Im spezifischen wurde Dehnströmung und einfacher Scherung verwendet, um die Assemblierungsprozesse von Proteinen mit verschiedenen hydrodynamischen Grössen und Oberflächen zu studieren. Es konnte gezeigt werden, dass vielmehr die Eigenschaften der Proteinoberfläche (d.h.: die Zunahme von anziehenden hydrophoben Wechselwirkungen) gekoppelt mit der Dehnströmung für die Aggregation von Proteinen verantwortlich sind, als die Proteingrösse selbst.
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Chapter 1

Introduction

1.1. Protein aggregation

The aggregation of proteins and peptides underlies a broad range of problems in medicine [1], in the pharmaceutical [2, 3] and food [4] industry as well as in biomaterials science [5]. The self-assembly process relies on the spontaneous incorporation of single building blocks into highly organized structures [6-8] and involves many intra- and intermolecular interactions such as hydrogen bonding, electrostatic forces, van der Waals and hydrophobic interactions [9, 10]. Proteins can form a broad range of several structures differing in size and architecture such as fibrils, amorphous aggregates, nanotubes or spherulites [11, 12].

Regular fibrillar structures formed by a large variety of peptides and proteins, known as amyloid fibrils, are associated to several neurodegenerative diseases such as Alzheimer’s, Huntington’s or Parkinson’s disease [1, 13]. These fibrils are characterized by a high amount of the cross-β structures [1, 14-16] and, remarkably, share similar morphology regardless of the primary sequence of the protein, suggesting that the amyloid state can represent an alternative general folding modality of proteins [11, 13, 17, 18]. The amyloid fibril is thus a generic self-assembly state accessible to a wide range of proteins and peptides under suitable conditions, and the primary sequence determines the propensity to aggregate and governs the fibril stability under given conditions [19]. In addition, most of the protein systems share analogies also in terms of self-assembly mechanism, namely a nucleated-polymerization aggregation, which consists of a series of several microscopic processes: primary and secondary nucleation, elongation and lateral aggregation [20-23]. After nuclei are formed in solution, a fast elongation phase takes place until all of the available monomer units are consumed, thus resulting in the formation of fibrils [21, 23]. Recently, it is emerging that the presence of the fibrils catalyzes the formation of additional secondary nuclei via a positive feedback mechanism that dramatically increases the kinetics of the reaction [24]. Lateral aggregation due to the fibril-fibril interactions leads to the formation of higher organized species such as long and branched fibril clusters or plaques [21, 23]. The deposits of such fibrillar aggregates were found in several organs as for instance brain, heart, kidney or liver inducing their
1. Introduction

damage and dysfunction [13, 25]. However, recent studies suggest that the oligomers rather than the mature fibrils represent the most toxic species [16, 26].

The peculiar mechanical properties of amyloid fibrils aroused scientific interest towards their application in designing novel biocompatible materials applied in many areas such as medicine, biology, biotechnology or nanotechnology [8, 15, 27]. In last years, short synthetic oligopeptides constituting of 8-16 amino acid residues, which are inspired by proteins naturally occurring in nature or de novo designed, started to be considered as precursors for such materials due to their ability to self-assemble in a controlled way in aqueous solutions [8, 27, 28]. “Amphiphilic” or “ionic-complementary peptides” belong to the family of these precursors, and are characterized by the simultaneous presence of charged and hydrophobic residues placed on the opposite sides of the chain [6, 8, 27]. In this case, the aggregation reaction occurs via both hydrophobic and electrostatic interactions resulting in the formation of β-sheet structures [6]. It was found that depending on the environmental conditions (e.g., pH, temperature, buffer composition) or the intrinsic properties of the peptides (e.g., amino acid sequence), they can adopt a rich variety of final structures including fibril networks [27], hydrogels characterized by extremely high water content (i.e., 99.5-99.9%) [29] or membranes [30]. All of these features resulted in their practical application in for instance 3D cell cultures [31-33], tissue repair and tissue engineering [30, 34, 35], cosmetic industry [36], drug release and drug delivery [37-39], biological surface engineering [40, 41], separation matrices [30] or membrane protein stabilization [8, 32]. However, despite the broad application of these peptides, the detailed knowledge about their aggregation mechanism is still unclear.

Additionally, the formation of amorphous aggregates is a tough nut to crack in particular in pharmaceutical industry. The production of therapeutic proteins involves several steps, where the protein is exposed to various stress conditions, such as high temperature, acidic pH, high salt concentration, presence of hydrophobic surfaces or shear force, which may possibly cause the protein instability [42]. The presence of such aggregates may lead to the decrease in drug efficiency, immune-response or even be lethal to the patient [43, 44]. Recently, the effect of shear flow gained a great scientific attention because of its presence in both intravascular body fluids and during the industrial production and formulation of protein-based drugs.

Despite many studies on protein stability are available in the literature, the detailed knowledge about the mechanism underlying their aggregation remains still
unclear. Its understanding is of interest not only in medicine in the context of developing suitable medical treatment against diseases associated with protein aggregation, but also in material and pharmaceutical sciences in particular during industrial production and storage of protein-based materials, as well as in quality of final product.

There are several physico-chemical factors known to induce protein aggregation, which are divided in two main groups. The first group is represented by intrinsic parameters such as protein amino acid sequence [45, 46]. It was found that as small change as single amino acid mutation can result in dramatic change in protein stability [47]. The second group consists of environmental parameters as for instance buffer composition (i.e., type and concentration of co-solutes [48, 49]), pH [2], temperature [50] as well as presence of shear flow [51, 52] and different types of surfaces [53]. Because of the significant impact of the protein aggregation in a broad range of fields as described in previous paragraphs, the understanding of the effect of the environmental stresses is particularly interesting. However, despite the fact that the impact of some of these factors is identified and widely studied, the detailed analysis remains for long phenomenological. Recently, mechanistic studies addressing the fundamental physical chemistry underlying the aggregation reaction are emerging as powerful tools in increasing our understanding of these aspects [54-57]. For instance, several concepts adopted from polymer [58-60] and colloid science [61] can serve as a valuable tool to describe the protein aggregation mechanism.

In this work, we investigate the role of intermolecular interactions on the aggregation kinetic of proteins and peptides (i.e., on the reaction rate constants) by applying concepts of colloid science to identify the fundamental physical forces governing the stability behavior of protein solutions. Colloidal particles are defined as small particles homogenously dispersed in a medium with the size ranging from 1 nm to 10 microns [62]. The colloidal stability is strictly related to intermolecular interactions between two molecules determining the energy barrier preventing their collision and aggregation [63, 64]. Because of their size and the good solubility in aqueous solution, proteins are often considered as lyophilic colloids [65]. However, as described previously, proteins are observed to aggregate in aqueous solutions under a broad range of conditions. The understanding of the aggregation mechanism of proteins is more challenging with respect to the typical colloidal particles due to simultaneous presence of the hydrophobic and hydrophilic patches as well as non-homogenous charge distribution [54]. The colloidal stability of protein solutions can be controlled by the environmental conditions
such as choosing the buffer pH far away from protein pI leading to the high surface charge causing the increase of repulsive forces and therefore inhibiting the aggregation process. In contrast, the addition of electrolytes (e.g., salts) results in the screening of the surface charges, hence in the decrease of the repulsive electrostatic barrier, thus enhancing the aggregation.

In addition to the colloidal stability, the conformational stability plays an important role in the protein aggregation behavior. The conformational stability is described by the ensemble of secondary and tertiary structures of a protein under given environmental conditions, which are dependent on the balance between intramolecular protein interactions and protein-solvent interactions [63, 64]. Namely, it is related to the energy barrier needed for a given protein to change its native structure (i.e., to unfold) [63, 64]. It is widely reported that the self-assembly process of proteins is often triggered by a change of the native secondary structure (i.e., protein conformation) under destabilizing conditions [11-13]. Such a destabilization of the protein native structure is observed when the solution pH is lowered [66], when the temperature is increased above the critical denaturation value [66] or when denaturants (e.g., guanidinium hydrochloride or urea) are added to the solution [67]. This change in secondary structure is often accompanied by exposure of hydrophobic patches that promote attractive hydrophobic intermolecular forces between two aggregating molecules.

When the protein aggregation behavior is considered, both colloidal and conformational stability have to be accounted simultaneously, since these two factors are strictly dependent on each other and often cannot be decoupled [2, 63, 64]. For instance, the attempt to increase colloidal stability can lead to decrease in conformational stability, thus potentially resulting in the occurrence of aggregation process. An example can be found in the aforementioned increase in the colloidal stability by lowering the solution pH far away from the protein pI (i.e., increase in the repulsive electrostatic interactions), which may result in the decrease in the conformational stability (i.e., protein unfolding) and enhancement of aggregation. Nevertheless, despite all of the limitations and approximations, several key concepts widely used in colloidal science such as Smoluchowski kinetic approach (Population Balance Equations), DLVO theory and fractal gel model can be successfully applied to describe the self-assembly behavior of protein solutions as shown in the next chapters of this thesis.
1.2. Outline of the work

In this thesis, the role of intermolecular forces on the kinetics and mechanism of self-assembly of proteins and peptides is investigated by studying their aggregation behavior under a broad range of environmental conditions in terms of buffer composition (i.e., type and concentration of co-solutes), pH, temperature and the presence of shear flow. The first part of the work (i.e., Chapters 2-5) is related to the aggregation of amyloidogenic proteins and peptides. In particular, we study the impact of the electrostatic forces on the aggregation mechanism of human insulin and of the model amphiphilic peptide RADA 16-I at different self-assembly levels. In the last part of this thesis (Chapter 6), we show the effect of the shear flow on the stability of proteins differing in the average hydrodynamic size and properties of protein surface.

The aggregation process is characterized by several experimental techniques such as light scattering, size exclusion chromatography, atomic force microscopy, electron microscopy, fluorescence and absorbance assays, circular dichroism, fourier transform infrared spectroscopy, diffusion wave spectroscopy and rheology. The experimental results are rationalized by using key concepts widely used in colloid science, such as Smoluchowski kinetic approach (Population Balance Equations), DLVO theory and fractal gel model.

In Chapter 2, we show the aggregation of human insulin at acidic pH. We apply a combination of experimental characterization to elucidate the mechanism and the kinetics of the aggregation process as a function of ion composition, which is a common strategy to investigate the role of electrostatics in the self-assembly process. Moreover, we study the effect of temperature and ionic strength on the aggregation. In particular, we focus on the sulfate anion, which is known for its peculiar effect on protein stability as well as its presence in several biologically relevant components.

In Chapters 3-5 we describe the aggregation behavior of a model amphiphilic peptide, representative of a larger family of ionic-complementary peptides. Here, we study intermolecular interactions and aggregation mechanism at different self-assembly levels: starting from the monomeric state to hydrogel formation at macro-scale through the nanofibrils at the nano- and microscales.

When monomeric peptide is considered, we investigate the role of electrostatic interactions in its self-assembly into fibrillar structures by combination of experimental characterization and metadynamics simulations as shown in Chapter 3. First, we
determine the relationship between peptide morphology as a function of global net charge of the peptide. Then, we quantify the contribution of electrostatics to the self-assembly process in the context of equilibrium constant determined at different net charges. Finally, we evaluate the effect of the increase of ionic strength as well as addition of charged denaturant (guanidinium hydrochloride) on monomer aggregation propensity.

In addition, we study the role of electrostatic interactions in the fibril-fibril aggregation process (Chapter 4). We investigate the aggregation stability of the fibrillar dispersions as a function of buffer composition in terms of salt concentration, anion and cation type, as well as the presence of organic solvent. The energy barrier represented by Fuchs stability ratio is obtained from Smoluchowski kinetic approach (Population Balance Equations) and compared with the theoretical value calculated from DLVO theory.

In Chapter 5, we focus on the electrostatics in sol-gel transition of the fibrillar dispersions. First, we show the mechanism of the gel formation. Then, we study the phase transition as a function of the fibril concentration and ionic strength. Finally, we describe the gelation behavior and the heterogeneous gel structure in the frame of the fractal gel theory.

Finally, in Chapter 6 we show the effect of shear flow on protein structure and stability. We investigate the impact of flow type and shear rate on proteins of various sizes and surface properties (i.e., altered by thermal denaturation) to determine the intermolecular forces governing the shear-induced protein aggregation.

The most important results obtained in this work together with outlook are presented in the last part of this thesis, Chapter 7.
Chapter 2

Sulfate anion delays the self-assembly of human insulin by modifying the aggregation pathway

2.1. Introduction

In the last decades, the interest in understanding the molecular mechanisms responsible for protein self-assembling has increased significantly due to the large number of implications in life sciences and technology. Examples range from the aberrant aggregation of peptides and proteins involved in several neurodegenerative diseases [1], to the stability of protein solutions in food and pharmaceutical industry [2-4] as well as the engineering of mechanically and chemically stable biomaterials [5].

A large number of aggregation pathways and aggregate structures has been observed depending on the specific protein and the environmental conditions under consideration: many proteins under suitable conditions are able to self-assemble into regular fibrillar structures known as amyloids [47, 68-71], while in other systems proteins aggregate into amorphous precipitates [54, 72, 73]. In addition, different aggregation pathways may occur simultaneously, thereby leading to a heterogeneous composition of the final products.

One of the most relevant external factors affecting protein aggregation pathways and aggregate morphologies is the buffer composition. Ions and other cosolutes, such as sugars, polyols and amino acids, mediate protein intra- and intermolecular interactions via a combination of several effects such as charge screening, ion binding, preferential exclusion and dipole interactions, therefore affecting the protein secondary and quaternary structure [65, 74-78] as well as the individual microscopic events underlying the aggregation process [79]. Peculiar effects such as ion specificity and re-stabilization behavior at large salt concentrations have been observed in the aggregation of many different proteins, ranging from short peptides to globular multi-domain proteins [80-84]. In the amyloid field, specific ion effects have been observed for instance in the

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aggregation of the Aβ peptide [85], α-synuclein [86], islet amyloid polypeptide (IAPP) [87], prion protein [88], and HypF-N [89]. The propensity of the different ions to induce protein aggregation is commonly related to either the electroselectivity or the Hofmeister series, a ranking which has been observed in a broad range of phenomena in physics and chemistry [90]. However, the exact order of the effectiveness of different ions in promoting aggregation is strongly dependent on the pH value, the salt concentration and the specific protein under consideration.

In this chapter the attention is concentrated on the effect of sulfate anion on the fibrillization process of human insulin under acidic conditions. Sulfate is a kosmotropic anion which stabilizes protein structure and reduces protein solubility in solution [88]. In the form of ammonium salt, sulfate is one of the most common anions used for inducing salting out of proteins in crystallography and purification processes [48, 91]. While the effect on the precipitation of native proteins is well characterized, the effect of sulfate on non-native protein aggregation behavior is more complex. On one hand, the salting-out property promotes aggregation due to the decrease of the total amount of exposed surface which accompanies the formation of the aggregates; on the other hand, the kosmotropic nature of the anion stabilizes the protein structure under non-native conditions, therefore reducing the formation of non-native aggregation-prone structures. In addition, the divalency of the anion confers peculiar properties in terms of charge screening, anion binding, and salt bridge formation. The overimposition of the different electrostatic effects can either promote [25, 92, 93] or inhibit [94] the protein aggregation propensity. In the case of an IgG2 monoclonal antibody a maximum of the aggregation propensity as a function of sulfate concentration has been recently reported [81].

The delicate balance between the different sulfate properties modulates not only the protein aggregation propensity, but also the morphology of the final aggregates. When monovalent anions are replaced by sulfate, either longer fibrils or, on the contrary, amorphous aggregates and shorter fibrils are observed [79, 93, 95]. Sulfate can also promote fibril lateral aggregation and increase fibril stability [93]. The different effect depends strongly on the anion concentration and the protein primary sequence. For instance, sulfate has been found to promote the fibril formation of the EAK16-II peptide [96], and to disrupt the regular fibrillar structure of the EMK16-II peptide [97].

The understanding of the complex effect of sulfate anion on protein aggregation may represent a first step towards the fundamental rationalization of the even more complex interactions between proteins and sulfonated biomacromolecules, such as
glycosaminoglycans (GAGs). These physiologically relevant components are polyelectrolyte macromolecules which are involved in the in vivo aggregation propensity of several amyloidogenic proteins [98-103].

In this chapter, the effect of sulfate on the aggregation behavior of human insulin, a hormone produced in pancreatic β cells and responsible for the metabolism of glucose [22], is analyzed. Insulin is one of the therapeutic proteins with the largest production volume due to the use as regulator of the level of sugar in the blood of people affected by diabetes [22]. Insulin is well known to form amyloid fibrils characterized by large β-sheet content under destabilizing conditions such as low pH and high temperature [22, 95, 104-106]. Several biophysical studies investigated the molecular pathway underlying the fibril formation process using monovalent anions, typically chloride [70, 79, 107-111]. When chloride is replaced by sulfate, destabilizing and stabilizing effects have been reported at low and high ionic strength, respectively [21], and a shortening of the fibril length has been observed [95].

In this chapter, the question, whether the changes in the kinetics and in the aggregate morphology observed when chloride is replaced by sulfate are connected to a change in the aggregation mechanism, is investigated. In particular, the aggregation of insulin in the presence of sodium sulfate under conditions where formation of amyloid fibrils is commonly observed, i.e., 25 mM HCl at pH 1.6 and 60 °C under quiescent conditions [112] is characterized in detail. At this pH value positive charges are largely dominating on the protein surface, and therefore the specific effect of monovalent cations can be considered negligible with respect to the anion effect. Indeed, the negligible effect of monovalent cations at low pH has been observed previously with other proteins [81, 89]. In addition, Hofmeister effects are normally dominated by anions with respect to cations.

The study is performed in parallel with the investigation of the aggregation behavior in the presence of sodium chloride, in order to allow the comparison with the well-known behavior reported in the literature [21, 70, 113-116].

The aggregation kinetics and the aggregate morphology are investigated using a combination of Thioflavin T fluorescence, dynamic light scattering, size exclusion chromatography, Fourier transform infrared spectroscopy and transmission electron microscopy. The results show that at concentrations larger than 5 mM sulfate ions induce an alternative aggregation pathway with respect to chloride ions. This alternative process involves an initial precipitation of amorphous aggregates, which during time undergo structural rearrangements into β-sheet structures which exhibit features of amyloid fibrils.
2. Sulfate anion delays the self-assembly of human insulin by modifying the aggregation pathway

The study shown in this chapter represents a relevant example where amorphous intermediates on-pathway to β-sheet rich structures could be isolated and characterized.

2.2. Materials and methods

2.2.1. Materials and aggregation conditions

Human insulin was kindly donated by NovoNordisk (Bagsvaerd, Denmark). Insulin solutions in the concentration range 0.5-5 g/L (86-860 μM) were freshly prepared prior to each experiment by dissolving insulin powder in 25 mM HCl solution (Fluka Chemika, Buchs, Switzerland) at pH 1.6 with 100 mM sodium chloride (VWR International BVBA, Leuven, Belgium) or 100 mM sodium sulfate (Merck kGaA, Darmstadt, Germany). In order to remove potential seeds, prior to the aggregation studies the solutions were filtered by Low Protein Binding Hydrophilic LCR, Millex®-LG syringe filters with 200 nm cut-off membrane (Merck Millipore, Merck KGaA, Darmstadt, Germany). The protein concentration after filtration was measured by UV absorbance at 280 nm. Aggregation was induced by incubating the protein solutions in the temperature range from 57 to 63 °C.

It is known that insulin fibrillation process can be affected by the presence of surfaces, in particular by hydrophobic surfaces [23, 117]. In order to probe for the presence of possible artifacts induced by this effect, the aggregation of insulin solutions incubated in cuvettes or tubes made of different materials (glass, polystyrene, polypropylene and polymethylmetacrylate) was monitored using different solution volumes. No significant difference was observed under the different conditions, indicating that, at least under the investigated conditions, both the material of the container and the total sample volume do not affect the conclusions of this work.

2.2.2. ThT fluorescence assay

Thioflavin T (ThT) (Sigma-Aldrich GmbH, Steinheim, Germany) fluorescence was measured in 96-well plate (96 Isoplate, Perkin Elmer, Waltham, MA, USA) using an EnSpire 2300 Multilabel Reader fluorometer (Perkin Elmer, Waltham, MA, USA). 10 μM ThT was added to 3-fold diluted samples taken at different time points and emission fluorescence values were measured at 485 nm after excitation at 450 nm.
On-line kinetic experiments were performed by incubating the protein solution in the presence of 10 μM ThT and monitoring the fluorescence signal during aggregation. No significant difference was observed between the off-line and the on-line kinetics (data reported in Figure A.1 in Appendix A).

### 2.2.3. Dynamic light scattering (DLS)

Dynamic light scattering measurements were performed on-line using a Zetasizer Nano (Malvern, Worcestershire, United Kingdom), operating in the backscattering mode at a fixed angle of 173° with a laser beam with wavelength of 633 nm.

### 2.2.4. Size exclusion chromatography (SEC) coupled with multi-angle static light scattering (MALS)

Size exclusion chromatography (SEC) analysis was performed with a Superdex Peptide 10/300 GL, 10 mm×300 mm size-exclusion column (GE Healthcare, Uppsala, Sweden) assembled on a Agilent 1100 series HPLC unit (Santa Clara, CA, USA). Each sample was eluted for 70 min at a constant flow rate of 0.4 mL/min using as mobile phase 10 mM HCl solution at pH 2.0. The pH of the mobile phase was slightly higher than the pH of the analyzed samples (pH = 1.6) to avoid damage of the equipment. UV absorbance was detected at 280 nm. To evaluate the amount of residual soluble insulin during the aggregation process, protein samples were centrifuged for 15 min at 10'000 rpm in order to precipitate the aggregates, and 40 μl aliquots of the supernatant were injected into the column after filtration by Low Protein Binding Hydrophilic LCR, Millex®-LG syringe filters with 200 nm cut-off membrane (Merck Millipore, Merck KGaA, Darmstadt, Germany).

Multi-angle static light scattering (MALS) of fractionated samples eluting from the SEC column was measured on-line by a Wyatt light scattering detector (Wyatt, Dernbach, Germany) with laser beam with wavelength of 658 nm and scattering angles from 14° to 163°.

### 2.2.5. 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,
2. Sulfate anion delays the self-assembly of human insulin by modifying the aggregation pathway

WI, USA). Aliquots of 10 µL were spotted on the crystal surface and dried under nitrogen flux. The spectra were collected in the wavenumber range from 1700 to 1600 cm\(^{-1}\) at 1 cm\(^{-1}\) resolution and smoothed using the Savitsky-Golay function after buffer and atmospheric subtraction.

2.2.6. Transmission electron microscopy (TEM)

The TEM pictures were taken using a FEI Morgagni 268 microscope. 5 µL of 75-fold diluted samples were spotted on a carbon support films - 300 mesh Cu grids (Quantifoil, Jena, Germany) for 30 seconds, washed with distilled water and negative stained with a 2 % uranyl acetate solution.

2.3. Results

2.3.1. Aggregation kinetics

Before inducing aggregation by incubating the protein samples at high temperature, the initial quaternary structure of insulin was analyzed under the investigated conditions by size exclusion chromatography (SEC) coupled with multi-angle static light scattering (MALS) measurements. A single peak corresponding to a species with a molecular weight of 5800±100 Da was observed in the SEC chromatograms (data reported in Figure A.2 in Appendix A). This value is in excellent agreement with the molecular weight of insulin monomer (5808 Da), thereby indicating that under the investigated conditions insulin is initially present as monomer.

The formation of the aggregates was monitored by ThT fluorescence assay and by dynamic light scattering (DLS), while the monomer conversion was measured by size exclusion chromatography (SEC). The time evolution of the SEC chromatograms is reported in Figure A.3 in Appendix A.

The ThT fluorescence values and the monomer conversion during the aggregation kinetics of a 5 g/L (860 µM) insulin solution with 100 mM NaCl or Na\(_2\)SO\(_4\) are shown in Figure 2.1a and 2.1b, respectively. ThT fluorescence assay is a common technique applied to monitor the formation of amyloid fibrils [118] which relies on the increase of the fluorescence yield of the dye upon binding to β-sheet structures.
Figure 2.1. Insulin aggregation kinetics in the presence of chloride and sulfate anion. a-b) Time evolution of the ThT fluorescence values (■) and of the residual monomer amount evaluated by SEC technique (▼) for a 5 g/L (860 μM) insulin solution at pH 1.6 and 60 °C with (a) 100 mM NaCl or (b) 100 mM Na₂SO₄; c) average hydrodynamic diameter measured by DLS for a 5 g/L (860 μM) insulin solution at pH 1.6 and 60 °C with 100 mM NaCl (▲) or Na₂SO₄ (●).
In the presence of sodium chloride the time evolution of the ThT signal shown in Figure 2.1a exhibits the sigmoidal profile commonly observed during the *in vitro* aggregation of several amyloidogenic proteins: a lag-phase is followed by rapid growth until a plateau corresponding to monomer depletion is reached [21]. The amount of soluble monomer as a function of time decreases according to a specular sigmoidal profile, being negligible during the lag-phase and decreasing rapidly during fibril growth until complete conversion is reached. This result indicates that essentially all the converted monomers are present in the form of fibrils characterized by β-sheet structures which are able to bind ThT.

In analogy with the behavior in the presence of sodium chloride, also in the presence of sodium sulfate the time evolution of the ThT fluorescence follows a sigmoidal profile, as shown in Figure 2.1b. However, in contrast with the situation with sodium chloride, after 6 h incubation about 20% of monomer conversion is observed despite the ThT fluorescence signal is still in the background noise level (Figure 2.1b). In addition, the increase of ThT fluorescence during time in the presence of sodium sulfate is slower than in the presence of sodium chloride. The results suggest that sulfate induces a first aggregation step leading to non-ThT binding aggregates which depletes about 20% of the monomer. This aggregation step is then followed by the formation of aggregates with amyloid-like content (refer also to Figure A.4 shown in Appendix A).

To confirm the initial aggregation step in the presence of sulfate, the aggregation kinetics were followed by dynamic light scattering (DLS) (Figure 2.1c). The ThT assay is unable to detect the formation of aggregates lacking β-sheet structures. In contrast, light scattering techniques are sensitive to the formation of aggregates independently of the morphology. The results reported in Figure 2.1c show that in the presence of NaCl the DLS signal increases after about 5 hours, in correspondence with the increase of ThT fluorescence (Figure 2.1a), indicating that all the aggregates formed in the presence of sodium chloride contain amyloid structures. Conversely, in the presence of sulfate the DLS signal increases already after 1 hour of incubation, indicating the formation of aggregates in the micron size range and confirming the monomer conversion measured by SEC. The formation of these aggregates is not accompanied by an increase of the ThT signal, which increases only after around 11 hours of incubation. This result confirms that in the case of sulfate the formation of ThT-binding aggregates is preceded by the appearance of aggregates which are not able to bind ThT.
The effect of temperature on the aggregation kinetics in the presence of both salts has been analyzed in the temperature range 57-63 °C. This small range of temperatures has been selected in order to analyze the aggregation kinetics without changing the aggregation mechanism or the aggregate morphology, which are strongly affected by the initial temperature value. The fibril formation measured by ThT assay and the time evolution of the monomer conversion at different temperatures are shown in Figure 2.2. In the investigated temperature range in the presence of chloride the kinetics are only slightly affected by the initial temperature value and, as expected, they increase as temperature increases (Figure 2.2a and 2.2c). In contrast, in the presence of sulfate the initial temperature value has significant impact on the aggregation kinetics: a difference of 3 degrees induces a difference in the lag-phase of several hours (Figure 2.2b and 2.2d). The monomer depletion corresponding to the initial aggregation step is independent of the temperature and equal to about 18-20%.

These observations indicate that in the presence of sulfate two different aggregation processes occur: i) an initial aggregation step which consumes about 18-20% of the initial monomer and leads to the formation of amorphous aggregates, and ii) a second aggregation phase which forms ThT-binding structures. This second aggregation process leading to amyloid-like structure is strongly affected by temperature, and is therefore characterized by larger activation energy with respect to the fibrillation process in the presence of sodium chloride.

To support the hypothesis of the formation of two populations of aggregates characterized by different structures, in addition to ThT binding assay, which provides indirect information about the β-sheet content of the aggregates, the aggregate morphology at different times was investigated by Fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM).
Figure 2.2. Temperature dependence of the aggregation kinetics. Time evolution of ThT fluorescence values with (a) 100 mM NaCl or (b) 100 mM Na\textsubscript{2}SO\textsubscript{4} and of residual monomer with (c) 100 mM NaCl or (d) 100 mM Na\textsubscript{2}SO\textsubscript{4} for a 5 g/L (860 µM) insulin solution at pH 1.6 and different temperatures: 57 °C (▲), 60 °C (●) and 63 °C (■). When non visible error bars are smaller than the symbols.
2. Sulfate anion delays the self-assembly of human insulin
by modifying the aggregation pathway

2.3.2. Aggregate morphology

The FTIR spectra of samples taken at different time points during the aggregation reaction are shown in Figure 2.3a and 2.3b. In the presence of both chloride and sulfate at time 0 the FTIR spectrum exhibits a peak with maximum intensity at the wavenumber 1656 cm\(^{-1}\), characteristic of the \(\alpha\)-helix content of the protein. In the presence of chloride after 6 h of incubation the position of the peak shifts towards 1632 cm\(^{-1}\), corresponding to the rich amount of intermolecular \(\beta\)-sheet structures of amyloid fibrils. This observation is consistent with the formation of fibrils monitored by ThT fluorescence (Figure 2.1a) and the almost complete monomer conversion measured by SEC at 6 h incubation (Figure 2.1a). Further analysis by TEM imaging confirms the presence of amyloid fibrils as unique final product (Figure 2.3c).

In the presence of sulfate, the FTIR spectrum of the sample collected after 12 hours of incubation is similar to the spectrum of the initial monomeric solution (Figure 2.3b). The absence of \(\beta\)-sheet structures is in agreement with the corresponding low ThT fluorescence value (Figure 2.1b). Considering that after 12 h of incubation about 25% of initial monomers is already converted into aggregates (Figure 2.1b), the FTIR analysis supports the hypothesis that the sulfate anion induces an initial precipitation of amorphous aggregates characterized by a secondary structure content which is similar to the initial monomeric insulin. The FTIR spectrum corresponding to 74% conversion shows a larger amount of \(\beta\)-sheet structures (Figure 2.3b), in agreement with the increase of ThT fluorescence (Figure 2.1b), confirming that the initial precipitation of amorphous aggregates is followed by the formation of amyloid-like structures. However, in the presence of sulfate, the samples corresponding to high conversion values retain a large amount of \(\alpha\)-helix content together with intermolecular \(\beta\)-sheet structures, suggesting a different morphology of the final aggregates with respect to the fibrils produced in the presence of sodium chloride. The polymorphism of the aggregates obtained in the presence of sulfate is confirmed by the TEM pictures of samples taken after 30 h of incubation (Figure 2.3d and 2.3e): the images show the presence of both fibrils and amorphous aggregates. Moreover, the fibrils obtained in the presence of sulfate anion are shorter and more branched than the fibrils produced in the presence of chloride anion (Figure 2.3c and 2.3d).
Figure 2.3. Change of the aggregate morphology during the aggregation. a-b) FTIR spectra of 5 g/L (860 µM) insulin samples collected at different time points during aggregation at 60 °C in 25 mM HCl at pH 1.6 with (a) 100 mM NaCl or (b) 100 mM Na₂SO₄. c-e) TEM pictures of the final aggregates formed in the presence of (c) 100 mM NaCl and (d-e) 100 mM Na₂SO₄. In the presence of sulfate both fibrils (d) and amorphous aggregates (e) are observed. f) Position of the maximum intensity in the FTIR spectra and (g) ThT fluorescence values at different temperatures (57 °C (▲), 60 °C (●) and 63 °C (■)) as a function of the monomer conversion during the aggregation of 5 g/L (860 µM) insulin in 25 mM HCl with 100 mM Na₂SO₄ at pH 1.6.

The behavior described above is confirmed by the analysis of the time evolution of the aggregate morphology obtained at different temperatures. In Figure 2.3f the wavenumber corresponding to the maximum intensity in the FTIR spectrum as a function
of monomer conversion is reported. The data corresponding to the different temperature values overlap on a single curve. This result indicates that, despite the aggregation kinetics differ significantly at different temperatures (Figure 2.2b and 2.2d), the aggregation mechanism is unaffected: at low conversion values insulin forms amorphous aggregates with α-helix content and a maximum peak in the FTIR spectrum in the range from 1650 to 1656 cm\(^{-1}\). At conversion values larger than 50% these aggregates evolve to β-sheet structures characterized by a maximum peak in the FTIR spectrum at about 1633 cm\(^{-1}\). The FTIR results are in excellent agreement with the ThT measurements, as shown in Figure 2.3g: the increase of ThT fluorescence with increasing conversion occurs simultaneously to the shift of the maximum peak in the FTIR spectra from 1655 to 1633 cm\(^{-1}\).

To investigate further the structure transition, the amorphous aggregates produced after 18% of monomer conversion (sample I in Figure 2.4a) were isolated by centrifugation and both the precipitated aggregates and the supernatant were analyzed. The supernatant contains monomeric proteins that after few minutes re-form the amorphous aggregates at the same amount of 18-20%, indicating that these aggregates are in equilibrium with the monomers. TEM images of the isolated aggregates (Figure 2.4a) confirm the amorphous morphology of the aggregates detected by ThT and FTIR analysis.

The stability of these amorphous aggregates was investigated in the absence of monomers by incubating the isolated aggregates at 60 °C in the presence of 10 µM ThT. Notably, the ThT fluorescence increases during incubation time, as shown in Figure 2.4b, indicating structure re-arrangements which lead to the formation of β-sheet structures able to bind ThT. It is interesting to note that the final ThT value is comparable but slightly smaller than the ThT fluorescence value corresponding to a sample with the same amount of mature amyloid fibrils produced under the same conditions but with 100 mM NaCl instead of sulfate (star symbol in Figure 2.4b). The structure rearrangement of the amorphous aggregates is confirmed by FTIR analysis, which shows the presence of β-sheet structures after incubation at 60 °C (insets in Figure 2.4a-b). In addition, the reactivity of the aggregates before and after incubation was tested by performing seeded kinetic experiments (Figure 2.4c). The amorphous aggregates have essentially no effect on the aggregation kinetics with respect to unseeded conditions, while the ordered aggregates obtained after the structure re-arrangements accelerate significantly the fibril formation.
2. Sulfate anion delays the self-assembly of human insulin by modifying the aggregation pathway

![Image of Figure 2.4](image)

**Figure 2.4. Structure re-arrangements of the aggregates formed in the presence of sulfate.**

a) Time evolution of monomer conversion of a 5 g/L (860 μM) insulin solution in 25 mM HCl with 100 mM Na₂SO₄ at 60 °C. The insets show a TEM image and the FTIR spectrum of the amorphous aggregates isolated by centrifugation from the sample corresponding to 18% monomer conversion (sample I). b) Time evolution of the ThT fluorescence of the amorphous aggregates collected from sample I in a); the insets show a TEM picture and the FTIR spectrum of the sample at the end of the incubation (sample II). The star symbol represents the ThT fluorescence value corresponding to a sample with the same amount of mature amyloid fibrils produced under the same conditions but with 100 mM NaCl instead of sulfate. c) Aggregation kinetics of solutions which were seeded with 1% amorphous aggregates isolated during the early stages (■) (sample I), and 1% ordered aggregates originating from the structure reorganization of the amorphous aggregates during incubation at 60 °C in the absence of monomer (▲) (sample II). Blue circles (●) represent the control unseeded experiment. Aggregation conditions are the same as in (a).
The results of the robust analysis obtained with the different applied techniques (ThT binding, SEC, DLS, FTIR, TEM and seeded kinetic experiments) suggest that in the presence of sulfate the amorphous aggregates formed during the early stages convert during time into amyloid-like structures. This structure change is likely driven by the minimization of the system free energy and could occur either internally or via continuous release and re-incorporation of the monomers from and into the aggregates. SEC measurements of samples taken during the incubation of the isolated aggregates at high temperature show no monomer in the system (data not shown). However, rapid exchange of monomers which is out of the detection limit of the SEC analysis cannot be excluded.

### 2.3.3. Effect of sulfate and insulin concentration

To investigate further the effect of sulfate on the insulin aggregation pathway, the analysis described in the previous sections was performed at different protein and sulfate concentrations. In Figure 2.5 the aggregation kinetics followed by ThT assay and SEC measurements at 60 °C is presented. The analysis was started by considering solutions at 100 mM Na₂SO₄ and varying the insulin concentration from 0.5 to 5 g/L (86-860 μM) (Figure 2.5a-b). As expected, the aggregation rate increases with increasing protein concentration. At concentration lower than 1 g/L (172 μM) the ThT signal after 30 h incubation is still in the background noise level, indicating the absence of significant amount of fibrils. The corresponding SEC measurements indicate that after 12 h incubation about 18-20% of the monomer is consumed, and no further aggregation occurs during the incubation time. The relative amount of monomer converted into the amorphous aggregates during the initial step is independent of the initial protein concentration and therefore the formation of these aggregates due to a solubility effect can be excluded.

After analyzing the effect of insulin concentration, the aggregation kinetics were recorded at the reference insulin concentration of 5 g/L (860 μM) changing the sulfate concentration from 2.5 to 100 mM (Figure 2.5c-d). The corresponding values of the lag-phase and of the half-time measured by means of ThT fluorescence assay are reported in Table 2.1.
Table 2.1. Lag-phases and half-times ($t_{50}$) of the aggregation of a 5 g/L (860 µM) insulin solution in 25 mM HCl at pH 1.6 and 60 °C at different sodium sulfate concentrations as measured by ThT fluorescence assays.

<table>
<thead>
<tr>
<th>SO$_4^{2-}$ Conc. [mM]</th>
<th>lag time [h]</th>
<th>$t_{50}$ [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.2</td>
<td>12.0</td>
</tr>
<tr>
<td>50</td>
<td>5.8</td>
<td>10.6</td>
</tr>
<tr>
<td>5</td>
<td>4.3</td>
<td>6.0</td>
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<tr>
<td>2.5</td>
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Figure 2.5. Effect of protein and Na$_2$SO$_4$ concentration on insulin aggregation in 25 mM HCl at pH 1.6 and 60 °C. Time evolution of ThT fluorescence (a,c) and monomer conversion (b,d) for: (a-b) 0.5 g/L (86 µM) (●), 1 g/L (172 µM) (▲), 3 g/L (516.6 µM) (★) and 5 g/L (860 µM) (●) insulin solutions with 100 mM Na$_2$SO$_4$; (c-d) 5 g/L (860 µM) insulin solutions with 2.5 mM (■), 5 mM (▼), 50 mM (▲) or 100 mM (●) Na$_2$SO$_4$. 
The rate of the aggregation process decreases with increasing the sulfate concentration. At 2.5 mM sodium sulfate, no precipitation of amorphous aggregates is observed, and the time evolution of monomer conversion and ThT signal is similar to the one observed in the presence of chloride (Figure 2.5c-d and Figure 2.1a). The increase of the sulfate concentration from 2.5 to 5 mM inhibits slightly the aggregation kinetics, while at sulfate concentrations of 50 and 100 mM the comparison between ThT and SEC data indicates clearly the formation of amorphous aggregates in the early stages. In this high sulfate concentration regime, the overall aggregation rate decreases significantly with increasing the sulfate concentration. Based on this analysis, the concentration of 5 mM sulfate, which, at the working insulin concentration of 860 μM, corresponds to a sulfate/insulin molar ratio equal to 6, can be considered as an approximate threshold concentration above which the alternative aggregation pathway overimposes the nucleation fibrillation pathway. The alternative nucleation pathway dominates at larger sulfate concentrations, and explains the decrease of the overall aggregation rate with increasing sulfate concentration.

2.4. Discussion

In the physiological range of salt concentrations typically considered in protein aggregation studies (0-150 mM), the increase in the concentration of monovalent salts induces commonly an increase of the aggregation rate, as verified in our system by kinetic experiments in the presence of sodium chloride (Figure A.5 in Appendix A). By contrast, it is found that the increase in sulfate concentration has an inhibitory effect on insulin aggregation kinetics at low pH. The results described in the previous sections of this chapter show that this effect is associated to a change in the insulin aggregation pathway with increasing the sulfate concentration.

At low sulfate concentrations (0-5 mM) insulin aggregation follows a nucleation polymerization mechanism which forms amyloid fibrils. This mechanism has been widely described in the literature in the presence of monovalent anions [21-23, 107, 108, 111-113, 115-117, 119] (Pathway 1 in Figure 2.6).
Figure 2.6. Schematic diagram showing the alternative aggregation mechanisms of insulin in the presence of sulfate. a) At low sulfate concentrations (0-5 mM) insulin forms amyloid fibrils following the nucleated polymerization mechanism commonly observed under acidic conditions in the presence of monovalent anions; b) When the sulfate concentration is increased above 5 mM, the sulfate anion induces the salt-out of about 18-20% of insulin molecules into reversible amorphous aggregates which retain a large content of α-helix structures. During time, these aggregates undergo structure re-arrangements into polymorphic species which exhibit β-sheet structures.

At sulfate concentrations larger than about 5 mM, about 18-20% of the insulin monomers are initially converted into reversible amorphous aggregates (Pathway 2 in Figure 2.6). When these amorphous aggregates are removed from the solution, they are reformed in few minutes at the same amount of 18-20% of the total protein concentration, indicating that the aggregates are in equilibrium with the monomers. During incubation, these aggregates undergo structure re-organization from α-helix to β-sheet structures, which are able to bind ThT dye and act as seeds which recruit soluble monomers. The aggregation process in the presence of sulfate is characterized by larger activation energy with respect to the aggregation in the presence of chloride. This larger activation energy may be related to either the structure rearrangements of the amorphous aggregates or the elongation reaction between monomeric proteins and the seed-competent aggregates.

Not only the aggregation pathway but also the morphology of the final aggregates obtained in the presence of the two anions is different. Although the final value of the
ThT signal is comparable for the two anions (Figure 2.1a-b), the TEM pictures show different macroscopic structures of the final aggregates: in particular, in the case of chloride anion only fibrils are detected (Figure 2.3c), while in the presence of sulfate anion both fibrillar and amorphous aggregates are observed (Figure 2.3d and 2.3e). These results are consistent with the FTIR analysis of the final aggregates, which shows a mixture of α-helix and β-sheet structures in the case of sulfate and reveals the presence of β-sheet structures in the case of chloride (Figure 2.3).

The alternative aggregation mechanism in the presence of sulfate which has been characterized in detail in this kinetic study is in agreement with previous findings reported in the literature which show a different morphology of insulin aggregates obtained in the presence of sulfate [21, 95, 120].

The microscopic mechanism responsible for the formation of the amorphous aggregates is likely due to a combination of the peculiar binding and salting-out properties of the sulfate anion. Sulfate anion has a large propensity to bind to insulin molecules [95] and to induce salt bridges [97, 121]. It has been recently demonstrated that, even at low salt concentrations (few-10 mM), ion-binding and additional ion-specific effects, on top of the Debye-Hückel charge screening effect, modify protein electrostatic interactions and aggregation kinetics [79]. It is expected that at larger salt concentrations (10-100 mM) the ion binding, together with the salting-out effect, can affect even more significantly both the protein structure and the intermolecular interactions.

According to circular dichroism analysis, the presence of sulfate does not modify either the initial protein structure or the thermal stability with respect to sodium chloride (Figure A.6 in Appendix A), although small changes not detectable by circular dichroism cannot be excluded.

The reduction of insulin net charge upon binding, together with the increase of hydrophobic interactions due to preferential exclusion, increases the net attractive intermolecular interactions between insulin molecules, thus promoting aggregation, and in particular the nucleation of aggregates characterized by native-like content. These aggregates are likely formed too rapidly to reach the most stable configuration. The kinetically trapped species reorganize during time into more thermodynamically favored β-sheet structures, which are able to bind ThT dyes and recruit additional monomers.

The system described in this chapter shows how the kinetic and the thermodynamic features of the protein aggregation process are highly sensitive to the
environmental parameters, which affect the intra- and intermolecular interactions driving the microscopic aggregation events and the overall reaction mechanism.

These aspects are relevant for instance in the biotechnology context for the development of strategies to improve the formulation of protein-based drugs. Salt-induced aggregation of proteins in aqueous solutions is a major problem which often limits the production and the storage of therapeutic proteins. The results shown in this work, although obtained under strong denaturing conditions, may suggest some caution in the use of sulfate and sulfonated compounds in the processing and formulation of insulin, since these compounds can rapidly trigger the formation of amorphous precipitates which cannot be detected by ThT fluorescence assay and can convert slowly into ordered aggregates with seeding properties.

In the context of protein aggregation involved in human diseases, it remains challenging to understand at a fundamental level the interactions between proteins and all the possible cellular components. On a long term, the results obtained on the effect of sulfate on \textit{in vitro} protein aggregation may represent a first step towards the understanding of the complex \textit{in vivo} interactions between proteins and sulfonated macromolecules such as GAGs.

### 2.5. Conclusions

In this study the effect of sulfate anion on the kinetics and the mechanism of insulin aggregation at low pH was investigated. It is found that the increase of sulfate concentration inhibits insulin aggregation kinetics.

This effect is associated to a change in the aggregation mechanism with increasing sulfate concentration. At low sulfate concentrations (0-5 mM) insulin monomers form amyloid fibrils following the nucleated-polymerization mechanism commonly observed with monovalent anions.

At sulfate concentrations larger than 5 mM, an alternative aggregation mechanism is observed: initially, 18-20% of monomers are converted into reversible aggregates which retain large amount of native $\alpha$-helix structure. During time, according to FTIR, ThT binding and seeded kinetic assays, these intermediate amorphous aggregates undergo reorganization into $\beta$-sheet structures which are able to bind to the ThT dye and seed fibril formation. TEM analysis shows that the final aggregates consist of a mixture of
amorphous and fibrillar aggregates, and differ from the regular amyloid fibrils obtained in the presence of chloride.

The system described in this chapter represents a case where amorphous aggregates on-pathway to the formation of structures with amyloid-like content could be detected and characterized.
Chapter 3

Contribution of electrostatics in the fibril stability of a model ionic-complementary peptide

3.1. Introduction

The self-assembly of peptides and proteins into regular fibrillar structures known as amyloid fibrils is involved in a large variety of research fields ranging from biological to material sciences. For instance, amyloid fibrils are increasingly recognized to be involved in the onset and the progression of several neurodegenerative diseases such as Alzheimer’s, Parkinson’s and Huntington's disease [1, 13]. Not only dysfunctional but also functional amyloids are observed in nature. An intriguing example is the storage of certain peptides, for instance hormones, as amyloid fibrils, which release the active monomeric form of the peptides in a controlled dissociation process [122]. This naturally occurring form of storing active biomolecules guarantees high mechanical and chemical stability, as well as the ability to release the active component in a controlled way, and may inspire formulations of long-acting drugs [123].

In the context of biotechnology, several small peptides have been rationally developed to spontaneously self-assemble in aqueous solutions into higher ordered structures depending on the environmental conditions [27, 32, 124-127]. One particular class of these functional materials is the family of ionic-complementary peptides, which exhibit hydrophobic residues on one side of the structure and charged amino acids on the other side [6, 27]. The controlled aggregation into chemically and mechanically resistant structures makes these peptides ideal building-blocks for the formation of biocompatible materials, which found applications in 3D cell cultures [128], tissue regeneration and engineering [34] as well as in drug release [38] and drug delivery [37, 39].

Both the kinetic and the thermodynamic stability of the fibrillar structures are regulated by a series of intermolecular forces, which can be tuned by modifying several operating parameters.

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3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

Amyloid fibrils are characterized by a high content of cross-β sheet structure organized into thin, long and unbranched fibrils [14, 15]. The network of hydrogen bonds in the peptide backbone plays a crucial role in stabilizing the amyloid structure [19]. Indeed, under suitable conditions a large number of peptides and proteins are able to form amyloid fibrils that share similar structure independently of the protein primary sequence [13, 129]. In addition to hydrogen bonding [19], several other intermolecular forces contribute to the fibril formation and stability, including aromatic [130, 131] and electrostatic interactions. Steric zippers formed by facing side chains of pair β-sheets [132] and curvature energies related to the twisting of the fibrils play also an important role, in particular in multi-stranded filaments [69, 133-135].

With this complex scenario, understanding the role of electrostatics in the self-assembly of peptides and proteins is a difficult task. This challenge arises largely from the heterogeneous distribution of charges on the protein surface, which involves the simultaneous presence of both positive and negative charges that are highly dependent on the environmental pH and buffer composition [65, 79, 97, 136-141]. Despite this heterogeneous distribution of charges on the protein surface, the global surface net charge has been recognized to be a crucial physicochemical parameter that determines the general aggregation behavior [9]. For instance, close to the isoelectric point (pI), corresponding to zero net charge, peptides and proteins tend to precipitate into amorphous aggregates, whereas higher net charges promote the formation of fibrillar structures [4, 142].

In this study, we provide a more quantitative framework of this qualitative behavior by evaluating the contribution of electrostatics in the free energy of fibril formation, considering the well-known amphiphilic peptide RADARADARADARADA (RADA 16-I) as a model system. We apply size exclusion chromatography, circular dichroism and electron microscopy to characterize the aggregate structure and quantify the monomer content at different pH values. In order to get insights into the structural conformation of the monomeric peptide at different pH values we complement the experimental characterization with metadynamics simulations, which have been proved to provide an efficient sampling of protein structures [143, 144], even in the presence of denaturant [145, 146]. We show that the global peptide net charge is a key property that correlates well with fibril stability, although the peptide conformation and the surface charge distribution also contribute to the self-assembly propensity.
3.2. Materials and methods

3.2.1. Material and sample preparation

RADA 16-I peptide (Ac-R-A-D-A-R-A-D-A-R-A-D-A-R-A-D-A-CONH₂ with MW 1712.8 Da and purity ~80% as assessed by mass spectrometry analysis as shown in Figure B.1 in Appendix B) was provided by Lonza Ltd (Visp, Switzerland) as lyophilized powder in the form of trifluoroacetic salt. The material was used without further purification. Before each experiment a stock solution of the peptide at a concentration of 5 g/L was freshly prepared by dissolving the peptide powder in 10 mM HCl at pH 2.0 as described elsewhere [56]. Briefly, the suitable amount of buffer was added to the weighted peptide powder and the solution was gently stirred at room temperature for 10 minutes for homogenization. The formation of fibrils was observed immediately after solubilization of the powder. Samples for analysis were prepared either by diluting 10-fold the stock solution in suitable buffers, or by dialyzing the stock solution against suitable buffers using the Dispo-Biodialyzer kit with 1 kDa molecular weight cut-off membrane (Sigma-Aldrich GmbH, Steinheim, Germany). Analysis of selected samples showed no significant difference between solutions prepared according to the two different applied protocols (data not shown). In order to guarantee proper buffering, the following solutions were used: 10 mM acetate buffer at pH from 3.0 to 5.0, 10 mM phosphate buffer at pH 6.0 and 7.0 and 10 mM TRIS-HCl at pH 8.5. The final pH value of the samples was checked by pH-Fix 0-14 color-fixed indicator sticks (Machery-Nagel GmbH & Co. KG, Düren, Germany).

3.2.2. Circular dichroism (CD)

Circular dichroism measurements were performed with a Jasco-815 CD spectrophotometer (Jasco, Easton, MD, USA). CD spectra were recorded in the far-UV region from 190 to 260 nm at 25 °C. A quartz cuvette with 0.1 cm path length was used. Spectra obtained after buffer subtraction were corrected and smoothed using the Savitsky-Golay function. Freshly prepared samples of RADA 16-I at 5 g/L in 10 mM HCl at pH 2.0 were diluted in 10 mM HCl (pH 2.0), 10 mM acetate buffer (pH 4.5), 10 mM phosphate buffer (both pH 6.0 and 7.0) and 10 mM TRIS-HCl buffer (pH 8.5) in order to obtain a peptide final concentration of 0.5 g/L. The pH value after sample dilution was
checked by pH-Fix 0-14 color-fixed indicator sticks (Machery-Nagel GmbH & Co. KG, Düren, Germany).

### 3.2.3. Transmission electron microscopy (TEM)

Peptide samples were imaged using a FEI Morgagni 268 microscope. Peptide solutions were diluted to a final concentration of about 0.05 g/L, and loaded on a carbon grid (Quantifoil, Jena, Germany). Samples at pH 2.0 and 4.5 were stained with a 2% uranyl acetate solution, whereas samples at pH ranging from 6.0 to 8.5 were stained with a 2% phosphotungstic acid solution (pH 6.5).

### 3.2.4. Size exclusion chromatography (SEC)

Size exclusion chromatography analysis was performed using a Superdex Peptide 10/300 GL, 10 mm×300 mm size-exclusion column (GE Healthcare, Uppsala, Sweden) mounted on a Agilent 1100 series HPLC unit (Santa Clara, CA, USA) consisting of an isocratic 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 a buffer at suitable pH as mobile phase. The UV absorbance peaks were detected at 217 nm.

### 3.2.5. Computational methods

#### 3.2.5.1. Guanidinium atomic charges

The guanidinium structure was optimized in vacuo by means of density functional theory (DFT) calculations at B3LYP/6-31G(d,p) level of theory [147-149]. The obtained geometry was further optimized in implicit water and modeled through the integral equation formalism polarizable continuum model (IEFPCM) [150] at 300 K, at the same level of theory.

Atomic charges were fitted by means of RESP formalism [151, 152], starting from the electrostatic potentials computed through DFT calculations at B3LYP/6-311+G(d,p) [153] level of theory in implicit water. Charge fitting was performed in two steps. First, atomic charges were determined assigning an overall charge equal to +1. In a second step, charge equivalence for chemically equivalent atoms was imposed. In particular, all hydrogen and nitrogen atoms have the same partial atomic charge because of resonance structures. Computations were performed by means of Gaussian 09 software [154].
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

3.2.5.2. Simulations protocol

Metadynamics simulations were performed following the approach proposed by Deighan et al. [155], who combined metadynamics and parallel tempering (PTMetaD) in the well-tempered ensemble (PTMetaD-WTE) in order to improve simulation efficiency. Metadynamics simulations allow to recover the free energy of a system by keeping track and discouraging the sampling of the conformations already visited during the simulations as a function of few conformational parameters (collective variables) [156, 157]. All simulations were carried out using GROMACS 5.0.2 [158] patched with PLUMED 2.1.0 plugin [159].

Linear RADA 16-I peptide structures were built using tleap module as implemented in AmberTools package [160]. Protonation states at pH 2.0 and pH 4.5 were determined according to PropKa calculations [161]. At pH 2.0, all arginine and aspartic acid side chains are protonated, thus leading to an overall net charge equal to +4; at pH 4.5, all arginine side chains are protonated, and three single aspartic acid moieties are dissociated, and the net charge is equal to +1. According to PropKa calculations, in the protein primary structure all aspartic acid residues exhibit the same pK$_a$ value. Although it can be expected that in the protein arrangement a dynamic exchange of proton among carboxyl groups takes place, a reliable constant pH method (needed to properly account for this dynamic equilibrium) is challenging to implement in GROMACS. Therefore, in the present simulations at pH 4.5, the protonation has been arbitrarily assigned to the first aspartic acid moiety in peptide sequence. Simulations were performed adopting ff14SB force field [162] for RADA 16-I peptide and General Amber Force Field (GAFF) [163] for guanidinium.

Each peptide was solvated using 5500 TIP3P water molecules [164] for simulations in water environment, or 7000 TIP3P water molecules and 80 guanidinium molecules when 0.6 M guanidinium hydrochloride solution was simulated. Electroneutrality was assured by adding Cl$^-$ ions, whose parameters (optimized for TIP3P water model) has been taken from Joung and Cheatham [165]. Starting system for PTMetaD-WTE simulations was obtained using the following protocol.

First, energy minimization was performed by means of conjugate gradient method in order to remove bad solvent/solvent and solvent/solute contacts. Temperature was raised from 0 to 300 K through 200 ps in NVT ensemble, applying a weak harmonic restraint on solute molecules in order to avoid wild fluctuation. The system was finally
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

equilibrated with 1 ns simulation in NPT ensemble at 300 K and 1 atm. Velocity rescale algorithm [166] and Parrinello-Rahman barostat [167] were employed to maintain the system at the desired values of temperature and pressure, respectively. Electrostatic long-range interaction were treated through the Particle Mesh Ewald (PME) method [168] using a cut-off value equal to 12 Å; the same cut-off was used for Lennard–Jones interactions. Neighbor list was updated every 5 fs, and all covalent bonds involving hydrogen were restrained by means of LINCS algorithm [169].

PTMetaD-WTE simulations were carried out using 12 replicas whose temperature ranges from 300 to 631 K. Before starting metadynamics simulations, a 1 ns molecular dynamics simulation was performed for each replica. In this way, every system could equilibrate according to its temperature and adjacent replicas exhibit different initial peptide structures.

PTMetaD-WTE simulations were then performed using a two steps protocol [155]. First, a 5 ns PTMetaD simulation [144] was carried out biasing only potential energy, chosen as collective variable; Gaussians were deposited every 0.5 ps, adopting an height value equal to 0.28 kcal mol⁻¹, a width value equal to 500 kcal mol⁻¹ and a bias factor equal to 24. In a second step, the radius of gyration related to α-carbon atoms in the protein backbone (R₉) and the intrachain hydrogen bonds (Hₖ) were chosen as collective variables (since they proved to be suitable for sampling protein secondary structures [143, 144, 146]) and biased according to well-tempered metadynamics algorithm [157]. No more Gaussians were added in order to bias potential energy; the cumulative bias was used as a static additional bias potential. Focusing on the radius of gyration and the intrachain hydrogen bonds, Gaussians were added every 1 ps, adopting a height value equal to 0.28 kcal mol⁻¹, width values equal to 0.01 and 0.1 for R₉ and Hₖ, respectively, and a bias factor equal to 8. Velocity rescale algorithm [166] was used in order to maintain the systems at the desired temperature values. Long – range interactions were treated as described above.

A simulation time equal to 250 ns was used for each replica, thus leading to a total simulation time of 3 μs for each system. Exchanges attempts between replicas were performed every 0.4 ps by means of the PTMetaD algorithm as implemented in GROMACS 5.0.2 patched with PLUMED 2.1.0 plugin. Convergence was verified by evaluating the folding free energy as a function of simulated time; details are reported in Figure B.2 in Appendix B.

Solvent Accessible Surface Area (SASA) has been calculated through g_sas utility as implemented in GROMACS package.
Electrostatic potentials have been computed through Adaptive Poisson Boltzmann Solver (APBS) [170]. Electrostatic potential is expressed in $k_B T e^{-1}$ units, where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature and $e$ is the electron charge.

### 3.3. Results and discussion

#### 3.3.1. pH effect on the RADA 16-I secondary structure and morphology

We start our analysis by investigating the effect of pH on the secondary structure of the peptide to compare our results with the broad range of data on amphiphilic peptides reported in several works in literature [9, 27, 171, 172]. The effect of the environmental pH on the peptide secondary structure was determined by circular dichroism analysis (Figure 3.1a). At pH 2.0, the minimum at 195 nm in the CD spectrum clearly indicates the presence of a large amount of random coil structures, which can be attributed to the monomeric peptide, whereas the shoulder visible at the wavelength of about 215 nm corresponds to the $\beta$-sheet structures of the fibrils. At pH 4.5, we observe mainly $\beta$-sheet structures, representative of a large amount of fibrils. An increase of the pH of the solution to neutral conditions (pH 6.0, 7.0 and 8.5) leads to the loss of $\beta$-sheet structures. Moreover, formation of amorphous precipitates in the micron size range was detected by macroscopic observations.

In parallel with CD analysis, we investigated the morphology of the RADA 16-I peptide by transmission electron microscopy. We observed the presence of long, thin and rather rigid fibrils at pH 2.0 and 4.5 (Figure 3.1b-c), and a mixture of fibrils and amorphous precipitates at higher pH values (Figure 3.1d-f). Our results are in excellent agreement with the findings reported for RADA 16-I by Ye et al. and Zhang et al. [171, 172].
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

![Graph](image)

**Figure 3.1. pH effect on the secondary structure and the morphology of RADA 16-I peptide.**

a) CD spectra of 0.5 g/L peptide solutions in 10 mM HCl at pH 2.0 (blue dashed line), 10 mM acetate buffer at pH 4.5 (red continuous line), 10 mM phosphate buffer at pH 6.0 (green dotted line) and 7.0 (violet dash-dotted line) and 10 mM TRIS-HCl buffer at pH 8.5 (black dashed line). b-f) TEM pictures of the RADA 16-I solutions in (b) 10 mM HCl at pH 2.0, (c) 10 mM acetate buffer at pH 4.5, (d) 10 mM phosphate buffer at pH 6.0, (e) 10 mM phosphate buffer at pH 7.0 and (f) 10 mM TRIS-HCl buffer at pH 8.5.

**3.3.2. Effect of electrostatic interactions on the monomer-fibril equilibrium**

The analysis of the secondary structure content of RADA 16-I peptide at different pH values indicates that the monomer fraction in the dispersions of RADA 16-I peptide at 0.5 g/L is predominant at pH 2.0, whereas at pH 4.5 the CD spectrum shows mainly the presence of fibrils (Figure 3.1a). We quantified in detail the dependence of the monomer amount on the pH value by measuring the monomeric peptide concentration in freshly prepared solutions of RADA 16-I at 0.5 g/L in the pH range 2.0-5.0 by size exclusion chromatography. We observed a decrease in the monomer percentage with increasing the pH from pH 2.0 to pH 4.5. Since precipitation of amorphous aggregates is observed at pH 5.0, as shown in Figure 3.2a, this analysis indicates that the optimal pH for the self-assembly of monomers into fibrils is equal to about 4.5.

The monomer content at different pH values can be correlated to the net charge of the peptide calculated from the amino acid composition and the pKₐ values of the single amino acids, as described elsewhere [173]. At low pH value, where the largest amount of
When a monomer is observed, the high amount of uncompensated charge induces repulsive interactions between monomer molecules, thus disfavoring the aggregation process and destabilizing the fibrils. The decrease in the net charge by increasing the pH value results in the increase of the fibril fraction in the solution, whereas close to the isoelectric point, which is equal to about 7.2 [171], the absence of stabilizing electrostatic interactions induces the precipitation of amorphous aggregates in the micron size range (Figure 3.2a). Actually, precipitates are already formed at pH 5.0 (star symbol in Fig. 3.2a). Although the net charge is not equal to zero, it is anyway not sufficient for stabilization. The maximum pH, at which the precipitation was not observed and the maximum aggregation propensity of monomers into fibrils occurred, is equal to 4.5 corresponding to a net charge equal to +0.75. This result is in excellent agreement with the value of ±1 reported by López de la Paz et al. [174] as well as Aggeli et al. [9] as the optimum net charge for amyloid fibril formation, indicating the possible presence of a certain level of universality in the aggregation behavior of peptides.

The effect of the surface net charge on the aggregation propensity of the peptide was further rationalized by performing metadynamics simulations of the structures of the monomeric peptides in solution. In particular, the monomer structure was calculated at two selected reference pH values, namely pH 2.0 and 4.5, at which, respectively, the minimum and the maximum of the monomer aggregation extent into fibrils were observed (Figure 3.2b-g). The number of intrapeptide hydrogen bonds and the radius of gyration of α-carbon atoms in the peptide backbone were used as collective coordinates to determine the minimum free energy surface under the considered conditions. In particular, in Figure 3.2b and c it is seen that such minimum occurs at a radius of gyration of about 0.56 nm at pH 2.0 and at about 0.66 nm at pH 4.5.

According to simulations, the global minimum of the free energy surface at pH 4.5 (which implies a low net charge value) corresponds to β-hairpin-like structures of the monomer (Figure 3.2d). Both positive and negative charges are exposed on monomer surface, thus promoting an electrostatic-driven aggregation between peptides (Figure 3.2f). More ordered β-hairpin structures as well as unordered open structures can be found in the neighbor regions of the minimum ($\Delta F \leq 4$ kJ/mol).
Figure 3.2. **Effect of pH and net charge on the peptide stability and the aggregate morphology.** a) Fractions of monomeric peptide (♦) and fibrils (●) as a function of the solution pH and the peptide net charge, \( z_p \), for RADA 16-I solutions at peptide concentration of 0.5 g/L. The red star (♦) corresponds to the critical pH \( = 5.0 \) and net charge \( z_p = 0.27 \) at which precipitation of amorphous aggregates is observed. If error bars are not visible, they are smaller than the symbol. b-c) Free energy surface calculated from PTMetaD-WTE simulations at (b) pH 4.5 and (c) pH 2.0; contour lines are plotted every \( k_bT \) unit (2.5 kJ/mol). d-e) Reference minimum energy structures at (d) pH 4.5 and (e) pH 2.0. f-g) Electrostatic surface potential expressed in \( k_bT \) units at (f) pH 4.5 and (g) pH 2.0. Representative SEC chromatograms are shown in Figure B.3 in Appendix B.
At pH 2.0, the global minimum of the free energy surface corresponds to compact random coil structures of the monomeric peptide, whose mean radius of gyration is equal to 0.56 nm. More open random coil structures, corresponding to a radius of gyration up to 0.67 nm are contained in the neighborhood of the minimum ($\Delta F \leq$ kJ/mol). In all sampled structures, the positively charged arginine side chains are located on the peptide surface and are fully exposed towards the water environment (Figure 3.2e), in order to minimize the favorable interactions between water molecules and the charged moieties. The resulting average surface potential is thus highly positive (blue-colored areas in Figure 3.2g), confirming the presence of a repulsive electrostatic barrier between two interacting monomers, which hinders the formation of fibrils.

The structures obtained by metadynamics simulations (Figure 3.2d-f) are in good agreement with both the secondary structure content measured by CD spectroscopy (Figure 3.1a) and the experimental observation of the aggregation behavior. Indeed, the $\beta$-sheet-like structure at pH 4.5 along with the low net charge is likely to promote fibril formation. By contrast, at pH 2.0 the aggregation is hindered by both electrostatic repulsion and the random coil geometry of the monomeric units.

After quantifying the monomer amount as a function of the pH value, we verified the presence of equilibrium between monomers and fibrils by measuring the dependence of the amount of free monomer as a function of the total peptide concentration. The fibril formation can be described as a linear polymerization process consisting of a series of reversible additions of monomers ($M$) to fibrils with length $n-1$ ($F_{n-1}$) to generate fibrils with length $n$ ($F_n$) [175]:

$$F_{n-1} + M \leftrightarrow F_n. \quad (3.1)$$

The equilibrium constant, $K_{eq}$, of this reversible association reaction of monomers to the fibrils ends can be defined as:

$$K_{eq} = \frac{[F_n]}{[F_{n-1}][M]} \quad (3.2)$$

where $[F_n]$, $[F_{n-1}]$, $[M]$ are the concentrations of fibrils with length $n$, fibrils with length $n-1$ and monomer, respectively. The model shown here is not accounting for the short fibrils (i.e., small $n$), but the approximation is valid for the long fibrils present in our system.
As described elsewhere [175], the total peptide concentration ($C_{\text{tot}}$) is equal to:

$$C_{\text{tot}} = \sum_{n=1}^{\infty} n \cdot [F_n] = \sum_{n=1}^{\infty} n \cdot K_{eq}^{n-1} \cdot [M]^n = \frac{[M]}{(1 - K_{eq} \cdot [M])^2}$$  \hspace{1cm} (3.3)

which can be derived based on the series for $(1-x)^{-2}$.

The equilibrium constant can be therefore defined as [175]:

$$K_{eq} = \frac{1}{[M]} - \frac{1}{\sqrt{[M] \cdot C_{\text{tot}}}}.$$  \hspace{1cm} (3.4)

Replacing monomer concentration with monomer fraction, $x_M = [M]/C_{\text{tot}}$, where $C_{\text{tot}}$ is the total peptide concentration, the equilibrium constant is equal to:

$$K_{eq} = \frac{1}{C_{\text{tot}}} \cdot \frac{1 - \sqrt{x_M}}{x_M}.$$  \hspace{1cm} (3.5)

From equation (3.5), which can be re-written as:

$$K_{eq} = \frac{1}{C_{\text{tot}}} \cdot \frac{1 - \sqrt{x_M}}{x_M},$$  \hspace{1cm} (3.6)

it can be seen that the monomer fraction is directly related to the total peptide concentration via the equilibrium constant. In Figure 3.3a we plot the monomer content as a function of the total peptide concentration for various pH values as measured by means of size exclusion chromatography according to equation (3.6). The observed linear relationship confirms the presence of the equilibrium between monomer and fibrils. The evaluated equilibrium constants are, respectively, 247.4 M$^{-1}$, 628.4 M$^{-1}$, 1575.7 M$^{-1}$ and 9639.4 M$^{-1}$ at pH 2, 2.5, 3.0 and 4.0. As shown in Figure 3.3b, the equilibrium constant increases together with the increase of the environmental pH and the decrease of the peptide net charge. These results indicate that the equilibrium is shifted towards fibrils at lower net charges, whereas the monomeric form is energetically favorable at higher net charges.
The Gibbs free energy is related to the equilibrium constant by the equation:

$$
\Delta G = -R \cdot T \cdot \ln\left( K_{eq} \right)
$$  \hspace{1cm} (3.7)

where $R$ is the gas constant and $T$ the temperature.

The electrostatic contribution of the Gibbs free energy between two charged objects can be approximated as $[79, 176]$:

$$
\Delta G_e = \frac{\left( z_p \cdot e \right)^2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon_r \cdot r} \cdot \exp\left( -\kappa \cdot r \right)
$$  \hspace{1cm} (3.8)
where $z_p$ is the net charge of the peptide, $e$ is the elementary charge, $\varepsilon_0$ is the vacuum permittivity, $\varepsilon_r$ is the relative dielectric permittivity, $r$ is the distance between the ions and $\kappa$ is the inverse Debye length.

According to equation (3.8), if the electrostatic contribution dominates in the Gibbs free energy, a linear relationship between the free energy and the square of the peptide net charge is expected. Indeed, we observed a linear correlation between these two values (R-squared value equal to 0.985) as seen in the Figure 3.4a, suggesting that electrostatics plays an important role in the peptide self-assembly process.

In order to further investigate this effect, we measured the monomer content at both pH 2.0 and pH 4.5 as a function of the salt concentration. In the presence of electrostatic interactions, the addition of salt at a concentration of 10-50 mM screens the repulsion forces, thus promoting aggregation. Indeed, as shown in Figure 3.4b, an initial increase of the salt concentration induces a decrease of the monomer content and a corresponding increase of the equilibrium constant (Figure 3.4c). Above 50 mM, where the screening effect is saturated, the monomer amount is independent of the salt concentration. The charge screening effect is confirmed by the calculation of the change in the Debye length as a function of salt concentration (inset in Figure 3.4b), which shows a decrease in the Debye length with increasing salt concentration. This decrease in the Debye length is significant in particular at low ionic strengths, while the dependence starts to flatten out at salt concentrations larger than 50 mM, suggesting that the most of the charges on the peptide surface are screened above this salt concentration.
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

Figure 3.4. Electrostatics in RADA 16-I self-assembly. a) Gibbs free energy as a function of the net charge, $z_p$, of RADA 16-I peptide. b-c) Effect of salt on (b) the monomer fraction and (c) the equilibrium constant calculated from equation (3.5) for RADA 16-I solutions at 1 g/L in 10 mM HCl at pH 2.0 (●, left y-axis) and in 10 mM acetate buffer at pH 4.5 (■, right y-axis). The inset in (b) shows the change in the Debye length as a function of salt concentration. If error bars are not visible, they are smaller than the symbol. Representative SEC chromatograms are shown in Figure B.5 in Appendix B.
3.3.3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

3.3.3. Perturbation of the monomer-fibril equilibrium by chemical denaturation

We further investigated the thermodynamic stability of the fibrils upon chemical denaturation at the two reference pH values of 2.0 and 4.5, where, respectively, the smallest and the largest amount of fibrils were observed. We monitored the monomer content in 10 mM HCl (pH 2.0) and 10 mM acetate buffer (pH 4.5) with guanidinium hydrochloride (Gnd-HCl) at concentrations ranging from 0 M to 6 M. The pH value was measured after the addition of the peptide and of Gnd-HCl.

As shown in Figure 3.5a, at both pH values we observe a non-monotonic behavior of the monomer amount as a function of the Gnd-HCl concentration: the monomer percentage initially decreases as small amounts of Gnd-HCl are added into the system, while a further increase in the concentration of Gnd-HCl destabilizes the fibrils and promotes the dissociation of monomers into the solution. Interestingly, at Gnd-HCl concentration of 6 M, the monomer content is similar at both pH 2.0 and 4.5 (41.9±2.3%) (Figure 3.5a), despite the stability of the fibrils in the absence of Gnd-HCl is different at the two pH values. Analysis by TEM imaging confirms that aggregates with fibrillar morphology are produced at all concentrations of denaturant (Figure B.7a-d in Appendix B). Moreover, the disruption of β-sheet structures at 6 M Gnd-HCl is confirmed by CD analysis (Figure B.7e in Appendix B).

In addition to a charge screening effect due to the ionic nature of Gnd-HCl, the initial decrease of the monomer content observed after the addition of a low amount of Gnd-HCl (0.3-1 M) can be correlated to a variation in the peptide conformation. To confirm this hypothesis, we performed metadynamics simulations of the structure of monomeric peptides at a reference concentration of denaturant equal to 0.6 M at both pH values under consideration.
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

Figure 3.5. Chemical stability of RADA 16-I fibrils. a) Amount of monomer in RADA 16-I solutions at 0.5 g/L in 10 mM HCl at pH 2.0 (♦) and 10 mM acetate buffer at pH 4.5 (●) as a function of Gnd-HCl concentration in the range 0-6 M. If error bars are not visible, they are smaller than the symbol. b-c) Free energy landscape as a function of the radius of gyration at (b) pH 2.0 and (c) pH 4.5. d) Simulation illustrating the formation of a Gnd-HCl salt bridge between two interacting monomers in 0.6 M Gnd-HCl solution at pH 4.5. Representative SEC chromatograms are shown in Figure B.6 in Appendix B.
At pH 2.0, we observed and increase in the radius of gyration of the structure with the minimum energy from about 0.56 to 0.62 nm, as seen in Figure 3.5b (the detailed free energy surface is shown in Figure B.8 in Appendix B). The global minimum of the free energy surface is still represented by random coil structures, which now exhibit a more open configuration due to the denaturing effect of guanidinium. The region of the free energy surface around the minimum ($\Delta F \leq 2$ kJ/mol) includes structures characterized by a wide span of $R_g$ values (0.58 – 0.78 nm). This result highlights that, although some compact structures are still present, guanidinium shifts the equilibrium towards unfolded configurations of the monomer, which can enhance the propensity of the peptide towards aggregation, as observed by Zhang et al. [172]. Regions of the free energy surface which are more distant from the minimum ($\Delta F \leq 4$ kJ/mol) contain a substantial presence of unfolded structures (Figure B.8 in Appendix B).

At pH 4.5, the addition of 0.6 M denaturant induces a change in the radius of gyration from 0.66 nm to 0.62 nm as shown in Figure 3.5c. This change corresponds to the disruption of the $\beta$-hairpin-like geometry into disordered and partially unfolded structures in the regions of the energy diagram close to the minimum ($\Delta F \leq 6$kJ/mol). The partially unfolded conformation corresponding to the global minimum in the free energy surface in the presence of guanidinium exposes a larger surface area to the solvent (1718 Å$^2$) with respect to the $\beta$-hairpin-like arrangement of the corresponding structure in pure water (1574 Å$^2$). This change is likely to be driven by the exposure of dissociated aspartic acid residues, which can easily bind guanidinium ions through hydrogen bonds. The interactions between aspartic acid residues and positively charged guanidinium ions are indeed more favorable at pH 4.5 than at pH 2.0, due to the unprotonated state of carboxyl groups.

In order to further investigate the role of guanidinium hydrochloride on the aggregation propensity at pH 4.5, we performed a 60 ns molecular dynamics simulation at 300 K and 1 atm by placing two partially unfolded monomers in a 0.6 M denaturant solution: the molecular trajectories showed that the guanidinium ions bind to the dissociated aspartic acid side chains, thereby acting as salt bridge between peptides (Figure 3.5d) and promoting aggregation. Although this simulation cannot clearly provide a comprehensive picture of the overall mechanism of the formation of fibrils, this result highlights a possible role of the guanidinium hydrochloride in the first stages of the self-assembly process.
The additional increase in the denaturant content from about 1 M to 6 M causes further denaturation of the peptide, which leads to the dissociation of the monomer from the fibrils into the solution. Overall, these results indicate that, in addition to electrostatic effects, the monomer conformation and the charge distribution plays a significant role in the fibril formation, and that extended conformations favor the self-assembly process of the RADA 16-I peptide.

3.4. Conclusions

We investigated the role of electrostatic interactions in the self-assembly of the model amphiphilic peptide RADA 16-I by using a combination of experimental characterization and molecular dynamic simulations.

It is found that the optimal net charge to promote the formation of regular amyloid fibrils corresponds to a value of +0.75, a result which is in agreement with previous findings reported in the literature [9, 174], indicating the possible presence of a certain level of universality in the aggregation behavior of peptides. Larger net charges, corresponding to low pH values, destabilize the fibrillar aggregates and promote the release of monomers that are in equilibrium with the fibrils, while lower net charge induces the formation of amorphous precipitates. The quantification of the fibril-monomer equilibrium constant as a function of pH and peptide net charge shows that electrostatic interactions contribute largely to the dependence of the free energy of fibril formation on the pH value.

The addition of both salt and a charged destabilizer (guanidinium hydrochloride) at moderate concentration (0.3-1 M) at both pH values shifts the monomer-fibril equilibrium towards the fibrillar aggregates. The effect of salt can be easily explained by the screening of electrostatic repulsion only. By contrast, the promotion of fibril formation in the presence of guanidinium hydrochloride is attributed to modifications of the peptide conformation in addition to charge screening effects.

The experimental and modeling results of this work indicate that intermolecular electrostatic interactions play a key role in the complex combination of the several intermolecular forces that contribute to fibril formation and stability. These additional interactions include largely hydrogen bonding [19] as well as “steric zippers” effects [132] and bending energies related to the twisting of the fibrils [69, 133-135]. Despite the complexity of this behavior, which is reflected by the role of the peptide conformation
3. Contribution of electrostatic in the fibril stability of a model ionic-complementary peptide

and the surface charge distribution on the aggregation stability, the peptide net charge appears to be a good indicator of the propensity of peptides towards aggregation.
Chapter 4

A colloidal description of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic peptide

4.1. Introduction

The colloidal stability and the self-assembly mechanisms of polymer and biopolymer nanofibrils have relevant implications in a wide range of recent technological applications including tissue engineering, sensors, textile industry and reinforcement in composite materials [177-180]. In particular, protein amyloid fibrils represent an attractive class of substrates in the context of nanotechnology and functional biomaterials because of their remarkable mechanical properties coupled with biocompatibility and degradability [181-186]. For instance, fibrillar hydrogels obtained from synthetic peptides have found many applications in fields ranging from 3D cell cultures [128] to drug release [38] and delivery [37, 39], as well as tissue engineering and tissue repair [34]. For the optimization of the conditions during manufacturing and the effective application of these materials, it is crucial to achieve a rational control of the stability of the fibril dispersions.

In addition to applications in material sciences, amyloid fibrils are widely investigated in the biomedical context because of their association with the onset and the progress of several neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Huntington disease [1, 13]. Increasing evidence indicates that fibrillar aggregates play a key active role in reactions leading to the formation of toxic species [24]. Therefore, it is of great interest to correlate the changes in the size and structure of the fibrils with the changes in their reactivity.

The self-assembly process of peptides and proteins into regular filamentous structures involves several microscopic steps, including nucleation and aggregate growth by either monomer addition or fibril-fibril aggregation. Several biophysical studies have recently shed light into the fundamental physics underlying the nucleation and the fibril elongation events [19, 71, 187]. However, the molecular mechanisms underlying the

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fibril-fibril aggregation into larger structures remain largely unknown, although this process is observed in many systems [188].

Despite the phase behavior of rod-like colloids has been widely investigated in the literature [189-192], the study of the stability of protein fibril dispersions is challenging, largely because of the inhomogeneous surface chemistry of proteins, which exhibit anisotropic distribution of charges as well as presence of both hydrophobic and hydrophilic patches [54]. As a consequence, intramolecular forces may be confined to specific regions of the surface [65, 137, 140]. However, in some systems, protein-protein interactions have been successfully described by coarse-grained interaction potentials [193-195]. The stability of colloidal systems is often rationalized in the frame of the Derjaguin-Landau-Verwey-Overbeck (DLVO) theory, which describes the aggregating units as homogeneously charged objects interacting via repulsive electrostatic interactions and attractive Van der Waals forces [196]. It is of interest to explore whether this simplified treatment can describe the more complex behavior of protein fibril dispersions, where additional orientation factors can affect the total interaction potential, because of the reduced number of patches available for the aggregation process [191].

To address this question, here we develop a kinetic study to quantify the intermolecular interactions underlying the colloidal stability of fibrils composed of the model amphiphilic peptide RADA 16-I at pH 2.0. The investigation of the peptide stability under these conditions is of great interest in the context of biotechnology, since acidic conditions represent operative parameters that are typically encountered in the industrial manufacturing of this class of peptides. Moreover, despite the results discussed in this work do not apply directly to aggregation occurring in biology, the general colloidal framework presented here could be of relevance for future studies addressing the stability of amyloid fibrils in living systems.

It has been previously shown that charged fibrils composed of this peptide aggregate during time into longer filaments with constant diameter via an irreversible end-to-end fibril-fibril aggregation mechanism [56]. This system represents therefore a rare example where the fibril-fibril aggregation is the only elementary process governing the self-assembly, while other reactions involving monomers are negligible. This feature opens the attractive possibility to investigate specifically the interactions underlying fibril-fibril aggregation reaction without the need of de-convoluting the global aggregation profiles into different elementary steps.
Kinetic analysis is emerging as a powerful tool to investigate aggregation mechanisms and intermolecular interactions in protein based systems [70, 71, 197, 198], in analogy with more established chemical systems. Here, we monitor the aggregation kinetics under a broad range of buffer compositions by light scattering technique, and we rationalize the experimental data based on Smoluchowski population balance equations. This analysis allows estimating the intermolecular potential in terms of Fuchs stability ratio ($W$).

When normalized on an appropriate dimensionless time weighted on the Fuchs stability ratio, the aggregation profiles under a broad range of conditions collapse on a single master-curve, indicating that the solvent composition modifies the aggregation kinetics without affecting the aggregation mechanism. A similar behavior was observed for water dispersions of polymer nanoparticles [199-202].

It is found that the dependence of the stability ratio on the ionic strength can be described quantitatively in terms of charge screening effects in the frame of the DLVO theory, indicating that electrostatic interactions play a key role in the stability of the system. However, specific anion and cation effects are also observed. While the anion effect is mainly related to the ion binding to the fibril surface and the consequent modification of the surface charge, the cation effect is more complex and involves additional contributions from solvation forces.

4.2. Materials and methods

4.2.1. Material and sample preparation

The RADA 16-I peptide (Ac-R-A-D-A-R-A-D-A-R-A-D-A-R-A-D-A-CONH$_2$) was provided by Lonza Ltd (Visp, Switzerland) as lyophilized powder in the form of trifluoroacetic salt. The material was used as received without further purification. Before each experiment, the peptide was freshly dissolved in 10 mM HCl (Sigma-Aldrich GmbH, Steinheim, Germany) solution at pH 2.0 following a protocol described previously [56]. For the experiments in the presence of organic solvent, the peptide was re-dissolved in 10 mM HCl solution at pH 2.0 with different concentrations of ethanol (Scharlab S.L., Sentmenat, Spain) or isopropanol (Scharlab S.L., Sentmenat, Spain). Suitable amount of sodium chloride (Merck KGaA, Darmstadt, Germany), sodium nitrate (Sigma-Aldrich GmbH, Steinheim, Germany), sodium phosphate (Fluka Analytical, Sigma-Aldrich GmbH, Steinheim, Germany) and calcium chloride (Sigma-Aldrich
GmbH, Steinheim, Germany) were added after peptide re-dispersion. The pH value of the final solutions was checked by pH-Fix 0-14 color-fixed indicator sticks (Machery-Nagel GmbH & Co. KG, Düren, Germany).

4.2.2. Atomic force microscopy (AFM)

Samples at the peptide concentration of 1 g/L were diluted 200- and 300-fold in the case of fresh and aggregated samples, respectively, and spotted on a freshly cleaved mica surface for 30 seconds before washing with Milli-Q deionized water to remove unattached materials and gently dried under nitrogen flux. Samples were imaged at room temperature by an Asylum Cypher Scanning Probe Microscope (Asylum Research, an Oxford Instruments Company, Santa Barbara, CA, USA) operating in tapping mode. A scan rate of 4 Hz was applied, using High Resonance Frequency Silicon Cantilever with resonance frequency of 1600 kHz and tip radius of 7 nm (Olympus, Japan).

4.2.3. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) measurements were performed at a fixed angle of $\theta = 173^\circ$ using a Zetasizer Nano (Malvern, Worcestershire, United Kingdom) with laser beam of wavelength $\lambda_0 = 633$ nm. Micro UV-Cuvettes with dimension $12.5 \times 12.5 \times 45$ mm (70 $\mu$L) and light path 1 cm (Brand GmbH, Wertheim, Germany) were used.

4.2.4. Smoluchowski kinetic approach and DLVO-interaction potential

We described the experimental results in the frame of a Smoluchowski kinetic approach (Population Balance Equations - PBE). In the presence of the end-to-end fibril-fibril aggregation mechanism, the time evolution of the number concentration of fibrils containing $i$ monomeric units, $N_i$, can be expressed as:

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

(4.1)

where $k_{ij}$ is the rate constant of the aggregation reaction between fibrils containing $i$ and $j$ monomeric units, respectively. The first term on the right side of equation (4.1) describes the formation of fibrils by aggregation of shorter filaments, while the second term represents the disappearance of fibrils which aggregate with other filaments to form longer units. The aggregation rate constant is equal to:
where $k_{ij}^{\text{Diff}}$ is the rate constant in the diffusion-limited conditions, i.e., aggregation is dominated by Brownian diffusion motion, since each collision leads to an aggregation event, and $W$ is the Fuchs stability ratio, which describes the energetic barrier between two interacting fibrils.

The rate constant for diffusion-limited aggregation for two rigid cylindrical rods of length $L_i$ and $L_j$ can be expressed as follows [203, 204]:

\[
k_{ij}^{\text{Diff}} = \frac{k_B \cdot T}{3 \cdot \eta} \left( \frac{\delta \cdot \omega}{L_i + L_j} \right)^2 \left( \ln \left( \frac{L_i}{b} \right) + \frac{\ln \left( L_j / b \right)}{L_i} + \frac{\ln \left( L_j / b \right)}{L_j} \right)
\]

\[
v_i = 0.312 + 0.565 \left( \frac{L_i}{b} \right)^{-1} - 0.1 \left( \frac{L_i}{b} \right)^{-2}
\]

where $k_B$ is the Boltzmann constant, $T$ is the temperature, $\eta$ is the solvent viscosity, $L_i$ is the length of fibril containing $i$ monomer units (equal to $L_i = iL_m$, where $L_m$ the length of the single monomer unit), $b$ is the fibril diameter, $\delta$ and $\omega$ are geometrical parameters which represent, respectively, the minimum allowed distance and angle between two colliding fibrils in order to have an aggregation event. The factor $\delta \cdot \omega$ was set equal to $1.0 \times 10^{-9} \text{ cm rad}$, which is the value reported for $\beta$-amyloid peptide filaments [204], and was assumed to be independent of solvent, salt type and salt concentration.

The Fuchs stability ratio, $W$, describes in a coarse-grained manner the sum of the intermolecular forces between two aggregating units, according to the relationship [205]:

\[
W = 2 \cdot b \cdot \int_{2b}^{\infty} \exp \left( \frac{V_{\text{tot}}}{k_B \cdot T} \right) \frac{dr}{r^2}
\]

where $V_{\text{tot}}$ is the total interaction potential between two interacting fibrils and $r$ is the distance between their centers.

Equations (4.2) to (4.4) represent the key to correlate the measured aggregation rate under a large variety of conditions to the interaction energy driving the colloidal stability of the fibril dispersion. In colloidal science, the interaction potential ($V_{\text{tot}}$) is commonly evaluated in the context of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which accounts for the presence of a repulsive electrostatic potential ($V_R$) and attractive van der Waals forces ($V_A$) [202]:

\[
V_{\text{tot}} = V_R + V_A
\]
Considering the nanofibrils as interacting crossed cylinders, the repulsive potential can be expressed as [191]:

$$V_R = 128 \cdot \pi \cdot I_{salt} \cdot N_A \cdot k_B \cdot T \cdot \gamma^2 \cdot \frac{b}{\kappa^2} \cdot \exp\left(-\kappa \cdot H_0\right)$$  \hspace{1cm} (4.6)

where $I_{salt}$ is the solution ionic strength, $H_0$ is the distance between the surfaces of the interacting fibrils, $\kappa$ is the inverse Debye length and $\gamma$ is equal to:

$$\gamma = \tanh\left(\frac{e \cdot \Psi_0}{4 \cdot k_B \cdot T}\right)$$  \hspace{1cm} (4.7)

with $e$ the elementary charge and $\Psi_0$ the surface potential. The inverse Debye length is given by:

$$\kappa = \sqrt{8 \cdot \pi \cdot I_{salt} \cdot N_A \cdot Q}$$  \hspace{1cm} (4.8)

where $Q$ is the Bjerrum length (equal to 0.7 nm in aqueous solution at room temperature [206]), defined as:

$$Q = \frac{e^2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon_r \cdot k_B \cdot T}$$  \hspace{1cm} (4.9)

where $\varepsilon_0$ is the vacuum dielectric permeability and $\varepsilon_r$ is the permittivity of the solvent.

The attractive van der Waals interactions can be expressed as [207, 208]:

$$V_A = -\frac{A_H \cdot \sqrt{b^2}}{6 \cdot H_0}$$  \hspace{1cm} (4.10)

where $A_H$ is the Hamaker constant.

We note that the calculation of the electrostatic contribution in the DLVO interaction potential assumes that the RADA 16-I fibrils interact with each other as crossed cylinders of infinite length. This approximation eliminates the dependence of the potential on the fibril length. This interaction is likely to represent the rate-limiting step of the series of events involved in the end-to-end fibril-fibril aggregation process. Indeed, after the fibrils have approached each other through this interaction, the balance between the electrostatic repulsion and the attraction arising from the hydrophobic patches would lead to the geometric alignment of the fibrils. This local rotational diffusion promotes the end-to-end self-assembly, since two free ends of fibrils of the non-homogenous length could now aggregate via the sliding diffusion mechanism proposed previously for the RADA 16-I system [28].
4.3. Results and discussion

4.3.1. Aggregation mechanism and role of the electrolyte concentration

We start our analysis by investigating the aggregation kinetics of RADA 16-I peptide at 1 g/L in the presence of sodium chloride at concentrations ranging from 25 to 175 mM at pH 2.0. In Figure 4.1a we show a representative AFM picture of freshly prepared peptide solution containing 25 mM salt. We identified the presence of short, rigid nanofibrils with average length of 100 nm. The stability of the fibril dispersion was monitored at 25 °C in a non-invasive manner by dynamic light scattering. As shown in Figure 4.1b, the increase of the average hydrodynamic radius observed within the first few hours of incubation indicates the presence of aggregation leading to the formation of larger structures. The aggregation rate is accelerated by increasing the salt concentration because of the screening of the positive net charges on the surface resulting in the decrease of the repulsive electrostatic barrier. Interestingly, after the fast initial increase of the average size, the hydrodynamic radius reaches a plateau value of about 75 nm, which is rather independent of the salt concentration (Figure 4.1c).

In parallel with DLS, the self-assembly was followed by atomic force microscopy. In the AFM pictures of samples incubated for 3 weeks in the presence of different concentrations of NaCl, we observed the presence of longer fibrils (Figure 4.1d), which elongate without changing their diameter (this result is supported by TEM analysis, the representative TEM pictures are shown in Figure C.1 in Appendix C). The length distributions of the aggregated samples are independent of the salt concentration (Figure 4.1e). Similarly to the situation observed in the absence of salt [56], size exclusion chromatography analysis (Figure C.2 in Appendix C) and dilution experiments (data not shown) confirm that the monomeric peptide does not participate into the aggregation process and that the fibril self-assembly is irreversible.

Overall, these results indicate that the salt concentration modifies the aggregation kinetics without affecting the aggregation behavior observed in the absence of salt [56]. Namely, the fibrils follow an irreversible end-to-end fibril-fibril aggregation mechanism, which generates longer fibrils with constant diameter according likely to the sliding diffusion mechanism [28]. This mechanism has been observed also at physiological conditions [8], and has been attributed to the presence of unpaired hydrophobic patches at the ends of the fibrils, which are highly reactive in aqueous environments [8].
4. A colloidal description of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic peptide

Figure 4.1. The NaCl effect on stability of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. a) A representative AFM picture of a freshly prepared sample at 1 g/L in 10 mM HCl with 25 mM NaCl at pH 2.0; b-c) The time evolution of the average hydrodynamic radius during (b) the first hours and (c) 3 weeks of incubation in the presence of 25 mM (▲), 50 mM (■), 75 mM (▲), 90 mM (●), 100 mM (★), 110 mM (▼), 125 mM (★), 150 mM (►) and 175 mM (x) NaCl; d) AFM picture of the peptide incubated for 3 weeks in 10 mM HCl solution with 25 mM NaCl at pH 2.0; e) fibril length distribution obtained from AFM pictures at time 0 (light blue bars) and after 3 weeks of incubation in the presence of 25 mM (dark blue bars), 50 mM (red bars), 75 mM (green bars) and 100 mM (violet bars) NaCl; f) The plateau value of the average hydrodynamic radius at long incubation time does not increase upon addition of a freshly prepared population of short reactive fibrils (same conditions as in (a) but with 100 mM NaCl).
Since the number of reactive patches is limited, the aggregation process arrests once the unpaired hydrophobic sites have all reacted, thereby explaining the plateau in the average size observed after several hours of incubation (Figure 4.1c). To prove this hypothesis, we added a sample of freshly prepared short reactive fibrils to an equal amount of longer fibrils that have already aggregated and reached the plateau value in the average radius, and we monitored their stability by DLS (Figure 4.1f). After an initial decrease in the size due to the addition of the short fibrils, the average hydrodynamic radius of the mixture increases during time, indicating that the added short fibrils aggregate into longer fibrils. After few hours, the average radius reaches the same plateau value of the original aggregated sample, suggesting that the aggregation occurs only among the freshly added fibrils and the aggregated ones remain inert. This result proves that the aggregation process is limited to a certain extent, which is likely fixed by the initial number of reactive unpaired patches.

To provide a quantitative picture of the aggregation behavior described in the previous paragraphs, we model the experimental data by using Smoluchowski population balance equations (see Material and Methods, equations (4.1)-(4.3)). In this kinetic model approach, we simulate the time evolution of the complete fibril length distribution obtained from AFM analysis and we compare the calculated values of the average hydrodynamic radius with the experimental results obtained by DLS. For polydispersed systems, the average hydrodynamic radius can be evaluated by using the Stokes-Einstein equation:

\[
\langle R_h \rangle = \frac{k_B \cdot T}{6 \cdot \pi \cdot \eta \cdot \langle D \rangle_z(q)}
\]  

(4.11)

where \( \langle D \rangle_z(q) \) is the z-averaged coefficient calculated from the length distribution of the single fibril [209]:

\[
\langle D \rangle_z(q) = \frac{\sum_{i=1}^{N} N_i \cdot D_i \cdot P_i(q) \cdot L_i^2}{\sum_{i=1}^{N} N_i \cdot P_i(q) \cdot L_i^3}
\]  

(4.12)

with \( D_i \) being the translational diffusion coefficient for the rigid rods calculated accordingly to Doi-Edwards relationship [210]:
A colloidal description of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic peptide

\[ D_i = \frac{k_B \cdot T}{3 \cdot \pi \cdot \eta \cdot L_i} \cdot \ln \left( \frac{L_i}{b} \right) \]  

(4.13)

and \( P_i(q) \) being the form factor of a rigid rod with length \( L_i \) at the scattering vector \( q \) \((q = 4\pi n / \lambda_0 \sin(\theta/2))\), with \( n \) the refractive index of the solvent, \( \lambda_0 \) the wavelength of the laser beam and \( \theta \) the scattering angle) expressed as [211]:

\[ P_i(q) = \frac{2}{q \cdot L_i} \cdot \int_0^{qL_i} \frac{\sin(x)}{x} \cdot dx - \left[ \frac{2}{q \cdot L_i} \cdot \sin \left( \frac{q \cdot L_i}{2} \right) \right]^2. \]  

(4.14)

For each single aggregation condition, the only fitting parameter considered in the model simulations is the Fuchs stability ratio (\( W \)), which contains information on the intermolecular interaction potential as described by equation (4.4). In Figure 4.2a it can be seen that the simulated curves are in good agreement with the experimental data measured in the presence of different concentrations of salt, in particular when the fibril length is relatively short. However, for longer fibril lengths, the simulations deviate from the experimental data, most likely because of the formation of fibril clusters and the increase in fibril flexibility, bending and curvature. These effects are not included in the expressions used in the kinetic model (equation (4.3)), which apply only for a rigid cylindrical shape.
Figure 4.2. The stability of RADA 16-I at 1 g/L in 10 mM HCl at pH 2.0 in the presence of NaCl. a-b) The evolution of the average hydrodynamic radius versus (a) absolute time and (b) dimensionless time normalized in terms of the characteristic aggregation time for 1 g/L RADA 16-I solutions in 10 mM HCl at pH 2.0 containing 25 mM (♦), 50 mM (○), 75 mM (▲), 90 mM (○), 100 mM (♦), 110 mM (▽), 125 mM (★), 150 mM (▸) and 175 mM (x) NaCl. The continuous lines in (a) and (b) correspond to model simulations according to the Smoluchowski kinetic approach (PBE). c) The experimental values of the Fuchs stability ratio as a function of the ionic strength are compared to model calculations based on DLVO theory.
4. A colloidal description of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic peptide

It is convenient to normalize the absolute aggregation time \(t\) measured under different conditions in terms of a characteristic aggregation time, \(t_c\), weighted on the Fuchs stability ratio as follows:

\[
\frac{1}{t_c} = \frac{\beta \cdot N_{1,0}}{8 \cdot W}
\]  

(4.15)

where \(N_{1,0}\) is the initial number concentration of peptide nanofibrils and \(\beta\) is the Smoluchowski rate constant for diffusion limited aggregation defined as:

\[
\beta = \frac{8 \cdot k_B \cdot T}{3 \cdot \eta}.
\]  

(4.16)

By introducing the so defined dimensionless time, \(\tau = t / t_c\), the PBE shown in equation (4.1) reduces to:

\[
\frac{dX_i}{d\tau} = \frac{1}{2} \sum_{j=1}^{i-1} B_{i-j} \cdot X_j \cdot X_{i-j} - \sum_{j=1}^{i} B_{ij} \cdot X_j \cdot X_i
\]  

(4.17)

where \(X_i\) is the dimensionless particle number concentration equal to \(X_i = N_i / N_{1,0}\) and \(B_{ij}\) is the dimensionless aggregation kernel defined as:

\[
B_{ij} = \frac{(\sigma \cdot \omega / b)^2}{(i + j) \cdot \alpha} \left( \frac{\ln(i \cdot \alpha) + v_i}{i \cdot \alpha} + \frac{\ln(j \cdot \alpha) + v_j}{j \cdot \alpha} \right)
\]  

(4.18)

where \(\alpha\) is the ratio of the monomer length to the fibril diameter equal to \(\alpha = L_m / b\).

It is seen that since the parameters \(\alpha\) and \((\sigma \omega / b)\) appearing in equation (4.18) are geometric parameters that are intrinsic of the fibril structure and independent of external factors, the solution of equations (4.17) and (4.18) leads to a master curve that is the same for all buffer compositions.

In addition, we can express the average aggregate size in the dimensionless form as a function of \(X_i\) by dividing the measured average hydrodynamic radius, \(<R_h>\), by a reference value. We selected this reference value, defined in following as \(R_{h0}\), as the radius \(<R_h>\) measured at time zero at the lowest salt concentration.

As shown in Figure 4.2b, we see that the curves at different ionic strengths collapse on a single master curve, indicating that the salt concentration modulates the aggregation kinetics without affecting the aggregation mechanism.

The dependence of the Fuchs stability ratio values estimated from the fittings of the experimental data in Figure 4.2a on the ionic strength (Figure 4.2c) contains crucial information about the intermolecular interactions involved in the fibril-fibril association
process. It is seen that the $W$ values decrease with increasing the ionic strength, approaching the value of 1 at the largest salt concentrations, indicating that at this point no energy barrier is present and aggregation occurs under diffusion-limited conditions. We attempt to model this behavior in the frame of the DLVO theory (equations (4.4) to (4.10)), which describes the colloidal stability of the system in terms of a van der Waals attractive potential and an electrostatic repulsive potential (see Material and Methods). The Hamaker constant, $A_H$, which for proteins is considered as poorly dependent on the amino acid sequence [212], has been set equal to $10.5k_BT$, a value reported for actin fibrils [213]. The only remaining unknown parameter, the surface potential, $\Psi_0$, has been estimated by comparison with experimental $W$ value at the reference ionic strength of 50 mM. It is worth noting that, while keeping fixed this parameter, we can describe well the dependence of the $W$ value on the salt concentration in the entire investigated range of ionic strength values, as shown in Figure 4.2c. Therefore, the dependence of the aggregation rate on the ionic strength predicted by the DLVO theory in the context of charge screening effects (equations (4.5)-(4.10)) is able to describe quantitatively the observed dependence of the aggregation kinetics on the salt concentration, indicating that electrostatic interactions play a key role in the aggregation of the RADA 16-I fibrils.

It should be mentioned that the application of DLVO theory to monomeric protein systems has been only partially successful and has been limited to small globular proteins [214, 215], since local intermolecular interactions related to the heterogeneous surface and the presence of specific aggregation-prone spots cannot be described in the frame of this coarse-grained approach. In contrast, the results of this work may indicate that for protein aggregates, where specific effects are averaged and the surface is more homogeneous, the DLVO theory can describe the stability behavior in a satisfactory manner.
4.3.2. Effect of the anion type

To investigate more in detail the role of electrostatic interactions on the stability of RADA 16-I fibril dispersions at low pH, we repeated the kinetic analysis discussed in the previous section in the presence of different types of anions and cations. First of all, we compare the time evolution of the average radius of fibril dispersions in the presence of sodium chloride, sodium nitrate and sodium phosphate at four different concentrations as shown in Figure 4.3a-d. For all anions, the aggregation rate increases with salt concentration (see Figure C.3 in Appendix C). In the ionic strength range 25-75 mM the kinetics depends on the type of anion according to the series: Cl$^-$ (less reactive) < H$_2$PO$_4^-$ < NO$_3^-$ (more reactive), whereas the difference between the different ions flattens at the concentration of 100 mM.

Following the same procedure described above, we apply the population balance equation platform to extrapolate the dependence of the Fuchs stability values on the salt concentration for the different considered anions. As shown in Figure 4.3a-d, the simulated curves reproduce the experimental data reasonably well. Moreover, also in the presence of different anions, when the absolute times are normalized with respect to the dimensionless time $\tau$, the aggregation curves collapse on a single master curve shown in Figure 4.3e, indicating that the type of anion influences the aggregation kinetic without affecting the self-assembly mechanism.

However, the dependence of the aggregation kinetics on the type of anion indicates that the charge screening is not the only electrostatic effect responsible for the fibril stability. Indeed, in the frame of the DLVO theory, the only key physicochemical parameter related to the presence of the salt is the total ionic strength (equations (4.5)-(4.10)), which is independent of the type of monovalent anion. However, an additional electrostatic effect that is expected at low pH values is the specific anion binding to the positively charged groups exposed on the surface of the fibrils. We described this effect by modifying the surface potential, $\Psi_0$, for the different anions in the simulations of the $W$ values as a function of the salt concentration (see Table 4.1). In Figure 4.3f it can be seen that the modification of the surface potential for the different anions is able to account alone for the dependence of the aggregation rate on the ionic strength for the different types of anion. From the estimated values of the surface potentials, the overall propensity of anions to bind to the RADA 16-I fibrils follows the series: Cl$^-$ > H$_2$PO$_4^-$ > NO$_3^-$.
note that this series is in disagreement with both electro-selectivity series [91, 216], which ranks the propensity of different anions to bind to the surface charge groups:

\[ \text{H}_2\text{PO}^- < \text{Cl}^- < \text{NO}_3^- < \text{ClO}_4^- < \text{SO}_4^{2-}, \]

and the ranking of anions to bind to polar and apolar groups as well as to the peptide bonds, which follows the order:

\[ \text{HSO}^- = \text{SO}_4^{2-} < \text{HPO}_2^- < \text{Cl}^- < \text{NO}_3^- < \text{ClO}_4^- . \]

These results indicate that, once the modification of the surface chemistry of the fibrils related to the specific anion binding is taken into account by changing the surface potential, the coarse-grained approach of the DLVO theory allows to describe quantitatively well the screening effect of electrostatic repulsions induced by the increase of salt concentration.

<table>
<thead>
<tr>
<th>Salt</th>
<th>( \Psi_o ) [mV]</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>54</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>48</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>51</td>
</tr>
</tbody>
</table>

*Table 4.1.* Values of the surface potential, \( \Psi_o \), used in the model calculations of the repulsive interaction potential in the presence of different salts according to equations (4.6) and (4.7).
Figure 4.3. The anion effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. a-d) Time evolution of the average hydrodynamic radius in the presence of NaCl (black, ●), NaNO₃ (blue, ▲) and NaH₂PO₄ (red, ▲) at ionic strength equal to (a) 25 mM, (b) 50 mM, (c) 75 mM and (d) 100 mM. e) Time evolution of the average hydrodynamic radius versus dimensionless time normalized with respect to the characteristic aggregation time of 1 g/L RADA 16-I in 10 mM HCl at pH 2.0 containing NaCl (black), NaNO₃ (blue) and NaH₂PO₄ (red) at ionic strength equal to 25 mM (●), 50 mM (○), 75 mM (▲), 90 mM (◆), 100 mM (★), 110 mM (△), 125 mM (■), 150 mM (▲) and 175 mM (x). The continuous lines in (a)-(e) represent model simulations according to the Smoluchowski kinetic approach (PBE). f) The experimental values of the Fuchs stability ratio as a function of the ionic strength in the presence of NaCl (●, continuous line), NaNO₃ (■, dash-dotted line) and NaH₂PO₄ (▲, dashed line) are compared to model calculations based on DLVO theory.
4.3.3. Effect of the cation type

The net charge of the peptide at pH 2.0 is equal to +4.4, and, therefore, positive charges dominate on the fibril surface. However, negative charges are present in the amount of -0.56 per monomeric unit of peptide and therefore effects of cations cannot be excluded. We investigated the cation effect on the aggregation propensity by comparing aggregation reactions monitored in the presence of calcium chloride with the results shown previously in the context of Figure 4.3a-d for sodium chloride. The comparison has been performed keeping constant either the ionic strength (concentrations of CaCl$_2$ equal to 8.3, 16.6, 25 or 33.3 mM) or the number of chloride anions (concentrations of CaCl$_2$ equal to 12.5, 25, 37.5 and 50 mM) (Figure 4.4a-d). In analogy with the sodium chloride salt, increasing the concentration of the calcium salt enhances the aggregation rate (see Figure C.4 in Appendix C). When compared at constant ionic strength, the aggregation rates in the presence of sodium and calcium chloride are similar at 25 and 50 mM salt, while at larger salt concentrations (75 and 100 mM) the kinetics are faster in the presence of sodium chloride with respect to calcium chloride. When compared at a fixed number of chloride anions, the aggregation rate is faster for calcium chloride with respect to sodium chloride at the lowest considered concentration (25 mM), and slower in the concentration range from 50 to 100 mM.

In analogy with the previous situations, we evaluated the Fuchs stability ratio as a function of the ionic strength from the population balance equation model analysis (equations (4.1)-(4.3)). The PBE simulations describe the experimental data reasonably well (Figure 4.4a-d). Also in this case, regardless of the cation type and concentration, all of the aggregation curves follow a master curve when normalized in terms of the characteristic aggregation time (Figure 4.4e), suggesting that the salt modulates the kinetics, but not the mechanism of the self-assembly.
Figure 4.4. The cation effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. a-d) The effect of NaCl (black, ●) compared to the effect of CaCl₂ at equal ionic strength (blue, ▲), and at equal concentration of Cl⁻ ions (red, ♦). The panels report the time evolution of the average hydrodynamic radius at the reference NaCl concentrations of (a) 25 mM, (b) 50 mM, (c) 75 mM and (d) 100 mM. e) Time evolution of the average hydrodynamic radius versus dimensionless time normalized with respect to the characteristic aggregation time of 1 g/L RADA 16-I in 10 mM HCl at pH 2.0 containing 25 mM (●), 50 mM (○), 75 mM (▲), 90 mM (◇), 100 mM (♦), 110 mM (▼), 125 mM (▲), 150 mM (►) and 175 mM (x) NaCl (black), CaCl₂ at equal ionic strength (blue) and CaCl₂ at equal concentration of Cl⁻ ions (red). The continuous lines in (a)-(e) represent model simulations according to the Smoluchowski kinetic approach (PBE). f) The experimental values of the Fuchs stability ratio as a function of the ionic strength in the presence of NaCl (●, continuous line), of CaCl₂ at equal ionic strength (▲, dashed line) and CaCl₂ at equal concentration of Cl⁻ ions (♦, dotted line) are compared to model calculations based on DLVO theory.
4. A colloidal description of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic peptide

Overall, the results indicate that calcium ions have a stabilizing effect with respect to sodium ions. However, the rationalization of the dependence of the $W$ values as a function of the ionic strength is more challenging with respect to the situations described in the previous paragraphs. In fact, a non-monotonic dependence of the $W$ values as a function of the salt concentration is observed, which cannot be clearly explained in the frame of DLVO theory only. As shown in Figure 4.4f, at high ionic strength an increase in the aggregation stability is observed with increasing the salt concentration. This complex behavior can result from the combination of a series of microscopic effects including anion and cation binding, which are more pronounced at larger salt concentrations. While anion binding decreases the surface potential and promotes aggregation, cation binding stabilizes the fibrils not only by increasing the net charge but also by promoting stabilizing solvation forces [82].

4.3.4. Effect of the solvent composition

Finally, we modulated the intermolecular interactions driving the colloidal stability of the fibril dispersion by changing the solvent composition. In particular, we monitored the aggregation kinetics of 1 g/L RADA 16-I peptide in 10 mM HCl at pH 2.0 with 25, 50, 75 or 100 mM sodium chloride in the presence of 10% isopropanol or 10 and 20% ethanol (see also Figure C.5 in Appendix C). While the addition of organic solvent enhances the aggregation kinetics in a concentration dependent manner, a negligible difference is detected between ethanol and isopropanol at the same concentration (Figure 4.5a-d). The experimental data can be well described in the frame of the Smoluchowski kinetic approach indicating that self-assembly occurs via the same mechanism discussed above also in the presence of organic solvents (Figure 4.5a-b and Figure 4.5e).

The introduction of organic solvents characterized by lower dielectric constant affects the electrostatic repulsion forces. In particular, the decrease in the dielectric constant results in the increase of the Debye length (equation (4.8) and (4.9)), which in turn decreases the repulsive electrostatic interactions (equation (4.6)). This effect explains the acceleration in the aggregation kinetics observed experimentally with increasing the concentration of organic solvent (Figure 4.5a-d). In Figure 4.5f we show the dependence of the $W$ values as a function of the ionic strength, evaluated according to the same procedure described in the previous paragraphs. In Table 4.2 we summarize the values of the dielectric constants and the surface potentials used in the model simulations for
different solvents. The experimental results are in a good agreement with the theoretical calculations, indicating that the change in the dielectric properties of the solvent can explain reasonably well the observed decrease in the repulsive barrier and the resulting acceleration of the aggregation kinetics with increasing the concentration of organic solvent. This result confirms that electrostatic repulsions play a key role in the colloidal stability of the nanofibril dispersions.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\varepsilon_r$</th>
<th>$\Psi_o$ [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM HCl</td>
<td>78.56</td>
<td>54</td>
</tr>
<tr>
<td>10mM HCl + 10% isopropanol</td>
<td>72.73 [217]</td>
<td>50</td>
</tr>
<tr>
<td>10mM HCl + 10% ethanol</td>
<td>73.95 [218]</td>
<td>51</td>
</tr>
<tr>
<td>10mM HCl + 20% ethanol</td>
<td>69.05 [218]</td>
<td>51</td>
</tr>
</tbody>
</table>

Table 4.2. Values of dielectric constant, $\varepsilon_r$, and surface potential, $\Psi_o$, used in the model calculations of the repulsive interaction potential in the presence of different salts according to equations (4.6)-(4.9).
Figure 4.5. The solvent effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. a-d) Time evolution of the hydrodynamic radius in the presence of 10 mM HCl without organic solvent (black, ●) and with 10% isopropanol (blue, □), 10% ethanol (red, ●) and 20% ethanol (green, ▲) containing (a) 25 mM, (b) 50 mM, (c) 75 mM and (d) 100 mM NaCl. e) Time evolution of the average hydrodynamic radius versus dimensionless time normalized with respect to the characteristic aggregation time of 1 g/L RADA 16-I solution in 10 mM HCl at pH 2.0 (black), containing 10% isopropanol (blue), 10% ethanol (red) and 20% ethanol (green) at ionic strength of 25 mM (●), 50 mM (□), 75 mM (▲), 90 mM (○), 100 mM (●), 110 mM (▲), 125 mM (●), 150 mM (▲) and 175 mM (x). The continuous lines in (a)-(e) represent model simulations according to the Smoluchowski kinetic approach (PBE). f) The experimental values of the Fuchs stability ratio as a function of the ionic strength for 10 mM HCl without organic solvent (●, continuous line) and with 10% isopropanol (□, dash-dotted line), 10% ethanol (●, dashed line) and 20% ethanol (▲, dotted line) are compared to model calculations based on DLVO theory.
4.4. Conclusions

We applied a kinetic approach to investigate the intermolecular interactions driving the self-assembly of charged fibrils composed of the model amphiphilic peptide RADA 16-I. We modulated the intermolecular forces by changing the solvent composition in terms of salt concentration, salt type and concentration of organic solvent. The experimental data were rationalized by a Smoluchowski kinetic model based on population balance equations, which allows to express the intermolecular interactions in a mean-field approach in terms of the Fuchs stability ratio (\( W \)). It is remarkable that all of the aggregation profiles normalized with respect to a characteristic aggregation time weighted on the Fuchs stability ratio collapse on a single curve. That is all data corresponding to different buffer compositions, when considering a suitable dimensionless time fall on the same curve, which is therefore referred to as the master curve since it reflects the behavior of this particular peptide in many different conditions. This observation suggests that the buffer composition affects the aggregation kinetics without modifying the self-assembly mechanism.

In conclusion, we have seen that the aggregation is not controlled purely by diffusion, but it is rather an activated process characterized by an activation energy barrier that is largely dominated by electrostatic interactions. Increasing the ionic strength or the concentration of organic solvent characterized by lower dielectric constants decreases the aggregation energy barrier because of the screening of the charges present on the peptide surface. Remarkably, both effects can be quantitatively described in the frame of DLVO theory. However, specific ion effects are also observed. Whereas the specific effect of the anion type can be explained mainly by the different binding affinity to the positively charged peptide surface, the cation effects are more complex and likely related to additional solvation forces.
Chapter 5

Sol-gel transition of charged fibrils composed of a model amphiphilic peptide

5.1. Introduction

The phase diagram of fibrils composed of peptides and proteins is involved in a large variety of problems in biology, biophysics and biochemistry: examples include the behavior of actin filaments, which are key components of the cytoskeleton [219-222], and of whey protein aggregates in the food industry [4, 223-226]. Despite the phase behavior of rod-like colloids has been widely investigated in the literature [189-192], the study of the phase diagram of protein solutions is often complicated by the presence of irreversible aggregation and metastable phases [4]. As a consequence, a rich and dynamic phase behavior is often observed, with formation of liquid-crystalline phase, gels and phase separation [65, 131, 227-229].

One example of the importance of controlling the stability of protein aggregates is found in the biotechnology context, where protein-based drugs and functional materials are increasingly developed [27, 230-232]. For instance, thanks to several advantages such as biocompatibility and degradability, hydrogels obtained from synthetic peptides have received large attention in fields ranging from 3D cell cultures [128] to drug release [38] and delivery [37, 39], as well as tissue engineering and tissue repair [34]. During the industrial processing of the material, protein solutions are exposed to several stresses, such as shear forces and incubation at low pH, which can potentially induce the degradation of the product. Obviously, the control of the aggregation state of peptides and proteins during their manufacturing is a prerequisite for the successful application of the material [55, 64].

The stability of protein fibril dispersions is involved also in biological systems, for instance in the aggregation of amyloidogenic peptides and proteins involved in several neurodegenerative disorders [1]. Amyloid fibrils associate in large bundles and plaques,

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4 The part of the material presented in this chapter has been published as: M. Owczarz, S. Bolisetty, R. Mezzenga, P. Arosio, Sol-gel transition of charged fibrils composed of a model amphiphilic peptide, *Journal of Colloid and Interface Science*, 437 (2015) 244-251
which are eventually observed in the tissues of the patients. Lateral association of protofilaments in multistranded fibrils and laminate structures has also been observed with both globular proteins [188] and synthetic peptides [233]. However, the understanding of the interactions between fibrils and of the phase diagram of amyloid fibril dispersions is still limited.

In this chapter, we investigate the stability behavior of positively charged fibrils composed of the RADARADARADARADA (RADA 16-I) peptide [8] as a model. RADA 16-I is a well-known example of the family of self-complementary peptides which have been designed to self-assemble in a controlled way into fibrils and higher ordered structures depending on the pH value [28, 171, 172]. Ionic-complementary peptides are typically 8-16 amino acid long with structures displaying hydrophobic residues on one side of the polypeptide chain and hydrophilic residues on the other side [6, 27, 234, 235]. The self-assembly process is driven both by the hydrophobic double sheet formation inside the fibril and the electrostatic interactions between charged residues on the hydrophilic side [6]. The inter- and intramolecular forces determining the secondary structure and the aggregate state of the peptides can be finely tuned by a series of intrinsic properties (e.g., amino acid periodicity and charge distribution) and environmental conditions, such as pH, salt and ionic strength [9, 27, 236].

Here a combination of microscopy, light scattering and rheology techniques is applied to characterize the gelation process of RADA 16-I fibril dispersions and the morphology of the resulting hydrogel at low pH. The fibril dispersions formed by RADA 16-I in water solutions at low peptide concentration under acidic conditions has been described elsewhere [56]. In this work, it is found that the fibrils aggregate irreversibly into longer fibrils and fibrillar aggregates which at sufficiently large volume fractions form a reversible gel network which can be destroyed upon dilution. The phase transition occurs directly from the isotropic liquid to the gel phase without the formation of a nematic phase at a critical peptide concentration which decreases with increasing the salt concentration. In this chapter it is also presented that the dependence of the critical percolation concentration on the ionic strength can be well rationalized in the frame of the fractal gel theory.
5. Materials and methods

5.2. Material and sample preparation

The RADA 16-I (Ac-R-A-D-A-R-A-D-A-R-A-D-A-R-A-D-A-CONH₂) peptide was provided by Lonza Ltd (Visp, Switzerland) as lyophilized powder in the form of trifluoroacetic or chloride salt. The material was used as received without further purification. Before each experiment the peptide was freshly dissolved in a suitable amount of solution. The concentration was checked by UV absorbance after mild stirring for 10 minutes for homogenization, and the pH value of the final solution was measured by a SevenEasy pH meter (Mettler Toledo). The results shown in this work refer to the peptide in the trifluoroacetic salt form. It is worth noting that very similar results have been obtained with peptides originating from three different production batches and with the peptide in the form of chloride salt [56].

5.2.2. Atomic force microscopy (AFM)

10 µL of RADA 16-I samples diluted 200-fold were deposited 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 an Asylum Cypher Scanning Probe Microscope (Asylum Research, Santa Barbara, CA, USA) operating in tapping mode. Scan rate of 1 Hz and antimony doped silicon cantilevers with resonance frequency in the range 300-360 kHz and tip radius of 8 nm (Veeco, Plainview, NY, USA) were used.

5.2.3. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) measurements at a fixed angle ranging from 20° to 90° were performed in situ using a goniometer BI-200SM (Brookhaven Instruments, Holtsville, NY, USA) equipped with a solid-state laser, Ventus LP532 (Laser Quantum, Manchester, UK) with a wavelength λ₀ = 532 nm as the light source. The average hydrodynamic radius of the sample was calculated from the measured diffusion coefficient based on the Stokes-Einstein equation. DLS was also measured at a fixed angle of θ = 173° using a Zetasizer Nano (Malvern, Worcestershire, UK) with laser beam of wavelength λ₀ = 633 nm.
5.2.4. **Diffusing wave spectroscopy (DWS)**

Diffusing wave spectroscopy measurements were done using a LS Instrument (Fribourg, Switzerland). Standard reference polystyrene particles with nominal hydrodynamic radius of 190 nm were mixed with the peptide solution at particles concentration of 2 g/L. Glass cuvettes with 0.2 cm light path were used (Hellma, Müllheim, Germany).

5.2.5. **Rheology**

Rheology experiments were performed using an ARES Rheometer (Rheometric Scientific, Piscataway, NY, USA) working under oscillatory, strain-controlled mode equipped with 25 mm cone-plane geometry. The gel was gently introduced between the cone and the plate and 10 minutes of re-equilibration were allowed before starting the measurements. Before measuring the frequency-dependent loss and storage modulus, the strain-dependent moduli were measured in a strain amplitude range from 0.1 to 200% at constant frequency of 200 rad/s in order to determine the linear viscoelastic region. Frequency-dependent measurements were performed at constant strain amplitude values ranging from 30 to 40% and within a frequency range of 0.05-500 rad/s. All measurements were performed at 25 °C at five different RADA 16-I concentrations (4.5, 5, 7, 9 and 10 g/L) dissolved in 10 mM HCl with 50 mM NaCl at pH 2.0.

5.2.6. **Phase diagram**

The phase diagram of RADA 16-I fibril dispersions at 25 °C was investigated as a function of the fibril concentration in the range 0.4-6.5 g/L and as a function of NaCl concentration in the range 0-200 mM. The possible presence of nematic phase was checked by observation under cross-polarized light using an optical microscope Zeiss Axioskop 2 (Carl Zeiss Jena GmbH, Jena, Germany) as described in Jung et al. [206]. The concentration of the RADA 16-I fibrils in the dispersion has been evaluated by subtracting from the total weighted peptide concentration the soluble monomer fraction, which has been quantified by size exclusion chromatography as described elsewhere [56].
5.3. Results and discussion

5.3.1. Kinetics and mechanism of gel formation

Before starting the stability studies, the aggregate state of the peptide at concentration of 10 g/L in 10 mM HCl solutions at pH 2.0 was analyzed. After re-dispersion of the peptide powder, a transparent solution is observed. Atomic force microscopy pictures of samples taken few minutes after re-dispersion show the presence of a rather homogeneous population of fibrils with an average length of about 100 nm (Figure 5.1a). In a recent work, Arosio et al. have shown that these fibrils contain β-sheet structures by FTIR and CD assays [56], and that AFM images are consistent with TEM pictures [56].

![Image](image_url)

Figure 5.1. Sol-gel transition of RADA 16-I nanofibril dispersion at 10 g/L in 10 mM HCl at pH 2.0. a) AFM image of a freshly prepared RADA 16-I fibril dispersion. A picture of the transparent solution is shown in the insert. b) Kinetics of gel formation monitored by following the time evolution of the average hydrodynamic radius measured by dynamic light scattering. The DLS signal has been recorded using a Zetasizer Nano (●) and a Brookhaven (□) instrument. c) Dynamic light scattering correlation function of the sample at time 0 (dashed line) and after 3 days (continuous line). d) Picture of the self-sustaining RADA 16-I fibrillar hydrogel after 3 days of incubation.
The kinetic stability of the fibril dispersion was followed in situ by dynamic light scattering (DLS) with both the Malvern Zetasizer Nano and the Brookhaven instrument by monitoring the relative changes in the average size with respect to the initial condition. As it can be seen in Figure 5.1b the results obtained with the two instruments are very similar. In addition, it was verified that the time evolution of the average $<R_h>$ relative to the initial value is independent of the scattering angle (Figure D.1 in Appendix D).

As shown in Figure 5.1b, the fibril dispersion is not stable during time. The time evolution of the average hydrodynamic radius follows two different regimes: initially, the average size increases almost linearly, indicating fibril growth and aggregation. After about 2 hours of incubation, the growth of the average size arrests and the average hydrodynamic radius oscillates during time around the plateau value of 60-80 nm. The corresponding intensity autocorrelation functions exhibit a shift of the baseline (Figure 5.1c), which is observed when the time average of the light scattering intensity oscillations differs from the ensemble average, indicating the presence of a non-ergodic phase. Therefore, the results indicate that after a first aggregation step the fibrillar aggregates are not able to diffuse freely and occupy all the available volume. The formation of a gel phase has been confirmed by macroscopic observation (Figure 5.1d).

In parallel with DLS, the kinetics of fibril aggregation and gel formation were analyzed by diffusing wave spectroscopy, which assesses the rheological properties of the network by measuring the diffusion of a tracer particle [237-239]. In Figure 5.2a the time evolution of the correlation functions of the tracer particles is shown. The decay of the correlation function of samples taken at increasing incubation times moves progressively towards larger delay times, indicating a decrease of the diffusion coefficient of the tracer particle due to the hindering of the movement occurring during the fibril aggregation process. The corresponding mean square displacement (MSD) profiles of the tracer particles, calculated from the intensity autocorrelation function, change during time (Figure 5.2b), confirming further the hindrance of the movement of the tracer particles caused by the formation of the fibrillar network. From the measurements of the mean square displacement, the storage and loss moduli ($G'$ and $G''$, respectively) of the viscoelastic phase can be calculated over a broad frequency range [237]. In Figure 5.2c and 5.2d the storage and loss moduli are reported at different frequencies for samples taken at 0.5 h and 2.5 days, respectively. Initially, the fibril dispersion exhibits a loss modulus larger than the storage modulus (Figure 5.2c), while after 2.5 days of incubation
the storage modulus is larger than the loss modulus at high frequencies, indicating a change of the rheological properties during time. In Figure 5.2e the time evolution of the two moduli is reported at the reference frequency of 100 rad/s: a cross-over between the $G'$ and the $G''$ can be observed at about 20 h of incubation, confirming further the formation of a gel.

The value of $G'$ measured at low pH in this work is lower than the values of $G'$ of RADA 16-I hydrogels observed at neutral pH [28, 172]. The increase of $G'$ with the decrease of the peptide net charge has been observed with other amphiphilic peptides [235], and can be explained by the decrease of electrostatic repulsion and the increase of lateral association between fibrils.

It is interesting to note that in the high frequencies regime the scaling of the $G''$ with the frequency follows the power law $G''(\omega) = \omega^\alpha$, with exponent $\alpha = 0.8 \pm 0.1$. In this regime the $G''$ is related to the dynamic fluctuations of the single unconfined filaments, which are smaller than the characteristic network mesh size [240]. The measured exponent of 0.8 is close to the value 0.75, which has been observed previously for actin filaments gel [219, 220], cardiac filaments [210] and amyloid fibrils [224], and has been predicted by theoretical models based on the bending modes of single wormlike filaments [240, 241], suggesting that this exponent in the high frequency regime is shared by a large range of semiflexible biopolymers.
Figure 5.2. Micro-rheology analysis of gel formation. DWS measurements of 10 g/L RADA 16-1 nanofibril dispersion at different incubation times at pH 2.0: a-b) Autocorrelation function (a) and (b) mean square displacement at time 0 (blue continuous line), 3 h (red dashed line), 10 h (green dotted line), 25 h (violet dash-dotted line), 3 days (black continuous line). c-d) The $G'(\omega)$ and $G''(\omega)$ values as a function of oscillating frequency at time (c) 0.5 h and (d) 2.5 days. The black dash-dotted lines represent the fitting of the $G''$ in the power law regime at high frequency values. e) Time evolution of the $G'(\omega)$ and $G''(\omega)$ at the reference frequency of 100 rad/s.
5.3.2. Reversibility of the gel

After characterizing the kinetics of gel formation, the reversibility of the gel during the aggregation process was investigated by diluting 2- and 10-fold samples taken at different incubation times (1 day, 2 days and 1 week). Macroscopic inspection indicates that the gel could be broken upon dilution, and, indeed, DLS analysis shows that the average hydrodynamic radius of the diluted samples is smaller than the value of the original gel as shown in Figure 5.3a (see also Figure D.2a and D.3a in Appendix D). The diluted dispersions originating from the gel exhibit comparable average hydrodynamic radius for the two different dilution extents applied and the different incubation times, although small differences in the order of few nanometers are observed (Figure D.2a and D.3a in Appendix D). The radius of the sample diluted after 1 day of incubation, equal to 38.8±5.6 nm (average of the 2- and the 10-fold diluted samples), is twice the average size measured immediately after re-dispersion of the peptide powder (equal to 22.2±2.9 nm), but smaller than the approximate critical size of about 60-80 nm corresponding to the formation of non-ergodic phase (Figure 5.1b). Moreover, all the samples obtained after the dilution of original gel were stable over one week independently of the dilution time and extent, as shown in Figure 5.3b (see also Figure D.2b and D.3b in Appendix D). This observation suggests the presence of irreversible aggregation during the early stages of incubation, followed by formation of reversible aggregates and gel which can be broken upon dilution. The reversibility experiment was repeated at selected time points diluting the gel sample directly in the cuvette (data not shown). The results of this experiment, which avoids aliquoting of the sample, are very similar to the data obtained with the previous procedure, confirming that the breakage of the weak gel is induced by the dilution and not by the aliquoting of the sample.
5. Sol-gel transition of charged fibrils composed of a model amphiphilic peptide

![Graph](attachment:Figure_5.3.png)

**Figure 5.3. Reversibility of the sol-gel transition upon dilution.** a) Comparison between the average hydrodynamic radius of the original gel sample at 10 g/L (blue bars) and the diluted sample (red bars) at different incubation times. Dilution has been performed immediately before the measurements; b) The stability of the diluted samples shown in (a) was followed by monitoring the time evolution of the average hydrodynamic radius. Diluted samples were prepared by taking aliquots of the original gel after 0 (●), 1 day (□), 2 days (▲), and 1 week (○) of incubation and the stability of the diluted samples was measured for 7 days.

In parallel with DLS, the effect of dilution on the fibril size was investigated by comparing atomic force microscopy images of freshly prepared samples at 10 g/L with images of 10-fold diluted gel samples at 10 g/L. In the case of the freshly prepared dispersion, short fibrils with an average length of about 100 nm are observed (Figure 5.4a), while in the case of the 10-fold diluted gel sample the images show longer fibrils with an average length of about 200-350 nm (Figure 5.4b). The corresponding fibril length distributions are reported in Figure 5.4c. The single fibrils elongate without changing their diameter. It is worth mentioning that the fibrils of the freshly prepared sample are present as single filaments (Figure 5.4a), whereas in the 10-fold diluted sample clusters of fibrils are often observed (Figure 5.4b). Although this result may be partially affected by the drying of the sample during the preparation for the AFM analysis, this observation confirms the formation of fibril clusters in the sample at large peptide concentrations.
5. Sol-gel transition of charged fibrils composed of a model amphiphilic peptide

Figure 5.4. Irreversibility of fibril-fibril aggregation. a) AFM picture of a fresh RADA 16-I sample at 10 g/L; b) AFM picture of a 10-fold diluted gel sample incubated for 48 h at room temperature; c) Fibril length distribution of the freshly prepared (blue bars) and the diluted (red bars) sample corresponding to the AFM pictures shown in (a) and (b), respectively.
Taken together, the data from DLS and AFM analysis indicate that RADA 16-I fibril dispersions incubated at 10 g/L at low pH are unstable, and that aggregation occurs according to a two-step mechanism which populates two classes of aggregates characterized by different bonding strength, as illustrated in the scheme in Figure 5.5.

![Scheme of the two-step aggregation/gelation process of RADA 16-I fibrils](image)

**Figure 5.5. Scheme of the two-step aggregation/gelation process of RADA 16-I fibrils**

Initially, aggregation occurs most likely via the same end-to-end elongation mechanism observed at lower volume fraction [56], and confirmed in this study by AFM pictures and SEC measurements of the soluble fraction (data not shown). The fibril-fibril aggregation involves strong interactions, possibly hydrophobic, and the elongated fibrils represent irreversible aggregates. The fibril elongation is accompanied by the formation of clusters of fibrils, which grow until eventually occupying all the available volume generating a non-ergodic phase. While the first elongation step is irreversible, the cluster and gel formation can be reverted upon dilution, indicating weak attractive interactions between the fibrillar aggregates. Such weak interactions are expected considering the large net positive charge (equal to +4 per monomeric peptide) of the fibril surface at pH 2.0 which generates electrostatic repulsion.

### 5.3.3. Gel structure and rheology

Protein aggregates and gels contain unordered structures which exhibit often self-similarity at sufficiently large length scales. Information on the fractal morphology is contained in the rheological behavior of the sample. The rheological properties of RADA 16-I gels were measured in 10 mM HCl at pH 2.0 in the presence of 50 mM NaCl in the peptide concentration range 4.5 – 10 g/L. At all the investigated concentrations and
frequencies \( (\omega) \) the storage modulus \( (G') \) is significantly larger than the loss modulus \( (G'') \): the loss tangent \( \tan \delta = G''(\omega)/G'(\omega) \) is always about 0.17, significantly smaller than the critical value of 1 required to define a gel phase according to the widely used Winter-Chambon criterion [242, 243]. The loss tangent is constant in the frequency range from 0.05 to 126 s\(^{-1}\) at peptide concentration 4.5-5 g/L and from 0.05 to 250 s\(^{-1}\) for peptide concentrations 9-10 g/L. The increase of the frequency interval where the \( \tan \delta \) value is independent of the frequency corresponds to the increase of the gel stiffness with increasing peptide concentration [242, 243].

In many systems, the storage modulus extrapolated at 0 frequencies \( (G') \) scales as a function of protein concentration \( (C) \) according to a power law function [244, 245]:

\[
G' \sim C^n
\]

The exponent \( n \) can be connected to the aggregate structure in the context of the fractal gel theory [4, 223, 244-246]. According to this model, the gel can be envisioned as a series of connected flocs represented by the fibril aggregates, as illustrated in Figure 5.5. Both the backbone and the aggregates in the gel network are characterized by fractal dimension \( D_b \) and \( D_f \), respectively. For a heterogeneous fiber network, the fractal dimension value of the aggregates can vary between 1 and 3 [192]. For protein gels, the elastic modulus is related to both the entropic elasticity due to the stretching of the aggregates under stress and the enthalpic contribution due to the bending of the gel strands. The storage modulus is given by the following expression [4]:

\[
G' \sim C^{\frac{1+D_b}{3-D_f}}
\]

The exponent \( n \) measured for the RADA 16-I hydrogels under the investigated conditions is equal to 2.41 (Figure 5.6). Due to the rod-like nature of the constitutive strand, the fractal dimension value of the backbone \( D_b \) can be assumed equal to 1. According to equation (5.2), the measured exponent of 2.41 corresponds therefore to a fractal dimension value of the aggregates \( D_f \) equal to 1.34. This low \( D_f \) value, related to the fibrillar nature of the aggregates, indicates a very low packing density of the fibrillar clusters constituting the heterogeneous gel network [192].
Figure 5.6. Gel rheology. $G'$ modulus as a function of RADA 16-I fibril concentration at pH 2.0 and 50 mM NaCl.

5.3.4. Phase diagram

After characterizing the kinetics of gel formation and the hydrogel morphology, the effect of two relevant operative parameters, i.e., the peptide concentration and the ionic strength, on the RADA 16-I phase behavior was analyzed. We investigate the possible presence of nematic phase by birefringence under polarized light, which is a property of liquid crystalline phase originating from the parallel alignment of peptide fibrils [221]. The gel formation was assessed by macroscopic observation and by diffusing wave spectroscopy measurements, performed as described in the previous paragraphs. Representative DWS measurements at the reference salt concentration of 75 mM are reported in Appendix D (Figure D.4-D.6). In these measurements gel formation is reflected by changes in the correlation function and in the mean square displacement of the tracer particles during time.

The results are summarized in the phase diagram shown in Figure 5.7. At fibril concentrations equal or smaller than 0.4 g/L no gelation was observed at any of the salt concentrations tested, while at fibril concentrations larger than 6.5 g/L gel formation occurs even in the absence of salt. At intermediate fibril concentrations gel formation is observed at a critical concentration which decreases with increasing the salt concentration, as expected by considering the increasing screening of electrostatic repulsion with increasing ionic strength.
5. Sol-gel transition of charged fibrils composed of a model amphiphilic peptide

Figure 5.7. Phase diagram of RADA 16-I nanofibril dispersion as a function of peptide concentration and NaCl concentration: isotropic phase (●), gel phase (■). Phase behavior has been experimentally characterized by light scattering, rheology, cross-polarized microscopy and visual observation. The continuous line represents the phase boundary described by equation (5.3).

We can rationalize the obtained phase diagram in the frame of the fractal gel theory. The critical percolation concentration required to form a heterogeneous network of fibrillar aggregates can be written as [192]:

$$\Phi = \frac{\alpha}{2} \frac{D_{\text{eff}}}{L_p} \left( \frac{R_c}{V_{\text{rod}}}^{1/3} \right)^{D_f - 3}$$  \hspace{1cm} (5.3)

where $L_p$ is the persistence length obtained from AFM images, $R_c$ is the characteristic radius of the fibrillar clusters, $D_f$ is the fractal dimension of the aggregates, $V_{\text{rod}}$ is the volume of the single fibril, $\alpha$ is the number of contact between two fibrils inside one aggregate, which has been considered equal to 1, and $D_{\text{eff}}$ is the effective diameter accounting for the double charge layer calculated using equation (5.4) [247]:

$$D_{\text{eff}} = D + \kappa^{-1}(\ln A + C + \ln 2 - \frac{1}{2})$$  \hspace{1cm} (5.4)

where $D$ is the diameter of the fibrils evaluated from TEM images [56], $\kappa^{-1}$ is the Debye length, $C$ is the Euler constant equal to 0.577 and $A$ is represented by the following expression:

$$A = 2 \cdot \pi \cdot v_{\text{eff}}^2 \cdot \kappa^{-1} \cdot Q \cdot \exp(-\kappa \cdot D)$$  \hspace{1cm} (5.5)

where $v_{\text{eff}}$ is the linear charge density equal to the ratio between the total charge and the total length of the monomer and $Q$ is the Bjerrum length equal to 0.7 nm in water at room temperature. The Debye length appearing in equations (5.4) and (5.5) is defined as [247]:

85
where $\varepsilon_0$ is the vacuum dielectric permeability, $\varepsilon_r$ the permittivity of the solvent, $k_B$ the Boltzmann constant, $T$ the temperature, $N_A$ the Avogadro number and $I$ the ionic strength of solution.

Most of the parameters appearing in equation (5.3) have been evaluated independently from the experiments described in the previous sections of this chapter. Due to the aggregation of the fibrils in the early stages of the aggregation process, some parameters, such as the contour length and the fractal dimension, are dependent on the incubation time and/or the salt concentration. As a first approximation, we considered the average values which are summarized in Table 5.1. The simulated phase transition predicted by the model is compared to the experimental data in Figure 5.7. We note that the model is able to describe the decrease of the critical gelation concentration with the increase of the ionic strength, in particular in the low salt concentration regime. However, at higher salt concentrations, an inconsistency between the model simulation and the experimental data is observed. From equations (5.3)-(5.6) it can be seen that the decrease of the critical percolation concentration with the increase of the ionic strength is related to the decrease in the effective diameter induced by the screening of electrostatic repulsion, which saturates at the salt concentration of 30 mM, as reflected by the constant value of the effective diameter above this critical salt concentration. Likely, in the high salt concentration range, a more detailed model accounting for the change in the fractal dimension and the characteristic radius of the fibrillar clusters with the salt concentration is required to describe accurately the gel boundary.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril effective diameter</td>
<td>$D_{\text{eff}}$</td>
<td>10-21 nm</td>
<td>Equation (5.4)</td>
</tr>
<tr>
<td>Fibril persistence length</td>
<td>$L_p$</td>
<td>100 nm</td>
<td>AFM images</td>
</tr>
<tr>
<td>Characteristic radius of fibrillar clusters</td>
<td>$R_c$</td>
<td>188 nm</td>
<td>Fitting parameter</td>
</tr>
<tr>
<td>Volume of single fibrils</td>
<td>$V_{\text{rod}}$</td>
<td>$1.57 \times 10^5$ nm$^3$</td>
<td>AFM/TEM images</td>
</tr>
<tr>
<td>Fractal dimension</td>
<td>$D_f$</td>
<td>1.34</td>
<td>Rheological measurements</td>
</tr>
</tbody>
</table>

Table 5.1. Values of the parameters in equation (5.3) for the calculation of the critical gelation concentration
The presence of nematic phase was not observed under any of the investigated conditions. The critical concentration required to have isotropic-nematic transition in a dispersion of charged amyloid fibers has been recently shown to follow the equation [247]:

\[
\phi = 6 \cdot \frac{D^2}{D_{eff} \cdot L_p} \cdot \rho_{fibril} \tag{5.7}
\]

where \( \rho_{fibril} \) is the density of the fibrils, assumed equal to 1350 g/L. Equation (5.7) has been proven successful to describe quantitatively the phase transition of \( \beta \)-lactoglobulin fibers in water under different conditions of pH and ionic strength [247]. In our system, the critical values of fibril concentrations \( c_{IN} \) calculated from equation (5.7) are in the range 390-810 g/L. These large values, which are due to the short length of the fibrils, are two orders of magnitude larger than the critical RADA 16-I peptide concentrations (3-6.5 g/L) at which gel phase is observed (Figure 5.7). In addition, the peptide concentration at which gel formation is observed is much lower than the critical concentration required to form a homogeneous gel network, \( \Phi=0.7/r \), where \( r \) is the aspect ratio of the fibrils (equal to about 5-10) [189]. This observation supports the conclusion that for the system under consideration excluded volume interactions alone are not able to account for the phase transition, and that attractive forces between the short fibrils are crucial for the formation of the heterogeneous gel network.

### 5.4. Conclusions

In this chapter, the sol-gel transition of dispersions of positively charged fibrils composed of the peptide RADA 16-I and the structure of the fibrillar hydrogels at low pH are characterized by a combination of light scattering, rheology and microscopy techniques. The peptide is initially present as a dispersion of short rigid fibrils with average length of about 100 nm. During incubation the fibrils aggregate irreversibly into longer fibrils via strong, likely hydrophobic, interactions. These longer fibrils aggregate further into fibrillar aggregates, which, at peptide concentrations of about 3-6.5 g/L, form a weak hydrogel which can be broken upon dilution.

The dependence of the critical percolation concentration as a function of the ionic strength could be well rationalized in the frame of the fractal gel theory considering the gel as a network of fibrillar clusters characterized by a fractal dimension value of 1.34.
Chapter 6

The impact of the flow type and shear rate on protein stability

6.1. Introduction

In the recent years, the stability and aggregation mechanism of proteins gained a large scientific attention because of several implications in medicine [1, 13], pharmaceutical industry [2, 42, 248, 249], food industry [4] as well as in production of chemically and mechanically stable biomaterials [5]. Currently several environmental factors are known to trigger protein aggregation. One of them is shear force, which can induce protein assembly leading to loss of drug activity and efficiency as well as being associated with many side effects and toxicity of the medicament [43, 44]. Therefore, the mechanistic understanding of this complex phenomenon is of great importance because of the presence of shear in both intravascular body fluids as well as during production and formulation of protein-based drugs.

In several studies reported in the literature it is shown that shear force may induce the changes in protein structure as well as in the alignment of protein molecules with respect to each other resulting in enhancement of protein aggregation [51, 52]. However, there is a large discrepancy in the reported results. For instance, shear-induced structure change was observed in case of bovine serum albumin [250], lysozyme [251] as well as von Willebrand factor [252, 253]. Nevertheless, not only the change in the protein structure but also the formation of aggregates was observed in case of whey proteins [254] or bovine insulin [255]. Moreover, the promotion in the formation of amyloid fibrils was found in case of insulin [256], β-amyloid [257], β-lactoglobulin [258-260], hen egg white lysozyme [261] as well as apolipoprotein C-II [262]. Additionally, the shear-induced inactivation of several enzymes as α-amylase [263], urease [264] and catalase [265] was reported in the literature. On the other hand, in some studies no significant effect of high shear rates in order of >10^5 s^{-1} on stability of such proteins as recombinant human growth hormone and recombinant human deoxyribonuclease [266], IgG1 monoclonal antibody [267] and cytochrome C [268] was observed. It is remarkable that the shear rate of 10^7 s^{-1} is shown as an obligatory threshold that induces unfolding of a small globular protein [267, 268]. Consequently, despite several studies available in the
6. The impact of the flow type and shear rate on protein stability

In this chapter, we investigate, in the presence of shear, the aggregation of native proteins of various sizes such as lysozyme, bovine serum albumin, ferritin and thyroglobulin as well as the pre-aggregates of BSA. The pre-aggregates were produced by incubating the protein at high temperature resulting in the modification of protein surface. The reference value of shear rate of $2 \times 10^5$ s$^{-1}$ chosen in this study is an order of magnitude higher than the value found in industrial applications ($2.0 \times 10^4$ s$^{-1}$ [267]) to ensure the pronounced effect of shear. Moreover, in the case of BSA pre-aggregates the effect of flow type (i.e., simple shear and elongational flow) and shear rate on their stability was studied. The simple shear, which relies on the uniform deformation occurring only parallel to the direction of velocity component [269, 270], was investigated at the shear rates ranging from 10 s$^{-1}$ to $2 \times 10^3$ s$^{-1}$. The effect of elongational flow, in which the deformation is proportional to the changes of the velocity field in streamwise direction (e.g., due to the presence of contraction) [269], was studied using shear rates ranging from 200 s$^{-1}$ to $2 \times 10^5$ s$^{-1}$. We observe that all the native proteins under investigation are stable in the presence of the shear conditions used. Moreover, whereas the aggregation occurs in the case of pre-denatured aggregates of BSA in elongational flow independently on the shear rate applied, the simple shear does not affect their stability. These results suggest that the shear is incapable of inducing the aggregation of proteins in their native, functional form, although, the modification of protein surface accompanied by the exposure of the aggregation-prone patches (i.e., increase in the attractive hydrophobic interactions) is enough to destabilize protein in elongational flow.

6.2. Materials and methods

6.2.1. Materials and sample preparation

Bovine serum albumin (>98%), type I ferritin from equine spleen and thyroglobulin from bovine thyroid with molecular mass of around 660 kDa were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Lysozyme from chicken egg white was supplied from Fluka Analytical (Sigma-Aldrich GmbH, Steinheim, Germany). All proteins were used as received without further purification.
The protein solutions were prepared by weighting the exact amounts of protein powder and adding suitable amount of buffer in order to obtain the final concentration of 5 g/L in the case of bovine serum albumin, and lysozyme or 1 g/L for thyroglobulin. The solutions were left for 10 minutes under gentle stirring for homogenization. The commercial solution of ferritin at concentration of 53 g/L was diluted in suitable buffer to obtain the final protein solution of 1 g/L. In order to guarantee proper buffering, 10 mM phosphate buffer at pH 7.0 and 10 mM HCl at pH 2.0 were used for experiments performed at physiological and low pH, respectively. Prior to use, all of the protein solutions were filtered with GHP Acrodisc® 13 mm syringe filters with 0.45 µm GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA).

6.2.2. Preparation of BSA pre-aggregates

The BSA pre-aggregates were prepared by using the protocol reported elsewhere [82]. Briefly, the suitable amount of protein was re-dispersed in 10 mM phosphate buffer at pH 7.0 in order to obtain the final protein concentration of 30 g/L and gently stirred for 10 minutes for homogenization. The solution was then incubated for 1.5 h at 90 °C. After incubation the solution was cooled down to the room temperature, diluted in 10 mM phosphate buffer at pH 7.0 to the final concentration of 5 g/L, filtered with GHP Acrodisc® 13 mm syringe filters with 0.45 µm cut-off GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA) and stored in refrigerator for the period of time not longer than 5 days.

6.2.3. Atomic force microscopy (AFM)

Protein solutions of freshly prepared BSA pre-aggregates at the concentration of 5 g/L were diluted 200-fold and spotted on a freshly cleaved mica surface for 30 seconds before washing with Milli-Q deionized water in order to remove unattached material and gently dried under nitrogen flux. Asylum Cypher Scanning Probe Microscope (Asylum Research, an Oxford Instruments Company, Santa Barbara, CA, USA) operating in tapping mode was used to image the samples. A scan rate of 4 Hz was applied, using High Resonance Frequency Silicon Cantilever with resonance frequency of 1600 kHz and tip radius of 7 nm (Olympus, Japan).

The morphology of freshly prepared BSA pre-aggregates obtained by using AFM is shown in Figure 6.1.
Figure 6.1. Morphology of freshly prepared BSA pre-aggregates. AFM pictures of BSA pre-aggregates at 5 g/L in 10 mM phosphate buffer at pH 7.0 at time 0 showing their worm-like structure.

6.2.4. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano (Malvern, Worcestershire, United Kingdom) equipped with the laser beam of wavelength, $\lambda_0 = 633$ nm, operating in backscattering mode at a fixed angle of $\theta = 173^\circ$. Micro UV-transparent cuvettes with light path of 1 cm (Brand GmbH, Wertheim, Germany) were used.

6.2.5. Static light scattering (SLS)

Static light scattering (SLS) measurements were performed offline using a goniometer BI-200SM (Brookhaven Instruments, Holtsville, NY, USA) equipped with a solid-state laser, Ventus LP532 (Laser Quantum, Manchester, United Kingdom), with the wavelength of $\lambda_0 = 532$ nm. 10 mL of protein solutions diluted 20 times in 10 mM phosphate buffer at pH 7.0 were measured at room temperature at the scattering angles covering the range from $\theta = 15^\circ$ to $145^\circ$.

6.2.6. Small angle light scattering (SALS)

Small angle light scattering (SALS) was measured by using Mastersizer 2000 (Malvern, Worcestershire, United Kingdom) equipped with the Hydro 2000µP cell (Malvern, Worcestershire, United Kingdom) at the angles ranging from $\theta = 0.014^\circ$ to $40.6^\circ$. 4 mL of protein solutions were slowly loaded in the cell and measured in the
6. The impact of the flow type and shear rate on protein stability

The presence of constant stirring at 650 rpm to ensure the proper mixing of the sample and to avoid the sedimentation of aggregates.

6.2.7. Zeta potential

Zeta potential measurements were performed using a Zetasizer Nano (Malvern, Worcestershire, United Kingdom). The electrophoretic mobility \( (U_E) \) was measured and related to the zeta potential \( (\zeta_p) \) by Henry equation:

\[
U_E = \frac{2 \cdot \varepsilon_0 \cdot \varepsilon_r \cdot \zeta_p \cdot f(ka)}{3 \cdot \eta}
\]

where \( \varepsilon_0 \) is the vacuum permittivity, \( \varepsilon_r \) is the solvent relative dielectric permittivity, \( \eta \) is the solvent viscosity, and \( f(ka) \) is the Henry function, which in our case is equal to 1.5 according to Smoluchowski approximation. Disposable capillary cells (Malvern, Worcestershire, United Kingdom) were used.

The measurements were performed at the protein concentrations of 1 g/L in the case of ferritin and thyroglobulin and 5 g/L in the case of lysozyme, bovine serum albumin and BSA pre-aggregates in suitable buffers at 25 °C. Each reported value was obtained from ten repeated measurements of two independent samples.

The parameters such as molecular weight, average hydrodynamic radius and zeta potential of each protein under consideration are presented in Table 6.1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight [kDa]</th>
<th>Average hydrodynamic radius [nm]</th>
<th>Zeta potential [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>14.3</td>
<td>pH 2.0 3.1</td>
<td>pH 7.0 3.2</td>
</tr>
<tr>
<td>BSA</td>
<td>66.4</td>
<td>pH 2.0 5.4</td>
<td>pH 7.0 28.2 -7.3</td>
</tr>
<tr>
<td>Ferritin</td>
<td>440</td>
<td>pH 2.0 -</td>
<td>pH 7.0 -13.5</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>660</td>
<td>pH 2.0 -</td>
<td>pH 7.0 -18.8</td>
</tr>
<tr>
<td>BSA pre-aggregates</td>
<td>-</td>
<td>pH 2.0 -</td>
<td>pH 7.0 -19.6</td>
</tr>
</tbody>
</table>

Table 6.1. The parameters of proteins under investigation.
6.2.8. **Intrinsic tryptophan fluorescence**

Intrinsic tryptophan fluorescence was measured by using an EnSpire 2300 Multilabel Reader Fluorometer (Perkin Elmer). 75 µL of the sample was introduced into a 384-well plate (Optiplate 384-F, Perkin Elmer, Waltham, MA) and excited at wavelength of 295 nm with emission signal recorded at 350 nm.

6.2.9. **Shear experiments**

In order to examine the effect of elongational flow on protein stability a contracting nozzle was placed between two 10 mL Omnifix Luer-Lock syringes (B. Braun Melsungen AG, Melsungen, Germany) and mounted into Lambda Vit-Fit programmable syringe pump (LAMBDA Instruments GmbH, Brno, Czech Republic). The effect of the shear rate was studied by using the nozzles with the length of 300 µm and diameter of 150, 300, 600 and 1200 µm corresponding respectively to the shear rates of $2.0 \times 10^5$, $2.0 \times 10^4$, $2.0 \times 10^3$ and $2.0 \times 10^2$ s$^{-1}$. The constant flow rate of 5 mL/min was applied to maintain the suitable shear rate. The absence of turbulent flow in all cases under consideration was confirmed by the calculation of Reynolds number equal to 707 for the highest shear rate used. The values of shear rate and Reynolds number for the contracting nozzle used in this work were calculated accordingly to the calibration reported elsewhere [271, 272].

The effect of simple shear was investigated in the Couette flow at the shear rates ranging from 10 s$^{-1}$ to 2000 s$^{-1}$ by using ARES rheometer (Rheometric Scientific, Piscataway, NY, USA) equipped with the basket with diameter of 34 mm and the bob with diameter of 33.3 mm.

In order to ensure the reproducibility, at least two experiments for two independent samples were performed.

6.3. **Results and discussion**

6.3.1. **The effect of the protein initial size on the aggregation behavior in the shear flow**

We start our analysis by investigating the stability of four different proteins of various average sizes (as listed in Table 6.1.) in the presence of the shear rate of $2 \times 10^5$ s$^{-1}$ by using the contracting nozzle with the diameter of 150 µm and length of 300 µm placed between two syringes as described in materials and methods section. At first, we focus on
the stability of proteins at physiological pH (i.e., in 10 mM phosphate buffer pH 7.0) by studying the time evolution of average hydrodynamic radius measured by DLS. As shown in Figure 6.2a, no substantial aggregation was detected over a time of 7 h of incubation under shear in case of all protein under consideration. In order to elucidate if the shear flow is inducing the structure changes of proteins, we compare the folding state of the protein before and after exposure to shear by using intrinsic tryptophan fluorescence. No evidence of the change in the protein structure was observed as a result of applied shear rate as can be seen in Figure 6.2b.

**Figure 6.2.** Shear effect on the stability of proteins differing in the molecular weight and average hydrodynamic radius. Time evolution of the average hydrodynamic radius of 5 g/L lysozyme (•), 5 g/L bovine serum albumin (■), 1 g/L ferritin (▲) and 1 g/L thyroglobulin (●) in (a) 10 mM phosphate buffer at pH 7.0 and (c) 10 mM HCl at pH 2.0 subjected to the shear rate of 2.0×10^5 s\(^{-1}\). The conformational stability given by intrinsic tryptophan fluorescence of 5 g/L lysozyme, 5 g/L bovine serum albumin and 1 g/L thyroglobulin in (b) 10 mM phosphate buffer at pH 7.0 and (d) 10 mM HCl at pH 2.0 before (blue bar) and after (red bar) incubation in the presence of shear rate of 2.0×10^5 s\(^{-1}\).
In addition to physiological pH, we study the stability of proteins at acidic pH to elucidate the effect of denaturation on the aggregation behavior under shear. In order to do so, we perform the experiments at shear rate equal to $2 \times 10^5 \text{ s}^{-1}$ for two reference proteins, lysozyme and BSA, dissolved in 10 mM HCl at pH 2.0. As shown in Figure 6.2c, also in this case no significant change in the average hydrodynamic radius was observed for the time period of 7 h. Moreover, the results obtained from intrinsic tryptophan fluorescence indicate that there was no structural change after both proteins were subjected to shear (Figure 6.2d).

Considering proteins as biocolloids, we can represent their aggregation behavior with respect to Péclet number, $Pe$, defined as [273]:

$$Pe = \frac{3 \cdot \pi \cdot \eta \cdot \gamma \cdot b^3}{k_B \cdot T}$$

where $\gamma$ is the shear rate, $b$ is the protein radius, $k_B$ is the Boltzmann constant and $T$ is the temperature.

The Péclet number represents the ratio of the shearing energy acting on a particle to the thermal energy. The low value of $Pe$ corresponds to the regime, where the aggregation behavior is driven by the thermal energy, in which the energy barrier between two interacting molecules controls the aggregation rate [274]. On the other hand, the increase in the $Pe$ number above the critical threshold induces the change of the aggregation regime into the shear-dominated one [274]. According to our previous studies, for the values of $Pe < 1$ the aggregation is controlled by the energy barrier in two-body interactions, whereas for $Pe > 10$ the system enters into the shear-dominated regime [273]. The regime in between those two values is so called transition regime, where the competition among the contribution of thermal and shearing energy arises.

The calculated values of the Péclet numbers for all proteins under consideration are presented in Table 6.2.
6. The impact of the flow type and shear rate on protein stability

<table>
<thead>
<tr>
<th>Protein</th>
<th>Péclet number</th>
<th>pH 2.0</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td></td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td>0.065</td>
<td>0.055</td>
</tr>
<tr>
<td>Ferritin</td>
<td>-</td>
<td></td>
<td>2.53</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>-</td>
<td></td>
<td>1.45</td>
</tr>
</tbody>
</table>

Table 6.2. The value of Péclet number calculated for all proteins under consideration corresponding to the shear rate of 2×10^5 s\(^{-1}\).

It is clearly seen that, while the values of \(Pe\) for BSA and lysozyme at both acidic and physiological pH are significantly smaller than 1, the values calculated for ferritin and thyroglobulin exceed the value of 1 indicating that the impact of shear becomes significant. However, the absence of the aggregation observed (Figure 6.2a and c) indicates that the energy barrier between two interacting protein molecules is higher than the energy arising from shearing, suggesting the presence of stabilizing forces.

The stability of the two aforementioned proteins in the shear flow can be explained by the properties of protein surface. Considering the surface charge given by zeta potential (Table 6.1), it is clearly seen that the high measured values correspond to the increase in the repulsive electrostatic interactions preventing two molecules from aggregation. This result implies that the electrostatic forces dominate the aggregation stability of native proteins in the shear flow, meaning that the shear force alone is not capable of inducing the assembly of proteins in their native, functional form.

6.3.2. The effect of the protein surface modification on protein aggregation behavior in the shear flow

In an effort to determine the effect of protein surface on protein stability in the shear flow, the pre-aggregates of BSA were prepared by thermal denaturation. As shown in Table 6.1, the final size and zeta potential of produced pre-aggregates were comparable to the properties of the two largest proteins (i.e., ferritin and thyroglobulin), which enable the direct comparison of their aggregation behavior under shear.

First of all, we investigated the stability of the produced pre-aggregates at 5 g/L in the presence of elongational flow at the shear rate of 2×10^5 s\(^{-1}\) in 10 mM phosphate buffer at pH 7.0 by using the contracting nozzle as described previously. The aggregation
kinetics of BSA pre-aggregates are followed by SLS and SALS, because of the size of formed aggregates exceeds the range suitable for DLS measurements. As shown in Figure 6.3a and b, the time evolution of the scattered intensity profiles as a function of scattering vector, \( q \) (\( q = \left( \frac{4 \pi n}{\lambda_0} \sin(\theta / 2) \right) \), with \( n \) being the refractive index of the solvent, \( \lambda_0 \) the wavelength of the laser and \( \theta \) the scattering angle), clearly suggests the formation of large aggregates generated in the elongational flow, as indicated by the increase of \( I(q) \) at small \( q \) values.

The size of formed aggregates in terms of radius of gyration, \( <R_g> \), was calculated from the Guinier region [275] of the normalized intensity curve, measured by SLS or SALS, plotted as a function of the scattering vector \( q \):

\[
\ln \left( \frac{I(q)}{I(0)} \right) = -\frac{q^2}{3} \langle R_g \rangle^2
\]

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

As shown in Figure 6.3c, we observe that the shear flow does not affect the stability of native proteins as indicated by no significant change in the average hydrodynamic radius, \( <R_h> \), obtained from DLS over the considered time period. However, the BSA pre-aggregates subjected to shear undergo assembly into the precipitates with size in the range of tens of microns as indicated by the values of radius of gyration, \( <R_g> \) as well as by macroscopic observations. Interestingly, the size of the formed aggregates is independent of the shearing time. It suggests that there are two aggregation events, i.e., aggregation and breakage of formed aggregates, taking place at the same. The balance between those two events results in the formation of species with the constant size over the time range under consideration – a behavior often observed in the case of colloidal particles [276-279].
6. The impact of the flow type and shear rate on protein stability

![Graphs showing the stability of native ferritin and thyroglobulin](image)

**Figure 6.3.** Stability of native ferritin and thyroglobulin as well as BSA pre-aggregates in the presence of elongational flow. a-b) The intensity profile as a function of scattering vector, $q$, measured by (a) SLS and (b) SALS at time 0 (black line) and 15 min (blue line), 30 min (red line), 35 min (green line), 40 min (violet line), 45 min (light blue line), 50 min (orange line), 55 min (grey line) and 60 min (pink line) of BSA pre-aggregates in 10mM phosphate buffer at pH 7.0 subjected to shear rate of $2\times10^5$ s$^{-1}$. c) Time evolution of the average hydrodynamic radius of native ferritin (▲) and thyroglobulin (●) at 1 g/L and average radius of gyration of BSA pre-aggregates at 5 g/L (■) in 10 mM phosphate buffer at pH 7.0 subjected to shear rate of $2\times10^5$ s$^{-1}$. 
In order to confirm the destabilizing effect of the shear flow on the BSA pre-aggregates, we performed the experiments in stagnant conditions. Namely, the protein solution was incubated in the glass vial and in the set-up used during experiments (i.e., two syringes connected with the nozzle with the diameter of 150 µm). We observe that the pre-aggregates in both cases are stable over the time period of 2 h being two times longer than in case of shearing experiment, indicating that the presence of shear is indeed required to induce the aggregation (Figure 6.4).

**Figure 6.4. Stability of BSA pre-aggregates at stagnant conditions.** The intensity profile as a function of scattering vector, \( q \), of BSA pre-aggregates at 5 g/L in 10 mM phosphate buffer at pH 7.0 in the absence of shear flow incubated in (a) glass vial or (b) two syringes connected with the nozzle with diameter of 150 µm. The measurements were done by using SLS at time 0 (blue line) and after 0.5 h (red line), 1 h (green line), 1.5 h (violet line) and 2 h (orange line) of incubation.

Let us now focus on the Péclet number being equal to, respectively, 6.9, 2.53 and 1.45 for BSA pre-aggregates, ferritin and thyroglobulin, suggesting similar behavior of all three proteins in the shear flow. Also since the comparable value of zeta potential was measured for all of three proteins (Table 6.1), the presence of similar electrostatic barrier is expected. However, considering the surface properties in case of pre-aggregates not only the surface charge was altered, but also the amount of hydrophobic patches on the protein surface is increased due to the denaturation induced by high temperature. This may result in the increase in attractive interactions overcoming the energy barrier given by the high value of surface electrostatic potential. In order to determine if the assembly of pre-aggregates is driven by the size or rather by the properties of surface we investigate the effect of the flow type and the shear rate as described in the next paragraph of this chapter.
6.3.3. The effect of the flow type and the shear rate on the stability of BSA pre-aggregates

To investigate more in detail the aggregation behavior of BSA pre-aggregates we study the effect of flow type on their stability by performing the experiments in the elongational flow (i.e., in the nozzle) using shear rates ranging from 200 s\(^{-1}\) to \(2 \times 10^5\) s\(^{-1}\) and simple shear (i.e., in the Couette flow) in the presence of shear rates ranging from 10 s\(^{-1}\) to \(2 \times 10^3\) s\(^{-1}\). The impact of the elongational flow was studied keeping constant either the total experiment time equal to 25 minutes or the total residence time, which protein solution spend in the nozzle throughout the whole experiment, with respect to the experiment performed at the highest shear rate (i.e., the nozzle with the smallest diameter). The study on the simple shear was conducted for the constant total experiment time equal to 25 minutes.

As shown in Figure 6.5a and b, the simple shear does not affect the protein stability in the whole range of shear rates under consideration. However, in the presence of the elongational flow the aggregation, accompanied by the formation of species in size range of tens of microns, is observed already at the shear rate of 200 s\(^{-1}\) in case of both sets of experiments, i.e., at equal total experiment time and total residence time, as can be seen in Figure 6.5.
Figure 6.5. The effect of flow type and shear rate on the stability of BSA pre-aggregates at 5 g/L in 10 mM phosphate buffer at pH 7.0. a) Time evolution of the average radius of gyration in the presence of simple shear in Couette flow incubated for 25 minutes (●) as well as elongational flow induced by the contracting nozzle for the same total experiment time (■) and the same total residence time (▲). b) The intensity profiles as a function of scattering vector, \( q \), measured by SLS at time 0 (blue line) and after 25 min of incubation in the Couette flow at shear rate of 10 s\(^{-1}\) (red line), 30 s\(^{-1}\) (green line), 100 s\(^{-1}\) (violet line), 300 s\(^{-1}\) (orange line), 1000 s\(^{-1}\) (grey line) and 2000 s\(^{-1}\) (pink line). c-f) The intensity profiles as a function of scattering vector, \( q \), measured by (c and e) SLS and (d and f) SALS at time 0 (blue line) and after the incubation at (c and d) the same total experiment time and (e and f) the same total residence time at shear rate of 200 s\(^{-1}\) (orange line), 2000 s\(^{-1}\) (violet line), 20000 s\(^{-1}\) (green line) and 200000 s\(^{-1}\) (red line).
It is worth mentioning that in case of the shear rate of $2 \times 10^2 \text{ s}^{-1}$ the pre-aggregates solution at constant residence time was subjected to the shear for the total time of 24 s corresponding to two passes through the nozzle at constant flow rate of 5 mL/min. These results indicate that the destabilization of the protein occurs immediately after entering the channel, meaning after being exposed to the elongational shear.

Let us now interpret the results with respect to Péclet number. The calculated values of $Pe$ for different experimental conditions are summarized in Table 6.3.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Shear rate [s$^{-1}$]</th>
<th>Péclet number [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA pre-aggregates</td>
<td>$10$</td>
<td>$3.45 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^2$</td>
<td>$6.90 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^3$</td>
<td>$6.90 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^4$</td>
<td>$6.90 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^5$</td>
<td>$6.90$</td>
</tr>
</tbody>
</table>

Table 6.3. The summary of corresponding Péclet numbers with the respect to shear rates used in the shearing experiments.

As can be seen in equation (6.2) the only variable parameter now is the shear rate. It means that decreasing the shear rate by three orders of magnitude the corresponding Péclet number is also smaller by three orders of magnitude. The value of $Pe$ is thus equal to $6.9 \times 10^3$ for the lowest shear rate considered in experiments in the elongational flow being much lower than the critical value of $1$ found for colloidal dispersions. However, the aggregation was observed in all of the cases under investigation independently on the shear rate applied, hence independently on the Péclet number. These results suggest that indeed, the shear-driven aggregation of proteins is not governed by their initial size, but rather the surface modification due to thermal denaturation together with the presence of an elongational flow causing the stretching of the protein chain may likely result in the unfolding of pre-aggregates and increase in the attractive hydrophobic interactions.
6.4. Conclusions and outlook

6.4.1. Conclusions

In this work, we apply the systematic study of shear-induced aggregation of proteins differing in the molecular weight and average hydrodynamic radius such as lysozyme, bovine serum albumin, ferritin and thyroglobulin as well as BSA pre-aggregates prepared by incubation at high temperature. In particular, we focus on the effect of the flow type and the shear rate on their aggregation behavior.

We observe that all of the native proteins are stable under shear flow regardless their size. The shear rate of $2.0 \times 10^5$ s$^{-1}$, applied in this study, is substantially higher than the values commonly met in the pharmaceutical industry [$2.0 \times 10^4$ s$^{-1}$ [267]] and intravascular blood system [$10^2-10^4$ s$^{-1}$ [256, 259]]. This suggests that the shear flow itself is incapable of inducing changes in the structure and leading to aggregation of proteins in their native form observed in the nature under typical industrial operation conditions.

However, the surface modification due to thermal denaturation, resulting in the exposure of hydrophobic patches, is enough to destabilize the pre-aggregates in the elongational flow independently of the shear rate applied. This observation is consistent with the aggregation behavior of colloid particles, suggesting the hydrophobicity of the particle surface as the determining factor in shear-induced aggregation. Moreover, no substantial aggregation was observed in the presence of simple shear (i.e., Couette flow) under all conditions tested indicating the strong effect of elongational flow on protein chain. This effect can be explained by the stretching properties of elongational flow, which may result in the unfolding of pre-aggregates further increasing attractive hydrophobic interactions, thus enhancing the assembly process.

6.4.2. Outlook

The detailed aggregation mechanism of BSA pre-aggregates in the elongational flow is planned to be investigated by in situ measurements using small angle X-ray scattering. This technique allows following the changes of the protein structure while flowing through the microchannel and thus elucidating the forces underlying the assembly.

Finally, we will study the aggregation behavior of the pre-aggregates formed out of other model protein (i.e., lysozyme) in the simple shear and elongational flow at
different shear rates. This work will allow us proving if the shear-driven aggregation of proteins is indeed not related to their size, but rather to the changes in their surface properties.
Chapter 7

Concluding remarks

In this thesis, we investigate the role of intermolecular interactions in the aggregation kinetics and mechanism of proteins and peptides. The aggregation behavior is studied under a broad range of environmental conditions in terms of buffer composition, pH, temperature and the presence of shear forces. The experimental results obtained by several techniques are rationalized by key concepts widely used in colloid science to determine the fundamental physical forces governing the stability of protein solutions.

First of all, we show the aggregation mechanism of human insulin in the presence of different anions (Chapter 2). In particular, we focus on the effect of the sulfate anion, which is present in several biological relevant components and has been reported to exhibit a peculiar effect on protein stability, which is yet poorly understood. It is found that the increase of sulfate concentration modifies the aggregation pathway and inhibits the incorporation of insulin molecules into aggregates. At low sulfate concentrations the self-assembly follows the nucleation polymerization mechanism typically observed for monovalent anions. However, above the threshold concentration of 5 mM, sulfate induces salting-out of around 18-20% of insulin into amorphous aggregates, which during time undergo the structure rearrangement into $\beta$-sheets. This aggregation scheme represents a case where amorphous aggregates on pathway to amyloid-like structure can be detected and characterized. These results indicate that in protein systems specific ion effects in addition to charge screening dramatically affect the aggregation rate, the aggregation mechanism and the morphology of the formed aggregates.

In Chapters 3-5 we analyze the role of the electrostatic interactions in the aggregation of the amphiphilic peptide RADA 16-I at different self-assembly levels. We start in Chapter 3 from the analysis of the self-assembly of monomers into fibrils by both experimental characterization and metadynamics simulations. It is found that the morphology of the peptide is strictly related to its global net charge. Namely, higher net charges are causing the destabilization of fibrillar structures and release of monomer molecules which are in equilibrium with remaining fibrils, while at lower net charges the precipitation of amorphous aggregates occurs. The optimal net charge for fibril formation is found to be equal to $+0.75$, what is in perfect agreement with the results reported in
Concluding remarks

The quantitative analysis of the equilibrium constant as a function of peptide net charge indicates that the electrostatic interactions contribute substantially to the free energy of fibril formation. The increase in ionic strength favors the formation of fibrils, a fact that is explained by the decrease of electrostatic repulsive barrier resulting from the charge screening. Moreover, the fibrillation process is also promoted by the addition of charged denaturant (guanidinium hydrochloride) at moderate concentrations (0.3-1 M), suggesting that also the peptide structure affects the self-assembly behavior. Overall, the combination of the results obtained by experimental observations and metadynamics simulations indicate that the global peptide net charge is a key property that correlates well with the fibril stability, although the peptide conformation and the surface charge distribution also contribute to the aggregation propensity.

In Chapter 4 we investigate the intermolecular forces underlying the end-to-end fibril-fibril aggregation of RADA 16-I into longer filaments. The aggregation kinetics are studied as a function of buffer composition including type and concentration of anion and cation as well as presence of organic solvent. It is found that both the increase of ionic strength and concentration of organic phase is enhancing aggregation rate due to the decrease in the repulsive barrier. The experimental results are rationalized with the Smoluchowski kinetic approach (Population Balance Equation), which is able to describe the aggregation process reasonably well. Moreover, by normalizing the aggregation kinetics with respect to characteristic aggregation time, the aggregation curves under broad range of conditions collapse on a single master curve. This result indicates that the buffer composition affects the aggregation rate, however, the self-assembly mechanism remains unchanged. Furthermore, it is found that the aggregation is not a diffusion-limited, but rather an activated process. The energy barrier represented in terms of Fuchs stability ratio is calculated accordingly to both PBE model and DLVO theory. We show that the experimental and simulated results are in reasonably good agreement suggesting that the aggregation process is dominated by repulsive electrostatic interactions, which can be reduced by increasing either the concentration of the salt or organic phase. The relationship between the energy barrier and ionic strength can be well described by DLVO theory, however, specific anion and cation effects are also observed. The anion effect can be explained by the specific binding to the positively charged groups on the peptide surface, whereas cation effect represents more complex problem and involve addition of non-electrostatic contributions.
The gel formation of the positively charged nanofibrils composed of amphiphilic peptide RADA 16-I at low pH is described in details in Chapter 5. The two-step gelation process consists of irreversible end-to-end fibril-fibril elongation followed by the reversible cluster formation, which eventually leads to the formation of a reversible weak gel. The percolation occurs directly from the isotropic to gel phase without the formation of nematic phase at critical percolation concentration decreasing with increasing ionic strength. The gelation behavior can be reasonably well described with the fractal gel model considering the gel as a collection of fibrillar interconnected aggregates characterized by self-similar structure with a fractal dimension of 1.34.

In the last chapter, (Chapter 6), we show the effect of shear flow (i.e., the flow type and the shear rate) on the structure and stability of various proteins that differ in the average hydrodynamic radius and properties of external protein surface altered by thermal denaturation. It is found that the shear in elongational flow is not capable of inducing the aggregation nor the structure changes of native proteins under as high shear rates as $10^5$ s$^{-1}$. However, in the case of BSA pre-aggregates formed by thermal denaturation, their assembly into agglomerates in the size range of tens of microns was observed in a wide range of the shear rate applied in the presence of elongational flow. In contrast, the experiments performed in the simple shear do not show any significant aggregation. The mechanism underlying the aggregation of BSA pre-aggregates may be the stretching properties of elongational flow possibly resulting in the further unfolding, thus in the increase in the attractive hydrophobic forces. In order to deeper investigate the mechanism of the assembly of proteins in elongational flow, we will study the changes in the pre-aggregates structure during shearing online by small angle X-ray scattering. Finally, the experiments in the presence of different flow types and shear rates will be conducted for pre-aggregates formed of another model protein (i.e., lysozyme) by its thermal denaturation to confirm the hypothesis that the shear-induced aggregation of proteins in the elongational flow is driven not only by the protein size, but also to the properties of protein surface.

Overall, the results of this thesis offer a colloidal perspective of the intermolecular interactions driving the self-assembly of peptides and proteins, and provide an engineering platform to rationalize the complex effect of different intermolecular forces, such as electrostatic and shear forces, on the kinetics and mechanisms of protein aggregation, with relevant implications in many practical systems in biology and biotechnology. The results show the potential of engineering and colloid approaches in
protein science, and at the same time highlight the complexity of the investigated systems, which still require further extensive research. For instance, in an attempt to prove the universality of the results shown in this thesis, similar studies could be performed on a broader range of model proteins and peptides. An interesting research line could involve the modification of the amino acid sequence of the model amphiphilic peptide RADA 16-I in order to investigate the effect of single amino acid mutation on the intermolecular forces underlying the protein self-assembly. This relatively simple system can represent a convenient model tool which can be analyzed by both experimental characterization and modelling activities based on molecular dynamics. The results obtained on this model system could offer fundamental insights into the physico-chemical principles driving the aggregation process which could be valid also in more complex and practical applications, including the aberrant aggregation of peptides and proteins associated with neurodegenerative diseases as well as the manufacturing of protein-based drugs and biomaterials.
Chapter 8

Appendix A

A.1. Aggregation kinetics with and without ThT dye in the reacting mixture

![Graph showing ThT fluorescence vs. time](image)

**Figure A.1.** Comparison of aggregation kinetics performed in the presence (○) and in the absence (●) of ThT dye performed at 5 g/L insulin (860 µM) in 25 mM HCl at pH 1.6 with 100 mM Na₂SO₄.

A.2. Insulin quaternary structure before aggregation

![SEC chromatograms of insulin solutions](image)

**Figure A.2.** SEC chromatograms of insulin solutions at 5 g/L (860 µM) in 25 mM HCl at pH 1.6 with (a) 100 mM NaCl and (b) 100 mM Na₂SO₄ before incubation at 60 °C. According to static light scattering the peak eluting between 28 and 35 min in the chromatogram corresponds to a species with molecular weight of 5800±100 Da, well in agreement with the theoretical molecular weight of monomeric insulin (5808 Da). Insulin was present as monomer at all protein and salt concentrations investigated in this work (data not shown).
A.3. Monomer conversion by SEC

During the aggregation process the area under the peak corresponding to insulin monomer in the chromatogram decreases due to monomer consumption. We observed a shift of the position of the peak to longer elution times during aggregation. This shift was observed also when different volumes of a sample at constant concentration were injected into the column: the peak moves to longer elution times when lower amount of protein is injected into the column. Despite the observed shift, the molecular weight of the peak measured by static light scattering is always in excellent agreement with the molecular weight of monomeric insulin.

**Figure A.3.** SEC chromatograms for insulin at 5 g/L (860 μM) in 25 mM HCl at pH 1.6 with: a) 100 mM NaCl, measured before incubation (black, solid line) and after 3 h (red, dashed line), 4.5 h (black, dash-dotted line) and 6 h (pink, solid line) incubation at 60 °C; b) 100 mM Na₂SO₄, measured before incubation (black, solid line) and after 6 h (black dashed line), 10.5 h (green, solid line), 18.5 h (black dash-dotted line), 24 h (pink dashed line) and 27.5 h (black dotted line) incubation at 60 °C.
A.4. ThT Fluorescence and monomer conversion in the presence of chloride and sulfate

Figure A.4. The time evolution of (a) ThT Fluorescence and (b) monomer conversion of 5 g/L (860 μM) insulin in 25 mM HCl at pH 1.6 with 100 mM NaCl (■) and 100 mM Na₂SO₄ at 60 °C (●).

A.5. Chloride concentration effect on aggregation kinetic of insulin (5 g/L, 25 mM HCl, pH 1.6)

Figure A.5. The time evolution of ThT Fluorescence of 5 g/L (860 μM) insulin in 25 mM HCl at pH 1.6 with 5 mM NaCl (●), 50 mM NaCl (●) and 100 mM NaCl (■) at 60 °C.
A.6. Circular Dichroism (CD)

CD spectra were collected using a Jasco-815 CD spectrophotometer (Jasco, Easton, MD) with a PFD-425S Peltier temperature controller. Far-UV CD spectra were recorded from 260 to 190 nm using a quartz cuvette with 0.1 cm path length. Spectra obtained after buffer subtraction were corrected for protein concentration and smoothed using the Savitsky-Golay function.

Figure A.6. Insulin thermal stability in the presence of 100 mM NaCl and 100 mM Na$_2$SO$_4$ measured by CD: temperature dependence of the molar ellipticity of 5g/L (860 µM) in 25 mM HCl at pH 1.6 in the presence of 100 mM NaCl at 210 nm (●) and 223 nm (■) and in the presence of 100 mM Na$_2$SO$_4$ at 210 nm (▼) and 223 nm (▲).
Chapter 9

Appendix B

B.1. Mass spectrometry

Figure B.1. Mass spectrometry analysis of monomeric RADA 16-I. The monomeric peptide was separated by size exclusion chromatography running in 0.1% formic acid at pH 2.6. For the details of the mass spectrometry analysis please refer to the Supplementary Information to the article by Arosio et al. [56].

B.2. Metadynamics simulations

Figure B.2. Convergence of the free energy for RADA 16-I at pH 2.0 with 0 M Gnd-HCl (blue, continuous line), pH 4.5 with 0 M Gnd-HCl (red, dash-dotted line), pH 2.0 with 0.6 M Gnd-HCl (green, dashed line) and pH 4.5 with 0.6 M Gnd-HCl (violet, dotted line).
B.3. Representative SEC chromatograms

![SEC Chromatogram](image)

**Figure B.3.** The SEC chromatograms of RADA 16-I at 0.5 g/L in 10 mM HCl at pH 2.0 (black line), 3.2 mM HCl at pH 2.5 (red line), 10 mM acetate buffer at pH 3.0 (green line), 10 mM acetate buffer at pH 3.5 (violet line), 10 mM acetate buffer at pH 4.0 (dark blue line) and 10 mM acetate buffer at pH 4.5 (light blue line).

![SEC Chromatogram](image)

**Figure B.4.** The SEC chromatograms of RADA 16-I at 0.25 g/L (black line), 0.5 g/L (dark blue line), 1 g/L (red line), 2 g/L (green line), 3 g/L (violet line), 3.5 g/L (light blue line) and 5 g/L (orange line) in (a) 10 mM HCl at pH 2.0, (b) 3.2 mM HCl at pH 2.5, (c) 10 mM acetate buffer at pH 3.0, and (d) 10 mM acetate buffer at pH 4.0.
Figure B.5. The SEC chromatograms of RADA 16-I at 1 g/L in (a) 10 mM HCl at pH 2.0 and (b) 10 mM acetate buffer at pH 4.5 containing 0 mM (black line), 25 mM (dark blue line), 50 mM (red line), 75 mM (green line) and 100 mM (violet line) NaCl.

Figure B.6. The SEC chromatograms of RADA 16-I at 1 g/L in (a) 10 mM HCl at pH 2.0 and (b) 10 mM acetate buffer at pH 4.5 containing 0 M (black line), 0.3 M (dark blue line), 0.6 M (red line), 1 M (green line), 2 M (violet line), 4 M (light blue line) and 6 M (orange line) Gnd-HCl.
Figure B.4. The effect of denaturant on the structure of RADA 16-I

Figure B.7. The effect of Gnd-HCl on the secondary structure and morphology of RADA 16-I peptide. a-d) TEM pictures of RADA 16-I solutions in 10 mM HCl at pH 2.0 with (a) 0.6 M Gnd-HCl and (b) 6 M Gnd-HCl and 10 mM acetate buffer at pH 4.5 containing (c) 0.6 M Gdn-HCl and (d) 6 M Gnd-HCl; e) CD spectra of 0.5 g/L peptide solutions in 10 mM HCl at pH 2.0 (blue lines), 10 mM acetate buffer at pH 4.5 (red lines) in the presence of 0.6 M Gnd-HCl (continuous lines) and 6 M Gnd-HCl (dash-dotted lines), showing the disruption of β-sheet structures with increasing the concentration of denaturant.
B.5. Free energy surface

Figure B.8. Free energy surface from PTMetaD-WTE at (a) pH 2.0 with 0M Gnd-HCl, (b) pH 4.5 with 0M Gnd-HCl, (c) pH 2.0 with 0.6M Gnd-HCl and (d) pH 4.5 with 0.6M Gnd-HCl. Contour lines are plotted every $k_B T$ unit (2.5 kJ/mol).
Chapter 10

Appendix C

C.1. Transmission Electron Microscopy

Peptide samples were recorded by using a FEI Morgagni 268 microscope. Peptide solutions were diluted to a final concentration of about 0.05 g/L, loaded on a carbon grid (Quantifoil, Jena, Germany), washed and stained with a 2% uranyl acetate solution.

![TEM images](image)

Figure C.1. TEM pictures of RADA 16-I fibrils at 1 g/L in the presence of NaCl in 10 mM HCl at pH 2.0. Representative TEM pictures of (a) freshly prepared solution of RADA 16-I peptide and aggregated solution of RADA 16-I peptide in the presence of (b) 25 mM NaCl and (c) 100 mM NaCl.
C.2. Size Exclusion Chromatography

Size exclusion chromatography analysis was performed using a Superdex Peptide 10/300 GL, 10 mm $\times$ 300 mm size-exclusion column (GE Healthcare, Uppsala, Sweden) mounted on a Agilent 1100 series HPLC unit (Santa Clara, CA, USA) consisting of an isocratic 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 10mM HCl at pH 2.0 as mobile phase. The fractionated samples were detected by UV absorbance at 217 nm.

Figure C.2. The time evolution of monomer conversion in the presence of NaCl in 10 mM HCl at pH 2.0. SEC chromatograms at zero time (blue, dash-dotted line), after 2 weeks (red, dashed line) and 4 weeks (green, continuous line) of incubation in the presence of (a) 25 mM and (c) 75 mM NaCl. The time evolution of the percentage of the residual monomer (♦) and peptide fragments eluting at longer elution times (■) in the presence of (b) 25 mM and (d) 75 mM NaCl.
C.3. Dynamic Light Scattering

Figure C.3. The anion effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. The time evolution of hydrodynamic radius in the presence of (a) NaNO₃ and (b) NaH₂PO₄ at ionic strength equal to 25 mM (♦), 50 mM (■), 75 mM (▲), 100 mM (●).

Figure C.4. The cation effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. The time evolution of hydrodynamic radius in the presence of (a) CaCl₂ at equal ionic strength and (b) CaCl₂ at equal concentration of Cl⁻ ions at ionic strength equal to 25 mM (♦), 50 mM (■), 75 mM (▲), 100 mM (●).
Figure C.5. The solvent effect on the aggregation of RADA 16-I peptide at 1 g/L in 10 mM HCl at pH 2.0. The time evolution of hydrodynamic radius in the presence of (a) 10% isopropanol, (b) 10% ethanol and (c) 20% ethanol in the presence of 25 mM (♦), 50 mM (■), 75 mM (▲), 100 mM (●) NaCl.
Chapter 11

Appendix D

D.1. Dynamic Light Scattering

![Diagram A](image1.png)

**Figure D.1. Aggregation process monitored by DLS analysis by acquiring the time evolution of the average $<R_h>$: (a) absolute values of the average $<R_h>$ versus time and (b) normalized values of the average $<R_h>$ with respect to the plateau value of $<R_h>$ versus time. Experiments have been performed at six different scattering angles: 20° ($\bullet$), 25° ($\ ■$), 30° ($\ ▲$), 45° ($x$), 90° ($*$) and 173° ($\ ●$).**

D.2. Reversibility of the gelation

![Diagram B](image2.png)

**Figure D.2. Reversibility of the sol-gel transition upon 2-fold dilution: a) Comparison between the average hydrodynamic radius of the original gel sample (blue bars) and the 2-fold diluted samples (red bars) few minutes after dilution at different incubation times. b) Stability of the 2-fold diluted samples obtained at different incubation times followed by monitoring the time evolution of the average hydrodynamic radius: diluted samples were prepared by taking aliquots of the original gel at time 0 ($\bullet$), after 1 day ($\ ■$), after 2 days ($\ ▲$), after 1 week ($\ ●$), as shown in (a).**
Figure D.3. **Reversibility of the sol-gel transition upon 10-fold dilution:** a) Comparison between the average hydrodynamic radius of the original gel sample (blue bars) and the 10-fold diluted samples (red bars) few minutes after dilution at different incubation times. b) Stability of the 10-fold diluted samples obtained at different incubation times followed by monitoring the time evolution of the average hydrodynamic radius: diluted samples were prepared by taking aliquots of the original gel at time 0 (●), after 1 day (■), after 2 days (▲), after 1 week (●), as shown in (a).

D.3. Diffusing Wave Spectroscopy

Figure D.4. **Microrheology of RADA 16-I nanofibrils:** DWS measurements of 1 g/L RADA 16-I nanofibrils sample in 10 mM HCl at pH 2.0 with 75 mM NaCl at different incubation times: (a) autocorrelation function, (b) mean square displacement at time 0 (●), 10.5 h (□), 25 h (▲), 40.5 h (○). The data at different incubation times overlap and are not distinguishable.
Figure D.5. Microrheology of RADA 16-I nanofibrils: DWS measurements of 3 g/L RADA 16-I nanofibrils sample in 10 mM HCl at pH 2.0 with 75 mM NaCl at different incubation times: (a) autocorrelation function, (b) mean square displacement at time 0 (●), 10 h (□), 22 h (▲).

Figure D.6. Micro-rheology of RADA 16-I nanofibrils: DWS measurements of 5 g/L RADA 16-I nanofibrils sample in 10 mM HCl at pH 2.0 with 75 mM NaCl at different incubation times: (a) autocorrelation function, (b) mean square displacement at time 0 (●), 22 min. (□), 52 min. (▲), 6 h (○), 3 days (★).
Chapter 12

Bibliography

12. Bibliography


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Bibliography


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Chapter 13

Curriculum Vitae

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Master thesis at the Institute of Chemical and Bioengineering in Prof. Morbidelli Group
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M. Owczarz, T. Casalini, A. C. Motta, M. Morbidelli, P. Arosio, Contribution
of electrostatics in the fibril stability of a model ionic-complementary peptide, accepted
for publication in *Biomacromolecules*

M. Owczarz, A. C. Motta, M. Morbidelli, P. Arosio, A colloidal description
of intermolecular interactions driving fibril-fibril aggregation of a model amphiphilic

L. Nicoud, M. Owczarz, P. Arosio, M. Morbidelli, A multiscale view of therapeutic
367-378

M. Owczarz, S. Bolisetty, R. Mezzenga, P. Arosio, Sol-gel transition of charged fibrils
composed of a model amphiphilic peptide, *Journal of Colloid and Interface Science*, 437
(2015) 244-251

M. Owczarz, P. Arosio, Sulfate Anion Delays the Self-Assembly of Human Insulin

P. Arosio, M. Owczarz, H. Wu, A. Butté, M. Morbidelli, End-to-End Self-Assembly
of RADA 16-I Nanofibrils in Aqueous Solutions, *Biophysical Journal*, 102 (2012) 1617-
1626

P. Arosio, M. Owczarz, T. Mueller-Spaeth, P. Rognoni, M. Beeg, H. Wu, M. Salmona,
M. Morbidelli, In Vitro Aggregation Behavior of a Non-Amyloidogenic lambda Light
Chain Dimer Deriving from U266 Multiple Myeloma Cells, *Plos One*, 7 (2012)

Oral and poster presentations:
(Presenting author is underlined)

*Marta Owczarz*, Paolo Arosio, Sreenath Bolisetty, Raffaele Mezzenga, Massimo
Morbidelli
“Self-assembly kinetics and mechanism of the amphiphilic peptide RADA 16-I”
Biological and Pharmaceutical Complex Fluids II: Novel Trends in Characterizing
Interactions, Microstructure and Rheology, August 10-14, 2014, Durham, North Carolina
(United States of America) – oral presentation
Marta Owczarz, Paolo Arosio and Massimo Morbidelli
“Sulfate anion delays human insulin aggregation by modifying the aggregation pathway”
ProtStab2014: 10th International Conference on Protein Stabilisation, May 7-9, 2014,
Stresa (Italy) – oral presentation

Marta Owczarz, Paolo Arosio, Sreenath Bolisetty, Raffaele Mezzenga, Massimo Morbidelli
“Self-assembling kinetics and mechanism of the amphiphilic peptide RADA 16-I”
ECIS 2013: 27th Conference of the European Colloid and Interface Society, September 1-6, 2013, Sofia (Bulgaria) – poster presentation

Paolo Arosio, Marta Owczarz, Hua Wu, Alessandro Butté, Massimo Morbidelli
“End-to-end self-assembly of RADA 16-I nanofibrils in aqueous solutions”
Nanopeptide 2012: Peptides as Nanomaterials & Biomaterials, November 12-14, 2012,
Manchester (United Kingdom) – oral presentation

Summer schools:

Short Course in Protein Chromatography: Engineering Fundamentals and Measurements for Process Development and Scale up, June 30th – July 5th 2013, BOKU, Vienna (Austria)