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Releasable and Traceless PEGylation of Arginine Residues

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Summary

Grafting methoxy poly(ethylene glycol) (mPEG) to proteins, a process referred to as PEGylation, has been widely exploited to slow down their renal clearance and reduce their recognition by the immune system. Currently, several PEGylated protein therapeutics are used in the clinic. From a developmental context, several synthetic strategies are available for preparing well-defined conjugates of peptides/proteins and polymers. Owing to its simplicity, one attractive grafting approach is to employ residue-specific reactions, which permit the selective modification of up to all solvent exposed amino acid residues of a given sort. However, in many cases, polymer-modification can lead to significant (or total) loss of peptide/protein activity. Although some solutions to this issue have been investigated, such as site-specific polymer conjugation, these solutions are not universally applicable. For instance, site-specific PEGylation may inefficiently mask all epitopes on immunogenic proteins. Site-specific PEGylation may also inactivate smaller therapeutic peptides.

One approach that is gaining momentum to address these challenges is to release the fully active and unmodified native peptide/protein from mPEG with time in the body. This process is termed releasable PEGylation (rPEGylation). rPEGylation incorporates an element of controlled release and is particularly useful for slowly regenerating the activity of proteins adversely affected by polymer-modification. In this Ph.D. thesis, a series of mPEG-phenylglyoxal (PGO) derivatives were synthesized and evaluated as agents for the rPEGylation at arginine residues in peptide or proteins. Arginine is an amino acid for which rPEGylation chemistry does not currently exist. The objective was to develop arginine-reactive mPEG bearing PGO derivatives, use these agents to modify peptides and proteins, and exploit the releasable nature of the coupling chemistry to release the unmodified and fully active agent over time. We also examined whether the rate of release can be tunable by changing the structure of the PGO group and the possibility to disrupt the protein-protein interactions in a high concentration protein solution

Chapter 1 provides a general introduction to protein therapeutics, the challenges associated with their use, and the strategies to overcome these limitations.

Chapter 2 provides an overview of the state-of-the-art of rPEGylation, with emphasis on the chemistry behind the release of the peptide/protein and the means for altering the rate of release in biological fluids.

Chapter 3 presents the first proof of concept of rPEGylation at the arginine residues, of arginine-rich antimicrobial peptides (AMPs). While arginine-rich AMPs are emerging therapeutics of interest, their applicability is limited by their short circulation half-life, caused in part by their small size and digestion by blood proteases (targeting arginine). To overcome these problems, a strategy to temporarily mask arginine residues within AMPs with mPEG bearing PGO was developed and evaluated. Based on the reagent used, release of AMPs can occur in a couple hours to days in a completely traceless fashion. Owing to its simplicity, this method should be applicable for the modification of the entire family of arginine-rich AMPs, which have great potential to be employed as the novel therapeutics.

In **Chapter 4**, mPEG-PGO-additives with different mPEG lengths were synthesized and used to disrupt protein–protein interactions within high-concentration immunoglobulin (IgG) solutions. The concentrated protein aqueous solutions are increasingly used for subcutaneous injections, however, high concentration are typically associated with formulation challenges such as protein aggregation and high viscosity. The addition of very small amounts of the rPEGylation agents based on PGO imparted a significant reduction of viscosity caused by the disruption of IgG self-assembly. In addition, the additives prevented IgG aggregation, caused by prolonged storage.

Chapter 5 provides a general conclusion and describes the major achievements of this Ph.D. thesis. A discussion of the limitations of our strategy is also given as well as the expected impact of our findings.

Zusammenfassung

Die Bindung von Methoxypoly(ethylene glycol) (mPEG) an Proteine (sog. PEGylierung) wird angewendet, um die renale Ausscheidung von Proteinen zu verlangsamen und deren Erkennung durch das Immunsystem zu vermindern. Verschiedene PEGylierte Proteine werden zurzeit therapeutisch verwendet. Es existieren unterschiedliche Synthesestrategien, um gut definierte Polymer-Protein- und Polymer-Peptid-Konjugate herzustellen. Eine häufig beschriebene und einfache Methode verwendet Reaktionen, die für bestimmte Aminosäuren spezifisch sind und diese, sofern frei zugänglich, selektiv modifizieren. Häufig führen PEGylierungen jedoch zu einem partiellen oder totalen Verlust der biologischen Aktivität. Gewisse Ansätze zur Behebung dieses Problems sind zwar in der Literatur beschrieben (z. B. die PEGylierung von spezifischen Aminosäureseitenketten), jedoch sind diese Strategien nicht universell einsetzbar und können auch (bei kleineren therapeutischen Peptiden zu Inaktivierung führen).

Ein immer häufiger benutzter Ansatz, um diesen Herausforderungen zu begegnen, ist die so genannte freisetzbare PEGylierung (rPEGylierung). Die rPEGylierung ermöglicht die kontrollierte Freisetzung *in vivo* von Peptiden und Proteinen aus PEG-Konjugaten und eignet sich besonders für die zeitabhängige Regeneration der Proteinaktivität im Falle einer Aktivitätsminderung aufgrund der PEGylierung. In dieser Doktorarbeit wurde eine Reihe von mPEG-phenylglyoxal (PGO) Derivaten synthetisiert und als Konjugationspartner für die rPEGylierung von Argininseitenketten in Peptiden und Proteinen verwendet. Für Arginin waren bis anhin keine chemischen Reaktionen zur rPEGylierung beschrieben. Ziel dieser Arbeit war die Synthese von argininreaktiven mPEG-modifizierten PGO-Derivaten, deren Anwendung in der PEGylierung von Peptiden und Proteinen und die Verwendung dieser Konjugate zur zeitabhängigen Freisetzung von vollständig aktiven Peptiden oder Proteinen. Es wurde zudem untersucht, ob die Freisetzungsgeschwindigkeit von Peptiden oder Proteinen aus dem PEG-Konjugat durch Strukturveränderung der PGO-Gruppe gesteuert und kontrolliert werden kann und ob Protein-Protein Interaktionen in hochkonzentrierten Proteinlösungen verhindert werden.

Kapitel 1 enthält eine Einführung in Proteintherapeutika mit einer Auflistung der Anwendungsproblemen und möglicher Lösungs strategien.

Kapitel 2 bietet einen Überblick über die rPEGylierung. Schwerpunkt sind die chemischen Reaktionen, die der Freisetzung von Peptiden/Proteinen aus PEG-Konjugaten

zugrunde liegen, und die Möglichkeiten, die Freisetzungsgeschwindigkeit in biologischen Flüssigkeiten zu beeinflussen.

Kapitel 3 präsentiert den ersten Machbarkeitsnachweis der rPEGylierung von Argininseitenketten argininreicher antimikrobieller Peptide. Argininreiche antimikrobielle Peptide sind interessante potenzielle Therapeutika, die aufgrund ihrer kurzen Halbwertszeit jedoch nur beschränkt therapeutisch anwendbar sind; die kurze Halbwertszeit rührt, teilweise von der kleinen Grösse und Anfälligkeit auf Zersetzung dieser Peptide durch argininerkennende Proteasen im Blut her. Um diese Probleme zu überwinden, wurde eine Strategie entwickelt, welche die Argininseitenketten vorübergehend mit mPEG-modifiziertem PGO maskiert. Abhängig vom benützten PEGylierungs-Molekül konnte eine *in vitro* Freisetzung der antimikrobiellen Peptide über Stunden bis Tagen erreicht werden, ohne dass Spuren der PEGylieurng auf den Peptiden zurückblieben. Aufgrund der Einfachheit und Spezifizität sollte die Methode prinzipiell auf alle argininreichen antimikrobiellen Peptide anwendbar sein.

In **Kapitel 4** wurden mPEG-PGO-Derivate mit unterschiedlicher mPEG-Länge synthetisiert, um Protein-Protein-Wechselwirkungen in hochkonzentrierten Immunoglobulinlösungen zu vermindern. Hochkonzentrierte Immunoglobulinlösungen, z. B. therapeutische monoklonale Antikörper, werden zunehmend therapeutisch eingesetzt, jedoch erweist sich die subkutane Injektion aufgrund von haüfig auftretender Proteinaggregation und hoher Viskosität als problematisch. Die Zugabe sehr kleiner Mengen von PGO-basierten rPEGylierungsagenzien führte zu einer signifikanten Reduktion der Viskosität infolge verminderter Immunoglobulinwechselwirkungen und verhinderte die Bildung mikroskopisch beobachtbarer Immunoglobulinaggregate, welche bei längerer Lagerung auftreten.

Kapitel 5 beinhaltet eine Schlussfolgerung und beschreibt die wichtigsten Erkenntnisse dieser Doktorarbeit. Insbesondere werden die Bedeutung und Grenzen des beschriebenen Ansatzes und der gewonnenen Erkenntnisse diskutiert.

Chapter 1.

Background and Purpose

1.1. Protein therapeutics

Proteins, which are composed of one or more chains of amino acid residues, are very versatile and fascinating biomacromolecules due to their diverse structures and biological functions. Furthermore, due to many technological advances enabling the manipulation of proteins, it is not surprising that this class of molecule has emerged as a major novel class of therapeutic agents since the 1980s.[1-3] Protein therapeutics have revolutionized the treatment of a broad range of diseases, such as autoimmune diseases, cardiovascular disorders, metabolic disorders, infections, and a variety of cancers. Over the past few years, the number of protein therapeutics available on the market or in clinical trials has followed an exponential increase.[4] Currently, more than 130 proteins or peptides have been approved by US Food and Drug Administration (FDA), meanwhile, many of others are in clinical trials.[5, 6] Protein therapeutics are highly attractive because of the specificity of the effect they incur in the body, such as the binding of a selected target, the catalysis of certain reaction and so on.[7, 8]

Based on their pharmacologic function, protein therapeutics can be classified into four categories: protein therapeutics with enzymatic or regulatory activity, protein therapeutics with special targeting activity, protein vaccines and protein diagnostics (Figure 1.1).[6] One of the most important uses is to correct an acquired or inherited deficiency or abnormality of a natural protein. For example, human recombinant insulin,[9] which was first generated and licensed protein therapeutic using recombinant DNA technology, is widely employed to manage diabetes mellitus. In addition, human growth hormone (GH) has been shown to efficiency treat GH deficiency, which causes a failure to grow.[10] Furthermore, it is possible to stimulate and strengthen the body's natural defense by immunogenic responses, using protein vaccines.[11-16] In addition, protein diagnostics, such as capromab pendetide, can be used as imaging agent to detect prostate cancer via the recognition of prostate specific membrane antigen,[17] while recombinant HIV antigens can be used as confirmatory tests of HIV infections via the detection of human antibodies to HIV.[18-20] These are valuable in the decision-making process for the diagnosis and treatment of many diseases. At present, antibody-based drugs are the largest and fastest growing class of protein therapeutics, with more than 75 billion dollars in global sales in 2014, and 47 monoclonal antibody (mAb) products on the market.[4, 21, 22] In the future, protein therapeutics are expected to benefit from advanced chemical modification strategies, novel artificial fusion partners, and innovative controlled release techniques. [23-26]
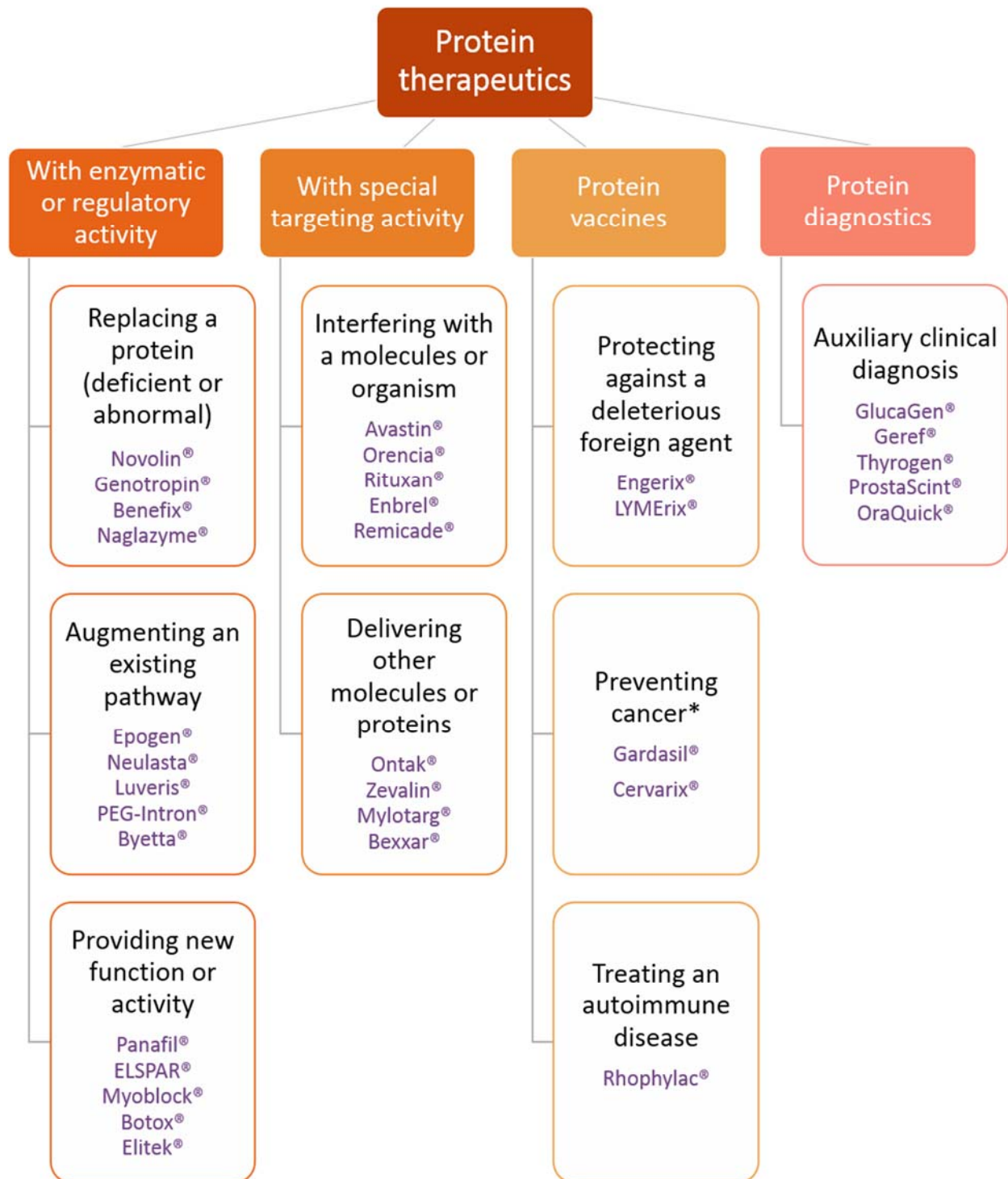
Figure 1.1. Pharmacological classification and examples of protein therapeutics. Adapted from original contributions. [6] * Gardasil® and Cervarix® are human papillomavirus (HPV) vaccines that help to prevent infection from HPV that probably cause cervical cancer, vaginal cancer, vulvar cancer, anal cancer, head and neck cancer cases.

1.2. Limitations and obstacles of protein therapeutics

Even though research in the field of protein therapeutics has made great advances and tremendous development, these molecules still possess certain limitations that need to be addressed.

High cost. Viewed from the perspective of therapeutics development, the overall research and development budget and testing timelines have dramatically risen in recent years,[6] however, the probabilities of success fully reaching the market are low. Viewed from the perspective of patients, the therapeutics are usually quite expensive, especially where multi-grams doses are required for the treatment regimen, which can limit its clinical applicability.[27, 28]

Immunogenicity. Many protein therapeutics are from non-human origin and can consequently induce immune responses. Furthermore, even human-derived proteins can illicit immune responses, usually due to the presence of a very small amount of particulate, caused by protein aggregation. Immunogenicity has become one of the most serious obstacles of protein therapeutics.[29] Unfortunately, its clinical consequences can be life-threatening. Reactions ranging from inflammation, mild skin reactions to anaphylaxis and immune-complex-mediated hypersensitivity. Immunogenicity can be caused by several factors, such as protein therapeutics structure, purity, dosage amount, delivery route and so on.[30]

Instability. Most of the protein therapeutics frequently possess worse stability within high concentration formulations and their activity can be lost as a result of proteolysis, hydrolysis, aggregation and chemical degradation.[31] Furthermore, due to their fast hepatic metabolism and rapid renal clearance, some protein therapeutics can possess short half-lives *in vivo*. Their instability can be a major drawback for widespread clinical use.

Size issue. Proteins are, in general, compact biomolecules that can be eliminated by renal filtration if the sizes are below 8 nm.[32] Nevertheless, in comparison to small molecules, protein are sufficiently large that their ability to penetrate the tissues can be limited grievously.

It has been widely recognized that the preclinical development of protein therapeutics is fraught with these obstacles. Methods to be addressed these challenges need to be developed urgently.

1.3. Solution to the obstacles faced by protein therapeutics

To overcome the challenges described above, several strategies have been exploited in the past few decades. For the sake of improving the stability and reducing immunogenicity, one attractive approach is to alter the amino acid sequence of the protein, which known as protein engineering.[8] This can be used, for instance to replace non-human domains in the protein by human ones (e.g., humanized antibodies).[33] In addition, the modification of proteins with synthetic polymers is also a widely investigated strategy to increase circulation half-life and reduce immunogenicity.[34, 35] The creation of such hybrid systems offers the potential to form new therapeutics possessing a beneficial combination of properties from the biological and polymeric synthetic components, which simultaneously overcome the intrinsic limitations of the components alone.[36, 37] The reduction of immunogenicity of protein therapeutics can be attributed to the shielding effect of the polymer chains, which prevents interactions with other proteins such as antibodies. Generally, the behavior of therapeutics can be changed significantly via the conjugation of hydrophilic polymers.[38]
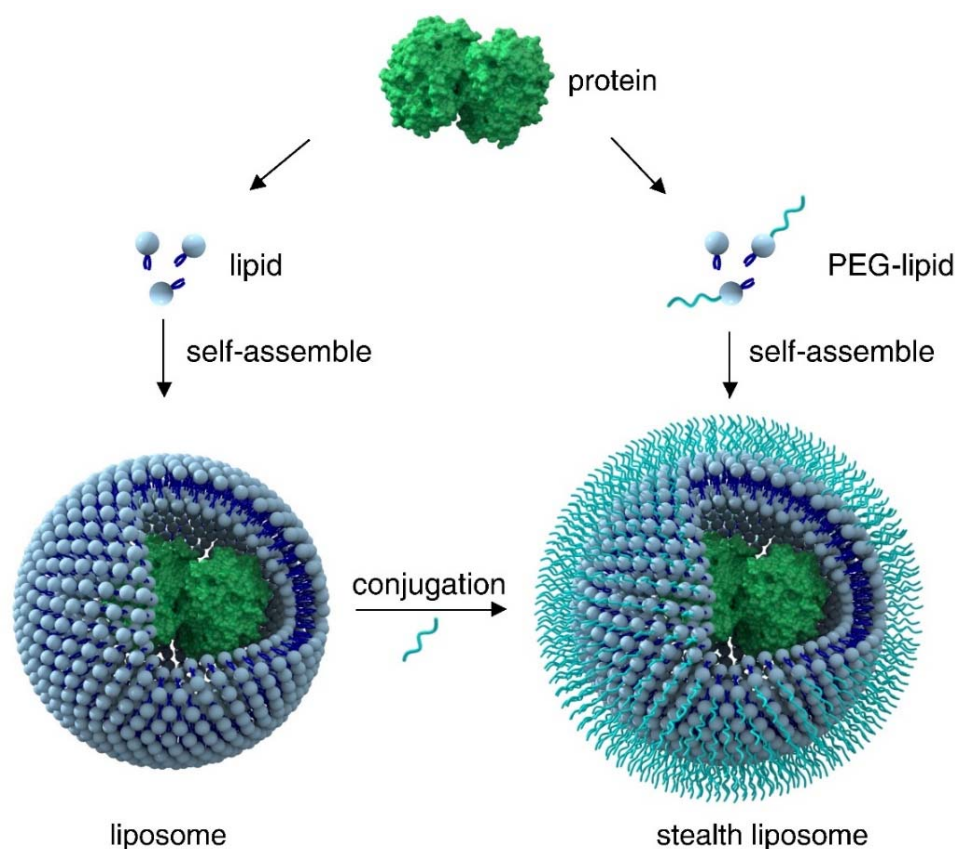

Figure 1.2. Illustration of preparing liposomes and 'stealth liposomes'.[41]

Concurrently, other strategies can be envisaged to improve the performance of protein therapeutics, such as the use of a drug delivery system. Liposomes, micelles, nanocapsules and microgels, amongst others, have been investigated for this purpose.[39, 40] For example, liposomes (nano-sized vesicles), which are produced with natural or artificial phospholipids, can stabilize encapsulated proteins (Figure 1.2). For example, the phosphotriesterase has been encapsulated in liposomes for the treatment of organophosphate intoxication.[41] To enhance the circulation half-life of liposome-wrapped protein therapeutics, 'stealth liposomes' have been prepared by constructing liposomes with poly(ethylene glycol) (PEG) conjugated lipids or by post-conjugating PEG on the surface of liposomes.

1.4. PEGylation

In 1977, Abuchowski et al. reported the first PEGylation of a protein.[42] Extended blood circulation and reduced immunogenicity was achieved by modifying bovine serum albumin (BSA) with methoxy poly(ethylene glycol) (mPEG) chains. Since then, attachment of one or more mPEG chains to proteins or peptides, has become a fast growing technique that has produced several therapeutics in clinical use. These possess advantages such as improved water solubility, enhanced bioavailability, prolonged half-life in bloodstream, specific distribution in some tissues *in vivo*, increased particle size to reduce the renal clearance, prevention of aggregation and degradation by enzymes and so on.[39, 43]

Developing efficient chemical strategies for the modification of biomolecules with high degree of specificity is of paramount importance in the field. Therefore, specifically controlling the site at which the PEG chain is attached to the protein has attracted substantial research interest. In comparison to the traditional non-specific PEGylation which leads to randomly distributed PEG chains on protein surface, site-specific PEGylation shows better ability to preserve protein activity when conjugating to a non-catalytic or non-active site. Currently, mPEGs with both linear and branched structures have been explored in site-specific PEGylation studies.[44]

Because of its natural abundance, almost all protein therapeutics possess exposed lysine residues on their surfaces. Therefore, conjugation to a lysine residue (Lys) or to the N-terminus of the protein therapeutics molecule is the most popular method for non-specific PEGylation.[45] Amine groups in Lys or N terminus commonly react with aldehyde and N-hydroxysuccinimide functionalized PEGs.[46] Reduced cysteine residues are less frequently observed on the surface of

proteins, and can be introduced by selective mutagenesis.[47] As a nucleophile, the thiol group of cysteine can be modified rapidly, efficiently and potentially reversibly depending on the electrophile used. One widely used coupling chemistry utilizes maleimide-functionalized mPEG, which selectively reacts with thiols by Michael addition. Furthermore, residue-specific modification of proteins/peptides at aspartic acid, glutamine, histidine, phenylalanine, tyrosine and some other residues are also part of the residue-specific PEGylation toolbox.[44] As there are usually more than one copy of a given amino acid in the sequence of a protein or peptide, choosing a less abundant amino acid as a target site can lead to a more controlled yet random PEGylation process.

As illustrated in Figure 1.3, a typical protein-mPEG conjugate structure usually contains three parts: 1) mPEG chains with protein-reactive functional groups; 2) a protein or peptide, and 3) an amino acid residue with complementary reactivity to the reactive polymer.
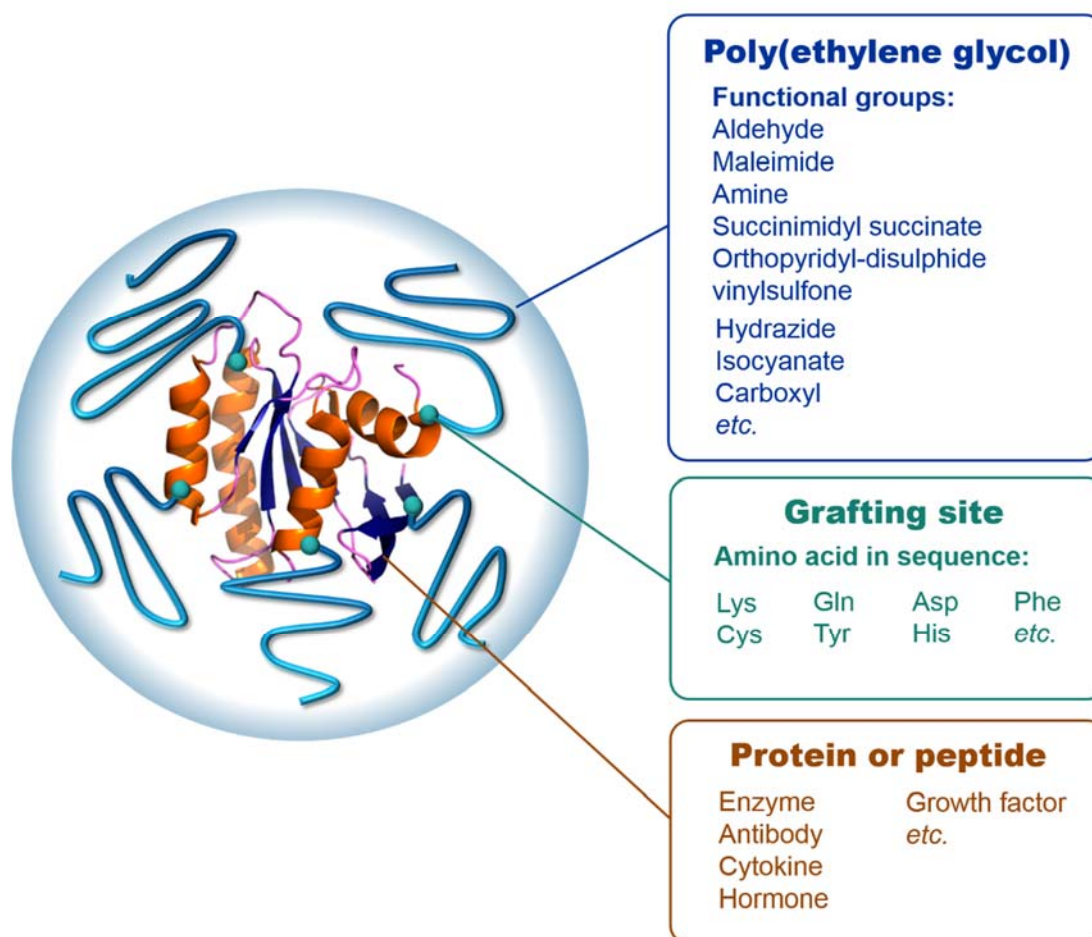

Figure 1.3. Structure of protein/peptide-PEG conjugates.

Table 1.1. Examples of PEGylated protein therapeutics that have received regulatory approval in the USA and/or the EU. Adapted from [42].

PEG therapeutics description	Company	Indication	Year of approval
Adagen® PEG-adenosine deaminase	Enzon Inc. (USA&EU)	Severe combined immunodeficiency	1990 (USA)
Cimzia® PEG-anti-TNFα	UCB S.A. (USA&EU)	Crohn's disease, rheumatoid arthritis	2008 (USA) 2009 (EU)
Krystexxa® PEG-uricase	Savient (USA&EU)	Chronic gout	2010 (USA) 2013 (EU)
Macugen® PEG-anti-VEGF-aptamer	Pfizer (EU)/ OSI Pharm. Inc. Pfizer (USA)	Age-related macular degeneration	2004 (USA) 2006 (EU)
Neulasta® PEG-G-CSF	Amgen Inc. (USA&EU)	Febrile neutropenia	2002 (USA&EU)
Oncospar® PEG-L-ASNase	Enzon Inc. (USA) Rhone-Poulenc Rorer (EU)	Acute lymphoblastic leukemia	1994 (USA)
PEG-Intron® PEG-interferon-α-2a	Schering-Plough Corp. (USA&EU)	Chronic hepatitis C	2000 (EU) 2001 (USA)
Pegasys® PEG-interferon-α-2b	Hoffmann-La Roche (USA&EU)	Chronic hepatitis C	2002 (USA&EU)
Plegridy® PEG-interferon-β-1a	Biogen (USA&EU)	Relapsing forms of multiple sclerosis	2014 (USA&EU)
Somavert® PEG-HG receptor antagonist	Pfizer (USA&EU)	Acromegaly	2002 (EU) 2003 (USA)

TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor, G-CSF: granulocyte-colony stimulating factor, HG: human growth.

To date, a large number of PEGylated therapeutics have been reported with higher efficiency and better stability than the corresponding native proteins. Several PEGylated therapeutics are used in the market as commercial products with approval in USA and Europe, as indicated in Table 1.1. [48-51] For example, mPEG for bovine adenosine deaminase (Adagen®), [52, 53] interferon α2a (Pegasys®),[54] and L-asparaginase (Oncaspar®) modification.[55, 56] In addition, it is interesting to note that polymers other than mPEG have been investigated and studied in clinical trial. For instance, poly(N-isopropylacrylamide) (PNIPAAm) has been used to modify mAbs to reduce Fc-dependent immunogenicity *in vivo*,[57] poly(N-acryloylmorpholine) (PAcM) has been used to modify uricase for the treatment of hyperuricemia and gout,[58, 59] and poly(N-vinyl pyrrolidone) (PVP) modified superoxide dismutase is used for the reactive oxygen species (ROS) associated disease.[40, 60]

1.5. Limitations and obstacles of PEGylation

The development of PEGylated therapeutics can be challenging for a number of reasons.[42]

Immunological response. While generally regarded as a weakly immunogenic polymer, the adverse reaction of PEG can sometimes occur through complement (C) activation, which may lead to hypersensitivity reactions that can provoke anaphylactic shock.[61, 62] Hypersensitivity reactions can not only occur when mPEG is injected, but has also been observed for oral administration and cutaneous applications.[63] For example, mPEG can be used as an oral laxative for preparing patients for colonoscopy. However, hypersensitivity and rash urticaria have been observed to be randomly induced occasionally.[64] The development of anti-mPEG antibodies is a subject of ongoing investigation.[65]

Pharmacokinetic changes. The development of antibodies towards mPEG can cause an accelerated rate of clearance of PEGylated therapeutics, and consequently lead to unpredictable circulation times.[66, 67] In addition, highly PEGylated therapeutics with hydrodynamic sizes higher than 30 nm has been found to be triggered, recognized and cleared rapidly by the cells upon the second administration, referred to accelerated blood clearance phenomenon.[68, 69]

Non-biodegradability. Another obstacle faced by mPEG is its non-biodegradability.[70, 71] The renal clearance of mPEG is limited to molecular weights lower than 20-60 kDa, with higher molecular weight mPEG accumulating in the body, mostly in the liver.[72, 73]

Therapeutics activity loss. A significant drawback of PEGylated proteins is their partial or total loss of bioactivity cause by the coupling with mPEG chains, especially when the number of PEG chains is high. Activity loss can be caused, for instance, by the permanent modification of the protein's catalytic or binding site.

1.6. Solution to the obstacles faced by PEGylation

To mitigate the limitations of PEGylation described above, many studies have been done and lots of efforts are currently underway.

First of all, to overcome the issue of non-biodegradability, the use multiple mPEG chains with lower molecular weights (below ca. 20 kDa) is advised. Such polymers can be eliminated from the body which avoids liver accumulating. It is need to mention that oligomeric mPEG with molecular weights <400 Da exhibits very weak toxicity in humans because of its sequential oxidation by alcohol/aldehyde dehydrogenase.[42, 70]

In order to reduce the loss of protein activity, several strategies have been explored. In addition to site-specific PEGylation describe above, one of the most recent strategies explored is to control the properties of the polymers, such as its architecture. For example, Liu. et al.[74] examined a comb-shaped analog of mPEG, poly(oligo(ethylene glycol) methyl ether methacrylate) (pOEGMA) because its three-dimensional shape can be controlled by main-chain length and comb-teeth length. Within a certain regime of polymer characteristics, they have shown that very high activity can be maintained concurrently with very low immunogenicity in mice. In addition, Fuhrmann et al.[75] have demonstrated that the functional groups on the polymer played a large role modifying the stability and retention of oral enzymes in the gastrointestinal tract.

Another possibly solution to prevent the loss of protein activity is to use biodegradable polymers instead of mPEG. However, the polymers with great biodegradability which will not to excite the accelerated blood clearance and a stealth effect are very few, for example, poly(amino acids) and polysaccharide dextrin.[76-80] Duncan et al. demonstrated that the biodegradable polysaccharide dextrin can be used in 'polymer-masking-unmasking-protein-therapy' (PUMPT) strategy. This PUMPT strategy may produce the conjugates of proteins/peptides with polymers and regenerate the bioactivity of proteins/peptides after the triggered polymer degradation at preferable target site.[81]

Finally, one strategy that is gaining momentum is to release the native proteins from the polymer conjugates with time in human body, a process referred to as releasable PEGylation (rPEGylation).[46, 82] One interesting example is the use of bifunctional bicine linkers containing carboxylic group, which can be modified with both a protein through the amide bond and an mPEG chain by an ester bond.[83, 84] Due to the hydrolysis of the ester bond under physiological conditions, the native proteins can be released *in vivo*. Making use of the rPEGylation technique, it is possible to control the rate of protein release, prevent the loss of activity to optimize the pharmacodynamics response. This approach is presented in detail in Chapter 2.

1.7. Major hypothesis of this thesis

In this thesis, the major hypothesis is that derivatives of phenylglyoxal (PGO) can be used as agents for the rPEGylation of arginine residues in peptide or proteins. Arginine is an amino acid for which reversibly PEGylation chemistry does not currently exist. The objective is to develop arginine-reactive mPEG bearing PGO derivatives, use these agents to modify peptides and proteins, and exploit the releasable nature of the coupling chemistry to release the unmodified and fully active agent over time. We have also examined whether the rate of release is tunable by changing the structure of the PGO group, as illustrated in Figure 1.4. Chapter 2 provides an overview of the current rPEGylation methods with emphasis on the chemistry behind the release of the peptide/protein and the means for altering the rate of release in biological fluids. Linkers discussed include those based on: substituted maleic anhydride and succinates, disulfides, thioesters, 1,6-benzyl-elimination, β-elimination, bicin, β-alanine, biodegradable polymers, E1cb elimination, zymogen activation, photo-immolation, host–guest interactions, and ion complexation.[75]

Chapter 3 presents the first proof of concept of rPEGylation, applied to arginine-rich antimicrobial peptides (AMPs). While arginine-rich AMPs are emerging therapeutics of interest, their applicability is limited by their short circulation half-life, caused in part by their small size and digestion by blood proteases (targeting arginine). Our method provides a strategy to temporarily mask arginine residues within AMPs with mPEG. Based on the reagent used, release of AMPs can occur in a couple hours to days in a completely traceless fashion. This implies that the native AMPs could, in principle, be recovered within the blood stream, with full retention of

biological activity. This coupling strategy is therefore highly relevant, and should be adaptable to the entire family of arginine-rich AMPs.

In Chapter 4, the concept of rPEGylation at arginine residues is tested for its ability to disrupt the protein–protein interactions within high-concentration protein formulations. Highly concentrated therapeutic mAbs solutions are of increasing importance in the pharmaceutics, however, high concentration typically leads to challenges such as protein aggregation and high viscosity. Our rPEGylation agents reversible couple to proteins, thereby lowering viscosity and preventing their aggregation by disrupting the formation of self-assembled protein species.
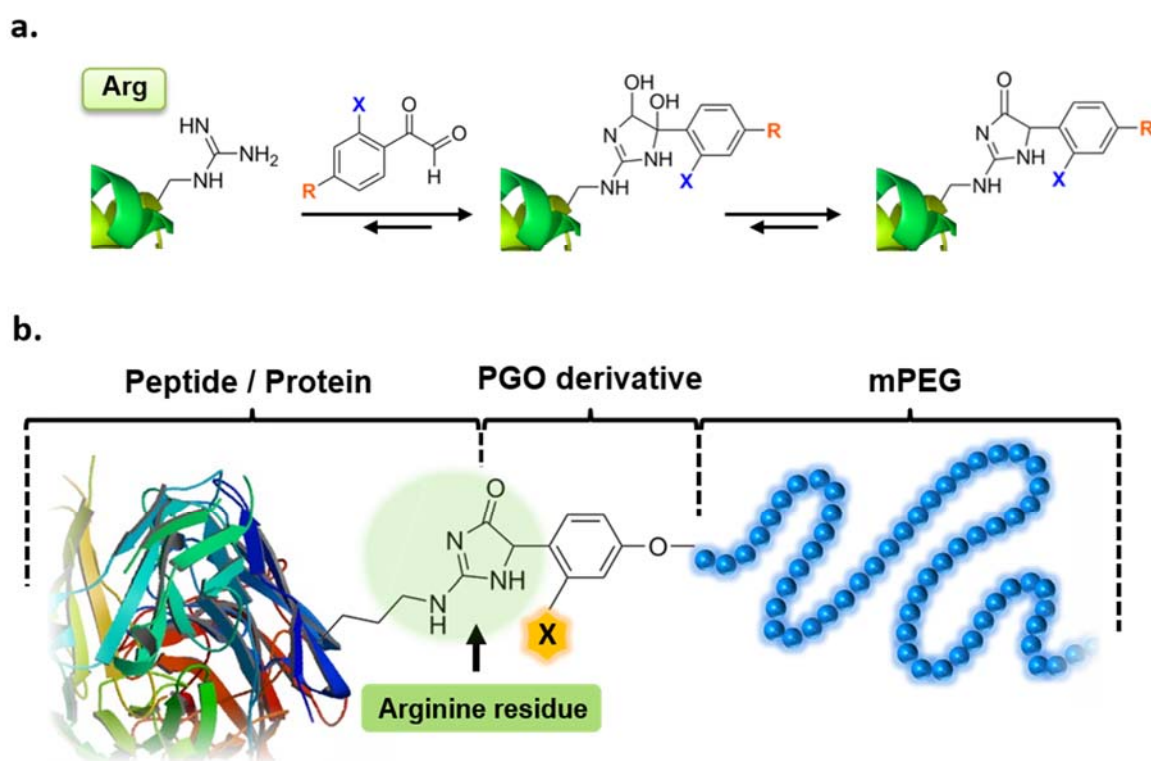

Figure 1.4. Strategy for rPEGylation of arginine residues using PGO derivatives. (a) Reaction of PGO with arginine; (b) Novel platform of protein-PGO-PEG system.

Chapter 2.

Releasable conjugation of polymers to proteins

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2.1. Introduction

For roughly four decades, grafting mPEG to proteins, a process referred to as PEGylation, has been exploited to prevent their renal clearance and their recognition by the immune system.[86-88] Currently, several PEGylated therapeutics are used in the clinic, including PegIntron® (interferon-α2b), Pegasys® (interferon-α2a), Neulasta® (granulocyte colony stimulating factor), Mircera® (epoietin-β), Somavert® (GH receptor antagonist), and Krystexxa® (porcine-like uricase).[89-91] From a developmental context, several synthetic strategies are available for preparing well-defined conjugates of peptides/proteins with polymers. Owing to its simplicity, one attractive grafting approach is to employ residue-specific reactions, which permit the selective modification of all solvent exposed amino acid residues of a given sort. There currently exist residue-specific reactions for permanently coupling polymers to at least 10 out of the 20 canonical amino acids found in proteins.[44, 92-94] However, in many cases, polymer-modification can lead to significant (or total) loss of peptide/protein activity. A classic example is lysozyme, whose ability to hydrolyze bacterial cell-wall polysaccharides is completely lost upon PEGylation with even a single 5 kDa mPEG chain.[95] One avenue for overcoming this challenge is to site-selectively PEGylate the protein in a region not involved in activity, based on an analysis of its 3D structure. For instance, Heredia et al.[96] have shown that the site-specific PEGylation of a V131C mutant of T4 lysozyme has no effect on activity, a dramatically different result from that achieved by random PEGylation. Nevertheless, protein engineering can be laborious, and introducing unpaired cysteine residues complicates oxidative re-folding of proteins. Alternatively, the site-specific modification of naturally-existing particularities on proteins, such as the solvent-exposed disulfide bonds on L-ASNase, can be used to a similar effect. In this example, full catalytic activity of the enzyme was preserved though, unfortunately, full immunogenicity was also maintained.[97, 98] Overall, even the best designed site-specifically PEGylated bioconjugates can demonstrate unacceptable biological properties. In addition, maintaining activity after site-specific PEGylation is especially challenging in the case of peptides/small proteins, proteins with macromolecular substrates, or those involved in binding events involving multiple cooperative interactions over a large area of its solvent-exposed surface. For instance, the site-specific coupling of mPEG to interferon-α2b at one of its native disulfide bonds imparts a 92% reduction of its antiviral activity, which is comparable to the 93% observed for random PEGylation with a single mPEG chain to a lysine residue.[99] Considering the ever-increasing number of therapeutic entities falling into the categories of proteins listed above, finding a solution for optimizing their pharmacological properties without compromising activity is of great interest, but remains a formidable task.

One approach that is gaining momentum to address these challenges is to release the native protein from the polymer with time in the body.[100-105] The PEGylated protein is thus considered as a pro-drug, whose reconversion releases the fully active and unmodified native peptide/protein. This approach, termed releasable rPEGylation, incorporates an element of controlled release and is particularly useful for slowly regenerating the activity of proteins adversely affected by polymer-modification. The two main challenges of rPEGylation are developing coupling chemistry that enable control of the rate of release of the protein from the polymer and, ideally, ensuring that the release mechanism leaves the protein completely unmodified (traceless) or minimally modified (nontraceless). Several excellent review articles exist which present how rPEGylation can be exploited to optimize the pharmacokinetics and pharmacodynamics of therapeutic peptides/proteins.[100-105] The scope of this contribution is to present the state-of-the-art linker chemistry available for the traceless reversible coupling of polymers to proteins, with emphasis on methods for tuning the rate of de-PEGylation of the bioconjugate. This chapter is limited to the conjugation of polymers to peptides/proteins, and will not discuss the conjugation of polymers to drugs, lipids, nucleic acids, other polymers, surfaces and so forth, nor will it discuss the reversible conjugation of drugs to proteins. Review of these topics can be found elsewhere.[106, 107]

2.2. Substituted maleic anhydride and succinate linkers

One of the first rPEGylation strategies investigated was inspired by anhydride reagents that have previously been used to reversibly mask amino groups on proteins, for temporary selective protection from tryptic digestion during peptide mapping.[108] Unfortunately, while presenting an original concept, these seminal articles generally did not provide sufficient analytical data for a detailed description of the rPEGylation process using this type of linker. Garman and Kalindjian have prepared a 5 kDa mPEG derivative of a substituted maleic anhydride, which they coupled to tissue-type plasminogen activator (**1** in Figure 2.1a).[109] However, the degree of PEGylation of the protein was not assessed, and the rate of polymer removal could not be calculated accurately from the supplied data. Roberts and Harris examined a similar approach in which lysozyme was modified with a 5 kDa mPEG bearing a substituted succinate linker (**2** in Figure 2.1b).[110] Hydrolysis of the ester between mPEG and the linker in turn triggers the hydrolysis of succinate from the protein, a process which is accelerated by intramolecular catalysis at acidic pH. Recovery of lysozyme activity was observed with a half-life of ~2.5 hours at 25 ℃

in a pH 8 buffer. However, a detailed analysis of the de-PEGylation process, including the analysis of full removal of the succinate from the protein, was not provided. It is interesting to note that removal of maleic anhydride itself from protein amino groups is typically achieved at acidic pH, and is generally not observed above a pH of 6. Thus, the long half-life at pH 6 ($>10^3$ hours) observed elsewhere,[111] suggests that at neutral pH elimination of this group from a protein at physiological pH will be very slow. rPEGylation using this chemistry is thus likely to be non-traceless, though evidence for this does not exist. Possibly due to limitations associated with linker immolation kinetics, and the requirement for acidic pH conditions to accelerate this process, rPEGylation based on this approach has, to the extent of our knowledge, not been further pursued.
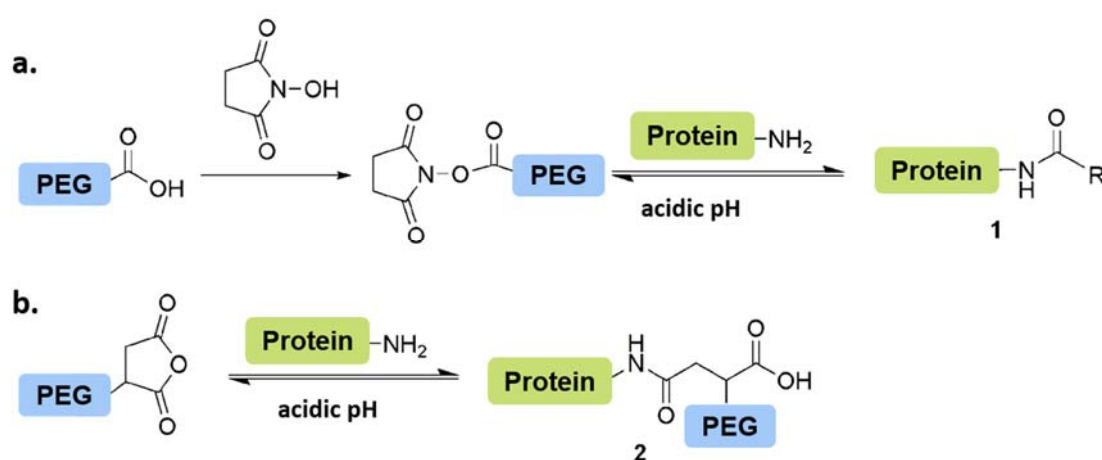
Figure 2.1. Reversible conjugation of protein with polymer via (a) maleic anhydride linker and (b) succinic anhydride linker. [109, 110]

2.3. Thiol–disulfide exchange

Another early rPEGylation strategy, examined in the early 1990s, relied on the formation of a disulfide bond between the PEGylation agent and a reduced thiol on the protein. To our knowledge, the first reported example of this strategy was reported by Woghiren et al. who modified the active-site cysteine residue of papain with a 4-pyridyl disulfide activated mPEG.[112] When the activated mPEG was added dropwise to papain during the conjugation step, the recovered product did not contain any detectable conjugate. The authors rationalize this to dimerization of papain caused by nucleophilic attack of the thiol on papain on the papain–mPEG bio-conjugate. This observation supports evidence of the potential for available free thiols to initiate de-PEGylation. Papain dimerization was not observed when the protein was added to

excess activated mPEG. Pomroy et al. employed a comparable strategy for promoting the solubility of artificial hydrophobic peptides bearing a cysteine residue.[113] In the presence of triscarboxyethylphosphine, PEGylation was fully reversible. In another application, rPEGylation of the active site of the cysteine proteases chymopapain and ficin has been employed for their purification in fully active forms.[114, 115] Purification of these enzymes is complicated by the existence of (iso) forms, of which some are catalytically inactive due to the absence of a thiol in the catalytic site. PEGylation of the active form, which possesses a free thiol group, allows for its selective isolation as a bio-conjugate. De-PEGylation releases the native and fully active protein. More recently, Bontempo et al.[116] have reported the synthesis of thiol-reactive polymers (**3** in Figure 2.2) produced by atom transfer radical polymerization (ATRP), and their reversible conjugation to BSA. More specifically, a pyridyl disulfide-functionalized initiator was prepared and used for ATRP of 2-hydroxyethyl methacrylate (HEMA) mediated by CuBr and 2, 2'-bipyridine. Conjugation was achieved in methanol/phosphate buffer solution at basic pH, and release of the protein could be achieved with dithiothreitol. More recently, Wang et al. modified inorganic pyrophosphatase near its active site with this same polymer. [117] Enzymatic activity was fully inhibited, yet restored in the presence of a reducing agent. Considering the versatility of this polymerization approach, substantial opportunities exist for preparing a variety of thiol-reactive polymers for bio-conjugation purposes.
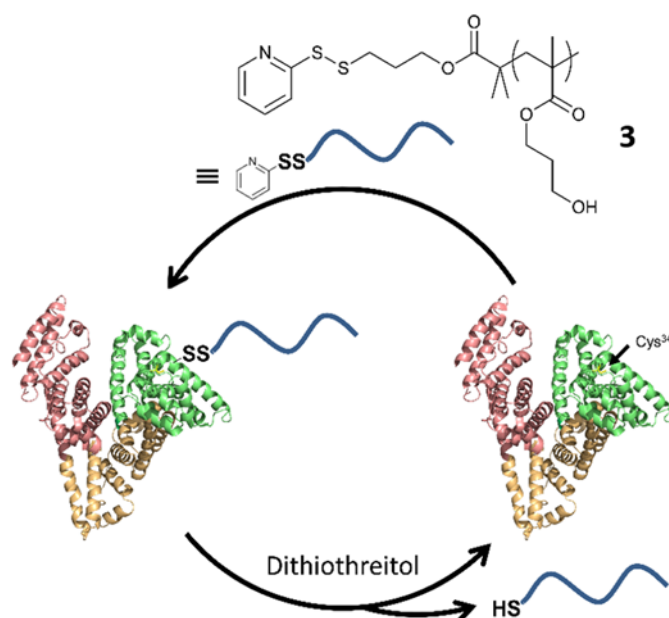

Figure 2.2. rPEGylation via thiol–disulfide exchange. Bontempo et al.[116] have synthesized a thiol-reactive pHEMA from a functional initiator for ATRP. This polymer was conjugated to cysteine-34 of BSA, and could be removed by exposure to reducing agents, such as dithiothreitol.

Adjusting the kinetics of thiol–disulfide exchange has been an extensively examined topic, which could be exploited for controlling the rate of de-PEGylation. Steric hindrance imparted by addition of chemical groups adjacent to the disulfide has a predictable and very large impact on the rate of exchange. For example, Kellogg et al. have systematically altered the number and position of methyl groups adjacent a disulfide bond in an antibody–drug conjugate and have evaluated its stability in a redox buffer.[118] While the presence of 1–2 methyl groups increased stability of the disulfide bond by 7- to 22-fold in comparison to the unhindered bond, further modification led to an increased stability of 170- (2 methyl), 980- (3 methyl), and >22000-fold (4 methyl) relative to the unhindered disulfide. Furthermore, a quantitative relationship between the local charge around the disulfide and its rate of reduction has also been established using model peptide disulfides.[119, 120] The authors report the possibility of adjusting the rate of thiol–disulfide exchange with model endogenous thiols over several orders of magnitude, with fine and predictable tunability over the entire range. Substantial opportunities thus exist for exploiting thiol–disulfide exchange for adjusting the rate of de-PEGylation. One caveat to this approach for traceless rPEGylation however, is the necessity that the protein naturally possess a solvent-accessible cysteine residue. This limits the applicability of this approach to a relatively small sub-set of proteins. Nevertheless, such a residue could be introduced by protein engineering, though the released protein will inevitably be a mutant of the wild-type protein.

2.4. Thiol–thioester exchange

Recently, Chen et al. have prepared a thioester derivative of mPEG containing an activated acid for conjugation to amino groups on proteins.[121] In the presence of thiols, thiol–thioester exchange at the thioester trigger exposes a free thiol, which in turn initiates its self-immolation by cyclization. The authors suggest that the release rate can be controlled via the steric hindrance of the thioester, as it does for thiol–disulfide exchange. In model studies, a series of thioester terminated PEGs with increasing steric hindrance were shown to be degraded monoexponentially in a phosphate buffered glutathione (5 mM) solution (Figure 2.3). Increased steric hindrance of the thioester functionality prolonged the half-lives from 0.4 to 9.7 hours. The authors PEGylated lysozyme and tumor necrosis-related apoptosis inducing ligand (TRAIL) and demonstrated the release of the native protein by mass spectrometry (MS). The cytotoxicity of the regenerated TRAIL was fully regenerated upon release.
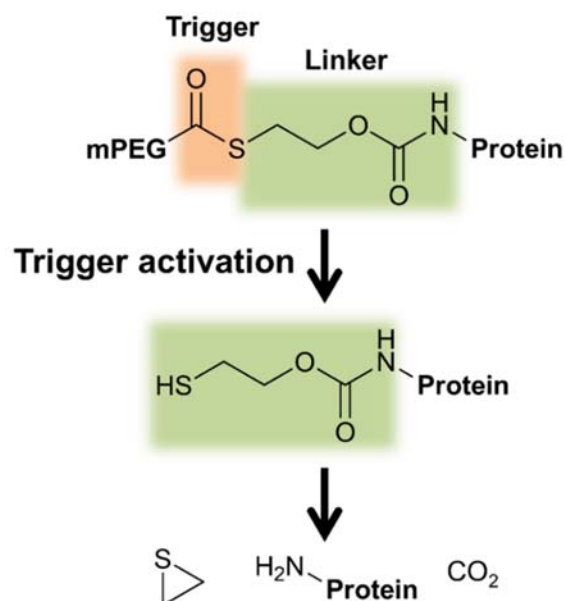
Figure 2.3. rPEGylation achieved by thiol–thioester exchange. Thiol–thioester exchange exposes a free thiol which initiates the release of the free protein by cyclization. De-PEGylation of conjugated peptide via thioester bond under reducing environment.[121]

2.5. 1, 6-Benzyl elimination linkers

2.5.1. Esterase-triggered

One of the most investigated rPEGylation strategies relies on the 1, 6-benzyl elimination of a linker molecule to release fully unmodified amino groups on proteins. This process is initiated by activation of a trigger group (Figure 2.4). Lee et al.[95] have exploited this chemistry for the preparation of a series of lysozyme–mPEG conjugates. De-PEGylation was triggered in rat plasma by the enzymatic cleavage of the ester bond trigger. While the conjugates themselves were stable in PBS pH 7.4, release was observed with a half-life of ~6 h in this buffer. The authors noted that de-PEGylation occurred three times more slowly than for a comparably rPEGylated small-molecule drug, suggesting the role of steric hindrance in enzyme-triggered reconversion. Complications could therefore potentially be observed for highly PEGylated proteins, due to restricted access to the trigger. To increase the rate of reconversion, Greenwald et al. developed more rapidly hydrolyzing linkers based on the concept of anchimeric assistance (intramolecular assistance to hydrolysis). [122] The authors designed a trigger consisting of an ester of a primary alcohol (**5** in Figure 2.4), which was more easily hydrolyzed than the ester trigger above (**4** in

Figure 2.4). Hydrolysis of the ester produced a carboxylate anion which in turn facilitated the rapid hydrolysis of the distal hindered ester link of the trigger, via a six-membered ring transition state. The rate of de-PEGylation was not affected by the presence of methyl substituents on the aromatic ring. The authors suggested that this is possibly because the anchimeric effect removed some of the steric effects on trigger activation. More recently Xie et al.[123] have reported the synthesis of a α,ω-hetero-bifunctional PEG bearing at one extremity the immolative linker **5**, and a second reactive group for decoration of the conjugate with ligands such as fluorophores peptides, antibodies, or other proteins. This report, however, does not describe the conjugation of the reactive polymer to a protein.

2.5.2. Disulfide-triggered

Zalipsky et al.[124] examined a variant of the approach above in which the trigger is changed from an ester to a disulfide (**6** in Figure 2.4). Thiol–disulfide exchange or reduction of the disulfide led to an unstable *p*-mercaptobenzyl carbamate intermediate, which broke down via 1, 6-benzyl elimination and decarboxylation to release the unmodified protein. The authors prepared an mPEG–lysozyme conjugate which spontaneously de-PEGylated upon exposure to thiols. However, a putative *p*-mercaptobenzyl carbamate lysozyme intermediate remained detectable in the reaction medium, indicating that self-immolation of the linker occurred relatively slowly. Following intravenous administration, the pharmacokinetics of mPEG–lysozyme conjugates was identical whether a cleavable and noncleavable linker was employed. This suggests that the low concentration of blood thiols may be insufficient in the given configuration of the disulfide to effectively trigger reconversion. Interestingly, after subcutaneous injection, a significantly higher area-under-the-curve was observed for the cleavable conjugate, suggesting disulfide trigger activation by thiols. Methods to alter the rate of thiol–disulfide exchange, which could be used to tune the rate of de-PEGylation, might be of use to optimize the rate of de-PEGylation. To further characterize the nature of thiols potentially involved in reconversion of the conjugate *in vivo*, the authors showed that overnight incubation of the conjugate in an albumin solution triggered disassembly. This suggests that this macromolecular thiol, with a moderately solvent-exposed cysteine residue, was able to interact with the disulfide trigger, despite expected steric hindrance considerations. Future work may be needed to accelerate the rate of immolation of the linker upon activation of the disulfide triggering, as this process appears to be slow at physiological pH.
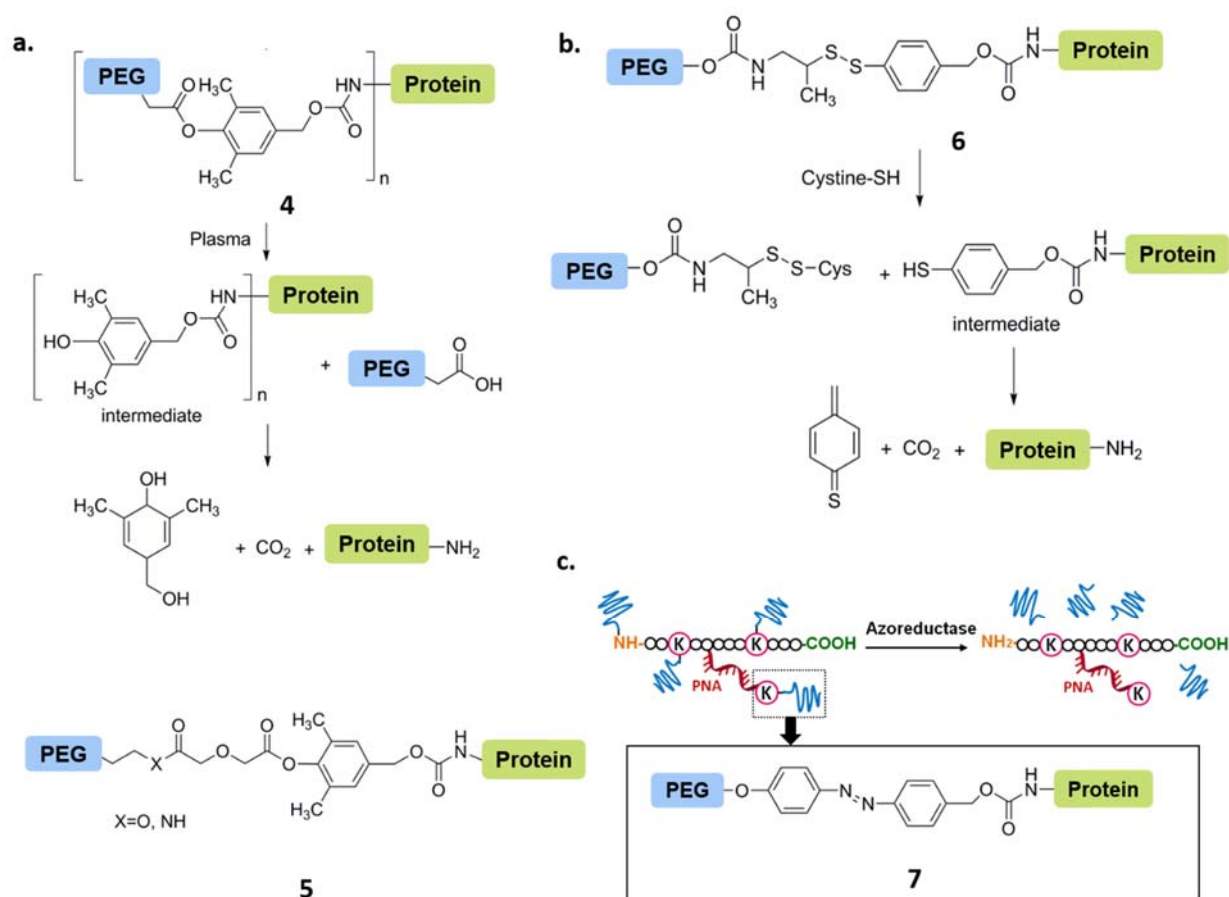
Figure 2.4. rPEGylation employing 1,6-Benzyl elimination linkers. (a) Examples of esterase-triggered linkers,[95, 122] (b) thiol–disulfide exchange-triggered linker,[124] and (c) azoreductase-triggered linker.[125]

2.5.3. Azoreductase-triggered

In a further adaptation of this linker design, Lee et al.[125] have recently prepared an activatable cell-penetrating peptide conjugate platform for the delivery of a peptide nucleic acid (PNA) drug to the colon (**7** in Figure 2.4). The concept relied on the rPEGylation of lysine residues of the cell-penetrating peptide (CPP) with a self-immolative aminobenzyl carbamate containing an azobenzene trigger. In its intact form, the PEGylated CPP is inactive, though reductive cleavage of the azobenzene in the colon by bacterial azoreductase is expected to initiate 1, 6-benzyl elimination. This releases the active CPP, which can then promote cellular uptake at this location. De-PEGylation releases 4-aminobenzyl alcohol, a byproduct reported to have low cytoxicity.[126] Since active azoreductases are not commercially available, the reconversion of the rPEGylated CPP–PNA was studied with a surrogate reducing agent, sodium dithionite. After incubation with this compound, disappearance of the PEGylated conjugate was observed with

reappearance of the free CPP–PNA. No evidence of a residual tag on the CPP was evidenced by MS, indicating that under these conditions the kinetics of linker immolation are rapid in comparison to the kinetics of trigger activation. Leriche et al.[127] recently conducted an extensive reactivity study to determine the key structural features that favor the dithionite-triggered reductive cleavage of the azo-arene group. Their work suggests that diazo triggers might potentially be tunable for controlling the rate of release by bacterial azoreductases via substitutions to the aromatic ring. This however remains to be examined experimentally.

2.6. β-eliminative linkers

Shechter and co-workers have developed a releasable linker for protein rPEGylation based on the β-elimination of a water-soluble sulfonated fluorenyloxycarbonyl *N*-hydroxysuccinimide ester (Figure 2.5a). This compound reacts with amino groups on proteins/peptides and also bears a maleimide group for connection to mPEG-SH by Michael addition. In a substantial body of work, bioconjugates of exendin-4, human GH, interferon-α2a, peptide YY$_{3-36}$, atrial natriuretic peptide, and insulin have been prepared and evaluated *in vitro* and *in vivo*.[128-132] The linker molecule itself absorbs light at 320 nm, which is convenient for determining the level of mPEG conjugation. The rate of de-PEGylation of the bio-conjugates was measured at pH 8.5, 37 ℃ which, for this type of linker, provided comparable cleavage results to that in serum. The peptides/proteins were released in a homogenous manner with an *in vitro* half-life of 12 hours and retention of biological activity. The constant rate of hydrolysis of the linker was due to the β-elimination reaction, which occurred at position 9 of the fluorenyl moiety, and is solely dependent on the pH of the surrounding medium. Furthermore, the released sulfonated fulvene moiety was reported to be nontoxic. While no effort to modulate the intrinsic rate of de-PEGylation has been attempted, such a modulation may be achievable for multiply-PEGylated proteins by varying the degree of polymer grafting.

Recently, Santi et al.[133] have developed a platform of β-eliminative linkers which incorporate a series of sulfone modulators that control the rate of de-PEGylation (Figure 2.5b). Release occurred on the basis of a nonenzymatic β-elimination reaction with preprogrammed and highly tunable cleavage rates. The linkers bore a succinimidyl carbonate group for attachment to an amine-containing peptide/protein and a tunable modulator that controls the rate of β-eliminative cleavage. The linkers provided predictable, tunable release rates of ligands from

macromolecular conjugates both *in vitro/vivo* with half-lives spanning hours to over one year at physiological pH. The rate of elimination was only modestly dependent on the basicity of the aliphatic amine component of the carbamate, suggesting only a small influence of the local chemical environment at the conjugation site. The co-product of the β-elimination is a PEGylated β-alkenyl sulfone, which is a Michael acceptor. The authors observed, however, that the reaction rates of such compounds with serum nucleophiles, such as 0.5 mM glutathione, were either competitive or slower than the rates of clearance of the polymer from the body. They further reported an excellent experimental Hammett correlation with σ constants for the substituents on the phenyl sulfone modulator (reported for pK_a values of substituted phenols), both *in vitro* and *in vivo*, using a model fluorophore rather than a protein. Release rates were two to three-fold faster *in vivo*. Predictable fine-tuning of *in vitro* β-elimination rates can thus be achieved by varying the substituent on the phenyl sulfone modulator by exploiting the availability of sigma constants for over 60 substituted phenols. More recently, the authors expanded this linker design for the modification of phenol groups rather than amino groups, though has not yet been tested for peptide/protein–polymer conjugation.[134]
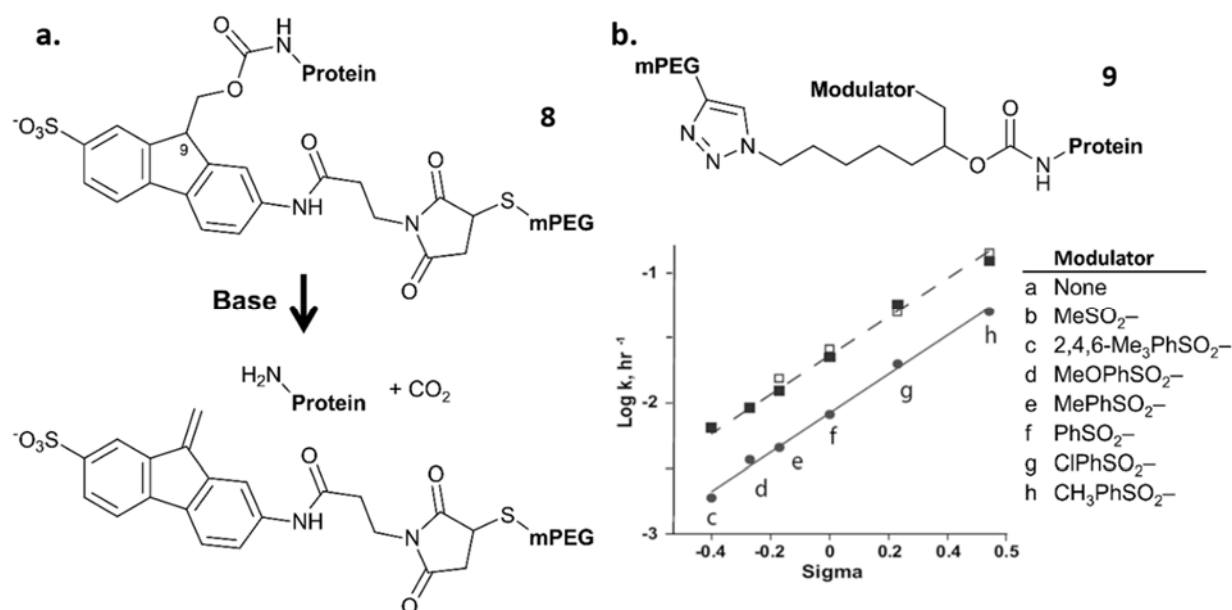
Figure 2.5. rPEGYlation employing β-eliminative linkers. (**a**) De-PEGylation of sulfonated fluorenyloxycarbonyl *N*-hydroxysuccinimide ester linkers is solely dependent on pH. (**b**) De-PEGylation of β-eliminative linkers is controlled *in vitro* and *in vivo* by a modulator group. An excellent Hammett correlation was observed, suggesting predictability in adjusting the rate of de-PEGylation by additional modification of the modulator group, using σ constants for the substituents (note: σ constant for *b* is not available). (*in vitro* (−●−), in rats (- -■- -), and mice (- -□- -). Adapted from original contributions with permission.[133]

2.7. Bicin linkers

Aliphatic esters structures synthesized from *N*-modified bis-2-hydroxyethylglycinamide (bicin) have been examined as self-immolating linkers for rPEGylation. Zhao et al. have prepared linear (**10** in Figure 2.6) and branched (**11** in Figure 2.6) mPEG bio-conjugates of both lysozyme and interferon β1b (Figure 2.6).[83] Bicin linkers de-PEGylate by cyclization after hydrolysis of an ester trigger. The rate of protein release from the linear PEG conjugate was faster than for the branched counterpart. This phenomenon could be due to reduced hindrance of the linear PEG conjugates toward esterase or non-enzymatic hydrolysis. The kinetics of protein release for proteins bearing multiple mPEG chains in rat plasma and *in vivo* correlated with the number of polymer chains, and the half-lives observed spanned from hours to days, within a therapeutically relevant window. The authors mentioned that the bicin linker bearing two mPEG chains sequentially release one mPEG after another, which could complicate the analysis of the resulting pharmacokinetics.
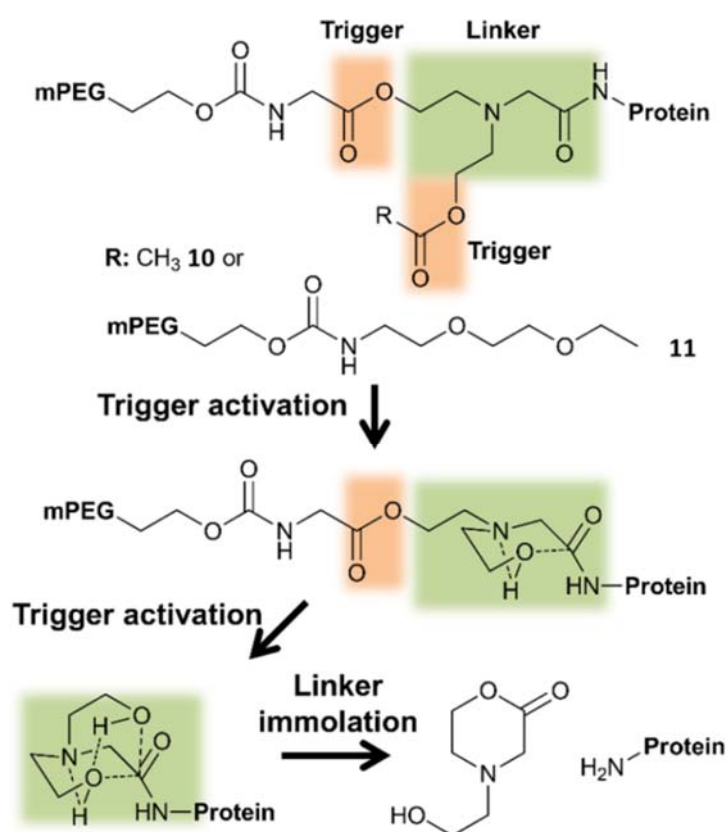
Figure 2.6. rPEGylation achieved using bicin linkers. Hydrolysis of the ester triggers induces release of the protein, via cyclization of bicin.[83]

Filpula *et al*. pursued work with this linker by PEGylating immunotoxins.[84] Immunotoxins produce potent antitumor responses, but the toxin domain may exhibit nonspecific binding to normal tissues. In addition, neutralizing antibodies can be formed resulting in dose-limiting toxicity or diminished therapeutic potency. Also, in contrast to antibodies, these systems do not possess an inherently long circulation time, due to the lack of an Fc domain. The authors examined conjugates containing the linear and branched bicin linkers. The conjugates exhibited prolonged blood residency time and greatly expanded the therapeutic exposure while reducing the non-specific toxicity of the immunotoxin. In addition to controlling the rate of protein release via the number of conjugated mPEG chains, in principle, altering the structure of the ester trigger could be used to control the intrinsic rate of de-PEGylation, which would be valuable in the case of mono-PEGylated bio-conjugates.

2.8. β-Alanine linkers

Pasut et al. have reported an rPEGylation strategy based on a β-alanine spacer between mPEG and the protein (**12** in Figure 2.7).[135] Following conjugation of mPEG to human GH, partial de-PEGylation was observed over a 3–5 day period in phosphate buffer, due to hydrolysis of the amide bond between the linker and the protein. In a follow-up study, the authors used model peptides to determine that the PEGylation of lysine and tyrosine residues using this linker is permanent. However, histidine residues were modified reversibly.[136] The use of this approach for effective rPEGylation and full release of the protein may therefore require that temporary protecting groups be installed on protein amino and phenol groups, so that the polymer is selectively conjugated to histidine residues.
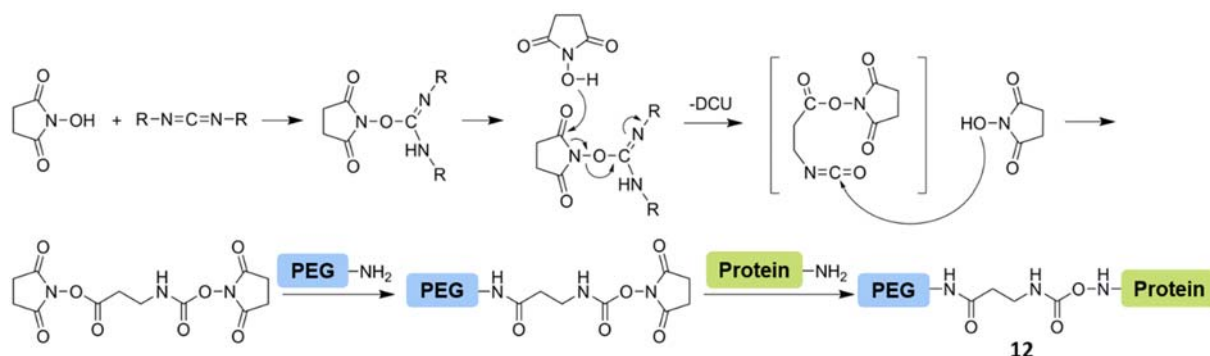

Figure 2.7. Mechanism of N-[(succinimidooxy)carbonyl]-β-alanine N-hydroxysuccinimide ester formation, and the following conjugation of PEG-NH$_2$ and protein-NH$_2$.[135]
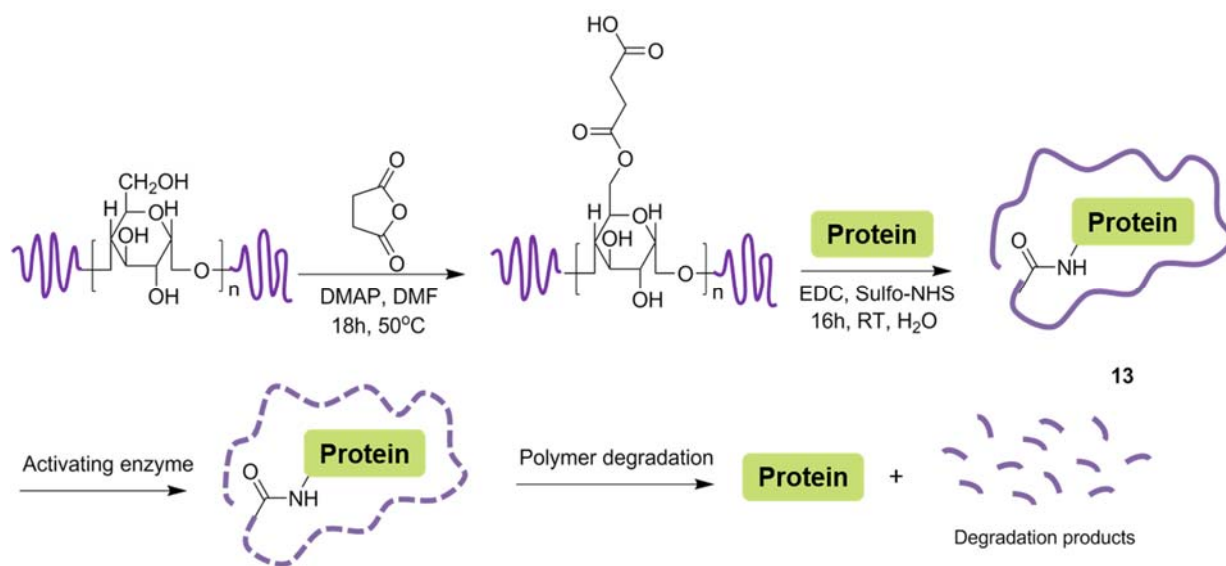
2.9. Biodegradable polymer

Figure 2.8. PUMPT strategy for reversible protein-polymer bio-conjugation.[81] Purple lines represent dextrin.

In 2008, Duncan and co-workers introduced in interesting strategy to recover protein activity from polymer-modified bioconjugates. This approach, referred to as PUMPT, resides in the use of a polymer that can be effectively hydrolyzed in biological fluids by enzymes to ultimately release the protein (**13** in Figure 2.8).[81] The polymer consisted of the polysaccharide dextrin, a linear α-1, 4-poly(glucose), which is degraded to maltose and isomaltose by α-amylases. This polymer can be modified by succinoylation for both conjugation to proteins, and to slow the rate of enzymatic degradation. The authors grafted this polymer to trypsin as well as melanocyte stimulating hormone, and demonstrated that coincubation with amylases partially regenerated protein activity. However, the extent of regeneration of protein/peptide activity in these *in vitro* models is complex to interpret because of the variability associated with the hydrolysis phenomenon. Subsequently, the authors developed a bioresponsive dextrin–phospholipase A$_2$ (PLA$_2$) conjugate which could reduce PLA$_2$ systemic toxicity but still retain its antitumor activity upon α-amylase-triggered release of the protein in the tumor interstitium.[80] In addition, Ferguson et al. investigated the replacement of dextrin with hyaluronic acid (HA), and prepared bioconjugates of trypsin with enhanced stability.[137] Restoration of biological activity was observed following exposure to hyaluronidase. HA conjugation did not alter trypsin's activity significantly though incubation of the conjugate with HAase increased its activity to 145 % compared to that of free enzyme. The underlying reason for this increase of activity remains to be

determined, though the results suggested that only partial removal of HA was occurring. Recently, biodegradable analogues of mPEG have been developed by incorporating hydrolysable linkers within the polymer's main chain.[138-140] Such an innovation represents an interesting opportunity for PUMPT of proteins using synthetic polyethers such as mPEG, rather than intrinsically biodegradable polymers such as those discussed above.

2.10. E1cb elimination linkers

Göfperich and co-workers have examined phenyl carbamates as hydrolysable linkers for conjugation of linear mPEG and 4-arm PEG to lysozyme and fluorescent BSA (Figure 2.9).[141, 142] The hydrolysis of phenyl carbamates in neutral and basic solutions proceeds by an E1cB elimination reaction involving the intermediate formation of an unstable isocyanate, which then disintegrates into a primary amine and carbon dioxide. This process releases the native protein from the polymer. The authors implemented this conjugation approach into the design of a hydrogel by mixing the multiarm polymer with protein. De-PEGylation induced disassembly of the network and ultimately the release of the native protein. In a model study, the rate of de-PEGylation was assessed using linear mPEG conjugated to lysozyme. For a lysozyme–mPEG conjugate bearing 3–5 mPEG chains, almost complete release of lysozyme was achieved within 24 hours at 50 ºC. Three linker structures were modified to control the rate of de-PEGylation. Electron-withdrawing carbamoyl groups in the *ortho*- or *para*-position (**14** in Figure 2.9) stabilize the phenolate ion by resonance and accelerate hydrolysis compared to the nonsubstituted analog. On the other hand, when the carbamoyl group was located in the *meta*-position (**16** in Figure 2.9), only a minor influence on rate of hydrolysis was observed. However, when an electron-releasing alkyl group was placed in the *para*-position (**15** in Figure 2.9), destabilization of the phenolate ion dramatically slowed hydrolysis kinetics. Unfortunately, analysis of the de-PEGylation process revealed only partial release of lysozyme.
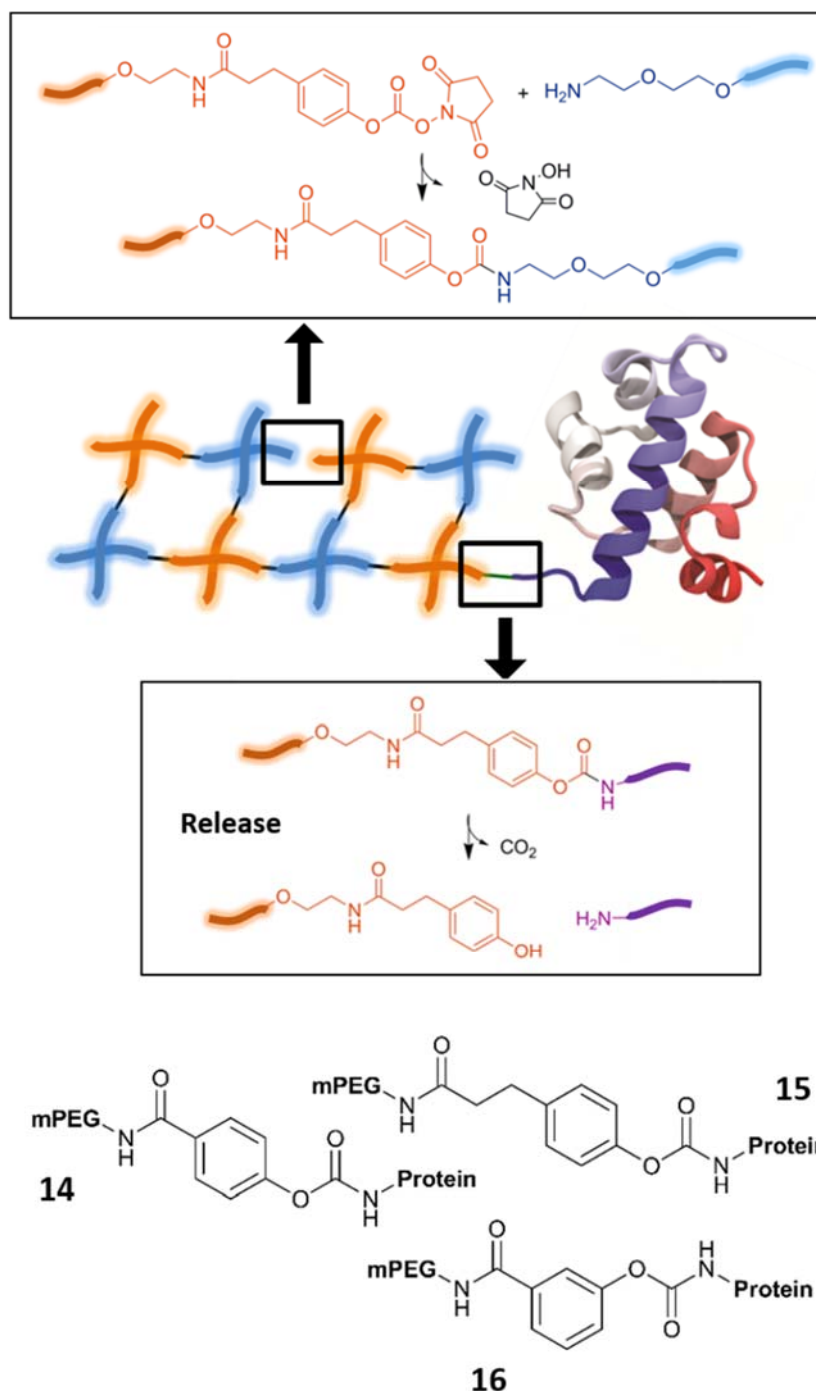
Figure 2.9. rPEGylation achieved with E1cb linkers. In the box, an example of the steps involved in protein release is given. An intermediate isocyanate is formed, which may be responsible for side-reactions.[141, 142]

The authors suggested that this could result on one hand from contaminants in the PEGylation reagent that permanently modify the protein, or from the reaction of nucleophiles on the protein with the isocyanate produced during the hydrolysis of the phenyl carbamate, which also produces stable chemical bond. The latter hypothesis was supported by evidence of protein dimer formation, due to reaction of nucleophiles on one protein with an isocyanate on another.

However, self-aggregation of lysozyme could not be discounted. This side-reaction could become problematic in the more complex environment of biological fluids due to the presence of numerous natural nucleophiles. This challenge should be further investigated and alterations to the carbamate implemented to minimize this phenomenon. An interesting aspect of this work is that, within the hydrogel, proteins act as cross-linking agents to which are connected to multiple polymer chains. While de-PEGylation of the hydrogel network can ultimately release a fully unmodified protein, is can also release partially PEGylated ones into circulation. The latter should have an extended circulation lifetime due to the attached polymer chains. This particularity may offer an addition degree of freedom for achieving sustained blood concentration of protein therapeutics from hydrogel implants. However, pharmacokinetics may be more complex, and this aspect should be investigated in future work.

2.11. Photoimmolative linker
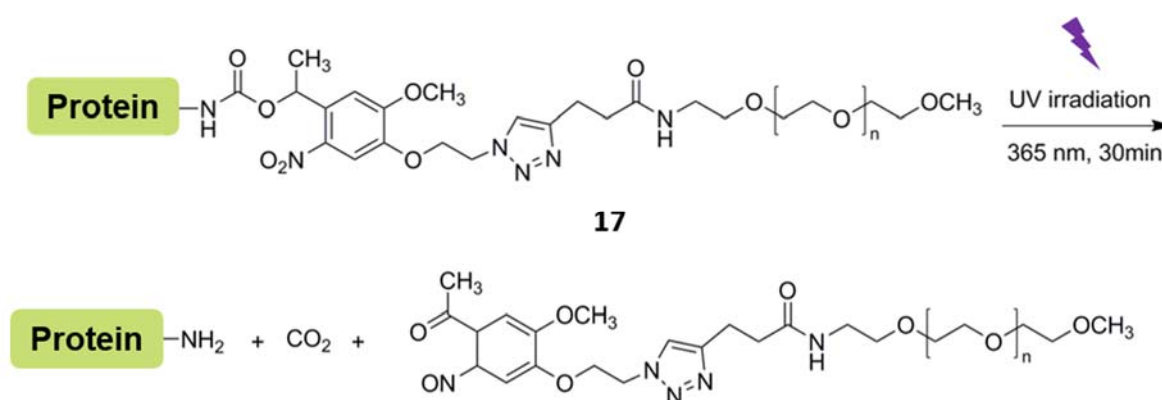
Figure 2.10. rPEGylation achieved by a photo-immolative linker. Exposure to light (365 nm) for 30 min leads to release of the protein.[143]

Light is a unique nonphysiological stimulus for releasing proteins from PEGylated adducts. Georgianna et al. have developed a photocleavable linker based on *ortho*-nitrobenzene that responds to non-phototoxic UV light (365 nm) to release the native protein (**17** in Figure 2.10).[143] Full release of lysozyme from a multi-PEGylated conjugate was achieved after 30 minute irradiation, using a 25 W source. However, the latter only possessed 50% of the activity expected of the protein. The authors indicated that an incomplete restoration of enzymatic activity is not uncommon in the light-activation of biological processes. While photo-control of reconversion of

mPEG–protein conjugates may be interesting for, e.g., localized therapies, pulsatile or controlled release, translation of this concept to *in vivo* studies will require that the opacity of biological tissues to UV light be addressed and the mechanism of protein deactivation be re-examined.
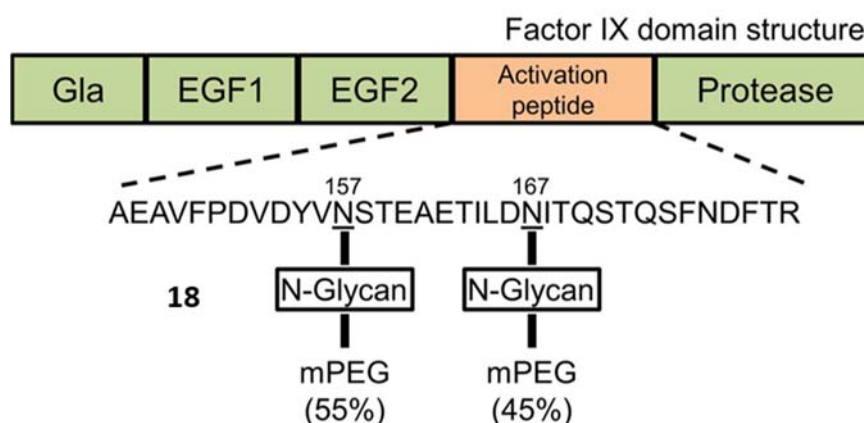
2.12. Zymogen activation

Figure 2.11. rPEGylation achieved by zymogen activation. Domain structure of FIX (Gla: γ-carboxyglutamic acid domain; EGF-1 and EGF-2: epidermal growth factor domains; Protease: serine protease domain). EGF-2 connects to the serine protease domain through a linker peptide that is required for a proper orientation and folding of serine proteases. To have a physiologically active FIX, two cleavages must occur to remove a 35 amino acid region that precedes the catalytic site.[144]

In an interesting example, Østergaard et al. described an rPEGylation strategy based on the activation of a protein zymogen.[144] Selective glyco-PEGylation was achieved using the substrate promiscuity of the sialyl transferase ST3Gal3, which allowed for the transfer of cytidine 5'-monophosphosialic acid-6'-mPEG (40 kDa) to the terminal galactoses of the *N*-glycans in the activation peptide of the desialylated recombinant Factor IX protein (FIX) (**18** in Figure 2.11). FIX is a vitamin K–dependent glycoprotein and an essential protease of the hemostatic system. FIX is converted to the 2-chain activated form by the tissue factor–factor VIIa complex or factor XIa. Activation occurs by limited proteolysis at Arg145 and Arg180 in the protease domain and liberates a 35-amino acid activation peptide that carries the only 2 N-linked glycans in the protein. The attached mPEG moiety is thus present only on the circulating zymogen form of the bioconjugate, and release of the final protein remains subjected to normal physiology regulation, while benefiting from enhanced circulation due to the mPEG component.
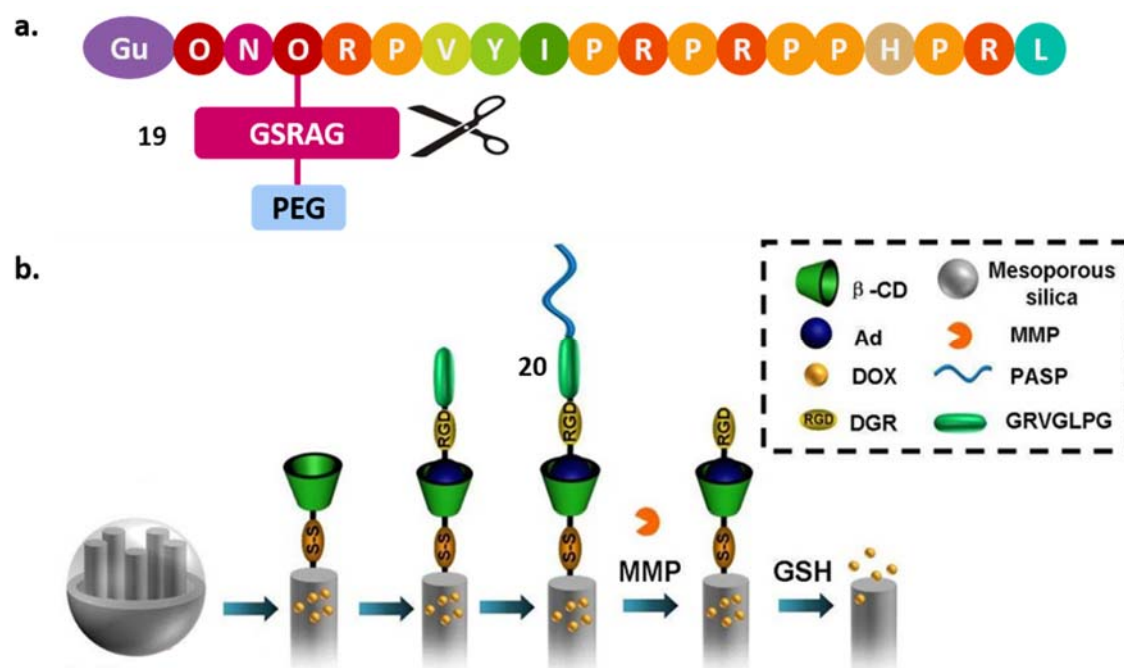
2.13. Proteolysis

Figure 2.12. (a) N-terminal rPEGylated AMP. Release is mediated by enzymatic hydrolysis of a peptide linker sequence;[145] (b) Structure of multifunctional envelop-type mesoporous silica nanoparticles for tumor-triggered drug delivery. The PASP protection layer can be removed by proteolysis in response to MMP at tumor site.[146]

Nollmann et al.[145] reported a strategy for the rPEGylation of proline-rich AMPs by *N*-terminal extension with three residues (glycine-alanine-arginine) and an mPEG chain (**19** in Figure 2.12a). The active peptide is rationalized to be released by trypsin-like activity in the body, due to digestion at the tripeptide. Interestingly, peptides were released at a similar rate, independently of the molecular weight of the mPEG moiety (0.75 and 5 kDa). This indicated that for this range of mPEG molecular weights, recognition of the cleavable peptide sequence was not hampered by the polymer. In addition, altering the sequence of the peptide linker was shown to control the rate of release. For instance, a second linker, glycine-alanine-arginine-serine-glycine, could be cleaved with a half-life of 40 min in mouse serum (37 °C), which is faster than the corresponding linker above. In another example, Zhang et al. conjugated poly(aspartic acid) to a cell-penetrating peptide via a matrix metalloproteinase (MMP)-cleavable peptide (PLGVR) (**20** in Figure 2.12b).[146] Upon exposure to MMP at tumor sites, the polyanion is expected to be removed by proteolysis, which would lead to exposure of cell-penetrating peptide and concurrent cellular uptake of the system. *In vitro*, the authors demonstrated the implication of MMP on

release. In general, because of the simplicity of solid-phase-peptide-synthesis, the approaches discussed in this section are easily implemented and adaptable. The rate of release in biological milieu may be complication by variability in the expression levels of the enzymes responsible for release of the peptide.
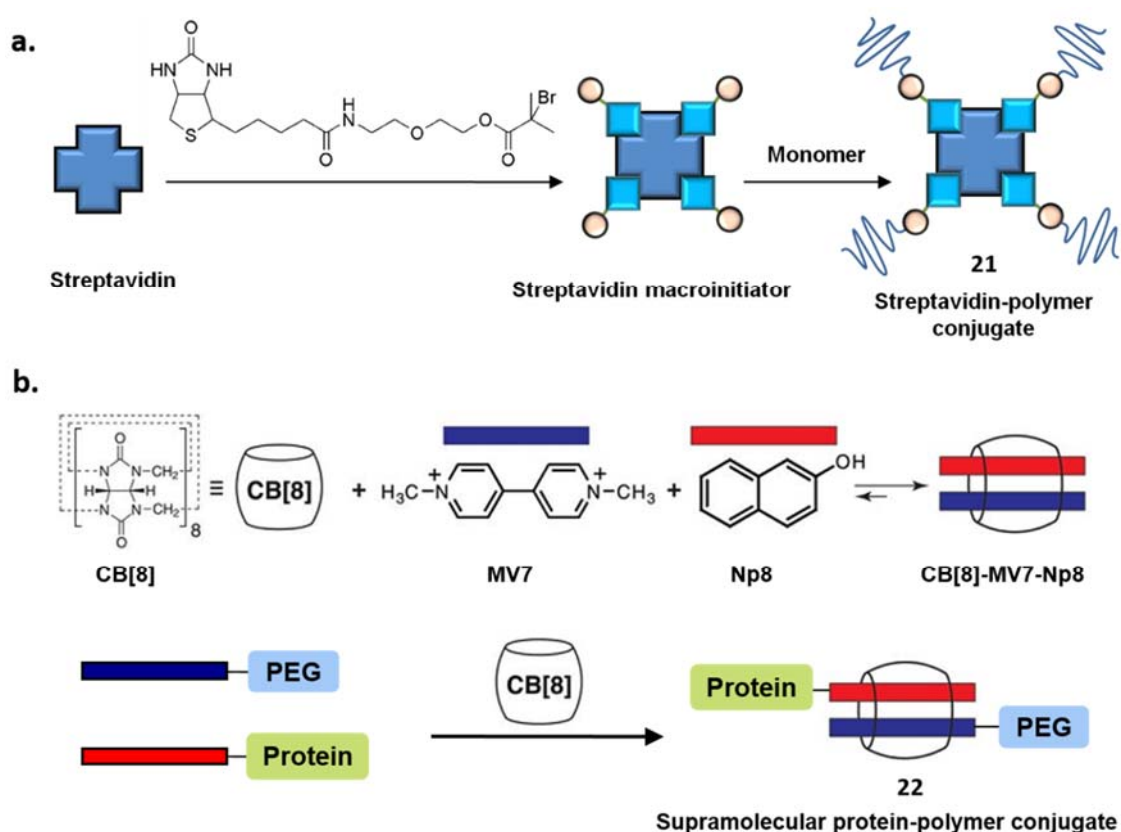
2.14. Host–guest interactions

Figure 2.13. (a) Streptavidin-polymer conjugate formed via ATRP. The PNIPAAm polymerization can be initiated by the modified biotins which are binded with streptavidin and streptavidin still remains bioactivity after polymerization.[147] (b) Supramolecular conjugated system of CB[8] with modified double guests. The macrocyclic host CB[8] can 'handcuff' an electron-poor guest and an electron-rich guest together forming a ternary complex.[148]

To the extent of our knowledge, the first example of rPEGylation based on host–guest interactions was from Bontempo et al.[147] who described the synthesis of poly(*N*-isopropylacrylamide) possessing a terminal biotin unit by atom transfer radical polymerization

(**21** in Figure 2.13a). This polymer readily conjugated to the corresponding binding sites on streptavidin, a process which could be reversed in *N,N*-dimethylformamide/water at 90 ºC within 1 h. More recently, Biedermann et al.[148] reported a traceless rPEGylation approach based on hydrophobic guests interacting with natural hydrophobic guest binding sites on proteins (**22** in Figure 2.13b). mPEG bearing a single or two dansyl groups were prepared, which when added to a salmon calcitonin solution decreased the susceptibility to aggregation of this protein. This effect was observed for protein/mPEG ratios from 100/1 to 1/1, but deteriorated at higher mPEG content. An mPEG with a molecular weight of 2 kDa was found to be optimal for preventing aggregation, and no added benefit between a single or two dansyl units was observed. This observation was suggested to result from hindered interaction between the protein and polymer, due to steric hindrance of the polymer. Unfortunately, binding constants between the two macromolecules were not reported.

2.15. Ion Complexation
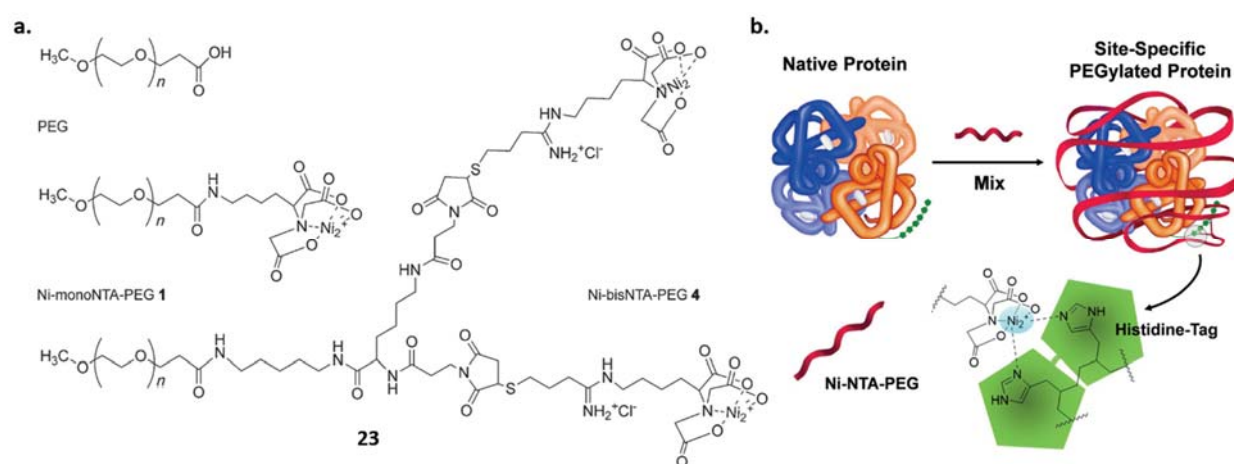
Figure 2.14. (a) Structures of mPEG and Ni-NTA-PEG analogues; (b) Site-specific PEGylation of a His-tag-fused protein with reactive Ni-NTA-PEG.[150]

In addition to exploiting reversible covalent coupling approaches for rPEGylation, recent efforts have been directed towards exploiting coordination chemistry. Early work by Kapanidis et al. have shown that nitrilotriacetic acid (NTA) has a k_d of 10 μM towards hexa-histidine tags on proteins.[149] NTA is able to associate with the imidazole side-chain of histidine residues in proteins through a reversible coordination bond, mediated by a metal ion. Szoka and co-workers

pursued this finding and developed an optimized a tri-NTA agent with a k_d of 20 nM for a hexa-histidine peptide.[150] In an interesting adaptation of this approach, Mero et al. have investigated the NTA-mediated rPEGylation of proteins not possessing hexa-histidine tags.[151] The authors synthesized an eight-arm PEG modified at its eight extremities with NTA, for cooperative complexation of isolated solvent-exposed histidine residues (Figure 2.14). It was rationalized that this structure would have sufficient flexibility to coordinate individual natural histidine residues dispersed on the protein surface. The authors examined their hypothesis with five different proteins, though only two efficiently complexed the PEG. Granulocyte colony stimulating factor strongly associated with the PEG in the presence of copper with a k_d of 4.3 nM, but the complex did not show a half-life prolongation *in vivo*. The authors attributed this result from either competition with plasma proteins or displacement *in vivo*. *In vitro*, human serum albumin did not compete for the polymer or cause de-PEGylation. More recently, Kim et al.[152] synthesized a bis-NTA mPEG reagent for the modification of hexa-histidine tagged proteins, a process which was mediated by Ni^{2+}. Using this reagent, the authors PEGylate TRAIL (k_d 0.27 μM). Improved *in vivo* properties were observed yielding three- to four-fold improved efficacy over native TRAIL in terms of solution stability and half-life versus the native protein. The authors observed that the bioactivity of the protein was not strongly deactivated by the polymer due to the benefits of the site-specific PEGylation. Future adaptations of the structure of the NTA ligand may potentially permit tuning of the rate of disassembly of the bioconjugate.

2.16. Outlook

This contribution highlights that numerous opportunities exist for the rPEGylation of peptides/proteins and several platforms already exist that permit their predictable and tunable release in biological milieu. De-PEGylation is typically triggered by hydrolysis, enzymatic processing, or reaction/exchange with endogenous nucleophiles. Future work may be directed toward identifying new types of dynamic covalent bonds that could exploit alternative stimuli or disease-associated stimuli. This could enable the targeted release of the proteins to specific regions in the body. In addition, one of the major challenges of this field is the development of self-immolating linkers which remove themselves from the protein upon activation. Rapid self-immolation is sometimes difficult to achieve, and can lead to the released protein possessing residual tags that persist and initiate unwanted side-reactions. This is a shared concern with non-traceless rPEGylation approaches. Additionally, most rPEGylation strategies have focused thus

far on the modification of amino groups on proteins and, arguably, strategies for the modification of other residues (or glycans) remain limited. Future work may be directed to the rPEGylation of other types of amino acids prone to degradation or associated with immune peptide sequences. This may enable the design of better protected rPEGylated protein therapeutics. Furthermore, while most work in this field has been performed using mPEG as polymer, recent studies have shown that structurally and functionally complex may confer new and exciting properties to protein bio-conjugates.[74-75, 153-155] Adapting rPEGylation to new polymer platforms may thus offer new opportunities for the development of functional bio-conjugates. Finally, while the specific case of reversible conjugation of polymers to peptides/proteins is discussed herein, lessons learned herein can be adapted for the reversible modification of other entities (drugs, surfaces, nanoparticles, etc.) with polymers.[85]

Chapter 3.

Releasable and traceless PEGylation of arginine-rich antimicrobial peptides

3.1. Introduction

Sepsis causes millions of deaths annually and is provoked by the immune system's response to a serious infection, most commonly from bacteria.[156-157] While the incidence of septic shock has increased over the years, mortality rates have remained constant or have decreased only slightly.[158] One major challenge is the development of multidrug-resistant bacterial strains that respond poorly to conventional antibiotics.[159-161] This points to the need for alternatives, such as AMPs.[162] Natural AMPs protect organisms against microbes by exerting a direct antibiotic activity, in addition to acting as effectors and regulators of the innate immune system. Synthetic variants are in development or in clinical evaluation. As pharmaceuticals, however, AMPs are small and have short circulatory half-lives, which can be on the order of tens of minutes, and are susceptible to proteolysis. Indeed, an important subset of AMPs are rich in arginine residues, which are necessary for interaction with bacterial membranes,[163] but, unfortunately, also the targets of trypsin-like proteases. These combined challenges can impose higher or more frequent dosing to maintain adequate blood levels, which is incompatible with the toxic side-effects observed at high doses, especially for lytic AMPs.

Established strategies for addressing such shortcomings, such as PEGylation, [39, 46, 82, 164-167] are inapplicable to AMPs because they can inactivate the small AMP. [86, 89, 91, 104, 168-169] However, the emerging concept of rPEGylation could provide the solution, as "native" AMPs would be released in the body with full recovery of biological activity.[85, 97, 100-101, 103-104, 111, 170-171] Nollmann et al.[145] have recently reported the rPEGylation of proline-rich AMPs by their N-terminal extension with mPEG and a linker that can be digested by human serum proteases. While exciting new rPEGylation chemistry is emerging, these almost exclusively involve protecting lysine residues or the N-terminus of peptides/proteins. In addition, strategies that do not rely on blood-borne triggers (enzymes), which might display patient-variability, could lead to a more reproducible behavior *in vivo*. Developing rPEGylation chemistry to temporarily masks other residues is therefore timely and of interest. Recently, we have reported the coupling chemistry for selective conjugation of polymers to arginine residues based on the selective coupling of arginine to methylglyoxal.[44] PGO, an aromatic α-oxo-aldehyde, which has the great selectivity reacting with arginine residues in a pH-dependent manner. In this study, a novel effective specific rPEGylation chemistry targeting in arginine residues, which may lead to long-circulating and stable formulation with the ability to release the conjugated native AMPs in a tunable rate, was developed and evaluated under various conditions (Figure 3.1). The synthesis route of PEGylation reagents can be found in Figure 3.1a. This arginine rPEGylation approach may provide a general

method for optimizing the pharmacological properties of peptides/proteins with high efficiency, especially for arginine-rich AMPs.
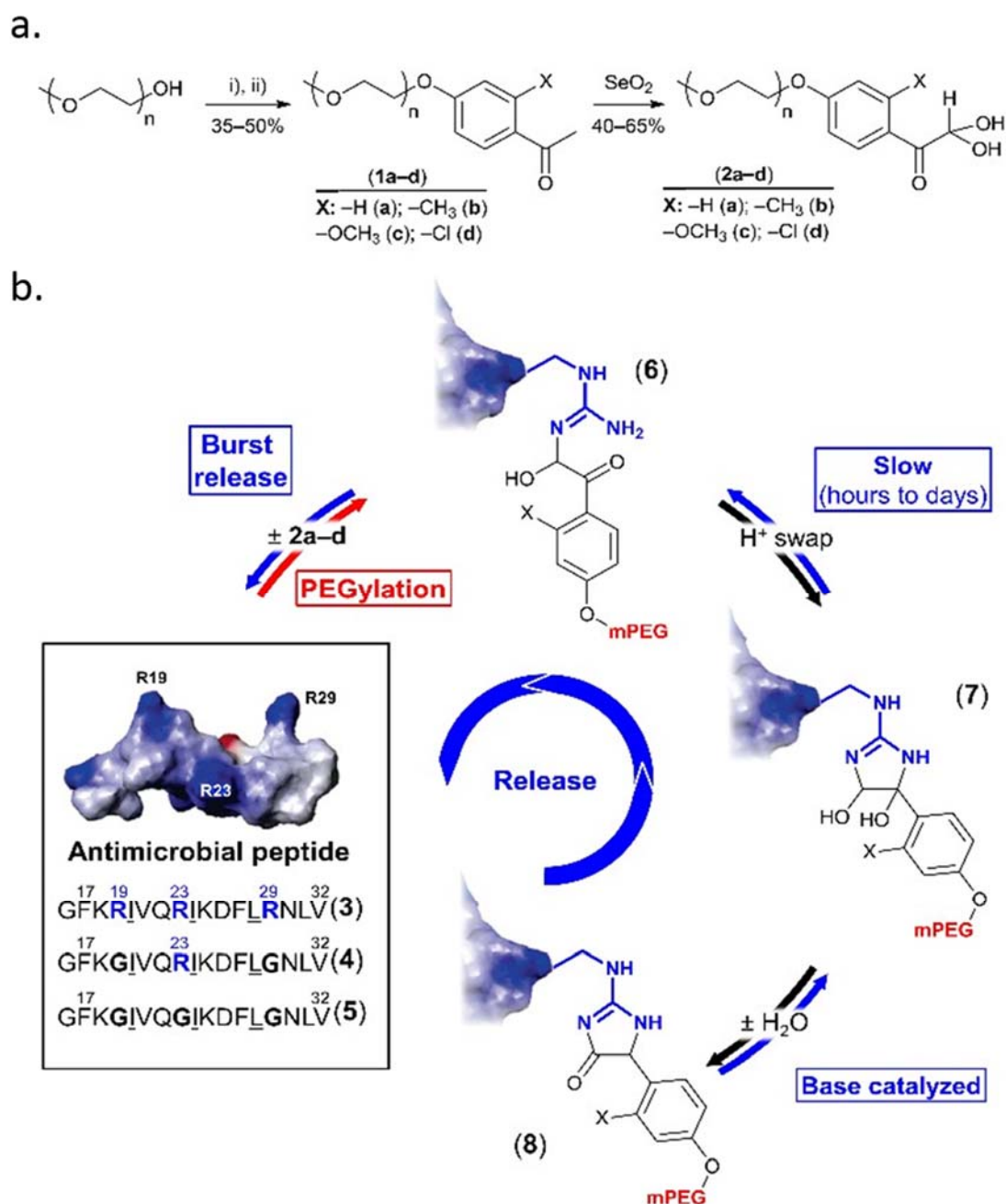

Figure 3.1. rPEGylation of arginine-rich AMPs. (a) Synthesis of arginine-reactive polymers (i: tosyl chloride, triethylamine; ii: o-substituted p-hydroxyacetophenone, K_2CO_3). (b) Sequence and 3D structure (inset from Ref. 11 with permission) of model AMP (3) and controls with one (4) or no arginine residues (5). Structure of three forms (**6–8**) of the conjugates obtained.

As the only human cathelicidin identified to date, LL-37,[172] which contains a few positive charges provided by arginines, has been demonstrated as an essential part in human innate immune system with ability to kill both Gram-positive and Gram-negative bacteria, enveloped viruses, fungi and even cancerous cells.[173] It also appears to play an important role in chemotaxis,[174] signal transduction[175-176] and angiogenesis. Li *et al.* studied the relationship between the structure and the activity of LL-37 and they suggested that the C-terminal fragment of LL-37 (17-32) with 3 arginine residues shown high antibacterial activity (Figure 3.1b). While the D-amino acids were used to substitute the L-diastereomer at isoleucine (position 20 and 24) and leucine (position 28), it exhibited quite similar antibacterial activity but lost the toxicity to normal human cells. Therefore, this short AMP containing three arginine residues (**3** in Figure 3.1b) derived from LL-37,[172] was selected as the model. LL-37 fragment derivatives have been considered for the chronic otitis media,[177] making this choice relevant. Variants containing a single (**4**) or no arginine residues (**5**) served as controls. The peptides containing arginine residues are supposed to react with **2a–d** reversibly and rapidly to form a hemiaminal structure (**6**), which could be cyclized to the dihemiaminal structure (**7**). Dehydration of **7** produces dehydrated product **8**.

3.2. Materials and Methods

3.2.1. Materials

4-Hydroxyacetophenone, mPEG-0.35 kDa, silica gel (60 Å, 230–400 mesh), and *p*-toluenesulfonyl chloride were obtained from Fluka (various global suppliers). Fetal bovine serum (FBS), hydrochloride acid (37%), 4'-hydroxy-2'-methylacetophenone, selenium dioxide, triethylamine, and trifluoroacetic acid were obtained from Sigma-Aldrich (various global suppliers). 4'-Hydroxy-2'-methoxyacetophenone was obtained from Apollo Scientific Limited (Cheshire, UK). 4'-Hydroxy-2'-chloroacetophenone was obtained from AK Scientific, Inc. (Union City, CA, USA). In addition to the chemicals above, all buffer salts and solvents were purchased as the highest possible grade, and used as received. Deuterium oxide and chloroform-d were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Peptides (from N- to C-terminus) GGRGGW, GEREGW, EEREEW, GKRKGW, KKRKKW, and GKGKGW were purchased N-terminally acetylated and C-terminally amidated from Selleck

Chemicals (Houston, TX, USA). Fluorescent peptides (from N- to C-terminus) (FITC)-GFKR{D-I}VQR{D-I}KDF{D-L}RNLV (**3**), (FITC)-GFKG{D-I}VQR{D-I}KDF{D-L}GNLV (**4**), and (FITC)-GFKG{D-I}VQG{D-I}KDF{D-L}GNLV (**5**) were purchased C-terminally amidated from Chempeptide Limited (Shanghai, PR China). All peptides were purchased at >95% purity and were supplied with analytical chromatograms and mass spectra.

3.2.2. Analysis equipment

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AV400 spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at 400 MHz for protons. Chemical shifts (δ) are reported in ppm relative to residual solvent signals.

Semi-preparative and analytical chromatography (for purification and monitoring kinetics, respectively) were performed with a high-performance liquid chromatography (HPLC) system (Merck HITACHI, Tokyo, Japan) equipped with an L-7100 pump, a Diode Array L-7455 detector and an FL L-7485 fluorescence detector [$\lambda_{ex/em}$ = 488 nm/525 nm]. The XBridge^{TM} BEH300 Prep C18 column (5 μm, 10 mm × 150 mm) was used for purification and XBridge^{TM} BEH C18 column (5 μm, 4.6 mm × 250 mm) was used for monitoring the kinetic process. The mobile phase was water and acetonitrile (ACN), both supplemented with 0.1 vol% trifluoroacetic acid (TFA), and the flow rate was 4.4 mL·min^{-1} for purification and 1.0 mL·min^{-1} for kinetic measurements, respectively. A gradient method (0–2 min 0% ACN; 25 min 100% ACN; 30 min 0% ACN was used for both purification and kinetic studies.

Liquid chromatography-mass spectrometry (LC-MS) was performed using a Rheos Allegro quaternary pump, Accela^{TM} PDA Detector, XBridge C18 column (5 μm, 4.6 mm × 250 mm) and a Thermo LTQ XL mass spectrometer (Thermo Fisher Scientific, Vernon Hills, IL, USA). Mobile phases and gradients were the same as above. Data was analyzed with XCalibur control software.

An Infinite M200$^{®}$ Tecan microplate reader (Männedorf, Switzerland) was used to record UV absorbance and fluorescence [UV: 452 nm; FL: $\lambda_{ex/em}$ =312 nm/395 nm] of the conjugates. 200 μL of sample solution was transferred into the 96-well UV plate (Costar$^{®}$ 3370, flat bottom, sterile polystyrene, Corning Lifesciences, Corning, NY, USA).

3.2.3. Synthesis of arginine-reactive mPEGs

3.2.3.1. Synthesis of mPEG tosylate
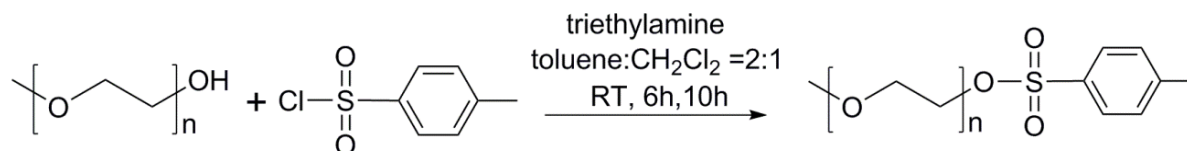
mPEG (4 g, 11.42 mmol) was dissolved in 120 mL of toluene and 60 mL of dichloromethane. *p*-Toluenesulfonyl chloride (11.44 g, 60 mmol) was added thereto, followed by the addition of 7.5 mL of triethylamine. The mixture was stirred for 6 h at r.t., and was followed by a second addition of *p*-toluenesulfonyl chloride (11.44 g, 60 mmol). The reaction was pursued for another 10 h, insoluble matter was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography using mixtures of dichloromethane and methanol as eluent, yielding 4.04 g of the desired product (8.08 mmol, 70.7%). The product was characterized by ^{1}H NMR and ^{13}C NMR.

3.2.3.2. Synthesis of 4'-mPEG-2'-substituted-acetophenone (1a–d)

X= -H (a); -CH$_3$ (b); -OCH$_3$ (c); -Cl (d)
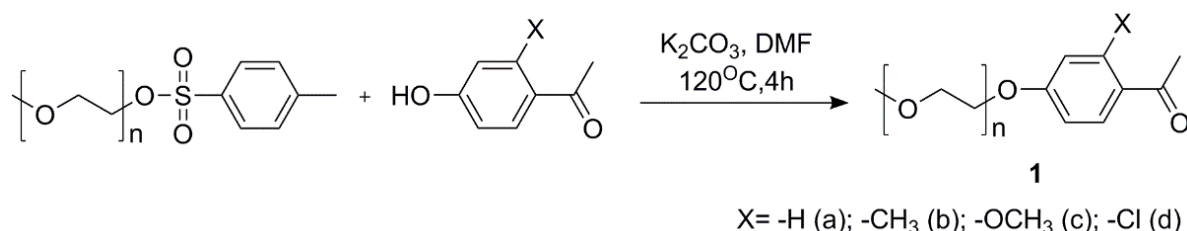
Compounds 1a–d were all prepared using the same protocol. As a representative example, mPEG tosylate (1 g, 2 mmol) and 4-hydroxyacetophenone (2.72 g, 20 mmol) were dissolved in 100 mL of N,N-dimethylformamide . Then, potassium carbonate (2.76 g, 20 mmol) was added and the mixture was stirred for 4 h at 120 °C. Following this, the insoluble matter was filtered off with filter paper and the filtrate concentrated under reduced pressure. The residue was purified by silica gel chromatography using mixtures of dichloromethane and methanol as eluent, and the desired product dried in vacuo. The recovered yields were 75%, 61%, 51%, and 65% for 1a–d, respectively. The product was characterized by ^{1}H NMR and ^{13}C NMR.

3.2.3.3. Synthesis of 4'-mPEG-2'-substituted-phenylglyoxal (2a–d)

X= -H (a); -CH₃ (b); -OCH₃ (c); -Cl (d)
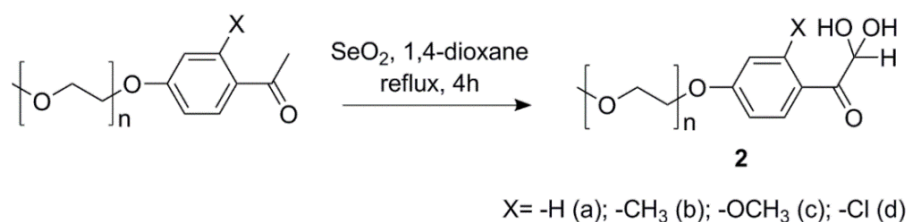
Compounds **2a–d** were all prepared using the same protocol. As a representative example, **1a** (0.50 g, 1 mmol) was dissolved in 30 mL 1,4-dioxane, and selenium dioxide (0.33 g, 3 mmol) then carefully to the solution at room temperature. Then solution was heated to reflux for 4 h, after which time it was cooled, the insoluble matter removed by filtration (paper) and the filtrate concentrated under reduced pressure. Compounds **2a–d** were purified from the resulting mixture by preparative HPLC and recovered by lyophilisation. The recovered yields were 41%, 61%, 56%, and 66% for **2a–d**, respectively. Fully assigned ^{1}H and ^{13}C NMR spectra of **2a–d**, as well as LC-MS chromatograms of the purified compounds can be found Figures 3.2–3.5.

3.2.4. PEGylation of 3–5 with 2a–d

The kinetics of PEGylation of **3–5** with **2a–d** were analyzed using the same protocol. As a representative example, 0.1 mg of **3** (1 eq.) was dissolved in 0.8 mL of 100 mM phosphate buffer pH 7.4. **2a** was dissolved into 100 mM sodium phosphate buffer pH 7.4 to prepare a 0.83 M stock solution. Then, 0.2 mL **2a** stock solution (4 eq. relative to arginine) was added using a micropipette to the peptide solution, which was then gently agitated and left to incubate for 24 h at r.t. (Note: control peptide **5** does not contain arginine, but was considered to have one arginine residue for the calculation purposes). During this period, 10 µL aliquots were withdrawn to monitor the progress of the reaction by HPLC using the fluorescence detector. Kinetics were monitored three times for each sample in order to obtain standard deviation. The resulting conjugates were purified after the 24 h period by preparative HPLC. Fully annotated LC-MS chromatograms of the purified conjugates [**4**+**2a–d**] can be found in Figures 3.7.

3.2.5. PEGylation of short peptides with 2a–d

The kinetics of PEGylation of the short arginine-containing peptides with **2a–d** was analyzed using the same protocol. As a representative example, 0.63 mg of peptide GGRGGW (1

µmol, 1 eq.) was dissolved in 1 mL 100 mM phosphate buffer pH 7.4, then **2a** (1.5 µmol, 1.5 eq., 1.80 µL of a 0.83 M **2a** stock solution) was added into the solution and then incubated at r.t. for 120 h. Small aliquots 10 µL were withdrawn to monitor the reaction by HPLC. The integration of each component was calculated and the reaction curves were made via Origin Program. The half-lives of all reactions were calculated and compared.

3.2.6. De-PEGylation of conjugated peptides

0.1 mg of purified conjugates were dissolved in 0.5 mL of 100 mM phosphate buffer pH 7.4 and the one containing 10% FBS. De-PEGylation under acidic and basic conditions were performed by adjusting the pH of the phosphate buffer to 4 and 9 using hydrochloric acid or sodium hydroxide, respectively. The de-PEGylation process was monitored over 24 h by taking small aliquots (10 µL) and quantifying the amount of peptide release by HPLC using the fluorescence detector.

3.2.7. Determination of the degree of PEGylation for [3+2a–c] conjugates

Determining the average degree of PEGylation of the conjugates **[3+2a–c]** requires quantification of the number of moles of the peptide component as well as the number of moles of the mPEG component, for a given dry weight of the conjugate.

3.2.7.1 UV absorbance standard curve preparation

To find out the unique wavelength for UV absorbance for **[3+2a–c]** conjugates, the UV absorbance of several initial substances water solution (AMP **3**, arginine, **2a-c**) with same concentration were introduced into 96-well plate and compared by using a plate reader at 24 °C. The strong absorbance at 452 nm of AMP **3** curve was caused by FITC group and this wavelength was chosen as the wavelength for **[3+2a–c]** conjugates.

AMP **3** was dissolved at different concentrations (0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4 mM) in water and the UV spectra of 200 uL solution were recorded in 96-well plate with the plate reader at 452 nm. The measured intensity was blank corrected. The standard curve was calculated with Origin Program.

3.2.7.2 Concentration calculation of [3+2a–c] conjugates

The D$_2$O solution of **[3+2a–c]** were prepared by dissolving purified **[3+2a–c]** into 500 µL D$_2$O respectively. Then 200 µL of each solution was transferred into the 96-well UV plate and measured with the plate reader at 24 °C with the wavelength of 452 nm. The measured intensity was blank corrected. The UV absorbance of each conjugates was recorded and the concentrations of the conjugates were determined by calculating with the equation of standard curve. The solution in plate reader could be reused in the NMR measurement.

3.2.7.3 NMR characterization of [3+2a–c] conjugates

The D$_2$O solution of **[3+2a–c]** conjugates were collected respectively. The volume of each solution was kept at 500 µL. Then 5 µL of fresh anhydrous DMSO were added into each solution and then the mixtures were transferred into NMR tubes. The solution was characterized by ^{1}H NMR. The integration of DMSO peak and PEG peak were performed and concentration of PEG in each solution could be calculated.

Comparing the integration of the peaks of the mPEG main-chain (~3.6 ppm) relative to those of DMSO provided access to the number of moles of mPEG within the solution. The degree of PEGylation is calculated by dividing the number of moles of mPEG by the number of moles of peptide.

3.2.8. Statistics

The reactivity of 2a-d towards short peptides was evaluated by one-way ANOVA followed by a Tukey post-hoc test. Differences were considered significant at $p < 0.05$.

3.3. Results and discussion

3.3.1. Synthesis of arginine-reactive mPEGs

rPEGylation agents bearing PGO units were designed and synthesized. PGO has been used for studying the function, local polarity, etc., of arginine (and citrulline) residues within proteins.[178-180] In contrast to aliphatic glyoxals, which permanently modify arginine,[94, 181] resonance of the ketone with the aromatic ring lowers the stability of the arginine–PGO adduct, making this a possible route for rPEGylation.[182-183]

All synthesized compounds were obtained as pure products. NMR spectra were acquired on a Bruker AV400 spectrometer (Bruker BioSpin, Fällanden, Switzerland), running at 400 MHz. Chemical shifts (δ) are reported in ppm relative to residual solvent signals (CDCl$_3$: 7.26 ppm for ^{1}H NMR, 77.16 ppm for ^{13}C NMR; D$_2$O: 4.79 ppm for ^{1}H NMR). The following abbreviations are used to indicate the multiplicity in NMR spectra: s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet; dd, double doublet; dt, double triplet; dq, double quartet; ddd, double double doublet; ddt double double triplet; tq, triple quartet; dp, double pentet; m, multiplet; bs, broad signal.

The ^{1}H and ^{13}C NMR data of tosylate-PEG and 1a-1d are shown as follows:

4-monomethoxypolyethylene glycol tosylate
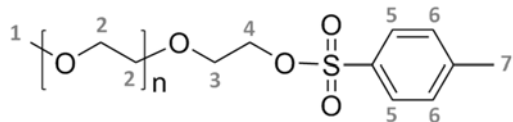
^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.80 (d, $J=$ 8.4 Hz, 2H, H-5), 7.35 (d, $J=$8.4 Hz, 2H, H-6), 4.17 (t, $J=$4.8 Hz, 2H, H-4), 3.69 (t, $J=$4.8 Hz, 2H, H-3), 3.60 (m, 16H, H-2), 3.38 (s, 3H, H-1), 2.45 (s, 3H, H-7); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 144.8, 133.0, 129.8 (2C), 128.0 (2C), 71.9, 70.6 (12C), 69.2, 68.6, 59.7, 21.6.

4-monomethoxypolyethylene glycol-acetophenone (1a)
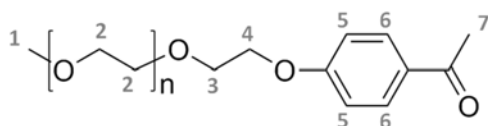
^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.85 (d, J $=$8.8 Hz, 2H, H-6), 6.86 (d, $J=$8.8 Hz, 2H, H-5), 4.12 (t, $J=$4.8 Hz, 2H, H-4), 3.82 (t, $J=$4.8 Hz, 2H, H-3), 3.58 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.48 (s, 3H, H-7); ^{13}C NMR (400 MHz, D$_2$O, δ ppm): 184.6, 162.8, 131.4 (2C), 129.9 (2C), 114.8, 71.1, 69.6(14C), 67.8, 67.2, 58.2, 25.8.

4-monomethoxypolyethylene glycol-2-methylacetophenone (1b)
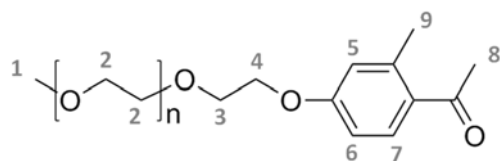
^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.67 (d, J=8.8 Hz, 1H, H-7), 6.69 (m, 2H, H-5, 6), 4.10 (t, J=4.8 Hz, 2H, H-4), 3.80 (t, J=4.8 Hz, 2H, H-3), 3.59 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.48 (d, J=3.6 Hz, 6H, H-8, 9); ^{13}C NMR (400 MHz, D$_2$O, δ ppm): 205.1, 161.8, 141.1, 133.3, 129.0, 118.2, 111.6, 71.1, 69.4 (12C), 67.8, 67.2, 58.2, 28.8, 21.3.

4-monomethoxypolyethylene glycol-2-methoxyacetophenone (1c)
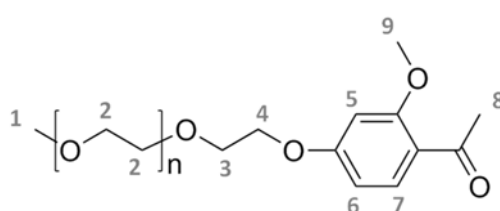
^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.76 (d, J=8.8 Hz, 1H, H-7), 6.46 (m, 2H, H-5, 6), 4.11 (t, J=4.8 Hz, 2H, H-4), 3.80 (m, 5H, H-3, 9), 3.59 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.50 (s, 3H, H-8); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 217.5, 163.7, 161.0, 132.6, 121.3, 105.5, 99.1, 70.9, 70.6 (12C), 69.6, 67.6, 59.0, 55.5, 31.8 .

4-monomethoxypolyethylene glycol-2-chloroacetophenone (1d)
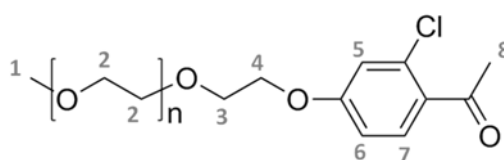
^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.59 (d, J=8.8 Hz, 1H, H-7), 6.89 (d, J=2.4 Hz, 1H, H-5), 6.78 (dd, J_1=8.8 Hz, J_2=2.4 Hz 1H, H-6), 4.10 (t, J=4.8 Hz, 2H, H-4), 3.79 (t, J=4.8 Hz, 2H, H-3), 3.58 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.57 (s, 3H, H-8); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 197.0, 161.2, 135.1, 132.9, 131.5, 117.0, 113.3, 70.9, 70.6(12C), 69.4, 67.9, 59.2, 29.4.

Four mPEG–PGO reagents (**2a–d**) bearing different substituents *ortho* to the glyoxal unit were prepared. A short mPEG (0.35 kDa) was chosen to facilitate analysis of the conjugates by MS. **2a-2d** were obtained in good yields, were conveniently purified, and identified by a variety of techniques, as shown in Figures 3.2-3.5. In neutral aqueous solution, the PGO units of **2a–d** were hydrated. Interestingly, in the course of all our experiments, **2a–d** did not significantly self-react or oligomerize, even upon prolonged incubation in solution at neutral pH, which contrasts to the instability of some earlier mPEG aldehydes and aliphatic glyoxals.

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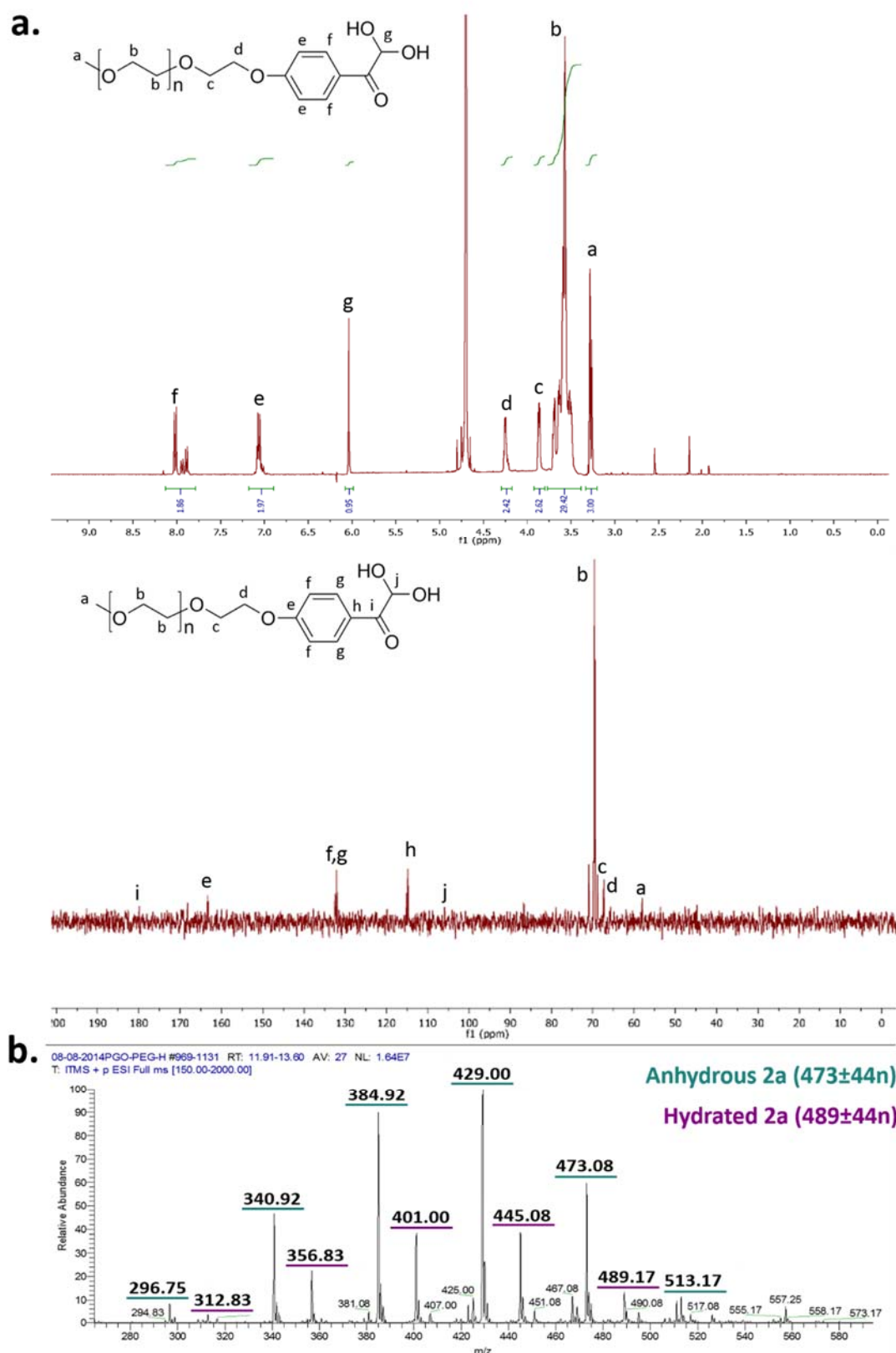
Figure 3.2. Assigned ^{1}H / ^{13}C NMR spectra in D$_2$O (a) and LC-MS (b) of compound **2a**. Two major peaks are observed in the chromatogram, which are assigned to the hydrated and anhydrous form of the glyoxal. Note that the anhydrous form is obtained under the acidic conditions used for its purification.
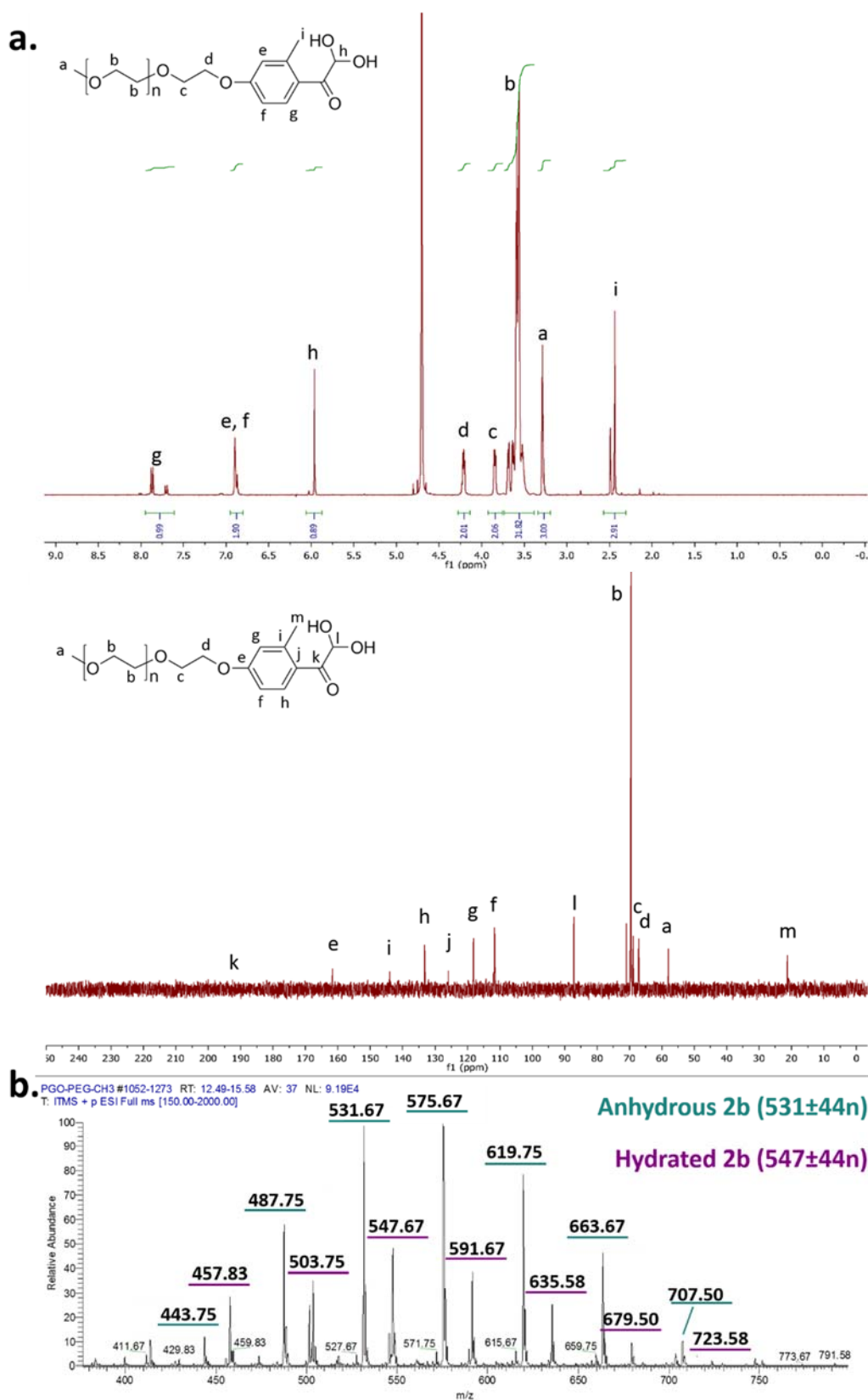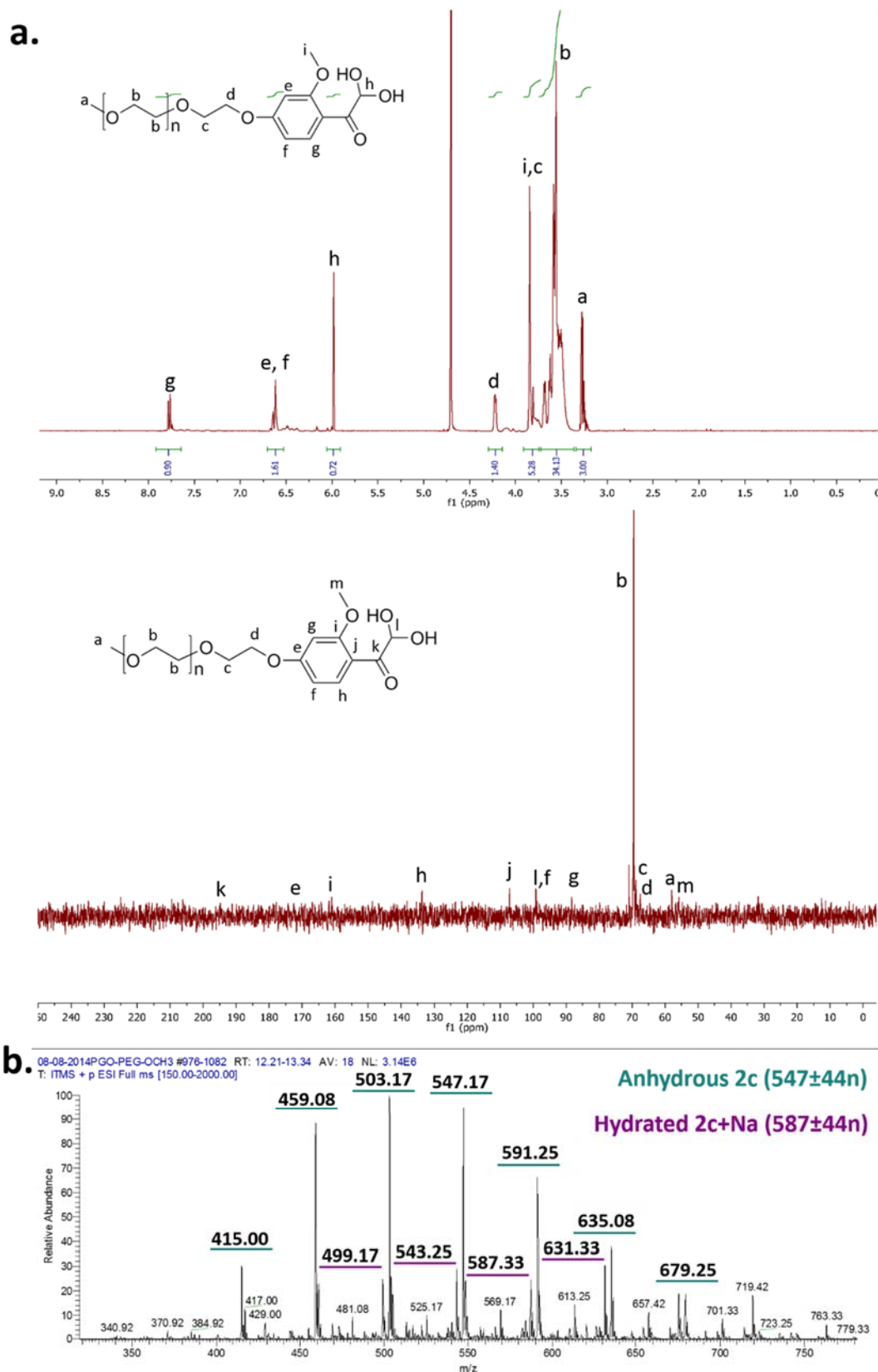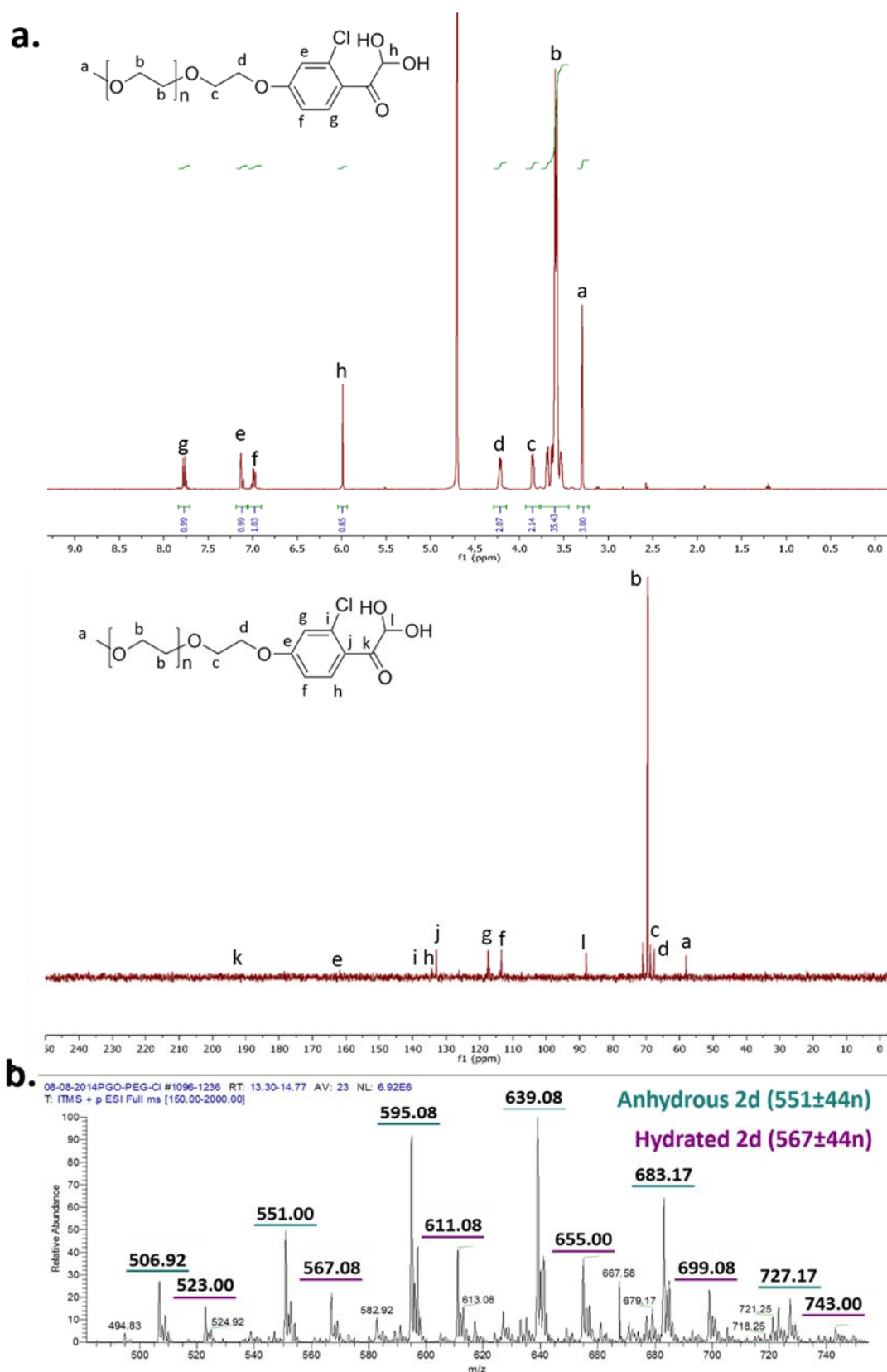
Figure 3.3. Assigned ^{1}H / ^{13}C NMR spectra in D$_2$O (a) and LC-MS (b) of compound **2b**. Two major peaks are observed in the chromatogram, which are assigned to the hydrated and anhydrous form of the glyoxal. Note that the anhydrous form is obtained under the acidic conditions used for its purification.

a.

b.

Figure 3.4. Assigned ^{1}H / ^{13}C NMR spectra in D$_2$O (a) and LC-MS (b) of compound **2c**. Two major peaks are observed in the chromatogram, which are assigned to the hydrated and anhydrous form of the glyoxal. Note that the anhydrous form is obtained under the acidic conditions used for its purification.

Figure 3.5. Assigned ^{1}H / ^{13}C NMR spectra in D$_2$O (a) and LC-MS (b) of compound **2d**. Two major peaks are observed in the chromatogram, which are assigned to the hydrated and anhydrous form of the glyoxal. Note that the anhydrous form is obtained under the acidic conditions used for its purification.

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3.3.2. PEGylation of 3–5/short peptides with 2a–d and statistics
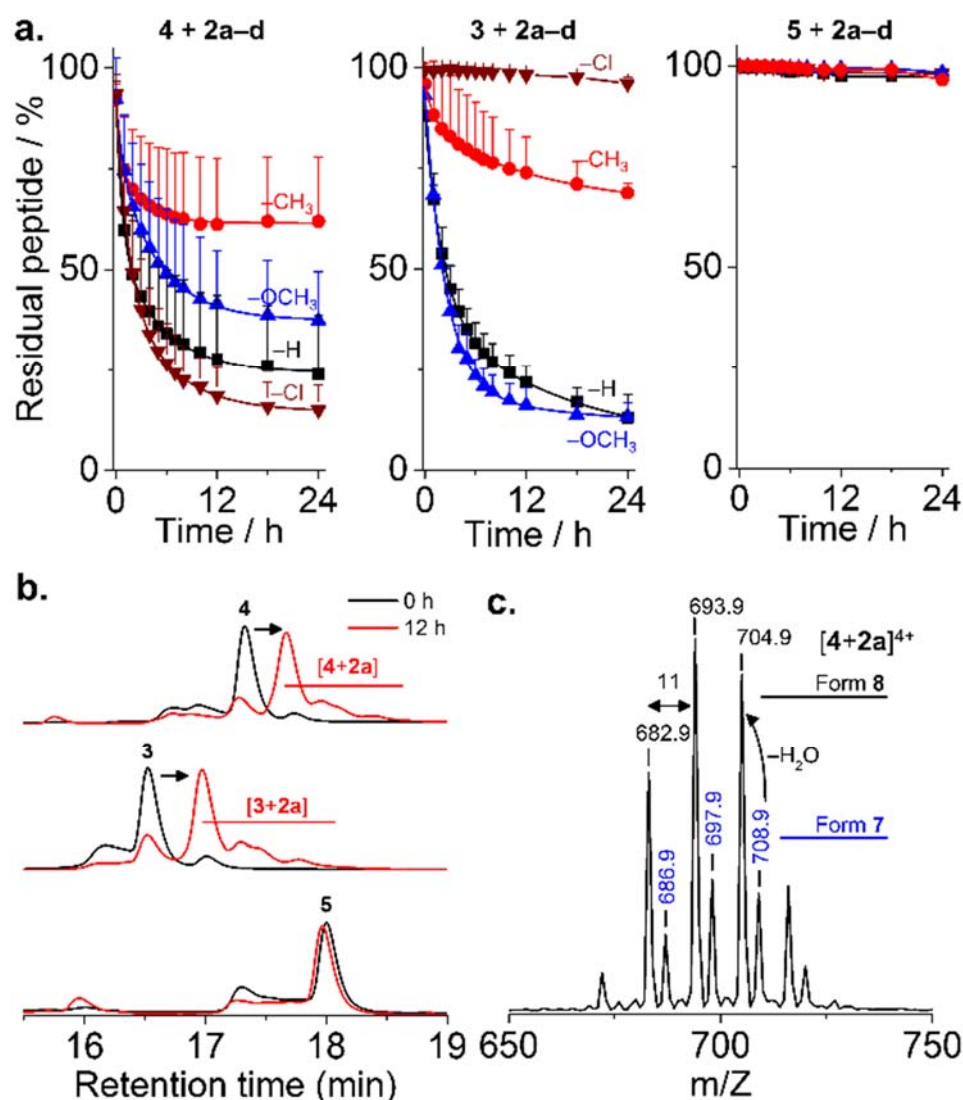
Figure 3.6. PEGylation of AMPs. (a) Kinetics of reaction of **3–5** with **2a–d**. Mean + SD (n = 3); (b) Chromatograms obtained at 0 h and 12 h; (c) Mass spectrum of purified conjugate [**4+2a**]$^{4+}$ with peaks corresponding to structures **7** and **8**.

With the existence of PGO group, the PEG chain is expected to react with peptides/proteins containing arginine residues. PEGylation using **2a–d** was first analyzed with AMP **4** as it contains only a single arginine. Conjugation with a small excess (4 eq. vs arginine) of **2a–d** proceeded smoothly over 24 h at pH 7.4 (Figures 3.6a). The disappearance of 4 was accompanied by the appearance of a series of peaks for the mono-PEGylated conjugate (Figure 3.6b), which were collectively isolated and identified by MS (Figure 3.7). With the exception of **4+2c**, both structures **7** and **8** could be observed for all other conjugates of **4**.
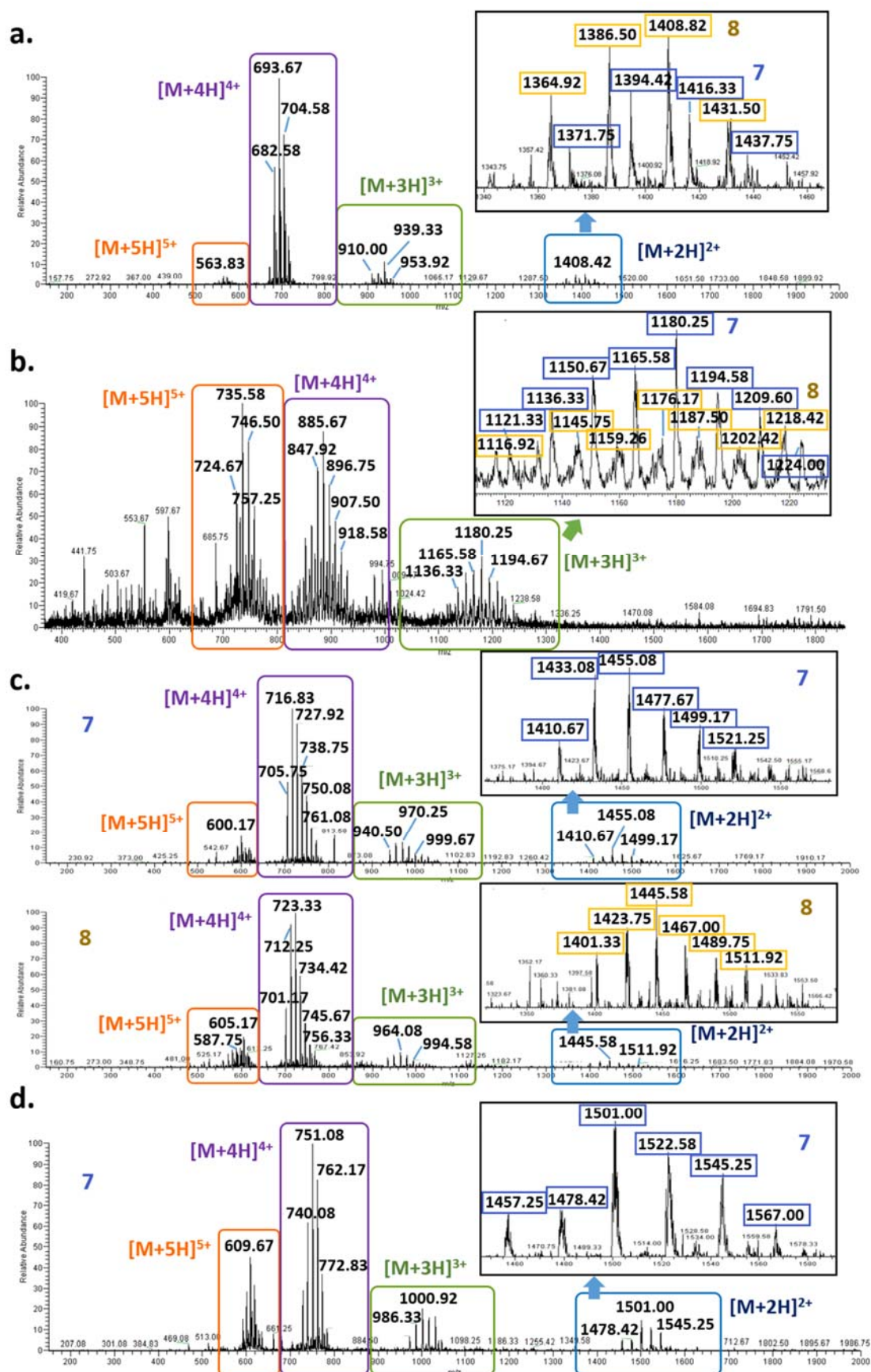
Figure 3.7. LC-MS of purified conjugate [**4**+**2a-2d**], with form **7** and form **8** assigned in the mass spectrum. (a) [**4**+**2a**]; (b) [**4**+**2b**]; (c) [**4**+**2c**]; (d) [**4**+**2d**].

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As depicted in Figure 3.1b, **2a–d** first reversibly (and rapidly) formed a hemiaminal (**6**), which then more slowly cyclized to the dihemiaminal (**7**) (Note: **6** and **7** are indistinguishable by MS; relative abundance discussed later). Dehydration of **7** produces **8** (Figure 3.6c), which was the "highest order" product observed. Multiple PEGylation of a single arginine residue was not observed, though this has been reported for PGO and other di-carbonyls.[184] In line with above, the PEGylation of AMP **3** (three arginine residues) proceeded smoothly (Figures 3.6a,b), yielding conjugated peptides after 24 h for **2a**, **2b**, and **2c**, respectively (2d, vide infra).
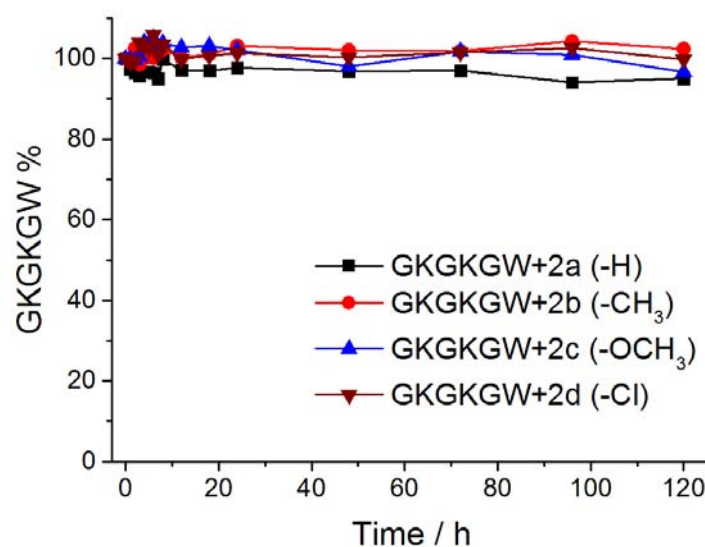
Figure 3.8. Reaction of GKGKGW with 4 eq. of **2a–d** in 100 mM phosphate buffer pH 7.4 over 5 days (n = 1).

PEGylation was selective to arginine residues, not surprisingly, no reaction of 2a–d occurred with peptide 5 (Figures 3.6a, b). In addition, a second model peptide with multiple lysine residues (GKGKGW) did not react with **2a–d**, even after five days at pH 7.4 (Figure 3.8). Thus **2a–d** can be employed for the arginine-specific PEGylation of short peptides within a reasonable timeframe. Of note, PEGylation can be accelerated by activation of the glyoxals using carbonate or borate buffers,[182, 185] though this aspect was not investigated herein.

The rate of reaction of **4** with **2a–d** was influenced by substituents on the aromatic ring. Baburaj et al.[185] have shown that a methoxy group para to the glyoxal decreases its reactivity towards arginine. A similar effect is therefore expected for mPEG, in addition to steric effects. Predicting "ortho-effects" for the other substituents is more complicated due to hindrance and potential interaction with the glyoxal. The relative reactivity of **2a–d** towards peptide **4** suggests that the electron-withdrawing properties of the chloro group (**2d**) are responsible for its greater

reactivity (versus **2a**), while the electron-donating properties of the methyl group (**2b**) are responsible for the lower reactivity. The reactivity of **2c** (OCH_3) was equivalent to the unsubstituted **2a**. A similar trend was observed for AMP **3**, with the exception of **2d** (Cl), which did not react. This intriguing result suggests some influence of peptide sequence on PEGylation. Indeed, it is known that while proteins feature many exposed arginine residues, invariably only few react.[186-187] It is not entirely clear why, but particularly reactive are arginine residues within anion recognition centers, which could be of relevance considering the cationic nature of many AMPs, including **3**.
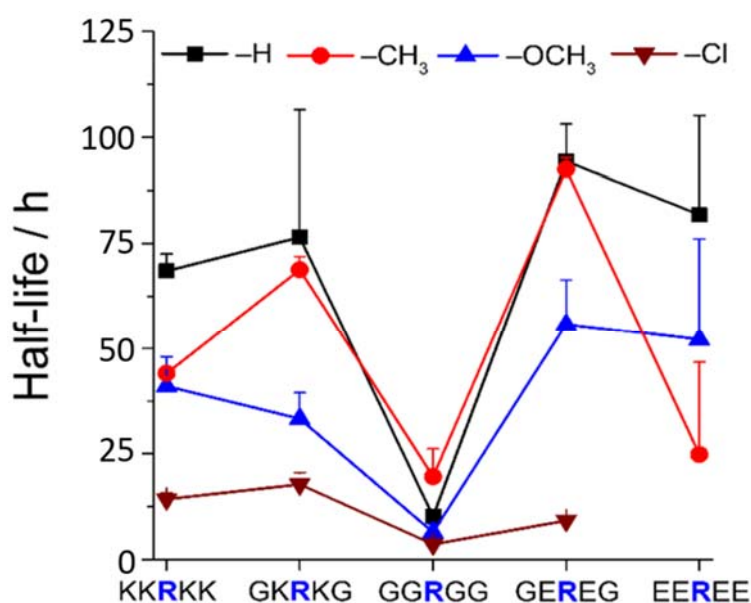
Figure 3.9. Influence of AMP sequence on PEGylation kinetics. Half-life of reaction of peptides with 1.5 eq. **2a–d** at pH 7.4 to assess the influence of steric hindrance and charge on reactivity. Mean + SD (n = 3).

To examine such environmental effects for AMPs, the reactivity of **2a–d** towards short peptides containing arginine and variable flanking residues was evaluated (Figure 3.9, Full ANOVA in Table 3.1). **2a–d** reacted fastest when the arginine was not sterically hindered (neighbors were glycine (G)) and slower when flanked by bulkier lysine (K) or glutamic acid (E). The charge of the microenvironment influenced the reactivity. When the first neighboring residues were negatively charged, peptides reacted more slowly than for correspondingly positively ones for **2a** (-H) and **2c** (**-OCH₃**) (in line with the observations with proteins) but the opposite was observed for **2d** (-Cl). **2a** was insensitive to the nature of the second neighboring residue. The influence of the second neighbor on the reactivity of **2b** (-CH₃) and **2c** ($-OCH_3$) was less predictable, and **2d** (-Cl) did not react at all with the peptide bearing four negative charges. Thus,

even though PEGylation is affected by the steric environment of the arginine residue, the reactivity of **2a** appeared to be less affected by the local charge and may be the most predictable PEGylation agent.

Table 3.1. Multiple pairwise means comparison table for Figure 3.9 (One-way ANOVA, Tukey post hoc $p = 0.05$). The value "1" indicates that the difference of the means is significant at the 0.05 level. The value "0" indicates that the difference of the means is not significant at the 0.05 level.

2a		Peptide				
		KKRKK	GKRKG	GGRGG	GEREG	EEGEE
Peptide	KKRKK	0				
	GKRKG	0	0			
	GGRGG	1	1	0		
	GEREG	0	0	1	0	
	EEREE	0	0	1	0	0

2b		Peptide				
		KKRKK	GKRKG	GGRGG	GEREG	EEGEE
Peptide	KKRKK	0				
	GKRKG	1	0			
	GGRGG	1	1	0		
	GEREG	1	1	1	0	
	EEREE	0	1	0	1	0

2c		Peptide				
		KKRKK	GKRKG	GGRGG	GEREG	EEGEE
Peptide	KKRKK	0				
	GKRKG	0	0			
	GGRGG	1	1	0		
	GEREG	0	1	1	0	
	EEREE	0	0	1	0	0

2d		Peptide			
		KKRKK	GKRKG	GGRGG	GEREG
Peptide	KKRKK	0			
	GKRKG	0	0		
	GGRGG	1	1	0	
	GEREG	1	1	1	0

3.3.3. De-PEGylation of conjugated peptides
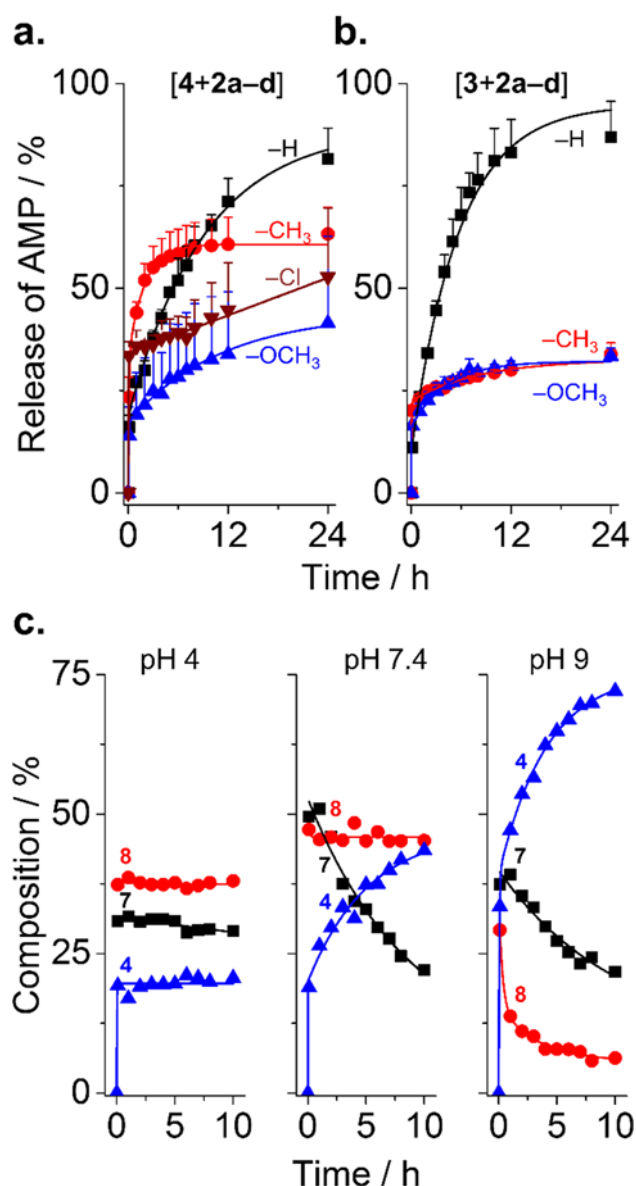
Figure 3.10. Release of AMPs from AMP–mPEG conjugates. (a,b) Influence of substituents on the release of peptide from conjugates of **4** or **3** with **2a–d**. Mean + SD (n = 3). (c) Effect of pH on the release of **4** from [**4+2c**] conjugates (n = 1).

Because substituents influenced the reactivity of **2a–d** towards arginine, it stands to reason that these should also influence the stability of the arginine–glyoxal adduct, though this effect has never been investigated before. Furthermore, because PEGylation was performed in phosphate buffer (vs borate buffer, *vide infra*), conjugates were present as a combination of forms **6–8** (Figure 3.1b), which was done intentionally to provide a more complete understanding of the equilibria involving all these species, and the opportunities they individually offer for rPEGylation. Release of the native peptide was triggered by dilution to 0.2 mg·mL^{-1} in buffer containing 10%

serum (higher serum content interfered with analysis) and monitored by HPLC (Figure 3.10). All profiles displayed burst release within minutes due to hydrolysis of form **6** of the conjugates (Figures 3.10a, b). Burst release was similar for all conjugates (~11–23%), reflecting the relative proportion of form **6** present at time zero. Thereafter, different release profiles were observed: For conjugates of **2a**, both peptides (**3** and **4**) were released almost completely over 24 h (82% for **4** and 87% for **3**). As the conjugate [**4+2a**] contained ~30% of form **8** (Figure 3.7a), complete release of peptide indicated that reactions **8 → 7** and **7 → 6** both occurred within the span of a couple hours. Interestingly, [**4+2d**] was uniquely present as form **7** (Figure 3.7d), indicating that Cl prevented dehydration to form **8**. Release of **4** from [**4+2d**] occurred in a sustained, even linear manner, to reach ~50% over 24 h. Finally, conjugates of **2b** (-CH₃) and **2c** (-OCH₃) displayed similar release profiles, which were incomplete and plateaued. This result prompted a more detailed analysis, which was possible because forms **7** and **8** of conjugate [**4+2c**] were distinguishable by HPLC. At near neutral pH, form **7** progressively disappeared to release **4** with a half-life of ~10 h, while form **8** was stable (Figure 3.10c). At acidic pH both forms **7** and **8** were stable. In basic media, the release process was accelerated, with recovery of ~75% of **4** in 10 h. This was possible because **8 → 7** is base catalyzed. These results indicate that [**4+2b**,**c**] were unable to hydrate from **8 → 7** at neutral pH, resulting in the plateaus in Figures 3.10a,b. Indeed, the plateaus are in line with the relative proportion of **8** within the mixture. Thus, with the exception of **2a**, form **8** of the conjugates should be avoided for complete release of AMP at neutral pH. From a practical standpoint, it is simple to selectively prepare form **7** of the conjugates by PEGylating in borate buffer. Borate promotes the formation of **7** (from **6**) by activating the glyoxal, and complexes the resulting 1,2-diol thereby preventing dehydration to **8**.
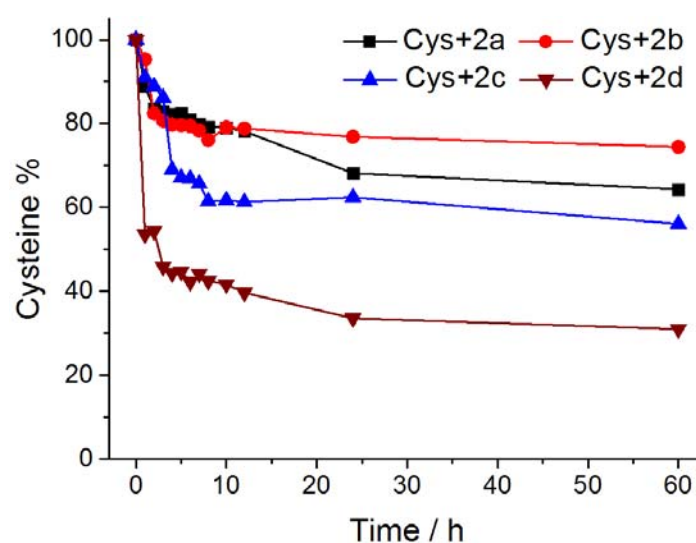

Figure 3.11. Reaction of cysteine with 1 eq. of **2a–d** in 100 mM phosphate buffer pH 7.4 over 60 h (n = 1).

Upon release **2a–d** are most likely to be eliminated from the body as-is, or possibly as a transient adduct with endogenous small nucleophiles (react faster than larger ones). In fact, incubation of **2a–d** with an equimolar amount of the bidentate nucleophile cysteine reached an equilibrium favoring free **2a–d** (Figure 3.11).

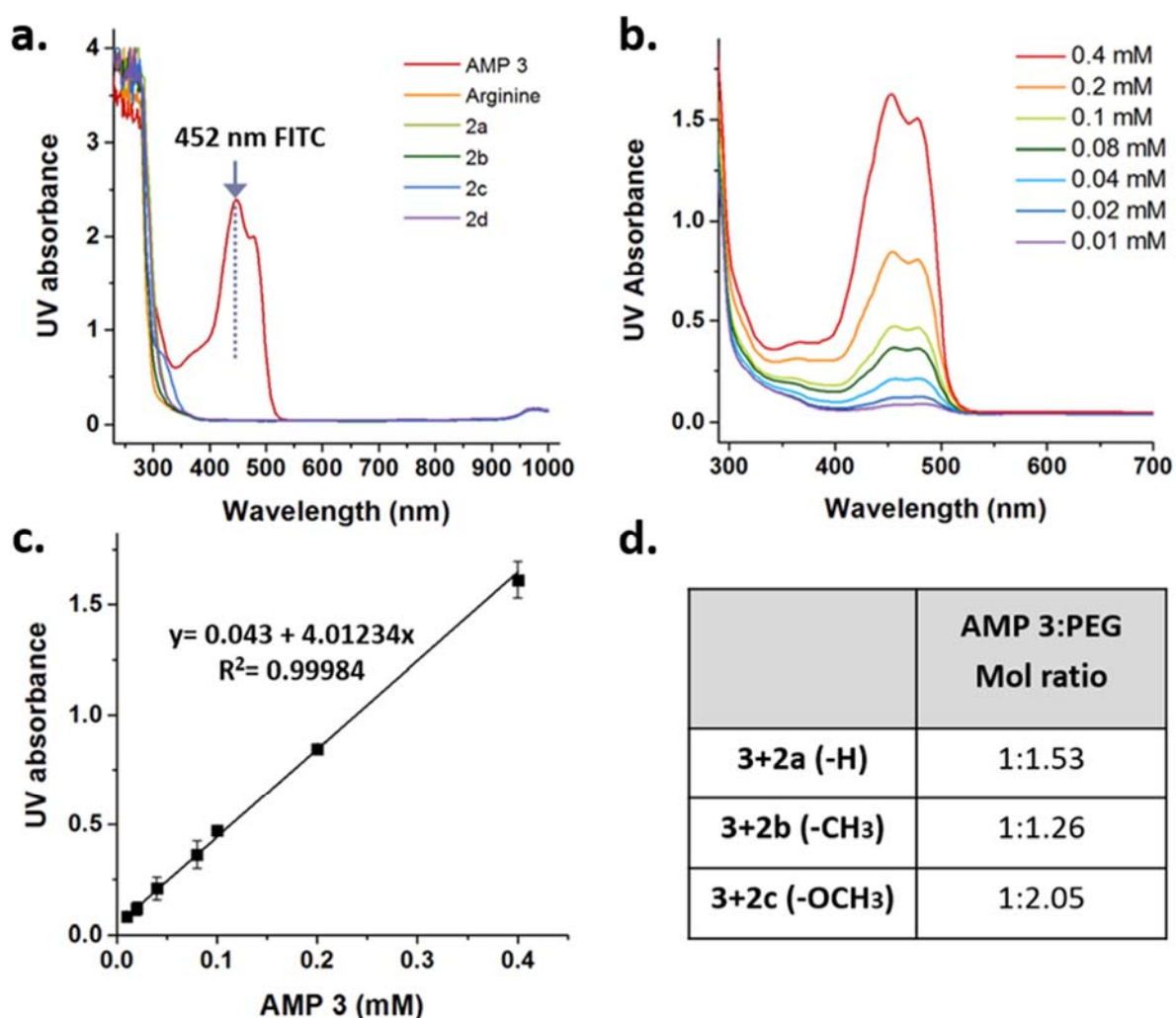## 3.3.4 Determination of the degree of PEGylation for [3+2a–c] conjugates

Figure 3.12. Characterization of degree of PEGylation of AMP **3** in **[3+2a-2c]** conjugates. (a) UV absorbance of different substances (**3**, arginine, **2a-2d**) in wavelength range of 250-1000 nm; (b) UV absorbance of AMP **3** with different concentrations at 452 nm; (c) standard curve of AMP **3,** mean ± SD (n = 3); (d) degree of PEGylation for **[3+2a-2c]**.

In order to calculate the molar ratio of AMP **3** and PEG in the conjugates, the purified conjugates **[3+2a-c]** with incubation of 2 days were prepared. The plate reader was used to record

the concentration of **3** in conjugates. To reduce the interference in UV absorbance spectra, it is important to find out a unique absorbance wavelength for **3**. Hence, AMP **3**, arginine and **2a-2d** solution with same concentration were detected with plate reader at the wavelength range of 250 nm to 1000 nm. Interestingly, at 452 nm, it has a strong absorbance only in AMP **3** spectrum due to the existence of FITC group, as shown in Figure 3.12a. Therefore, 452 nm was selected as the wavelength for the measurement of conjugates. The standard curve was obtained after the measurement of **3** with different concentrations, as in Figure 3.12b,c. With the standard curve, the concentration of **3** in the purified conjugates solution can be calculated. The PEGylation degrees of AMP **3** are 1.5, 1.3, and 2.0 mPEG units per peptide after 24 h for **2a**, **2b**, and **2c**, respectively (**2d**, *vide infra*).

3.4. Conclusions

In summary, this study presents four aromatic glyoxal mPEGs that selectively couple with arginine residues in AMPs, and release them in the span of a couple hours to days (pharmaceutically-relevant range) in a traceless fashion. Complete release was possible from the dihemiaminal form (**7**) of the conjugates, but only possible from the dehydrated form (**8**) for **2a** (-H). With the exception of **2d** (-Cl), the reagents show tolerance to AMP sequence, and no reaction was observed at residues other than arginine. As arginine residues are major cleavage sites for blood proteases, this rPEGylation strategy is highly relevant and should be adaptable to the entire family of arginine-rich AMPs. In addition, arginine is also statistically over-expressed within binding sites of many proteins.[44] This chemistry may therefore be of interest to transiently disrupt e.g., protein–protein interactions.

Chapter 4.

Sub-stoichiometric dynamic PEGylation disrupts immunoglobulin self-assembly in concentrated solution

4.1. Introduction

The increasing number of approved mAb therapies suggests that this new class of drug, as well as their derivatives, will be the focus of the pharmaceutical industry for years to come.[188-192] In many cases, mAbs are administered at high dose because of, for instance, their penetration into cancerous tumors can be inefficient.[193] High doses are usually achieved for cancer therapy by intravenous infusion in the hospital setting. However, it would be very desirable to formulate high concentration mAb solutions for self-administration at home, via the subcutaneous route. This would be very convenient for the treatment of chronic inflammatory diseases such as rheumatoid arthritis.[194] However, because subcutaneous administration is limited to a low injection volume ($< \sim 2$ mL), very high mAb concentrations must be used. Unfortunately, at high concentration, mAbs reversibly self-associate causing an important increase of viscosity, beyond that which is acceptable for injection. Furthermore, protein–protein interactions, which are inevitable at high concentration, can lead to formulation instability such as the formation of soluble and eventually insoluble aggregates.[194, 195]

The origin of the high viscosity in concentrated mAb solutions is thought to be linked to multivalent interactions between their target-binding regions, although other regions can also be involved. Because of the variability of these regions, the nature of the underlying interactions can be mAb-specific. For this reason, disrupting these interactions with inorganic/organic salt additives to lower viscosity has led to divergent results.[196-199] In addition, while the viscosity-lowering effect of certain additives was sometimes very pronounced, their high concentrations (typically ~ 0.25–1 M) could raise other issues such as colloidal instability, inappropriate osmolarity for injection, or potential toxicity.[200] Furthermore, other common excipients such as carbohydrates (to prevent the formation of insoluble aggregates or to protect mAbs during drying) can increase viscosity by promoting reversible mAb self-association.[83, 201-202] As a consequence, it remains very challenging to identify simple additives that effectively reduce the viscosity of concentrated mAb solutions containing relevant amounts of salt and protective osmolytes. One of the most efficient and general means for disrupting protein–protein interactions has been to graft polymers such as mPEG to the surface of proteins, mostly to prevent recognition by the immune system.[37, 103, 203] Several "PEGylated" protein therapeutics are used clinically, and the performance of this strategy is well-documented.
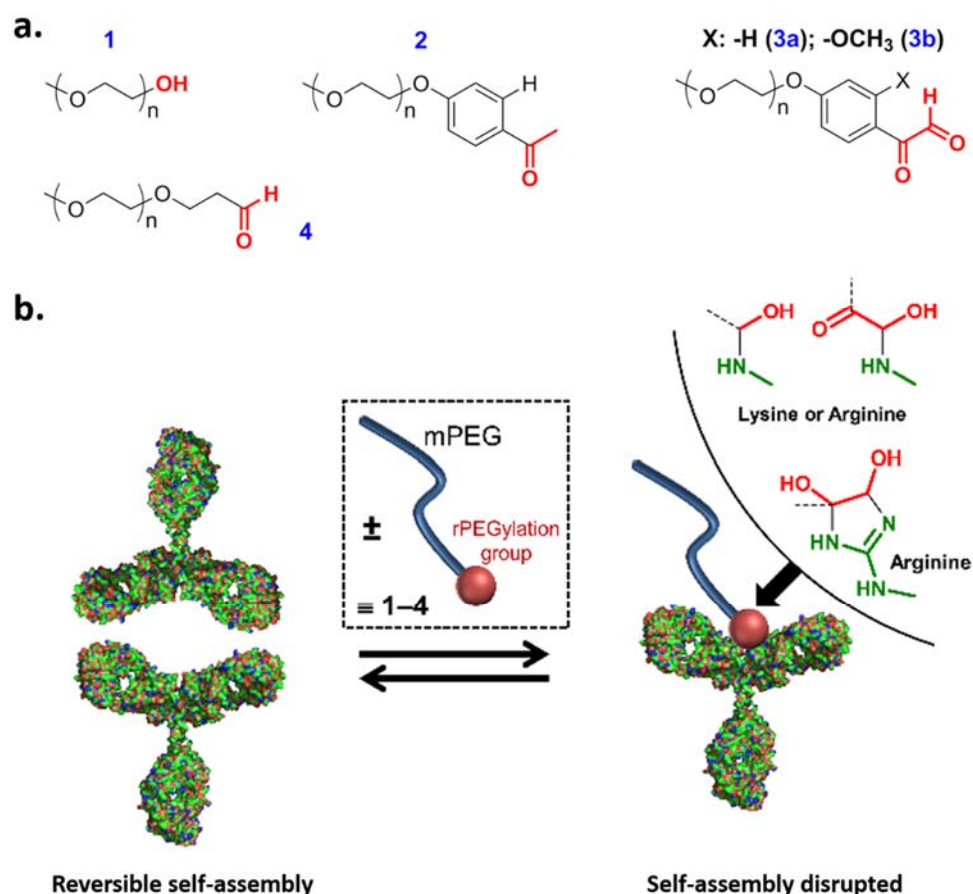
Figure 4.1. Dynamic rPEGylation of IgG to disrupt protein–protein interactions. (a) Synthesis and structure of rPEGylation agents tested. The structure of the protein-reactive group (in red) can modulate the equilibrium between its "protein-coupled" and "free" states and hence protein–protein interactions. (b) rPEGylation agents can dynamically and reversibly couple to IgG, thereby transiently disrupting interactions. In the scheme, an IgG dimer is shown as an example of one of the possible self-assembled solution structures.

However, PEGylation is largely inapplicable for disrupting mAb–mAb interactions for two important reasons: firstly, as certain mAb–mAb interactions can result from mutual interaction of their target-binding domains,[196, 204-205] PEGylation of these areas would intrinsically abolish their activity. Secondly, at high concentration, mPEG can induce protein precipitation due to excluded volume effects.[206] One possible solution to these challenges is rPEGylation, which could provide the means for transiently grafting mPEG to mAbs as well as a mechanism for releasing them completely unmodified after injection. Several innovative rPEGylation approaches have been reported, most relying on cleavable linker strategies.[85, 100-101, 103] Only a few strategies are based on an equilibrium,[112, 116-117, 207] which could allow PEGylation and de-PEGylation reactions to occur repetitively and dynamically. The dynamic nature of this

process might be interesting because a single mPEG chain could, over time, couple to many different mAbs and disrupt their self-association. Arginine is a particularly interesting rPEGylation target because it is statistically over-represented near binding sites on proteins (vs. natural abundance).[44] Based on the last chapter which reported a dynamic rPEGylation strategy based on PGO to arginine residues in an equilibrium fashion, this study demonstrates that very small amounts of PGO-based rPEGylation agents significantly reduce viscosity and prevent aggregation by disrupting protein self-assembly.

The viscosity-lowering effect of dynamic rPEGylation was examined for several additives with different affinities for nucleophilic residues on proteins. As illustrated in Figure 4.1a, mPEG (**1**), mPEG–acetophenone (**2**), PGO-mPEG derivatives (**3**), and mPEG–propionaldehyde (**4**) were analyzed. **2–4** are expected to dynamically and reversibly form hemiaminals (which dehydrate to Schiff bases) with nucleophilic amino acid residues, such as arginine. In addition to this, the PGO derivatives **3a** and **3b** will form more stable di-hemiaminal structures with arginine residues. Thus, the nature of the protein-reactive group will allow the kinetics and equilibrium of the rPEGylation process to be modulated. Three different lengths of mPEG were selected (0.35, 1 and 2 kDa), and **1** is a control for non-specific polymer–protein interactions involving mPEG. Note that in that absence of a large externally applied pressure, mPEG chains do not absorb to proteins.[208] For analysis, commercial lyophilized human immunoglobulin G (IgG) was selected because of its clinical relevance for the treatment of antibody deficiencies, and because it contains IgG variants from all four subclasses.[209] As such, polyclonal IgG may provide a more general view of the performance of dynamic rPEGylation compared to the analysis of individual mAbs, which can possess additional specific interactions.[209] This IgG formulation contains residual salts and osmolytes from the manufacturing process (*vide infra*).

4.2. Materials and Methods

4.2.1. Materials

4-Hydroxyacetophenone, mPEG-0.35 kDa (**1**-0.35 kDa), mPEG-2 kDa (**1**-2 kDa), silica gel (60 Å, 230–400 mesh), and p-toluenesulfonyl chloride were obtained from Fluka (various global suppliers). mPEG-1 kDa (**1**-1 kDa) and mPEG-propionaldehyde-0.55 kDa (**4**-0.55 kDa)

were purchased from Creative PEGworks (Chapel Hill, NC, USA). mPEG-propionaldehyde- 1 kDa (**4**-1 kDa) and mPEG-propionaldehyde-2 kDa (**4**-2 kDa) were obtained from Nanocs (New York, NY, USA). FBS, hydrochloride acid (37%), selenium dioxide, triethylamine, and trifluoroacetic acid were obtained from Sigma-Aldrich (various global suppliers). 4'-Hydroxy-2'-methoxyacetophenone was obtained from Apollo Scientific Limited (Cheshire, UK). In addition to the chemicals above, all buffer salts and solvents were purchased as the highest possible grade, and used as received. Deuterium oxide and chloroform-d were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Whole human IgG was obtained from Equitech-Bio Inc. (Kerrville, TX, USA). 10 kDa centrifugal filters (Amicon Ultra-4, Merck Millipore Ltd., Tullagreen, Ireland)

4.2.2. Analysis equipment

NMR spectra were acquired on a Bruker AV400 spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at 400 MHz for protons. Chemical shifts (δ) are reported in ppm relative to residual solvent signals.

Semi-preparative and analytical chromatography (for purification and SEC tests, respectively) were performed with an HPLC system (Merck HITACHI, Tokyo, Japan) equipped with an L-7100 pump, a Diode Array L-7455 detector. The XBridgeTM BEH300 Prep C18 column (5 μm, 10 mm × 150 mm) was used for purification and TSK-Gel G4000SWXL column, 7.8 mm ID × 30.0 cm (Tosoh Bioscience GmbH, Germany) was used for SEC tests. For the purification, the mobile phase was water and ACN, both supplemented with 0.1 vol% trifluoroacetic acid (TFA), and the flow rate was 4.4 mL·min^{-1}. A gradient method (0–2 min 0% ACN; 25 min 100% ACN; 30 min 0% ACN was used. The mobile phase for SEC test was 0.1 M sodium phosphate (pH 6.7) with the flow rate of 0.8 mL·min^{-1}.

LC-MS was performed using a Rheos Allegro quaternary pump, AccelaTM PDA Detector, XBridge C18 column (5 μm, 4.6 mm × 250 mm) and a Thermo LTQ XL mass spectrometer (Thermo Fisher Scientific, Vernon Hills, IL, USA). Mobile phases and gradients were the same as above. Data were analyzed with XCalibur control software.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) in the MS service of the Laboratory of Organic Chemistry in ETH Zurich was performed using Bruker's UltraFlex II system with Compass 1.5 control software.

Solution viscosity was measured with a Haake VT 550 PK100 cone-plate viscometer and a mechanical chip-based (MEMS) capillary rheometer (m-VROC™, RheoSence, San Ramon, CA, USA) equipped with an m-VROC2.5-GA05 chip, a 500 µL glass syringe (Hamilton 1010 C SYR500 µL), and a VROC-700-4 Peltier-chilled water bath. The syringe containing 100 µL the sample was placed inside the thermal jacket (22 ± 0.5 °C) until the temperature was stable, and 70 µL sample was pumped through the flow channel of the chip at shear rate 200 s^{-1}. The pressure changing was detected by a sensor (cell m-VROC A-10 or D-10). Viscosity was calculated from the raw data using the m-VROC-RP3.0 Control Software™.

The changes in the average particle hydrodynamic radius with time was monitored by dynamic light scattering (DLS) instrument performed with a DelsaNano C particle analyzer with the fixed angle of θ = 165º by using a Class 3B laser (Beckman Coulter, Brea, CA, USA). At least 500 µL sample was needed to be loaded into cuvette. The data were analyzed with Delsa Nano Beckman Coulter Control Software.

The IgG was characterized by applying thermogravimetric analysis (TGA), Q500, TA Instruments (Eschborn, Germany). The weight changes was measured by the instrument and analyzed by a TGA Q500 V5.3 program.

Circular dichroism spectroscopy was performed using a ChirascanTM CD spectrometer (Applied Photophysics, Surrey, United Kingdom). Measurements were recorded in a high transparency quartz cuvette (Hellma Analytics, Müllheim, Germany) with path length of 1 mm.

4.2.3. Synthesis of arginine-reactive mPEGs

4.2.3.1. Synthesis of mPEG tosylate 0.35 kDa, 1 kDa and 2 kDa

mPEG tosylate of different molecular weights were all prepared using the same protocol. As a representative example, mPEG-350 (4 g, 11.42 mmol) was dissolved in 120 mL of toluene and 60 mL of dichloromethane. p-Toluenesulfonyl chloride (11.44 g, 60 mmol) was added thereto, followed by the addition of 7.5 mL of triethylamine. The mixture was stirred for 6 h at r.t., and was followed by a second addition of p-toluenesulfonyl chloride (11.44 g, 60 mmol). The reaction was pursued for another 10 h, insoluble matter was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography using mixtures of dichloromethane and methanol as eluent, and the desired product dried in vacuo. Recovered yields

were 71%, 93%, and 60% for mPEG tosylate-0.35 kDa, 1 kDa and 2 kDa, respectively. The product was characterized by ^{1}H NMR, ^{13}C NMR and MS.

4.2.3.2. Synthesis of 4'-mPEG-2'-substituted-acetophenone 0.35 kDa, 1 kDa and 2 kDa (2)

Compounds **2** were all prepared using the same protocol. As a representative example, mPEG tosylate-0.35 kDa (1 g, 2 mmol) and 4-hydroxyacetophenone (2.72 g, 20 mmol) were dissolved in 100 mL of N,N-dimethylformamide. Then, potassium carbonate (2.76 g, 20 mmol) was added and the mixture was stirred for 4 h at 120 °C. Following this, the insoluble matter was filtered off with filter paper and the filtrate concentrated under reduced pressure. The residue was purified by silica gel chromatography using mixtures of dichloromethane and methanol as eluent, and the desired product dried in vacuo. The recovered yields were: 75% (**2**-0.35 kDa), 74% (**2**-1 kDa), 81% (**2**-2 kDa). Besides, the derivatives of compounds **2** containing methoxy group were also synthesized and the yields were: 51% (0.35 kDa), 70% (1 kDa), 83% (2 kDa). The product was characterized by ^{1}H NMR, ^{13}C NMR and MS.

4.2.3.3. Synthesis of 4'-mPEG-2'-substituted-phenylglyoxal 0.35 kDa, 1 kDa, and 2 kDa (3a, 3b)

Compounds **3a** (2' substituent: –H) and compounds **3b** (2' substituent: –OCH₃) were all prepared using the same protocol. As a representative example, **2**-0.35 kDa (0.50 g, 1 mmol) was dissolved in 30 mL 1,4-dioxane, and selenium dioxide (0.33 g, 3 mmol) then carefully to the solution at room temperature. Then solution was heated to reflux for 4 h under nitrogen, after which time it was cooled, the insoluble matter removed by filtration (paper) and the filtrate concentrated under reduced pressure. Compounds **3a**, **3b** were purified from the resulting mixture by preparative HPLC and recovered by lyophilization. The recovered yields were 41%, 92%, 87%, 56%, 96%, and 96% for **3a**-0.35 kDa, 1 kDa, 2 kDa and **3b**-0.35 kDa, 1 kDa, 2 kDa, respectively. The product was characterized by ^{1}H NMR and ^{13}C NMR. MS results of **3a**, **3b** are illustrated in Figures 4.2-4.4.

4.2.4. Thermogravimetric analysis (TGA)

6.7670 mg dry commercial IgG sample was characterized with a TGA, Q500, TA Instruments (Eschborn, Germany). The sample was heated at a rate of 20°C/min from 35 °C to 900 °C under air atmosphere with a flow rate of 25 mL·min^{-1}. The change of sample weight was measured and recorded by the instrument and then analyzed by a TGA Q500 V5.3 program. Result can be found in Figure 4.5a.

4.2.5. NMR characterization for quantification of IgG and sucrose

5.00 mg commercial IgG was dissolved into 498 µL D$_2$O. Then 2 µL dry DMSO was added and the solution was mixed gently. The solution was characterized with ^{1}H NMR. The integrations of DMSO peak and sucrose peaks were calculated and compared. The ^{1}H NMR result is shown in Figure 4.5b.

4.2.6. Viscosity measurements

4.2.6.1 Viscosity measurements IgG with different concentrations

The solid commercial lyophilized IgG formulation was freshly reconstituted to 100 mg·mL^{-1}, 200 mg·mL^{-1}, 300 mg·mL^{-1}, 400 mg·mL^{-1} and 500 mg·mL^{-1} (based on dry weight) in 10 mM sodium phosphate, pH 7.4. Different concentrations of IgG samples were measured with a Haake VT 550 PK100 cone-plate viscometer. The influence of shear rate (0 s^{-1}-1000 s^{-1}) on IgG viscosity was also measured with 500 mg·mL^{-1} sample. The results are shown in Figure 4.6.

4.2.6.2 Viscosity measurements of mixtures of IgG with PEG additives

The solid commercial lyophilized IgG formulation was freshly reconstituted to 526 mg·mL^{-1} (based on dry weight) in 10 mM sodium phosphate, pH 7.4. Then a second solution containing the additives **1–4** (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, or 10^{-1} M) in 10 mM sodium phosphate, pH 7.4, was prepared. As a representative example to prepare 100 µL of test solution, 95 µL of the IgG solution was added to 5 µL of the additive solution, or to 5 µL of additive-free

buffer. This solution was gently mixed and left to stand at room temperature for 20 min prior to analysis. Then the process was same as above. Experiments were performed in triplicate. The results are illustrated in Figure 4.7.

4.2.7. Dynamic light scattering (DLS)

Measurement of the Z-average hydrodynamic radius was performed with a DelsaNano C particle analyzer with the fixed angle of $\theta = 165°$ (Beckman Coulter, Brea, CA, USA). The solid commercial lyophilized IgG formulation was freshly reconstituted to 63 mg·mL^{-1} (based on dry weight) in 10 mM sodium phosphate, pH 7.4. Then a second solution containing the additives **1–4** (10^{-4} M) in 10 mM sodium phosphate, pH 7.4, was prepared. As a representative example to prepare 100 µL of test solution, 95 µL of the IgG solution was added to 5 µL of the additive solution, or to 5 µL of additive-free buffer. This solution and 5 µM **1–4**, was gently mixed and left to stand at room temperature for 20 min prior to analysis. The Z-average hydrodynamic radius and average diffusion coefficient was calculated using the Stoke-Einstein equation, and were corrected for solution viscosity, which varied from $0.88 \times 10^{-3} – 1.53 \times 10^{-3}$ Pa·s at maximum. Thereafter, the test solution was diluted with 10 mM sodium phosphate, 100 mM NaCl, 100 mM sucrose (pH 7.4) and re-analyzed. This process was repeated three times and results are illustrated in Figure 4.8.

4.2.8. Storage stability

The solid commercial lyophilized IgG formulation was freshly reconstituted to 84.8 mg·mL^{-1} (based on dry weight) in 10 mM sodium phosphate, pH 7.4. Then a second solution containing the additives **1–4** (10^{-4} M) in 10 mM sodium phosphate, pH 7.4, was prepared. As a representative example to prepare 100 µL of test solution, 95 µL of the IgG solution was added to 5 µL of the additive solution, or to 5 µL of additive-free buffer. Each test solution was divided into two equal-volume groups (~50 µL each) in 0.5 mL Eppendorf vials so as to be stored statically or under agitation (IKA KS 250 shaker; 400 rpm) at room temperature (protected from light). The photos of all samples can be found in Figure 4.9.

4.2.9. Size-exclusion chromatography (SEC)

An HPLC system (Merck HITACHI, Tokyo, Japan) equipped with an L-7100 pump, a Diode Array L-7455 detector and TSK-Gel G4000SWXL column, 7.8 mm ID × 30.0 cm (Tosoh Bioscience GmbH, Germany) was used to monitor the aggregation of IgG for 7 days. The mobile phase for SEC test was 0.1 M sodium phosphate buffer (pH 6.7) with a flow rate of 0.8 mL·min^{-1}. At each time point, 2 µL aliquots (storage ability test samples) were withdrawn and diluted with 45 µL 10 mM sodium phosphate (pH 7.4) to obtain SEC samples. Then, 10 µL diluted samples were injected and separated on tandem TSK-Gel column with the eluent being monitored by spectrophotometry at 280 nm at room temperature (Figure 4.10).

4.2.10. Gel electrophoresis

The diluted IgG incubated with additives samples (storage stability test samples) were analyzed with SDS gel electrophoresis and native gel electrophoresis. The sample solution with 2 × sample buffer (30% (v/v) glycerol, 0.02 % (w/v) bromophenol blue, 250 mM Tris HCl, pH 6.8, with or without 10% (w/v) SDS) was loaded on the SDS protein gel (5 % of stacking gel + 10 % separating gel) for SDS-PAGE analysis and native protein gel (5 % of stacking gel + 10 % separating gel) for native-PAGE analysis. After the running, the gel was fixed with fixing solution (25% (v/v) isopropanol, 10% (v/v) acetic acid in water) and stained with Rapid Coomassie blue staining solution (10% (v/v) acetic acid, 0.006% (w/v) Coomassie brilliant blue G-250 in water). Then the gel was de-stained in de-staining solution (10% (v/v) acetic acid in water) for 24 h. The photos can be found in Figure 4.11.

4.2.11. Circular dichroism spectroscopy (CD)

Circular dichroism spectroscopy was performed and the measurements were recorded in a high transparency quartz cuvette with path length of 1 mm. The spectra were recorded at 23 ºC, between 190–270 nm, and magnitude scaling was set to 1. An average of 3 scans were taken for each sample. A buffer scan was also obtained under the same measurement conditions, and was subtracted from the sample scans prior to analysis. For analysis, the soluble fraction of IgG

solutions aged for 25 days was diluted to 0.5 µM with 10 mM sodium phosphate, pH 7.4, containing 100 mM NaCl and 100 mM sucrose (Figure 4.12).

4.2.12. Characterization of the IgG conjugates

The solid commercial lyophilized IgG formulation was freshly reconstituted to 84.8 mg·mL^{-1} (based on dry weight) in 10 mM sodium phosphate, pH 7.4. Then a second solution containing the additives **1-4** (10^{-4} M) in 10 mM sodium phosphate, pH 7.4, was prepared. As a representative example, to prepare 100 µL of test solution, 95 µL of the IgG solution were added to 5 µL of the additive solution, or to 5 µL of additive-free buffer. This solution contains 5 µM **1-4**. The samples were incubated at room temperature in the dark for a few days. The IgG conjugates were then purified with 10 kDa centrifugal filters (Amicon Ultra-4, Merck Millipore Ltd., Tullagreen, Ireland) with water containing 0.1 vol % TFA at room temperature. The purified conjugates were lyophilized. To check the UV absorbance of IgG-mPEG conjugates, purified IgG conjugates and fresh IgG were dissolved into pH 7.4 phosphate buffer (containing 10 mM sodium phosphate, 100 mM NaCl and 100 mM sucrose) to obtain 1 mg·mL^{-1} solutions. Each sample was analyzed with plate reader (Infinite M200$^{®}$ Tecan microplate reader) with UV absorbance scan mode (230-500 nm) at room temperature. The measurements were performed triplicates.

4.3. Results and discussion

4.3.1. Synthesis of arginine-reactive mPEGs

The ^{1}H and ^{13}C NMR data of mPEG tosylate, **2**-0.35 kDa, 1 kDa, 2 kDa and **3a, 3b**-0.35 kDa, 1 kDa, 2 kDa are shown as follows. The MS data of **3a, 3b** can be found in Figures 4.2-4.4.

4-monomethoxypolyethylene glycol tosylate (0.35 kDa, 1 kDa, 2 kDa)
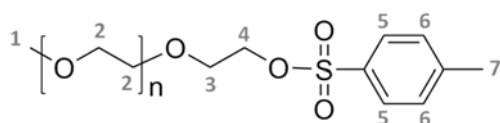
0.35 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.80 (d, J=8.4 Hz, 2H, H-5), 7.35 (d, J=8.4 Hz, 2H, H-6), 4.17 (t, J=4.8 Hz, 2H, H-4), 3.69 (t, J=4.8 Hz, 2H, H-3), 3.60 (m, 16H, H-2), 3.38 (s, 3H, H-1), 2.45 (s, 3H, H-7); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 144.8, 133.0, 129.8 (2C), 128.0 (2C), 71.9, 70.6 (12C), 69.2, 68.6, 59.7, 21.6.

1 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.80 (d, J=8.4 Hz, 2H, H-5), 7.35 (d, J=8.4 Hz, 2H, H-6), 4.17 (t, J=4.8 Hz, 2H, H-4), 3.69 (t, J=4.8 Hz, 2H, H-3), 3.60(m, 80H, H-2), 3.38 (s, 3H, H-1), 2.40 (s, 3H, H-7); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 144.8, 133.0, 129.8 (2C), 128.0 (2C), 71.9, 70.6 (40C), 69.2, 68.6, 58.8, 21.6.

2 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.80 (d, J=8.4 Hz, 2H, H-5), 7.35 (d, J=8.4 Hz, 2H, H-6), 4.17 (t, J=4.8 Hz, 2H, H-4), 3.69 (t, J=4.8 Hz, 2H, H-3), 3.60 (m, 160H, H-2), 3.38 (s, 3H, H-1), 2.40 (s, 3H, H-7); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 144.8, 133.0, 129.8 (2C), 128.0 (2C), 71.9, 70.6 (80C), 69.2, 68.6, 58.8, 21.6.

4-monomethoxypolyethylene glycol-acetophenone (2-0.35 kDa, 1 kDa, 2 kDa)
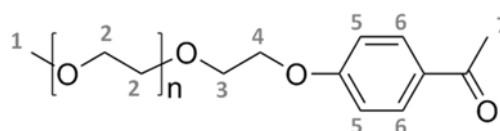
2-0.35 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.85 (d, J=8.8 Hz, 2H, H-6), 6.86 (d, J=8.8 Hz, 2H, H-5), 4.12 (t, J=4.8 Hz, 2H, H-4), 3.82 (t, J=4.8 Hz, 2H, H-3), 3.58 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.48 (s, 3H, H-7); ^{13}C NMR (400 MHz, D$_2$O, δ ppm): 184.6, 162.8, 131.4 (2C), 129.9 (2C), 114.8, 71.1, 69.6(14C), 67.8, 67.2, 58.2, 25.8.

2-1 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.85 (d, J=8.8 Hz, 2H, H-6), 6.86 (d, J=8.8 Hz, 2H, H-5), 4.12 (t, J=4.8 Hz, 2H, H-4), 3.82 (t, J=4.8 Hz, 2H, H-3), 3.58 (m, 80H, H-2), 3.31 (s, 3H, H-1), 2.48 (s, 3H, H-7); ^{13}C NMR (400 MHz, D$_2$O, δ ppm): 184.6, 162.8, 131.4 (2C), 129.9 (2C), 114.8, 71.1, 69.6(40C), 67.8, 67.2, 58.2, 25.8.

2-2 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.85 (d, J=8.8 Hz, 2H, H-6), 6.86 (d, J=8.8 Hz, 2H, H-5), 4.12 (t, J=4.8 Hz, 2H, H-4), 3.82 (t, J=4.8 Hz, 2H, H-3), 3.58 (m, 160H, H-2), 3.31 (s, 3H, H-1), 2.48 (s, 3H, H-7); ^{13}C NMR (400 MHz, D$_2$O, δ ppm): 184.6, 169.8, 131.4 (2C), 129.9 (2C), 114.8, 71.0, 69.6(80C), 67.8, 67.1, 58.2, 25.8.

4-monomethoxypolyethylene glycol-2-methoxyacetophenone (0.35 kDa, 1 kDa, 2 kDa)
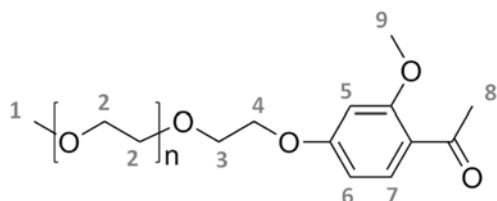
0.35 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.76 (d, J=8.8 Hz, 1H, H-7), 6.46 (m, 2H, H-5, 6), 4.11 (t, J=4.8 Hz, 2H, H-4), 3.80 (m, 5H, H-3, 9), 3.59 (m, 20H, H-2), 3.31 (s, 3H, H-1), 2.50 (s, 3H, H-8); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 217.5, 163.7, 161.0, 132.6, 121.3, 105.5, 99.1, 71.9, 70.6 (12C), 69.6, 67.6, 59.0, 55.5, 31.8.

1 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.76 (d, J=8.8 Hz, 1H, H-7), 6.46 (m, 2H, H-5, 6), 4.11 (t, J=4.8 Hz, 2H, H-4), 3.80 (m, 5H, H-3, 9), 3.59 (m, 80H, H-2), 3.31 (s, 3H, H-1), 2.50 (s, 3H, H-8); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 197.5, 163.7, 161.0, 132.6, 121.3, 105.5, 99.1, 71.9, 70.6 (40C), 69.6, 67.6, 59.0, 55.5, 31.8.

2 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 7.76 (d, J=8.8 Hz, 1H, H-7), 6.46 (m, 2H, H-5, 6), 4.11 (t, J=4.8 Hz, 2H, H-4), 3.80 (m, 5H, H-3, 9), 3.59 (m, 160H, H-2), 3.31 (s, 3H, H-1), 2.50 (s, 3H, H-8); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 197.5, 163.7, 161.0, 132.6, 121.3, 105.5, 99.1, 71.9, 70.6 (80C), 69.6, 67.6, 59.0, 55.5, 31.8.

4-monomethoxypolyethylene glycol-phenylglyoxal (PGO(-H)-PEG, 3a-0.35 kDa, 1 kDa, 2 kDa)
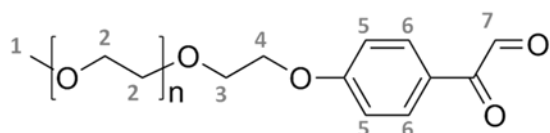
3a-0.35 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.58 (s, 1H, H-7), 8.25 (m, 2H, H-6), 6.98

(m, 2H, H-6), 4.23 (t, J＝4.8 Hz, 2H, H-4), 3.87 (t, J＝4.8 Hz, 2H, H-3), 3.57 (m, 20H, H-2), 3.28 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 201.3, 190.2, 169.8, 132.5, 132.0 (2C), 115.2, 71.9, 70.5 (12C), 68.8, 68.2, 58.1.

3a-1 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.57 (s, 1H, H-7), 8.25 (m, 2H, H-6), 6.98 (m, 2H, H-6), 4.23 (t, J＝4.8 Hz, 2H, H-4), 3.87 (t, J＝4.8 Hz, 2H, H-3), 3.57 (m, 80H, H-2), 3.28 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 201.3, 190.2, 169.8, 132.5, 132.0 (2C), 115.2, 71.9, 70.5 (40C), 68.8, 68.2, 59.0.

3a-2 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.60 (s, 1H, H-7), 8.25 (m, 2H, H-6), 6.98 (m, 2H, H-6), 4.23 (t, J＝4.8 Hz, 2H, H-4), 3.87 (t, J＝4.8 Hz, 2H, H-3), 3.57 (m, 160H, H-2), 3.28 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 190.3, 187.2, 169.8, 132.5, 132.0 (2C), 115.2, 71.9, 70.5 (80C), 68.8, 68.3, 59.0.

4-monomethoxypolyethylene glycol-2-methoxyphenylglyoxal (PGO(-OCH$_3$)-PEG, 3b-0.35 kDa, 1 kDa, 2 kDa)
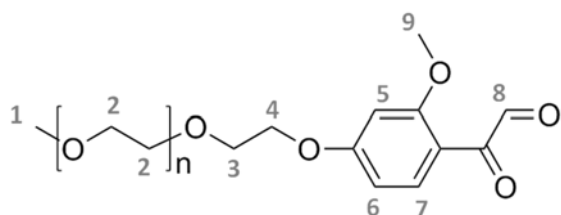
3b-0.35 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.68 (s, 1H, H-8), 7.77 (d, J＝8.8 Hz, 1H, H-7), 6.62 (d, 1H, H-5), 6.48 (s, 1H, H-6), 4.22 (t, J＝4.8 Hz, 2H, H-4), 3.83 (m, 5H, H-3, 9), 3.57 (m, 20H, H-2), 3.27 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 191.8, 185.2, 169.8, 161.1, 134.1, 111.3, 107.1, 99.1, 71.1, 69.5 (12C), 67.8, 67.2, 58.2, 55.7.

3b-1 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.68 (s, 1H, H-8), 7.76 (d, J＝8.8 Hz, 1H, H-7), 6.62 (d, 1H, H-5), 6.48 (s, 1H, H-6), 4.22 (t, J＝4.8 Hz, 2H, H-4), 3.83 (m, 5H, H-3, 9), 3.58 (m, 80H, H-2), 3.27 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 191.8, 185.2, 169.8, 161.1, 134.1, 111.3, 107.1, 99.1, 71.0, 69.5 (40C), 67.8, 67.2, 58.2, 55.7.

3b-2 kDa: ^{1}H NMR (400 MHz, CDCl$_3$, δ ppm): 9.68 (s, 1H, H-8), 7.77 (d, J＝8.8 Hz, 1H, H-7), 6.62 (d, 1H, H-5), 6.48 (s, 1H, H-6), 4.22 (t, J＝4.8 Hz, 2H, H-4), 3.83 (m, 5H, H-3, 9), 3.57 (m, 160H, H-2), 3.27 (s, 3H, H-1); ^{13}C NMR (400 MHz, CDCl$_3$, δ ppm): 191.8, 185.2, 169.8, 161.1, 134.1, 111.3, 107.1, 99.1, 71.1, 69.5 (80C), 67.8, 67.2, 58.2, 55.7.
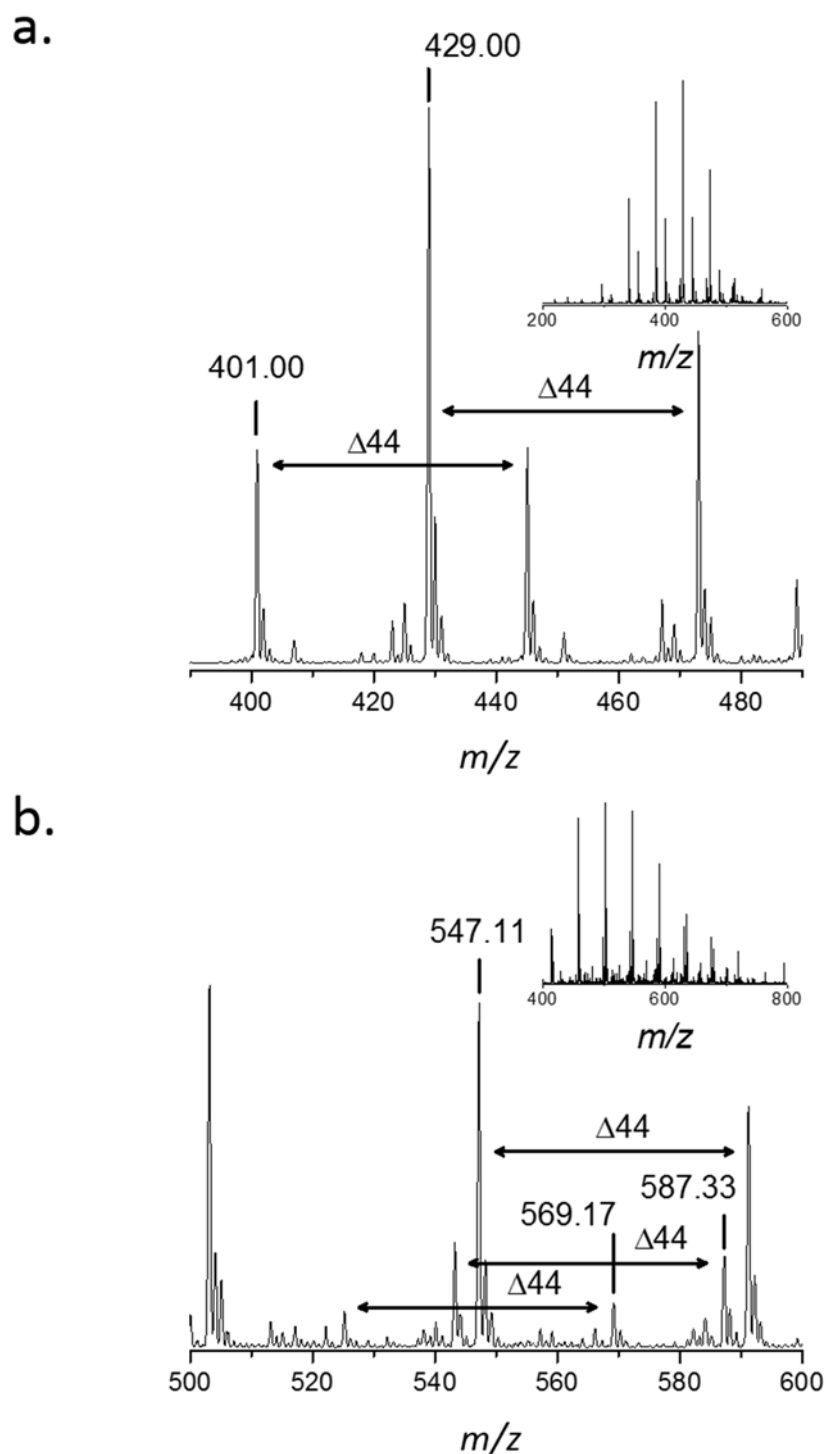
Figure 4.2. Mass spectra of compound **3a**-0.35 kDa and **3b**-0.35 kDa. HRMS (ESI-ion trap) m/z: Parent ions: $[M + H]^+$ Calcd. for **3a**-0.35 kDa: $C_{21}H_{33}O_9$ 429.21 (Found 429.00). Fragment ions: $[M + H]^+$ (α-cleavage at aldehyde); Calcd. for $C_{20}H_{33}O_8$ 401.22 (Found 401.00). Calcd. for **3b**-0.35 kDa: $C_{26}H_{43}O_{12}$ 547.27 (Found 547.11); $[M + Na]^+$ Calcd. for $C_{26}H_{43}O_{12}Na$ 569.26 (Found 569.17); $[M + H_2O + Na]^+$ Calcd for $C_{26}H_{44}O_{13}Na$ 587.27 (Found 587.33).
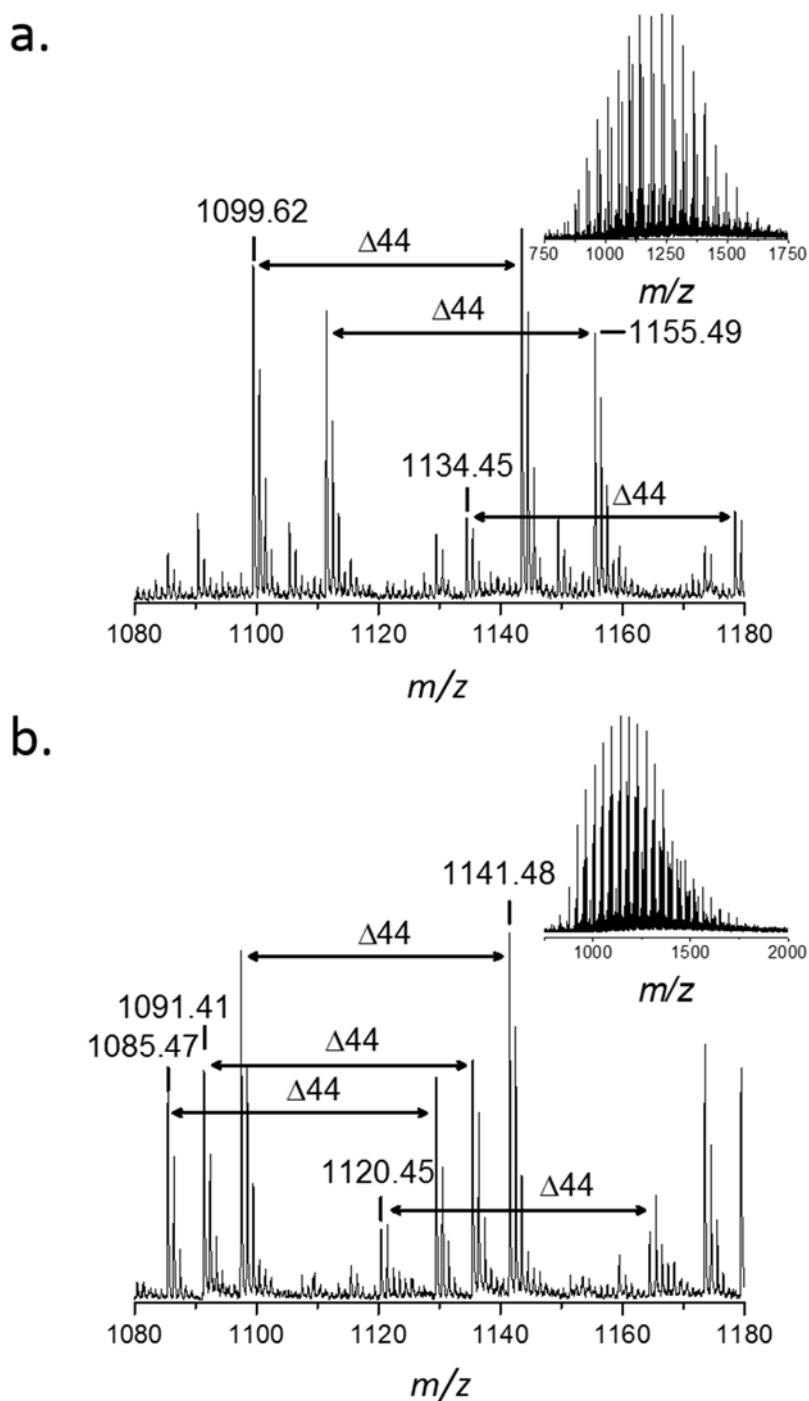
Figure 4.3. Mass spectra of compound **3a**-1 kDa and **3b**-1 kDa. HRMS (MALDI-TOF) m/z: Parent ions: $[M + H]^+$ Calcd. for **3a**-1 kDa: $C_{53}H_{97}O_{25}$ 1133.63 (Found 1134.45); $[M + Na]^+$ Calcd. for $C_{53}H_{96}NaO_{25}$ 1155.61 (Found 1155.49). Fragment ions: $[M + Na]^+$ (α-cleavage at ketone) Calcd. for $C_{51}H_{96}NaO_{23}$ 1099.62 (1099.62). Calcd. for **3b**-1 kDa: $C_{52}H_{95}O_{25}$ 1119.62 (Found 1120.45); $[M + Na]^+$ Calcd. for $C_{52}H_{94}O_{25}Na$ 1141.60 (Found 1141.48). Fragment ions: $[M + H]^+$ (α-cleavage at aldehyde) Calcd. for $C_{51}H_{94}O_{24}$ 1090.61 (Found 1091.41); $[M + Na]^+$ (α-cleavage at ketone) Calcd. for $C_{50}H_{94}NaO_{23}$ 1085.61 (Found 1085.47).
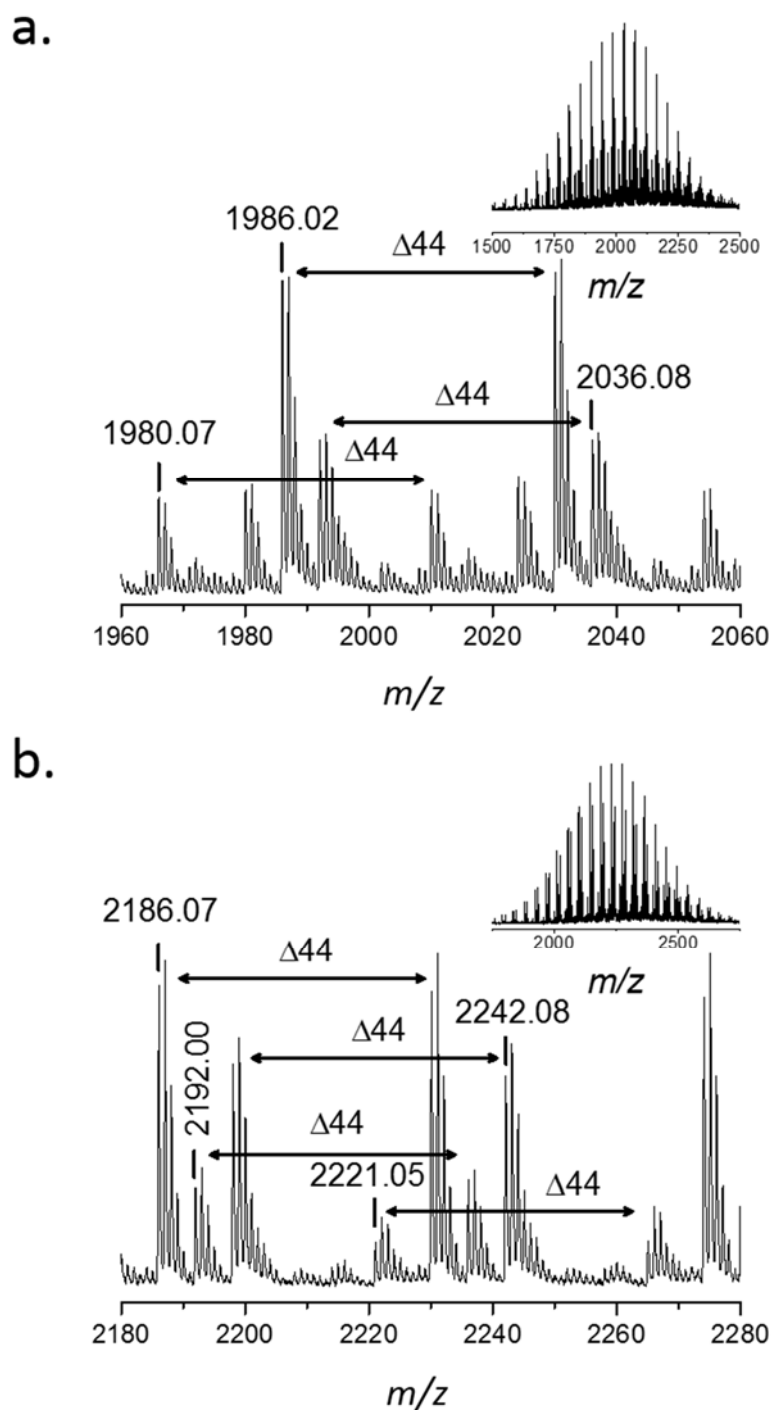
Figure 4.4. Mass spectra of compound **3a**-2 kDa and **3b**-2 kDa. HRMS (MALDI-TOF) m/z: Parent ions: $[M + Na]^+$ Calcd. for **3a**-2 kDa: $C_{93}H_{176}NaO_{45}$ 2036.14 (Found 2036.08). Fragemnt ions: $[M + H]^+$ (α-cleavage at aldehyde) Calcd. for $C_{92}H_{177}O_{44}$ 1986.16 (Found 1986.02); $[M + Na]^+$ (α-cleavage at ketone) Calcd. for $C_{91}H_{176}NaO_{43}$ 1980.15 (Found 1980.07). For 3b-2 kDa: Parent ions: $[M + H]^+$ Calcd. for $C_{102}H_{195}O_{50}$ 2221.27 (Found 2221.05); $[M + Na]^+$ Calcd. for $C_{102}H_{194}NaO_{50}$ 2242.25 (Found 2242.08). Fragment ions: $[M + H]^+$ (α-cleavage at aldehyde) Calcd. for $C_{101}H_{195}O_{49}$ 2192.28 (Found 2192.00); $[M + Na]^+$ (α-cleavage at ketone) Calcd. for $C_{100}H_{194}NaO_{48}$ 2186.26 (Found 2186.07).

81

4.3.2. TGA and NMR characterization of IgG
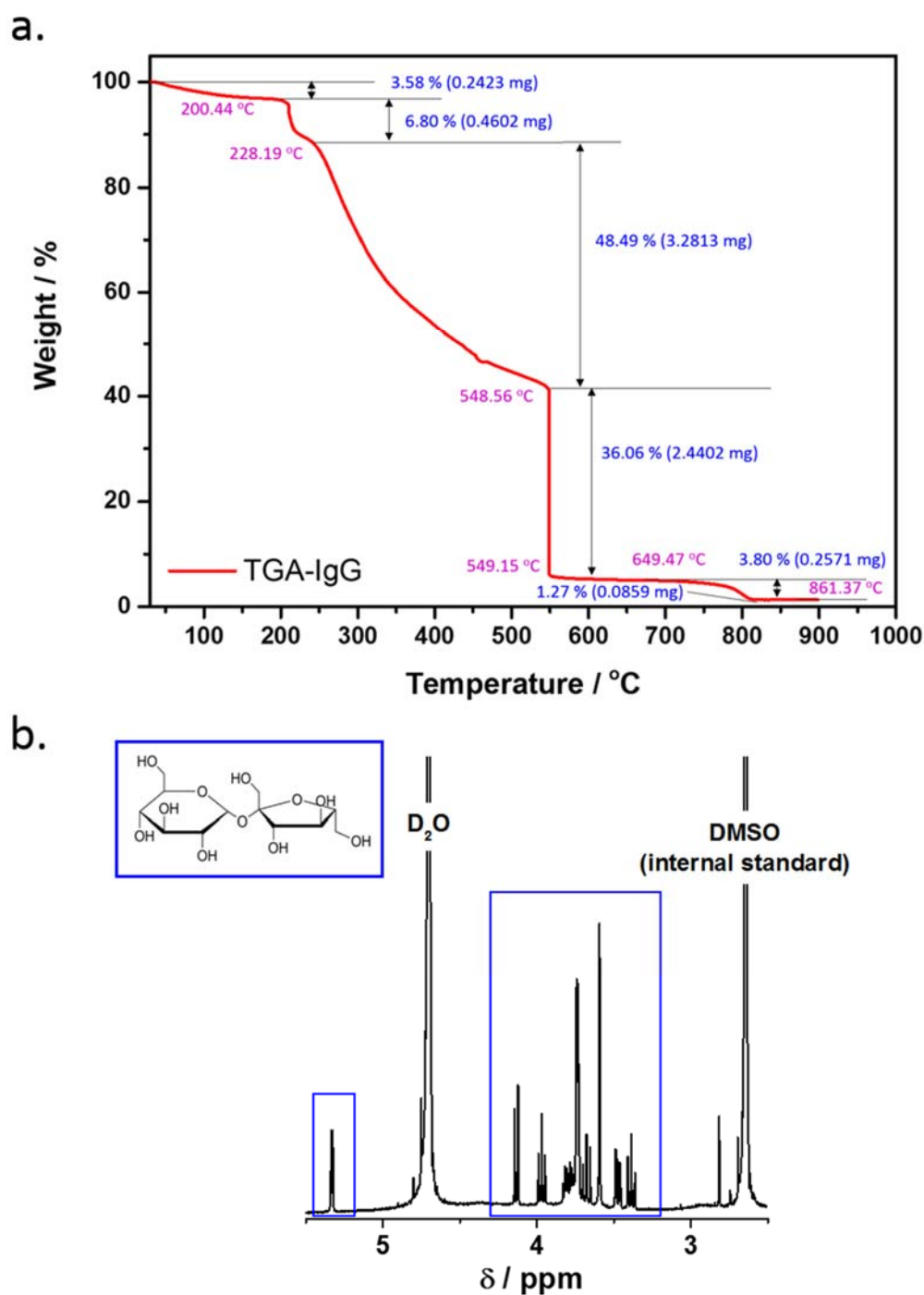
Figure 4.5. TGA and NMR result of IgG sample. (a) TGA of IgG formulation in air at a heating rate of 20 °C·min^{-1}. Residual buffers salts account for 1.27 % of the dry weight of the commercial IgG preparation. (b) ^{1}H NMR spectrum of IgG in D$_2$O containing a known concentration of DMSO. Integration of peaks of sucrose relative to that of DMSO provides the concentration of sucrose in solution, which can then be related to the dry weight of the IgG formulation dissolved.

The commercial IgG formulation was characterized by both TGA and NMR spectroscopy (Figure 4.5). It was supplied as a powder that was lyophilized by the manufacturer from a solution containing 10 mM sodium phosphate, 150 mM NaCl and an unknown amount of sucrose (based on datasheet). From the calculation of the TGA result, 1.27 wt% (0.0859 mg) residual buffer salts left indicated that it contains 0.17 wt% Na_2HPO_4 and 1.10 wt% NaCl (based on buffer composition prior to lyophilization). According to the NMR integration of DMSO standard and sucrose, it was calculated that it contains 6.66 wt% sucrose. Therefore, the IgG is 92.07 wt% (=1 - 1.27 wt% - 6.66 wt%) in the lyophilized powder. This composition is close to being isotonic with blood.

4.3.3. Viscosity measurements
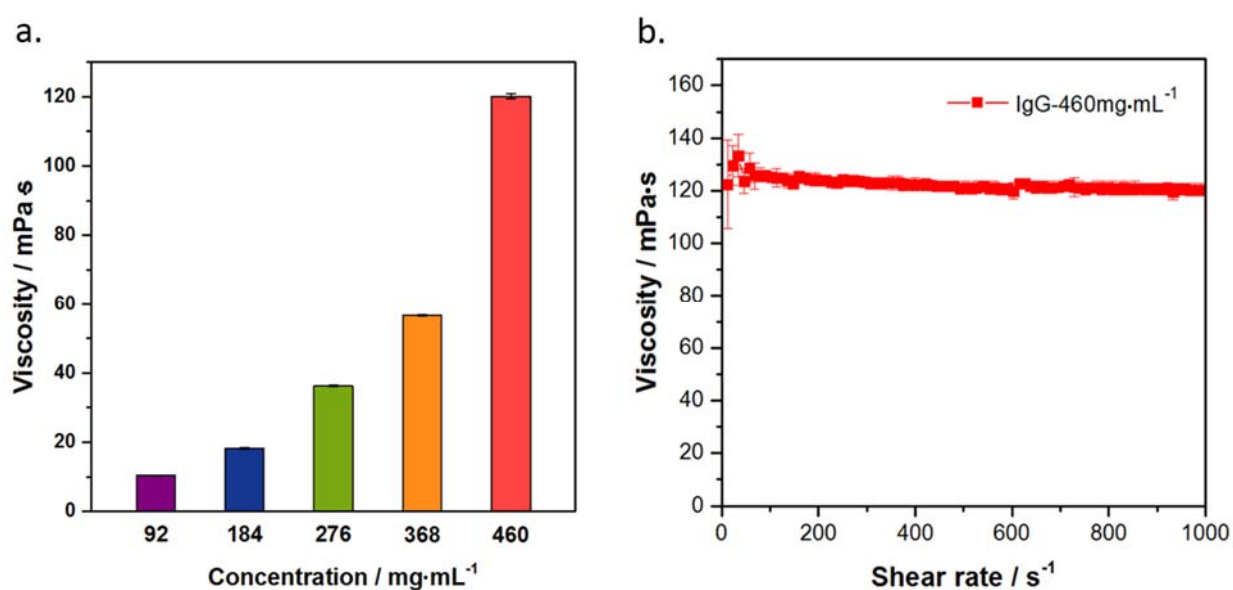
Figure 4.6. The viscosities of IgG. (A) Viscosities of different concentrations of IgG in PBS (pH 7.4) at shear rate of 200 s^{-1}. (b) Viscosity of IgG with 460 mg·mL^{-1} with changing shear rate (0 s^{-1}-1000 s^{-1}). Mean ± SD (n=3).

The viscosity of IgG solutions with different concentrations were first studied. The result is illustrated in Figure 4.6a. The viscosities of the IgG solutions increase with the concentration increase. The viscosity increased exponentially with concentration. Before the analysis of IgG with additives, IgG containing 460 mg·mL^{-1} IgG (= 500 × 0.92), with the highest viscosity (120 mPa·s), was further studied with the changing shear rate (Figure 4.6b). It shown that the fluid was

within the Newtonian plateau at a shear rate of 200 s^{-1}. Because of the presence of excipients in the commercial IgG formulation, reconstitution of 500 mg in 1 mL 10 mM sodium phosphate (pH 7.4) yields a solution containing 460 mg·mL^{-1} IgG, 100 mM sucrose, 16 mM sodium phosphate, and 100 mM NaCl which is close to being isotonic with blood. Therefore, IgG concentration of 460 mg·mL^{-1} was chosen for next experiments.
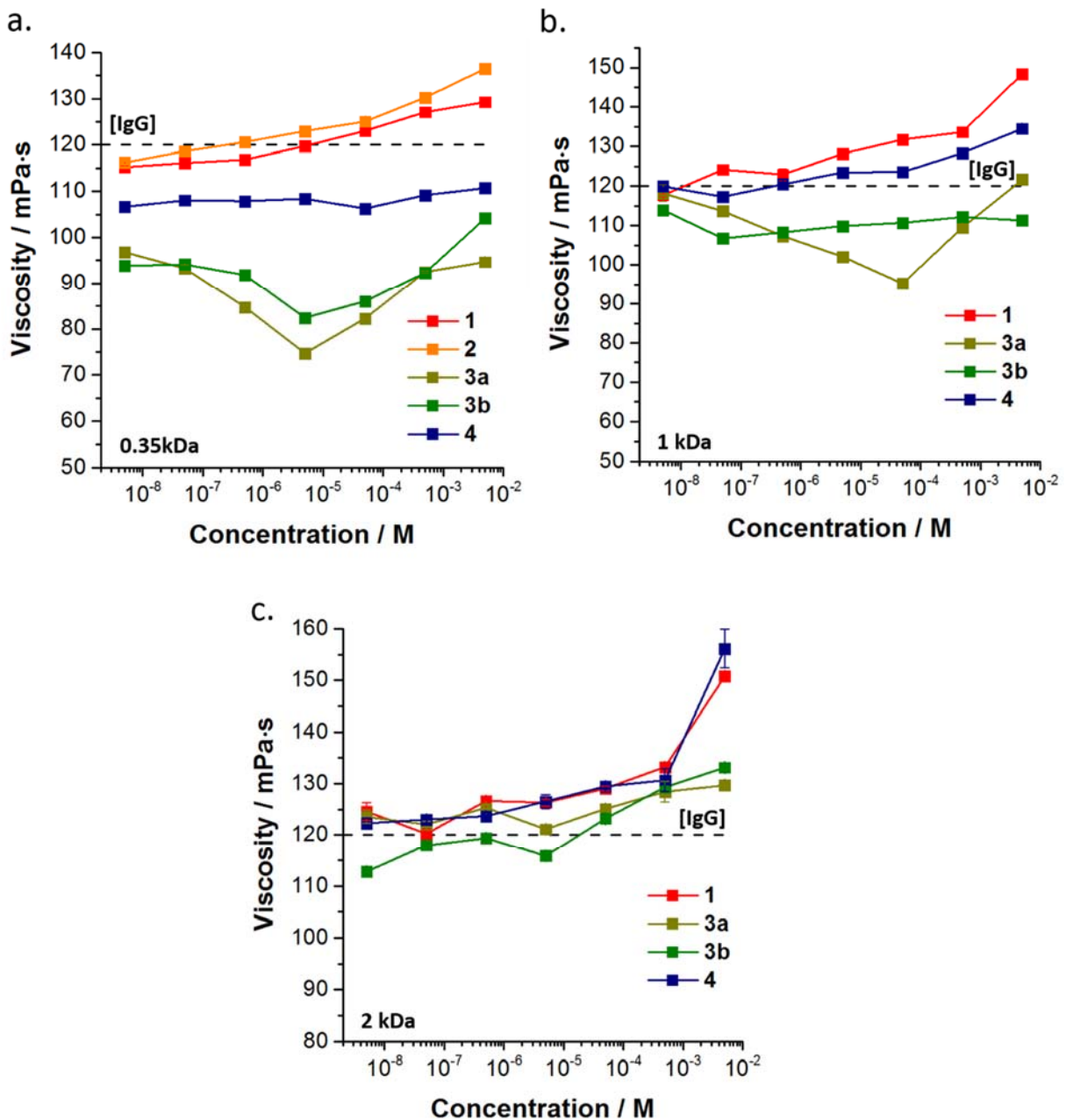
Figure 4.7. rPEGylation reduces the viscosity of concentrated IgG solutions. Effect of **1–4** concentration and mPEG length (a) 0.35 kDa, (b) 1 kDa, and (c) 2 kDa on the viscosity of 460 mg·mL^{-1} IgG solution. Note: in (a) the molecular weight of **4** was 0.55 kDa, due to its commercial availability. Horizontal dashed lines at 120 mPa·s represent the viscosity in the absence of additive. All data shown as mean ± SD (n = 3).

Then, the viscosity of IgG solutions containing a broad range of additive concentrations was evaluated (Figure 4.7). According to the theory of excluded volume, proteins are excluded from regions of solvent containing mPEG and sucrose.[210, 211] This concentrates the proteins thereby promoting protein–protein interactions, and exerts a pressure on them to adopt their most compact structure (e.g., folded vs. denatured).[210] In fact, at high additive concentrations, proteins can be concentrated beyond their solubility, a property which has been harnessed in the form of mPEG-induced precipitation, or even the crystallization of mAbs.[206, 211] To gauge the extent of these effects, the viscosity of 460 mg·mL^{-1} (= 526 × 0.95 × 0.92) IgG solutions containing **1–4** was evaluated using a capillary rheometer (Figure 4.7). When mPEG (**1**) was added as a control for non-specific interactions between mPEG and IgG, an increase of viscosity was observed and was more pronounced with increasing mPEG molecular weight. This is consistent with expectations arising from excluded volume theory. The acetophenone derivative, **2**, produced a similar result to **1**, suggesting that the hydrophobic aromatic group is not contributing to any specific interaction with IgG, under conditions of near-neutral pH. mPEG–propionaldehyde **4** was expected to more readily react with nucleophilic residues on IgG than **2**, because its carbonyl is not in resonance with an aromatic group (making the latter a poorer electrophile).[35] Indeed, low molecular weight **4** (550 Da) had a small effect on viscosity (~10% vs. IgG alone) when present at as little as 5 nM. This effect was persistent up to 10 mM, at which point the viscosity of the additive-free IgG solution was recovered. It is very interesting to note that the molar concentration of IgG itself is ~3 mM (assuming a molecular weight of 150 kDa), which implies that extremely low (highly sub-stoichiometric) concentrations of dynamic rPEGylation agents can affect the bulk properties of the solution. Increasing the molecular weight of **4** to the 1–2 kDa range, however, attenuated any beneficial effect on viscosity, and results akin to mPEG (**1**) were observed.

Additives **3a, 3b** bear aromatic glyoxals can form more stable di-hemiaminal structures with arginyls (Figure 4.1b). These structures can resist hydrolysis for hours at neutral pH, even upon dilution. Interestingly, even at the lowest concentration of **3a, 3b** tested, 5 nM, both additives reduced the viscosity of the IgG solution by 20%. This effect became more pronounced when the concentration of **3a, 3b** was increased, and was most important at 5 μM, where solution viscosity was reduced by ~35% compared to the additive-free IgG solution. Beyond this point the viscosity increased in accordance with expectations from excluded volume theory. For perspective, at 5 μM, **3a, 3b** is ~600-fold more dilute than IgG on a molar basis, suggesting that a single molecule of **3a, 3b** is reversibly coupling with many IgG molecules over time. Only small differences were observed between the viscosity-lowering effect of two PGOs **3a** and **3b**, which differ by a

methoxy substituent on the aromatic ring (used to modulate reactivity). **3a** has been previously shown to be slightly more protein-reactive than **3b,** which is consistent with its slightly greater effect on viscosity. The viscosity-lowering effect of **3a, 3b** persisted when the molecular weight of its mPEG moiety was increased to 1 kDa, though the magnitude of the effect was less pronounced. This effect became largely negligible when further increased to 2 kDa, suggesting that the reactivity of **3a, 3b** towards IgG is diminishing due to steric or kinetic considerations. Overall, dynamic rPEGylation with PGO–mPEG effectively reduced viscosity of concentrated IgG at highly sub-stoichiometric amounts (vs. IgG).

4.3.4. Dynamic light scattering (DLS)

IgG within concentrated solutions is reported to reversibly self-associate into multimeric structures via their Fab regions, mostly by electrostatic and hydrogen bonding interactions.[209] To examine the influence of additives on protein–protein interactions, and explain lowered viscosity, concentration-dependent dynamic light scattering was performed (Figure 4.8). A 55 mg·mL^{-1} (= $63 \times 0.95 \times 0.92$) IgG solution containing 5 µM additive was analyzed and repeatedly diluted with a buffer of same salt and sucrose composition. This upper IgG concentration was chosen due to complications of multiple scattering, and the concentration of **1–4** was very close to that yielding minimum viscosity in Figure 4.7. In accordance with the observations of others,[212] the Z-average hydrodynamic radius ($<R_h>$) of IgG increased dramatically upon repetitive dilution due to the presence of self-assembled, yet soluble, clusters. This type of response arises because the intensity of the scattered light scales with size to sixth power. Thus the signal from smaller structures (i.e., monomers) decreases more rapidly by dilution than that from larger ones. The presence of **1–4** disrupted these assemblies in a manner that was proportional to their viscosity-lowering effect. These data therefore suggests that the viscosity-lowering effect observed lies in disrupted IgG self-assembly, caused by the presence of very small amounts of rPEGylating agents. In fact, very low concentrations of **3a, 3b** (~75-fold less than IgG) prevented assembly beyond the monomeric species, as only a slight increase of $<R_h>$ was observed upon dilution. This is suggestive that these may be very potent stabilizers for preventing aggregation within concentrated IgG solutions, in addition to lowering their viscosities.
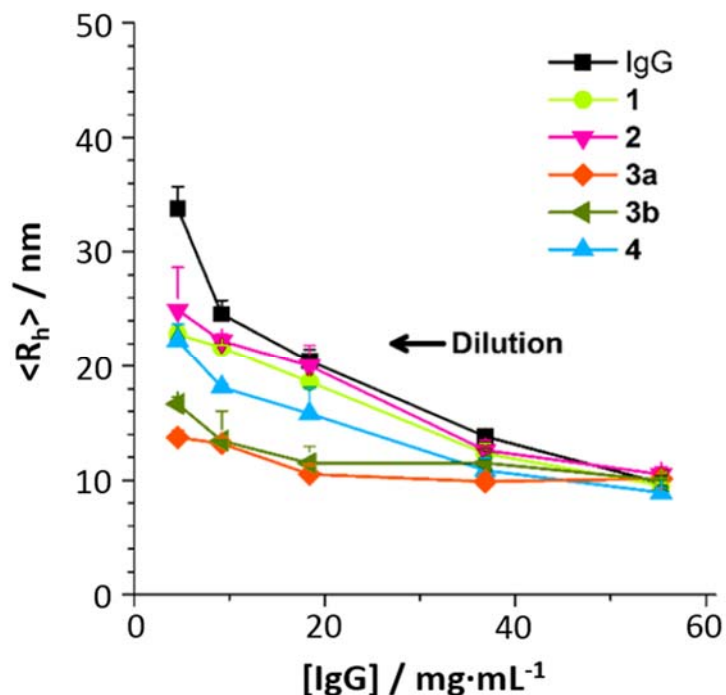
Figure 4.8. Concentration-dependent Z-average hydrodynamic radius $\langle R_h \rangle$ reveals the presence of self-assembled structures. This process is attenuated by some of the additives. All data plotted as Mean + SD (n = 3).

4.3.5. Storage stability

In the storage stability test, the aggregates of IgG controls without any additives both under static environment and agitation environment can be observed after 20 days incubation (Figure 4.9). Meanwhile, the IgG sample with **1** and **4** all formed the aggregates, just like IgG controls at both conditions. In previous viscosity measurements, **3a** and **3b** can effectively decrease the viscosities of high concentrated IgG solutions. Therefore, the IgG mixture samples with **3a, 3b** are expected to prevent the formation of the aggregation in this storage stability test. As shown in the photos, there was no visible aggregation formed in IgG samples with **3a, 3b,** which present the consistent results as the previous viscosity measurements.
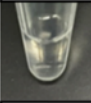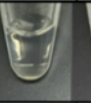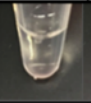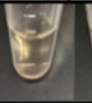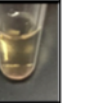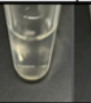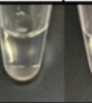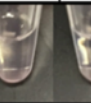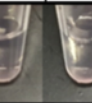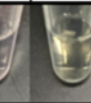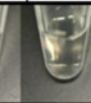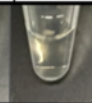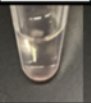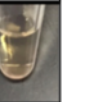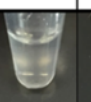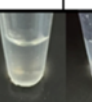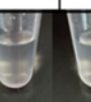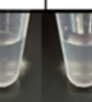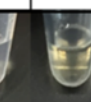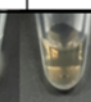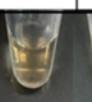
Statically	IgG	IgG+mPEG			IgG+PEG-aldehyde			IgG+PGO(-H)-PEG			IgG+PGO(-OCH₃)-PEG		
	control	0.35 kDa	1 kDa	2 kDa	0.55 kDa	1 kDa	2 kDa	0.35 kDa	1 kDa	2 kDa	0.35 kDa	1 kDa	2 kDa
0 d	-	-	-	-	-	-	-	-	-	-	-	-	-
20 d	+	+	+	+	+	+	+	-	-	-	-	-	-

Agitation	IgG	IgG+mPEG			IgG+PEG-aldehyde			IgG+PGO(-H)-PEG			IgG+PGO(-OCH₃)-PEG		
	control	0.35 kDa	1 kDa	2 kDa	0.55 kDa	1 kDa	2 kDa	0.35 kDa	1 kDa	2 kDa	0.35 kDa	1 kDa	2 kDa
0 d	-	-	-	-	-	-	-	-	-	-	-	-	-
20 d	+	+	+	+	+	+	+	-	-	-	-	-	-

Figure 4.9. Optical images of IgG with different PEG additives after 20 days incubation under both static and agitation environment. (-) no aggregation observed; (+) aggregation observed. Note: the yellow color of **3a** and **3b** is a hallmark of the PGO group.

4.3.6. Size exclusion chromatography and gel electrophoresis

As **3a, 3b** appeared to effectively disrupt IgG self-assembly, its ability to prevent the formation of insoluble aggregates was analyzed. ~74 mg·mL^{-1} (= 84.8 × 0.95 × 0.92) IgG solutions and 5 µM **1–4** containing (or no mPEG additives) were stored statically at room temperature, or subjected to mechanical stress in the form of orbital agitation. Size-exclusion chromatography performed after 24 h shows that while the average size of additive-free IgG increased, the sample containing **3a** retained the location of its maxima, but now possessed a tail. This is suggestive of enthalpic interactions with the column, due to rPEGylation (Figure 4.10). This was confirmed by performing denaturing gel electrophoresis (non-reducing) after 1 week incubation (Figure 4.11). No differences between IgG without and with **3–4**, were observed and the relative amount of monomeric versus dimeric IgG was also unchanged. Furthermore, because the denaturing conditions employed may have disrupted aggregates or released **3–4** from IgG, native gel electrophoresis was performed. With the exception of samples containing **3a, 3b**, the

band associated with IgG increased in size to somewhere between the monomeric and dimeric species, suggesting (at least partial) denaturation. Furthermore, larger aggregates that were unable to penetrate the gel were also observed for the samples showing denaturation. (Figure 4.11). It is also interesting to observe that because **3a, 3b** are present in such small amounts, IgG–mPEG conjugates are not even visible on the gel, because of their low abundance relative to free IgG.
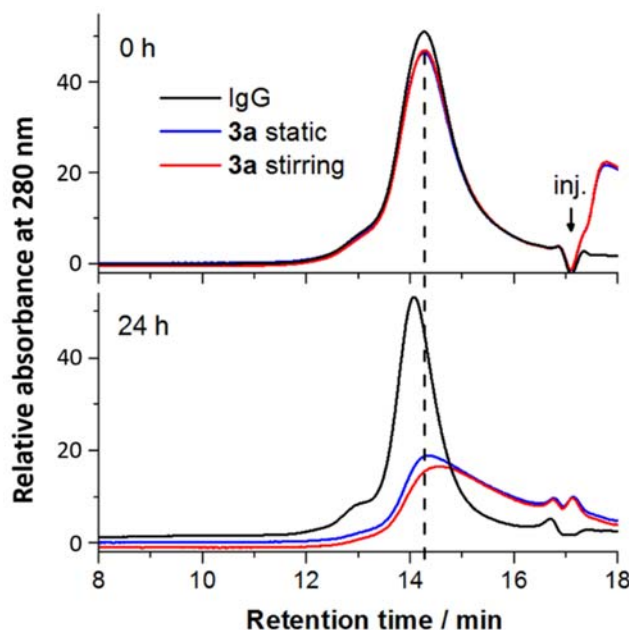
Figure 4.10. Size-exclusion chromatograms of IgG without and with **3a** after 24 h (inj.: injection solvent peak).
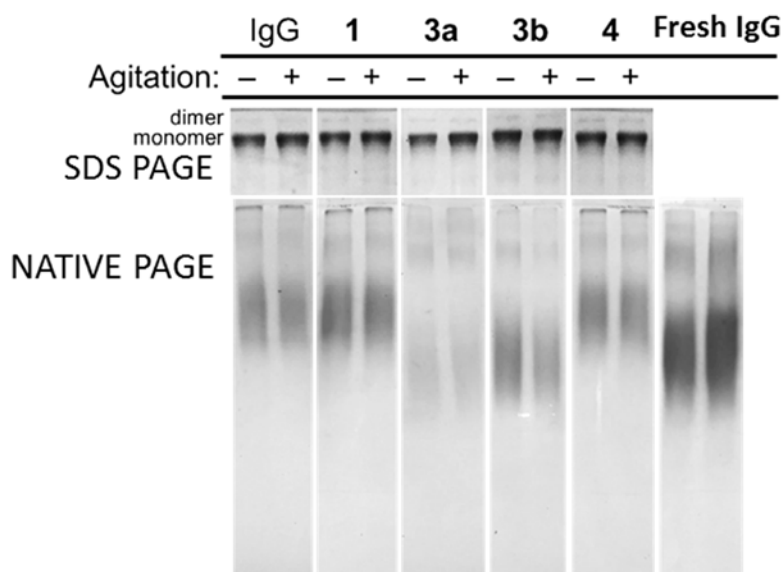
Figure 4.11. SDS and native gel electrophoresis of IgG without and with **1–4** after one week and 20 days incubation.

4.3.7. Circular dichroism spectroscopy

Circular dichroism spectroscopy of the aged samples confirmed that IgG maintained its native folded structure only in the presence of **3a**, and that partial unfolding was occurring for other additives (Figure 4.12). This was evidenced by the decrease in IgG's characteristic negative peak at ~218 nm (β-sheet structure). Interestingly, a more pronounced negative peak was not observed, because such a result has been attributed to mAb aggregation.
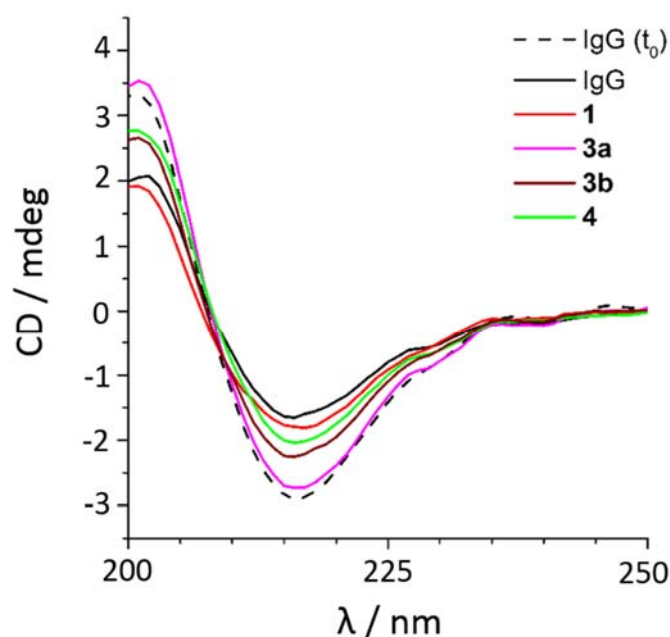
Figure 4.12. Circular dichroism (CD) spectroscopy after 25 days confirms partial denaturation of IgG. 3a completely preserved the native folded structure of IgG.

4.3.8. Characterization of the IgG conjugates

As the IgG used in this project is the commercial whole human IgG, the purification and demonstration of the conjugates can be more complicated than the monoclonal antibody. Here, the purified IgG conjugates were characterized with the UV absorbance. As the PGO group has specific absorbance at wavelength of 340 nm, this wavelength was used to determine the existence of the conjugates of IgG with **3a**, **3b**. The absorbance results of wavelength scanning from 280 nm to 500 nm are illustrated in Figure 4.13. At 320 nm, there is significant absorbance observed within the purified conjugates. The shift from 340 to 320 nm is probably caused by the PGO

structure changing after the reaction with arginine residues. Meanwhile, there was no absorbance observed in the IgG samples or IgG with addition of **1**, **4**. Therefore, the existence of conjugates of IgG with **3a, 3b** was demonstrated.
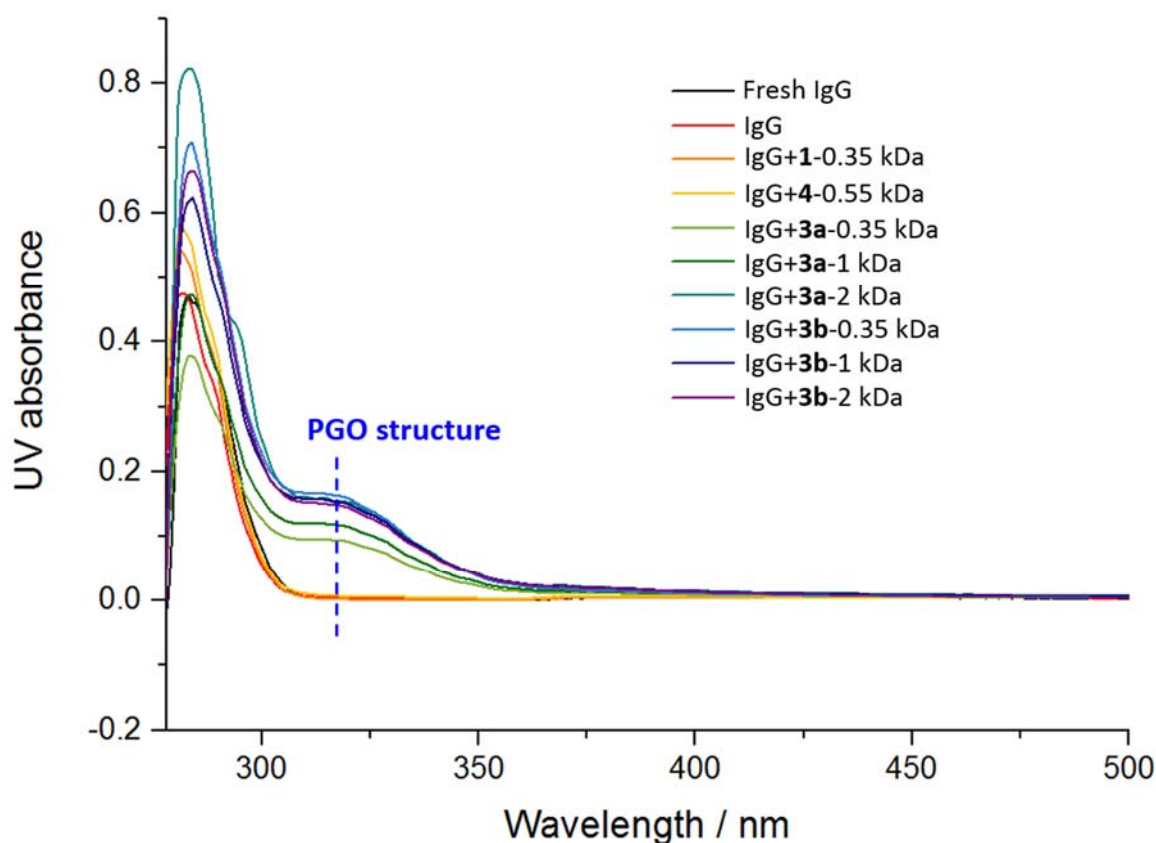
Figure 4.13. UV absorbance of IgG samples and purified conjugates. At 320 nm, the specific absorbance demonstrated the existence of the IgG-PGO-mPEG conjugates. The concentration of the IgG samples and conjugates was 1 mg·mL^{-1}.

4.4. Conclusions

In summary, this study demonstrates the highly-sub-stoichiometric amounts of arginine-specific rPEGylation agents effectively reduce viscosity, disrupt the self-assembly, and prevent insoluble aggregation of IgG within very concentrated solutions containing therapeutically-relevant amounts of salts and osmolytes. This finding provides exciting new perspectives for the design of additives subcutaneously-injectable solutions of concentrated IgG or mAbs.

Chapter 5.

General conclusion and outlook

5.1. Most significant finding of this thesis
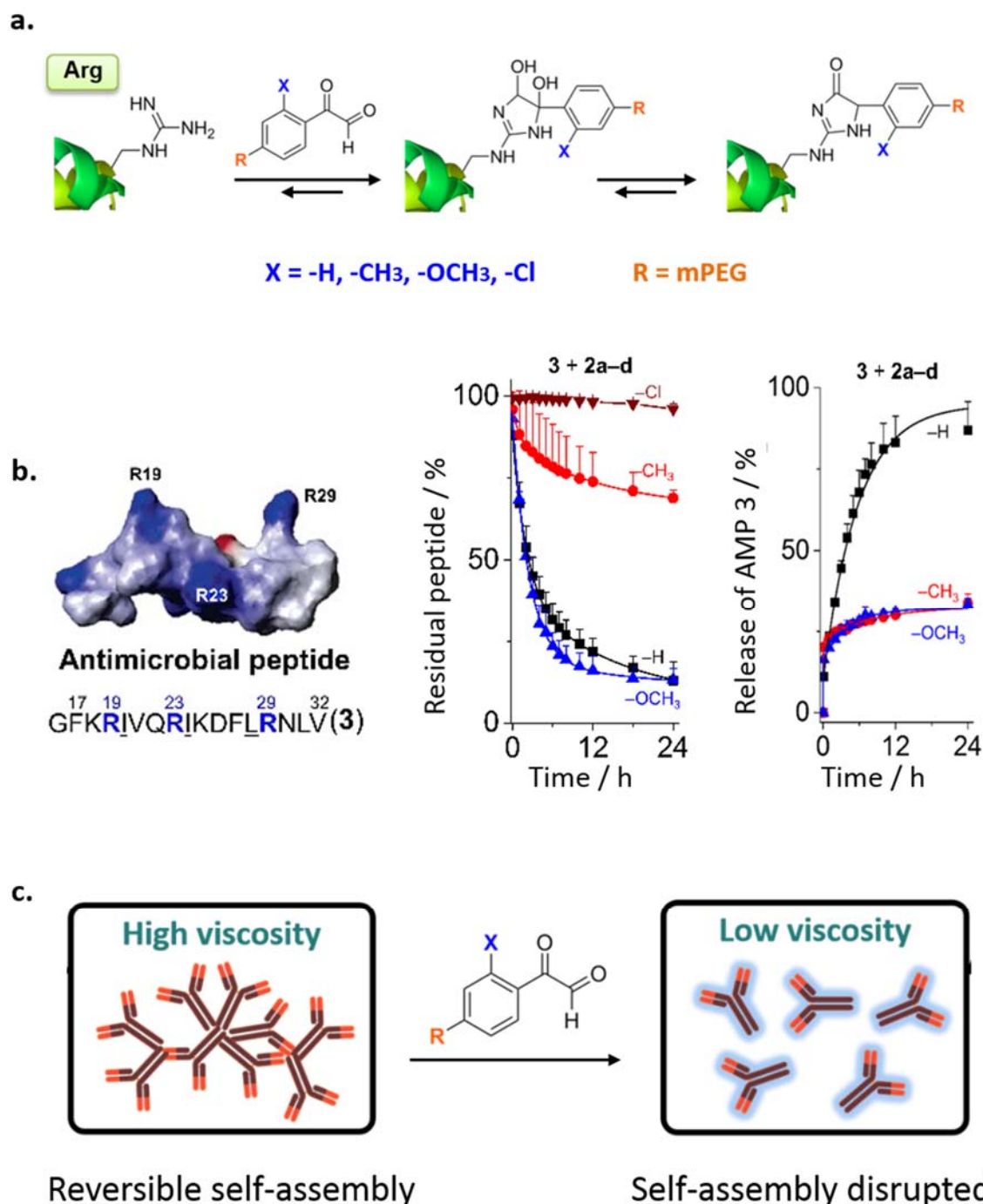
Figure 5.1. Summary of the major findings of the thesis. (a) Arginine-reactive mPEG bearing PGO derivatives were synthesized and they may reversibly conjugate with arginine residues in proteins/peptides; (b) Arginine-rich AMPs could be modified with PGO-mPEG to increase the circulation half-life and control the rate of release the with a traceless fashion; (c) This arginine specific rPEGylation can be applied to disrupt the protein–protein interactions within high concentration mAbs solutions. The aggregation and viscosity of mAbs can be prevented and reduced significantly.

The most significant finding of this thesis is the demonstration that PGO derivatives can be used for rPEGylation at arginine residues on peptides and proteins. This platform may have great utility for pharmaceutical applications and it was specifically shown to be useful for two classes of therapeutic agents herein. This finding could be obtained via the contributions as illustrated in Figure 5.1. More specifically:

1) With our rPEGylation method, it is possible to temporarily conjugate mPEG to arginine-rich AMPs, which should increase their circulation half-life effectively while providing a means of release AMPs in physiological conditions with a traceless fashion.

2) The peptide/protein rate of release can be modified by changing the structure of the PGO group. For example, the substitution of electron-donating and electron-withdrawing groups on PGO.

3) Very small amounts of rPEGylation agent efficiently disrupt protein–protein interactions within high concentration IgG solutions. This significantly reduces viscosity, and prevented IgG precipitation under prolonged storage and stress. Because mAbs are emerging as an important new class of therapeutic agent, this finding may broadly impact the way they will be formulated.

5.2. Major limitations of this work

First of all, the major limitation of the presented strategy is that the reactive PGO group may also react with other nucleophilic amino acid residues, such as cysteine. Therefore, the selectivity of rPEGylation may be compromised when the protein sequence contains both arginine and cysteine residues. In the case of synthetic peptides containing cysteine residues, selective side-group protection strategies could be considered to overcome this caveat.

Secondly, the reactivity of the targeted arginine residue is sensitive to its local environment. For example, as the conjugation can be influenced by steric hindrance, there should be no bulky neighboring residues around. In line with this idea, arginine residues that are not solvent-exposed will unlikely react with the described rPEGylation agents. Therefore, while the described strategy remains highly applicable to short arginine-containing peptides, or for proteins for which lower

degrees of conjugation are desired, it may be less applicable to extensively modify the surface of a protein.

Finally, one last limitation may lie in large-scale production. In Chapter 3, the reaction between the peptide and the mPEG-PGO proceeds over a period of ca. 24 h, which is slow on an industrial scale. While this reaction could be accelerated by increasing reactant ratios, this could potentially complicate purification. It should however be mentioned that similar limitations are expected for conventional PEGylation.

5.3. The differences of this work from that of others

The first difference is that we target a residue for which no rPEGylation chemistry currently exists. Thus our work has added value as it provides new opportunities for rPEGylation peptides/proteins, which may not have e.g., lysine residues. The second major differentce is that rPEGylation using glyoxals does not involve linker chemistry but rather relies on an equilibrium. This particularity is responsible for the result observed in Chapter 4 where highly sub-stoichiometric amounts of rPEGylation agent affect the bulk properties of concentrated IgG solutions.

5.4. Future work to be continued

This thesis presents the *in vitro* proof-of-concept for arginine-specific rPEGylation. The next logical step will be evaluate rPEGylation in the more complex environment of the body, to examine the influence of serum components on release. In addition, it will be important to better characterize the potential toxicity of PGO-mPEG. Indeed, common glyoxals can be cytotoxic in the mM range due to the scavenging of thiols.[213, 214] The extent to which this will be important in the context of a therapy will, however, inevitably relate to the potency of the therapeutic peptide/protein, the dose at which it is administered, and the duration of the treatment. It will be also important to study the biological activity of AMPs after release from the conjugates.

In addition, it would be interesting to examine additives to accelerate the rate of the conjugation reaction, so as to improve scalability. Borate and carbonate buffers appear to be good starting points considering their known activation of the glyoxal groups. [93, 94]

Next, the storage stability of the antibody conjugates will be investigated. It will be interesting to optimize the formulation of IgG conjugates, which may provide the possibility for the clinical applications.

Finally, considering the recent interest in replacing mPEG with other polymers, it would be interesting to design a PGO reagent that can be conveniently appended to different types of synthetic or natural polymers.

5.5. How this work may benefit society

This thesis has shown two plausible applications of arginine-specific rPEGylation. The most direct way of this research will benefit society will be to pursue pre-clinical development.

1) For AMPs, with the tests of biological activity and stability of AMPs, the best course of action would be to contact an industrial partner that is already pursuing the pre-clinical development of an AMP. In a collaborative effort, we can explore and compare on a head-to-head basis the performance of the native and rPEGylated AMP.

2) For mAbs, the formulation of IgG conjugates will be further optimized and safety issues needs to be investigated. Then, the next step will be to contact manufacturers of mAbs to obtain ones that in solution suffer from unusually high viscosity, and destined for the treatment of chronic diseases such as rheumatoid arthritis. In a rapid timeframe, it will be possible to evaluate the viscosity-lowering effect of rPEGylation, which could contribute to accelerating the development of an injectable mAb formulation.

List of abbreviations

ACN	acetonitrile
AMPs	antimicrobial peptides
anti-TNFα	anti-tumor necrosis factor α
Arg (R)	arginine
ASNase	L-asparaginase
ATRP	atom transfer radical polymerization
Bicin	bis-2-hydroxyethylglycinamide
BSA	bovine serum albumin
CD	circular dichroism
$CDCl_3$	deuterated chloroform
Cys (C)	cysteine
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
D_2O	deuterium oxide
EGF	epidermal growth factor
FBS	fetal bovine serum
FDA	US Food and Drug Administration
FITC	fluorescein isothiocyanate
HEMA	2-hydroxyethyl methacrylate
HIV	Human immunodeficiency virus
G-CSF	granulocyte colony-stimulating factor
GH	growth hormone
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
IgG	immunoglobulin G
LC-MS	liquid chromatography-mass spectrometry
Lys (K)	lysine
mAb	monoclonal antibody
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MS	mass spectrometry

mPEG	methoxy poly(ethylene glycol)
NHS	N-hydroxysuccinimide
NTA	nitrilotriacetic acid
NMR	nuclear magnetic resonance
OspA	outer surface protein A
PBS	phosphate-buffered saline
PEG	poly(ethylene glycol)
PGO	phenylglyoxal
PLA$_2$	phospholipase A$_2$
PNA	peptide nucleic acid
PUMPT	polymer-masking-unmasking-protein-therapy
rPEGylation	releasable PEGylation
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
TFA	trifluoroacetic acid
TGA	thermogravimetric analysis
TRAIL	tumor necrosis-related apoptosis inducing ligand
UV	ultraviolet
VEGF	vascular endothelial growth factor

Scientific contributions

Publications:

- **Yuhui Gong**, Jean-Christophe Leroux, Marc A. Gauthier. Releasable and traceless PEGylation of arginine-rich antimicrobial peptides. *In revision.*

- **Yuhui Gong**, Andreas Niederquell, Martin Kuentz, Jean-Christophe Leroux, Marc A. Gauthier. Highly sub-stoichiometric dynamic PEGylation disrupts protein-protein interactions in concentrated immunoglobulin solutions. *In preparation.*

- **Yuhui Gong**, Jean-Christophe Leroux, Marc A. Gauthier. Releasable conjugation of polymers to proteins. *Bioconjugate Chem.*, **2015**, 26, 1172. **(IF=4.513)**

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- Juan Yang, Huiyuan Wang, Wenjie Yi, **Yuhui Gong**, Xiang Zhou, Renxi Zhuo, Xianzheng Zhang. PEGylated peptide based reductive polycations as efficient nonviral gene vectors. *Adv. Healthcare Mater.*, **2013**, 2, 481. **(IF=5.797)**

- Cao Li, Kang Wang, **Yuhui Gong**, Zeyong Li, Jing Zhang, Guofeng Luo, Renxi Zhuo, Xianzheng Zhang. Fabrication of photosensitive multilayered films via layer-by-layer assembly with well controlled porous structure. *J. Mater. Chem.*, **2012**, 12, 2045. **(IF=6.626)**

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- **02. 2015**   Doktorandentag, Zürich, Switzerland

- **01. 2015**   Zürich-Basel-Geneva Joint Seminar Series in Drug Formulation & Delivery, Basel, Switzerland

- **12. 2014**   9th Chunhui Cup Innovation & Entrepreneurship Conference, Guangzhou, China

- **08. 2014**   7th Swiss Pharma Science Day, Bern, Switzerland

- **07. 2014**   Annual Controlled Release Society Meeting, Chicago, USA

- **06. 2014**   Materials and Process (MaP) Graduate Symposium of ETH Zürich, Zürich, Switzerland

- **07. 2013**   Zürich-Basel-Geneva Joint Seminar Series in Drug Formulation & Delivery, Geneva, Switzerland

- **06. 2013**   8th Graduated Symposium of the Materials Research Center of ETH Zürich, Zürich, Switzerland

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