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## Development of novel PEGylation approaches based on non-covalent interactions

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**Development of novel**  
**PEGylation approaches based on**  
**non-covalent interactions**

THÈSE

présentée à la Faculté des Sciences de l'Université de Genève  
pour obtenir le grade de Docteur ès Sciences,  
mention Sciences Pharmaceutiques

par

**Claudia MUELLER**

de

Suhl (Allemagne)

***To my family.***

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# Chapter 1

## Introduction of the Thesis

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### **PEGylation and Drug Modifying Techniques for Sustained Drug Action**

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## 1 Introduction

In the late 1970's, the development of protein therapeutics was still in its infancy. Moreover, therapeutics of non-human origin were prone to elicit adverse immunogenic reactions after parenteral injection, and suffered from poor pharmacokinetic profiles. The covalent attachment of polyethylene glycol (PEG), coined "PEGylation", initially developed by Davies and co-workers at Rutgers University (NJ, USA) for the solubilisation of lipophilic small molecular weight therapeutics [1], appeared to be an applicable technology to mitigate these obstacles for effective therapy using protein therapeutics. Not only reduced or even absent immunogenicity was demonstrated for such modified compounds; prolonged residence time of the modified compounds in the body was described, as well [2].

Since then, the availability of a broad range of protein therapeutics of human origin and of high purity has increased considerably thanks to genetic engineering, with the biotech/biopharmaceutical industry being a major player in modern drug development [3,4]. At the same time, PEGylation technology advanced tremendously, gaining importance in the generation of novel compounds, now including also nucleic acid-based therapeutics, and in the life-cycle management of existing therapeutics. Today, various PEG-conjugated molecules have been approved for therapeutic use in humans or are in the development pipeline, and various approaches for PEGylation exist or are under development [5]. However, clinical reports of the development of anti-PEG antibodies observed in patients treated with PEGylated therapeutics and drug carrier systems are available now [6-11], and therefore alternative technologies are being also pursued.

This chapter aims to discuss the state-of-the-art of PEGylation and point out its benefits and limits, and recent alternatives.

## **2 PEG and general aspects of PEGylation**

Ring opening polymerization of ethylene oxide monomers results in a polydisperse sample of polyethylene glycol (PEG). The obtained PEG is subsequently modified to linear monofunctional (one end is rendered non-reactive by methoxylation while the second end contains a reactive moiety), linear homobifunctional (both ends carry the same reactive moiety), and linear heterobifunctional (each end possesses a different reactive moiety) PEGs. The terminal moiety (“linker”) is designed to react with functional groups on the target moiety, sometimes endowed with a certain specificity, to form a covalent bond between the PEG residue and the target molecule. In addition to these linear PEG residues, two or more polyethylene glycol chains may be connected via a central core molecule, forming branched or multi-arm designs, which again can be tethered to the target molecules. The subsequent derived polymers are highly water-soluble (by virtue of binding of several water molecules by each ethylene unit via hydrogen bonding), expected to have low toxicity and are therefore approved by FDA [12, 13]. The PEGylation process itself encompasses the attachment of one or several of these polyethylene glycol chains of different molecular weights to proteins and peptides, low molecular weight drugs or oligonucleotides by various available chemical or enzymatic strategies.

## **3 PEGylation Chemistry**

With regard to PEGylation chemistry, first generation PEGylation processes and second generation PEGylation chemistry have to be distinguished [12, 14]. Since amino groups are the most prevalent functional groups and are functional groups easily accessible in proteins, they were the preferential targets for modification via first generation chemistry. Though problems may arise from this strategy, several conjugates using this chemistry were approved by the FDA from the early 1990's on,

e.g., Oncaspar® (PEG-asparaginase), Adagen® (PEG-adenosine deaminase) or PEG-Intron® (PEG-Interferon- $\alpha$ -2a). Characteristic for first generation PEG chemistry was the high number of isomers obtained due to multiple conjugation events, resulting in challenges concerning the batch-to-batch reproducibility and purification processes. Furthermore, mostly linear and small molecular weight PEGs ( $\leq 12$  kDa) were used. During the reaction, the formation of weak linkages was possible, leading to unstable products, or even side reactions such as cross-linkage occurred, due to a relatively high PEG diol content [5, 12, 14].

With second generation PEGylation technology, the problems that arose from first generation chemistry were attempted to be avoided. In particular, few or even only one high molecular weight PEG chain(s) are being conjugated in a site-specific manner by means of various newly developed conjugation technologies. Furthermore, PEG diol can be removed to a large extent by formation of PEG carboxylic acid intermediates and subsequent purification using ion-exchange chromatography [15, 16]. By virtue of all of these processes, improved pharmacokinetic and pharmacodynamic properties, lower immunogenicity, and higher homogeneity of the PEG-protein entity compared to the unmodified molecule are obtained.

In the following section, we would like to provide a short overview of first generation processes, and mainly concentrate on the second generation processes and recent developments.

### **3.1 First generation chemistry**

As already stated above, the main target functional groups for PEGylation in proteins with first generation processes were alpha and epsilon amino groups. Since peptides and proteins are too susceptible to any kind of activation reaction and protein



degradation may occur, usually the PEG is converted into more a reactive species for the linking process. The first reports on PEGylation from Davies and coworkers described the transformation of PEG to the dichlorotriazine derivative using cyanuric chloride [2, 17]. The two remaining chlorine atoms of the dichlorotriazine-PEG both are able, although to a different degree of reactivity, to non-selectively react with different nucleophilic groups like amines, sulfhydryles, hydroxyl- and imidazole-moieties [18, 19]. To avoid cross-linking of proteins and multiple non-selective conjugations, the group around Inada produced the monovalent and less reactive chlorotriazine mPEG [20]. Being more selective, the derivative was supposed to be targeted to amino- and thiol-groups, but as was shown by Ono et al. [21], dichlorotriazine-PEG and mPEG<sub>3</sub>-triazine were generated as well during the synthesis providing again the possibility of side reactions. Today, both derivatives are no longer used for PEGylation of therapeutics due to their toxicity [5].

Using PEG–tresylate (2,2,2-trifluoroethanesulfonate), a more reactive sulfonate ester than the tosylate or the mesylate, secondary amine linkages are generated during reaction with amino groups of proteins. Although the tresylate also reacts with present thiol moieties of proteins and peptides, it is already more specific to amino-groups than the dichlorotriazine derivative [14].

Even more selective towards primary amino groups, PEG-acetaldehyde forms secondary amine linkages in a two step reaction: the Schiff's base generated in a first step is subsequently reduced *in situ* with sodium cyanoborohydrate [22]. The product is relatively unstable, since rapid dimerization by aldol condensation or oxidation to carboxylic acids takes place, providing a challenge for the preparation of highly pure batches.

Attachment of carboxyl-PEG or activated carboxyl-esters as well as reactive carbonate-derivatives of PEG to proteins result in the formation of amide linkages.

The use of these so-called acylating agents leads to the loss of positive charge in the final conjugate, whereas after reaction with the above described arylating and alkylating compounds the charge of the native protein remains conserved in the final PEGylated product [5]. Preserving the charge in the conjugate may be of fundamental importance for proteins, since their activity might be related to their overall charge and resulting folding.

The class of reactive carbonates of mPEG comprises a wide range of compounds: PEG-succinimidyl carbonate [23,24], PEG-benzotriazolyl carbonate [25,26], PEG-*p*-nitrophenyl carbonate [27], PEG-2,3,5-trichlorophenyl carbonate [27] and PEG-carbonylimidazole [28, 29] are yielding relatively stable carbamate/urethane linkages after being attached to amino groups of proteins and peptides. Very important is the difference of reactivity, decreasing gradually from the most reactive succinimidyl carbonate and the benzotriazolyl derivative over to the *p*-nitrophenyl carbonate, trichlorophenyl carbonate and carbonylimidazole, the latter being the least reactive [30]. The more reactive the derivative, the less selective and specific the PEGylation becomes, and consequently the more heterogeneous the final product will be. Thus, it is not surprising that succinimidyl carbonate and the benzotriazolyl-PEG are able to react with histidine and tyrosine moieties besides lysine residues [31, 32].

A very well known example of a reactive carboxyl-ester remaining from first-generation chemistry is succinimidyl succinate-PEG [33, 34]. First, mPEG-carboxyl is produced from reaction of the mPEG-hydroxyl with succinic anhydride. Secondly, the acid is activated by reaction with N-hydroxysuccinimide and a carbodiimide. After reaction with a protein a carbamate linkage is formed. However, the ester linkage between the PEG and the succinate moiety is a source of severe stability problems, since esters are easily hydrolyzed. Furthermore, antibody formation against the remaining succinic acid group on the protein was observed [35]. This undesired effect

of PEGylation, adding hapten properties of succinate moieties to the protein, may also be applied as an 'adjuvant' property for protein vaccines: proteins for vaccination are rendered more immunogenic after modification with succinic acid [36, 37]. A non-exhaustive list of first generation PEGylation reagents is shown in Table 1.

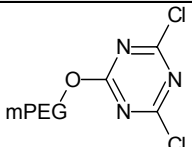
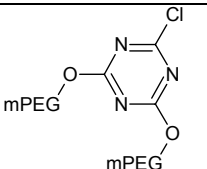
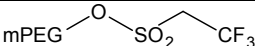
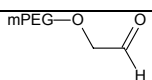
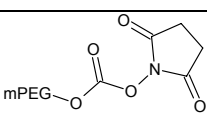
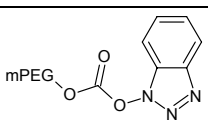
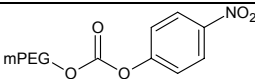
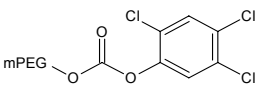
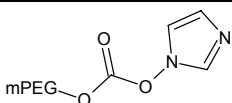
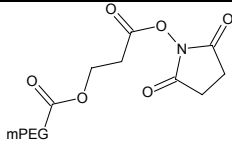
## **3.2 Second generation chemistry**

### **3.2.1 PEG chemistry for amine modification**

One of the earliest PEG-derivatives of the second generation, mPEG-propionaldehyde, was developed by Harris et al. [38]. PEG-propionaldehyde is highly selective towards N-terminal  $\alpha$ -amines when working in an acidic environment of a pH at around 5, as Kinstler et al. observed in several publications [39, 40]. Having a lower  $pK_a$  than other nucleophiles of proteins at this pH, conjugation mainly takes place at the N-terminal amino group [41]. Nevertheless, also other amines may be modified [42, 43], however the degree of PEGylation is clearly reduced when compared to first generation techniques.

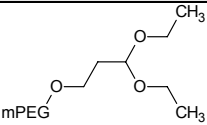
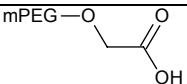
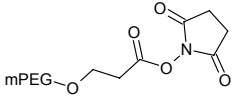
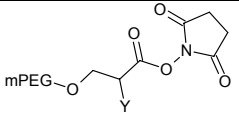
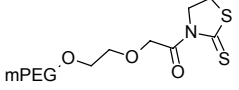
Another possibility is represented by the use of aldehyde hydrates formed *in situ* from PEG-aldehyde diethyl acetals at acidic pH. The obtained aldehyde hydrates are not further purified or isolated, hence condensation and oxidation is prevented, but are directly used for conjugation with the target protein in solution [44, 45].

Recently, a method of rendering conjugation with PEG-aldehyde even more specific was presented by Lee and co-workers [46]. Although using mPEG-aldehyde, a site-specific PEGylation of the N-terminus of interferon  $\alpha$ -2a was obtained by adsorption of the protein to a solid phase during the conjugation. Although the yield of mono-PEGylated interferon was only around 60% for different molecular weight PEGs used, the removal of impurities, product isolation and purification was simplified when compared to liquid phase conjugation.

Activated PEG	Structure	Resulting linkage
dichlorotriazine-PEG		secondary amine
chlorotriazine-PEG		secondary amine
PEG-tresylate		secondary amine
mPEG-acetaldehyde		secondary amine
mPEG-succinimidyl carbonate		carbamate/urethane
mPEG-benzotriazolyl carbonate		carbamate/urethane
mPEG- <i>p</i> -nitrophenyl carbonate		carbamate/urethane
mPEG-2,3,5-trichlorophenyl carbonate		carbamate/urethane
mPEG-carbonylimidazole		carbamate/urethane
mPEG-succinimidyl succinate		amide

**Table 1:** PEGylation reagents for first generation chemistry.

Further derivatives for amino group modification are reactive esters of PEG-carboxyls. With the introduction of carboxymethylated PEG [47], propionic or butanoic acid PEGs and branched derivatives thereof [16], hydrolyzation as seen with succinimidyl succinate is avoided. Another important property of propionic- and butanoic acid-PEGs is that their reactivity is dependent on the number of CH<sub>2</sub>-groups between the PEG and the activated carboxyl moiety. The more groups, the slower and thus more selective the reaction takes place. Thus, the butanoic acid-PEG is less reactive than the propionic derivative and may be even more decreased by the introduction of branching moieties in  $\alpha$ -position to the carboxyl group. For each derivative the carboxylic acid needs to be activated, whether as N-hydroxysuccinimidyl ester, benzotriazole or imidazole [5].

Activated PEG	Structure	Resulting linkage
mPEG-aldehyde hydrate		secondary amine
carboxymethylated mPEG		amide
NHS ester of propionic acid mPEG		carbamate
NHS ester of $\alpha$ -branched propionic acid mPEG		carbamate
thiazolidine-2-thione activated mPEG		carbamate

**Table 2:** Selected PEGylation reagents for amine specific second generation chemistry.

Another approach impeding formation of hydrolyzable conjugates is the use of PEGs conjugated with an activated amino acid, such as norleucine (Nle) or  $\beta$ -alanine ( $\beta$ Ala), or a short activated peptide, such as Met-Nle or Met- $\beta$ Ala [48]. Conjugation results in stable amide bonds and quantification and localization of the PEGylation may easily be performed by amino acid analysis of Nle and  $\beta$ Ala as reporters.

Greenwald and co-workers described the synthesis of thiazolidine-2-thione activated mPEGs that form amide bonds during the reaction with proteins and peptides [49]. Synthesized by activating mPEG carboxylate to its acid chloride and reacting it subsequently with 2-mercaptothiazoline, the obvious advantage of this derivative is its rate of hydrolysis. Having a tremendously increased half-life of 240 min at pH 7.4 and a temperature of 22°C compared to other derivatives like succinimidyl carbonate-PEG (129 min), or succinimidyl succinate-PEG (65 min), it is not only more stable in aqueous solutions but also allows protein modification without pH alteration, minimizing the possibility of denaturation of pH sensitive proteins.

Another means to avoid multiple PEG-conjugation is the replacement of reactive lysines with inert amino acids by genetic engineering, as described for TNF  $\alpha$  [50], the immunotoxin anti-Tac(dsFv)-PE38 [51], and acetylcholinesterase [52].

Recently, growth hormone-releasing factor (1-29) [GRF(1-29)], the shortest and fully active form of GRF, was site-specifically PEGylated at lysine<sup>21</sup> under Fmoc-protection of other reactive amino acids, tyr<sup>1</sup> and lys<sup>12</sup> [53]. By optimization of the reaction conditions, a 95 % yield of mono-lys<sup>21</sup>-PEGylated GRF(1-29) was obtained, which is around 3.8-fold higher than obtained by non-specific PEGylation.

Rendering concurrent amino acids inert during the PEGylation process by means of *tert*-butoxycarbonyl (Boc) protection has been described for insulin, as well [54]. Maleic anhydride-protection/-deprotection was shown for glucagon-like peptide-1 (GLP-1), creating different protected isomers in a first step, which were separated by

reversed phase HPLC and subsequently conjugated with mPEG-succinimidyl propionate at the remaining free amino function [55]. Table 2 shows selected PEGylation reagents for amine-specific second generation chemistry.

### **3.2.2 PEG chemistry for sulfhydryl modification**

Site-specific PEGylation focusses to a great extent on the conjugation with cysteines. However, the number of free cysteines present on the surface of proteins is generally marginal, very often the cysteines are buried inside a hydrophobic patch of the protein, rendering the access of conjugation reagents difficult. In such cases, free and better amenable cysteines may be introduced into the protein by genetic engineering, which are subsequently conjugated to PEG [56-60]. Chapman et al. reported on a site-specific conjugation of an  $F_{ab}$  at hinge region cysteines that are usually involved in inter-heavy chain disulfide bridging by reducing them with a mild reducing agent [61]. Humphreys et al. recently published the use of strong reducing agents, resulting in a reduction of the disulfide bond between the heavy and light chain moiety that is believed to be crucial for stability and following therapeutic effect of the  $F_{ab}$ . The group was able to show that functionality was unaffected and thus PEGylation was performed at these new cysteines presenting a new alternative for site-specific antibody fragment PEGylation [62].

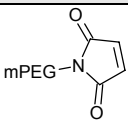
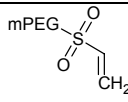
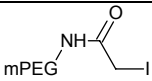
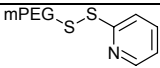
Another modification technique was described by Veronese and co-workers by PEGylation of the free, but rather inaccessible Cys<sup>17</sup> of G-CSF by partial and reversible denaturation [63].

Recently, site-specific PEGylation of disulfide bonds [64-66] was described as an even more original method. Conjugation is performed by reduction of the protein's disulfide bridges and subsequent sequential-bisalkylation with  $\alpha$ ,  $\beta$ -unsaturated  $\beta'$ -

monosulfone. The two thiols formed during reduction are reconnected via a PEGylated three-carbon bridge.

To attach PEG site-specifically to cysteine residues of proteins and peptides, several derivatives were developed: PEG-maleimide [67], PEG-vinyl sulfone [68], PEG-iodoacetamide [69], and PEG orthopyridyl disulfide [70]. Unlike the more reactive PEG-maleimide, PEG-vinyl sulfone is stable in aqueous solutions, however, both may undergo reactions with amines at an elevated pH. The pyridyl disulfide forms a disulfide bond during reaction with a protein that is amenable to reduction *in vitro* and *in vivo*.

PEGylation reagents for thiol specific second generation chemistry are listed in Table 3.

Activated PEG	Structure
mPEG-maleimide	
mPEG-vinyl sulfone	
mPEG-iodoacetamide	
mPEG-o-pyridyl disulfide	

**Table 3:** PEGylation reagents for thiol specific second generation chemistry.



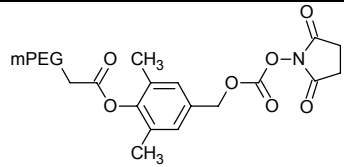
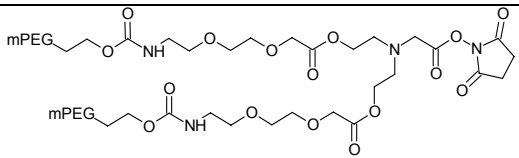
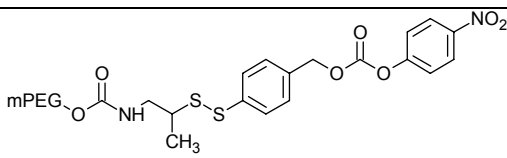
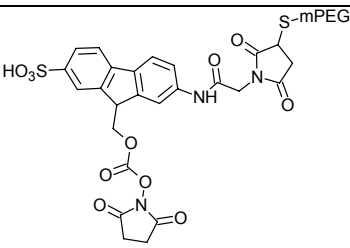
### 3.2.3 Chemistry for releasable PEGylation

The success of a PEGylated protein or peptide drug depends largely on its bioactivity. It is generally known that PEGylation may result in reduced bioactivity, which in most cases can be compensated by a prolonged pharmacokinetic profile or higher dose compared to the non-PEGylated molecule. Increasing the molecular size by conjugation with linear PEG, the use of branched and multiarm PEGs and different conjugation chemistries may all have an influence on the final pharmacokinetic profile and bioactivity of the resulting compound [71-75].

Releasable PEGylation results in a sustained release of the fully active API by virtue of degradable bonds between the PEG and the active principle. Several techniques have been developed and described for a modified release of proteins.

In 2000, Schering-Plough marketed a PEG-interferon- $\alpha 2b$  consisting mainly of an isomer having an unstable acyl imidazole linkage, which is formed between succinimidyl carbonate-PEG and His<sup>34</sup> at acidic pH [76, 77]. Other techniques include the use of succinimidyl activated PEGs with further instable ester groups in the backbone [78,79], leaving a possibly antigenic tag within the protein structure. The application of PEG-substituted maleic anhydride [80] or thiol specific PEG-pyridyl disulfide [70], resulting in a reducible linkage, prevent the remaining of tags. Greenwald and co-workers presented a drug releasing technology based on a 1,6-elimination mechanism, wherein the protein is attached via a labile carbamate bond to a p-substituted benzyl alcohol, which is in turn bound to a PEG via a cleavable ester, carbamate or carbonate linkage [81, 82]. The double prodrug design is first subject to hydrolysis releasing the PEG, and subsequently undergoes a fast benzyl elimination. Another technique developed by Greenwald's group is based on linear bicin linkers that were further improved by Zhao et al. into branched bicin linkers undergoing an anchimeric assisted hydrolysis [83, 84]. Recently, Filpula et al.

investigated four different releasable PEGylation systems of mesothelin targeted immunotoxin SSP1 and found reduced non-specific toxicity while pharmacokinetics were improved compared to the native immunotoxin [85]. Furthermore, antitumor efficacy was tremendously ameliorated compared to native and permanent conjugated immunotoxin.

Activated PEG	Structure
Example structure for a releasable linker employing 1,6-elimination as proposed by Greenwald <i>et al.</i> [77, 78]	
Releasable PEG based on branched bicin technology [79, 80]	
example structure for a releasable linker employing 1,6-elimination as proposed by Zalipsky <i>et al.</i> [82]	
FMS-linked PEG [83-85]	

**Table 4:** Selected PEGylation reagents used for reversible PEGylation.

Zalipsky et al. reported on a thiolytically cleavable linker based on a dithiobenzyl urethane bond between mPEG and the protein, which breaks down by a 1,6-elimination mechanism and decarboxylation after the disulfide bond of the linker is reduced or thiolytically cleaved [86].

A 2-sulfo-9-fluorenylmethoxycarbonyl spacer (FMS) was prepared and conjugated at one end to PEG-SH and at the other to different protein drugs [87-89]. The conjugates slowly released the proteins within 8-14 hours due to a  $\beta$ -elimination at position 9 of the fluorenyl moiety.

Since the field of releasable PEGylation develops fast, only the most important conjugation strategies are mentioned here. More detailed information is found in a recent review [90].

Table 4 lists selected PEGylation reagents employed for reversible PEGylation.

### **3.2.4 Other techniques for PEGylation**

An enzymatic approach employing transglutaminase for site-specific modification with PEG has been described by Sato et al. [91], and more recently by Fontana [92]. Furthermore, Ritter Jones et al. used a tissue transglutaminase to transfer peptides and peptide conjugated PEG to cartilage [93]. Using transglutaminase, an acyl transfer between  $\gamma$ -carboxamide groups of glutamine residues in proteins and a PEG-amine is catalyzed yielding a covalent linkage.

Non-glycosylated proteins expressed in *E. coli* were successfully and selectively glycosylated at serine and threonine hydroxyl moieties using a recombinant N-acetylgalactosamine-transferase [94]. A sialyltransferase subsequently introduced a sialic acid PEG to the before inserted GalNAc groups.

Chemical conjugation of the carbohydrate moiety of the ricin A-chain was obtained after oxidation of the former by sodium m-periodate and following ligation with hydrazide-PEG [95].

Other groups incorporate non-natural amino acids by genetic engineering, which are subsequently PEGylated. For example, p-azidophenylalanine was introduced into proteins and site-specifically modified with an alkyne bearing PEG [96].

A site-specific PEGylation at the C-terminus was obtained after engineering an azido-methionine into a human thrombomodulin mutant and subsequent reaction of this amino acid with PEG-triarylphosphine via Staudinger ligation [97].

Aminoxy-functionalized PEG was conjugated to N-terminal serine or threonine residues of proteins after generation of reactive carbonyl groups through periodate or metal-catalyzed transamination resulting in a stable oxime linkage [98].

## **4 Properties of PEGylated proteins**

### **4.1 Influence of PEGylation on formulation stability and shelf-life**

Since the therapeutic activity of a protein is highly dependent on its conformation, the successful development of a protein therapeutic depends to a large extent on its physico-chemical formulation stability. Physical and chemical degradation processes of proteins are frequently encountered during manufacturing, storage and handling of biopharmaceutics, resulting in poor stability and limited shelf-lives of the final formulation [99, 100].

Physical degradation encompasses phenomena like adsorption to surfaces, protein unfolding, and aggregation, being the most prevalent and cumbersome instability. Chemical degradation adverts modification of covalent bonds and may be seen in form of deamidation, oxidation, disulfide bond breakage and formation, as well as non-disulfide crosslinking [99–105]. Various factors, such as pH [106-108],

temperature [107-111], protein concentration [107, 112], additives and buffers used [108,113,114], as well as agitational stress [115, 116], thawing and freezing processes [117-119] and light [120] have been shown to influence protein stability. Until now, inhibition of protein drug aggregation is mostly achieved by empirical approaches, such as usage of different buffer systems, different pH values, ionic strengths, excipients, among others. Since the cause of aggregation is not yet totally clear and processes involved may be very complex, formulations either have to be maintained constantly at low temperature or have to be lyophilized to achieve an acceptable shelf-life [118, 121].

Furthermore, the majority of proteins used in biopharmaceutics are subject to post translational modifications [122], such as glycosylation (e.g., erythropoietin) [123], amidation (e.g., salmon calcitonin) [124], carboxylation or hydroxylation (e.g., blood coagulation factors) [125,126], which may influence their *in vitro* and *in vivo* properties. Recombinant production of proteins, for example in *E. coli* as expression system, does not allow for post translational modifications. As Runkel et al. showed for example for human interferon- $\beta$ , the lack of glycosylation lead to increased aggregation, increased sensitivity to thermal denaturation and diminished *in vitro* activity due to the absence of stabilization of the carbohydrate backbone of interferon and an increased solvent exposure of hydrophobic amino acids on the protein's surface [127]. For *E. coli* derived erythropoietin (EPO), an increased susceptibility against the destabilizing influence of guanidin HCl, pH and temperature was shown compared to glycosylated erythropoietin produced in mammalian cells [128, 129].

For several proteins, such as human recombinant interferon  $\alpha_{2a}$  [130], recombinant interferon- $\beta_{1b}$  [131], insulin [54,132], a human immunoglobulin G [133] and granulocyte colony stimulating factor [134], a decrease in aggregation tendency was reported after conjugation to PEG. A reduction of aggregation tendencies may be

explained by the steric shielding of hydrophobic patches within the molecular structure, thereby reducing intermolecular interactions of the protein molecules.

Increase in solution viscosity due to PEGylation may cause reduction of protein aggregation by decreasing molecular diffusion velocity.

On the other hand, however, an elevated viscosity poses a problem with regard to the injection of highly concentrated protein formulations. Therefore, a compromise between the degree of PEGylation and the consecutive increase in solution viscosity needs to be considered on a case-by-case basis.

PEGylation may not only affect the aggregation tendency of proteins, but also their solubility. Able to bind several water molecules per ethylene oxide unit via hydrogen bonding, PEG is highly hydrophilic, and it confers this property to the molecules it is conjugated to. The first observation of this phenomenon was published in 1977 by Abuchowski *et al.* who described an improved solubility of PEGylated bovine serum albumin over a wide pH range [17]. Later this effect was reported for other molecules, as well. For example, interleukin-2 usually precipitates at pH 7, which may cause side effects after administration. Conjugated to PEG, the molecule remains soluble at this pH [135,136]. Furthermore, Katre and Knauf registered a patent in 1990 wherein an improved solubility for interferon  $\beta$ , amongst other target molecules, after conjugation to PEG was claimed [137]. Giutto *et al.* successfully PEGylated the natural antimicrobial peptide nisin A, which is frequently used as a food preservative. The conjugation improved its solubility and resistance to enzymes remaining in food, although its antimicrobial effect was abolished [138]. Protamine, which is a heparin neutralizing protein applied to circumvent toxic inflammatory responses after cardiopulmonary bypass surgery was shown to have significantly improved pharmaceutical properties due to an increased solubility after PEGylation [139].

Furthermore, improved solubility is also a desired property in the field of biotech products used for industrial purposes. Here, PEGylation is used to render enzymes soluble and active in organic solvents, like benzene or chloroform. Thus, very stable enzyme preparations are obtained that are used for catalytic processes in non-aqueous media [140, 141].

#### **4.2 Influence of PEGylation on immunogenicity and antigenicity of protein drugs**

Although recombinantly produced protein therapeutics have the same or at least similar structure to their native human counterparts, immunogenicity and antigenicity remain a major concern [142].

Antigenicity refers to the ability of a compound to react with pre-existing antibodies, whereas immunogenicity defines the ability of compounds to elicit an immune response. Clinically, immunogenicity is expressed by the formation of neutralizing antibodies (NABs) and non-neutralizing antibodies (NNABs) [143-145]. Both kinds of antibodies bind to their target, but unlike NNABs, NABs are able to impair the ligation of the protein and its receptor, thus leading to a reduced efficacy or even inactivation of the therapeutic compound. By contrast, NNABs may show no effect at all or reduce bioavailability due to formation of immune complexes, which exhibit a higher clearance by the reticuloendothelial system (RES). Furthermore, the possibility of neutralization of the native protein by NABs can occur, as was described, for example, for EPO. The formation of neutralizing antibodies against both, the natural and the recombinant protein, have been considered causative for the occurrence of pure red-cell aplasia (PRCA) after subcutaneous injection of a specific recombinant EPO product [146, 147].

Factors considered to have a potential influence on the formation of antibodies are manyfold and can originate for example from the protein structure, the formulation and manufacturing processes, or the route of application [142, 148-151].

Following conjugation with PEG, the immunogenicity and antigenicity patterns of proteins and enzymes are altered.

Commonly, a reduction in immunogenic properties of PEGylated proteins compared to their non-conjugated forms is observed. Attachment of PEG to proteins is thought to lead to a formation of a protective, highly hydrated polymer shell, which sterically hinders recognition by immune cells and binding of antibodies by shielding antigenic epitopes [152,153]. This, in turn, induces a prolongation of the conjugated protein's circulation time in the body.

In 1977, Abuchowski *et al.* reported reduced immunogenicity of bovine liver catalase after PEG conjugation when injected into rabbits [2]. They even observed a dependence on the PEGylation degree: the more PEG chains attached to the target, the lower its asserted immunogenicity. Since then, PEGylation has been conducted extensively and almost every PEG-modified protein drug displayed a diminished immunogenicity.

One example is L-asparaginase, an enzyme produced in *E. coli* or *Erwinia caratovora* cultures, having found clinical application for the treatment of acute lymphoblastic leukemia (ALL) [154, 155]. Due to its bacterial origin, therapy suffered from a short half-life of L-asparaginase and a high rate of immunogenicity. With the conjugation of this enzyme to mPEG, not only the immunological side effects were significantly reduced, but also the circulation half-life was increased from 2.9 hours for non-modified L-asparaginase to 56 hours of PEG-asparaginase [156].

Yet another successfully applied PEGylated enzyme is bovine adenosine deaminase. Inherited deficiency of adenosine deaminase (ADA) causes one form of the fatal



affliction of Severe Combined Immune Deficiency (SCID). Accumulation of the toxic substrates of ADA impairs lymphoid maturation and thus leads to lymphoid apoptosis causing lethal immunodeficiency. Children suffering from ADA-SCID have to be kept in a sterile environment, therapeutic options consist of transfusion of normal erythrocytes and transplantation of bone marrow to reconstitute the immune system. After Davies *et al.* reported on the abrogation of immunogenicity of bovine ADA after PEG attachment in mice, Hershfield *et al.* successfully treated two children suffering from ADA-SCID by injection of PEGylated ADA in 1987 [157,158]. Until 1997, in a follow-up of all patients treated, no allergic or hypersensitivity reactions were observed [159].

A further example is superoxide dismutase (SOD). SOD represents a key enzyme in scavenging the toxic and highly reactive oxygen species (ROS), which originate from superoxide ions in cells, and transforms them into non-toxic compounds [160]. ROS seem to be involved in several inflammatory processes, as well as other pathological developments like lung injuries and cerebral and myocardial ischemia [160]. Nucci *et al.* were able to show a decrease in immunogenicity of PEG-SOD after injection in mice [161]. Nevertheless, they observed a maintenance of the ability of PEG-SOD to react with preformed antibodies against unmodified SOD. They assumed that the extent of PEGylation was not sufficient to reduce antigenicity, whereas it was sufficient to abate immunogenicity. However, it was later successively demonstrated that the attachment of a few high molecular weight strands of PEG were superior in the reduction of antigenicity and immunogenicity than low molecular weight PEG chains [162].

The research group around Gamez and Wang tested the influence of the conjugation ratio of a phenylalanine ammonia lyase (PAL) of *Rhodospiridium toruloides* with linear and branched PEGs of different molecular weights on immunogenicity

[163,164]. They not only applied different *in vitro* tests, but also evaluated PEG-PAL immunogenicity in mice. A reduced immunogenicity of the conjugate, dependent on the conjugation degree of PAL with PEG was described *in vitro*, while the *in vivo* activity of the enzyme was preserved. It was concluded that PEG-PAL was a better candidate for treatment of phenylketonuria, a metabolic disorder usually treated with a special diet to prevent the accumulation of neurotoxic phenylalanine, than the non-protected enzyme [165].

The above mentioned non-recombinant enzymes are just selected examples that have been subjected to PEGylation in order to decrease or eliminate immunogenicity and antigenicity. Table 5 lists further examples of PEGylated recombinant enzymes. Modification of these non-human enzymes minimizes or even abates their immunogenicity, rendering them valuable therapeutics.

Enzyme	Reference
PEG-methioninase	[173,174]
PEG-uricase	[174, 175]
PEG-arginine deiminase	[174,176,177]
PEG-histaminase	[178]
PEG-acetylcholinesterase	[179]
PEG-formate dehydrogenase	[180]
PEG-catalase	[161]

**Table 5:** Further examples of PEGylated recombinant enzymes.

Conjugation of PEG to recombinantly produced human proteins also changes their immunogenic properties depending on the size and amount of PEG-molecules attached to the therapeutic. Thus, human interleukin-2 [136], insulin [54,166], cyanovirin N [60], a soluble tumor necrosis factor receptor type I [167], and interferon- $\beta_{1b}$  [131] were proven to be less immunogenic after their modification with PEG.

In addition to therapeutic molecules, PEGylation has also been applied to attenuate immunorecognition of erythrocytes [168], peripheral blood mononuclear cells [169] and splenocytes [170]. Pancreatic islets transplanted into rats were protected via PEGylation against infiltration of host immune cells, which then were no longer able to destroy the transplant [171]. A survival of one year in the recipient animals was shown under supportive cyclosporine administration, given at a reduced dose than usually needed for immunosuppression. However, in the absence of additive cyclosporine dosing, long term viability of the transplants was most probably impaired by cytotoxic cytokine release from host immune cells. An increase in survival time in the absence of immunosuppressive therapeutics was obtained after increase in the PEG conjugation degree [172].

#### **4.3 Influence of PEGylation on pharmacodynamics and pharmacokinetics of protein drugs**

Today, delivery of protein and peptide drugs remains mainly restricted to parenteral injection and – depending on the therapeutic protein - associated with short circulation half-lives. A short plasma half-life is mainly due to susceptibility towards enzymatic degradation, as well as fast renal elimination [181].

Consequently, different strategies are applied to prolong the systemic circulation of therapeutic proteins and peptides, among them PEGylation being successfully implemented [181,182].

In general, most substances applied systemically having a molecular weight below a threshold of 70 kDa are excreted into the glomerular filtrate [183]. Attachment of polymers, such as PEG, increases the molecular weight and hydrodynamic volume of the target molecules, consequently resulting in decreased glomerular excretion.

Furthermore, highly hydrated PEG molecules shield the target molecule against proteolysis [181, 183, 184] and, therefore, often tremendously improved plasma half-life times are obtained for the conjugates.

It is therefore not surprising that Pegfilgrastim<sup>®</sup>, which is obtained after modification of recombinantly produced methionyl granulocyte colony-stimulating factor (G-CSF) with a 20-kDa PEG covalently attached to the methionine moiety, shows a reduced elimination via the kidneys leading to a longer systemic exposure as compared to the non-conjugated cytokine [185,186]. Thus, administration only once per chemotherapy cycle is made possible with the PEGylated compound as compared to daily injections of the non-modified protein, stimulating maturation, differentiation and proliferation of neutrophils and their precursors at the same efficacy.

The pharmacokinetic profile of a PEGylated molecule is governed primarily by the polymer moiety. Distribution and elimination of PEG itself depends on its molecular size and the site of application [187,188]. Yamaoka et al. [187] were able to show in mice that plasma half-life of PEG is directly correlated to its molecular weight. Furthermore, the polymers were retained longer at the site of injection after subcutaneous (s.c.) and intramuscular (i.m.) administration, again showing a molecular weight dependency, whereas after intraperitoneal (i.p.) application, fast

elimination from the injection site, being independent on molecular weight, was observed [188].

Depending on the size of the polymer and resulting degree and site of PEGylation, activity and/or binding affinity of the protein to its receptor may be altered [189-191]. Often affinity constants or enzymatic activities measured *in vitro* of PEGylated compounds are shown to be lower for the modified compounds, if not absent at all [192,193]. However, due to the improved systemic exposure of the therapeutic after modification, the decrease in activity is often more than compensated for. Consequently, the same or even improved biological activity is obtained after PEGylation, as was shown for PEG-Interferon- $\alpha_{2a}$ , Pegvisomant and growth hormone releasing factor [53, 194, 195]. This also means that for PEGylated therapeutics rather poor *in vitro/in vivo* correlation concerning their bioactivity exists, and activity must therefore be determined *in vivo*.

For example, a long-acting form of recombinant human interleukin 11 (rhIL11) was prepared by Tagaki *et al.* by conjugation with PEG [196]. Attaching a branched 20 kDa PEG to rhIL11, a residual biologic activity of 14.3 % *in vitro*, but a prolonged mean residence time (MRT) in mice being 50- to 60-times higher than the MRT of non-conjugated rhIL11, was observed.

As the thrombopoietic activity of the cytokine is supposed to compensate hematological toxicity during chemotherapy, the level of peripheral platelets after administration of the conjugates was measured. An effect comparable to the subcutaneous infusion of rhIL11 for 3 days was obtained by a single bolus administration of the branched 20 kDa rhIL11.

Another example is a 20 kDa mPEG-O-2-methylpropionaldehyde derivative of interferon- $\beta$ -1a, which has recently been produced and tested for its *in vitro* antiproliferative and antiviral activity, as well as for its inhibitory potential against the

formation of tumor induced blood vessels in the periphery of human melanoma tumors grown in mice [197]. The protein being predominantly modified with a single PEG chain at the N-terminus retained 50% of the native *in vitro* antiviral and antiproliferative activity of the non-conjugated IFN- $\beta$ -1a, and showed improved pharmacokinetics. Clearance of the PEGylated compound decreased by 30-fold, the elimination half-life increased by 13-fold and the AUC increased by 10-fold.

Furthermore, the conjugate was shown to be more effective in its ability to inhibit the formation of tumor induced blood vessels compared to its non-conjugated counterpart.

Nevertheless, use of genetic engineering and/or site-specific PEGylation methods already made possible to obtain only marginal or even no loss of *in vitro* bioactivity [57, 72,191,198].

## **5 Limitations and challenges for PEGylation technology**

In this book chapter we mainly concentrated on an outline of PEGylation technology concerning proteins, but did not mention the potential of the technology to improve the therapeutic potential of other molecules and devices, such as small drug molecules, cofactors, biomaterials, nanoparticles and liposomes or oligonucleotides. Interested readers are referred to publications discussing some of these topics [5, 12, 199-203]. Although PEGylation is regarded as a technology of clear benefits and advantages over other formulation strategies, some limitations still remain to be addressed.

PEGylation is applied to improve the pharmacokinetic, immunogenic or physico-chemical properties of proteins and peptide drugs. Nevertheless, it is important to stress that all aspects have to be seen as a whole, since the final aim is a product

applied in human subjects. Thus, potential changes of all properties need to be carefully examined.

Although progress has been made in the development of new technologies for site specific conjugation of PEG to proteins since the birth of this technology, mostly heterogeneous mixtures of conjugates are obtained, which first need to be separated. Generally, the higher the heterogeneity, the more difficult the separation and characterization of each single conjugate. Separation of products with a different extent of PEGylation can be best obtained using methods such as gel filtration or ion exchange chromatography [204,205]. Currently, ultrafiltration is examined for its utility in the separation of mono-PEGylated protein from its by-products [206, 207]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page) or gel filtration may help to roughly estimate the PEGylation degree, since PEG is heavily hydrated it often shows a higher hydrodynamic volume than to be expected from its molecular weight.

More significant information is gained from matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry and capillary electrophoresis, both used for the characterization of the extent of conjugation [208-212]. The colorimetric determination of the remaining free and accessible lysine residues with trinitrobenzenesulfonic acid (TBNA) [213] was found to overestimate conjugation with PEG [212]. The use of mass spectrometry is restricted to MALDI-TOF when polydisperse PEGs are used for modification, which mostly is the case because also polydisperse conjugates are obtained rendering electrospray mass spectroscopy analysis difficult.

Analysis of the PEGylation site may result in some challenges: usually the conjugates and the free proteins are enzymatically digested, and the resulting peptides are identified after purification by mass spectrometry or amino acid analysis. The missing

peptide of the conjugate reveals the PEGylation site. The issue of this procedure lies in the enzymatic degradation, since the PEG may prevent access of the enzyme to the protein leading to incomplete digestion and difference in the analysis result obtained for free and modified proteins.

As already outlined before, differences in conjugation chemistry, size and form of PEGs (branched, multiarm or linear) may all affect the features of the obtained conjugate and need to be compared [71-75]. Furthermore, a limiting property of PEG is its lack of functionality. Other multivalent polymers, like poly(N-vinyl pyrrolidone) (PVP), may couple more proteins to one polymer-molecule. Furthermore, reactive groups may be more easily introduced into other polymers than into PEG [214]. Recently, Chandna *et al.* [215] synthesized a multivalent drug delivery system, consisting of PEG connected via a spacer to citric acid which in turn was linked to luteinizing hormone-releasing hormone (LHRH) to target the system to cancer cells, camptothecin as cell death inducer, and BH3 peptide acting as a suppressor of cellular antiapoptotic defense. This approach shows a possible method to partially overcome the limited functionality of PEG.

Other issues that were recently reported are the formation of anti-PEG antibodies. Kiwada and coworkers found a decrease in half-life of liposomes after multiple injections into rats and were able to show this to be due to IgM antibodies developed against PEG itself [6-11]. Cheng already reported the same phenomenon of anti-PEG IgM binding the polymer moiety of PEGylated  $\beta$ -glucuronidase in mice [9]. Recently, anti-PEG antibodies have also been detected in humans after application of PEGylated urate oxidase [10] and PEG-asparaginase [11] rendering therapy with the latter in most cases ineffective. Since modification with PEG is also applied to reduce immunogenicity, these findings have to be taken seriously and need to be further



investigated. In consequence, the statement that PEG is non-immunogenic may be subject to reconsideration.

PEGylation has also been discussed in the context of biosimilars or so-called follow-up biologics. Biosimilars are follow-on products of biopharmaceuticals, whose patent protection has expired or is due to expire soon [216]. An issue discussed is the potential of less expensive biosimilar drugs versus the potentially increased risks of side-effects of these products compared to the “originator products”. Regulatory authorities in Europe (EMA) passed first guidelines in 2005 concerning development and approval of biosimilars, whereas the FDA is still hesitant [217, 218]. The properties of a biopharmaceutical are highly dependent on the manufacturing process; slight changes might result in tremendous changes of efficacy, immunogenicity and additional safety features [219, 220]. Further post-processing modifications like PEGylation again may lead to different features of the product that may not be detectable by existing analytical methods. Thus PEGylated biosimilars are facing the same rigorous regulatory approval process.

## **6. Conclusion**

PEGylation opened up the path to new drugs and therapeutic approaches previously not thought possible. Modification of protein drugs improving their pharmacokinetic and immunogenic properties is a commonly used technology in today's drug development process. PEGylation is accepted by the industry and regulatory authorities as the technology of choice in macromolecular engineering, and is currently developed into its next generation of reversible PEGylation reagents. However, as reports of immunogenicity of PEG are surfacing, other polymers and conjugation technologies offering extended features with regard to functionality and biodegradability, should seriously be considered.

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## **Chapter 2**

### **Objectives of the Thesis**

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## General

Proteins are macromolecules being fundamental components of all organisms and play an essential role in the structure, function and organization of cells, tissues and organs. With the dawn of genetic engineering in the 1970's protein therapeutics (biopharmaceuticals) became available and since then the number and frequency of application increased tremendously. Nowadays, proteins represent important therapeutics due to several reasons: i) generally, a superior target specificity and efficacy is observed in comparison to small molecular drugs, ii) due to this specificity, adverse reactions are less probable, and iii) external protein replacement is possible for severe diseases originating from mutated/deleted genes. Despite this tremendous success of biopharmaceuticals, several challenges remain: i) fast enzymatic degradation and renal elimination leading to short circulation half-lives, ii) observed *in vivo* immunogenicity and antigenicity, and iii) physical and chemical degradation processes resulting in a poor stability and limited shelf-lives.

PEGylation is a technology allowing to adress the above described challenges. After PEGylation, the hydrodynamic volume and the molecular weight of the biopharmaceutical are increased resulting in reduced renal filtration. Shielding of the molecule due to the highly hydrated PEG leads to decreased proteolysis. Decreased *in vivo* immunogenicity/antigenicity is also observed as a consequence of shielding of antigenic/immunogenic patches. Steric shielding caused by the PEG reduces intermolecular interactions resulting in a decreased aggregation and this in turn leads to improved stability. However, in spite of the great potential of PEGylation, also here limitations still persist. Although vast progress has been made in PEGylation chemistry, mostly heterogeneous products originate from synthesis requiring subsequent separation and characterization. Furthermore, the chemical processing needed in order to attach the PEG might represent additional stress for the

biopharmaceutical, which may lead to increased aggregation and partial or complete loss of activity. Although PEGylation techniques are under development resulting in only marginal or no loss of *in vivo* bioactivity, these are more the exception than the rule and are relatively difficult to employ. Furthermore, chemical processing and subsequent purification still are required. Thus, new strategies are needed in order to overcome the remaining challenges of PEGylation. A possible approach might be novel PEGylation techniques based on non-covalent interactions.

### **Objectives of the thesis**

- i. To synthesize and characterize PEG-based excipients.
- ii. To study the influence of varying concentrations and molecular weights of the synthesized and other purchased PEG-based excipients on the aggregation of biopharmaceuticals *in vitro*.

### **Innovation of the thesis**

To our best knowledge, we are the first to describe the development of novel PEGylation approaches based on non-covalent interactions. On 2<sup>nd</sup> November 2009, a provisional US patent application has been filed with the US patent and trademark office. On 6<sup>th</sup> July 2010 a publication on the synthesis and characterization of different dansyl-PEGs and their effect on the aggregation of salmon calcitonin was submitted to the Journal of Pharmaceutical Sciences.

More precisely, this thesis focused on the preparation of different hydrophobic headgroups to PEGs of varying molecular weight. Subsequently, thorough physico-chemical analysis was performed. Furthermore, several excipients were purchased and physico-chemically characterized.

Thereafter, the interaction with salmon calcitonin, a biopharmaceutical, was investigated. The influence of the PEG-based excipients on the aggregation of salmon calcitonin was evaluated. Finally, hen egg white lysozyme, another biopharmaceutical, was employed to study the effect of the various PEG-based excipients on its aggregation.

## **Organization of the thesis**

**Chapter 3** describes the synthesis and characterization of dansyl-mPEG 2 kDa, bis-dansyl-PEG 3 kDa and dansyl-mPEG 5 kDa. A stable solution of dansyl-mPEG 2 kDa and salmon calcitonin (sCT) was analyzed for possible interactions. The different polymers were evaluated for their effect on the aggregation of sCT and possible cytotoxicity and hemolysis potentials were assessed.

The synthesis and physico-chemical characterization of Tryptophan-mPEG 2 and 5 kDa (Trp-mPEGs) is described in **Chapter 4**. Furthermore, comprehensive studies of their influence on the aggregation of sCT, and cytotoxicity and hemolysis tests were performed.

In **chapter 5** the preparation of phenylbutylamino-mPEG 2 kDa is presented. Characterization of phenylbutylamino-mPEG 2 kDa and further purchased excipients, namely benzyl-mPEGs 2 and 5 kDa and cholesteryl-PEGs 2 and 5 kDa, is described. The excipients were tested for their potential to reduce the aggregation of sCT. Out of the results on the stabilization of sCT against aggregation and with help of molecular modelling, a possible interaction leading to aggregation of sCT was proposed. Furthermore, all the excipients (dansyl-, Trp-, phenylbutylamino-, benzyl- and cholesteryl-PEGs) were evaluated for their potential to reduce aggregation of hen egg white lysozyme.



## Chapter 3

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### **Non-covalent PEGylation by dansyl-PEGs as a new means against aggregation of salmon calcitonin**

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## **Abstract**

During all stages of protein drug development, aggregation is one of the most often encountered problems. Covalent conjugation of poly(ethylene glycol), also called PEGylation, to proteins has been shown to reduce aggregation of proteins.

In this paper new excipients based on poly(ethylene glycol) are presented that are able to reduce aggregation of salmon calcitonin (sCT). Several PEG-polymers consisting of a hydrophobic dansyl-headgroup attached to PEGs of different molecular weight have been synthesized and characterized physico-chemically. After addition of dansyl-mPEG 2 kDa to a 40 times molar excess of sCT resulted in an increase in dansyl-fluorescence and a decrease in 90° light scatter suggesting possible interactions. The aggregation of sCT in different buffer systems in presence or absence of the different dansyl-PEGs was measured by changes in Nile Red fluorescence and turbidity. Dansyl-mPEG 2 kDa in a 1:1 molar ratio to sCT strongly reduced aggregation. Reduction of sCT aggregation was also measured for the bivalent dansyl-PEG 3 kDa in a 1:1 molar ratio. Dansyl-mPEG 5 kDa deteriorated sCT aggregation. Potential cytotoxicity and hemolysis were investigated.

This paper shows that dansyl-PEGs are efficacious in reducing aggregation of sCT.

## **1 Introduction**

The growing of market shares of biologics observed during the last decade is not surprising considering the tremendous progress that has been made since the advent of genetic engineering and recombinant DNA technology in the 1970's, enabling and facilitating the large-scale production of biopharmaceuticals [1-3]. Nevertheless, several challenges remain, in particular poor pharmacokinetic profiles due to fast enzymatic degradation and renal excretion, *in vivo* immunogenicity and

physical and/or chemical instability of the protein or peptide drug in its final formulation [4, 5].

Among physical instabilities, protein aggregation is one of the most frequent concerns occurring during all stages of protein drug manufacturing, thus challenging the development of safe, efficacious, and stable formulations [6, 7]. Aggregation is a complex process and is dependent on various factors, such as pH [8-10], temperature [8, 9, 11, 12], protein concentration [10, 13], additives and buffers used [9, 14, 15], or stresses applied like freezing, thawing or shaking [16-18]. Its suppression is usually achieved by application of empirical approaches, i.e. testing of different buffer systems, pH values or ionic strengths.

For many proteins, a nucleation-dependent mechanism has been described, during which aggregation-prone folding/unfolding intermediates are formed due to conformational changes. These intermediates favour protein/protein interactions, and thus initiate aggregation, most probably by liberation and approximation of hydrophobic patches usually buried inside the intact protein structure [7, 19].

PEGylation, which is the covalent conjugation of poly(ethylene glycol), is used to overcome remaining challenges of biopharmaceutics and has led to the approval of various very successful therapeutics, such as Oncaspar<sup>®</sup>, Adagen<sup>®</sup> or PegIntron<sup>®</sup> [5]. Conjugation of PEG has also been reported to reduce aggregation of proteins [20-24], which may be explained by steric shielding of hydrophobic patches on the proteins surface by the polymer chains.

To attach the PEG polymer to the protein drug, various chemical approaches exist. In particular, site-specific conjugation or degradable PEG linkers were developed to circumvent the formation of heterogenous PEG-conjugates and to overcome the observed reduction of *in vivo* bioactivity [4, 5, 25-27].

However, conjugation of the PEG polymers to the biopharmaceutics needs chemical processing and subsequent purification. This additional step during the formulation process imposes further stress on the protein and may eventually lead to a partial or complete loss of bioactivity.

The aim of this work was to develop a novel PEGylation approach by which chemical processing of the biopolymer is avoided. Additionally, key benefits of PEGylation on aggregation shall be maintained. To attach the PEG non-covalently to a biopharmaceutical we opted for hydrophobic interactions. Hydrophobic headgroups, which shall interact with hydrophobic patches on the protein surface were conjugated to PEG-polymers. By means of the PEG, shielding of the biopharmaceutical shall be obtained and render protein/protein interactions less probable. As a consequence, aggregation of the biopharmaceutical in liquid formulations should be reduced. A successful patent application has been filed [28].

For this, PEG-polymers possessing the hydrophobic dansyl head-group were synthesized, characterized and tested for their potency to inhibit the aggregation of salmon calcitonin.

## **2 Materials and Methods**

### *2.1 Materials*

The chemicals required for preparing the buffer solutions, the dyes 5-(dimethylamino)-1-naphthalenesulfonamide and Nile Red (9-diethylamino-5H-benzo[ $\alpha$ ]phenoxazine-5-one), anhydrous toluene and dansyl chloride were provided by Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The buffers used consisted of: (1) acetic acid – sodium acetate pH 5, (2, 3) citric acid – sodium citrate pH 5 and 6, (4) sodium phosphate monobasic – sodium phosphate dibasic pH 8. Diethyl ether, dichloromethane, anhydrous triethyl amine and iso-propanol were

purchased from Acros (Acros Organics BVBA; Geels, Belgium). The mPEG-amine 2 kDa, 5 kDa and the PEG-diamine 3 kDa were obtained from Iris Biotech (Iris Biotech GmbH, Marktredwitz, Germany). All solvents and compounds used were of analytical grade. The salmon calcitonin was obtained from Therapeomic (Therapeomic Inc., Basel, Switzerland). UV transparent 96-well or 384-well Costar<sup>®</sup> Corning microplates and UV-Vis transparent and pressure sensitive Corning<sup>®</sup> Universal Optical sealing tape were supplied by Corning (Corning Life Sciences, Schiphol, Netherlands).

## *2.2 Characterization of dansyl-PEGs*

<sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were recorded on a Varian VXR 300MHz spectrometer (Varian, Switzerland). All polymers were dissolved in DMSO. The MALDI-TOF mass spectrometry was carried out on an Axima CFR+, Shimadzu mass spectrometer, using 2-(4-hydroxyphenylazo)-benzoic acid (HABA) as matrix. FTIR spectra were recorded on a Perkin Elmer 100 FT-IR spectrometer (PerkinElmer, Switzerland) in the range of 4000–400 cm<sup>-1</sup> using KBr pellets (1% w/w of product in KBr). UV analysis was performed on a Varian Cary 50 spectrophotometer (Varian, Switzerland).

## *2.3 Synthesis of dansyl-mPEG 2 kDa*

The method was adapted from [29]. In short, 0.33 mMol of dried mPEG-amine 2 kDa were dissolved in anhydrous toluene and 0.98 mMoles of dansyl chloride and 0.13 mMoles of dry triethyl amine were added. The reaction was performed at 100 °C under reflux for 24 hours. Toluene was evaporated and the solid was redissolved in dichloromethane, precipitated from cold diethyl ether, and collected via filtration. Reprecipitation from isopropyl alcohol was repeated twice for further purification. A slightly yellowish powder was obtained and dried under vacuum.

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 2.82 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 2.96 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 3.23 ppm, PEG CH<sub>3</sub>-O- (s); 3.50 ppm, PEG -O-CH<sub>2</sub>- (s); 7.27 ppm, dansyl naphthalene structure (d); 7.60 ppm, dansyl naphthalene structure (t); 8.10 ppm, dansyl naphthalene structure (d); 8.36 ppm, dansyl naphthalene structure (d); 8.45 ppm, dansyl naphthalene structure (d). <sup>13</sup>C-NMR (300 Mhz, DMSO-d-6): 42.04 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 44.90 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 57.85 ppm, PEG CH<sub>3</sub>-O- (s); 69.59 ppm, PEG -O-CH<sub>2</sub>- (m); 114.89 ppm, dansyl naphthalene structure (s); 119.08 ppm, dansyl naphthalene structure (s); 123.41 ppm, dansyl naphthalene structure (s); 127.80 ppm, dansyl naphthalene structure (s); 128.99 ppm, dansyl naphthalene structure (s); 136.14 ppm, dansyl naphthalene structure (s); 151.12 ppm, dansyl naphthalene structure (s). FTIR: 2886; 2741; 2695; 2238; 1968; 1575; 1467; 1412; 1360; 1343; 1280; 1242; 1148; 1113; 1060; 963; 842; 793; 683; 625; 571; 529 cm<sup>-1</sup>. MS (MALDI-TOF): m/z 2224 (M<sup>+</sup>).

#### 2.4 Synthesis of bis-dansyl-PEG 3 kDa

The reaction was performed as described for the dansyl-mPEG 2 kDa, whereas 0.033 mMol of dried PEG-diamine 3 kDa, 0.2 mMoles of dansyl chloride and 0.27 mMoles of dry triethyl amine were used. A slightly yellowish powder was obtained.

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 2.83 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 2.95 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 3.23 ppm, PEG- CH<sub>3</sub>-O- (s); 3.50 ppm, PEG -O-CH<sub>2</sub>- (s); 7.24 ppm, dansyl naphthalene structure (d); 7.59 ppm, dansyl naphthalene structure (t); 8.10 ppm, dansyl naphthalene structure (d); 8.28 ppm, dansyl naphthalene structure (d); 8.45 ppm, dansyl naphthalene structure (d). <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 42.43 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 45,27 ppm, dansyl (CH<sub>3</sub>)<sub>2</sub>-N- (s); 69,98 ppm, PEG -O-CH<sub>2</sub>- (m); 115.27 ppm, dansyl naphthalene structure (s); 119.47 ppm, dansyl

naphthalene structure (s); 123.77 ppm, dansyl naphthalene structure (s); 128.01 ppm, dansyl naphthalene structure (s); 129.36 ppm, dansyl naphthalene structure (s); 136.56 ppm, dansyl naphthalene structure (s); 151.49 ppm, dansyl naphthalene structure (s). FTIR: 2885; 2741; 2695; 1969; 1575; 1467; 1413; 1360; 1344; 1280; 1242; 1148; 1113; 1060; 963; 841; 793; 625; 571; 529  $\text{cm}^{-1}$ . MS (MALDI-TOF):  $m/z$  3462 ( $\text{M}^+$ ).

## 2.5 Synthesis of dansyl-mPEG 5 kDa

The reaction was performed as described for the dansyl-mPEG 2 kDa, whereas 0.02 mMol of dried mPEG-amine 5 kDa, 0.06 mMoles of dansyl chloride and 0.08 mMoles of dry triethyl amine were used. A slightly yellowish powder was obtained.

$^1\text{H}$ -NMR (300 MHz, DMSO- $d_6$ ): 2.82 ppm, dansyl  $(\text{CH}_3)_2\text{-N-}$  (s); 2.95 ppm, dansyl  $(\text{CH}_3)_2\text{-N-}$  (s); 3.23 ppm, PEG-  $\text{CH}_3\text{-O-}$  (s); 3.50 ppm, PEG - $\text{O-CH}_2\text{-}$  (s); 7.27 ppm, dansyl naphthalene structure (d); 7.59 ppm, dansyl naphthalene structure (t); 8.10 ppm, dansyl naphthalene structure (d); 8.34 ppm, dansyl naphthalene structure (d); 8.43 ppm, dansyl naphthalene structure (d).  $^{13}\text{C}$ -NMR (300 MHz, DMSO- $d_6$ ): 42.60 ppm, dansyl  $(\text{CH}_3)_2\text{-N-}$  (s); 45.71 ppm, dansyl  $(\text{CH}_3)_2\text{-N-}$  (s); 58.77 ppm, PEG  $\text{CH}_3\text{-O-}$  (s); 70.42 ppm, PEG - $\text{O-CH}_2\text{-}$  (m); 115.78 ppm, dansyl naphthalene structure (s); 119.91 ppm, dansyl naphthalene structure (s); 124.21 ppm, dansyl naphthalene structure (s); 128.51 ppm, dansyl naphthalene structure (s); 129.81 ppm, dansyl naphthalene structure (s); 137.01 ppm, dansyl naphthalene structure (s); 151.94 ppm, dansyl naphthalene structure (s). FTIR: 2885; 2741; 2695; 2166; 1968; 1575; 1467; 1412; 1360; 1343; 1280; 1242; 1148; 1111; 1060; 963; 842; 795; 625; 571; 529  $\text{cm}^{-1}$ . MS (MALDI-TOF):  $m/z$  5130 ( $\text{M}^+$ ).

## *2.6 Sensitivity of dansyl-PEGs to solvent polarity*

Solutions of dansyl-PEGs at 0.04 mM concentration in 0, 25, 50, 75 and 100 % ethanol were prepared and analyzed at 25°C by UV and intrinsic fluorescence, using a temperature-controlled Cintra 40 spectrophotometer (GBC, Melbourne, Australia), and Fluoromax spectrofluorometer (Spex, Stanmore, UK), respectively. The dielectric constant for water is 78.54 and for ethanol 96 % is 24.3. For the dansyl-mPEG 2 kDa, the fluorescence emission spectra were recorded after excitation at 332 nm using bandwidths of 0.5 nm for excitation and 1 nm for emission. Fluorescence emission spectra of bis-dansyl-PEG 3 kDa were measured after excitation at 336 nm using bandwidths of 0.3 and 1 nm for excitation and emission, respectively. Solutions of dansyl-mPEG 5 kDa were excited at 335 nm, using bandwidths of 0.5 nm at the excitation and 1 nm at the emission side. All fluorescence emission spectra were recorded between 400 and 650 nm.

## *2.7 Studies of potential of self-association of dansyl-PEGs*

The different aqueous polymer solutions (1 mg/ml) were diluted to give solutions with concentrations between 0.6 mg/ml and 0.1 µg/ml. Fluorescence spectra were obtained using an excitation wavelength of 332 nm. Depending on the concentration, slit widths were adapted to obtain a sufficiently high fluorescence signal.

Various concentrations above 0.1 mg/ml were furthermore analyzed by dynamic light scattering (DLS) at an angle of 173° (back-scatter) at 25°C using a Zetaziser Nano ZS (Malvern Instruments), equipped with a 4 mW He–Ne laser operating at a wavelength of 633 nm. As a control experiment, the micellization of Tween 20® was measured.



## *2.8 Interaction of dansyl-mPEG 2 kDa with human serum albumin (HSA) in 50 mM HEPES buffer 154 mM NaCl pH 7.4*

Interaction of DNSA and dansyl-mPEG 2 kDa were measured as described by [30] using a Tecan Safire™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Constant concentrations of 2  $\mu$ M DNSA or dansyl-mPEG 2 kDa were used, while the HSA concentration varied from 100 nM to 200  $\mu$ M. The fluorescence measurements were performed at 26 °C using an excitation wavelength of 350 nm and an emission wavelength of 493 nm. The bandwidths were set to 12 nm for both, excitation and emission side. The measured values were corrected for HSA contribution. The studies were performed using 96-well Costar® Corning microplates, which were sealed with UV-Vis transparent and pressure sensitive Corning® Universal Optical sealing tape.

## *2.9 Interaction of dansyl-mPEG 2 kDa with salmon calcitonin (sCT) in 10 mM sodium acetate buffer pH 5*

### Steady-state fluorescence

Stock solutions of sCT (5 mg/ml) and dansyl-mPEG 2 kD (0.09 mg/ml) were freshly prepared in 10 mM sodium acetate buffer pH 5. The tested solutions were (1) 2.5 mg/ml sCT and 0.045 mg/ml dansyl-mPEG 2 kD (40:1 molar ratio peptide to dansyl-mPEG 2 kDa), (2) 2.5 mg/ml sCT, and (3) 0.045 mg/ml dansyl-mPEG 2 kDa in 10 mM sodium acetate buffer pH 5.

The steady-state fluorescence measurements were performed with a Fluoromax spectrofluorometer (Spex, Stanmore, UK) at 25°C in a thermostated cuvette holder. The tyrosine fluorescence of sCT was monitored at emission wavelengths between 280 and 400 nm, using an excitation wavelength of 273 nm. The spectra were

recorded with a 0.1 s integration time and bandwidths of 0.3 nm and 1 nm for excitation and emission, respectively.

The dansyl-moiety was excited at 330 nm using slit widths of 0.5 nm for excitation and 1 nm for emission and an integration time of 0.1 s. The emission spectra were recorded between 400 and 700 nm.

### 90° light-scattering

The intensity of light scattering was determined as described by [31] using the Fluoromax spectrofluorometer (Spex, Stanmore, UK). The light scatter was measured between 400 and 750 nm using synchronized excitation and emission monochromators, slits were set to 1 nm and the spectra were recorded with 0.01 s integration time.

### Fluorescence lifetime measurements

An IBH 5000U fluorescence lifetime spectrophotometer (Glasgow, United Kingdom) equipped with a 279 nm NanoLED as excitation source and a monochromator at the emission side was used to determine fluorescence lifetimes by time-correlated single-photon counting (TCSPC). DAS6 software (IBH, Glasgow, United Kingdom) was used to analyze the data by linear and non-linear least-squares fitting. Calculation of the average lifetimes was done using the equation given in [32]:

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

where  $\bar{\tau}$  is the calculated fluorescence decay time and  $\alpha$  the pre-exponential factor.

sample	10 mM sodium acetate buffer pH 5		10 mM sodium citrate buffer pH 5		10 mM sodium citrate buffer pH 6		10 mM sodium phosphate buffer pH 8	
	addition of Nile Red		addition of Nile Red		addition of Nile Red		addition of Nile Red	
respective buffer	-	+	-	+	-	+	-	+
sCT 2.5 mg/ml	-	+	-	+	-	+	-	+
sCT:dansyl-mPEG = 1:1	-	+	-	+	-	+	-	+
dansyl-mPEG	-	+	-	+	-	+	-	+
sCT:mPEG-OH = 1:1	-	+	-	+	-	+	-	+
mPEG-OH	-	+	-	+	-	+	-	+
sCT + DNSA 1 $\mu$ M	-	+	-	+	-	+	-	+
DNSA 1 $\mu$ M	-	+	-	+	-	+	-	+

**Table 1.** Formulations prepared for high throughput spectroscopic analysis of sCT:dansyl-mPEG 2 kDa = 1:1 molar ratio. A total of 64 samples (32 without and 32 with Nile Red 1  $\mu$ M) were prepared in four different buffers (10 mM sodium acetate

buffer pH 5, 10 mM sodium citrate buffer pH 5 and 6, and 10 mM sodium phosphate pH 8) and pipetted in triplicate into a microplate for simultaneous analysis in time. + and – indicate the presence or absence of Nile Red.

### *2.10 High Throughput Spectroscopic Analysis*

The method has been adapted from [33]. A stock solution of 12.5 mg/ml salmon calcitonin in purified MilliQ™ Millipore water was freshly prepared. The four different buffer solutions, (1) 10 mM sodium acetate buffer pH 5, (2, 3) 10 mM sodium citrate buffer pH 5 and 6, and (4) 10 mM sodium phosphate pH 8, were prepared using sterile filtered 100 mM buffer solutions. The sCT formulations were prepared in 96-well plates or 384-well plates. The tested formulations were composed of 2.5 mg/ml sCT in 10 mM buffer and each sample was prepared in duplicate. To one of each duplicate 1  $\mu$ M Nile Red was added. DNSA concentration tested was kept constant at 1  $\mu$ M. Each formulation was measured in triplicate, the respective controls, i. e. the non-conjugated PEG and DNSA in the presence and absence of sCT, were measured simultaneously. An example of the formulations prepared for tests of sCT:dansyl-mPEG 2 kDa = 1:1 molar ratio is shown in Table 1. Table 2 shows the different molar ratios and respective concentrations of sCT and dansyl-PEGs tested. After sample preparation, the 96-well or 384-well Costar® Corning microplates were sealed using UV-Vis transparent and pressure sensitive Corning® Universal Optical sealing tape.

Every 10 minutes a cycle of the following was measured at 26°C with a Tecan Safire™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland): i) turbidity at 450 nm, ii) fluorescence emission of Nile Red at a high and iii) low detector sensitivity, and iv) DNSA fluorescence emission.

The fluorescence emission of DNSA over time was determined at 550 nm after excitation at 330 nm using bandwidths of 7.5 and 12 nm for excitation and emission, respectively. After 6 days the kinetics were stopped and fluorescence emission spectra of DNSA were recorded between 500 and 600 nm using the same settings as during the kinetics. The fluorescence emission of Nile Red was studied over time at 620 nm after excitation at 550 nm using bandwidths of 12 nm for both excitation and emission. The kinetics were stopped after 6 days, and the fluorescence emission spectra of Nile Red were measured between 590 and 690 nm using the same settings as during the kinetics. The data output of the XFluor® software of the Tecan Safire™ microplate reader was in Microsoft® Excel™ format. Visual basic macros were developed by the authors to facilitate high throughput data analysis.

sample	molar ratio	concentration (mg/ml)
sCT:dansyl-mPEG 2 kDa	1:1	2.5mg/ml sCT; 1.86 mg/ml dansyl-mPEG 2 kDa
	1:2	2.5mg/ml sCT; 3.72 mg/ml dansyl-mPEG 2 kDa
	100:1	2.5mg/ml sCT; 0.019 mg/ml dansyl-mPEG 2 kDa
	5:1	2.5mg/ml sCT; 0.372 mg/ml dansyl-mPEG 2 kDa
sCT:bis-dansyl-PEG 3 kDa	1:1	2.5mg/ml sCT; 2.89 mg/ml bis-dansyl-PEG 3 kDa
sCT:dansyl-mPEG 5 kDa	1:1	2.5mg/ml sCT; 4.36 mg/ml

**Table 2.** Formulations tested by high throughput spectroscopic analysis. Different molar ratios and the respective concentrations of sCT and dansyl-PEGs used.

### *2.11 Evaluation of dansyl-PEGs' cytotoxicity*

To determine potential cytotoxicity of the polymers, a screening of polymer solutions at different concentrations (0.1 µg/ml – 10 mg/ml) was done on HaCaT cells (immortalized human keratinocyte cell line). Viability was analyzed by using the cell proliferation kit II (XTT assay) from Roche Diagnostics (Basel, Switzerland). In brief, defined numbers of up to  $8 \times 10^3$  washed viable cells per well were incubated with serial dilutions of the polymers in 96-well plates (Nuncclon TM  $\Delta$  Surface by Nunc, Roskilde, Denmark) for a total of 24 and 48 hours. Absorption at 490 nm was measured after 1, 3, 6, and 24 h by using a BioTek PowerWave XS reader (Witec AG, Littau, Switzerland). As a positive control for cytotoxicity, 0.02 % solutions of SDS (sodium dodecyl sulfate) were used, and as a negative control cells incubated with medium only. Calculated cell survival was normalized against the negative control. All measurements were performed in quadruplicate.

### *2.12 Evaluation of dansyl-PEGs' hemocompatibility*

The potential of hemolysis due to membrane interactions and membrane lysis was assessed by incubation of the same concentrations of the compounds tested in the cytotoxicity assay. In brief, serial dilutions of polymers were prepared in sterile 0.9 % NaCl. Human blood of a fasted volunteer was collected in citrate tubes and immediately used. The blood was added to the samples at a 1:3 (v/v) ratio and put into an incubator at 37 °C for 1 and 24 hours under shaking. After centrifugation for 10 minutes at 770 x g, 40 µl of the supernatant were diluted 4 times with sterile 0.9% NaCl and pipetted into a 96-well Costar® microplate (Product Number 3635, Corning Life Sciences, Schiphol, The Netherlands). Absorbance of released hemoglobin was measured at 575 nm by using a Tecan Safire microplate reader (Tecan Group Ltd,

Männedorf, Switzerland). A positive control, 1% Triton X 100, as well as a negative control (0.9% sterile NaCl) were tested as references. All measurements were performed in triplicates.

### 3 Results

#### 3.1 Synthesis and characterization of the different dansyl-PEGs

Successful conjugation of dansyl chloride to the different PEGs and purity of the resulting dansyl-PEGs were analyzed by  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, FTIR, MALDI-TOF and UV.  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, and FTIR showed characteristic peaks from both, the PEG-polymer and the dansyl-group (see sections 2.3-2.5 of synthesis of the different dansyl-PEGs in Materials and Methods). MALDI-TOF indicated an increase in molecular weight as expected (Table 3). UV spectroscopy was used as a means to determine the degree of conjugation of dansyl-groups to PEG as an orthogonal method to  $^1\text{H}$ -NMR and to exclude impurities remaining from excess dansyl-chloride from the synthesis. Thus, a degree of conjugation of 93 % for the dansyl-mPEG 2 kDa, 96 % for the dansyl-mPEG 5 kDa and 91 % of the bis-dansyl-PEG 3 kDa was determined.

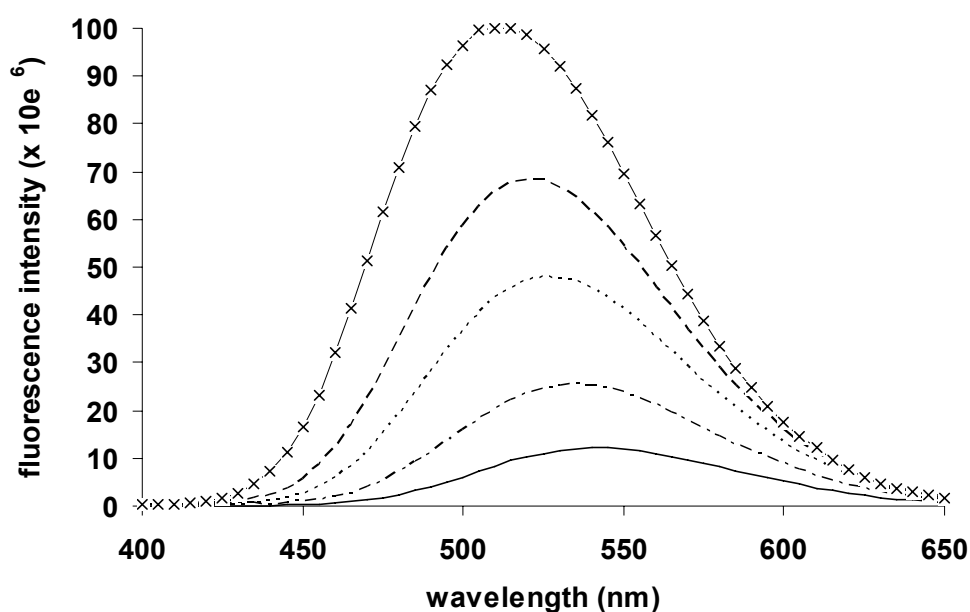
compound	observed m/z ( $\text{M}^+$ )
mPEG-NH <sub>2</sub> 2 kDa	1900
dansyl-mPEG 2 kDa	2224
mPEG-NH <sub>2</sub> 5 kDa	4900
dansyl-mPEG 5 kDa	5130
NH <sub>2</sub> -PEG-NH <sub>2</sub> 3 kDa	2970
bis-dansyl-PEG 3 kDa	3462

**Table 3.** Masses of the used PEG-amines and the resulting dansyl-PEGs measured

by MALDI-TOF mass spectrometry.

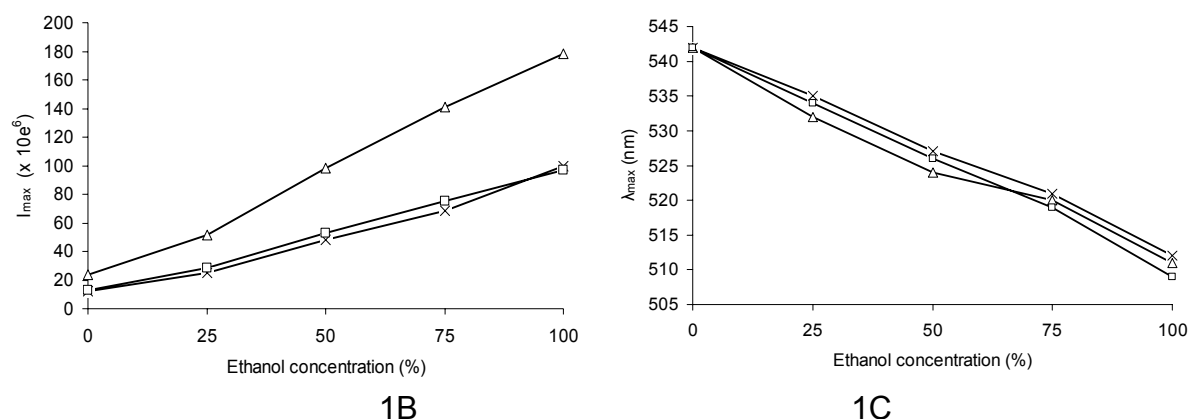
### 3.2 Sensitivity of dansyl-PEGs to solvent polarity

With increasing ethanol concentration, a shift of the fluorescence maximum (542 nm in water) towards lower wavelengths (512 nm in 100 % ethanol) was detected for the dansyl-mPEG 2 kDa. The fluorescence intensity of the dansyl-moiety of the 2 kDa dansyl-mPEG polymer increased with increasing ethanol concentration (Figure 1A). Similar behaviour was observed for the 5 kDa dansyl-mPEG and the bis-dansyl-PEG 3 kDa (Figure 1B). A shift of fluorescence maxima was observed for dansyl-mPEG 5 kDa from 542 nm in water to 509 nm at 100 % ethanol and from 542 nm at 0 % to 511 nm at 100 % ethanol for the bis-dansyl-PEG 3 kDa (Figure 1C).



**Figure 1A.** Fluorescence emission spectra of dansyl-mPEG 2 kDa at different concentrations of ethanol. — fluorescence emission spectrum water; — • — fluorescence emission spectrum 25 % ethanol; – – – fluorescence emission spectrum 50 % ethanol; — — — fluorescence emission spectrum 75 % ethanol; -x- fluorescence emission spectrum 100 % ethanol.





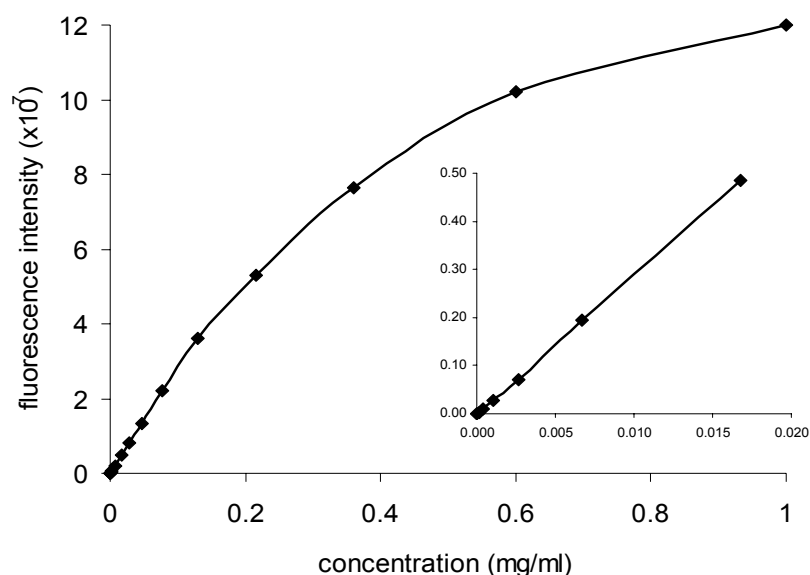
**Figure 1B.** Fluorescence intensity of dansyl-mPEG 2 kDa at the respective maxima against ethanol concentration (%) of the different dansyl-PEGs. –x– dansyl-mPEG 2 kDa; – $\Delta$ – bis-dansyl-PEG 3 kDa; – $\square$ – dansyl-mPEG 5 kDa.

**Figure 1C.** Wavelength of the fluorescence maxima against ethanol concentration (%) of the different dansyl-PEGs. –x– dansyl-mPEG 2 kDa; – $\Delta$ – bis-dansyl-PEG 3 kDa; – $\square$ – dansyl-mPEG 5 kDa.

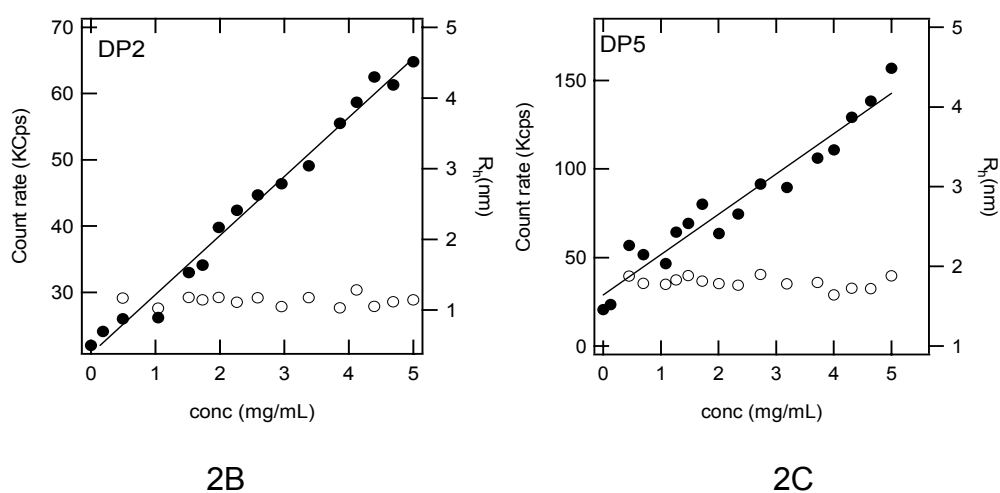
### 3.3 Studies of potential of self-association of dansyl-PEGs

Sensitivity towards solvent polarity of the intrinsic dansyl-fluorescence was used to test for self-assembly of the dansyl-mPEGs of concentrations up to 1 mg/ml. Figure 2A shows the dependency of fluorescence intensity against concentration of dansyl-mPEG 2 kDa. Below 0.13 mg/ml linearity was observed (Figure 2A, lower concentrations tested are enlarged in the insert). For concentrations between 0.13 mg/ml to 1 mg/ml quenching of the fluorescence occurred. Various concentrations of each polymer were analysed by dynamic light scattering (DLS) to test for formation of large micelles. A linear increase in the count rate as a function of concentration was obtained for all samples up to 5 mg/ml of dansyl-mPEG 2 kDa (Figure 2B) and dansyl-mPEG 5 kDa (Figure 2C). Bis-dansyl-PEG 3 kDa showed the same linearity up to 30 mg/ml (data not shown). The mean hydrodynamic radii calculated according

to number distribution for all samples throughout the whole concentration ranges were between 1-2 nm. As control, DLS experiment correctly detected the CMC (critical micelle concentration) of Tween 20<sup>®</sup> in water (data not shown).



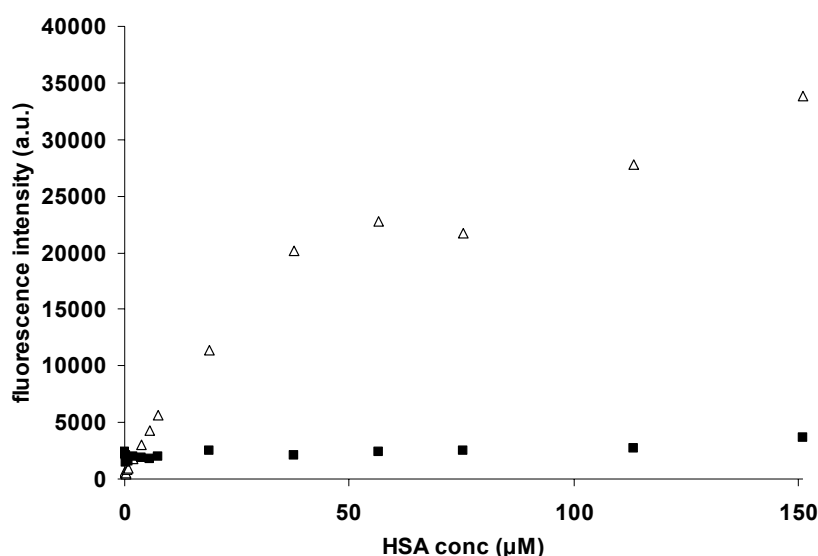
**Figure 2A.** Fluorescence intensity of maximum at 542 nm versus concentration of dansyl-mPEG 2 kDa.



Count rate and hydrodynamic radius( $R_h$ ) at various concentrations of dansyl-mPEG 2 kDa (**Figure 2B**) and dansyl-mPEG 5 kDa (**Figure 2C**) measured at 173° angle by dynamic light scattering (DLS).

### 3.4 Interaction of dansyl-mPEG 2 kDa with human serum albumin (HSA) in 50 mM HEPES buffer 154 mM NaCl pH 7.4

With increasing concentration of HSA the fluorescence intensity of DNSA at 2  $\mu$ M is steadily increasing, while the fluorescence intensity for dansyl-mPEG 2 kDa at 2  $\mu$ M remains stable (Figure 3).



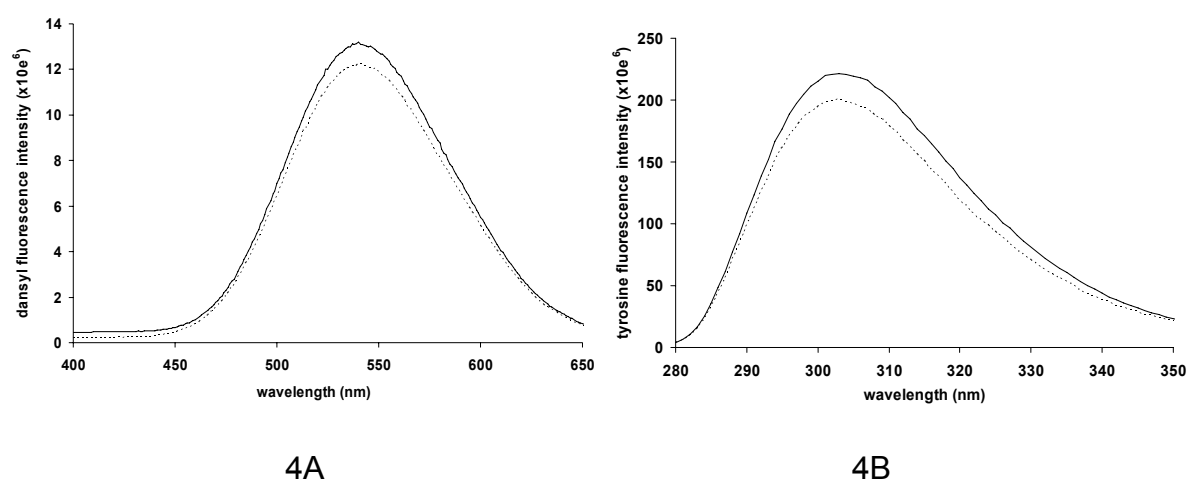
**Figure 3.** Dependency of fluorescence intensity of DNSA and dansyl-mPEG 2 kDa on concentration of human serum albumin (HSA).  $\Delta$  DNSA 2  $\mu$ M;  $\blacksquare$  dansyl-mPEG 2 kDa 2  $\mu$ M.

### 3.5 Interaction of dansyl-mPEG 2 kDa with salmon calcitonin (sCT) in 10 mM sodium acetate buffer pH 5

The dansyl-fluorescence emission spectrum of the dansyl-mPEG 2 kDa showed an increase in presence of salmon calcitonin (Figure 4A). The tyrosine fluorescence of salmon calcitonin decreased when dansyl-mPEG 2 kDa was added to sCT (Figure 4B). Figure 4C shows that the 90° light scatter was the lowest for 10 mM sodium acetate buffer pH 5. Increasing count rates were observed for the samples in the

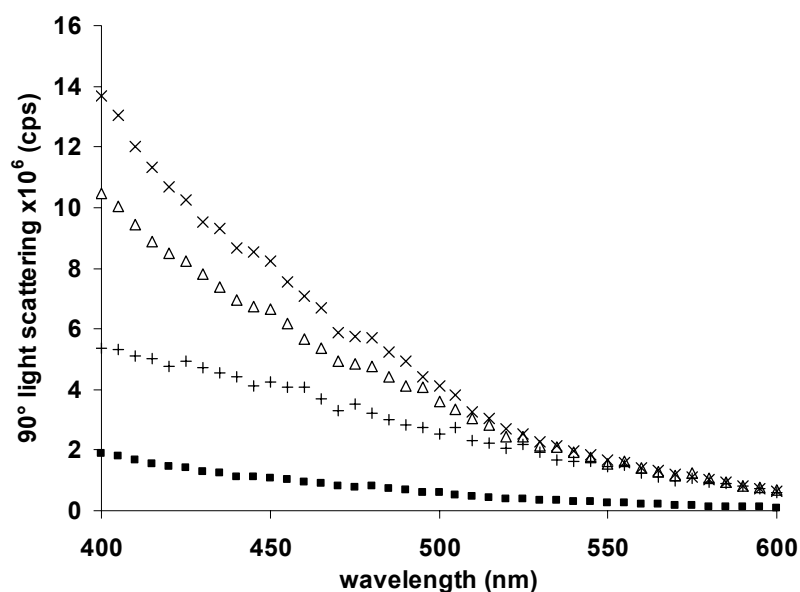
following order: i) dansyl-mPEG 2 kDa, ii) the mixture of salmon calcitonin with dansyl-mPEG 2 kDa at a 40:1 molar ratio and iii) salmon calcitonin. The count rate of sCT decreased strongly after the addition of dansyl-mPEG 2 kDa.

The mean lifetimes of tyrosine of sCT in absence and presence of the dansyl-mPEG 2 kDa polymer were similar (Table 4).



**Figure 4A.** Fluorescence emission spectra of dansyl-mPEG 2 kDa in absence and in presence of salmon calcitonin (sCT:dansyl-mPEG 2 kDa = 40:1) in 10 mM sodium acetate buffer pH 5. — fluorescence emission spectrum sCT:dansyl-mPEG 2 kDa = 40:1; – – – fluorescence emission spectrum dansyl-mPEG 2 kDa.

**Figure 4B.** Fluorescence emission spectra of tyrosine of sCT in absence and in presence of dansyl-mPEG 2 kDa (sCT:dansyl-mPEG 2 kDa = 40:1) in 10 mM sodium acetate buffer pH 5. — fluorescence emission spectrum sCT; – – – fluorescence emission spectrum sCT:dansyl-mPEG 2 kDa = 40:1.



**Figure 4C.** 90° light scatter. ■ 10 mM sodium acetate buffer pH 5; + dansyl-mPEG 2 kDa; Δ sCT:dansyl-mPEG 2 kDa = 40:1; x sCT.

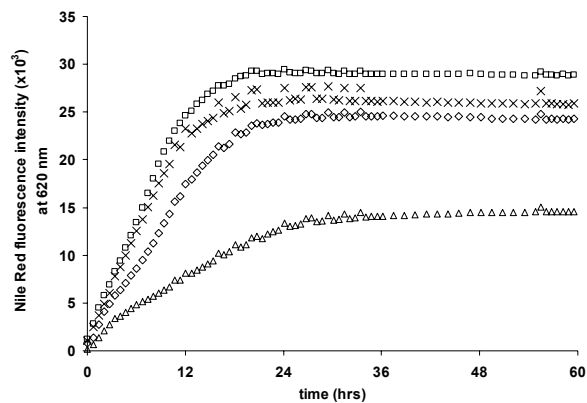
Sample	$\alpha_1$	$\tau_1$ (ns)	$\alpha_2$	$\tau_2$ (ns)	$\chi^2$	$\bar{\tau}$ (ns)
sCT	10.3	0.2	89.6	1.4	1.04	1.4
sCT:dansyl-mPEG 2 kDa =40:1	9.3	0.2	90.6	1.4	1.05	1.4

**Table 4.** Mean fluorescence lifetimes of tyrosine of sCT. Parameters obtained for 2.5 mg/ml sCT in absence and presence of 0.045 mg/ml dansyl-mPEG 2 kDa and thereof calculated mean fluorescence lifetimes in 10 mM sodium acetate buffer pH 5.

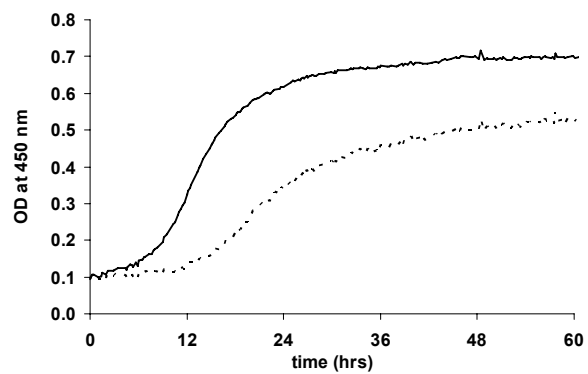
### 3.6 High Throughput Spectroscopic Analysis

#### *Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa at 1:1 molar ratio*

Aggregation of sCT in 10 mM sodium citrate buffer pH 6 is reduced in the presence of dansyl-mPEG 2 kDa, as shown by Nile Red fluorescence measurements at 620 nm (Figure 5A) and by turbidity measurements at 450 nm (Figure 5B) in time. The slope of the Nile Red fluorescence curve of sCT without dansyl-mPEG 2 kDa was steeper and also the fluorescence intensity of Nile Red was higher compared to the curve of the mixture of sCT and dansyl-mPEG 2 kDa. The control of mPEG-OH 2 kDa added to sCT showed an aggregation similar to that of salmon calcitonin. A mixture of sCT with dansylamide (DNSA) resulted in a slight decrease in the slope, but fluorescence intensity remained comparable to the sCT solution (Figure 5A). Turbidity in time of sCT measured at 450 nm in 10 mM sodium citrate buffer pH 6 showed immediate increase and a steep slope of the curve. A maximum in turbidity was reached after approximately 44 hours. In presence of dansyl-mPEG 2 kDa, turbidity remained stable for about 6 hours before it slowly increased. The slope of the curve in the presence of dansyl-mPEG 2 kDa was less steep than the slope of turbidity measured for sCT without dansyl-mPEG 2 kDa. A plateau was reached after approximately 60 hours for the mixture of sCT with dansyl-mPEG 2kDa (Figure 5B). In 10 mM sodium phosphate buffer pH 8, the slope of the Nile Red fluorescence and fluorescence intensity were decreased when dansyl-mPEG 2 kDa was added to sCT (Figure 5C). Nile Red fluorescence at 620 nm in time recorded for i) sCT, ii) sCT in mixture with mPEG-OH 2 kDa and iii) sCT with DNSA was comparable. Turbidity at 450 nm in time in 10 mM sodium phosphate buffer pH 8 of sCT and sCT with dansyl-mPEG 2 kDa remained stable for the first 17 hours. After this period a fast increase in turbidity was detected for sCT, resulting in a higher final value of turbidity than the mixture of sCT with dansyl-mPEG 2 kDa (Figure 5D).

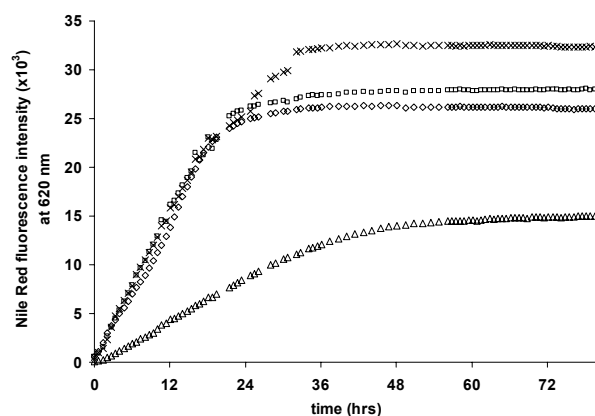


5A

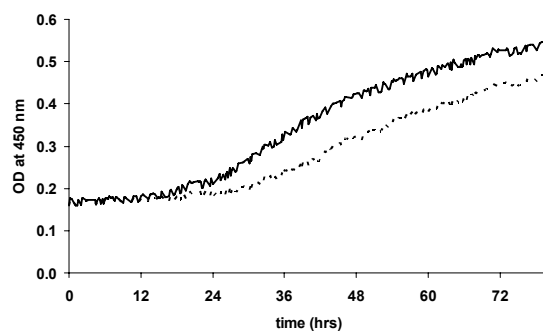


5B

**Figures 5A and B.** Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa against time at a 1:1 molar ratio. **Figure 5A** shows the Nile Red fluorescence measured at 620 nm in 10 mM sodium citrate buffer pH 6. □ sCT:mPEG-OH 2 kDa = 1:1; x salmon calcitonin; ◇ sCT:DNSA; △ sCT:dansyl-mPEG 2 kDa = 1:1. **Figure 5B** represents turbidity measured at 450 nm in 10 mM sodium citrate buffer pH 6. - - - sCT:dansyl-mPEG 2 kDa = 1:1; — salmon calcitonin.



5C



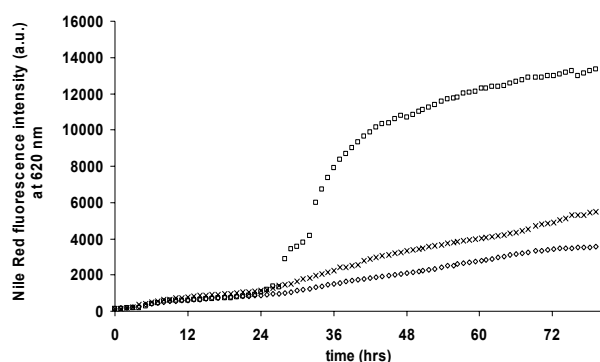
5D

**Figures 5C and D.** Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa against time at a 1:1 molar ratio. **Figure 5C** shows the Nile Red fluorescence measured at 620 nm in 10 mM sodium phosphate buffer pH 8. □ sCT:mPEG-OH 2 kDa = 1:1; x salmon calcitonin; ◇ sCT:DNSA; △ sCT:dansyl-mPEG 2 kDa = 1:1.

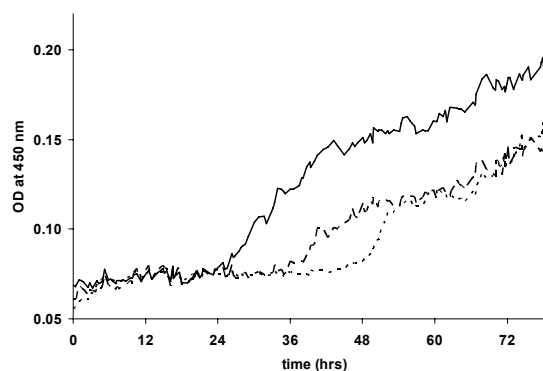
**Figure 5D** represents the turbidity measured at 450 nm in 10 mM sodium phosphate buffer pH 8. - - - sCT:dansyl-mPEG 2 kDa = 1:1; — salmon calcitonin.

### 3.7 Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa at different molar ratios

Changes in Nile Red fluorescence at 620 nm in time in 10 mM sodium citrate buffer pH 5 was measured for sCT and mixtures of sCT with dansyl-mPEG 2 kDa at ratios of 100:1 and 5:1. A reduction was observed in the following order: i) sCT, ii) sCT:dansyl-mPEG 2 kDa = 100:1, and iii) sCT:dansyl-mPEG 2 kDa = 5:1 (Figure 6A). Turbidity data showed the same result (Figure 6B). The lag phase of aggregation was prolonged depending on the amount of dansyl-mPEG 2 kDa added, being the shortest for i) sCT, followed by ii) sCT:dansyl-mPEG 2 kDa=100:1 molar ratio, and iii) sCT:dansyl-mPEG 2 kDa=5:1 molar ratio.



6A



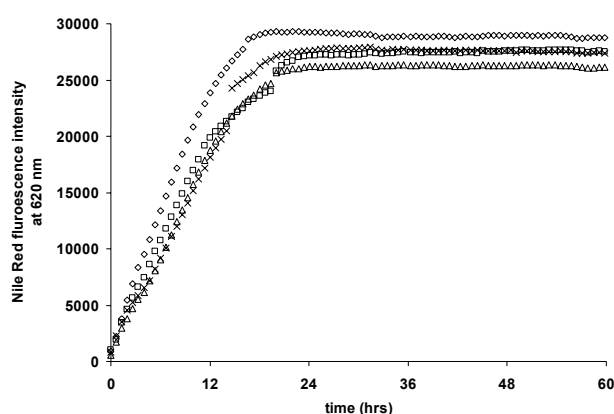
6B

**Figures 6A and B.** Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa against time at different molar ratios. **6A** shows the Nile Red fluorescence at 620 nm in 10 mM sodium citrate buffer pH 5. □ salmon calcitonin; x sCT:dansyl-mPEG 2 kDa = 100:1; ◇ sCT:dansyl-mPEG 2 kDa = 5:1. **6B** represents the turbidity at 450 nm in

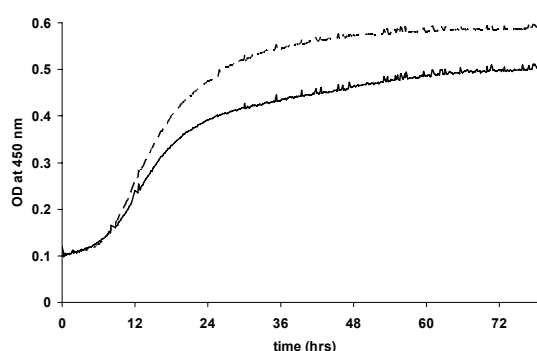


10 mM sodium citrate buffer pH 5. — salmon calcitonin; - - - sCT:dansyl-mPEG 2 kDa = 100:1; ••• sCT:dansyl-mPEG 2 kDa = 5:1.

Increasing the amount of dansyl-mPEG to a 1:2 molar ratio of sCT:dansyl-mPEG 2 kDa in 10 mM sodium citrate buffer pH 6 lead to a stronger increase in turbidity in time for the mixture of sCT with dansyl-mPEG 2 kDa compared to sCT (Figure 6D). An increased Nile Red fluorescence in time was also observed for the mixture of sCT:dansyl-mPEG 2 kDa at 1:2 molar ratio (Figure 6C). During these kinetics, an increase of the dansyl fluorescence in time was observed in the mixture of sCT with dansyl-mPEG 2 kDa compared to the control of dansyl-mPEG 2 kDa without sCT (Figure 6E). Spectra acquired after 140 hours showed an increase in the fluorescence intensity and a blue shift of the emission maximum of the dansyl-mPEG 2 kDa when mixed with aggregated sCT (Figure 6F).



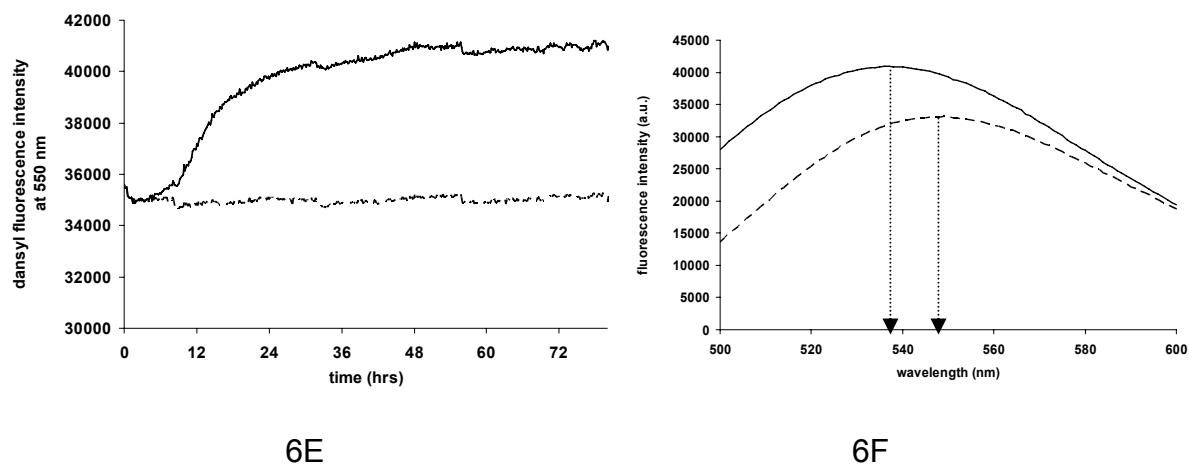
6C



6E

**Figures 6C and 6D.** Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa against time at different molar ratios. **6C** shows the Nile Red fluorescence at 620 nm in 10 mM sodium citrate buffer pH 6. □ sCT:mPEG-OH 2 kDa = 1:2; x salmon calcitonin; ◇ sCT:DNSA; △ sCT:dansyl-mPEG 2 kDa = 1:2. **6D** represents the

turbidity at 450 nm in 10 mM sodium citrate buffer pH 6. — salmon calcitonin; - - - sCT:dansyl-mPEG 2 kDa = 1:2.



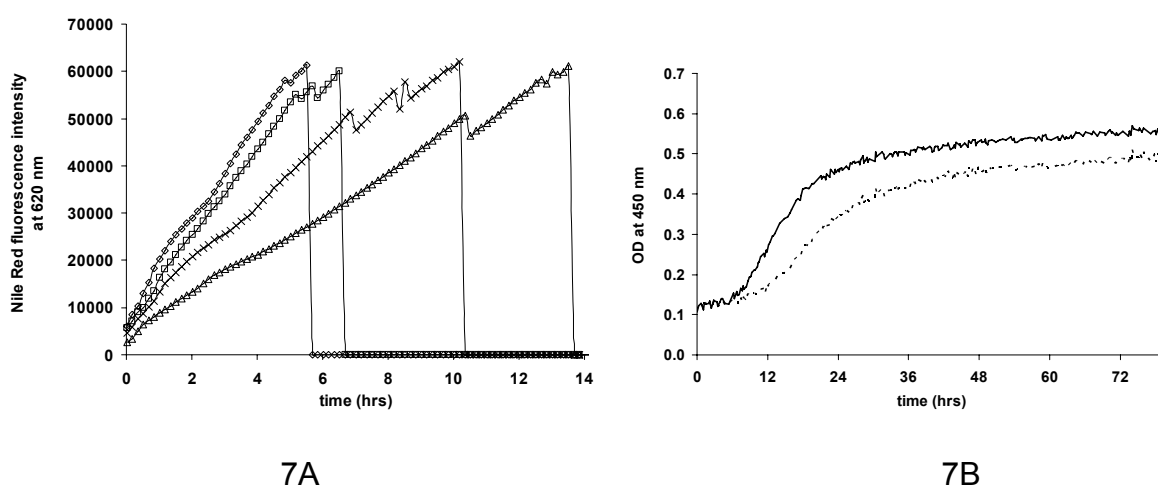
**Figures 6E and 6F.** Aggregation of salmon calcitonin with dansyl-mPEG 2 kDa against time at different molar ratios. **6E** represents the dansyl fluorescence at 550 nm in 10 mM sodium citrate buffer pH 6. — salmon calcitonin:dansyl-mPEG 2 kDa = 1:2; - - - dansyl-mPEG 2 kDa. **6F** shows the dansyl fluorescence emission scan after the aggregation kinetics were stopped (4 days) in 10 mM sodium citrate buffer pH 6. — sCT:dansyl-mPEG 2 kDa = 1:2; - - - dansyl-mPEG 2 kDa.

### 3.8 Aggregation of salmon calcitonin with bis-dansyl-PEG 3 kDa and dansyl-mPEG 5 kDa at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6

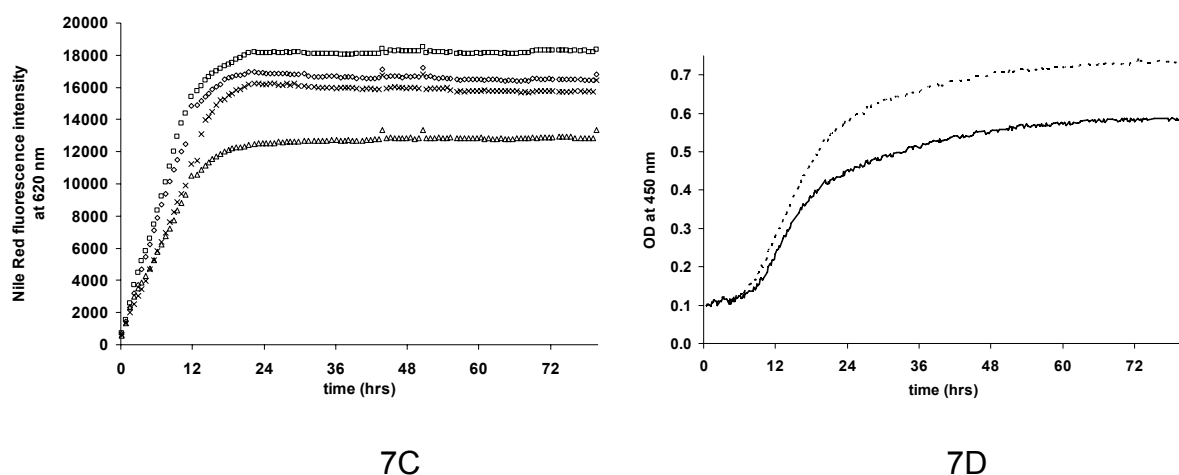
Nile Red fluorescence at 620 nm in time showed small differences between solutions of sCT with DNSA and sCT with OH-PEG-OH 3 kDa (Figure 7A). In both samples faster aggregation was observed than for sCT alone. It took i) 5.5 hours for sCT with DNSA, ii) 6.5 hours for sCT with OH-PEG-OH 3 kDa, and iii) 10 hours for sCT until a saturation of the detector was reached (shown as zero fluorescence intensity). The fluorescence signal of the mixture of sCT with bis-dansyl-PEG 3 kDa took 13.5 hours to reach saturation of the detector. Turbidity at 450 nm in time remained stable during

the first 5 hours for sCT alone and the mixture of sCT with bis-dansyl-PEG 3 kDa (Figure 7B). After 5 hours, turbidity of sCT strongly increased and resulted in a more turbid sample after 140 hours than the 1:1 molar mixture of sCT with bis-dansyl-PEG 3 kDa.

Differences were small between the Nile Red fluorescence at 620 nm in time of i) a 1:1 molar mixture of sCT and dansyl-mPEG 5 kDa ii) sCT, iii) sCT with mPEG-OH 5 kDa, and iv) sCT with DNSA (Figure 7C). Turbidity in time of sCT in combination with dansyl-mPEG 5 kDa showed a steeper slope and a higher final turbidity as compared to sCT (Figure 7D).



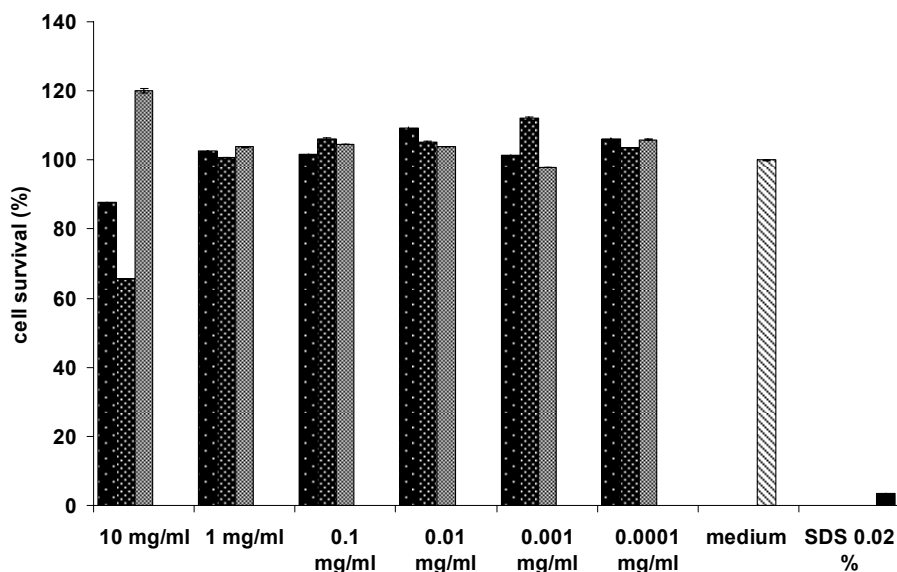
**Figures 7A and B.** Aggregation of salmon calcitonin with bis-dansyl-PEG 3 kDa and dansyl-mPEG 5 kDa against time. **7A** represents the Nile Red fluorescence measured at 620 nm in 10 mM sodium citrate buffer pH 6. -□- sCT:OH-PEG-OH 3 kDa = 1:1; -x- salmon calcitonin; -◇- sCT:DNSA; -△- sCT:bis-dansyl-PEG 3 kDa = 1:1. A higher sensitivity of the fluorescence detector has been used during measurements of these data compared to those of Figures 4A and 4C. **7B** shows the turbidity measured at 450 nm in 10 mM sodium citrate buffer pH 6. - - - sCT:bis-dansyl-PEG 3 kDa = 1:1; — salmon calcitonin.



**Figures 7C and D.** Aggregation of salmon calcitonin with bis-dansyl-PEG 3 kDa and dansyl-mPEG 5 kDa against time. **7C** shows the Nile Red fluorescence measured at 620 nm in 10 mM sodium citrate buffer pH 6.  $\square$  sCT:mPEG-OH 5 kDa = 1:1;  $\times$  salmon calcitonin;  $\diamond$  sCT:DNSA;  $\triangle$  sCT:dansyl-mPEG 5 kDa = 1:1. **7D** represents the turbidity measured at 450 nm in 10 mM sodium citrate buffer pH 6. - - - sCT:dansyl-mPEG 5 kDa = 1:1; — salmon calcitonin.

### 3.9 Evaluation of dansyl-PEGs' cytotoxicity

Figure 8 shows the cytotoxic potential of the different dansyl-PEGs. Neither dansyl-mPEG 2 kDa, bis-dansyl-PEG 3 kDa, nor dansyl-mPEG 5 kDa did decrease the viability of HaCaT cells after 48 hours of incubation at all tested concentrations, whereas the positive control of 0.02 % SDS showed severe cytotoxicity. The same results were obtained after incubation of HaCaT cells for 24 hours (data not shown).



**Figure 8.** Cytotoxicity of dansyl-PEGs tested on HaCaT cells after 48 hours of incubation with polymers. Serial dilutions of ■ dansyl-mPEG 2 kDa, ▤ bis-dansyl-PEG 3 kDa, ▨ dansyl-mPEG 5 kDa with positive (■ cells with 0.02% SDS) and negative control (▧ cells with medium only).

### 3.10 Evaluation of dansyl-PEGs' hemocompatibility

Hemocompatibility of the different dansyl-PEG polymers after 24 hour incubation with full human blood are shown in Table 5. The positive control, 1% Triton X, was 100 % hemolytic, whereas no hemolysis was measured for all tested concentrations of dansyl-mPEG 2 kDa, bis-dansyl-PEG 3 kDa, and dansyl-mPEG 5 kDa. Similar results were obtained after incubation of blood samples with the same polymer concentrations and controls for 1 hour (data not shown).

	20	10	1	0.1	0.01	0.001	0.0001	NaCl	Triton
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	0.9 %	X 1%
dansyl- mPEG 2 kDa	99.28	99.93	100.04	100.18	100.10	100.31	99.98	100.00	0.00
bis-dansyl- PEG 3 kDa	100.10	99.93	100.34	100.57	100.56	100.75	100.82		
dansyl- mPEG 5 kDa	100.11	100.33	100.32	100.37	100.34	100.39	100.60		

**Table 5.** Hemocompatibility of dansyl-PEGs. Different concentrations of dansyl-mPEG 2 and 5 kDa and bis-dansyl-PEG 3 kDa were incubated for 24 hours with full human blood. 0.9 % NaCl and 1 % Triton X 100 were taken as negative and positive control, respectively.

#### 4 Discussion

Covalent attachment of poly(ethylene glycol) or methoxypoly(ethylene glycol), also known as PEG and mPEG, has been a very successful technique to overcome remaining challenges like poor pharmacokinetics and increased *in vivo* immunogenicity of biopharmaceuticals. For several cases, a decreased protein aggregation has been reported, which is most probably due to steric hindrance exerted by the PEG, subsequently inhibiting approximation of protein molecules [34-39]. These different positive features of PEGylation resulted in the successful approval and use of various therapeutics since the beginning of the 1990's [5].

In this paper we describe dansyl-PEG-derivatives that are able to reduce aggregation of sCT without the need for covalent conjugation. A dansyl-group was conjugated to PEG polymers of different molecular weight in a mono- or bivalent manner. The

bivalent bis-dansyl-PEG was prepared in order to analyze, whether the presence of two dansyl-headgroups might be beneficial for reduction of aggregation. Twice the amount of hydrophobic headgroups might better shield large hydrophobic patches than one dansyl-headgroup. Dansylamide (DNSA) is a naphthylamine sulfonic acid like 8-Anilino-1-naphthalenesulfonic acid (1,8-ANS) or 6-(p-Toluidino)-2-naphthalenesulfonic acid (2,6-TNS). These hydrophobic molecules have a weak fluorescence in aqueous solutions, but the fluorescence is strongly increased when dissolved in apolar solvents or binding to hydrophobic surfaces. The fluorophores are used to characterize changes in protein tertiary structure during unfolding, to assess surface hydrophobicity, and for labeling or drug interaction studies [40-43]. Dansylamide possesses similar properties and was, therefore, used for this study.

The synthesized dansyl-PEGs all maintained the sensitivity of the dansyl-group towards environmental polarity (**Fig. 1**). With increasing ethanol concentrations (decreasing polarity) an increase of the dansyl-fluorescence intensity and a blue shift of its emission maximum were observed. The attachment of the different PEG-polymers did not significantly change the fluorescence properties of the dansyl head-group. However, the basic dansyl-fluorescence intensity in aqueous solutions was increased due to improved solubility, which can be ascribed to the PEG-moiety.

The sensitivity to polarity was subsequently used to analyze self-assembly, e.g., formation of micelles, of the different dansyl-PEGs at low concentrations. When the fluorophore is located in a more hydrophobic environment such as inside the micellar core, an increase of the intrinsic dansyl-fluorescence and a possible blue shift of the emission maximum may occur. No evidence was observed for the presence of micelles at low dansyl-mPEG 2 kDa polymer concentrations since the fluorescence intensity increased linearly with concentration (e.g., between 0 and 0.13 mg/ml). At high polymer concentrations (e.g., 0.13 mg/ml in the case of dansyl-mPEG 2 kDa)

fluorescence quenching occurred (**Fig. 2A**). Thus, for the higher concentrations dynamic light scattering (DLS) was used to investigate association of dansyl-mPEG 2 kDa molecules. When molecular complexes are forming, e.g. micelles, an increase should occur in the count rates, intensity of scattered light and measured hydrodynamic radii. The DLS experiment did not detect the formation of aggregates or micelles in the case of the different dansyl-PEGs (**Figures 2B and 2C**). In addition, the hydrodynamic radii measured under these conditions for all samples throughout the whole concentration range were between 1-2 nm similarly to those reported in literature [44].

Micelles may also be detected by addition of fluorophores, such as Nile Red [45]. Addition of Nile Red to dansyl-PEGs at different concentrations, resulted only in a poor Nile Red fluorescence, as seen for example at the beginning of the aggregation kinetics of sCT (see **Figures 5A, 5C and 6C**). The absence of a significant Nile Red fluorescence intensity further indicated that there were no aggregates/micelles formed at the different concentrations of dansyl-PEGs used during the aggregation kinetics experiments of sCT.

DNSA is reported to have a tight binding site on human serum albumin (HSA) [46]. The changes in fluorescence intensity upon binding to HSA were used to determine the binding affinity of DNSA and dansylated amino acids [30, 46, 47]. Performance of a titration of DNSA 2  $\mu$ M with increasing concentrations of HSA resulted in an increase of fluorescence intensity as expected (**Fig. 3**). With increasing HSA concentration more DNSA bound to HSA, resulting in increased DNSA fluorescence emission intensity. On the contrary, no changes in fluorescence intensity were observed for dansyl-mPEG 2 kDa at 2  $\mu$ M with increasing HSA concentration indicating that binding of the dansyl-headgroup to HSA is no longer possible. This



might most probably be assigned to a sterical shielding by the PEG impeding the interaction between the dansyl-headgroup and its binding site on HSA.

The influence of dansyl-mPEG 2 kDa on sCT was tested in 10 mM sodium acetate buffer pH 5, which is a stable solution (**Fig. 4**). An increase in the dansyl-fluorescence emission in the mixture with 40 times more sCT was detected as compared to dansyl-mPEG 2 kDa alone, indicating that the polymer was in a more hydrophobic environment in the mixture (**Fig. 4A**). The tyrosine fluorescence of sCT was decreased indicating that tyrosine was in a more hydrophobic environment after dansyl coupling to sCT (**Fig. 4B**). The 90° light scatter of sCT decreased after addition of dansyl-mPEG 2 kDa to sCT compared to sCT alone (**Fig. 4C**). These results show an interaction is taking place between sCT and dansyl-mPEG 2 kDa molecules. One possibility is that dansyl-mPEG 2 kDa interacts with transitory aggregates of sCT in the solution. A further explanation is based on the hypothesis that part of the sCT molecules in solution exist as dimers formed by the interaction of amphipathic  $\alpha$ -helices of each monomer [44]. The percentage of such dimers should be low since CD spectroscopy of sCT in solution shows mainly unordered structures [48-50]. Adding the dansyl-mPEG 2 kDa to sCT may dissociate these sCT dimers contributing in this way to a reduction of the 90°light scatter.

sCT is known to be stable in 10 mM sodium acetate buffer pH 5 [33]. This stability was not changed by the addition of of the different dansyl-PEGs in a 1:1 molar ratio over 140 hours (data not shown).

sCT strongly aggregates in 10 mM sodium citrate buffer pH 5, pH 6 and 10 mM sodium phosphate buffer pH 8 at room temperature [33]. The aggregation of sCT in 10 mM sodium citrate buffer pH 6 and 10 mM sodium phosphate buffer pH 8 was reduced after addition of dansyl-mPEG 2 kDa in a 1:1 molar ratio (**Fig. 5**). Measurements of Nile Red fluorescence and turbidity in time indicated a decrease in

aggregation velocity for sCT:dansyl-mPEG 2 kDa=1:1. The slopes of the curves were less steep compared to the curves of sCT without dansyl-mPEG 2 kDa. Furthermore, the final degree of aggregation is diminished, as presented in the lower final values of both parameters. Turbidity curves showed a prolongation in the onset of aggregation of sCT in mixture with dansyl-mPEG 2 kDa. Similar, though less pronounced behaviour was observed in citrate buffer pH 5 (data not shown).

Aggregation of sCT at different molar ratios to dansyl-mPEG 2 kDa in 10 mM sodium citrate buffer pH 5 revealed that the reduction of aggregation is observed even at a 100:1 molar ratio of sCT:dansyl-mPEG 2 kDa (**Fig. 6**). Turbidity measurements showed a dependency of the rate of aggregation of sCT on the quantity of dansyl-mPEG 2 kDa added: i) 43 hours of lag time were observed for a 5:1 molar ratio of sCT:dansyl-mPEG 2 kDa, ii) 33 hours for a 100:1 molar ratio of sCT:dansyl-mPEG 2 kDa, and iii) 24 hours for sCT alone (**Fig. 6B**). Nile Red fluorescence for these different ratios was comparable to the turbidity data (**Fig. 6A**).

Increasing the amount of dansyl-mPEG 2 kDa to a molar ratio of sCT:dansyl-mPEG 2 kDa = 1:2 deteriorated the aggregation of sCT in 10 mM sodium citrate buffer pH 6, observed by turbidity and Nile Red fluorescence measurements (**Figures 6C and 6D**). During the kinetics an increase in the dansyl-mPEG 2 kDa fluorescence was observed in the mixture with sCT (**Fig. 6E**). The increase in dansyl-fluorescence appeared simultaneously with the increase in turbidity. The emission spectra of dansyl-mPEG 2 kDa in absence and in presence of aggregated sCT were measured after the kinetics were stopped (**Fig. 6F**). An increase in the dansyl-fluorescence intensity and blue shift of the emission maximum were detected, which indicates that the dansyl-group was in a more hydrophobic environment in presence of aggregated sCT. These data show that the sensitivity of the dansyl-moiety to polarity may be used as a further parameter to detect and follow the aggregation of sCT.

As a conclusion, a decrease of sCT aggregation was detected for molar ratios of sCT:dansyl-mPEG 2 kDa of 100:1 up to 1:1, whereas a ratio of 1:2 deteriorated the aggregation of the peptide.

The molecular weight of the mPEG polymer appeared to play an important role in reducing the aggregation of sCT. While the best reduction of aggregation was obtained with the dansyl-mPEG 2 kDa at a 1:1 molar ratio (**Figures 5A and 5B**), stabilization to a lesser degree was detected when using the bis-dansyl-PEG 3 kDa at a 1:1 molar ratio (**Figures 7A and 7B**). An faster aggregation was observed using the dansyl-mPEG 5 kDa at a 1:1 molar ratio (**Figures 7C and 7D**). While present covalent PEGylation technology uses the conjugation of few high molecular weight PEGs (typically 20 or 40 kDa) [27], the use of higher molecular weight dansyl-PEGs was not beneficial in inhibiting sCT aggregation. A possible explanation for these effects may be that the interaction of the dansyl-headgroup with the peptide drug is sterically inhibited, due to the large and highly hydrated PEG. Another possibility might be that the hydrophilic-lipophilic balance of the dansyl-PEGs plays a role, as reported for nonionic detergents [51]. Accordingly, with increasing molecular weight of the PEG-moiety, the influence of the polymeric part on aggregation increased, at the disadvantage of dansyl headgroup-sCT interactions. This hypothesis is further supported, by the observation that the control group of nonconjugated PEG-diamine accelerated sCT aggregation. A steeper slope was also observed in the Nile Red fluorescence curve of the mixture of sCT:dansyl-mPEG 5 kDa = 1:1 compared to sCT alone (**Fig. 7C**). A further possible explanation might be a water exclusion effect due to the PEGs. PEGs remove the solvation water from the outer shell of biopharmaceuticals and are, therefore, used as precipitating agents. Due to hydrophobic interactions with the dansyl-headgroup might allow sCT to remain in solution instead of precipitating, although solvation water was removed.

An improved effect on aggregation by the bis-dansyl-PEG 3 kDa compared to the monovalent dansyl-mPEG 2kDa was not observed. The presence of two hydrophobic dansyl-headgroups was not superior to one dansyl-headgroup.

Excipients consisting of a hydrophobic and a hydrophilic moiety, such as detergents, are known to interact with and destabilize cellular membranes. To address the potential use of the dansyl-PEGs as excipients for protein drug formulations, we investigated their potential cytotoxicity and hemolysis properties. Immortalized keratinocytes were used to examine cytotoxicity, because peptide and protein drugs are often applied subcutaneously. No cytotoxic effects were observed after 48 hours of incubation with various concentrations of the different dansyl-PEGs. (**Fig. 8**).

The dansyl-PEGs were also shown to have low hemolytic properties (**Table 5**).

## Conclusions

In this paper, dansyl-PEGs were shown to decrease aggregation of sCT. Mono- and bifunctional dansyl-PEG polymers of 2, 3 and 5 kDa were synthesized and characterized by various techniques. Reduction of the 90° light scatter of a 40:1 molar mixture of sCT and dansyl-mPEG 2 kDa compared to sCT alone and an increase in dansyl-fluorescence after sCT addition may suggest possible interactions. We have shown that the dansyl-mPEG 2 kDa reduced aggregation best at a 1:1 molar ratio, although stabilization was observed down to molar ratios of sCT:dansyl-mPEG 2 kDa of 100:1. Bis-dansyl-PEG 3 kDa also stabilized sCT against aggregation, though to a lesser extent than observed for the monovalent 2 kDa dansyl-mPEG at 1:1 molar ratio. Deteriorated aggregation of sCT was observed for the dansyl-mPEG 5 kDa. Our data have shown that the changes in dansyl-fluorescence of the dansyl-PEGs may also be used to detect and follow aggregation of sCT. The polymers were non-toxic and non-hemolytic at all concentrations tested.

Ongoing studies may be revealing whether other key benefits of covalent PEGylation are maintained by our approach.

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## Chapter 4

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### **Tryptophan-mPEGs: Novel excipients that stabilize salmon calcitonin against aggregation by non-covalent PEGylation**

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## **Abstract**

Protein aggregation, which is triggered by various factors, is still one of the most prevalent problems encountered during all stages of protein formulation development. In this publication, we present novel excipients, tryptophan-mPEGs (Trp-mPEGs) of 2 and 5 kDa molecular weight and their use in protein formulation. The synthesis and physico-chemical characterization of the excipients is described. Possible cytotoxic and hemolytic activity of the Trp-mPEGs was examined. Turbidity, 90° static light scatter, intrinsic fluorescence, fluorescence after staining the samples with Nile Red and fluorescence microscopy were used to study the inhibitory effect of the Trp-mPEGs on the aggregation of salmon calcitonin (sCT) in different buffer systems and at various molar ratios. Aggregation of sCT was reduced significantly with increasing concentrations of Trp-mPEG 2 kDa. A 10-fold molar excess of Trp-mPEG 2 kDa suppressed almost completely the aggregation of sCT in 10 mM sodium citrate buffer (pH 6) for up to 70 hours. Trp-mPEG 5 kDa also reduced aggregation of sCT, though less pronounced than Trp-mPEG 2 kDa. Low aggregation of sCT was measured after ~10 days in 10 mM sodium citrate buffer pH 5 with a 10-fold molar excess of Trp-mPEG 2 kDa. This paper shows that Trp-mPEGs are potent excipients in reducing aggregation of sCT. Trp-mPEGs are superior to dansyl-PEGs concerning the stabilization of sCT in a harsh environment, wherein sCT is prone to aggregation. Trp-mPEGs might therefore also be used for stabilization of other biopharmaceuticals prone to aggregation.

## 1 Introduction

Biologics considerably gained increased market share during the last decade [1-3]. However, chemical and physical degradation, among which aggregation is one of the main concerns, limit the rapid commercialization of protein-based pharmaceuticals [4]. During their manufacturing and formulation processes, proteins are subjected to various stresses, which may lead to aggregation of the biopharmaceutical drug. Processing steps, e.g., freeze drying, pumping, agitation, shear stress, changes in temperature and environmental conditions like buffers, pH or additives, or packaging materials are a few parameters that may affect the stability of a protein [4-6]. Due to the many factors triggering aggregation, and which vary from drug to drug, suppression or reduction of aggregation is most often obtained experimentally by testing various parameters, such as different buffer systems or pH values, under accelerated aggregation conditions [4, 7].

For many proteins, aggregation starts by formation of partially unfolded intermediates. The fraction of unfolded intermediates is usually relatively small but favours protein association, because of an increased amount of exposed hydrophobic patches [8-10]. Native monomers may also aggregate, if 'sticky' patches are present on the protein's surface. Aggregation can then proceed through hydrophobic or electrostatic forces between those patches [9, 11].

Covalent conjugation of poly(ethylene glycol), commonly referred to as PEGylation, has been successfully applied to reduce aggregation of biopharmaceuticals [12-14]. The protective effect of PEG on protein aggregation may be explained by sterical shielding of hydrophobic patches on the protein's surfaces. Further benefits that may be obtained by PEGylation are an increased half-life *in vivo* and a decreased *in vivo* immunogenicity of the biopharmaceutical [15-17]. These positive attributes led to the

successful approval of different PEGylated biopharmaceuticals since the beginning of the 1990's [18].

However, several challenges concerning covalent PEGylation remain, which are i) the chemical reaction needed in order to attach the PEG-polymer, and ii) the loss of *in vivo* bioactivity observed after PEGylation. Although various techniques have been developed for covalent PEGylation, none has been found that circumvents the chemical reaction and subsequently needed purification. These additional processes during formulation of the final drug product represent additional stresses for biopharmaceuticals and may lead to aggregation, resulting in a partial or complete loss of *in vivo* bioactivity and increased *in vivo* immunogenicity.

In order to reduce the imposed stresses on biopharmaceuticals during formulation, we presented in a recent publication the new method of non-covalent PEGylation against aggregation by hydrophobic interaction (J Pharm Sci, accepted for publication). The concept is based on non-covalent interaction between the hydrophobic headgroup of a synthesized PEG derivative and hydrophobic patches on the surface of biopharmaceuticals. A sterical shielding of the latter by the PEG moiety shall be obtained. Protein/protein interactions should thus be rendered less probable, and consequently aggregation of the biopharmaceutical in liquid formulations should be reduced. A patent application related to this technology has recently been filed [19].

We were able to show that aggregation of salmon calcitonin (sCT) in liquid formulations was reduced after addition of dansyl-PEGs of varying molecular weights. The reduction of aggregation was dependent on the molecular weight of the dansyl-PEG and the molar ratio of sCT to dansyl-PEG used. It was also shown that the fluorescence of the dansyl headgroup can be used as a further tool to follow the aggregation of salmon calcitonin. In this publication, we present the preparation and

physico-chemical characterization of PEG conjugates with the amino acid tryptophan (Trp) as hydrophobic headgroup. Although in our studies no hemolysis was observed for both the Trp-PEGs and the dansyl-PEGs, Trp holds the advantage of being used as nutritional supplement for humans [20]. The influence of the monovalent Trp-mPEG polymers of 2 and 5 kDa on the aggregation of sCT was evaluated by performance of accelerated aggregation studies. Trp-mPEGs were superior to dansyl-PEGs in stabilizing sCT against aggregation in a harsh environment, in which sCT in the absence of stabilizing excipients aggregates very fast.

## **2 Materials and Methods**

### *2.1 Materials*

The chemicals employed to prepare the buffer solutions, Nile Red (9-diethylamino-5H-benzo[*a*]phenoxazine-5-one), anhydrous toluene and trifluoroacetic acid (TFA) were supplied by Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The used buffers were constituted of: (i) acetic acid – sodium acetate pH 5; (ii, iii) citric acid – sodium citrate pH 5 and pH 6, respectively; (iv) sodium phosphate monobasic – sodium phosphate dibasic pH 8. Anhydrous DMSO, anhydrous dichloromethane, and L-tryptophan were purchased from Fluka (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Chloroform was supplied by Chimie-Plus (Chimie Plus Laboratoires, Denicé, France). Concentrated hydrochloric acid and anhydrous Na<sub>2</sub>SO<sub>4</sub> were obtained from Riedel de Haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany). Diethyl ether, dichloromethane, anhydrous triethyl amine, iso-propanol, and *p*-nitrophenyl chloroformate were provided by Acros (Acros Organics BVBA; Geels, Belgium). The mPEG-OH 2 kDa and 5 kDa were obtained from Iris Biotech (Iris Biotech GmbH, Marktredwitz, Germany). All solvents and compounds used were of analytical grade. The salmon calcitonin was provided



by Therapeomic (Therapeomic Inc., Basel, Switzerland). UV transparent 96-well or 384-well Costar<sup>®</sup> Corning microplates and UV-Vis transparent and pressure sensitive Corning<sup>®</sup> Universal Optical sealing tape were purchased from Corning (Corning Life Sciences, Schiphol, Netherlands).

## *2.2 Characterization of tryptophan-PEGs*

All polymers were dissolved in deuterated DMSO and analyzed on a Varian VXR 300MHz spectrometer (Varian, Switzerland) to obtain <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra. MALDI-TOF mass spectrometry was performed on an Axima CFR+, Shimadzu mass spectrometer, using 2-(4-hydroxyphenylazo)-benzoic acid (HABA) as matrix. A Perkin Elmer 100 FT-IR spectrometer (PerkinElmer, Switzerland) was used to measure FTIR spectra in the range of 4000–400 cm<sup>-1</sup>. The used pellets were made of 1% w/w of product in KBr. UV spectra were obtained on a Varian Cary 50 spectrophotometer (Varian, Switzerland). The tryptophan-mPEGs were further analyzed for their specific optical rotation properties according to European Pharmacopeia 5.6 using a Perkin Elmer 241 Polarimeter.

## *2.3 Synthesis of mPEG-p-nitrophenyl carbonate 2 kDa*

The method was adapted from [21]. In short, 1.76 mMol of dried mPEG-OH 2 kDa were dissolved in anhydrous dichloromethane and 5.27 mMoles of p-nitrophenyl chloroformate and 3.52 mMoles of dry triethyl amine were added (1:3:2 ratio). The pH was adjusted to a value between 7.5 - 8 and the reaction was left to proceed at room temperature for 24 hours. The reaction was stopped by adding several drops of TFA until the solution got colourless, then dichloromethane was partially evaporated and precipitation from cold diethyl ether was performed. The solid collected by filtration

was twice redissolved in dichloromethane, precipitated from cold diethyl ether, and collected by filtration. A white powder was obtained and dried under vacuum.

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 3.23 ppm, PEG CH<sub>3</sub>-O- (s); 3.50 ppm, PEG -O-CH<sub>2</sub>- (m); 7.55 ppm, p-nitrophenyl-aromatic (d); 8.31 ppm, p-nitrophenyl-aromatic (d). <sup>13</sup>C-NMR (300 Mhz, DMSO-d-6): 58.06 ppm, PEG CH<sub>3</sub>-O-; 69.52 ppm, PEG -O-CH<sub>2</sub>-; 122.59 ppm, p-nitrophenyl-aromatic; 125.34 ppm, p-nitrophenyl-aromatic; 144.21 ppm, PEG -O-CH<sub>2</sub>-C=O; 151.99 ppm, aromatic C<sub>5</sub>H<sub>4</sub>=C-NO<sub>2</sub>; 155.27 ppm, PEG -CH<sub>2</sub>-OCO-. FTIR: 3435; 2888; 2739; 2678; 2493; 1967; 1769; 1617; 1594; 1527; 1468; 1360; 1343; 1281; 1242; 1113; 1060; 963; 841; 663; 529 cm<sup>-1</sup>. MS (MALDI-TOF): m/z 2201 (M<sup>+</sup>).

#### *2.4 Synthesis of tryptophan-mPEG 2 kDa*

The method was adapted from [21]. 0.018 Mol L-tryptophan were dissolved in anhydrous DMSO and the pH was adjusted to a value of ~8.3. 1.76 mMol of dried mPEG-p-nitrophenyl carbonate 2 kDa were added. The pH was maintained at ~8.3 and the reaction was left to proceed at room temperature for 4 hours. The reaction was stopped by cooling to 0°C and adjusting the pH to a value of 3 with 2 M HCl. The aqueous phase was extracted with chloroform. The obtained organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and partially evaporated. Precipitation from cold diethyl ether was performed and the solid collected by filtration. The solid was once reprecipitated from cold diethyl ether, and twice from cold iso-propanol. A white powder was obtained and dried under vacuum.

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 3.17 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (d); 3.24 ppm, PEG -CH<sub>3</sub>-O- (s); 3.51 ppm, PEG -O-CH<sub>2</sub>- (m); 4.17 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (q); 6.98 ppm, Trp-indole (t); 7.06 ppm, Trp-indole (t); 7.16 ppm, Trp-indole (s); 7.32 ppm, Trp-

indole (d); 7.51 ppm, Trp-indole (d); 10.82 ppm Trp –COOH (s).  $^{13}\text{C}$ -NMR (300 MHz, DMSO-d-6): 54.78 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 58.58 ppm, PEG CH<sub>3</sub>-O-; 63.28 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 69.70 ppm, PEG -O-CH<sub>2</sub>-; 110.02 ppm, Trp-indole; 111.33 ppm, Trp-indole; 117.79 ppm, Trp-indole; 120.80 ppm, Trp-indole; 123.65 ppm, Trp-indole; 126.88 ppm Trp-indole; 136.17 ppm, Trp-indole; 156.26 ppm, PEG -CH<sub>2</sub>-OCO-NH-; 173.87 ppm, -COOH. FTIR: 3412; 2886; 2741; 2695; 2167; 1970; 1721; 1526; 1467; 1413; 1360; 1343; 1280; 1242; 1110; 963; 842; 745, 529 cm<sup>-1</sup>. MS (MALDI-TOF):  $m/z$  2266 (M<sup>+</sup>).  $[\alpha]_D^{20} = -0.005$ .

## 2.5 Synthesis of mPEG-*p*-nitrophenyl carbonate 5 kDa

The reaction was performed as described for the mPEG-*p*-nitrophenyl carbonate 2 kDa, whereas 0.68 mMol of dried mPEG-OH 5 kDa, 2.03 mMoles of *p*-nitrophenyl chloroformate and 1.36 mMoles of dry triethyl amine were used. A white powder was obtained.

$^1\text{H}$ -NMR (300 MHz, DMSO-d-6): 3.23 ppm, PEG CH<sub>3</sub>-O- (s); 3.50 ppm, PEG -O-CH<sub>2</sub>- (m); 7.55 ppm, *p*-nitrophenyl-aromatic (d); 8.31 ppm, *p*-nitrophenyl-aromatic (d).  $^{13}\text{C}$ -NMR (300 Mhz, DMSO-d-6): 58.27 ppm, PEG CH<sub>3</sub>-O-; 69.70 ppm, PEG -O-CH<sub>2</sub>-; 122.62 ppm, *p*-nitrophenyl-aromatic; 125.33 ppm, *p*-nitrophenyl-aromatic; 145.93 ppm, PEG -O-CH<sub>2</sub>-C=O; 152.10 ppm, aromatic C<sub>5</sub>H<sub>4</sub>=C-NO<sub>2</sub>; 154.76 ppm, PEG -CH<sub>2</sub>-OCO-. FTIR: 3447; 2889; 2741; 2694; 2603; 2494; 1971; 1769; 1642; 1526; 1468; 1360; 1343; 1281; 1242; 1219; 1113; 1060; 963; 842; 529 cm<sup>-1</sup>. MS (MALDI-TOF):  $m/z$  4698 (M<sup>+</sup>).

## 2.6 Synthesis of tryptophan-mPEG 5 kDa

The reaction was performed as described for tryptophan-mPEG 2 kDa. 0.68 mMol of dried mPEG-*p*-nitrophenyl carbonate 5 kDa and 6.78 mMoles of L-tryptophan were used. A white powder was obtained.

$^1\text{H-NMR}$  (300 MHz, DMSO- $d_6$ ): 3.21 ppm, Trp indole- $\text{CH}_2\text{-CH}_2\text{-}$  (d); 3.24 ppm, PEG  $\text{-CH}_3\text{-O-}$  (s); 3.51 ppm, PEG  $\text{-O-CH}_2\text{-}$  (m); 4.18 ppm, Trp indole- $\text{CH}_2\text{-CH}_2\text{-}$  (q); 6.96 ppm, Trp-indole (t); 7.02 ppm, Trp-indole (t); 7.14 ppm, Trp-indole (s); 7.32 ppm, Trp-indole (d); 7.51 ppm, Trp-indole (d); 10.81 ppm Trp  $\text{-COOH}$  (s).  $^{13}\text{C-NMR}$  (300 MHz, DMSO- $d_6$ ): 54.85 ppm, Trp indole- $\text{CH}_2\text{-CH}_2\text{-}$ ; 58.04 ppm, PEG  $\text{CH}_3\text{-O-}$ ; 63.46 ppm, Trp indole- $\text{CH}_2\text{-CH}_2\text{-}$ ; 69.72 ppm, PEG  $\text{-O-CH}_2\text{-}$ ; 110.83 ppm, Trp-indole; 111.34 ppm, Trp-indole; 117.99 ppm, Trp-indole; 120.90 ppm, Trp-indole; 124.11 ppm, Trp-indole; 127.04 ppm Trp-indole; 136.62 ppm, Trp-indole; 156.24 ppm, PEG  $\text{-CH}_2\text{-OCO-NH-}$ ; 173.68 ppm,  $\text{-COOH}$ . FTIR: 3438; 2885; 2741; 2695; 1969; 1719; 1647; 1467; 1360; 1343; 1281; 1242; 1112; 1060; 963; 842; 746; 529  $\text{cm}^{-1}$ . MS (MALDI-TOF):  $m/z$  4772 ( $\text{M}^+$ ).  $[\alpha]_D^{20} = -0.002$ .

## 2.7 Sensitivity of tryptophan-mPEGs to solvent polarity

0.04 mM solutions of tryptophan-mPEGs in 0, 25, 50, 75 and 100 % ethanol were prepared. The dielectric constants are 78.54 for water and 24.3 for ethanol 96 %. The solutions were analyzed by intrinsic fluorescence and UV-Vis absorbance at 25°C, using a Fluoromax spectrofluorometer (Spex, Stanmore, UK), and temperature-controlled Cintra 40 spectrophotometer (GBC, Melbourne, Australia), respectively. For tryptophan-mPEG 2 kDa and 5 kDa, the fluorescence emission spectra were recorded between 290 and 450 nm after excitation at 280 nm using bandwidths of

0.3 mm at the excitation and 1 mm at the emission side. The integration time used was 0.01 s.

### *2.8 Studies of potential of self-association of tryptophan-PEGs*

Fluorescence emission of solutions below 0.6 mg/ml and dynamic light scattering at concentrations above 0.1 mg/ml were used to analyze self-association of the Trp-mPEGs. Aqueous polymer solutions of 1 mg/ml were further diluted to obtain solutions with concentrations between 0.6 mg/ml and 0.1  $\mu$ g/ml and their fluorescence emission spectra were measured using an excitation wavelength of 280 nm. In order to obtain a sufficiently high fluorescence signal, slit widths were adapted depending on the concentration. Dynamic light scattering (DLS) at an angle of 173° (back-scatter) at 25°C was employed to analyze solutions with concentrations above 0.08 mg/ml by using a Zetaziser Nano ZS (Malvern Instruments), equipped with a 4 mW He–Ne laser that operates at 633 nm. The micellization of Tween 20® was followed as a control experiment for DLS measurements.

### *2.9 High Throughput Spectroscopic Analysis*

The method was adapted from [22]. The four different buffer solutions, (i) 10 mM sodium acetate buffer pH 5; (ii, iii) 10 mM sodium citrate buffer pH 5 and 6, respectively; and (iv) 10 mM sodium phosphate pH 8 were prepared using sterile filtered 100 mM buffer stock solutions. A stock solution of 12.5 mg/ml salmon calcitonin in purified MilliQ™ Millipore water was freshly prepared. The final solutions tested contained 2.5 mg/ml sCT in 10 mM buffer. Each sample was prepared in duplicate and to one of each duplicate Nile Red (1  $\mu$ M in final formulation) was added. Each formulation was measured in triplicate. Table 1 shows the different molar ratios and respective concentrations of sCT and Trp-mPEGs.

All sCT solutions were prepared in 96- or 384-well Costar<sup>®</sup> Corning microplates. Afterwards, the plates were sealed using UV-Vis transparent and pressure sensitive Corning<sup>®</sup> Universal Optical sealing tape.

sample	molar ratio	concentration (mg/ml)
sCT:Tryptophan-mPEG 2 kDa	1:0.5	2.5mg/ml sCT; 0.93 mg/ml Trp-mPEG 2 kDa
	1:1	2.5mg/ml sCT; 1.86 mg/ml Trp-mPEG 2 kDa
	1:2	2.5mg/ml sCT; 3.72 mg/ml Trp-mPEG 2 kDa
	1:5	2.5mg/ml sCT; 9.29 mg/ml Trp-mPEG 2 kDa
	1:10	2.5mg/ml sCT; 18.59 mg/ml Trp-mPEG 2 kDa
sCT:Tryptophan-mPEG 5 kDa	1:1	2.5mg/ml sCT; 4.36 mg/ml Trp-mPEG 5 kDa
	1:5	2.5mg/ml sCT; 21.79 mg/ml Trp-mPEG 5 kDa

**Table 1.** Solutions tested by high throughput spectroscopic analysis. Different molar ratios and the respective concentrations of sCT and Trp-PEGs used.

Every 7.5 minutes a cycle of the following was measured at 26°C with a Tecan Safire™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland): i) UV-Vis absorbance at 500 nm (turbidity); ii) fluorescence emission of the hydrophobic dye Nile Red; and iii) intrinsic Trp fluorescence emission.

The fluorescence emission of Trp over time was determined at 360 nm after excitation at 280 nm using bandwidths of 5 and 12 nm for excitation and emission, respectively. The fluorescence emission of Nile Red was studied over time at 620 nm after excitation at 550 nm using bandwidths of 12 nm for both excitation and emission. After 3 days, the intrinsic Trp fluorescence emission spectra (300 – 400 nm) and Nile Red fluorescence emission spectra (590 – 700nm) were recorded using the same settings as during the kinetics. The data output of the XFluor<sup>®</sup> software of

the Tecan Safire™ microplate reader is in Microsoft® Excel™ format. To facilitate high throughput data analysis, visual basic macros were developed by the authors.

#### *2.10 Interaction of Trp-mPEG 2 kDa with salmon calcitonin (sCT) at different molar ratios in 10 mM sodium citrate buffer pH 5*

##### Steady-state fluorescence and anisotropy

sCT (12.5 mg/ml) and Trp-mPEG 2 kD (37.17 mg/ml) stock solutions were freshly prepared in 10 mM sodium citrate buffer pH 5. The tested solutions were (i) 2.5 mg/ml sCT and 18.59 mg/ml Trp-mPEG 2 kDa (1:10 molar ratio protein to excipient), (ii) 2.5 mg/ml sCT and 1.86 mg/ml Trp -mPEG 2 kDa (1:1 molar ratio protein to excipient), and (iii) 2.5 mg/ml sCT in 10 mM sodium citrate buffer pH 5.

Steady-state fluorescence measurements were performed using a Fluoromax spectrofluorometer (Spex, Stanmore, UK) at 25°C in a thermostated cuvette holder. The tyrosine fluorescence of sCT was monitored with a 0.01 s integration time at emission wavelengths between 290 and 450 nm, using an excitation wavelength of 275 nm and 280 nm. Bandwidths of 1 nm and 2 nm were used for excitation and emission, respectively, while an optical filter (10 % light transmission) was placed at the emission site in front of the detector.

To measure tryptophan fluorescence the samples were excited at 295 nm using the same settings and filter arrangement as for tyrosine. Nile-Red fluorescence was measured between 590 and 750 nm using an excitation wavelength of 550 nm and slits of 1 nm and 2 nm for excitation and emission, respectively.

Steady-state anisotropy measurements were performed using the above described Fluoromax spectrophotometer with prism polarizers and anisotropy was calculated as described in [23]. The anisotropy value for tyrosine was calculated from fluorescence spectra between 300 and 320 nm, using an excitation wavelength of 275 nm, with 1

second integration time per 1 nm increment and excitation and emission slits of 1 and 2 mm, respectively. The anisotropy value for Nile Red was calculated from fluorescence spectra between 610 and 630 nm, using an excitation wavelength of 550 nm, with 2 seconds integration time per 1 nm increment. The excitation and emission slits were 1 and 2 mm, respectively.

### 90° light scattering

The intensity of scattered light at a 90 degree angle was determined as described by [23] using the Fluoromax spectrofluorometer (Spex, Stanmore, UK). The light scatter was measured between 450 and 700 nm using synchronized excitation and emission monochromators. Slits of 2 mm at the excitation and emission side and an integration time of 0.01 s were used. An optical filter (10 % light transmission) was put in front of the detector.

### Fluorescence lifetime measurements

Time-correlated single-photon counting (TCSPC) was used to determine fluorescence lifetimes on an IBH 5000U fluorescence lifetime spectrophotometer (Glasgow, United Kingdom) equipped with a 279 nm (Tyr and Trp) or 560 nm (Nile Red) NanoLED as excitation sources and a monochromator at the emission side. Linear and non-linear least-squares fittings were performed using DAS6 software (IBH, Glasgow, United Kingdom) to analyze the data. The average lifetimes were calculated using the equation given in [24]:

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

where  $\bar{\tau}$  is the calculated fluorescence decay time and  $\alpha$  the pre-exponential factor.



## UV-Vis measurements

UV-Vis absorbance was measured at 25 °C on a Cintra 40 UV-Vis spectrophotometer (GBC, Melbourne) equipped with a thermostatted cuvette holder.

## Brightfield and fluorescence microscopy

Aliquots of the samples were placed on Kova Glasstic slides (Hycor, Garden Grove, USA) and observed by microscopy using an Axiovert 200 microscope (Zeiss, Göttingen, Germany) equipped with a Tungsten lamp and a Mercury discharge lamp. Observations were done using 10x, 20x, and 40x A-Plan LD objectives (Zeiss, Göttingen, Germany). The photos were taken using a cooled Retiga 1300C colour CCD camera (QImaging, Burnaby, Canada) and processed with Openlab version 3.1.7 software (Improvision, Coventry, UK).

For Nile Red microscopy, 1.5 µl of Nile Red stock solution (100 µM in ethanol) were added to 50 µl sample and aliquots were immediately measured. The final Nile Red concentration in the tested solutions was 3 µM. For Nile Red fluorescence, a Zeiss filter cube n° 15 was used (EX BP 546/12, BS FT 580, EM LP 590) additionally to the already described materials.

### *2.11 Evaluation of Trp-PEGs' hemocompatibility cytotoxicity*

The cell proliferation kit II (XTT assay) from Roche Diagnostics (Basel, Switzerland) was used to determine potential cytotoxicity of the two polymers on HaCaT cells (immortalized human keratinocyte cell line). Concentrations between 0.1 µg/ml and 20 mg/ml were tested. Defined numbers of  $8 \times 10^3$  washed viable cells per well were incubated with serial dilutions of the polymers in 96-well plates (Nuncloen TM  $\Delta$  Surface by Nunc, Roskilde, Denmark) for a total of 24 and 48 hours. Absorption at

490 nm was measured after 1, 3, 6, and 24 h by using a BioTek PowerWave XS reader (Witec AG, Littau, Switzerland). As a positive control for cytotoxicity, a 0.02 % solution of SDS (sodium dodecyl sulfate) was used, and as a negative control cells incubated with medium only. Calculated cell survival was normalized against the negative control. All measurements were performed in quadruplicate.

### *2.12 Evaluation of Trp-PEGs' hemocompatibility*

The same concentrations of the compounds tested in the cytotoxicity assay were used to assess the potential of hemolysis due to membrane interactions and membrane lysis. After preparation of serial dilutions of polymers in sterile 0.9 % NaCl, full human blood of a fasted volunteer (collected in citrate tubes) was added to the samples at a 1:3 (v/v) ratio. The positive control consisted of a 1% Triton X 100 solution and the negative control of 0.9% sterile NaCl. All the samples were put into an incubator at 37 °C for 1 and 24 hours under shaking. After centrifugation (10 minutes at 770 x g), 40 µl of the supernatant were diluted 4 times with sterile 0.9% NaCl. After pipetting into a 96-well Costar® microplate (Product Number 3635, Corning Life Sciences, Schiphol, The Netherlands), the absorbance of released hemoglobin was measured at 575 nm employing a Tecan Safire microplate reader (Tecan Group Ltd, Männedorf, Switzerland). All measurements were performed in triplicates.

### 3 Results

#### 3.1 Synthesis and characterization of the *p*-nitrophenyl carbonate mPEGs and Trp-mPEGs

The successful activation by *p*-nitrophenyl chloroformate and the successful conjugation of tryptophan to the mPEGs of 2 and 5 kDa molecular weight were analyzed by  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, FTIR, MALDI-TOF and UV (see sections 2.3-2.6 of synthesis of the different *p*-nitrophenyl mPEGs and Trp-mPEGs in Materials and Methods).  $^1\text{H}$ -NMR indicated complete activation of mPEG-OH by *p*-nitrophenyl chloroformate, since the triplet at 4.56 ppm originating from the hydroxyl group of mPEG-OH was no longer detected [25]. After conjugation of L-Trp to *p*-nitrophenylcarbonate-mPEG FTIR spectra revealed a shift of the ketone peak at  $\sim 1769\text{ cm}^{-1}$  to  $\sim 1721\text{ cm}^{-1}$ , indicating the successful conjugation of Trp (see sections 2.3-2.6 of synthesis of the different *p*-nitrophenyl mPEGs and Trp-mPEGs in Materials and Methods). MALDI-TOF indicated an increase in molecular weight as expected for *p*-nitrophenyl carbonate mPEG 2 kDa (pnp-PEG 2 kDa) and Trp-PEG 2 kDa (Table 2). pnp-PEG 5 kDa and Trp-PEG 5 kDa represented a molecular mass below the non-conjugated mPEG-OH. This may result from the purification process during the pnp-mPEG 5 kDa synthesis. The increase in molecular weight observed from pnp-mPEG 5 kDa to Trp-mPEG 5 kDa was again as expected. The degree of conjugation of Trp to PEG was determined by UV spectroscopy as an orthogonal method to  $^1\text{H}$ -NMR. Hence, impurities and excess L-Trp remaining from the synthesis can be excluded. A degree of conjugation of 98 % was determined by UV spectroscopy for both Trp-mPEGs of 2 and 5 kDa molecular weight, while  $^1\text{H}$ -NMR again did no longer show the –OH triplet from mPEG-OH at 4.56 ppm or peaks from *p*-nitrophenol. Measurements of the specific optical rotation showed that a

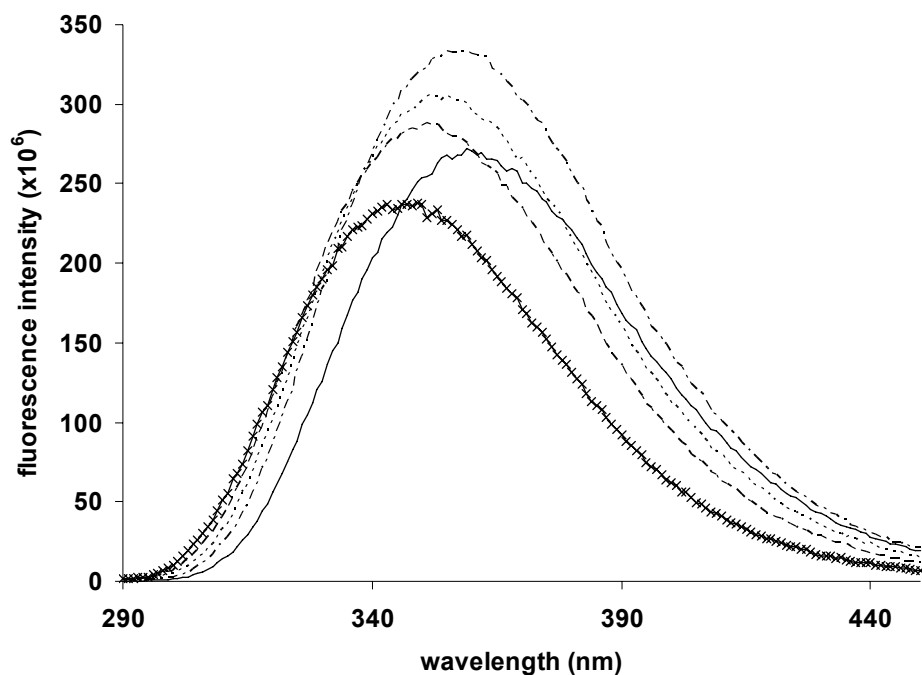
racemization of the chiral C-atom of L-Trp occurred on successful conjugation to mPEG.

compound	observed m/z ( $M^+$ )
mPEG-OH 2 kD	2080
<i>p</i> -nitrophenyl carbonate-mPEG 2 kD	2201
Trp-mPEG 2 kD	2266
mPEG-OH 5 kD	4839
<i>p</i> -nitrophenyl carbonate-mPEG 5 kD	4698
Trp-mPEG 5 kD	4772

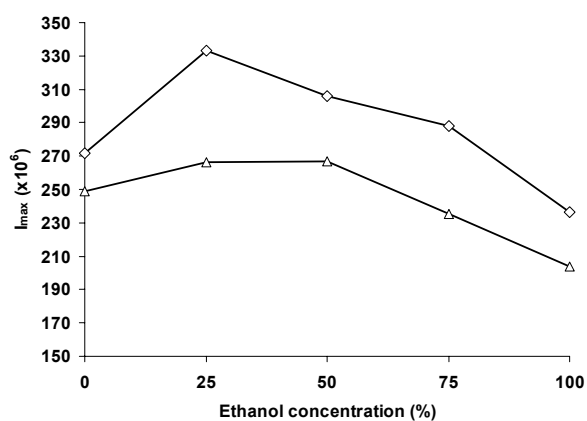
**Table 2.** Masses of the used mPEG-OHs and the resulting Trp-PEGs measured by MALDI-TOF mass spectrometry.

### 3.2 Sensitivity of tryptophan-mPEGs to solvent polarity

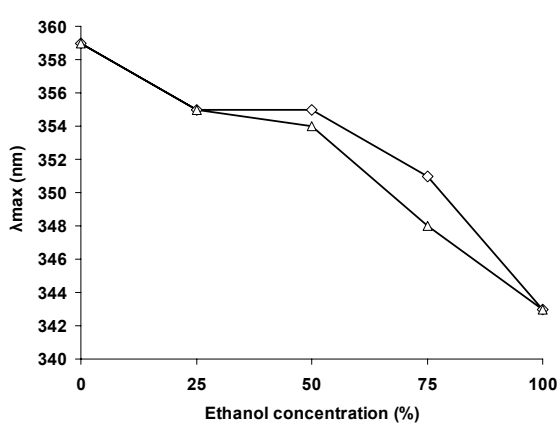
Figures 1A and 1C show a blue-shift of the fluorescence maximum with increasing ethanol concentration for Trp-mPEG 2 kDa (from 357 nm in water to 343 nm in 100 % ethanol). The fluorescence intensity of the 2 kDa Trp-mPEG polymer first increased from water to 25 % ethanol concentration, then constantly decreased from 25% to 100 % ethanol concentration (Figure 1A, 1B). A similar shift in fluorescence maximum and change of fluorescence intensity were observed for the Trp-mPEG 5 kDa (Figure 1B, C).



**Figure 1A.** Fluorescence emission spectra of Trp-mPEG 2 kDa. — fluorescence emission spectrum water; — • — fluorescence emission spectrum 25 % ethanol; – – – fluorescence emission spectrum 50 % ethanol; — — — fluorescence emission spectrum 75 % ethanol; -x- fluorescence emission spectrum 100 % ethanol.



**1B**



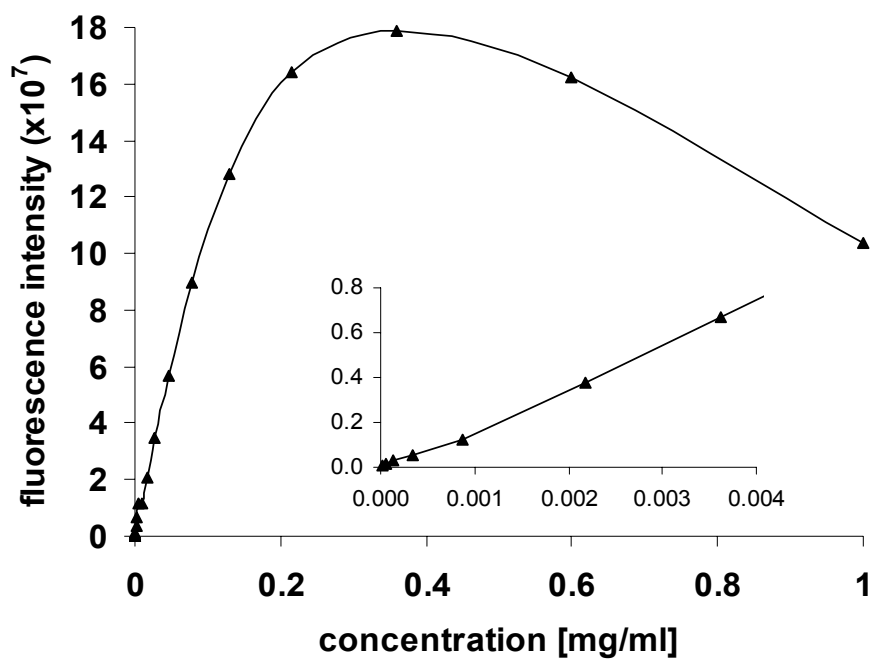
**1C**

**Figure 1B.** Fluorescence intensity at the respective maxima against ethanol concentration (%) of the different Trp-PEGs. —◇— Trp-mPEG 2 kDa; —△— Trp-mPEG 5 kDa. **Figure 1C.** Wavelength of the fluorescence maxima against ethanol

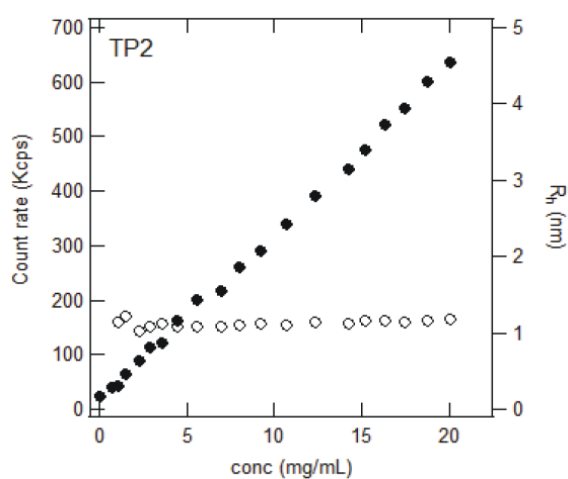
concentration (%) of the different Trp-PEGs. —◇— Trp-mPEG 2 kDa; —△— Trp-mPEG 5 kDa.

### *3.3 Studies of potential of self-association of tryptophan-PEGs*

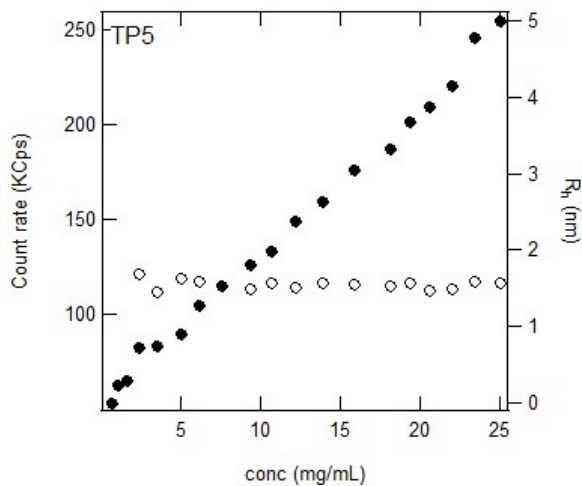
Self-association of the Trp-mPEGs was tested by measuring changes of the intrinsic Trp fluorescence depending on concentration using the Trp sensitivity towards solvent polarity. The dependency of fluorescence intensity against concentration of Trp-mPEG 2 kDa up to 1 mg/ml is shown in Figure 2A. Low concentrations tested are enlarged in the insert where a small kink was measured at 0.0009 mg/ml (Figure 2A). Up to 0.08 mg/ml linearity in the fluorescence versus concentration was observed. Fluorescence quenching of Trp occurred for concentrations between 0.08 mg/ml to 1 mg/ml. Therefore, different concentrations of the two Trp-mPEGs were analysed by dynamic light scattering (DLS) to test for formation of large micelles. For Trp-mPEG 2 kDa a linear increase in the intensity of scattered light as a function of concentration was obtained for concentrations of up to 20 mg/ml (Figure 2B). Calculation of the mean hydrodynamic radii  $R_h$  (according to number distribution) for the different concentrations of Trp-mPEG 2 kDa resulted in values between 1.0 and 1.5 nm. For Trp-mPEG 5 kDa a linear increase in the intensity of scattered light as a function of concentration was observed, as well (Figure 2C). The calculated mean hydrodynamic radii  $R_h$  remained stable for all concentrations at a value of around 1.5 nm. As a control, the critical micelle concentration (CMC) of Tween 20<sup>®</sup> in water was correctly detected by DLS measurements (data not shown).



**Figure 2A.** Fluorescence intensity of fluorescence maximum at 350 nm versus concentration of Trp-mPEG 2 kDa.



**2B**



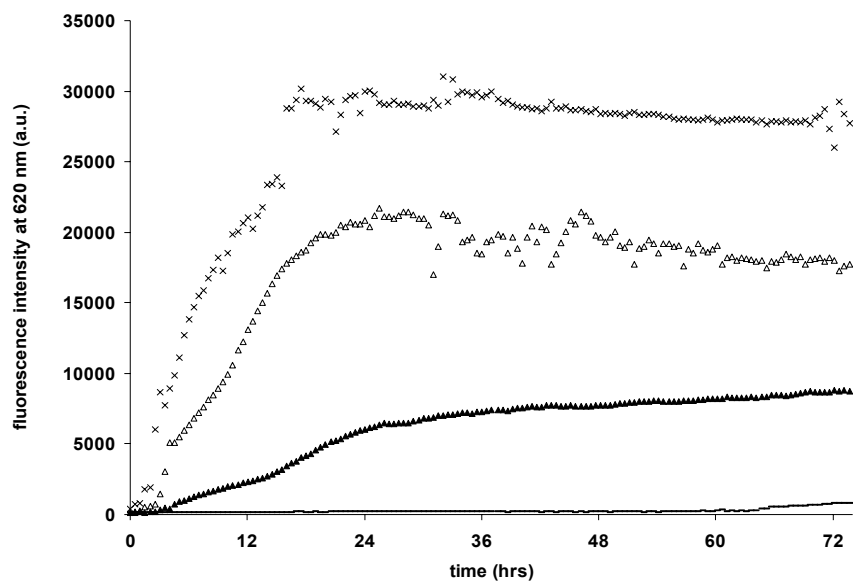
**2C**

Count rate and hydrodynamic radius ( $R_h$ ) at various concentrations of Trp-mPEG 2 kDa (**Figure 2B**) and Trp-mPEG 5 kDa (**Figure 2C**) measured at 173° angle by dynamic light scattering (DLS).

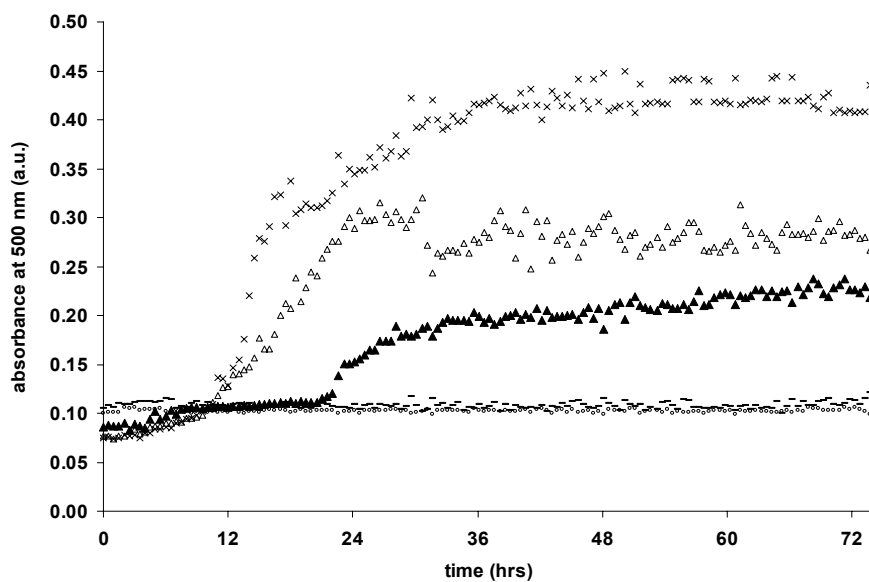
### *3.4 High Throughput Spectroscopic Analysis*

Nile Red fluorescence intensity at 620 nm and turbidity at 500 nm over time were measured for sCT and mixtures of different molar ratios of sCT and Trp-mPEG 2 kDa in 10 mM sodium citrate buffer pH 6. Reduced turbidity and Nile Red fluorescence intensity were observed for the various samples in the following order: i) sCT; ii) sCT:Trp-mPEG 2 kDa = 1:1 and iii) sCT:Trp-mPEG 2 kDa = 1:5; iv) sCT:Trp-mPEG 2 kDa = 1:10. With increasing amounts of Trp-mPEG 2 kDa, the lag phase of sCT aggregation was prolonged (Figures 3A and 3B). Increasing lag phases were measured in the following order: i) sCT; ii) sCT:Trp-mPEG 2 kDa = 1:1 and iii) sCT:Trp-mPEG 2 kDa = 1:5; iv) sCT:Trp-mPEG 2 kDa = 1:10. The Nile Red fluorescence intensity remained low and did not change for up to 64 hours for the sample (Trp-mPEG 2 kDa concentration is 18.59 mg/ml). After 64 hours a small increase in Nile Red fluorescence intensity was observed. Turbidity for this solution did not change during the 74 hours of the experiment although an increased absorbance of  $\sim 0.1$  at 500 nm was observed directly at the beginning of the kinetics. The control solution containing 18.59 mg/ml Trp-mPEG 2 kDa showed a similar absorbance.



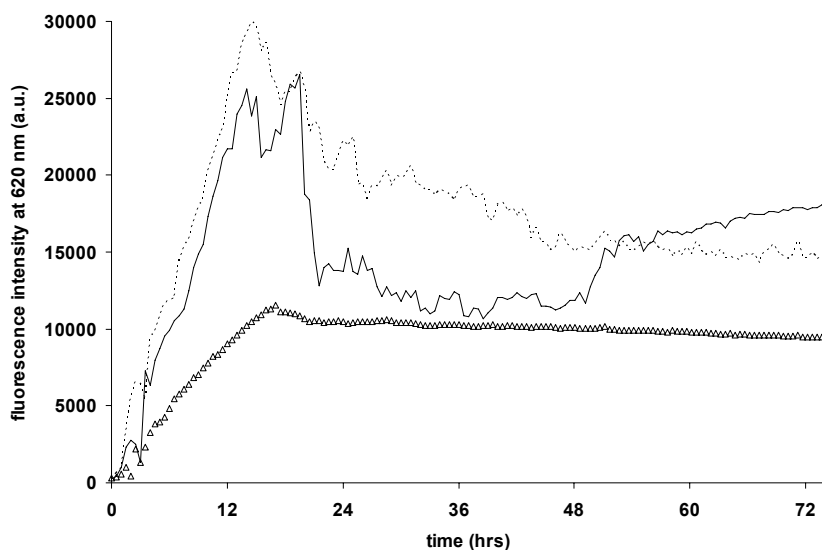


**Figure 3A.** Aggregation of salmon calcitonin with Trp-mPEG 2 kDa in time at different molar ratios. Nile Red fluorescence at 620 nm in 10 mM sodium citrate buffer pH 6. x salmon calcitonin;  $\Delta$  sCT:Trp-mPEG 2 kDa = 1:1;  $\blacktriangle$  sCT:Trp-mPEG 2 kDa = 1:5; - - - sCT:Trp-mPEG 2 kDa = 1:10.

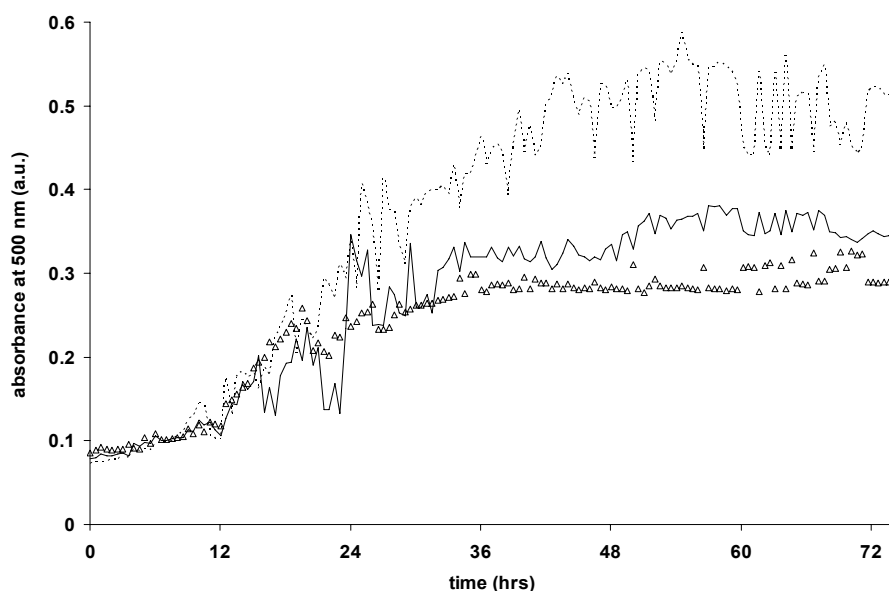


**Figure 3B.** Turbidity at 500 nm in 10 mM sodium citrate buffer pH 6. x salmon calcitonin;  $\Delta$  sCT:Trp-mPEG 2 kDa = 1:1;  $\blacktriangle$  sCT:Trp-mPEG 2 kDa = 1:5; - - - sCT:Trp-mPEG 2 kDa = 1:10;  $\circ$  Trp-mPEG 2 kDa 18.59 mg/ml.

The influence of Trp-mPEG 5 kDa on the aggregation of sCT was studied. The Nile Red fluorescence intensity at 620 nm and turbidity at 500 nm over time of sCT and mixtures of sCT with of Trp-mPEG 5 kDa at various molar ratios in 10 mM sodium citrate buffer pH 6 were measured (Figures 4A and 4 B). Up to ~25 hours turbidity remained similar for all samples tested (Figure 4B). After 25 hours a decrease in turbidity in the following order was observed: i) sCT; ii) sCT:Trp-mPEG 5 kDa = 1:1; and iii) sCT:Trp-mPEG 5 kDa = 1:5. Nile Red fluorescence over time during the 74 hours of experiment was similar for sCT and the mixture of sCT and Trp-mPEG 5 kDa at a 1:1 ratio, although turbidity at 500 nm after 74 hours was reduced for this mixture compared to sCT alone (Figures 4A and B). The slope of the Nile Red fluorescence curve of the mixture of sCT and :Trp-mPEG 5 kDa at a ratio of 1:5 was less pronounced compared to sCT and sCT:Trp-mPEG 5 kDa = 1:1. A plateau was reached for sCT:Trp-mPEG 5 kDa = 1:5 after about 17 hours (Figure 4A). The aggregation after 74 hours for this solution was reduced compared to sCT alone and sCT:Trp-mPEG 5 kDa = 1:1, as observed by Nile Red fluorescence and turbidity measurements (Figures 4A and B).



**Figure 4A.** Aggregation of salmon calcitonin with Trp-mPEG 5 kDa in time at different molar ratios in 10 mM sodium citrate buffer pH 6 represented by Nile Red fluorescence at 620 nm in 10 mM sodium citrate buffer pH 6. - - - salmon calcitonin; — sCT:Trp-mPEG 5 kDa = 1:1;  $\Delta$  sCT:Trp-mPEG 5 kDa = 1:5.



**Figure 4B.** Aggregation of salmon calcitonin with Trp-mPEG 5 kDa in time at different molar ratios in 10 mM sodium citrate buffer pH 6 showed by following the turbidity at 500 nm in 10 mM sodium citrate buffer pH 6. - - - salmon calcitonin; — sCT:Trp-mPEG 5 kDa = 1:1;  $\Delta$  sCT:Trp-mPEG 5 kDa = 1:5.

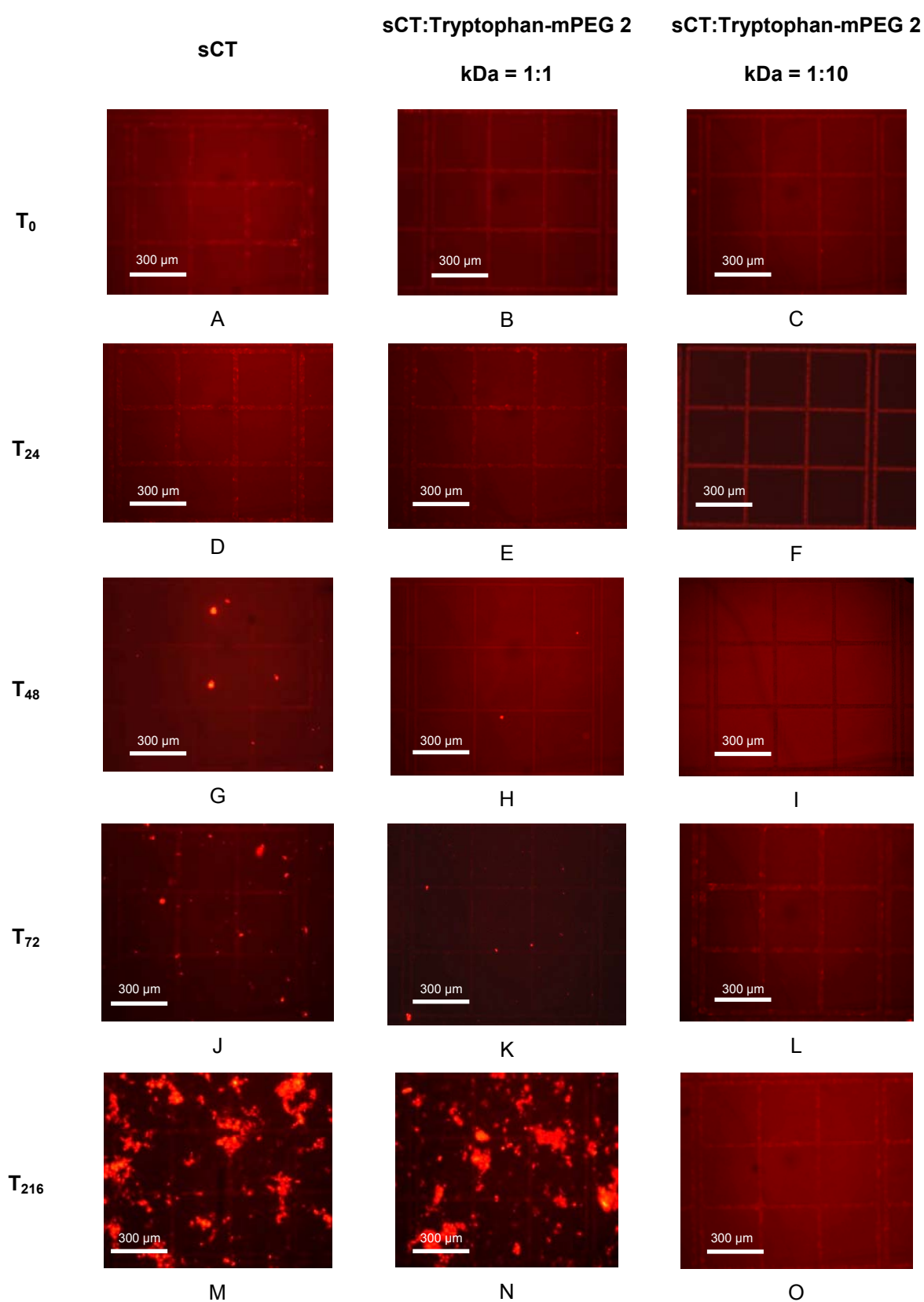
### *3.5 Interaction of Trp-mPEG 2 kDa with salmon calcitonin (sCT) at different molar ratios in 10 mM sodium citrate buffer pH 5*

No significant changes over time were seen in the fluorescence emission intensity of Tyr at 314 nm for the sCT sample in 10 mM sodium citrate buffer pH 5. The tyrosine anisotropy remained constant during the aggregation of the sCT solution in 10 mM sodium citrate buffer pH 5 (data not shown). Over time, a small increase in tyrosine lifetime was observed for this sample (Table 3). The 90° light scatter of sCT at 48 hours showed only a small increase (data not shown), but was significantly increased after 72 hours and increased further up to 216 hours (Table 3). The UV-Vis absorbance of sCT at 500 nm slightly decreased after 24 hours and then steadily increased up to 216 hours (Table 3). Aggregates were observed for sCT after 48 hours by fluorescence microscopy with Nile Red staining (Figure 5G). The aggregation continued to increase for sCT as seen by the Nile Red photomicrographs from 72 and 216 hours (Figures 5J, 5M) and resulted in a strongly aggregated sample after 216 hours (Figure 5M). Significant increase in Nile Red fluorescence emission and a blue shift of its emission maximum were detected for this sample after 72 hours (Table 4).

When Nile Red fluorescence intensity is too low ( $< 2.5 \times 10^6$ ), neither Nile Red anisotropy nor mean fluorescence lifetime can be measured (e.g., for 0 to 48 hours of sCT). With the significant increase in Nile Red fluorescence intensity after 72 hours, anisotropy and mean fluorescence lifetime of Nile Red could be measured. Both parameters increased from 72 hours to 216 hours (Table 4).

sample	Time (hours)	absorbance (a.u.)	LS (cps x 10 <sup>6</sup> )	I <sub>max</sub> (a.u. x10 <sup>6</sup> )	lifetime $\bar{\tau}$ (ns)
sCT	0	0.08	0.05	2.7	1.48
	24	0.03	0.05	3.0	1.50
	72	0.07	0.36	2.9	1.50
	216	0.17	2.80	3.1	1.52
sCT:Trp- mPEG 2 kDa =1:1	0	0.04	0.07	57	7.91
	24	0.07	0.06	55	7.91
	72	0.07	0.18	48	7.94
	216	0.13	1.74	50	7.96
sCT:Trp- mPEG 2 kDa =1:10	0	0.16	0.23	63	7.36
	24	0.06	0.23	65	7.23
	72	0.06	0.28	54	7.27
	216	0.09	0.50	44	7.10

**Table 3.** Aggregation of salmon calcitonin with Trp-mPEG 2 kDa in time at different molar ratios in 10 mM sodium citrate buffer pH 5. UV-Vis absorbance at 500 nm, 90°light scatter at 450 nm (LS), fluorescence maxima (I<sub>max</sub>) measured after excitation at 280 nm, and mean fluorescence lifetimes  $\bar{\tau}$  measured in the emission maximum during the kinetics of sCT, sCT:Trp-mPEG 2 kDa = 1:1, and sCT:Trp-mPEG 2 kDa = 1:10 at time points 0, 24, 72 and 216 hours.



**Figure 5.** Aggregation in time of salmon calcitonin with Trp-mPEG 2 kDa at different molar ratios in 10 mM sodium citrate buffer pH 5 followed by Nile Red microscopy.

Nile Red photomicrographs taken at time points 0, 48, 72 and 216 hours during the aggregation of sCT, sCT:Trp-mPEG 2 kDa = 1:1, and sCT:Trp-mPEG 2 kDa = 1:10. Contrast and brightness of the photomicrographs were varied, to improve visibility of aggregates.

**Figure 5A.** Time point 0: sCT 2.5 mg/ml. 11 s exposure time.

**Figure 5B.** Time point 0: sCT:Trp-mPEG 2 kDa = 1:1. 4 s exposure time.

**Figure 5C.** Time point 0: sCT:Trp-mPEG 2 kDa = 1:10. 5 s exposure time.

**Figure 5D.** Time point 48 hours: sCT 2.5 mg/ml. 6 s exposure time.

**Figure 5E.** Time point 48 hours: sCT:Trp-mPEG 2 kDa = 1:1. 5 s exposure time.

**Figure 5F.** Time point 48 hours: sCT:Trp-mPEG 2 kDa = 1:10. 3 s exposure time.

**Figure 5G.** Time point 72 hours: sCT 2.5 mg/ml. 7 s exposure time.

**Figure 5H.** Time point 72 hours: sCT:Trp-mPEG 2 kDa = 1:1. 5 s exposure time.

**Figure 5L.** Time point 72 hours: sCT:Trp-mPEG 2 kDa = 1:10. 10 s exposure time.

**Figure 5M.** Time point 216 hours: sCT 2.5 mg/ml. 4 s exposure time.

**Figure 5N.** Time point 216 hours: sCT:Trp-mPEG 2 kDa = 1:1. 3 s exposure time.

**Figure 5O.** Time point 216 hours: sCT:Trp-mPEG 2 kDa = 1:10. 8 s exposure time.

The fluorescence intensity of Trp in the sCT:Trp-mPEG 2 kDa = 1:1 sample decreased from 0 to 72 hours and increased at 216 hours. The lifetime of Trp in this mixture was stable for up to 48 hours, then slightly increased up to the 216 hour timepoint (Table 3). The Nile Red fluorescence emission at 630 nm for this solution slowly increased up to 72 hours, but was too low to measure Nile Red anisotropy and mean fluorescence lifetime (Table 4). After 216 hours a strong increase in Nile Red fluorescence emission at 630 nm was detected. The Nile Red anisotropy for sCT:Trp-mPEG 2 kDa = 1:1 at 216 hours was comparable to the value obtained for sCT after

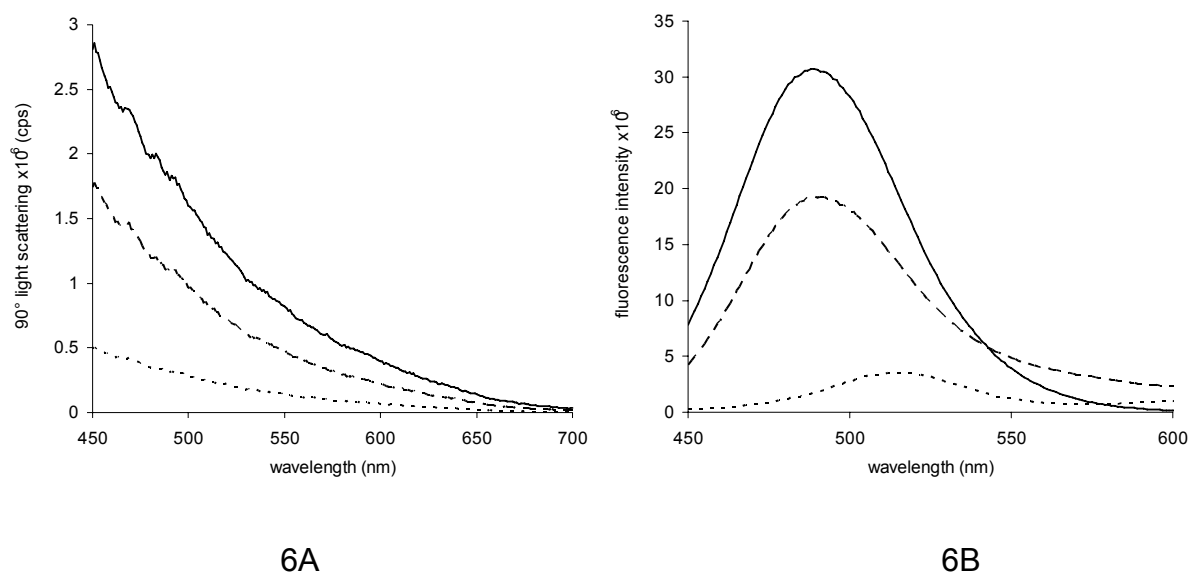
216 hours, while the mean fluorescence lifetime of Nile Red after 216 hours was lower compared to sCT (Table 4). After 216 hours, the Nile Red fluorescence emission intensity at 630 nm, the 90° light scatter, and UV-Vis absorbance at 500 nm for the mixture of sCT:Trp-mPEG 2 kDa = 1:1 were lower than for sCT alone (Figure 6A and Figure 6B, Table 3 and Table 4). Nile Red microscopy showed some aggregates after 48 hours for sCT:Trp-mPEG 2 kDa = 1:1 (Figure 5H). After 72 hours, the amount of aggregates for sCT:Trp-mPEG 2 kDa = 1:1 (Figure 5K) was much smaller compared to sCT alone (Figure 5J). Strong aggregation was observed for sCT:Trp-mPEG 2 kDa = 1:1 after 216 hours by Nile Red microscopy (Figure 5N). After 24 hours a small increase in Trp fluorescence intensity was observed for sCT:Trp-mPEG 2 kDa = 1:10, which then constantly decreased up to 216 hours (Table 3). The 90° light scatter of this sample was stable until 48 hours, then increased after 72 hours and further increased after 216 hours. The 90° light scatter of sCT:Trp-mPEG 2 kDa = 1:10 at 216 hours was significantly lower than the values measured at this time point for sCT alone and sCT:Trp-mPEG 2 kDa = 1:1 (Figure 6A). The UV-Vis absorbance of sCT:Trp-mPEG 2 kDa = 1:10 at 500 nm decreased between 0 and 24 hours, then remained stable until 72 hours, and increased slightly after 216 hours (Table 3). The mean Trp lifetime of the mixture of sCT:Trp-mPEG 2 kDa = 1:10 decreased after 24 hours, then remained 7.25 ns up to 72 hours and decreased again after 216 hours (Table 3). The Nile Red fluorescence for this sample slightly increased after 24 hours, decreased again after 72 hours, and remained stable until 216 hours (Table 4). The Nile Red fluorescence intensity after 216 hours was the smallest for i) sCT:Trp-mPEG 2 kDa = 1:10, followed by ii) sCT:Trp-mPEG 2 kDa = 1:1, and then by iii) sCT (Figure 6B). The Nile Red fluorescence emission up to 216 hours of the sCT:Trp-mPEG 2 kDa = 1:10 mixture was very low, and therefore neither Nile Red anisotropy nor the mean Nile Red lifetime could be measured (Table



4). Nile Red fluorescence microscopy did not detect any aggregates up to 216 hours (Figures 5C, F, I, L, O).

sample	Time (hours)	Nile Red fluorescence intensity at 630 nm (a.u. $\times 10^6$ )	anisotropy of Nile Red (a.u.)	mean lifetime $\bar{\tau}$ of Nile Red (ns)
sCT	0	0.59	n.d.	n.d.
	24	1.01	n.d.	n.d.
	72	2.79	0.31	3.48
	216	30.61	0.34	3.59
sCT:Trp-mPEG 2 kDa =1:1	0	1.08	n.d.	n.d.
	24	1.13	n.d.	n.d.
	72	1.78	n.d.	n.d.
	216	19.22	0.35	3.51
sCT:Trp-mPEG 2 kDa =1:10	0	1.42	n.d.	n.d.
	24	2.35	n.d.	n.d.
	72	1.83	n.d.	n.d.
	216	1.85	n.d.	n.d.

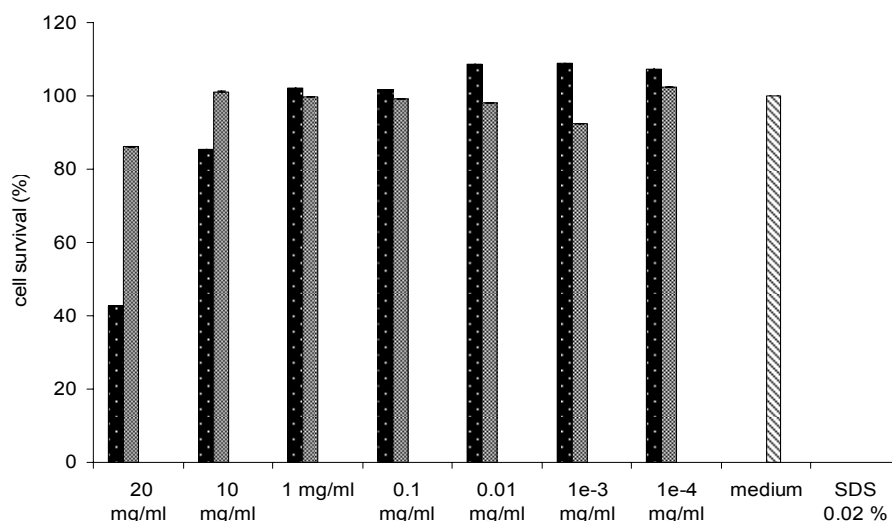
**Table 4.** Aggregation of salmon calcitonin with Trp-mPEG 2 kDa in time at different molar ratios in 10 mM sodium citrate buffer pH 5. Mean fluorescence lifetimes, anisotropy and fluorescence intensity at 630 nm of Nile Red measured for sCT, sCT:Trp-mPEG 2 kDa = 1:1, and sCT:Trp-mPEG 2 kDa = 1:10 at time points 0, 24, 72 and 216 hours. N.d. refers to not detectable.



**Figure 6.** Aggregation of salmon calcitonin with Trp-mPEG 2 kDa in time at different molar ratios in 10 mM sodium citrate buffer pH 5. **6A** represents the 90° light scatter after 216 hours in 10 mM sodium citrate buffer pH 5. — salmon calcitonin; – – – sCT:Trp-mPEG 2 kDa = 1:1; - - - sCT:Trp-mPEG 2 kDa = 1:10. **6B** shows the Nile Red fluorescence emission after 216 hours in 10 mM sodium citrate buffer pH 5. — salmon calcitonin; – – – sCT:Trp-mPEG 2 kDa = 1:1; - - - sCT:Trp-mPEG 2 kDa = 1:10.

### 3.6 Evaluation of Trp-mPEGs' cytotoxicity

A concentration of 20 mg/ml Trp-mPEG 2 kDa was cytotoxic after 48 hours of incubation with HaCaT cells (Fig 7). All other concentrations of Trp-mPEG 2 kDa down to 0.0001 mg/ml and all concentrations of Trp-mPEG 5 kDa (20 mg/ml included) were non-toxic after 48 hours. The same results were observed after 24 hours (data not shown).



**Figure 7.** Cytotoxicity of Trp-mPEGs tested on HaCaT cells after 48 hours of incubation. Serial dilutions of ■ Trp-mPEG 2 kDa, ▨ Trp-mPEG 5 kDa with positive (▤ cells with 0.02% SDS) and negative control (■ cells with medium only).

### 3.7 Evaluation of Trp-mPEGs' hemocompatibility

All solutions of Trp-mPEG 2 kDa and 5 kDa were hemocompatible. None of the tested solutions did lyse erythrocytes after a maximum of 24 hours of incubation with full human blood (Table 5).

	20 mg/ml	10 mg/ml	1 mg/ml	0.1 mg/ml	0.01 mg/ml	0.001 mg/ml	0.0001 mg/ml	NaCl 0.9 %	Triton X 1%
Trp-mPEG 2 kDa	100.12	100.47	100.35	100.81	100.27	100.61	100.72	100.00	0.00
Trp-mPEG 5 kDa	100.26	100.76	100.28	100.68	100.18	100.27	100.54		

**Table 5.** Hemocompatibility of Trp-mPEGs. Different concentrations of Trp-mPEG 2 and 5 kDa were incubated for 24 hours with full human blood. 0.9 % NaCl and 1 % Triton X 100 were taken as negative and positive control, respectively.

## 4 Discussion

Covalent PEGylation has been employed as a successful technique in order to extend plasma half-lives *in vivo*, to reduce *in vivo* immunogenicity and to decrease the aggregation of biopharmaceuticals [12, 14, 15, 17]. Since the early 1990's several PEGylated biopharmaceuticals have been approved that hold important therapeutic value in the treatment of severe diseases like cancer (Oncaspar<sup>®</sup>), hepatitis C (PEGIntron<sup>®</sup>) or severe combined immunodeficiency disease (Adagen<sup>®</sup>) [18]. The aim of this project was to prevent chemical processing of the biopharmaceutical, which is needed to covalently attach the PEG, by developing a novel PEGylation approach based on non-covalent hydrophobic interaction. The key benefits of PEGylation on aggregation shall be maintained. In an earlier project, we have observed that dansyl-PEGs are able to reduce the aggregation of sCT in accelerated aggregation studies most probably due to non-covalent interactions (paper submitted to J Pharm Sci).

In this publication, we investigated the potential of Trp-mPEGs of 2 and 5 kDa to reduce the aggregation of sCT. Tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are aromatic amino acids that possess an intrinsic fluorescence. Out of these three, Trp has the strongest molar extinction coefficient and its fluorescence spectrum depends on its local environment. Therefore, it is often used as an intrinsic fluorescence probe to study protein conformation and intermolecular interactions [26, 27]. Besides its hydrophobic nature, the fluorescence characteristics of Trp were further important reasons to evaluate Trp as a hydrophobic headgroup for our concept of non-covalent PEGylation. Furthermore, Trp is used as a nutritional supplement and its maximum recommended therapeutic dose in humans is 100 mg/kg bodyweight/ day [20].

The sensitivity of Trp-mPEGs to changes in the polarity of the environment was analyzed. As expected, with decreasing solvent polarity a blue-shift of the emission maximum occurred for both Trp-mPEGs (**Fig. 1A, 1C**) [24]. Changing from water to 25 % ethanol solution caused an increase in fluorescence intensity which may be explained by a reduced water quenching (**Fig. 1A, 1B**) [28]. Increasing the ethanol concentration from 25 to 100 %, a constant decrease in fluorescence intensity was observed. Since the photophysics of tryptophan and its underlying indole structure are complex, the exact reason for the observed decrease in fluorescence intensity is unknown. Several possible quenching mechanisms might apply, such as quenching by the carbamate bond between Trp and PEG, the free negatively charged carboxylate or resonance energy transfer among the Trp residues [24, 27-29].

Potential self association of the Trp-mPEGs at low concentration was analyzed by using the sensitivity of Trp to changes in environmental polarity (**Fig. 2A**). An increase in fluorescence intensity accompanied by a possible blue shift of the emission maximum might be observed when Trp is included into a more hydrophobic environment like a micellar core. Above 0.0009 mg/ml of Trp-mPEG 2 kDa a steeper slope is observed in the curve showing the dependency of the fluorescence intensity on increasing concentration (**Fig. 2A**), which might indicate association of Trp-mPEG molecules. Quenching occurred for solutions with concentrations higher than 0.08 mg/ml (**Fig. 2A**). To test for association of larger molecular complexes, like micelles, solutions above 0.08 mg/ml were analyzed by dynamic light scattering (DLS). Formation of aggregates or micelles was not detected for any of the Trp-mPEGs by DLS as can be seen by the linear increase of the intensity of scattered light/count rates with concentration (**Fig. 2B and 2C**). The hydrodynamic radii measured were ranging between 1 and 2 nm, similar to reported values in literature [30], further indicating the absence of large complexes or micelles.

Hydrophobic fluorophores like Nile Red possess a weak fluorescence in aqueous solutions. Being dissolved in apolar solvents or bound to hydrophobic surfaces, the fluorescence of Nile Red is strongly increased. Therefore, the fluorophore is used to follow the aggregation of proteins [22, 23, 31, 32] and for the detection of micelles [33]. **Fig. 3A** and **4A** showed only a weak fluorescence of Nile Red at the beginning of the kinetics even in the presence of high concentrations of Trp-mPEGs. Since no significant Nile Red fluorescence at various Trp-mPEG concentrations was detected, rather an absence of large micelles or aggregates of Trp-mPEGs is indicated. Therefore, the observed changes in fluorescence intensity of Trp-mPEG 2 kDa at 0.0009 mg/ml might be due to the association of two or three Trp-mPEG molecules, forming complexes that are too small to include a Nile Red molecule.

Capelle et al. have shown that sCT strongly aggregates at room temperature in 10 mM sodium citrate buffer pH 6 [22]. Aggregation of sCT at different molar ratios of Trp-mPEG 2 kDa was reduced with increasing concentrations of Trp-mPEG 2 kDa as shown by changes of Nile Red fluorescence and turbidity in time (**Fig. 3A** and **3B**). With increasing Trp-mPEG 2 kDa concentrations the slopes of the Nile Red fluorescence intensity over time got less, indicating a decreased aggregation velocity (**Fig. 3A**). Turbidity over time showed a prolongation in the onset of aggregation of sCT with increasing Trp-mPEG 2 kDa concentration (**Fig. 3B**). In the solution of sCT:Trp-mPEG 2 kDa = 1:10 a stable and low Nile Red fluorescence suggested the absence of aggregation up to 64 hours, while the turbidity remained stable during all of the 74 hours of the kinetics. The increased turbidity (~0.1) of this mixture at time point 0 of the kinetics can be assigned to the high concentration of Trp-mPEG 2 kDa. The reduction of sCT aggregation was also concentration dependent for Trp-mPEG 5 kDa, though to a lesser extent than for Trp-mPEG 2 kDa (**Fig. 4A** and **4B**). **Fig. 4B** indicates a reduction in aggregation velocity and in the final degree of aggregation

with increasing concentration of Trp-mPEG 5 kDa. Changes of Nile Red fluorescence over time did not reveal differences between the aggregation of sCT and sCT:Trp-mPEG 5 kDa = 1:1 as clear as turbidity (**Fig. 4A** and **4B**). For the mixture of sCT:Trp-mPEG 5 kDa = 1:5 the highest value of Nile Red fluorescence was already reached after 17 hours, as compared to 25 hours observed by turbidity measurements. However, a reduction in aggregation velocity and in the final degree of aggregation were observed by both methods for sCT:Trp-mPEG 5 kDa = 1:5. For both Trp-mPEG polymers aggregation of sCT was decreased in a harsh environment, where sCT rapidly aggregates. This stabilization was also visible by eye: the wells containing sCT:Trp-mPEG 2 kDa = 1:10 were clear while those containing sCT solution were turbid. In our studies, Trp-mPEG 2 kDa was superior to dansyl-mPEG 2 kDa in stabilizing sCT against aggregation. Though aggregation of sCT was reduced with dansyl-mPEG 2 kDa, no suppression of aggregation was obtained as was the case for Trp-mPEG 2 kDa in a 10-fold molar excess over sCT. The headgroup used appears to be important for inhibiting sCT aggregation.

The molecular weight of the used Trp-mPEG polymers also seems to have an effect on the observed reduction of sCT aggregation, as was also the case for the dansyl-PEGs. A significant reduction in aggregation of sCT was obtained with Trp-mPEG 2 kDa at a 1:1 molar ratio. A reduction of aggregation of sCT to a lesser extent was obtained with sCT:Trp-mPEG 5 kDa = 1:1. These results are comparable to observations made with the dansyl-PEGs and are contradictory to present covalent PEGylation, where preferably few and high molecular weight PEGs (e.g., 20 or 40 kDa) are employed [34]. Three possible explanations might apply: i) the interaction of the Trp headgroup with sCT is reduced due to sterical hindrance caused by the PEG moiety, or ii) with increasing molecular weight of the PEG the hydrophilic-lipophilic balance of Trp-mPEG is biased towards an increased influence of the PEG moiety on

aggregation, which is detrimental for the interaction between the Trp headgroup and sCT [35], or iii) with increasing molecular weight of the PEG the hydrophobic interactions between sCT and the dansyl-headgroup that allow sCT to remain in solution are decreased and, furthermore, increasing amounts of the solvation water from the outer shell of sCT are removed. As a result sCT precipitates.

In 10 mM sodium citrate buffer pH 5 sCT was reported to aggregate strongly [22]. Therefore, we investigated in this buffer system more profoundly the aggregation at different molar ratios of sCT:Trp-mPEG 2 kDa. Measurement of various parameters over time, all showed consistently increasing aggregation of sCT (**Table 3, Table 4, Fig. 5A, 5D, 5G, 5J, 5M**). No changes were measured in the Tyr fluorescence emission and anisotropy, which suggested that aggregation did not directly involve Tyr. However, the increasing Tyr lifetime might indicate increasing conformational restrictions in the Tyr environment due to the aggregation.

Aggregation of sCT in the mixture of sCT:Trp-mPEG 2 KDa = 1:1 was already observed after 48 hours, comparable to sCT without the excipient. However, 90° light scatter, UV-Vis absorbance, Nile Red fluorescence microscopy, Nile Red fluorescence emission, and mean lifetime of Nile Red indicated sCT was less aggregated in presence of an equimolar amount of Trp-mPEG 2 kDa compared to sCT alone at time points 48, 72 and 216 hours (**Table 3, Table 4, Fig. 6A and B, Fig. 5B, 5E, 5H, 5K, 5N**). Therefore, it can be concluded that Trp-mPEG 2kDa at an equimolar amount stabilized sCT against aggregation.

The inhibition of sCT aggregation by Trp-mPEG 2 kDa is more pronounced at a molar ratio of sCT:Trp-mPEG 2 KDa 1:10. The 90° light scatter and Nile Red fluorescence emission were significantly reduced even after 216 hours compared to sCT and sCT:Trp-mPEG 2 KDa at a 1:1 ratio (**Fig. 6A, 6B**). Nile Red fluorescence microscopy did not detect any aggregates up to 216 hours (**Fig. 5C, 5F, 5I, 5L, 5O**).



UV-Vis absorbance at 500 nm is in agreement with the fluorescence and microscopy results (**Table 3**). For both solutions, sCT:Trp-mPEG 2 kDa = 1:1 and sCT:Trp-mPEG 2 kDa = 1:10, a decrease in Trp fluorescence intensity was observed over time (**Table 3**). The sensitivity of the Trp emission to solvent polarity is affected by hydrogen bonding to the imino group and the surrounding electrical field of the solvent and solutes [24, 36]. One explanation for the decrease in Trp fluorescence intensity of Trp-mPEG over time might be due to possible changes in hydrogen bonding or changes in the surrounding electrical field of the Trp-headgroup with progressing aggregation. However, increasing concentration of the Trp-mPEG 2 kDa might also be an influencing factor leading to increased resonance energy transfer. Since the Trp-mPEGs are composed of a hydrophilic and a hydrophobic moiety, showing a detergent-like structure, destabilization of cellular membranes due to solubilization appears possible. To investigate the potential application of the Trp-PEGs as excipients for protein formulations, their cytotoxic and hemolytic activities were examined. Since proteins and peptides are often injected subcutaneously, immortalized keratinocytes were used to assess cytotoxicity. After 48 hours of incubation of various concentrations of the two Trp-mPEGs, only the solution of 20 mg/ml Trp-mPEG 2 kDa was cytotoxic (**Fig. 7**). The same concentrations of both Trp-mPEGs demonstrated no hemolytic properties in our assay (**Table 5**). Aggregation of biopharmaceuticals is dependent on concentration and elevated concentrations are known to increase aggregation [4, 5]. A concentration of 2.5 mg/ml of sCT was used as a model for studying the inhibitory effect on the aggregation within a reasonable time span. In marketed formulations, the highest concentration of sCT is 0.37 mg/ml. Thus, toxic concentrations of 20 mg/ml Trp-mPEG 2 kDa would not be clinically applied, if a 1:10 ratio of sCT: Trp-mPEG 2 kDa was maintained.

## 5 Conclusions

In this paper, monofunctional Trp-PEGs of 2 and 5 kDa were synthesized and characterized by various techniques. These novel excipients reduced and suppressed the aggregation of sCT in different buffers dissolved in which sCT without excipients is unstable and prone to aggregation. Stabilization of sCT by Trp-mPEG 2 kDa was concentration dependent: with increasing Trp-mPEG 2 kDa concentration, sCT aggregation decreased. Trp-mPEG 5 kDa also stabilized sCT against aggregation in a concentration dependent manner in 10 mM sodium citrate buffer pH 6, although to a minor extent than the 2 kDa Trp-mPEG. Trp-PEGs were non-hemolytic at all concentrations tested, while cytotoxicity was observed for Trp-mPEG 2 kDa at 20 mg/ml concentration.

Ongoing studies may reveal whether further properties of covalent PEGylation, such as reduced *in vivo* immunogenicity or improved pharmacokinetics, are maintained by our approach. In this paper we presented novel excipients that can be easily prepared at low costs. Trp-mPEGs might be used for stabilizing aggregation prone and difficult to handle proteins as a promising alternative to current formulation strategies. With this approach proteins might be formulated and marketed at higher concentrations resulting in a more patient friendly and economic drug product.

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# Chapter 5

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## **Non-covalent PEGylation: various PEG-based excipients with different effects on aggregation inhibition of biopharmaceuticals.**

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## **Abstract**

Protein aggregation is a major instability that can occur during all stages of protein drug production and development. Protein aggregates compromise the safety and efficacy of the final protein formulation. In this paper, several new excipients (phenylbutylamino-, benzyl-, and cholesteryl-PEGs) and their use for non-covalent PEGylation of salmon calcitonin (sCT) and hen egg white lysozyme (HEWL) are presented. The ability to suppress aggregation of sCT in various buffer systems at a 1:1 molar ratio was assessed by following changes in protein conformation and aggregation state over time. The results are compared to the stabilizing effects of dansyl- and tryptophan-PEGs described in earlier publications. Furthermore, the influence of various PEG-based excipients on the aggregation of HEWL was measured: aggregation was completely suppressed in the presence of cholesteryl-PEGs 2 and 5 kDa, while aggravation was observed using benzyl-PEGs 2 and 5 kDa. Phenylbutylamine- and tryptophan-mPEG 2 kDa, as well as dansyl-PEGs of different molecular weights prolonged the lag phase of aggregation, and reduced the aggregation velocity of HEWL. In this publication, the varying influence on aggregation of sCT and HEWL by PEG-based derivatives is presented.

## **1 Introduction**

Biopharmaceuticals exhibit in general a superior target specificity and efficacy compared to small molecular drugs [1]. Therefore, they became very potent tools in the treatment of severe diseases, such as cancer, diabetes or hemophilia. However, rapid market authorization is often hindered due to various chemical and physical degradation processes, among which aggregation is a significant concern [2]. Protein aggregates compromise the safety and efficacy of the final formulation since they may possess a decreased or complete loss of *in vivo* bioactivity, an increased



immunogenicity and increased cellular toxicity [2, 3]. During all stages of protein drug manufacturing and formulation, biopharmaceuticals are subject to mechanical stresses (e.g., shearing or shaking), temperature changes, changing buffer compositions or different packaging materials that all may trigger aggregation [2-4]. Since the causes for aggregation are manifold and vary from drug to drug, reduction and/or suppression of aggregation in a formulation is mostly obtained experimentally [2].

Many proteins aggregate by formation of partially unfolded intermediates that possess an increased amount of exposed hydrophobic patches. Although the number of unfolded intermediates is usually relatively small, they trigger aggregation due to these conformational changes. However, „sticky“ patches on the surface of a native protein may also lead to attracting hydrophobic or electrostatic interactions resulting in aggregation of native monomers [5, 6].

PEGylation, the covalent conjugation of poly(ethylene glycol), has been reported to reduce aggregation of biopharmaceuticals, which is most probably due to sterical shielding of hydrophobic patches on the protein's surface [7-9]. Other positive effects that may be observed after PEGylation are an increased *in vivo* plasma half-life and/or reduced *in vivo* immunogenicity of the biopharmaceutical. These diverse conveniences of PEGylation led to various successful market authorizations of PEGylated therapeutics since the early 1990's [10-13]. However, mainly two major challenges for covalent PEGylation remain: i) the attachment of the PEG needs chemical processing and following purification steps, and ii) decreased *in vivo* bioactivity is observed after covalent PEGylation. Although a large range of conjugation techniques have been developed, none prevents chemical processing and subsequent purification, which may represent additional stresses for the

biopharmaceutical. This in turn may result in a partial or complete loss of *in vivo* bioactivity.

In a successful patent application and two recent publications (accepted by J Pharm Sci and submitted to EJPB), we presented the new method of non-covalent PEGylation based on hydrophobic interactions [14]. A hydrophobic headgroup attached to PEG shall interact non-covalently with hydrophobic patches on the surface of biopharmaceuticals thus leading to a sterical shielding of the latter. Consequently, the aggregation of biopharmaceuticals in liquid formulations should be decreased due to reduced protein/protein interactions.

We have shown that dansyl-mPEG 2 kDa and tryptophan-mPEG 2 kDa effectively reduced the aggregation of salmon calcitonin (sCT) in liquid formulations, while dansyl-mPEG 5 kDa and tryptophan-mPEG 5 kDa were less efficient. In this publication, we present the influence of PEG-derivatives of varying molecular weight possessing a benzyl-, phenylbutylamine- or cholesterol-headgroup on the aggregation of sCT in accelerated aggregation studies. Comparison to dansyl-PEGs and tryptophan-mPEGs was conducted, as well. Furthermore, the influence of the dansyl-, tryptophan-, benzyl-, phenylbutylamine- and cholesteryl-PEGs on the aggregation of the ~14 kDa hen egg white lysozyme was evaluated and compared for the different excipients.

## **2 Materials and Methods**

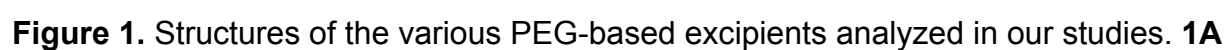
### *2.1 Materials*

The chemicals employed to prepare the buffer solutions, Nile Red (9-diethylamino-5H-benzophenoxazine-5-one), anhydrous toluene, phenylbutylamine, hen egg white lysozyme (HEWL) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The used buffers were

composed of: (i) acetic acid – sodium acetate pH 5, (ii, iii) citric acid – sodium citrate pH 5 and pH 6, respectively (iv) sodium phosphate monobasic – sodium phosphate dibasic pH 8, (v) sodium phosphate dibasic – sodium hydroxide pH 12.2. Anhydrous dichloromethane was supplied by Fluka (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Chloroform was obtained from Chimie-Plus (Chimie Plus Laboratoires, Denicé, France). Concentrated hydrochloric acid and anhydrous Na<sub>2</sub>SO<sub>4</sub> were purchased from Riedel de Haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany). Diethyl ether, dichloromethane, anhydrous triethyl amine, iso-propanol, and *p*-nitrophenyl chloroformate were supplied by Acros (Acros Organics BVBA; Geels, Belgium). The mPEG-OH 2 kDa was provided by Iris Biotech (Iris Biotech GmbH, Marktredwitz, Germany). The cholesteryl-PEGs were obtained from NOF corporation (Sunbright® series, NOF Europe, Grobbendonk, Belgium) and the benzyl-mPEGs were custom synthesized by JenKem Technology (JenKem Technology USA Inc. Allen, Texas, U.S.A.). The cholesteryl-PEGs and benzyl-mPEGs were used as supplied after characterization by various techniques (see section 2.2). All solvents and compounds used were of analytical grade. The salmon calcitonin was obtained from Therapeomic (Therapeomic Inc., Basel, Switzerland). UV transparent 96-well or 384-well Costar® Corning microplates and UV-Vis transparent and pressure sensitive Corning® Universal Optical sealing tape were obtained from Corning (Corning Life Sciences, Schiphol, Netherlands).

## *2.2 Characterization of the PEG derivatives*

The polymers (see Figure 1 for the respective structures) were analyzed by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR on a Varian VXR 300MHz spectrometer (Varian, Switzerland) after dissolution in deuterated DMSO. For FT-IR spectroscopy, pellets of 1% w/w of product in KBr were prepared and examined on a Perkin Elmer 100 FT-IR



The synthesis of *p*-nitrophenyl carbonate mPEG was adapted from [15] and

Na<sub>2</sub>SO<sub>4</sub> and partially evaporated. Precipitation from cold diethyl ether was performed and the solid collected via filtration. The solid was once reprecipitated from cold diethyl ether, and once from cold iso-propanol. A white powder was obtained, dried under vacuum and redissolved in milliQ water. The solution was filtered through a 0.22 µm Millex-GV filter (Millipore, Carrigtwohil, Co. Cork, Ireland) and freeze dried (Freeze dryer Micro Modulyo, Edwards High Vacuum Int., Crawley Sussex, UK).

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 1.40 ppm phenylbutylamine –CH<sub>2</sub>-; 1.54 ppm phenylbutylamine –CH<sub>2</sub>-; 3.24 ppm, PEG –CH<sub>3</sub>-O-; 3.51 ppm, PEG –O-CH<sub>2</sub>-; 7.19 ppm phenylbutylamine –C<sub>6</sub>H<sub>5</sub>. <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 28.02 ppm phenylbutylamine –CH<sub>2</sub>-; 29.07 ppm phenylbutylamine –CH<sub>2</sub>-; 34.66 ppm phenylbutylamine –CH<sub>2</sub>-; 58.10 ppm, PEG CH<sub>3</sub>-O-; 69.50 ppm, PEG –O-CH<sub>2</sub>-; 125.49 ppm phenylbutylamine –C<sub>6</sub>H<sub>5</sub>; 128.40 ppm phenylbutylamine –C<sub>6</sub>H<sub>5</sub>; 141.87 ppm phenylbutylamine –C<sub>6</sub>H<sub>5</sub>; 155.76 ppm phenylbutylamine –C<sub>6</sub>H<sub>5</sub>. FTIR: 2883; 1964; 1719; 1537; 1466; 1359; 1341; 1279; 1240; 1146; 1098; 1059; 959; 841; 749; 700. MS (MALDI-TOF): m/z 2124 (M<sup>+</sup>).

#### *2.4. logP calculations of the headgroups used for the PEG-derivatives*

KOWWIN software V. 1.67 (EPI Suite<sup>TM</sup>, US Environmental Protection Agency) was used to calculate the logP values of the different headgroups employed.

#### *2.5 Studies of potential self-association of the PEG-derivatives*

Aqueous polymer solutions of varying concentrations were prepared and analyzed by Dynamic light scattering (DLS) at an angle of 173° (back-scatter). A Zetaziser Nano ZS (Malvern Instruments) was employed at 25°C, equipped with a 4 mW He–Ne laser operating at 633 nm. The photon count rates were measured as a function of concentration of all samples at constant measurement position and constant

attenuation of the laser, so that the count rates will be comparable for different polymers. The mean hydrodynamic radii ( $R_h$ ) were calculated according to number distribution. The micellization of Tween 20<sup>®</sup> was followed as a control experiment for DLS measurements. The critical micelle concentration (CMC) of the two cholesteryl-PEGs were determined as described in [17].

## *2.6 Aggregation of Salmon Calcitonin (sCT)*

The method has been adapted from [18]. Four different buffers were prepared using sterile filtered 100 mM buffer stock solutions: (i) 10 mM sodium acetate buffer pH 5, (ii, iii) 10 mM sodium citrate buffer pH 5 and 6, and (iv) 10 mM sodium phosphate pH 8. A freshly prepared stock solution of 12.5 mg/ml salmon calcitonin in purified MilliQ<sup>™</sup> Millipore water was used to prepare the samples. The final solutions tested contained 2.5 mg/ml sCT in 10 mM buffer. Each sample was prepared in duplicate. To one of each duplicate Nile Red was added (final concentration of 1  $\mu$ M). Each solution was measured in triplicate. All PEG-derivatives were tested in a 1:1 molar ratio to sCT. All solutions were prepared in 96- or 384-well Costar<sup>®</sup> Corning microplates. After sample preparation the plates were sealed with UV-Vis transparent and pressure sensitive Corning<sup>®</sup> Universal Optical sealing tape.

Every 5 to 10 minutes a cycle of the following was measured at 26°C employing a Tecan (Tecan Group Ltd., Männedorf, Switzerland) Safire<sup>™</sup> microplate reader: i) UV-Vis absorbance at 500 nm, ii) UV-Vis absorbance at 350 nm, and iii) fluorescence emission of Nile Red. Since some excipients absorbed at 350 nm, UV-Vis absorbance was also measured at 500 nm. Nile Red was excited at 550 nm and fluorescence emission was measured at 620 nm over time, while bandwidths were set to 12 nm at excitation and emission side. The kinetics were stopped after 3 days and Nile Red fluorescence emission spectra were measured between 590 and 700

nm using the same settings as during the kinetics. Since the data output of the XFluor<sup>®</sup> software of the Tecan Safire<sup>™</sup> microplate reader is in Microsoft<sup>®</sup> Excel<sup>™</sup> format, visual basic macros were developed by the authors to facilitate high throughput data analysis.

### *2.7 Aggregation of Hen Egg White Lysozyme (HEWL)*

A freshly prepared stock solution of 300.3 mg/ml HEWL in purified MilliQ<sup>™</sup> Millipore water was used. The final solutions tested contained 30.3 mg/ml HEWL in sterile filtered 0.05 M sodium phosphate buffer pH 12.2. HEWL was described to aggregate rapidly under these conditions [19, 20]. Each sample was measured in triplicate. All PEG-derivatives were tested in a 1:1 molar ratio to HEWL, while the phenylbutylamine-mPEG 2 kDa and the benzyl-mPEG 2 and 5 kDa were also tested in a 10-fold molar excess to HEWL. All solutions were prepared in 96- or 384-well Costar<sup>®</sup> Corning microplates, which were sealed as described in section 2.6. Every 5 to 10 minutes, turbidity at 500 nm was measured at 26°C using the above described Tecan Safire<sup>™</sup> microplate reader. After 20 hours the kinetics were stopped and absorbance spectra were measured. Nile Red fluorescence in time was not measured since HEWL samples rapidly become turbid under these conditions, leading to a high sensitivity of UV-Vis absorbance.

## **3 Results**

### *3.1 Characterization of the PEG derivatives*

After reaction of *p*-nitrophenylcarbonate-mPEG 2 kDa (pnp-mPEG 2 kDa) with phenylbutylamine a shift of the ketone peak from 1769 cm<sup>-1</sup> to 1719 cm<sup>-1</sup> was observed by FT-IR, suggesting successful conjugation of phenylbutylamine to PEG. <sup>1</sup>H-NMR did no longer detect the hydroxyl triplet at 4.56 ppm originating from mPEG-

OH 2 kDa [21], indicating complete conjugation of phenylbutylamine to PEG. UV spectroscopy was used as an orthogonal method for determining the degree of conjugation with phenylbutylamine and to exclude impurities remaining from synthesis. Thus, a conjugation degree of 98 % was measured. The mass determined by MALDI-TOF of phenylbutylamine-mPEG 2 kDa was smaller ( $m/z$  2124) than the mass expected ( $m/z$  ~2270). This could be due to the final filtration using a 0.22  $\mu$ m filter, stronger retaining high molecular weight species.

After characterization of the benzyl-mPEGs and cholesteryl-PEGs by the methods described under 2.2 (data not shown), the derivatives were used as supplied without any further purification.

### *3.2 logP calculations of the headgroups used for the PEG-derivatives*

The conjugation to PEG was not taken into consideration for the calculations of the logP values. Therefore, the obtained logPs account for the underlying structure of the respective headgroup, e.g., benzyl alcohol as underlying structure for the benzyl-headgroup or dansylamide as the underlying structure for the dansyl-headgroup. The following logP values were calculated: 1.08 for benzyl alcohol, 2.54 for phenylbutylamine, 8.74 for cholesterol, -1.84 for Tryptophan and 1.72 for dansylamide.

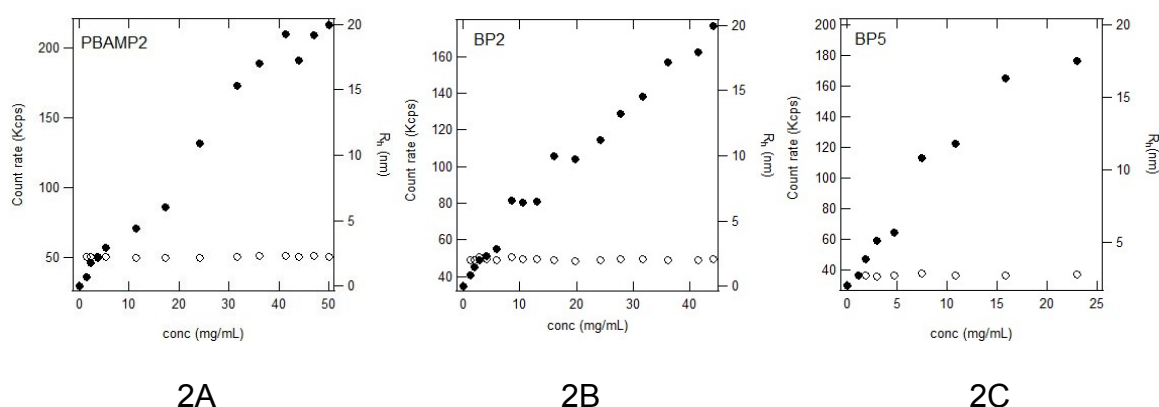
### *3.3 Studies of potential self-association of PEG-derivatives*

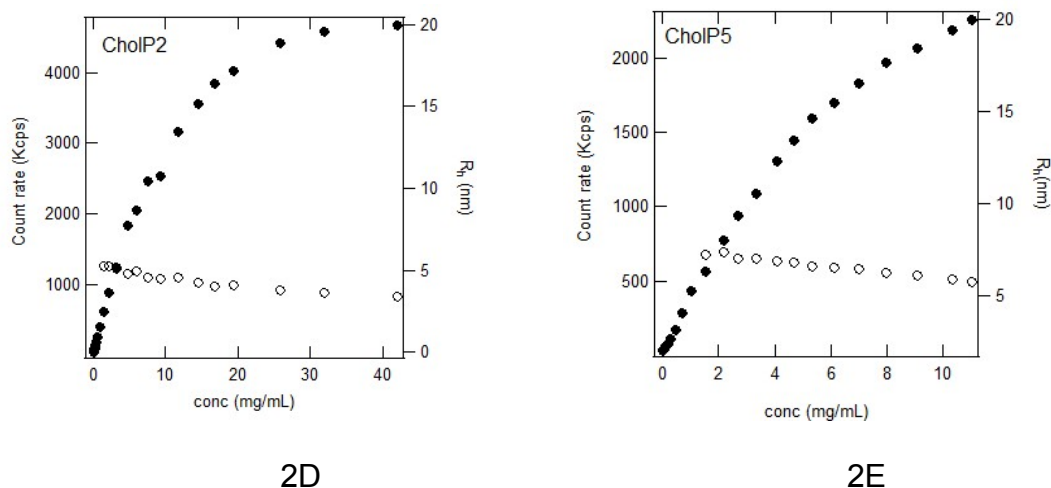
Dynamic light scattering was used to test for formation of large micelles. Phenylbutylamine-mPEG 2 kDa, benzyl-mPEG 2 kDa and benzyl-mPEG 5 kDa showed a linear increase of the count rate vs. concentration (Figures 2A, 2B, and 2C). The calculated mean hydrodynamic radii  $R_h$  of the three derivatives were between 2 and 3 nm (Figures 2A, 2B, and 2C). For the cholesteryl-PEGs the count



rates of scattered light were much higher compared to the other PEG derivatives (Figures 2D and 2E). The calculated  $R_h$  values for cholesteryl-PEGs were in the range of 4-7 nm, which is higher than observed for other polymers. Both cholesteryl-PEG 2 kDa and 5 kDa displayed non-linearity with increasing concentrations above 8-10 mg/mL. This non-linearity is a consequence of multiple scattering at high sample concentrations with such high scattered light intensity.

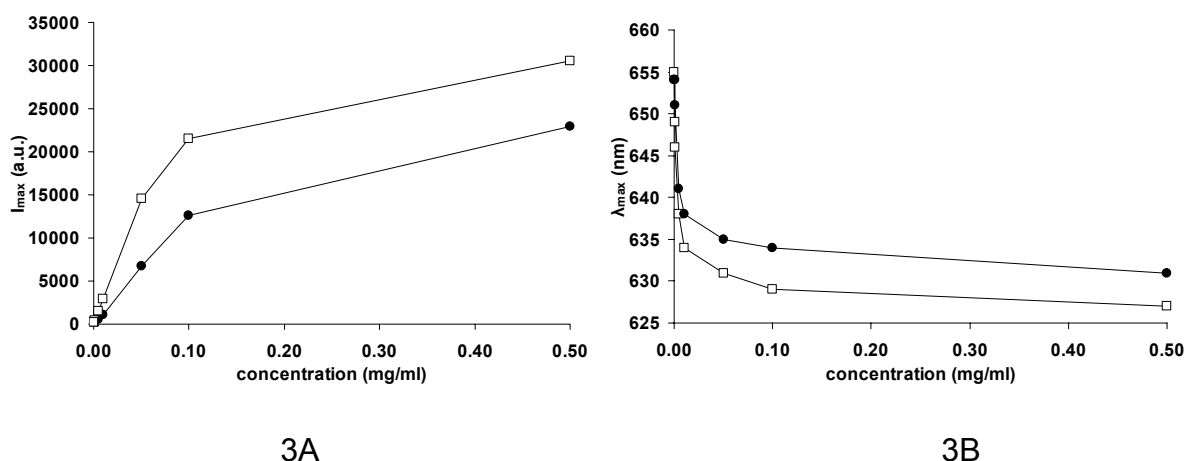
Changes in Nile Red fluorescence depending on concentration were used to determine the critical micelle concentration (CMC) of the two cholesteryl-PEGs. At 0.005 mg/ml of cholesteryl-PEG 2 kDa and 0.01 mg/ml of cholesteryl-PEG 5 kDa the fluorescence intensity of Nile Red increased strongly (Figure 3A). With increasing concentration of the PEG-derivatives, a blue-shift of the emission maximum of Nile Red was observed (Figure 3B). The CMC was obtained from the inflection point of the curve showing the Nile Red emission maximum against concentration. A critical micelle concentration of 0.007 mg/ml was determined for cholesteryl-PEG 2 kDa, while for cholesteryl-PEG 5 kDa a CMC of 0.01 mg/ml was obtained.





**Figure 2.** Dynamic light scattering (DLS) measured at an  $173^\circ$  angle: Count rates of scattered light and hydrodynamic radius ( $R_h$ ) at various concentrations. **2A** phenylbutylamine-mPEG 2 kDa; **2B** benzyl-mPEG 2 kDa; **2C** benzyl-mPEG 5 kDa; **2D** cholesteryl-PEG 2 kDa; **2E** cholesteryl-PEG 5 kDa.

Changes in Nile Red fluorescence depending on concentration were used to determine the critical micelle concentration (CMC) of the two cholesteryl-PEGs. At 0.005 mg/ml of cholesteryl-PEG 2 kDa and 0.01 mg/ml of cholesteryl-PEG 5 kDa the fluorescence intensity of Nile Red increased strongly (Figure 3A). With increasing concentration of the PEG-derivatives, a blue-shift of the emission maximum of Nile Red was observed (Figure 3B). The CMC was obtained from the inflection point of the curve showing the Nile Red emission maximum against concentration. A critical micelle concentration of 0.007 mg/ml was determined for cholesteryl-PEG 2 kDa, while for cholesteryl-PEG 5 kDa a CMC of 0.01 mg/ml was obtained.

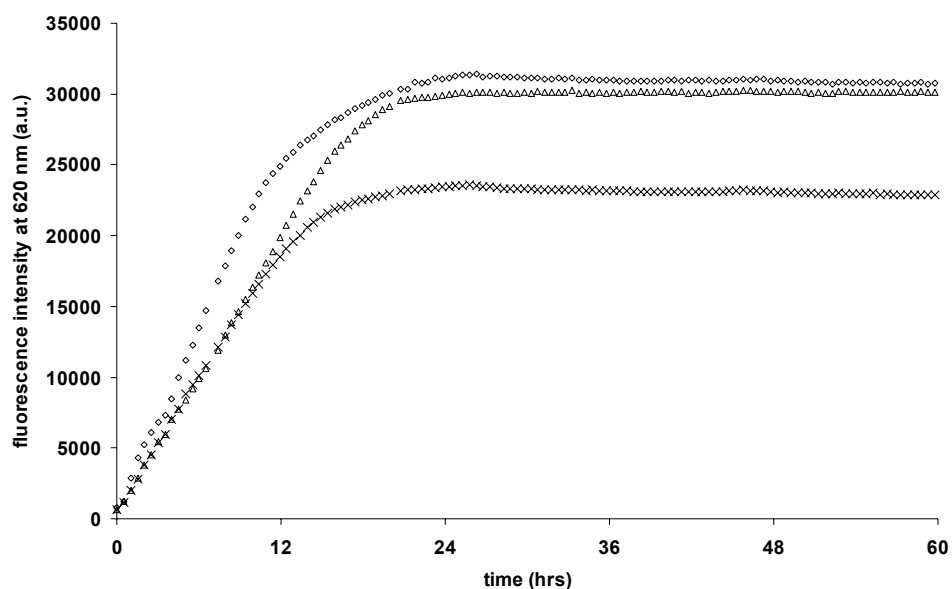


**Figure 3.** Dependency of Nile Red fluorescence emission maximum and Nile Red fluorescence intensity in the emission maximum on cholesteryl-PEG concentration. **Fig. 3A** shows the fluorescence emission intensity  $I_{\max}$  of Nile Red vs. cholesteryl-PEG concentration. -□- Cholesteryl-PEG 2 kDa; -●- Cholesteryl-PEG 5 kDa. **Fig. 3B** represents the fluorescence emission maximum  $\lambda_{\max}$  of Nile Red vs. cholesteryl-PEG concentration. -□- Cholesteryl-PEG 2 kDa; -●- Cholesteryl-PEG 5 kDa.

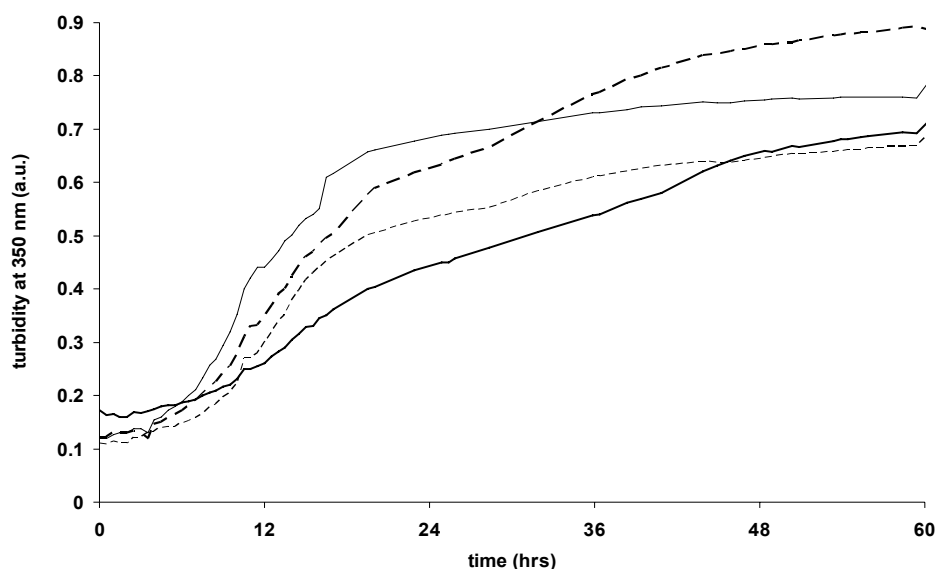
### 3.4 Aggregation of Salmon Calcitonin (sCT)

The aggregation of sCT was analyzed by following the changes in Nile Red fluorescence and turbidity over time. sCT aggregated stronger in presence of equimolar amounts of phenylbutylamine or phenylbutylamine-mPEG 2 kDa compared to sCT (Figure 4A). For phenylbutylamine:sCT = 1:1 the slope of the curve of Nile Red fluorescence intensity over time increased stronger than for sCT and the mixture of phenylbutylamine-mPEG 2 kDa:sCT = 1:1. The lag-time of aggregation was reduced as shown by turbidity over time in the following order i) sCT; ii) phenylbutylamine-mPEG 2 kDa:sCT=1:1; iii) phenylbutylamine:sCT=1:1; and iv) mPEG-OH 2 kDa:sCT=1:1 (Figure 4B). The final degree of aggregation was elevated for i) phenylbutylamine:sCT = 1:1, for ii) mPEG-OH 2 kDa:sCT=1:1 and for iii)

phenylbutylamine-mPEG 2 kDa:sCT = 1:1 compared to sCT only (Figures 4A and 4B).

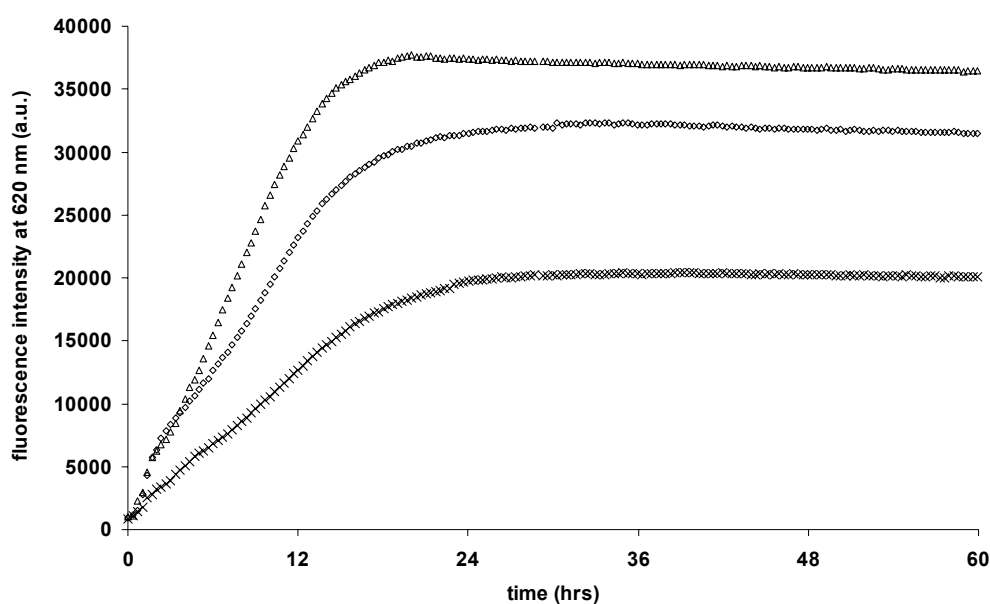


**Figure 4A.** Aggregation of salmon calcitonin with phenylbutylamine-mPEG 2 kDa over time at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6. Nile Red fluorescence at 620 nm: x salmon calcitonin; Δ sCT:phenylbutylamine-mPEG 2 kDa = 1:1; ◇ sCT: phenylbutylamine = 1:1.

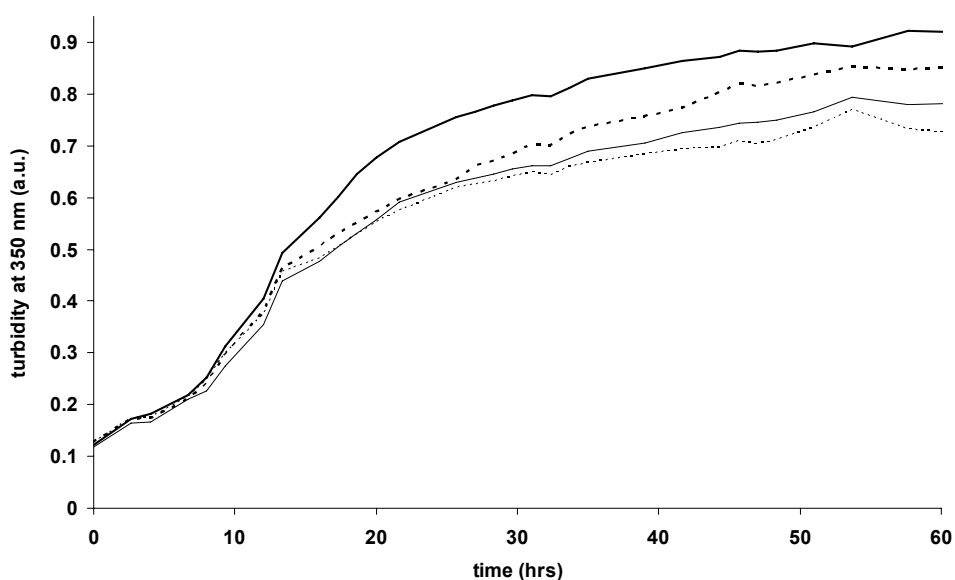


**Figure 4B.** Aggregation of salmon calcitonin with phenylbutylamine-mPEG 2 kDa over time at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6. Turbidity at 350 nm: — salmon calcitonin; - - - sCT:phenylbutylamine-mPEG 2 kDa = 1:1; — sCT:phenylbutylamine = 1:1; - - - sCT: mPEG-OH 2 kDa = 1:1.

In presence of an equimolar ratio of benzyl-mPEG 2 kDa and benzyl alcohol the aggregation of sCT was aggravated (Figures 5A and 5B). The slope of the curve of Nile Red fluorescence intensity over time increased in the following order: i) sCT, ii) sCT: benzyl alcohol = 1:1, and iii) sCT: benzyl-mPEG 2 kDa = 1:1 (Figure 5A). After 60 hours the final degree of aggregates was the strongest for the mixture of sCT: benzyl-mPEG 2 kDa = 1:1. Turbidity over time also showed stronger aggregation for the mixtures of i) sCT: benzyl-mPEG 2 kDa = 1:1, ii) sCT: benzyl alcohol = 1:1, and iii) sCT:mPEG-OH 2 kDa = 1:1 compared to sCT only (Figure 5B). Similar results were obtained for sCT: benzyl-mPEG 5 kDa = 1:1 (data not shown).



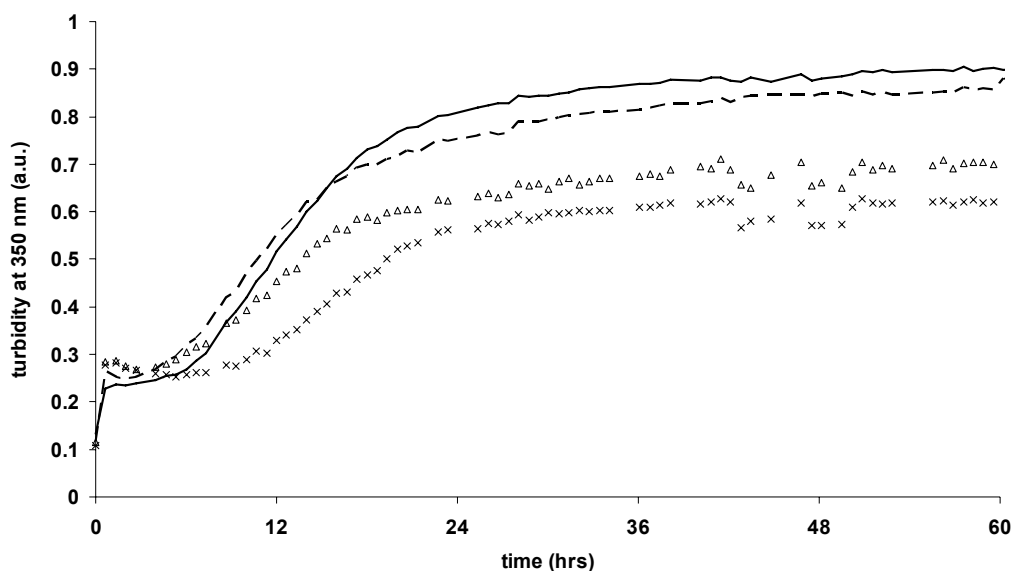
**Figure 5A.** Aggregation of salmon calcitonin with benzyl-mPEG 2 kDa over time at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6. Nile Red fluorescence at 620 nm: x salmon calcitonin; Δ sCT:benzyl-mPEG 2 kDa = 1:1; ◇ sCT:benzyl alcohol= 1:1.



**Figure 5B.** Aggregation of salmon calcitonin with benzyl-mPEG 2 kDa over time at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6. Turbidity at 350 nm: - - - salmon

calcitonin; - - - sCT:benzyl-mPEG 2 kDa = 1:1; — sCT:mPEG-OH 2 kDa = 1:1; — sCT:benzyl alcohol= 1:1.

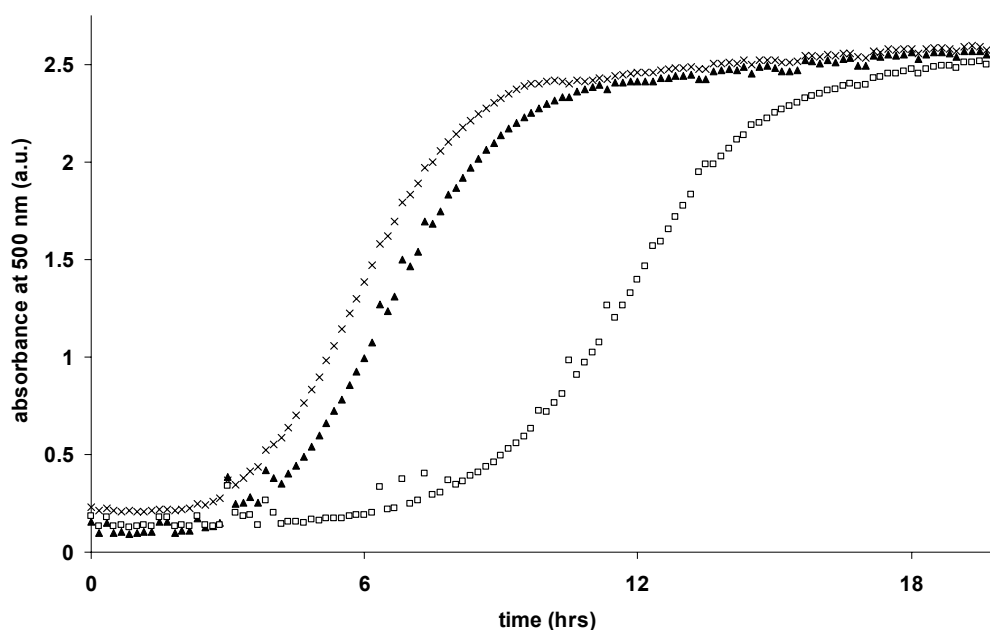
For the mixtures of cholesteryl-PEG 2 kDa:sCT = 1:1, OH-PEG-OH 2 kDa:sCT = 1:1, and cholesteryl-PEG 5 kDa:sCT = 1:1 the final degree of aggregation was higher than for sCT (Figure 6). In presence of the two cholesteryl-PEGs the lag-time of aggregation was reduced, being the shortest for cholesteryl-PEG 5 kDa:sCT = 1:1, followed by OH-PEG-OH 2 kDa:sCT = 1:1, and cholesteryl-PEG 2 kDa:sCT = 1:1, and sCT. The slope of the turbidity over time of sCT only inclined less strongly compared to cholesteryl-PEG 2 kDa:sCT = 1:1, OH-PEG-OH 2 kDa:sCT = 1:1, and cholesteryl-PEG 5 kDa:sCT = 1:1, indicating a more rapid aggregation in the presence of both cholesteryl-PEGs and non-conjugated PEG.



**Figure 6.** Aggregation of salmon calcitonin with cholesteryl-mPEGs over time at 1:1 molar ratio in 10 mM sodium citrate buffer pH 6. Turbidity at 350 nm: x salmon calcitonin; — sCT:cholesteryl-PEG 2 kDa = 1:1; - - - sCT:cholesteryl-PEG 5 kDa = 1:1; Δ sCT:mPEG-OH 2 kDa=1:1.

### 3.5 Aggregation of Hen Egg White Lysozyme (HEWL)

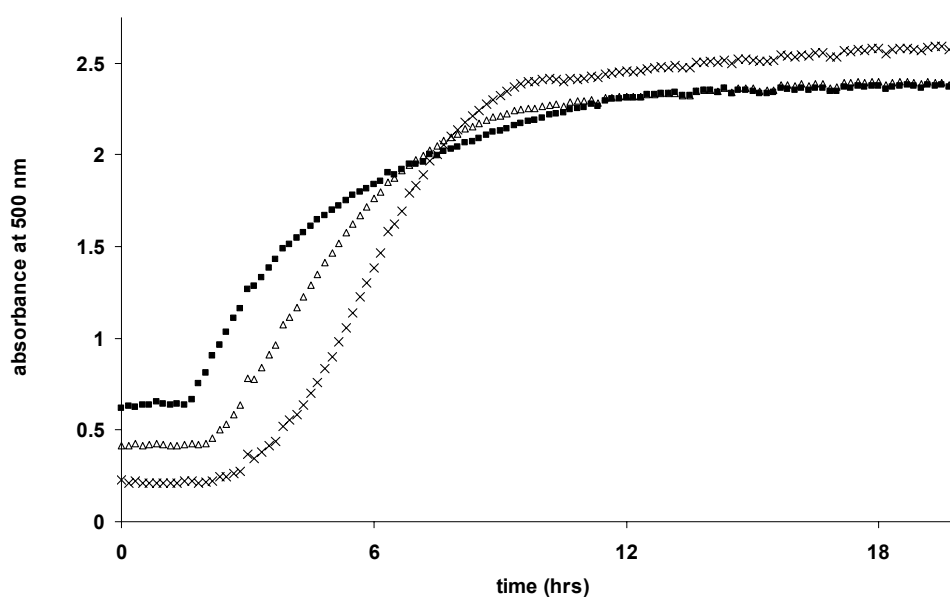
Aggregation of HEWL was analyzed by following changes of turbidity over time. Depending on the molar ratio of HEWL to phenylbutylamine-mPEG 2 kDa the onset of aggregation of HEWL was prolonged, being the shortest for: i) HEWL (2.3 hours), followed by ii) HEWL:phenylbutylamine-mPEG 2 kDa = 1:1 (3 hours), and iii) HEWL:phenylbutylamine-mPEG 2 kDa = 1:10 (6 hours) [Figure 7]. With increasing concentration of phenylbutylamine-mPEG 2 kDa the slope of the turbidity curve decreased, suggesting a deceleration of the HEWL aggregation.



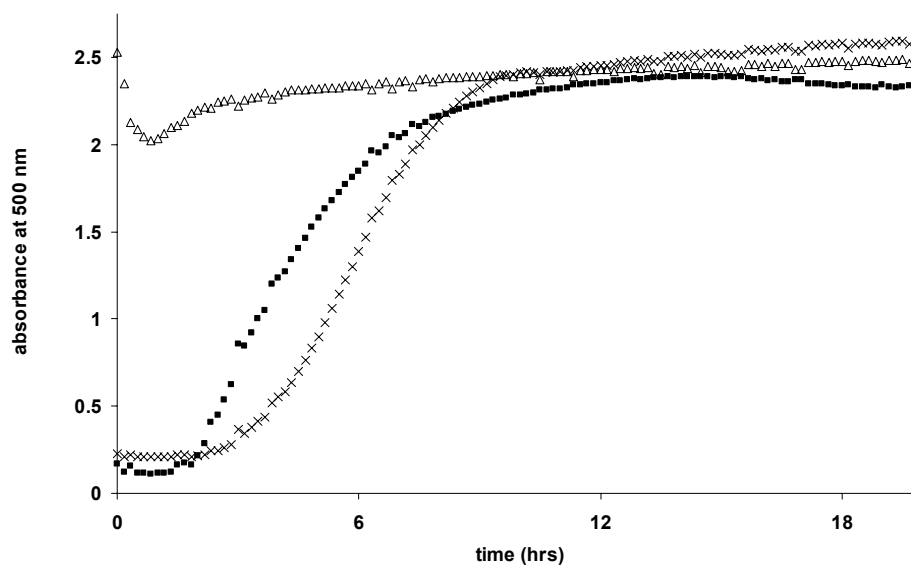
**Figure 7.** Aggregation of hen egg white lysozyme (HEWL) with phenylbutylamine-mPEG 2 kDa over time at different molar ratios in 50 mM sodium phosphate buffer pH 12.2. Turbidity at 500 nm: x hen egg white lysozyme; ▲ HEWL:phenylbutylamine-mPEG 2 kDa = 1:1; □ HEWL:phenylbutylamine-mPEG 2 kDa = 1:10.



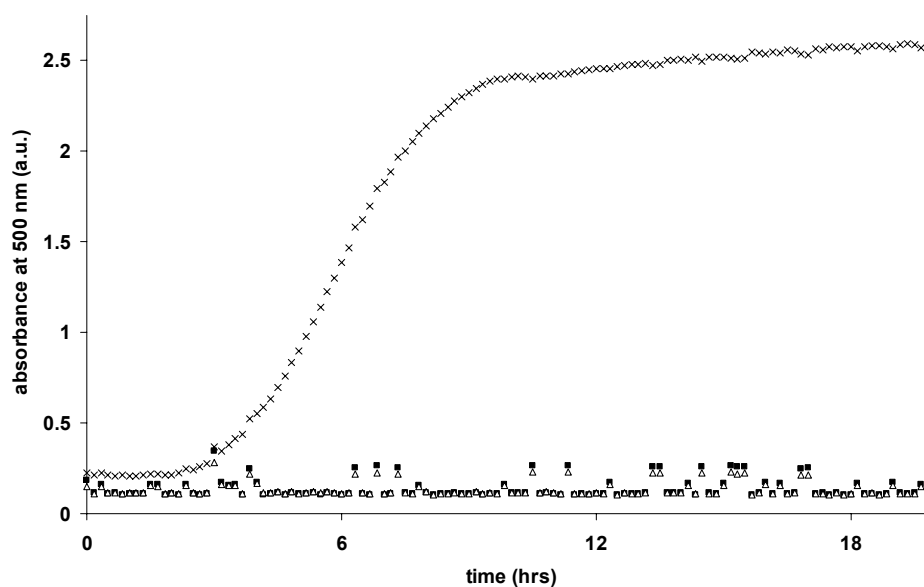
Benzyl-mPEG 2 kDa was found to enhance the aggregation of HEWL (Figure 8A). For the solution of benzyl-mPEG 2 kDa:HEWL = 1:1 the lagtime of aggregation was shortest (1.5 hours), followed by benzyl-mPEG 2 kDa:HEWL = 10:1 (2 hours), and being the longest for HEWL alone (2.3 hours). The final amount of aggregates for both molar ratios with benzyl-mPEG 2 kDa was decreased compared to HEWL. The turbidity at time point 0 was strongly increased for i) benzyl-mPEG 2 kDa:HEWL = 1:1, being smaller for ii) benzyl-mPEG 2 kDa:HEWL = 10:1, and being the smallest for HEWL. Benzyl-mPEG 5 kDa also aggravated the aggregation of HEWL (Figure 8B). Very strong aggregation was observed for benzyl-mPEG 5 kDa:HEWL =10:1. Cholesteryl-PEGs of 2 and 5 kDa in a 1:1 molar ratio suppressed aggregation of HEWL completely over the 20 hours of the kinetics (Figure 9).



**Figure 8A.** Aggregation of hen egg white lysozyme (HEWL) with benzyl-mPEG 2 kDa and 5 kDa in time at different molar ratios in 50 mM sodium phosphate buffer pH 12.2. Turbidity at 500 nm: x hen egg white lysozyme; ■ HEWL:benzyl-mPEG 2 kDa = 1:1; △ HEWL:benzyl-mPEG 2 kDa = 1:10.



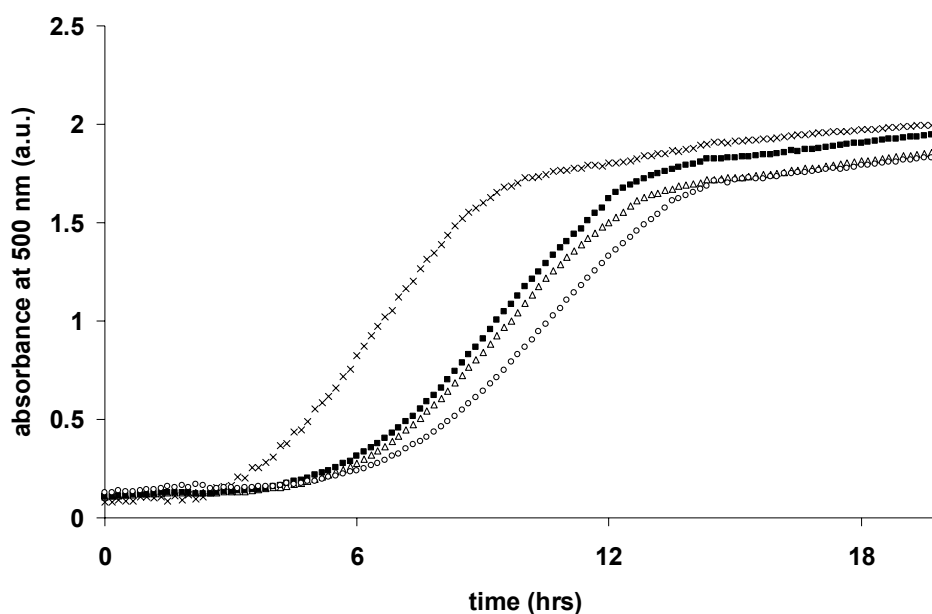
**Figure 8B.** Aggregation of hen egg white lysozyme (HEWL) with benzyl-mPEG 2 kDa and 5 kDa over time at different molar ratios in 50 mM sodium phosphate buffer pH 12.2. Turbidity at 500 nm: x hen egg white lysozyme; ■ HEWL:benzyl-mPEG 5 kDa = 1:1; Δ HEWL:benzyl-mPEG 5 kDa = 1:10.



**Figure 9.** Aggregation of hen egg white lysozyme (HEWL) with cholesteryl-PEG 2 kDa and 5 kDa over time at 1:1 molar ratio in 50 mM sodium phosphate buffer pH

12.2. Turbidity at 500 nm: x hen egg white lysozyme; ■ HEWL:cholesteryl-PEG 2 kDa = 1:1; △ HEWL:cholesteryl-PEG 5 kDa = 1:1.

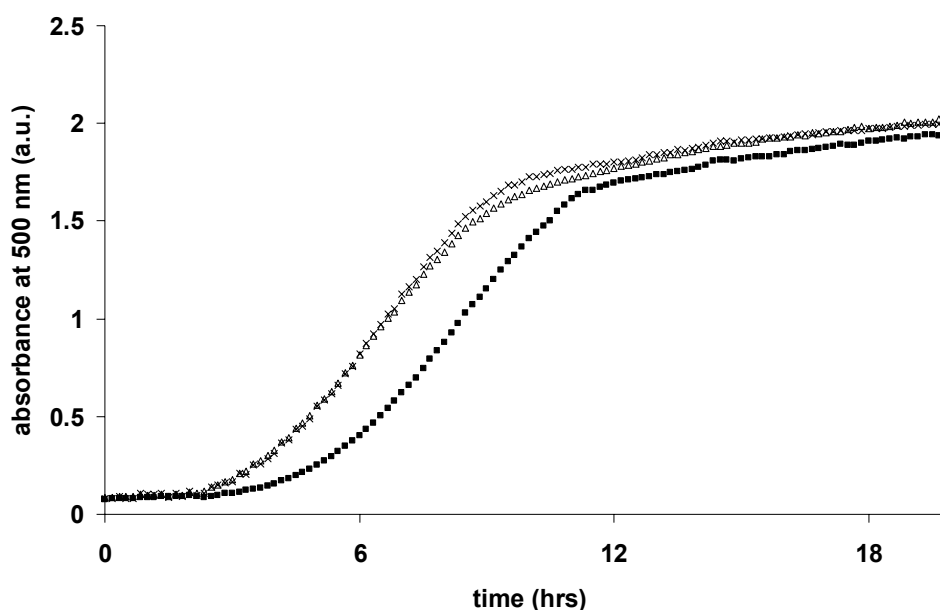
The influence of dansyl-PEGs of different molecular weight on the aggregation of HEWL was analyzed (Figure 10). The lag-time until the onset of aggregation increased as follows: i) HEWL (2.3 hours), ii) HEWL:dansyl-mPEG 5 kDa = 1:1 (3.5 hours), iii) HEWL:dansyl-mPEG 2 kDa = 1:1 (3.8 hours), being the longest for iv) HEWL:bis-dansyl-PEG 3 kDa = 1:1 (4.3 hours). The aggregation of HEWL was slowed down in the presence of the dansyl-PEGs. Also, the final degree of aggregation was decreased in the presence of the different dansyl-PEGs compared to HEWL alone, being the smallest for HEWL:dansyl-mPEG 2 kDa = 1:1 and HEWL:bis-dansyl-PEG 3 kDa = 1:1, then slightly increasing for ii) HEWL:dansyl-mPEG 5 kDa = 1:1, and being the highest for iii) HEWL alone (Figure 10).



**Figure 10.** Aggregation of hen egg white lysozyme (HEWL) with dansyl-mPEG 2 kDa, dansyl-mPEG 5 kDa, and bis-dansyl-PEG 3 kDa over time at 1:1 molar ratio in 50 mM sodium phosphate buffer pH 12.2. Turbidity at 500 nm: x hen egg white

lysozyme;  $\triangle$  HEWL:dansyl-mPEG 2 kDa = 1:1;  $\blacksquare$  HEWL:dansyl-mPEG 5 kDa = 1:1;  
 $\circ$  HEWL:bis-dansyl-PEG 3 kDa = 1:1.

Tryptophan-mPEG 5 kDa (Trp-mPEG 5 kDa) at a 1:1 molar ratio had no influence on the aggregation of HEWL, while tryptophan-mPEG 2 kDa at an equimolar amount slightly increased the lag-time until the onset of aggregation to 3.3 hours (Figure 11). The aggregation velocity and the final degree of aggregation of HEWL were reduced in presence of Trp-mPEG 2 kDa at a 1:1 molar ratio.



**Figure 11.** Aggregation of hen egg white lysozyme (HEWL) with tryptophan-mPEG 2 kDa and 5 kDa over time at 1:1 molar ratio in 50 mM sodium phosphate buffer pH 12.2. Turbidity at 500 nm: x hen egg white lysozyme;  $\blacksquare$  HEWL:tryptophan-mPEG 2 kDa = 1:1;  $\triangle$  HEWL:tryptophan-mPEG 5 kDa = 1:1.

## 4 Discussion

PEGylation has been a successful technique for various protein therapeutics in order to prolong their *in vivo* circulation half-life, to reduce *in vivo* immunogenicity, and to ameliorate formulation stability by decreasing aggregation and precipitation of biopharmaceutics [7, 9, 10, 12, 13]. Although PEGylation represented a tremendous progress in protein formulation, challenges still remain today. To address the need of PEGylation approaches circumventing remaining drawbacks of covalent PEGylation, we have presented the new principle of non-covalent PEGylation in previous publications (manuscripts accepted by JPS and submitted to EJPB). Excipients were prepared consisting of a hydrophobic headgroup attached to PEG. The hydrophobic headgroup shall interact non-covalently with hydrophobic patches present on the surface of the biopharmaceutical. The aim of non-covalent PEGylation is to i) prevent stress generated by chemical processing and the subsequent purification needed for covalent PEGylation, ii) reduce aggregation of the biopharmaceutical due to sterical shielding by the PEG, and iii) to maintain the key benefits of covalent PEGylation (e.g., prolonged pharmacokinetics or reduced immunogenicity). In previous publications we have shown that 2 kDa PEGs possessing a dansyl- or tryptophan-headgroup reduced the aggregation of salmon calcitonin (sCT) in accelerated aggregation studies. The molar ratio of excipient to sCT used as well as the molecular weight of the employed PEG-based excipient were important for the reduction of sCT aggregation. The aim of this paper was i) to test other hydrophobic headgroups (benzyl-, phenylbutylamino-, cholesteryl-) besides dansyl- and Trp- for their influence on sCT aggregation, and ii) to test all PEG-based excipients (see Fig. 1) for their influence on the aggregation of hen egg white lysozyme (HEWL). Since various reports exist in literature on the interaction of benzyl alcohol with biopharmaceutics, the benzyl-moiety was chosen as a candidate headgroup [22-27].

Opting for hydrophobic interactions, the phenylbutylamino-headgroup was selected as another headgroup because of its increased hydrophobicity compared to the benzyl-moiety. Major hydrophobicity was the reason for employing the cholesterol-headgroup.

Dynamic light scattering (DLS) was used to investigate the association of phenylbutylamino-mPEG 2 kDa, the benzyl-mPEGs and the cholesteryl-PEGs of 2 and 5 kDa. No evidence was observed by DLS on the formation or presence of micelles for phenylbutylamino-mPEG 2 kDa, and benzyl-mPEGs 2 and 5 kDa (**Figures. 2A, 2B, 2C**). However, DLS indicated the presence of bigger sized particles in the samples of both cholesteryl-PEGs (**Figures 2D, 2E**) that display elevated count rates of scattered light and increased hydrodynamic radii  $R_h$  compared to the other polymers. It was not possible to determine the critical micelle concentration with these measurements, as cholesteryl-PEGs are readily forming micelles at very low concentrations (see below), such that the concentration at which transition to micelles takes place is too low for detection by DLS. Since micelles can also be detected by the addition of hydrophobic fluorophores, cholesteryl-PEG solutions of varying concentrations containing the Nile Red fluorophore were prepared and analyzed as described in [17]. Thus, CMCs of 0.01 mg/ml and 0.007 mg/ml were determined for cholesteryl-PEG 5 kDa and cholesteryl-PEG 2 kDa, respectively (**Fig. 3**). While Nile Red fluorescence was already visible in this assay by the naked eye for concentrations of cholesteryl-PEGs of 0.05 mg/ml and below, no significant fluorescence was detected neither for phenylbutylamino-mPEG 2 kDa nor the benzyl-mPEGs even at elevated concentrations (see for example **Figures 4A and 5A** in the beginning of the kinetics).

The aggregation of sCT was followed by measuring changes in Nile Red fluorescence and turbidity in time. Equimolar amounts of phenylbutylamino-mPEG 2

kDa, benzyl-mPEGs 2 and 5 kDa, and cholesteryl-PEGs of 2 and 5 kDa to sCT all enhanced the aggregation compared to sCT without a PEG based excipient. In our previously performed studies, we observed stabilization of sCT against aggregation by dansyl-mPEG 2 kDa and Trp-mPEG 2 kD (submitted manuscripts). All excipients tested contained PEG-moieties of either 2 or 5 kDa. Therefore, the observed differences are likely due to the respective headgroup. The only apparent difference between excipients reducing sCT aggregation and excipients increasing the latter was the presence or absence of charged functions included in the headgroup. Dansyl- and Trp-PEG both possess charged functions and stabilized sCT against aggregation. In contrast, phenylbutylamino-mPEG, both benzyl-mPEGs, and both cholesteryl-PEGs do not have charged groups and all deteriorated sCT aggregation. At pH values  $\leq 7$  sCT is positively charged, having an isoelectric point of 10.4 [28, 29]. Therefore, electrostatic repulsion might be due to a reduced aggregation of sCT compared to human calcitonin (hCT) [28, 30]. It was reported in literature that ethylenediamine tetraacetic acid (EDTA) leads to aggregation of sCT due to electrostatic interactions occurring between the negatively charged EDTA and the positively charged sCT. EDTA binds to sCT monomers and connects them among each other resulting in aggregation [30]. Electrostatic interactions between the dansyl- or Trp-headgroup with sCT monomers might be possible. This in turn may result in the observed reduced sCT aggregation due to sterical shielding obtained by the PEG attached to the respective headgroup. Native DNSA, L-Trp or PEG were not effective in reducing sCT aggregation. The superior stabilization of Trp-mPEG 2 kDa compared to dansyl-mPEG 2 kDa might result from the negatively charged carboxyl-function ( $pK_a \sim 2.1 - 2.4$  [31]) enabling stronger interactions. The deteriorated aggregation of sCT in presence of phenylbutylamino-mPEG, benzyl-mPEG or cholesteryl-PEG might not necessarily originate from the respective headgroups. As

can be seen in **Figures 4B, 5B and 6** already the addition of PEG to sCT resulted in an aggravated aggregation. This might be caused by a removal of solvation water by the PEG leading to increased precipitation/aggregation of sCT. Overall, the differences in aggregation were relatively small comparing i) sCT with PEG only to ii) sCT with PEG-based excipients having either a benzyl-, phenylbutylamino- or cholesteryl-headgroup (**Figures 4B, 5B and 6**).

Swaminatham *et al.* have shown that the aggregation of HEWL at pH 12.2 proceeds by the formation and exposure of hydrophobic surfaces [32]. In our studies, benzyl-PEGs increased the aggregation of HEWL under these conditions, while increasing concentrations of phenylbutylamine-PEG 2 kDa slowed the aggregation down (**Figures 7, 8A, 8B**). Cholesteryl-PEGs completely suppressed HEWL aggregation (**Fig. 9**). With increasing hydrophobicity of the headgroup (benzyl- < phenylbutylamino- < cholesteryl-), aggregation of HEWL was reduced. Here dansyl- and Trp cannot be taken into account since also electrostatic interactions might occur. A possible explanation might be an increased shielding of the liberated hydrophobic surfaces of HEWL with increasing hydrophobicity of the headgroup. However, the absence of aggregation observed in presence of the two cholesteryl-PEGs might also be due to surfactant-like properties, e.g., micellization.

Concerning the stabilization of HEWL against aggregation by the dansyl-PEGs and tryptophan-mPEGs, there seems to be a dependency on the molecular weight of the PEG used: the 2 kDa PEG-derivatives were more effective in reducing HEWL aggregation than the 5 kDa PEG-derivatives (**Figures 10 and 11**). Similar observations were already made for the stabilization of sCT by dansyl- and Trp-PEGs. In the previous publications, several possible explanations were given. Increasing the molecular weight of the PEG-moiety also increases sterical hindrance exerted by the PEG, which in turn might reduce the interactions between the



headgroup and the biopharmaceutical. However, increasing the molecular weight of the PEG might imbalance the hydrophilic-lipophilic balance of the PEG-based excipients. This in turn leads to an increased impact of the PEG-moiety on aggregation [33]. A further possibility is that increasing the molecular weight of the PEG leads to an increased water exclusion from the biopharmaceutical by the PEG resulting in a decreased stabilization. The two latter theories are supported by an observed enhanced precipitation/aggregation of HEWL in presence of native PEG (data not shown).

From the studies of these two different biopharmaceutical drugs we may draw several conclusions. i) Although dansyl- and Trp-headgroups are hydrophobic, they also contain charged groups, which enable electrostatic interactions. As observed for sCT electrostatic interactions between those headgroups and sCT might be fundamental for the observed stabilization against aggregation rather than only hydrophobic interactions. Due to these electrostatic interactions between the dansyl- or Trp-PEGs and sCT a steric shielding by the PEG-moiety might impede interactions with other sCT molecules leading to the observed reduced aggregation. ii) HEWL aggregation was described to proceed by liberation of hydrophobic surfaces under the tested conditions [32]. Hydrophobic interactions between the phenylbutylamino- or cholesteryl-headgroups resulting again in a sterical shielding conferred by the PEG-moiety might be one possible reason for the observed stabilization of HEWL. iii) Micellization of cholesteryl-PEGs might be another reason for the observed stabilization of HEWL. iv) The best excipient for stabilization of sCT is not the best for HEWL. Most probably for each biopharmaceutical a specific stabilizer/headgroup is needed. No general stabilizer was found up to now. Therefore, a screening for each biopharmaceutical is needed to find the optimal conditions for stabilization against aggregation.

We were able to show that non-covalent PEGylation results in reduced aggregation of biopharmaceuticals. The maintenance of bioactivity and further key benefits of covalent PEGylation, such as prolonged pharmacokinetics or reduced immunogenicity, will be evaluated in further ongoing studies.

## **5 Conclusions**

In this paper, the effect of different PEG-based excipients on the aggregation of two biopharmaceuticals, sCT and HEWL, was shown. The performed aggregation studies of sCT with the different PEG-based excipients lead to the conclusion that dansyl- and Trp-mPEGs possibly interact electrostatically with sCT due to charged functions contained in the respective headgroups. This in turn leads to a sterical shielding by the PEG-moiety and results in the observed stabilization of sCT against aggregation by dansyl- and Trp-mPEGs. No beneficial effect on sCT aggregation was obtained for PEG-excipients possessing only hydrophobic headgroups, like benzyl-, phenylbutylamino- or cholesteryl-PEGs. By contrast, HEWL aggregation was reduced with increasing hydrophobicity of the headgroup employed (benzyl- < phenylbutylamino- < cholesteryl-) under conditions where HEWL aggregation proceeds by hydrophobic interactions. Dansyl-PEGs and Trp-mPEGs also reduced the aggregation of HEWL depending on the molecular weight. Ongoing studies are performed in order to analyze, whether further key benefits (e.g., reduced immunogenicity or prolonged pharmacokinetics) of covalent PEGylation are maintained by non-covalent PEGylation. In this publication, we presented the varying efficacy of PEG-based excipients due to non-covalent interactions depending on the type of headgroup and the type of biopharmaceutical used. The PEG-based excipients can be easily obtained at low costs. With the approach of non-covalent PEGylation, aggregation prone proteins might be easier formulated at higher

concentrations. Thus, more economic drug products and an improved patient compliance might be obtained.

## **6 Acknowledgements**

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## **Chapter 6**

### **Discussion and future perspectives**

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## **Covalent bonds and non-covalent interactions**

Although weaker than covalent bonds, non-covalent interactions are very important for the three-dimensional structures of biomacromolecules and play, furthermore, a fundamental role in biochemical processes, *e.g.*, intra-and extra-cellular signaling, antibody-antigen binding, formation of the DNA double-helix, or enzymatic reactions. The most important types of non-covalent interactions are electrostatic interactions, hydrogen bonds, van der Waals interactions, and hydrophobic interactions [1]. Electrostatic interactions occur between two oppositely charged molecules leading to attractive forces. Hydrogen bonds and van der Waals interactions are basically electrostatic interactions. Hydrogen bonds are formed between two electronegative atoms, called hydrogen-bond donor and hydrogen-bond acceptor. The donor atom is covalently bonded to a hydrogen atom, from which it pulls electron density away. The thus partially positively charged hydrogen atom then interacts non-covalently with another partially negative atom. Van der Waals interactions are based on fluctuations of the electronic charge around an atom resulting in an unsymmetrical charge distribution around the atom. This assymetrical charge distribution induces complementary assymetrical charge distributions in its neighboring atoms leading to electrostatic interactions [1].

Hydrophobic interactions are based on the nonpolar properties of molecules and their incapability to establish hydrogen bonds or electrostatic interactions with water. Hydrophobic or nonpolar molecules being dissolved in water are surrounded by water molecules forming ordered cage-like structures. Maintaining this ordered structure is energetically unfavorable for the system regarding thermodynamics. Furthermore, water molecules energetically favor interactions among themselves rather than with the nonpolar molecules unable to participate in hydrogen bonding or electrostatic interactions. Thus, the release of the water molecules from the nonpolar surfaces



thermodynamically results in an increase in entropy, which in turn is energetically favourable for the system. The release of the water molecules from the nonpolar surfaces results in an increased association of the nonpolar molecules based on nonpolar interactions [1].

### **Protein therapeutics**

With the approval of insulin by the FDA in 1982, the first recombinantly produced protein therapeutic became commercially available [2] and became a major breakthrough for the therapy of diabetes mellitus. Since then, the application of recombinant protein based therapeutics and diagnostics increased tremendously. Biopharmaceuticals became of major importance in the treatment of severe diseases, such as cancer, infectious diseases or autoimmune diseases [3]. However, formulation scientists are confronted by various problems, trying to develop a safe and stable protein formulation being highly accepted also by the patients: i) proteins suffer from short plasma half-lives due to fast enzymatic degradation and renal filtration, ii) proteins were observed to be immunogenic, although human homologues are nowadays preferably used, iii) biopharmaceutical formulations are affected from poor stability and shelf-lives of due to physical and chemical degradation processes of proteins [3, 4].

### **Protein aggregation - pathways and inducing factors**

Recently, Philo has described different possible mechanisms causing protein aggregation being a major physical degradation process [5]. In principle, conformational changes, partial unfolding, chemical degradation or modification of the native protein may lead to oligomerization and result in aggregation [5, 6]. Furthermore, the adsorption of native protein monomers onto container surfaces or

air-liquid interfaces may result in partial unfolding leading to aggregation whether directly at the surface/interface or even in solution after liberation of the altered protein [7, 8]. These processes are mostly called „non-native“ aggregation, since an altered protein structure is involved. In addition, native proteins may also self-associate and aggregate [5].

Regardless of the mechanism, non-covalent interactions of electrostatic and hydrophobic nature are most often implicated [9]. Unfolded intermediates are highly flexible and expose hydrophobic patches usually buried in the native protein. Though present only to a tiny extent, these intermediates are supposed to trigger aggregation by attracting hydrophobic interactions [6, 10]. When a native protein possesses sticky patches on its surface, aggregation may proceed by attractive forces of hydrophobic, electrostatic or van der Waals nature [9, 11].

Aggregation may be induced by a myriad of „stress“ conditions occurring during the production, purification, and formulation processes of a biopharmaceutical. The protein structure itself, protein concentration, temperature changes, freezing/thawing cycles, changes in pH or ionic strength, additives such as preservatives or polyols, shaking or shearing processes all may induce aggregation [6, 9, 11]. Thus, stabilization of a biopharmaceutical against aggregation is most often an empirical process testing various processing and formulation conditions.

### **Protein formulation – covalent PEGylation**

PEGylation represents the covalent conjugation of poly(ethylene glycol) to biopharmaceuticals and might be employed in order to obtain one or several of the following effects: i) prolongation of the *in vivo* circulation half-life, ii) decrease of *in vivo* immunogenicity, and iii) decrease of aggregation [4]. However, several challenges remain for covalent PEGylation: i) after synthesis, heterogeneous

products are obtained needing separation and characterization, ii) the chemistry employed to attach the PEG may represent additional stress for the protein, which might lead to increased aggregation and partial or complete loss of activity, iii) because of sterical shielding by the PEG, reduced *in vivo* bioactivity is most often obtained resulting from decreased interactions of the drug with its receptor.

### **Development of novel PEGylation approaches based on non-covalent interactions**

In order to address the need of PEGylation approaches circumventing one or several remaining drawbacks of covalent PEGylation, this work deals with the development of novel PEGylation techniques based on non-covalent interactions. Non-covalent interactions are crucial in nature and play a key role during the aggregation and interaction of proteins. We opted for the development of non-covalent PEGylation techniques based on hydrophobic interactions, since the latter have been predominantly described in literature to be a key parameter during aggregation.

At first, we synthesized PEG-based excipients of different molecular weight bearing dansyl- as hydrophobic headgroup (see **chapter 3**). Although dansylamide (the underlying structure for the headgroup employed) is not approved for use in humans, it offered the advantage of having a further diagnostic tool by following changes in its fluorescence characteristics. The successful conjugation of the dansyl-headgroup to PEG and the purity degree were analyzed using  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR, FTIR, MALDI-TOF and UV-Vis spectroscopy. Thus, three different PEG-based excipients were obtained: monovalent dansyl-mPEGs of 2 kDa (93 % conjugation degree) and 5 kDa (96 % conjugation degree) and a bivalent bis-dansyl-PEG of 3 kDa (91% conjugation degree). The sensitivity of the dansyl-fluorescence towards the polarity of its environment was maintained after PEG-conjugation. This property and dynamic

light scattering measurements (DLS) were employed to check for the ability to associate (micellization). Both techniques suggested the absence of micelles. Furthermore, neither cytotoxic nor hemolytic effects were observed for any of the dansyl-PEGs at all concentrations tested. By fluorimetric titration DNSA was found to bind to human serum albumin (HSA) while for dansyl-mPEG 2 kDa binding to HSA was absent.

Dansyl-mPEG 2 kDa was added to a stable solution of salmon calcitonin (sCT). Reduction of the 90° light scatter and an increase in dansyl-fluorescence of this mixture may suggest possible interactions between sCT and dansyl-mPEG 2 kDa. Subsequently, the influence of all excipients on the aggregation of sCT in various buffer systems was analyzed by following changes in Nile Red fluorescence (added as an extrinsic fluorophore to follow aggregation of sCT) and turbidity in time.

Dansyl-mPEG 2 kDa reduced aggregation best at a 1:1 molar ratio, although stabilization was observed down to molar ratios of sCT:dansyl-mPEG 2 kDa of 100:1. Bis-dansyl-PEG 3 kDa also stabilized sCT against aggregation in a 1:1 molar ratio, though less effectively than dansyl-mPEG 2 kDa. Dansyl-mPEG 5 kDa deteriorated aggregation of sCT. Furthermore, we were able to show, that the changes in dansyl-fluorescence of the dansyl-PEGs may also be used to detect and follow aggregation of sCT.

In **chapter 4**, Tryptophan-mPEGs (Trp-mPEGs) of 2 and 5 kDa were synthesized and physicochemically characterized, and their influence on sCT aggregation was evaluated. Trp is approved for use as nutritional supplement offering furthermore the advantage of its fluorescence being susceptible to environmental polarity. Successful conjugation and purification were analyzed using the same methods as described for the dansyl-PEGs. A conjugation degree of 98 % was obtained for both Trp-mPEGs. The susceptibility of Trp fluorescence intensity to polarity of its environment was

maintained after PEG-conjugation. Again, this property and DLS were used to evaluate possible association. Following the fluorescence intensity vs. concentration suggested a possible association of few Trp-mPEG 2 kDa molecules resulting in complexes too small to include hydrophobic dyes like Nile Red. No association was observed for Trp-mPEG 5 kDa. Furthermore, the hemolytic and cytotoxic potentials were analyzed for both excipients. Only Trp-mPEG 2 kDa at 20 mg/ml showed to be cytotoxic.

With increasing Trp-mPEG 2 kDa concentration, sCT aggregation was decreased in 10 mM sodium citrate buffer pH 6, being a harsh environment, wherein sCT alone aggregates very fast. A 10-fold molar excess even suppressed aggregation of sCT up to 64 hours under these conditions. Again, Trp-mPEG 5 kDa was less effective than Trp-mPEG 2 kDa. With a 10-fold molar excess of Trp-mPEG 2 kDa in the presence of sCT, few aggregation was measured after ~10 days in 10 mM sodium citrate buffer pH 5 by various techniques such as 90° light scattering, Nile Red fluorescence, Nile Red fluorescence microscopy, fluorescence lifetime and fluorescence anisotropy. Trp-mPEG 2 kDa significantly reduced sCT aggregation in harsh environments, wherein sCT alone aggregated fast. Trp-mPEG 2 kDa was superior to dansyl-mPEG 2 kDa in reducing the aggregation of sCT.

In **chapter 5** of this thesis commercially obtained benzyl-mPEGs 2 and 5 kDa and cholesteryl-PEGs 2 and 5 kDa were physicochemically characterized and subsequently analyzed for their influence on sCT aggregation. Furthermore, phenylbutylamino-mPEG 2 kDa was synthesized and characterized as described above for the dansyl-PEGs. A conjugation degree of 98 % was determined. DLS measurements indicated absence of micelles for phenylbutylamino-mPEG 2 kDa and benzyl-mPEGs 2 and 5 kDa. For the cholesteryl-PEGs increased count rates of scattered light and hydrodynamic radii indicated the presence of micelles. The critical

micelle concentrations (CMCs) for cholesteryl-PEG 2 kDa and 5 kDa were determined to be at 0.007 mg/ml and 0.01 mg/ml from measurements following the changes in fluorescence intensity and emission maximum after inclusion of the hydrophobic Nile Red fluorophore.

The aggregation of sCT was increased after the addition of equimolar amounts of phenylbutylamino-mPEG 2 kDa, benzyl-mPEGs 2 and 5 kDa, and cholesteryl-PEGs 2 and 5 kDa. Based on the observations of the varying efficacy of all the PEG-based excipients on the aggregation of sCT, it is proposed that electrostatic interactions between sCT and the dansyl-or Trp-headgroup are of importance for the observed stabilization against aggregation. Due to these interactions, the PEG gets attached non-covalently to sCT and can perform a sterical shielding of the latter resulting in decreased aggregation.

Furthermore, the different excipients were evaluated for their influence on the aggregation of hen egg white lysozyme (HEWL) by following turbidity. Benzyl-PEGs enhanced HEWL aggregation, while phenylbutylamino-mPEG 2 kDa prolonged the lag phase of HEWL aggregation with increasing concentration. Both cholesterol-PEGs suppressed HEWL aggregation over 20 hours. Trp-mPEG 2 kDa reduced the aggregation velocity, Trp-mPEG 5 kDa had no effect on HEWL aggregation. After addition of the dansyl-PEGs HEWL aggregation was decreased, the aggregation velocity reduced, and the lag time of aggregation increased to a varying degree.

From the different studies performed we can draw several conclusions: i) in the most cases the stabilization of the PEG-based excipients against aggregation is dependent on molecular weight, ii) the headgroup and the biopharmaceutical itself are of significance, iii) the molar ratio of biopharmaceutical to PEG-based excipient used is important. Concerning conclusion number i) it was observed that 2 kDa excipients bearing the same headgroups were most often more efficient than 5 kDa

excipients. With regards to conclusion ii) it was noticed that Trp-mPEG 2 kDa was superior to dansyl-mPEG 2 kDa at a 1:1 molar ratio of sCT to PEG-based excipient. Phenylbutylamino-mPEG 2 kDa, benzyl-mPEGs 2 and 5 kDa, and cholesteryl-PEGs 2 and 5 kDa all enhanced sCT aggregation at equimolar amounts. By contrast, equimolar amounts of phenylbutylamino-mPEG 2 kDa prolonged the lag time of HEWL aggregation and cholesteryl-PEGs both even suppressed HEWL aggregation completely.

Conclusion iii) renders the matter even more complicated. A two-fold molar excess of dansyl-mPEG 2 kDa deteriorated sCT aggregation, while a 10-fold molar excess of Trp-mPEG suppressed sCT aggregation for over 64 hours. Similar tendencies were observed concerning the influence of phenylbutylamino-mPEG 2 kDa on HEWL aggregation velocity and HEWL aggregation lag time: a ten-fold molar excess of the excipient was superior than an equimolar amount.

Covalent PEGylation is performed in order to prolong the *in vivo* circulation half-life, to decrease *in vivo* immunogenicity, and/or to decrease aggregation [4]. In this thesis, the development of novel PEG-based excipients and their influence on the aggregation of two biopharmaceuticals is described. These excipients were able to weakly interact with biopharmaceuticals based on non-covalent interactions. By this approach chemical reactions needed to attach the PEG to the biopharmaceutical are circumvented. Simple addition to a liquid formulation is sufficient in order to obtain interactions. We were further able to show that aggregation of biopharmaceuticals can be reduced and in some cases even suppressed over a certain period of time in accelerated stress tests by the described PEG-based excipients. However, depending on the headgroup, the biopharmaceutical and the molar ratio of excipient to biopharmaceutical used, stabilization or even destabilization might be obtained. Therefore, these influencing factors need to be carefully investigated and evaluated.

The next step will be the evaluation of the influence of dansyl-mPEG 2kDa and Trp-mPEG 2 kDa on sCT stability in a stable formulation under stressing conditions such as shaking at room temperature or elevated storing temperature.

Prolongation of the *in vivo* circulation half-life and decrease of *in vivo* immunogenicity are further benefits obtained by covalent PEGylation. Most probably, these characteristics will not be delegated on the biopharmaceutics by the described PEG-based excipients. This conclusion may be drawn from fluorescence titration measurements of the dansyl-mPEG 2 kDa with human serum albumin (HSA), where only a marginal binding affinity was detected. Interaction of the dansyl-headgroup with the hydrophobic binding pocket on HSA was probably reduced due to steric interactions from the PEG. In order to obtain prolonged pharmacokinetics and decreased immunogenicity *in vivo* by non-covalent PEGylation, specific interactions need to be employed resulting in a high binding affinity of the excipient to the biopharmaceutical although the PEG might sterically interfere.

A further application of phenylbutylamino-, benzyl- or cholesteryl-PEGs in the future might be the possible stabilization of human calcitonin (hCT). The efficient use of hCT as a drug is limited due to its marked tendency to fibrillate and precipitate in aqueous solutions [12, 13]. It has been speculated that the enhanced tendency of hCT aggregation and fibrillation might be due to aromatic contributions from phenylalanine side chains [12, 14, 15]. Interaction of these phenylalanine rings with benzyl- or phenylbutylamino-mPEGs might be possible, which might result in a reduced hCT aggregation.



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# Chapter 7

## Summary of the Thesis

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This thesis describes the development of novel PEGylation techniques based on non-covalent interactions.

Therapeutics based on protein and peptide drugs hold important value for the treatment of severe diseases like cancer, infectious diseases or autoimmune diseases. Their medical application increased tremendously over the past 20 years because biopharmaceuticals are regarded to be superior to small molecular drugs. Biopharmaceuticals may show an increased target specificity, a decreased probability of adverse events, and are used in replacement therapies of innate deficiencies of endogeneous proteins. However, stability concerns remain one of the main obstacles for successful market authorization of such drugs. Generally, physical instabilities and in particular aggregation pose major challenges encountered during the production and formulation of biopharmaceuticals. Furthermore, protein therapeutics suffer from short circulation half-lives due to enzymatic degradation, followed by fast renal elimination and *in vivo* immunogenicity.

PEGylation represents the covalent conjugation of poly(ethylene glycol) to biopharmaceuticals, which has become the technology of choice for macromolecular engineering. After PEGylation, decreased enzymatic degradation and prolonged plasma half-lives are observed due to decreased renal filtration. Also, a reduced *in vivo* immunogenicity was observed in several cases. Additionally, the stability of the formulation may be increased due to a steric shielding obtained by the PEG, leading to a decreased aggregation.

However, several challenges still remain for covalent PEGylation:

- i) the chemical processing and subsequent purification needed in order to attach the PEG, which may represent further stress on the protein, resulting in increased aggregation and partial or complete loss of activity

- ii) the issue of obtaining heterogeneous products requiring to be separated and characterized
- iii) an observed reduced *in vivo* bioactivity resulting from reduced interaction of the drug with its receptor due to steric shielding by the PEG.

Thus, new strategies are needed in order to overcome the remaining challenges of covalent PEGylation.

As described in this thesis, we addressed the need for alternative PEGylation approaches by development of novel PEGylation techniques based on non-covalent interactions, since the latter are the underlying forces during protein-protein interactions, which includes aggregation.

Various PEG-based excipients possessing a hydrophobic headgroup were synthesized: dansyl-mPEG 2 kDa and 5 kDa, bis-dansyl-PEG 3 kDa, tryptophan-mPEGs (Trp-mPEGs) of 2 and 5 kDa, and phenylbutylamino-mPEG 2 kDa. Yield of synthesis, purity and the degree of conjugation were determined by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR, FTIR, MALDI-TOF and UV-Vis spectroscopy. Cytotoxic and hemolytic potentials were evaluated for dansyl-PEGs and Trp-mPEGs at various concentrations. Only Trp-mPEG 2 kDa at a concentration of 20 mg/ml was shown to be cytotoxic. Furthermore, benzyl-mPEGs of 2 and 5 kDa and cholesteryl-PEGs of 2 and 5 kDa were commercially obtained. The potential of these excipients to associate in solution forming micellar complexes was evaluated for all excipients. Only the two cholesteryl-PEGs formed micelles at critical micelle concentrations of 0.007 mg/ml and 0.01 mg/ml, respectively for cholesteryl-PEG 2 kDa and 5 kDa.

The influence of all the PEG-based excipients on the aggregation of salmon calcitonin (sCT) in various buffer systems was successively evaluated. At a 1:1 molar ratio of PEG-based excipient to sCT, dansyl-mPEG 2 kDa reduced sCT aggregation well, while bis-dansyl-PEG 3 kDa was less effective. Dansyl-mPEG 5 kDa

aggravated aggregation as was a two-fold molar excess of dansyl-mPEG 2 kDa. With increasing Trp-mPEG 2 kDa concentrations sCT aggregation was reduced. The 5 kDa Trp-mPEG also acted in a concentration-dependent fashion, but was less effective than the 2 kDa Trp-mPEG. Trp-mPEG 2 kDa was superior in stabilizing sCT against aggregation compared to dansyl-mPEG 2 kDa.

Phenylbutylamino-mPEG 2 kDa, benzyl-mPEGs 2 and 5 kDa, and cholesteryl-PEGs 2 and 5 kDa all deteriorated sCT aggregation. Native PEG was also increasing sCT aggregation. We propose electrostatic interactions of dansyl- or Trp-PEGs with sCT resulting in decreased sCT aggregation due to steric shielding obtained by the PEG-moiety. Phenylbutylamino-mPEG, benzyl-mPEG, and cholesteryl-PEG did not contain charged functions and were, therefore, not able to interact with sCT resulting in a deteriorated aggregation being comparable to native PEG.

Analyzing the influence of the various excipients on aggregation of hen egg white lysozyme (HEWL), dansyl-PEGs, Trp-mPEG 2 kDa, and phenylbutylamino-mPEG 2 kDa were shown to decrease the aggregation velocity. Benzyl-mPEGs deteriorated HEWL aggregation and both cholesteryl-PEGs suppressed HEWL aggregation over 20 hours under the tested conditions.

In this thesis the influence of novel PEG-based excipients on aggregation of different biopharmaceuticals was evaluated: **Chapter 3** describes the interactions of dansyl-PEGs with sCT. **Chapter 4** presents the impact of Trp-mPEGs on the aggregation of sCT. **Chapter 5** shows the influence of all excipients on aggregation of HEWL besides the performance of aggregation studies of sCT in presence of phenylbutylamino-mPEG 2 kDa, benzyl-mPEGs, and cholesteryl-PEGs.

In **chapter 6** the results were discussed and a strategy was proposed in order to maintain further benefits of covalent PEGylation, such as improved pharmacokinetics and decreased immunogenicity *in vivo*.

# Chapter 8

## Résumé

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La thèse traite du développement de nouvelles techniques de PEGylation basées sur des interactions non-covalentes.

L'utilisation de protéines ou peptides comme principes actifs est aujourd'hui primordiale pour le traitement de maladies sévères telles que le cancer, les maladies infectieuses ou auto-immunitaires. Leurs applications médicales ont augmenté de façon significative pendant ces vingt dernières années parce que ces protéines ou peptides sont considérés comme supérieurs aux petites molécules. En effet, le ciblage de ces produits pharmaceutiques peut être amplifié, les effets secondaires sont diminués, et ils sont utilisés dans des thérapies de remplacement des défauts congénitaux des protéines endogènes. Néanmoins, le principal enjeu pour leur légitimisation est leur stabilité. Les instabilités physiques en général et l'agrégation en particulier posent des problèmes majeurs pendant la production et la formulation de produits biopharmaceutiques. En outre, les protéines thérapeutiques souffrent d'une immunogénicité *in vivo* et d'une biodisponibilité courte du fait des dégradations enzymatiques et d'une élimination rénale rapide.

La PEGylation consiste en la conjugaison covalente de poly(éthylène glycol) sur les produits biopharmaceutiques, et est actuellement la technologie favorisée pour le développement de macromolécules innovantes. Après leur PEGylation, la demi-vie de ces composés pharmaceutiques dans la circulation sanguine est augmentée grâce à la diminution de la filtration rénale et de la dégradation enzymatique. En outre, la diminution du caractère immunogénique *in vivo* a été observée pour plusieurs produits. Enfin, la stabilité de la formulation peut-être accrue par le cloisonnement stérique dû au PEG, conduisant à une réduction de l'agrégation. Néanmoins, les enjeux de la PEGylation covalente sont encore nombreux:



- i) les réactions chimiques pour conjuguer le PEG et les étapes de purification peuvent représenter un stress pour la protéine, conduisant à une agrégation et/ou une perte partielle ou complète d'activité;
- ii) les produits de la PEGylation sont hétérogènes et doivent être séparés, purifiés et caractérisés;
- iii) une bioactivité *in vivo* réduite, due à des interactions diminuées entre le principe actif et son récepteur à cause d'un cloisonnement stérique par le PEG.

De ce fait, de nouvelles stratégies doivent être mises en place afin de surmonter ces enjeux de la PEGylation covalente.

Comme décrit dans cette thèse nous avons abordé ce besoin de techniques alternatives en essayant de développer de nouvelles méthodes de PEGylation basées sur des interactions non-covalentes, puisque celles-ci sont les forces sur lesquelles reposent les interactions protéiques, l'agrégation incluse.

Différents excipients basés sur du PEG et possédant un groupe hydrophobe ont été synthétisés: les dansyl-mPEG 2 kDa et 5 kDa, le bis-dansyl-PEG 3 kDa, les Tryptophan-mPEGs (Trp-mPEGs) de 2 et 5 kDa, et le phenylbutylamino-mPEG 2 kDa. Le rendement de synthèse, la pureté et le degré de conjugaison ont été déterminés par  $^1\text{H}$ -RMN et  $^{13}\text{C}$ -RMN, FTIR, MALDI-TOF et spectroscopie UV-Vis. Leur potentiel cytotoxique et hémolytique ont été évalués pour les dansyl-PEGs et les Trp-mPEGs à différentes concentrations. Seul le Trp-mPEG 2 kDa à 20 mg/ml a été cytotoxique. En outre, les benzyl-mPEGs de 2 et 5 kDa et les cholesteryl-PEGs de 2 et 5 kDa ont été achetés. Le potentiel associatif en solution, formant des complexes micellaires, a été évalué pour tous ces excipients. Seuls les deux cholesteryl-PEGs ont formé des micelles aux concentrations critiques micellaires de 0.007 mg/ml et 0.01 mg/ml respectivement pour le cholesteryl-PEG 2 kDa et 5 kDa.

L'influence de tous ces excipients basés sur le PEG sur l'agrégation de salmon calcitonin (sCT) a été évaluée dans différents tampons. Pour un ratio molaire de 1:1, le dansyl-mPEG 2 kDa a bien réduit l'agrégation de sCT, tandis que le bis-dansyl-PEG 3 kDa a été moins efficace. Au contraire, le dansyl-mPEG 5 kDa a accru l'agrégation de sCT, tout comme le dansyl-mPEG 2 kDa en double excès de sCT. Avec des concentrations croissantes de Trp- mPEG 2 kDa, l'agrégation de sCT a été réduite. L'effet de Trp-mPEG 5 kDa est également croissant avec une augmentation de concentration, mais reste moins efficace que le Trp- mPEG 2 kDa. De plus, le Trp- mPEG 2 kDa stabilise davantage le sCT de l'agrégation que le dansyl-mPEG2 kDa. En revanche, le phenylbutylamino-mPEG 2 kDa, les benzyl-mPEGs de 2 et 5 kDa, et les cholesteryl-PEGs de 2 et 5 kDa ont tous amplifié l'agrégation de sCT, tout comme le PEG natif.

Nous avons proposé que des interactions électrostatiques de dansyl- ou Trp-PEGs avec le sCT mènent à une diminution de l'agrégation de sCT grâce à un cloisonnement stérique obtenu par la partie moléculaire polymérique (PEG). Le phenylbutylamino-mPEG, les benzyl-mPEGs, et les cholesteryl-PEGs ne contiennent pas de fonctions chargées et n'ont par conséquent pas été capables de créer des interactions avec le sCT, ce qui a résulté à une aggravation de l'agrégation, comparable au PEG natif.

En analysant l'influence de ces différents excipients sur l'agrégation du lysozyme de blanc d'œuf (HEWL), les dansyl-PEGs, le Trp-mPEG 2 kDa, et le phenylbutylamino-mPEG 2 kDa ont montré une diminution de la vitesse d'agrégation. Les benzyl-mPEGs ont aggravé l'agrégation d'HEWL tandis que les deux cholesteryl-PEGs ont complètement supprimé cette dernière pendant 20 heures, sous les conditions évaluées.

Dans cette thèse l'influence de nouveaux excipients basés sur le PEG sur l'agrégation de produits biopharmaceutiques différents a été évaluée : le **chapitre 3** décrit l'interaction entre les dansyl-PEGs avec le sCT. Le **chapitre 4** présente l'influence de Trp-PEGs sur l'agrégation de sCT. Le **chapitre 5** décrit l'influence de tous les excipients sur l'agrégation de HEWL et les études d'agrégation de sCT en présence du phenylbutylamino-mPEG 2 kDa, des benzyl-mPEGs, et des cholesteryl-PEGs.

Le **chapitre 6** discute les résultats et propose des stratégies pour surmonter les dernières difficultés de la PEGylation covalente par la PEGylation non-covalente, comme la prolongation de la pharmacocinétique ou une diminution de l'immunogénicité *in vivo*.

## List of Abbreviations

ADA	adenosine deaminase
ALL	acute lymphoblastic leukemia
1,8-ANS	8-anilino-1-naphthalenesulfonic acid
AUC	area under the curve
BAlc	benzyl alcohol
Boc	<i>tert</i> -butoxycarbonyl
bPEG	benzyl-mPEG
βAla	β-alanine
Cys	L-cysteine
DNSA	dansyl amide / 5-(dimethylamino)-1-naphthalenesulfonamide
dPEG	dansyl-PEG
E.coli	Escherichia coli
EMA	European Medicines Agency
EPO	erythropoietin
F <sub>ab</sub>	antigen binding fragment
FDA	Food and Drug Administration
Fmoc	fluorenylmethoxycarbonyl
FMS	2-sulfo-9-fluorenylmethoxycarbonyl
GalNAc	<i>N</i> -acetylgalactosamine
G-CSF	granulocyte colony stimulating factor
GLP-1	glucagon-like peptide-1
GRF	growth hormone releasing factor
HCl	hydrochloride
hCT	human calcitonin
HEWL	hen egg white lysozyme

His	L-histidine
HPLC	high performance liquid chromatography
IFN	interferon
Ig	immuno globulin
kDa	kilodalton
LHRH	luteinizing hormone-releasing hormone
Leu	L-leucine
Lys	L-lysine
mPEG	methoxypoly(ethylene glycol)
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MRT	mean residence time
Nle	norleucine
NABs	neutralizing antibodies
NNABs	non-neutralizing antibodies
NR	Nile Red
PAL	phenylalanine ammonia lyase
pBAm-PEG	phenylbutylamine-PEG
pBAm	phenylbutylamine
PEG	poly(ethylene glycol)
phe	L-phenylalanine
pnp-PEG	<i>p</i> -nitrophenyl carbonate mPEG
PRCA	pure red-cell aplasia
PVP	poly(N-vinyl pyrrolidone)
RES	reticuloendothelial system
rhIL11	recombinant human interleukin 11
ROS	reactive oxygen species

SCID	severe combined immune deficiency
sCT	salmon calcitonin
SDS-page	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TBNA	trinitrobenzenesulfonic acid
TNF $\alpha$	tumor necrosis factor $\alpha$
2,6-TNS	6-(p-Toluidino)-2-naphthalenesulfonic acid
Trp	L-tryptophan
Trp-mPEG	tryptophan-mPEG
Tyr	tyrosine
WT	wild type

## **Scientific Publications, Patents and Poster Presentations**

### **Publications**

*Non-covalent PEGylation: various PEG-based excipients with different effects on aggregation inhibition of biopharmaceuticals.* C. Mueller, M. A. H. Capelle, S. Martel, P. A. Carrupt, T. Arvinte, G. Borchard. To be submitted (2010).

*Tryptophan-mPEGs: Novel excipients that stabilize salmon calcitonin against aggregation by non-covalent PEGylation.* C. Mueller, M. Capelle, T. Arvinte, E. Seyrek, G. Borchard. Submitted to European Journal of Pharmaceutics and Biopharmaceutics (2010).

*Non-covalent PEGylation by dansyl-PEGs as a new means against aggregation of salmon calcitonin.* C. Mueller, M. Capelle, T. Arvinte, E. Seyrek, G. Borchard. Accepted to Journal of Pharmaceutical Sciences (2010).

*PEGylation and Drug Modifying Techniques for Sustained Drug Action.* C. Mueller and G. Borchard in: H.-C. Mahler, G. Borchard, H. L. Luessen (eds.), Protein Pharmaceuticals. Formulation, Analytics and Delivery, Edition Cantor Verlag, Aulendorf, Germany, 312-352 (2009).

### **Poster Presentations**

*Novel excipients stabilizing Salmon Calcitonin against Aggregation.* C. Müller, G. Borchard, T. Arvinte, M. Capelle. Poster presentation at Science to Market Conference, Vienna (2010).

*Non-covalent PEGylation: Excipients stabilizing Salmon Calcitonin against Aggregation.* C. Müller, G. Borchard, T. Arvinte, M. Capelle. Poster presentation at 2010 Workshop on Protein Aggregation and Immuno-genicity, Breckenridge.

*Non-covalent PEGylation: Excipients stabilizing Salmon Calcitonin against Aggregation.* C. Müller, G. Borchard, T. Arvinte, M. Capelle. Poster presentation at Swiss Pharma Science Day 2010, Bern.

### **Oral Presentations**

*Non-covalent PEGylation-Stabilization of Salmon Calcitonin against Aggregation.* C. Müller, G. Borchard, T. Arvinte, M. Capelle. Oral presentation at 7<sup>th</sup> World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Malta (2010).

### **Patents**

*Stabilized protein formulations and use thereof.* G. Borchard, C. Müller, T. Arvinte, M. Capelle. US 61257054. 02/11/2009



## Curriculum vitae

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hard times and shared enjoying the good times. Thank you mom and dad, thanks to my grand-parents, who unfortunately cannot read this anymore. Thank you Markus, Steffi and Maxim. Thank you Kay, you entered my life and you changed it to the better, I am happy to have you. Thanks also go to Christina, Stefan, Jutta und Jürgen for being my second home in Germany and to Vroni und Rainer. Without the support of all of you this work would not have been possible.

## **Annexes**

### ***STABILIZED PROTEIN FORMULATIONS AND USE THEREOF***

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PCT/US61/257054, to be submitted by November 1<sup>st</sup> 2010.

#### Field of the invention

The present invention is directed to pharmaceutical formulations of therapeutic peptides and proteins, in particular peptides and proteins having a propensity to form aggregates.

#### Background of the invention

The development of a large variety of therapeutic proteins and peptides, notably through the progresses in gene recombinant technologies, has to face severe safety and efficacy problems implying the delicate understanding and control of protein misfolding. In particular, protein aggregation has been for a long time a recurrent problem to address when developing biopharmaceuticals (*Cleland et al., 1993, Crit. Rev. Ther. Drug. Carrier Syst., 10, 307-377*). The formation of protein and peptide aggregates ends up in a broad panel of drawbacks for the producer and the patients spanning from affecting the elegance of the product, its shelf stability, increasing the severity of potential side effects to the rendering of the substance completely unsuitable for use.

Therapeutic protein and peptide aggregation is also a source of batch to batch variabilities in the production chain and its control leads to regulatory and quality control burden which have extremely costly consequences.

Further, aggregation propensity of biopharmaceuticals affects their stability in storage, including shelf-life and their useable administration time, once removed from optimum

storage conditions which often undesirably impose restrictions on their conditioning and administration protocol.

Among potential side effects often associated with the use of biopharmaceuticals having a propensity to aggregate, the decrease in pharmacokinetics of the protein or peptide, the enhancement of the immune response to the protein or peptide and toxicity of aggregates are widely known (Rosenberg, 2006, *The AAPS Journal*, 8(3), E501-E507; Demeule et al., 2006, *Eur. J. Pharm. Biopharm.*, 62:121-30; Bucciantini et al., 2004, *J. Biol. Chem.*, 279:31374-31382). The formation of protein or peptide aggregate-induced antibodies often inhibits drug efficacy and may cause life-threatening complications, especially when directed against endogenous proteins.

PEGylation technology is one of the strategies used in the pharmaceutical industry to improve the pharmacokinetic, pharmacodynamic, and immunological profiles of biopharmaceuticals, and thus enhance their therapeutic effects. This technology involves the covalent attachment of polyethylene glycol (PEG) to a drug and thereby changes the physical and chemical properties of the host biomedical molecule, electrostatic binding, and hydrophobicity, and results in an improvement in the pharmacokinetic profile of the drug.

Currently, PEGylation is used to modify proteins, peptides, oligonucleotides, antibody fragments, and small organic molecules. In general, PEGylation improves drug solubility and decreases immunogenicity, increases drug stability and the retention time of the conjugates in blood, and reduces proteolysis and renal excretion, thereby allowing a reduced dosing frequency (Veronese et al., 2008, *Biodrugs*, 22(5), 315-29; Bailon et al., 2009, *Expert Opin. Drug Deliv.*, 6(1), 1-16). However, the use of PEGylation technology faces some limitations or drawbacks such as being dependent on the presence of specific amino acids in the sequence of the target protein or peptide, implying covalent modifications of the primary structure of the protein, which may also affect its secondary structure and/or its biological activity, involving the use of reactants such as thiols which remain present in the medium as reactive residues after the protein coupling steps and may crosslink with the protein.

Since stability is a major issue for the production, formulation and/or administration of therapeutic proteins and peptides, as protein and peptide instability such as aggregate formation can lead to loss of biological activity, loss of solubility and even increased immunogenicity, the development of a method of stabilizing and/or stable formulations of proteins and peptides, for example for proteins and peptides having a propensity to aggregate that would lead to an increased stability of those bioproducts would be highly desirable.

### Summary of the invention

The invention relates to the unexpected finding of the non-covalent stabilization of proteins such as instable proteins, in particular those having a high propensity to aggregate when formulated in liquid solution, notably in the form of a formulation suitable for administration to a mammal. The invention further relates to the unexpected finding of the stabilizing effects of PEG derivatives on proteins and peptides such as therapeutic proteins and peptides when used in a non-covalent combination, e.g., down to PEG excipients/protein ratios below unity in a process for the preparation of such proteins. Stabilizing effects of proteins according to the invention are supported in particular by the observed reduced propensity of those proteins to form aggregates.

A first aspect of the invention provides a stable protein formulation, said formulation comprising a non-covalent combination of an aqueous carrier, a protein and a PEG derivative, wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group.

A second aspect of the invention provides a pharmaceutical formulation such as a formulation formulated for administration to a mammal (e.g. human) comprising a stable protein formulation according to the invention or a stabilized protein according to the invention.

A third aspect of the invention provides a pharmaceutical unit dosage form suitable to a mammal comprising formulation according to the invention.

A fourth aspect of the invention provides a kit comprising in one or more container(s) a formulation according to the invention together with instruction of use of said formulation.

***A fifth aspect of the invention provides a formulation according to the invention for use as a medicament.***

A sixth aspect of the invention provides a formulation according to the invention for the prevention or treatment of a disease or a disorder.

A seventh aspect of the invention provides a method of stabilizing a protein or peptide in aqueous solution.

An eighth aspect of the invention provides a process for the preparation of a protein or peptide in aqueous solution or a formulation thereof according to the invention.

A ninth aspect of the invention provides a stabilized protein or peptide or a formulation thereof obtainable by a process or a method according to the invention.

A tenth aspect of the invention provides a method of preventing, treating or ameliorating a disease or a disorder, said method comprising administering in a subject in need thereof a



prophylactic or therapeutically effective amount of a formulation according to the invention or of a stabilized protein or peptide according to the invention.

An eleventh aspect of the invention provides a use of a formulation according to the invention or of a stabilized protein or peptide according to the invention for the preparation of a pharmaceutical formulation for the prevention and/or treatment of a disease or disorder.

A twelfth aspect of the invention provides a process for the preparation of a PEG derivative according to the invention.

A thirteenth aspect provides a PEG derivative according to the invention.

#### Description of the figures

**Figure 1** shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 2 via aggregation kinetics. **A:** salmon calcitonin (sCT) alone (x), sCT with dansylamide (◇) 1:1 molar ratio, sCT with dansyl-mPEG 2 kD (△) 1:1 molar ratio, sCT with mPEG-amine 2 kD (□) 1:1 molar ratio measured by fluorescence of Nile red at 620 nm in 10 mM sodium citrate buffer pH 6; **B:** salmon calcitonin (sCT) alone (-), sCT with dansyl-mPEG 2 kD (---) 1:1 molar ratio, measured by turbidity at 450 nm in 10 mM sodium citrate buffer pH 6.

**Figure 2** shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 2 via aggregation kinetics in the early phase of the experiment. **A:** salmon calcitonin (sCT) alone (x), sCT with dansylamide (◇) 1:1 molar ratio, sCT with bis-dansyl-PEG 3 kD (△) 1:1 molar ratio, sCT with PEG-diamine 3 kD (□) 1:1 molar ratio measured by fluorescence of Nile red at 620 nm in 10 mM sodium citrate buffer pH 6; **B:** salmon calcitonin (sCT) alone (-), sCT with bis-dansyl-PEG 3 kD (---) 1:1 molar ratio, measured by turbidity at 450 nm in 10 mM sodium citrate buffer pH 6.

**Figure 3** shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 2 via aggregation kinetics in the early phase of the experiment. **A:** salmon calcitonin (sCT) alone (x), sCT with Tryptophan-mPEG 2 kDa (Δ) 1:1 molar ratio, sCT with Tryptophan-mPEG 2 kDa (▲) 1:5 molar ratio, sCT with Tryptophan-mPEG 2 kDa (-) 1:10 molar ratio, measured by fluorescence of Nile Red at 620 nm in 10 mM sodium citrate buffer pH 6; **B:** salmon calcitonin (sCT) alone (x), sCT with Tryptophan-mPEG 2 kDa (Δ) 1:1 molar ratio, sCT with Tryptophan-mPEG 2 kDa (▲) 1:5 molar ratio, sCT with Tryptophan-mPEG 2 kDa (-) 1:10 molar ratio, measured by turbidity at 500 nm in 10 mM sodium citrate buffer pH 6.

Figure 4 shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 2 via aggregation kinetics in the early phase of the experiment. salmon

calcitonin (sCT) alone (---) measured by fluorescence of Nile Red at 620 nm, sCT with Tryptophan-mPEG 5 kDa (—) 1:5 molar ratio measured by fluorescence of Nile Red at 620 nm; turbidity at 500 nm of salmon calcitonin (sCT) alone (▲), turbidity at 500 nm of sCT with Tryptophan-mPEG 5 kDa (◆) 1:5 molar ratio. All experiments were done in 10 mM sodium citrate buffer pH 6.

**Figure 5** shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 3 via aggregation kinetics in the early phase of the experiment. Hen egg white lysozyme (HEWL) alone (x), HEWL with phenylbutylamine-mPEG 2 kDa (▲) 1:1 molar ratio, HEWL with phenylbutylamine-mPEG 2 kDa (□) 1:10 molar ratio, measured by turbidity at 500 nm in 50 mM sodium phosphate buffer pH 12.2.

**Figure 6** shows the stabilizing effect of PEG derivatives according to the invention such as described in Example 4 via aggregation kinetics in the early phase of the experiment. Hen egg white lysozyme (HEWL) alone (x), HEWL with cholesterol-PEG 2 kDa (■) 1:1 molar ratio, HEWL with cholesterol-PEG 5 kDa (Δ) 1:1 molar ratio, measured by turbidity at 500 nm in 50 mM sodium phosphate buffer pH 12.2.

#### Description of the tables

**Table 1** shows a list of some PEG compounds.

**Table 2** shows the optical density (OD) at 450 nm and Nile Red fluorescence at 620 nm at selected time points during the aggregation kinetics of salmon calcitonin (sCT) alone and sCT with dansyl-mPEG 2 kD in 1:1 molar ratio in 10 mM sodium citrate buffer pH 6 as shown in Figures 1A and B.

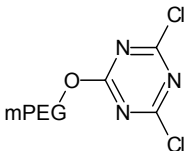
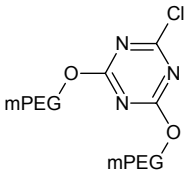
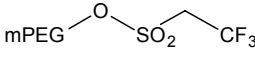
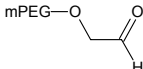
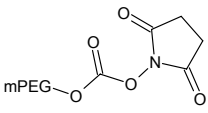
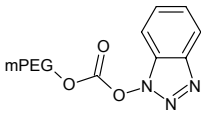
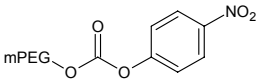
**Table 3** shows the optical density (OD) at 450 nm and Nile Red fluorescence at 620 nm at selected time points during the aggregation kinetics of salmon calcitonin (sCT) alone and sCT with bis-dansyl-PEG 3 kD in 1:1 molar ratio in 10 mM sodium citrate buffer pH 6 as shown in Figures 2A and B. A higher sensitivity of the fluorescence detector has been used during measurements of these data compared to those of Table 2 and Figure 1A.

#### Detailed description of the invention

The term “PEG” or “polyethylene glycol” refers to a polyethylene glycol polymer comprising polymers of the Formula (I):  $R^1-(OCH_2CH_2)_n-X$ , wherein  $R^1$  is selected from H, optionally substituted  $C_1-C_6$  alkyl such as optionally substituted methyl, optionally substituted ethyl and optionally substituted propyl, such as optionally substituted amino  $C_1-C_6$  alkyl (e.g. 5-Dimethylamino-naphthalene-1-sulfonyl ethylamine);  $n$  is selected from 10-500;  $X$  is selected from  $-OR^2$  and  $-C(O)-OR^2$ ;  $R^2$  is selected from H, optionally substituted heteroaryl, optionally

substituted sulfonyl, optionally substituted acyl C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted alkoxycarbonyl such as para-nitrophenoxycarbonyl and optionally substituted alkoxycarbonyl C<sub>1</sub>-C<sub>6</sub> alkyl. In a particular embodiment, “PEG” refers to compounds listed in Table 1 below. Other examples of PEGs are described in *Roberts et al., 2002, Adv. Drug Del. Rev. 54, 459-476*. In particular, the term includes linear PEGs, such as PEGs of Formula (I), wherein R<sup>1</sup> and R<sup>2</sup> are H, monofunctional methyl ether PEG (methoxypoly(ethylene glycol)), abbreviated mPEG (wherein R<sup>1</sup> is CH<sub>3</sub>-, X is –OH), branched PEGs having 2 to 10 PEG chains emanating from a central core group such as an amino acid (e.g., lysine), including linear, forked or branched PEGs. Typically, the molecular weight of the PEGs is about 2 to about 50’000 Daltons (e.g. n is selected from 40 to 1200). In a particular embodiment, the molecular weight of the PEGs that can be used in the context of the invention is about 200 to about 20,000 Daltons. In another particular embodiment, the molecular weight of the PEGs is about 500 to about 1’000 Daltons. In yet another embodiment, the molecular weight of the PEGs is about 1’000 to 8’000 Daltons.

**Table 1**

PEG	Structure	Resulting linkage
dichlorotriazine-PEG		secondary amine
chlorotriazine-PEG		secondary amine
PEG-tresylate		secondary amine
mPEG-acetaldehyde		secondary amine
mPEG-succinimidyl carbonate		carbamate/urethane
mPEG-benzotriazolyl carbonate		carbamate/urethane
mPEG- <i>p</i> -nitrophenyl carbonate		carbamate/urethane

PEG	Structure	Resulting linkage
mPEG-2,3,5-trichlorophenyl carbonate		carbamate/urethane
mPEG-carbonylimidazole		carbamate/urethane
mPEG-succinimidyl succinate		amide
mPEG-aldehyde hydrate		secondary amine
carboxymethylated mPEG		amide
NHS ester of propionic acid mPEG		carbamate
PEG	Structure	Resulting linkage
NHS ester of $\alpha$ -branched propionic acid mPEG		carbamate
thiazolidine-2-thione activated mPEG		carbamate

wherein “Y” represents any branching group.

The term “PEG derivative” refers to a compound comprising at least one polyethylene glycol covalently grafted to a hydrophobic group, wherein the PEG derivative exhibits a stabilizing effect on a protein when combined non-covalently with such protein.

The term “pharmaceutically acceptable derivative” of a specific PEG derivative refers to a PEG derivative which is substituted with from 1 to 5 substituents selected from the group consisting of “C<sub>1</sub>-C<sub>6</sub> alkyl”, amino, halogen, cyano, hydroxy, mercapto, nitro, and the like.

The term “pharmaceutically acceptable salts” refers to salts or complexes of the PEG derivatives according to the invention. Examples of such salts include, but are not restricted, to sodium, potassium, ammonium, hydrochloride, magnesium, calcium.

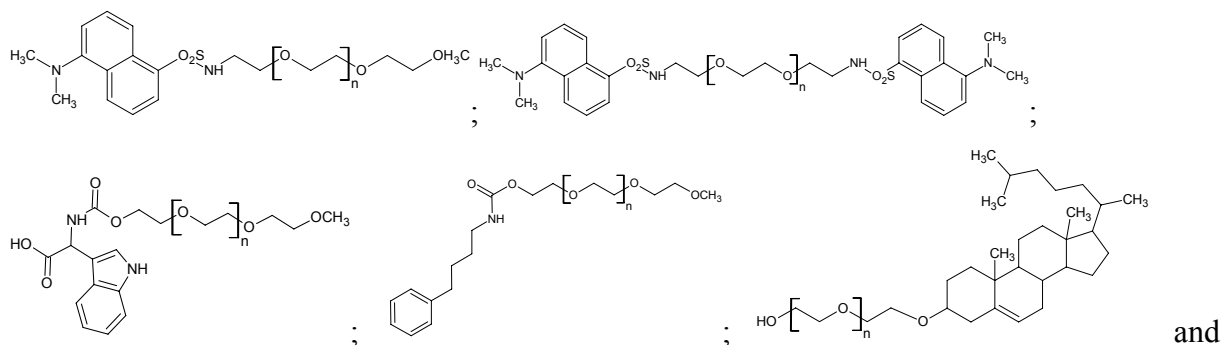
The term “hydrophobic group” comprises any chemical group, which is hydrophobic under following conditions: pH 4-7.5, temperatures between 4°C and 100°C, water, buffer systems used for protein formulations, ethanol and other organic solvents, for example such that its hydrophobicity, expressed as  $\log D$  is of about 0 to about 8 (*Testa et al., 2001, Pharmacokinetic Optimization in Drug Research. Biological, physicochemical, and computational strategies. Editor: Pekka Jäckli, Verlag Helvetica Chimica Acta, Zürich, Switzerland and Wiley-VCH, Weinheim, Germany*). Examples of hydrophobic groups include naphthylamine sulphonic acid groups such as dansylamide, benzyl groups such as benzyl amine, benzyl alcohol, benzyl amide, phenylbutylamine, phenylbutylamide, steroid groups such as cholesterol, triterpenes, saponins, steroid hormones, amino acids such as tryptophan, phenylalanine, leucine, isoleucine, tyrosine, proline, methionine, alanine and peptides thereof. Examples of peptides as hydrophobic groups according to the invention typically range from about 2 to about 50 amino acids. The grafting of a hydrophobic group to a polyethylene glycol to lead to a PEG derivative according to the invention can be obtained through the reaction of a PEG according to the invention (e.g. a polyethylene glycol to wherein the OH side has been activated) with a hydrophobic group as described below.

In a particular embodiment, a PEG derivative refers to at least one polyethylene glycol covalently grafted to a hydrophobic group selected from dansylamide, phenylbutylamine, cholesterol and an amino acid such as tryptophan.

In another particular embodiment, a PEG derivative refers to at least one polyethylene glycol covalently grafted to a hydrophobic group selected from phenylbutylamine, cholesterol and an amino acid such as tryptophan. In a further particular embodiment, a PEG derivative refers to compounds of Formula (II):  $R^1-(OCH_2CH_2)_n-R^3$ , wherein  $R^3$  is selected from  $OR^4$  wherein  $R^4$  is selected from substituted heteroaryl such as optionally substituted indolyl or optionally substituted naphthyl or optionally substituted cyclopentanaphthalenyl groups (e.g. 3-(1,5-Dimethyl-hexyl)-3a,6,6-trimethyl-2,3,3a,4,5,5a,6, 9,9a,9b-decahydro-1H-cyclopenta[a]naphthalene), substituted amide (e.g. formylamino-(1H-indol-3-yl)-acetic acid or N-(4-Phenyl-butyl)-formamide), and substituted amine such as optionally substituted sulfonyl amino (e.g. 5-dimethylamino-naphthalene-1-sulfonyl amine);  $n$  is selected from 40 to 122;  $R^1$  is as defined above. In a particular embodiment,  $R^1$  is methyl.

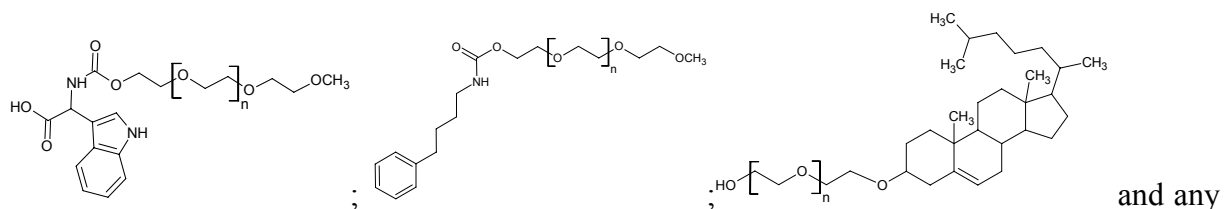
In another particular embodiment,  $R^1$  is H.

In another particular embodiment, “PEG derivative” refers to compounds selected from the group consisting of:



any pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof.

In another particular embodiment, “PEG derivative” refers to compounds selected from the group consisting of:



pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof.

Synthesis of PEG derivatives of the invention may be carried out by known methods, for example as described in *US 5,286,637* or *Miyajima et al., 1987, Colloid Polym. Sci., 265, 943*.

The term “stabilized protein” refers to a protein stabilized by a method according to the invention.

The term “C<sub>1</sub>-C<sub>6</sub> alkyl” when used alone or in combination with other terms, comprises a straight chain or branched C<sub>1</sub>-C<sub>6</sub> alkyl which refers to monovalent alkyl groups having 1 to 6 carbon atoms.

The term “alkoxy C<sub>1</sub>-C<sub>6</sub> alkyl” refers to C<sub>1</sub>-C<sub>6</sub> alkyl groups having an alkoxy substituent, including methoxyethyl and the like.

The term “heteroaryl” refers to a monocyclic heteroaromatic, or a bicyclic or a tricyclic fused-ring heteroaromatic group. For example, heteroaryl refers to indolyl, or naphthyl or cyclopentanaphthalenyl groups.

The term “acyl C<sub>1</sub>-C<sub>6</sub> alkyl” to C<sub>1</sub>-C<sub>6</sub> alkyl groups having an acyl substituent, including 2-acetyethyl and the like.

The term “sulfonyl” refers to group “-SO<sub>2</sub>-R” wherein R is selected from “aryl,” “heteroaryl,” “C<sub>1</sub>-C<sub>6</sub> alkyl,” “C<sub>1</sub>-C<sub>6</sub> alkyl” substituted with halogens, *e.g.*, an -SO<sub>2</sub>-CF<sub>3</sub> group, “C<sub>2</sub>-C<sub>6</sub>

alkenyl,” “C<sub>2</sub>-C<sub>6</sub> alkynyl,” “C<sub>3</sub>-C<sub>8</sub>-cycloalkyl,” “heterocycloalkyl,” “aryl,” “heteroaryl,” “aryl C<sub>1</sub>-C<sub>6</sub> alkyl,” “heteroaryl C<sub>1</sub>-C<sub>6</sub> alkyl,” “aryl C<sub>2</sub>-C<sub>6</sub> alkenyl,” “heteroaryl C<sub>2</sub>-C<sub>6</sub> alkenyl,” “aryl C<sub>2</sub>-C<sub>6</sub> alkynyl,” “heteroaryl C<sub>2</sub>-C<sub>6</sub> alkynyl,” “cycloalkyl C<sub>1</sub>-C<sub>6</sub> alkyl,” or “heterocycloalkyl C<sub>1</sub>-C<sub>6</sub> alkyl”.

The term “sulfonylamino” refers to a group –NRSO<sub>2</sub>-R’ where R and R’ are independently H, “C<sub>1</sub>-C<sub>6</sub> alkyl,” “C<sub>2</sub>-C<sub>6</sub> alkenyl,” “C<sub>2</sub>-C<sub>6</sub> alkynyl,” “C<sub>3</sub>-C<sub>8</sub>-cycloalkyl,” “heterocycloalkyl,” “aryl,” “heteroaryl,” “aryl C<sub>1</sub>-C<sub>6</sub> alkyl,” “heteroaryl C<sub>1</sub>-C<sub>6</sub> alkyl,” “aryl C<sub>2</sub>-C<sub>6</sub> alkenyl,” “heteroaryl C<sub>2</sub>-C<sub>6</sub> alkenyl,” “aryl C<sub>2</sub>-C<sub>6</sub> alkynyl,” “heteroaryl C<sub>2</sub>-C<sub>6</sub> alkynyl,” “C<sub>3</sub>-C<sub>8</sub>-cycloalkyl C<sub>1</sub>-C<sub>6</sub> alkyl,” or “heterocycloalkyl C<sub>1</sub>-C<sub>6</sub> alkyl”.

The term “alkoxycarbonyl” refers to the group –C(O)OR where R includes “C<sub>1</sub>-C<sub>6</sub> alkyl,” “aryl,” “heteroaryl,” “aryl C<sub>1</sub>-C<sub>6</sub> alkyl,” “heteroaryl C<sub>1</sub>-C<sub>6</sub> alkyl” or “heteroalkyl”.

Unless otherwise constrained by the definition of the individual substituent, the term “substituted” refers to groups substituted with from 1 to 5 substituents selected from the group consisting of “C<sub>1</sub>-C<sub>6</sub> alkyl,” “C<sub>2</sub>-C<sub>6</sub> alkenyl,” “C<sub>2</sub>-C<sub>6</sub> alkynyl,” “C<sub>3</sub>-C<sub>8</sub>-cycloalkyl,” “heterocycloalkyl,” “C<sub>1</sub>-C<sub>6</sub> alkyl aryl,” “C<sub>1</sub>-C<sub>6</sub> alkyl heteroaryl,” “C<sub>1</sub>-C<sub>6</sub> alkyl cycloalkyl,” “C<sub>1</sub>-C<sub>6</sub> alkyl heterocycloalkyl,” “amino,” “aminosulfonyl,” “ammonium,” “acyl amino,” “amino carbonyl,” “aryl,” “heteroaryl,” “sulfinyl,” “sulfonyl,” “alkoxy,” “alkoxy carbonyl,” “carbamate,” “sulfanyl,” “halogen,” trihalomethyl, cyano, hydroxy, mercapto, nitro, and the like.

The term “amphipathic peptide” comprises peptides containing both hydrophilic and hydrophobic amino acid residues, where spatial separation of these residues, such as for example through the secondary structure of the peptide, result in their ability to partition at an interface between a polar and an apolar medium such as a lipidic interface, an air/water interface, hydrophilic solvent/hydrophobic solvent interface and air/packaging material interface. Typically, amphipathic peptides present an amphipathicity defined by a mean hydrophobic moment between about 0 and about 0.9, according to the Eisenberg plot (*Eisenberg et al., 1984, J. Mol. Biol. 179, 125-142*). Typical amphipathic peptides used in the context of the invention include samples from reference *McLean. et al., 1991, Biochemistry 30, 31-37*.

The term “protein” includes any natural, synthetic or recombinant protein or peptide, in particular proteins, notably therapeutic proteins (e.g., polypeptides, enzymes, antibodies, hormones) which are unstable in solution such as for example hydrophobic proteins. Typically, molecular weight of the peptides and proteins according to the invention range from about 200 D to about 1’000 kD. Examples of proteins in the context of the invention are

salmon calcitonin (sCT), interferon-beta and granulocyte-colony stimulating factor (G-CSF). In another embodiment, an example of a protein according to the invention comprises hen egg white lysozyme (HEWL).

As used herein, “treatment” and “treating” and the like generally mean obtaining a desired pharmacological and physiological effect. The effect may be prophylactic in terms of preventing or partially preventing a disease, symptom or condition thereof and/or may be therapeutic in terms of a partial or complete cure of a disease, condition, symptom or adverse effect attributed to the disease. The term “treatment” as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it such as a preventive early asymptomatic intervention; (b) inhibiting the disease, i.e., arresting its development; or relieving the disease, i.e., causing regression of the disease and/or its symptoms or conditions such as improvement or remediation of damage.

The term “subject” as used herein refers to mammals. For examples, mammals contemplated by the present invention include human, primates, domesticated animals such as cattle, sheep, pigs, horses, laboratory rodents and the like.

The term "effective amount" as used herein refers to an amount of at least one protein or a pharmaceutical formulation thereof according to the invention that elicits the biological or medicinal response in a tissue, system, animal or human that is being sought. In one embodiment, the effective amount is a "therapeutically effective amount" for the alleviation of the symptoms of the disease or condition being treated. In another embodiment, the effective amount is a "prophylactically effective amount" for prophylaxis of the symptoms of the disease or condition being prevented. The term also includes herein the amount of active polypeptide sufficient to reduce the progression of the disease thereby elicit the response being sought (i.e. an "inhibition effective amount").

The term “efficacy” of a treatment according to the invention can be measured based on changes in the course of disease in response to a use or a method according to the invention.

The term “stable” or “stabilized” refers in the context of the invention to formulations in which the protein therein retains its physical stability (e.g. level of aggregation or aggregation propensity decreased, absence of precipitation or denaturation) and/or chemical stability (e.g. absence of chemically altered forms by disulfide bond formation or exchange) upon formulation or storage. Stability of the protein formulations according to the invention may be measured by various techniques known to the skilled person in the art. For example, stability can be measured by aggregation state measurements (e.g., by field flow fractionation, light



scattering, high performance size exclusion, ultracentrifugation, turbidity measurements, fluorescence microscopy, electron microscopy, others named in *Mahler et al.*, 2008, *J. Pharm. Sci.*, 98(9):2909-2934. Preferably, the stability of the formulation is measured at a selected temperature and/or for a selected period of time storage.

The term “stabilizing amount” according to the invention refers to an amount of at least one PEG derivative according to the invention that elicits the stabilizing effect on a protein. The stabilizing effect of a PEG derivative or a method according to the invention on a protein can be measured by a reduction in the rate and extent of aggregation of the protein once non-covalently combined with a PEG derivative according to the invention, such as described in (*Capelle et al.*, 2009, *Pharm. Res.*, 26 :118-128). Alternatively, the stabilizing effect of a PEG derivative or a method according to the invention on a protein can be measured by an increased bioavailability and/or a decrease of immunogenicity of the protein once non-covalently combined with a PEG derivative according to the invention, such as described in *Graham*, 2003, *Adv. Drug Del. Rev.*, 55: 1293-1302 or *Caliceti et al.* 2003, *Adv. Drug Del. Rev.*, 55: 1261-1277.

The term “pharmaceutical formulation” refers to preparations which are in such a form as to permit biological activity of the active ingredient(s) to be unequivocally effective and which contain no additional component which would be toxic to subjects to which said formulation would be administered.

#### PEG derivatives according to the invention

According to an embodiment, is provided a PEG derivative according to the invention wherein said at least one polyethylene glycol is covalently grafted to a hydrophobic group, wherein the PEG is above defined. In a particular embodiment, PEG is selected from m-PEGs, in particular m-PEGs of molecular weight of 2kDa or 3kDa. In another particular embodiment, PEG is an m-PEG of molecular weight of 5kDa.

According to another embodiment, is provided a PEG derivative according to the invention wherein the hydrophobic group is selected from groups having a log *D* between 0 and 8. In another particular embodiment, the hydrophobic group is a dansyl group (DNS). In another particular embodiment, the hydrophobic group is selected from phenylbutylamine, cholesterol and an amino acid such as tryptophan.

#### Formulations according to the invention

According to an embodiment, is provided a stable protein formulation, said formulation comprising a non-covalent combination of an aqueous carrier, a protein and a PEG derivative,

wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group.

According to another embodiment, is provided a stabilized protein or a formulation thereof obtainable by a process or a method according to the invention.

According to further embodiment, the invention provides a formulation according to the invention wherein the protein formulation thereof is at a concentration in the range from about 0.01 ng/ml to about 500 mg/ml.

According to another further embodiment, the invention provides a formulation according to the invention wherein the PEG derivative is at a concentration in the range from about 0.001 ng/ml to about 1 g/ml.

According to another further embodiment, the invention provides a formulation according to the invention wherein the molar ratio PEG derivative to protein is in the range from about 1:0.001 molar ratio to about 1:1'000.

According to another further embodiment, the invention provides a formulation according to the invention wherein the molar ratio PEG derivative to protein is in the range from about 1:1 molar ratio to about 1:100.

According to another further embodiment, the invention provides a formulation according to the invention wherein the molar ratio PEG derivative to protein is 1:1.

According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is an mPEG.

According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is an mPEG of molecular weight of 2 kDa.

According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is an mPEG of molecular weight of 5 kDa.

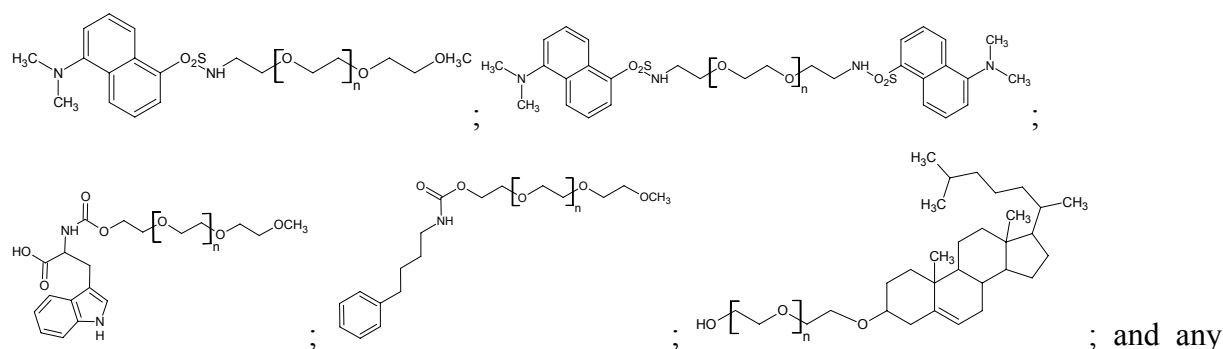
According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is such that the said at least one polyethylene glycol moiety is covalently grafted to a hydrophobic group selected from dansylamide, tryptophan, phenylbutylamine, cholesterol, and an amphipathic peptide.

According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is such that the said at least one polyethylene glycol moiety is covalently grafted to a hydrophobic group selected from tryptophan, phenylbutylamine and cholesterol.

According to another further embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is of Formula (II):  $R^1-(OCH_2CH_2)_n-R^3$ , wherein

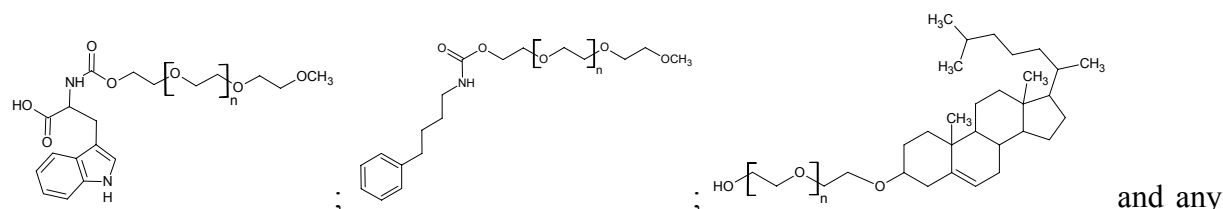
$R^3$  is selected from  $OR^4$  wherein  $R^4$  is selected from substituted heteroaryl such as optionally substituted indolyl or optionally substituted naphthyl or optionally substituted cyclopentanaphthalenyl groups (e.g. 3-(1,5-Dimethyl-hexyl)-3a,6,6-trimethyl-2,3,3a,4,5,5a,6,9,9a,9b-decahydro-1H-cyclopenta [a]naphthalene), substituted amide (e.g. formylamino-(1H-indol-3-yl)-acetic acid or N-(4-phenyl-butyl)-formamide), and substituted amine such as optionally substituted sulfonyl amino (e.g. 5-dimethylamino-naphthalene-1-sulfonyl amino); n is selected from 40 to 120;  $R^1$  is as defined above.

In another particular embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is selected from the group consisting of:



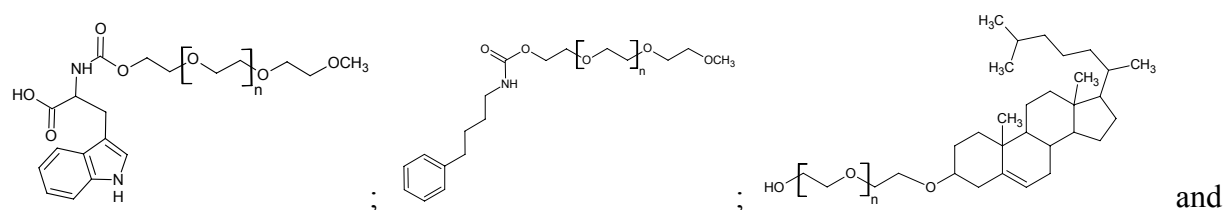
pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof.

In another particular embodiment, is provided a stable protein formulation according to the invention wherein the PEG derivative is selected from the group consisting of:



pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof.

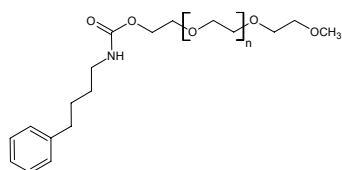
According to another further embodiment, is provided a stable protein formulation according to the invention wherein the protein is selected from sCT and HEWL and the PEG derivative is selected from the group consisting of:



any pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof

According to another further embodiment, the invention provides a formulation according to the invention further comprising an excipient.

In another particular embodiment, is provided a PEG derivative according to the invention, wherein the PEG derivative is:



Formulations of the invention, together with a conventionally employed adjuvant, carrier, diluent or excipient may be placed separately into the form of pharmaceutical compositions and unit dosages thereof, and in such form may be employed as liquids such as solutions, suspensions, emulsions, elixirs, or capsules filled with the same, all in the form of sterile injectable solutions. Such pharmaceutical compositions and unit dosage forms thereof may comprise ingredients in conventional proportions, with or without additional active compounds or principles, and such unit dosage forms may contain any suitable effective amount of the active ingredient commensurate with the intended daily dosage range to be employed.

Such liquid preparations may contain additives including, but not limited to, suspending agents, emulsifying agents, non-aqueous vehicles and preservatives. Suspending agents include, but are not limited to, sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminum stearate gel, and hydrogenated edible fats. Emulsifying agents include, but are not limited to, lecithin, sorbitan monooleate, and acacia. Injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art.

In another particular aspect, the formulation is adapted for delivery by repeated administration.

Further materials as well as formulation processing techniques and the like are set out in *Part 5 of Remington's Pharmaceutical Sciences, 21<sup>st</sup> Edition, 2005, University of the Sciences in Philadelphia, Lippincott Williams & Wilkins*, which is incorporated herein by reference.

Formulations according to the invention, stabilized protein and formulations thereof obtainable by a process or a method according to the invention are useful in the prevention and/or treatment of a disease or a disorder.

#### Methods of preparation according to the invention

According to one aspect of the invention, is provided a method of stabilizing a protein in aqueous solution by non-covalently combining said protein with a PEG derivative according to the invention.

According to another embodiment, is provided a process for the preparation of a protein or a formulation thereof comprising the steps of:

- (i) non-covalently combining said protein with a PEG derivative into a liquid mixture or forming said protein in a liquid medium containing a PEG derivative, wherein the said PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group;
- (ii) collecting the liquid mixture or liquid medium obtained under step (i) containing the stabilized protein non-covalently combined with the said PEG derivative, wherein the percentage of monomers of protein is increased as compared to said protein prepared in absence of the said PEG derivative.

Typically, for a PEG derivative being PEG-DNS, the percentage of aggregates of stabilized protein formulation is reduced by about at least 30% after ca. 7 days at 26°C at 2.5 mg/ml.

Typically, for a PEG derivative being PEG-Trp, the percentage of aggregates of stabilized

sCT formulation is reduced by about at least 90% after ca. 2.5 days at 26°C at 2.5 mg/ml for a molar ratio sCT/PEG derivative of 1:10.

Typically, for a PEG derivative being Cholesteryl-PEG, the percentage of aggregates of stabilized protein formulation is reduced by about at least 100%. For a PEG derivative being phenylbutylamine, the onset of the aggregation process is shifted of about at least 3.5 hours for a molar ratio HEWL/PEG derivative of 1:10.

In a particular embodiment, is provided a method according to the invention wherein the said PEG derivative is an mPEG derivative.

According to another further embodiment, is provided a method according to the invention wherein the said PEG derivative is an mPEG derivative of molecular weight of 2 kDa.

According to another further embodiment, is provided a method according to the invention wherein the said PEG derivative is an mPEG derivative of molecular weight of 5 kDa.

In a particular embodiment, is provided a method according to the invention wherein the said PEG derivative is such that the said at least one polyethylene glycol covalently grafted to a hydrophobic group selected from dansylamide, tryptophan, phenylbutylamine, cholesterol, and an amphipathic peptide. In a particular embodiment, the hydrophobic group is selected from phenylbutylamine, dansylamide, cholesterol and an amino acid such as tryptophane.

In a further embodiment, the invention provides a method or a process according to the invention wherein the aqueous solution is a pharmaceutical formulation and the protein is in a therapeutically effective amount.

In a further embodiment, the invention provides a method, a process, a use or a formulation according to the invention wherein the protein is selected from sCT and HEWL.

In a further aspect of the invention, the method or process according to the invention may be useful in decreasing the aggregation ability of a protein during its production process.

In another aspect the method or process according to the invention may be useful in preparing stable formulations of proteins presenting an increased shelf-life and enabling multiple dosing conditioning.

In another aspect is provided a process for the preparation of a PEG derivative according to the invention comprising the step of reacting an mPEG-p-nitrophenyl carbonate with

phenylbutylamine in an anhydrous solvent, typically selected from dichloromethane, chloroform, Dimethylformamide (DMF) and Dimethyl Sulfoxide (DMSO) at a pH between about 9 and about 11 at room temperature.

#### Mode of administration

Formulations of this invention may be administered in any manner including parenterally, transdermally, rectally, transmucosally, intra-ocular or combinations thereof. Parenteral administration includes, but is not limited to, intravenous, intra-arterial, intra-peritoneal, subcutaneous, intramuscular, intra-theal, and intra-articular. The compositions of this invention may also be administered in the form of an implant, which allows slow release of the compositions as well as a slow controlled i.v. infusion.

#### Methods according to the invention

According to another aspect, the invention provides a method of preventing, treating or ameliorating a disease or a disorder, said method comprising administering in a subject in need thereof a prophylactic or therapeutically effective amount of a stable protein formulation or a formulation of a stabilized protein obtainable by a process or a method according to the invention.

The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factors, including pharmacokinetic properties, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired.

#### Patients

In an embodiment, patients according to the invention are patients suffering from a disease or a disorder for which the protein of the invention is therapeutically beneficial. The stabilized formulation according to the invention of the said protein allows the use of lower doses of said protein, and/or increases the protein therapeutic efficacy and/or leads to a decrease in side effects as compared to the protein administered in the form of known formulations.

References cited herein are hereby incorporated by reference in their entirety. The present invention is not to be limited in scope by the specific embodiments and drawings described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. The

examples illustrating the invention are not intended to limit the scope of the invention in any way.

## EXAMPLES

### GENERAL PROCEDURES & CONDITIONS

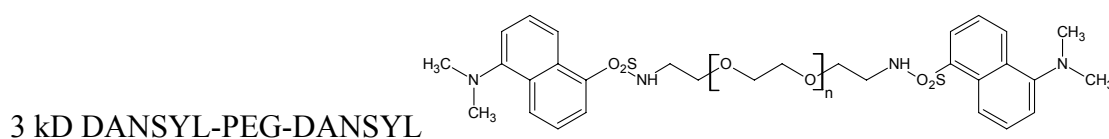
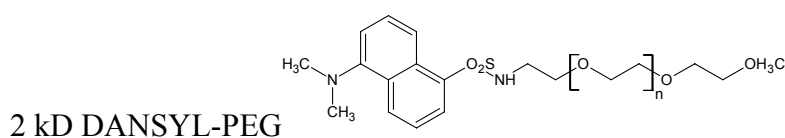
The following studies are conducted to support the influence of a PEG derivative according to the invention on the stability of proteins. Aggregation (reduction or absence of which) of the protein is measured to determine whether its non-covalent association with a PEG derivative according to the invention into a single formulation influences the aggregation state of this protein. Since aggregates have been observed to cause severe side-effects, this study is of great importance for anticipating beneficial effects in clinical use. Further, bioavailability and immunogenicity studies are conducted to support further stabilizing effects.

The following abbreviations refer respectively to the definitions below:

**a.u.** (arbitrary units), **hr** (hours), **i.v.** (intravenous), **kD** or **kDa** (kilo Dalton), **MHz** (Megahertz), **mM** (millimolar), **nm** (nanometer), **ppm** (parts per million), **qs** (quantum satis), **s.c.** (subcutaneous) **Ar** (aromatic), **FFF** (flow field-flow fractionation), **DMSO** (Dimethyl Sulfoxide), **DNS** (Dansyl), **DANSA** (dansylamide), **FTIR** (Fourier Transform Infrared), **LS** (light scattering), **MS** (mass spectrometry), **NMR** (Nuclear Magnetic resonance), **OD** (optical density), **TFA** (Trifluoroacetic acid), **UV** (Ultraviolet).

#### Example 1: Synthesis of DNS-PEG derivatives

The following PEG derivatives according to the invention (of Formula (II), wherein  $R^3$  is substituted sulfonyl amino (e.g. 5-dimethylamino-naphthalene-1-sulfonyl amine);  $n$  is selected from 40 to 120 and  $R^1$  is optionally substituted  $C_1$ - $C_6$  alkyl (e.g. methyl) or substituted amino  $C_1$ - $C_6$  alkyl (e.g. 5-Dimethylamino-naphthalene-1-sulfonyl ethylamine), respectively) were synthesized as follows:



*Synthesis of dansyl-PEG 2 kDa (Method adapted from Pendri et al., 1995, Bioconjugate Chem., 6, 596)*



0.33 mMol of dried mPEG-amine 2 kDa (Iris Biotech, Germany) were dissolved in 34 ml of anhydrous toluene and 0.98 mMoles of dansyl chloride and 0.13 mMoles of dry triethyl amine were added. The reaction was performed at 100 °C under reflux for 24 hours. Toluene was evaporated and the solid was redissolved in dichloromethane. After precipitation from cold diethyl ether, the solid was collected via filtration and reprecipitated from isopropyl alcohol. A slightly yellowish powder was obtained that was dried under vacuum and characterized by NMR, UV and FTIR spectrometry. <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 2.82 ppm, CH<sub>3</sub>-N- (s); 2.96 ppm, CH<sub>3</sub>-N- (s); 3.23 ppm, CH<sub>3</sub>-O- (s); 3.50 ppm, -O-CH<sub>2</sub>- (s); 7.27 ppm, aromatic (d); 7.60 ppm, aromatic (t); 8.10 ppm, aromatic (d); 8.36 ppm, aromatic (d); 8.45 ppm, aromatic (d). <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 42.04 ppm, CH<sub>3</sub>-N- (s); 44.90 ppm, CH<sub>3</sub>-N- (s); 57.85 ppm, CH<sub>3</sub>-O- (s); 69.59 ppm, -O-CH<sub>2</sub>- (m); 114.89 ppm, aromatic (s); 119.08 ppm, aromatic (s); 123.41 ppm, aromatic (s); 127.80 ppm, aromatic (s); 128.99 ppm, aromatic (s); 136.14 ppm, aromatic (s); 151.12 ppm, aromatic (s).

*Synthesis of bis-dansyl-PEG 3 kDa (Method adapted from Pendri. et al., 1995, above)*

0.033 mMol of dried PEG-diamine 3 kDa (Iris Biotech, Germany) were dissolved in 30 ml of anhydrous toluene and 0.2 mMoles of dansyl chloride and 0.27 mMoles of dry triethyl amine were added. The reaction was performed at 100 °C under reflux for 24 hours. Toluene was evaporated and the solid was redissolved in dichloromethane. After precipitation from cold diethyl ether, the solid was collected via filtration and reprecipitated from isopropyl alcohol. A slightly yellowish powder was obtained that was dried under vacuum and characterized by NMR, UV and FTIR spectrometry. <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 2.83 ppm, CH<sub>3</sub>-N- (s); 2.95 ppm, CH<sub>3</sub>-N- (s); 3.23 ppm, CH<sub>3</sub>-O- (s); 3.50 ppm, -O-CH<sub>2</sub>- (s); 3.50 ppm, -O-CH<sub>2</sub>-; 7.24 ppm, aromatic (d); 7.59 ppm, aromatic (t); 8.10 ppm, aromatic (d); 8.28 ppm, aromatic (d); 8.45 ppm, aromatic (d). <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 42.43 ppm, CH<sub>3</sub>-N- (s); 45.27 ppm, CH<sub>3</sub>-N- (s); 69.98 ppm, -O-CH<sub>2</sub>- (m); 115.27 ppm, aromatic (s); 119.47 ppm, aromatic (s); 123.77 ppm, aromatic (s); 128.01 ppm, aromatic (s); 129.36 ppm, aromatic (s); 136.56 ppm, aromatic (s); 151.49 ppm, aromatic (s).

**Example 2: Comparison of the aggregation propensity of calcitonin alone and in combination with DNS-mPEGs**

In order to assess the stabilizing effect of PEG derivatives according to the invention, the aggregation propensity of salmon calcitonin (sCT) is assayed in presence or absence of PEG derivatives according to the invention. Salmon calcitonin is a 32-amino acid polypeptide hormone (Martha et al., 1993, *Biotechnology*, 11, 64 - 70). It acts to reduce blood calcium

(Ca<sup>2+</sup>), is used for the treatment of various bone associated disorders (*Capelle et al., 2009, Pharm. Res., 26: 118-128*) and has a lower propensity to aggregate in solution than the human form (*Gaudiano et al., 2005, Biochim Biophys Acta 1750:134-145*).

#### *Aggregation of salmon calcitonin (sCT)*

Salmon calcitonin (*Therapeomic Inc., Switzerland*) in a final concentration of 2.5 mg/ml per well with and without the respective excipients to be tested, i.e. non-conjugated mPEG-amines and non-conjugated hydrophobic headgroups were prepared in 4 different buffer systems: 10 mM sodium acetate pH 5, 10 mM sodium citrate pH 5, 10 mM sodium citrate pH 6, 10 mM sodium phosphate buffer pH 8. Samples are prepared two times and Nile Red in a final concentration of 1 µM is added to one of each. Aggregation is followed in UV-transparent 96-well plates or 384-well Costar® plates from Corning (Corning Life Sciences, Schiphol, Netherlands) by a microplate reader (Tecan Safire™ microplate reader, Tecan Group Ltd, Männedorf, Switzerland) by monitoring turbidity, Nile Red fluorescence and intrinsic fluorescence of the protein/peptide drug or the hydrophobic head-group. After finishing the aggregation kinetics, final spectra of Nile Red fluorescence, UV and protein/peptide drug or hydrophobic head group fluorescence were measured.

#### *Aggregation propensity of calcitonin alone and in combination with mPEGs*

Salmon calcitonin (sCT) was aggregated using different buffer systems in which sCT was shown to be unstable (*Capelle et al., 2009, Pharm. Res., 26:118-128*). Aggregation was checked in absence of any excipient, in presence of equimolar amounts of dansylamide, mPEG-amine 2 kD and dansyl-mPEG 2 kD, respectively. Lower aggregation was seen for the equimolar mixture of sCT with dansyl-mPEG 2 kD in citrate buffer pH 6 by checking Nile Red fluorescence at 620 nm over time (Fig. 1A, 2A) and turbidity at 450 nm (Fig. 1B, 2B). It can be clearly seen by both techniques that the final level of aggregation is lower. Furthermore, turbidity shows that the onset of aggregation has been prolonged. The same tendency was observed in phosphate buffer pH 8.

**Table 2**

time (hrs)	OD at 450 nm		time (hrs)	Nile red fluorescence at 620 nm	
	sCT	sCT + D-PEG2		sCT	sCT + D-PEG2
0	0.10	0.10	0	1058	181
9.5	0.22	0.12	9.5	18581	6375
20	0.63	0.37	20	27276	11818
100	0.68	0.55	100	25328	14469
166	0.64	0.57	166	24729	14144
lag time of aggregation	3.5 hours	10.9 hours	slope of aggregation curve	1843 a.u./hrs	488 a.u./hrs

**Table 3**

time (hrs)	OD at 450 nm		time (hrs)	Nile red fluorescence at 620 nm	
	sCT	sCT + bis-D-PEG3		sCT	sCT + bis-D-PEG3
0	0.11	0.11	0	4560	2599
6.2	0.15	0.13	5	38483	24967
10	0.22	0.15	10	61019	49030
30.5	0.49	0.39	13.5	> 65000	61143
140	0.56	0.50	20	> 65000	> 65000
lag time of aggregation	4.2 hours	8.0 hours	slope of aggregation curve	5546 a.u./hrs	4351 a.u./hrs

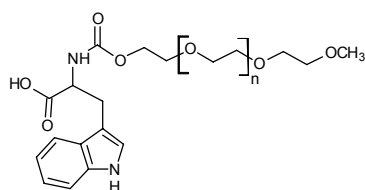
*Bis-D-PEG3* = *bis-DNS-PEG 3kDa*; *D-PEG2* = *DNS-mPEG 2kDa*

Experiments with sCT (salmon calcitonin) formulations according to the invention at various SCT/Dansyl-PEG 2kDa molar ratios (100:1, 5:1 and 1:1) show an increasing stabilizing effect

with increasing molar ratios, the higher stabilizing effects being obtained for a molar ratio of 1:1. Further, the results show that there is a stabilizing effect occurring at very early stage of the mixture between the PEG derivative according to the invention and the protein and the formulations are stable over time (at least up to 72 hours).

### **Example 3: Synthesis of Trp-PEG derivatives**

The following PEG derivatives according to the invention (2 kDa and 5 kDa Trp-PEGs) (of Formula (II) wherein  $R^3$  is  $OR^4$  wherein  $R^4$  is substituted amide (e.g. formylamino-(1H-indol-3-yl)-acetic acid); n is selected from 40-120;  $R^1$  is optionally substituted  $C_1$ - $C_6$  alkyl (e.g. methyl)) were synthesized as depicted in Scheme 1 below:



#### *Synthesis of mPEG-p-nitrophenyl carbonate 2 kDa (method adapted from US 5,286,637)*

1.76 mMol of dried mPEG-OH 2 kDa (Iris Biotech GmbH, Marktredwitz, Germany) were dissolved in anhydrous dichloromethane and 5.27 mMoles of p-nitrophenyl chloroformate (Acros Organics BVBA; Geels, Belgium) and 3.52 mMoles of dry triethyl amine were added (1:3:2 ratio). The pH was adapted between 7.5 - 8 and reaction was left to proceed at room temperature for 24 hours. Reaction was stopped by adding several drops of TFA until the solution was colourless, then dichloromethane was partially evaporated and precipitation from cold diethyl ether was performed. The solid collected via filtration was twice redissolved in dichloromethane, precipitated from cold diethyl ether, and collected via filtration. A slightly yellowish powder was obtained and dried under vacuum.  $^1H$ -NMR (300 MHz, DMSO- $d_6$ ): 3.23 ppm, PEG  $CH_3$ -O- (s); 3.50 ppm, PEG -O- $CH_2$ - (m); 7.55 ppm, p-nitrophenyl-aromate (d); 8.31 ppm, p-nitrophenyl-aromate (d).  $^{13}C$ -NMR (300 Mhz, DMSO- $d_6$ ): 58.06 ppm, PEG  $CH_3$ -O-; 69.52 ppm, PEG -O- $CH_2$ -; 122.59 ppm, p-nitrophenyl-aromate; 125.34 ppm, p-nitrophenyl-aromate; 144.21 ppm, PEG -O- $CH_2$ -C=O; 151.99 ppm, aromatic  $C_5H_4=C-NO_2$ ; 155.27 ppm, PEG - $CH_2-OCO$ -. FTIR: 3435; 2888; 2739; 2678; 2493; 1967; 1769; 1617; 1594; 1527; 1468; 1360; 1343; 1281; 1242; 1113; 1060; 963; 841; 663; 529  $cm^{-1}$ . MS (MALDI-TOF):  $m/z$  2201 ( $M^+$ ).

#### *Synthesis of Tryptophan-mPEG 2 kDa (method adapted from US 5,286,637)*

0.018 Mol L-Tryptophan (Fluka (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland)) were dissolved in anhydrous DMSO and pH was adapted to ~8.3. Then, 1.76 mMol of dried

mPEG-*p*-nitrophenyl carbonate 2 kDa obtained as described above were added. The pH was maintained at ~8.3 and reaction was left to proceed at room temperature for 4 hours. Reaction was stopped by cooling to 0°C and adapting pH to 3 with 2 M HCl. The aqueous phase was extracted with chloroform. The obtained organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and partially evaporated. Precipitation from cold diethyl ether was performed and the solid collected via filtration. The solid was once reprecipitated from cold diethyl ether, and twice from cold iso-propanol. A slightly yellowish powder was obtained and dried under vacuum. <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 3.17 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (d); 3.24 ppm, PEG -CH<sub>3</sub>-O- (s); 3.51 ppm, PEG -O-CH<sub>2</sub>- (m); 4.17 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (q); 6.98 ppm, Trp-indole (t); 7.06 ppm, Trp-indole (t); 7.16 ppm, Trp-indole (s); 7.32 ppm, Trp-indole (d); 7.51 ppm, Trp-indole (d); 10.82 ppm Trp -COOH (s). <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 54.78 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 58.58 ppm, PEG CH<sub>3</sub>-O-; 63.28 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 69.70 ppm, PEG -O-CH<sub>2</sub>-; 110.02 ppm, Trp-indole; 111.33 ppm, Trp-indole; 117.79 ppm, Trp-indole; 120.80 ppm, Trp-indole; 123.65 ppm, Trp-indole; 126.88 ppm Trp-indole; 136.17 ppm, Trp-indole; 156.26 ppm, PEG -CH<sub>2</sub>-OCO-NH-; 173.87 ppm, -COOH. FTIR: 3412; 2886; 2741; 2695; 2167; 1970; 1721; 1526; 1467; 1413; 1360; 1343; 1280; 1242; 1110; 963; 842; 745, 529 cm<sup>-1</sup>. MS (MALDI-TOF): *m/z* 2266 (M<sup>+</sup>).  $[\alpha]_D^{20} = -0.005$ .

*Synthesis of mPEG-p-nitrophenyl carbonate 5 kDa (method adapted from US 5,286,637)*

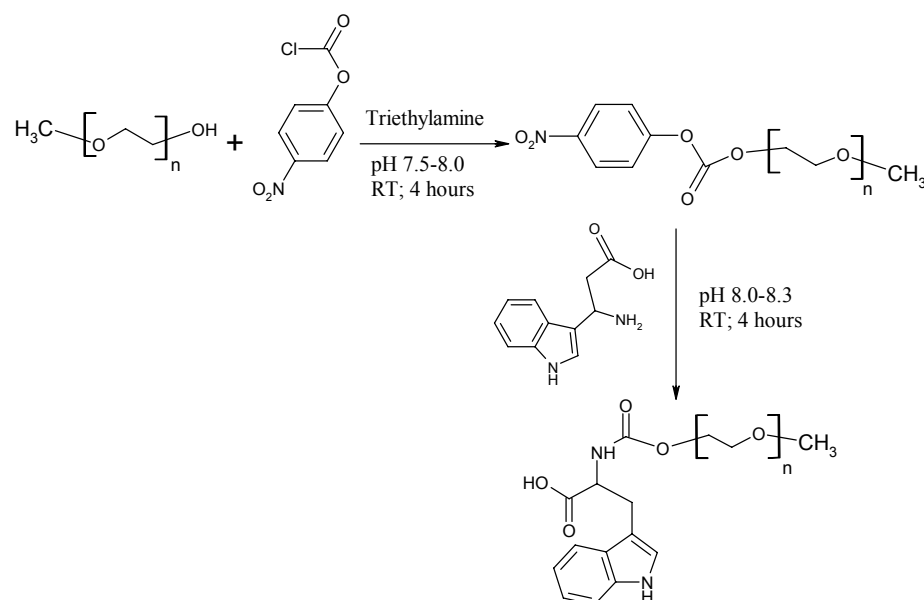
The reaction was performed as described for the mPEG-*p*-nitrophenyl carbonate 2 kDa, where 0.68 mMol of dried mPEG-OH 5 kDa, 2.03 mMoles of *p*-nitrophenyl chloroformate and 1.36 mMoles of dry triethyl amine were used. A slightly yellowish powder was obtained. <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 3.23 ppm, PEG CH<sub>3</sub>-O- (s); 3.50 ppm, PEG -O-CH<sub>2</sub>- (m); 7.55 ppm, *p*-nitrophenyl-aromate (d); 8.31 ppm, *p*-nitrophenyl-aromate (d). <sup>13</sup>C-NMR (300 Mhz, DMSO-d-6): 58.27 ppm, PEG CH<sub>3</sub>-O-; 69.70 ppm, PEG -O-CH<sub>2</sub>-; 122.62 ppm, *p*-nitrophenyl-aromate; 125.33 ppm, *p*-nitrophenyl-aromate; 145.93 ppm, PEG -O-CH<sub>2</sub>-C=O; 152.10 ppm, aromatic C<sub>5</sub>H<sub>4</sub>=C-NO<sub>2</sub>; 154.76 ppm, PEG -CH<sub>2</sub>-OCO-. FTIR: 3447; 2889; 2741; 2694; 2603; 2494; 1971; 1769; 1642; 1526; 1468; 1360; 1343; 1281; 1242; 1219; 1113; 1060; 963; 842; 529 cm<sup>-1</sup>. MS (MALDI-TOF): *m/z* 4698 (M<sup>+</sup>).

*Synthesis of Tryptophan-mPEG 5 kDa (method adapted from US 5,286,637)*

The reaction was performed as described for the Tryptophan-mPEG 2 kDa, where 0.68 mMol of dried mPEG-*p*-nitrophenyl carbonate 5 kDa (synthesized as described above) and 6.78 mMoles of L-Tryptophan were used. A slightly yellowish powder was obtained. <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 3.21 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (d); 3.24 ppm, PEG -CH<sub>3</sub>-O- (s);

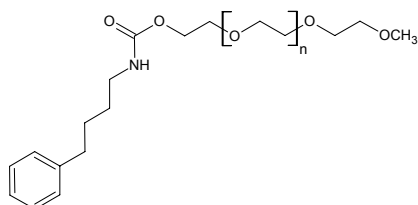
3.51 ppm, PEG -O-CH<sub>2</sub>- (m); 4.18 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>- (q); 6.96 ppm, Trp-indole (t); 7.02 ppm, Trp-indole (t); 7.14 ppm, Trp-indole (s); 7.32 ppm, Trp-indole (d); 7.51 ppm, Trp-indole (d); 10.81 ppm Trp -COOH (s). <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 54.85 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 58.04 ppm, PEG CH<sub>3</sub>-O-; 63.46 ppm, Trp indole-CH<sub>2</sub>-CH<sub>2</sub>-; 69.72 ppm, PEG -O-CH<sub>2</sub>-; 110.83 ppm, Trp-indole; 111.34 ppm, Trp-indole; 117.99 ppm, Trp-indole; 120.90 ppm, Trp-indole; 124.11 ppm, Trp-indole; 127.04 ppm Trp-indole; 136.62 ppm, Trp-indole; 156.24 ppm, PEG -CH<sub>2</sub>-OCO-NH-; 173.68 ppm, -COOH. FTIR: 3438; 2885; 2741; 2695; 1969; 1719; 1647; 1467; 1360; 1343; 1281; 1242; 1112; 1060; 963; 842; 746; 529 cm<sup>-1</sup>. MS (MALDI-TOF): *m/z* 4772 (M<sup>+</sup>).  $[\alpha]_D^{20} = -0.002$ .

### Scheme 1



### Example 4: Synthesis of phenylbutylamine-PEG derivative

The following PEG derivative according to the invention (2 kDa phenylbutylamine-PEG) (of Formula (II) wherein R<sup>3</sup> is OR<sup>4</sup> wherein R<sup>4</sup> is substituted amide (e.g. N-(4-phenyl-butyl)-formamide); n is selected from 40-50; R<sup>1</sup> is optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl (e.g. methyl)) was synthesized as follows:

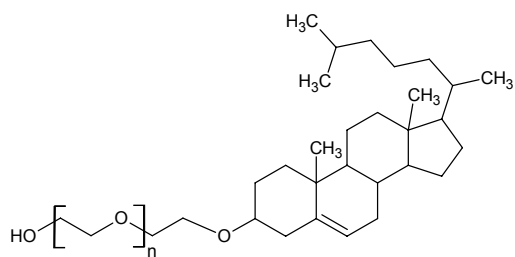


*Synthesis of phenylbutylamine-mPEG 2 kDa*

0.069 Mol phenylbutylamine (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were dissolved in anhydrous dichloromethane 1.39 mMol of dried mPEG-*p*-nitrophenyl carbonate 2 kDa (synthesized as described in Example 3) were added. The pH was maintained at ~10.4 and reaction was left to proceed at room temperature for 6 hours. Reaction was stopped by evaporation of dichloromethane. The residue was redissolved in 2 M HCl and pH was adapted to 2. The aqueous phase was extracted with dichloromethane. The obtained organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and partially evaporated. Precipitation from cold diethyl ether was performed and the solid collected via filtration. The solid was once reprecipitated from cold diethyl ether, and once from cold iso-propanol. A white powder was obtained, dried under vacuum and redissolved in milliQ<sup>TM</sup> water. The solution was filtered through a 0.22 µm Millex-GV<sup>TM</sup> filter (Millipore, Carrigtwohil, Co. Cork, Ireland) and freeze dried (Freeze dryer Micro Modulyo<sup>TM</sup>, Edwards High Vacuum Int., Crawley Sussex, UK). <sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 1.40 ppm phenylbutylamine –CH<sub>2</sub>–; 1.54 ppm phenylbutylamine –CH<sub>2</sub>–; 3.24 ppm, PEG –CH<sub>3</sub>-O–; 3.51 ppm, PEG –O-CH<sub>2</sub>–; 7.19 ppm phenylbutylamine Ar. <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 28.02 ppm phenylbutylamine –CH<sub>2</sub>–; 29.07 ppm phenylbutylamine –CH<sub>2</sub>–; 34.66 ppm phenylbutylamine –CH<sub>2</sub>–; 58.10 ppm, PEG CH<sub>3</sub>-O–; 69.50 ppm, PEG –O-CH<sub>2</sub>–; 125.49 ppm phenylbutylamine Ar; 128.40 ppm phenylbutylamine Ar; 141.87 ppm phenylbutylamine Ar; 155.76 ppm phenylbutylamine Ar. FTIR: 2883; 1964; 1719; 1537; 1466; 1359; 1341; 1279; 1240; 1146; 1098; 1059; 959; 841; 749; 700. MS (MALDI-TOF): *m/z* 2124 (M<sup>+</sup>).

#### **Example 5: Synthesis of Cholesterol-PEG derivatives**

The following PEG derivatives according to the invention (2kDa and 5 kDa Cholesterol-PEGs) (of Formula (II) wherein R<sup>3</sup> is OR<sup>4</sup> wherein R<sup>4</sup> is substituted heteroaryl (e.g. 3-(1,5-Dimethyl-hexyl)-3a,6,6-trimethyl-2,3,3a,4,5,5a,6,9,9a,9b-decahydro-1H-cyclopenta[a]naphthalene), *n* is selected from 40-120; R<sup>1</sup> is H) were purchased to NOF Corporation, Tokyo, Japan (Sunbright CS-020 and -050).



Cholesteryl-PEG 2 kDa

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 0.69 ppm; 0.87 ppm; 0.89 ppm; 0.98 ppm; 1.14 ppm; 2.34 ppm; 3.14 ppm; 3.55 ppm; 5.34 ppm,. <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 11.61 ppm; 18.48 ppm; 19.00 ppm; 20.56 ppm; 22.34 ppm; 22.61 ppm; 23.15 ppm; 23.81 ppm; 27.35 ppm; 27.74 ppm; 27.98 ppm; 31.35 ppm; 35.19 ppm; 35.60 ppm; 36.23 ppm; 36.64 ppm; 41.79 ppm; 49.55 ppm; 55.52 ppm; 56.13 ppm; 60.13 ppm; 66.56 ppm; 69.72 ppm; 72.28 ppm; 78.36 ppm; 99.56 ppm; 120.97 ppm; 140.44 ppm.

FTIR: 2883; 1967; 1466; 1359; 1341; 1279; 1240; 1146; 1102; 1060; 958; 841; 735. MS (MALDI-TOF): m/z 1994 (M+).

Cholesteryl-PEG 5 kDa

<sup>1</sup>H-NMR (300 MHz, DMSO-d-6): 0.68 ppm; 0.84 ppm; 0.88 ppm; 0.97 ppm; 1.11 ppm; 2.33 ppm; 3.13 ppm; 3.54 ppm; 5.34 ppm. <sup>13</sup>C-NMR (300 MHz, DMSO-d-6): 11.61 ppm; 18.48 ppm; 19.00 ppm; 20.54 ppm; 22.34 ppm; 22.62 ppm; 23.13 ppm; 23.81 ppm; 27.34 ppm; 27.74 ppm; 27.99 ppm; 31.36 ppm; 35.15 ppm; 35.59 ppm; 36.64 ppm; 41.78 ppm; 49.54 ppm; 55.52 ppm; 56.13 ppm; 60.13 ppm; 66.57 ppm; 69.71 ppm; 72.28 ppm; 78.34 ppm; 121.01 ppm; 140.39 ppm.

FTIR: 2882; 2740; 1969; 1466; 1359; 1340; 1279; 1240; 1145; 1102; 1059; 957; 841; 735. MS (MALDI-TOF): m/z 4858 (M+).

**Example 6: Comparison of the aggregation propensity of calcitonin or hen egg white lysozyme alone and in combination with further PEG derivatives**

In order to assess the stabilizing effect of PEG derivatives according to the invention, the aggregation propensity of salmon calcitonin (sCT) or hens egg white lysozyme (HEWL) is assayed in presence or absence of PEG derivatives according to the invention.

Hen egg white lysozyme is a 130-amino acid polypeptide of 14.4 kDa (*EC 3.2.1.17, Jolles, 1969, Angewandte Chemie, International Edition, 8, 227-239*) which can be separated by high-speed countercurrent chromatography using a reverse micellar system as described in *Xue-li Cao et al., 2007, Journal of Liquid Chromatography & Related Technologies, 30(17), 2593-2603*. It presents bacteriolytic and immunological modulating properties (*Mine et al., 2004, J. Agric. Food Chem., 52:1088–1094; Eun-Ha Kim et al., 2002, Immunopharmacology and Immunotoxicology, 24(3), pp. 423–440*).

Aggregation studies of sCT were performed as described in Example 2. Aggregation studies of HEWL were performed as follows: HEWL (Sigma-Aldrich Chemie GmbH, Buchs,



Switzerland) in a final concentration of 2.1 mM per well with and without the respective excipients to be tested, and non-conjugated PEG-derivatives were prepared in 50 mM sodium phosphate buffer pH 12.2. Aggregation is followed in UV-transparent 384-well Costar® plates as described in Example 2. The protein formulations according to the invention in Table 4 below were tested:

**Table 4**

<b>Protein</b>	<b>PEG-derivatives</b>	<b>Molar ratios Protein/PEG Derivative</b>
sCT	Trp-PEG 2 kDa	2:1; 1:1; 1:2; 1:5; 1:10
sCT	Trp-PEG 5 kDa	1:1; 1:5
HEWL	phenylbutyl amine PEG 2 kDa	1:1; 1:10
HEWL	Cholesterol-PEG 2 kDa	1:1
HEWL	Cholesterol-PEG 5 kDa	1:1

*Aggregation propensity of proteins alone and in combination with PEG derivatives*

**Figures 3A and 3B show that with increasing amounts of Trp-mPEG 2 kDa added, the lag phase of aggregation was prolonged and the aggregation of sCT was reduced. Reduced turbidity and Nile Red fluorescence intensity were observed in the following order: i) sCT, ii) sCT:Trp-mPEG 2 kDa molar ratio = 1:1, and iii) sCT:Trp-mPEG 2 kDa molar ratio = 1:5, iv) sCT:Trp-mPEG 2 kDa molar ratio = 1:10, demonstrating a reduction of sCT aggregation. For sCT:Trp-mPEG 2 kDa molar ratio = 1:10 the aggregation was completely suppressed up to 64 hours. Figure 4 shows that the aggregation of sCT in 10 mM sodium citrate buffer pH 6 was also reduced in presence of Trp-mPEG 5 kDa in a molar ratio sCT:Trp-mPEG 5 kDa of 1:5.**

**With increasing concentration of phenylbutylamine-mPEG 2 kDa a prolongation in the onset of HEWL aggregation was observed (Figure 5). Cholesterol-PEGs of 2 and 5 kDa completely suppressed the aggregation of HEWL (Figure 6) in a molar ratio protein:PEG derivative of 1:1.**

**Example 7: Comparison of the stability of sterile solution for injection of calcitonin alone and in combination with PEG derivatives**

Stability of formulations according to the invention is compared to the stability of a sterile solution for injection containing 0.033 mg/ml (resp. 200 I.U.) of sCT (Miacalcin®, Novartis, Switzerland) which compositions are described under Table 5 below. The formulations from Table 5 below are prepared as follows: first a solution of the respective amounts of acetic acid, phenol, sodium acetate trihydrate, and sodium chloride in a fraction of water for injection (less than 1 ml) are prepared. In the case of formulations containing DNS-mPEG or Trp-mPEG 2 kDa, the respective amounts of the PEG derivatives are added to and dissolved in the solution prepared in the first step. Then, sCT is added and dissolved. Finally, the volume is completed with water for injection to 1 ml.

**Table 5**

Composition	Miacalcin®	sCT: D- PEG2 1:1	sCT: T- PEG2 1:1	sCT: T- PEG2 1:10	Control D- PEG2	Control T- PEG2	Control T- PEG2
sCT (mg)	0.033	0.033	0.033	0.033	-	-	-
acetic acid (mg)	2.25	2.25	2.25	2.25	2.25	2.25	2.25
phenol (mg)	5.0	5.0	5.0	5.0	5.0	5.0	5.0
sodium acetate trihydrate (mg)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
sodium chloride (mg)	7.5	7.5	7.5	7.5	7.5	7.5	7.5
water for injection	qs to 1 ml	qs to 1 ml	qs to 1 ml	qs to 1 ml	qs to 1 ml	qs to 1 ml	qs to 1 ml
PEG-derivative (mg)	-	0.021	0.021	0.21	0.021	0.021	0.21

*D-PEG2= DNS-PEG 2kDa; T-PEG2 = Trp-PEG 2kDa*

All formulations are prepared in glass vials protected from light and stressed by two methods, i) horizontal shaking at room temperature (25°C) and by ii) storage at 37°C. At preselected time points (e.g. bi-weekly), one or more of the following measurements is performed:

- UV absorbance scan (230-550 nm), Nile red fluorescence emission spectra and the intrinsic fluorescence emission spectra of the dansyl- or Trp-headgroup is measured by a microplate reader on a 96-well plate as described above. The intrinsic tyrosine emission of sCT is measured with samples containing the dansyl-PEGs to follow conformational changes.
- Intrinsic fluorescence emission/excitation spectra of the dansyl- or Trp-headgroup, intrinsic tyrosine fluorescence emission/excitation of sCT, 90° light scatter, anisotropy, UV absorbance spectra is measured. Furthermore, Nile Red fluorescence emission/excitation, 90° light scatter, anisotropy are measured. Brightfield and Nile Red fluorescence microscopy are performed. All measurements are performed at various settings.

The extent of aggregation of sCT is used as a measure of the stabilizing effect of the PEG derivatives according to the invention as compared to a commercial formulation of this protein.

#### **Example 8: Pharmacokinetic studies**

In order to assess the stabilizing effect of PEG derivatives according to the invention, the bioavailability of a protein is assayed in presence or absence of PEG derivatives according to the invention. The stabilized protein formulation is injected i.v. and s.c. in suitable animals (mice, rats, rabbits). Blood samples are drawn at pre-determined intervals and subjected to treatment allowing quantitative measurement of protein concentration by standard assay (e.g., ELISA). Protein solution in the absence of stabilizing PEG-derivative serves as control. Pharmacokinetic parameters, including  $t_{\max}$ ,  $c_{\max}$ , AUC,  $t_{1/2}$ , and  $k_{el}$  is determined for the stabilized protein and the control group and for both application routes and compared to each other.

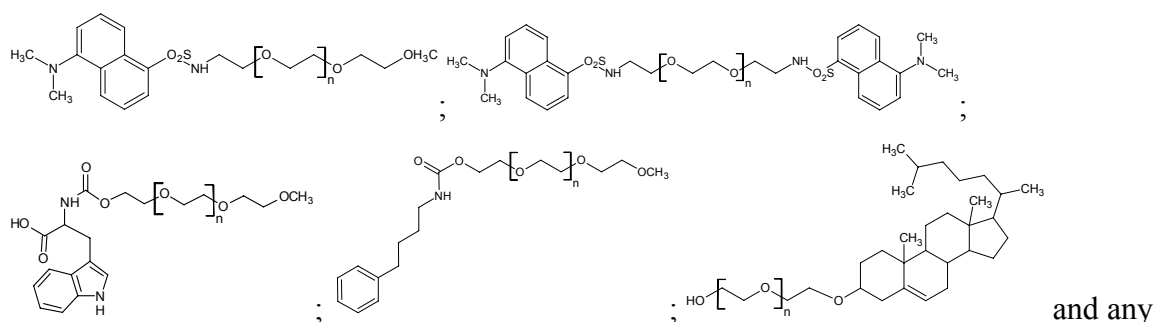
#### **Example 9: Immunogenicity studies**

In order to assess the stabilizing effect of PEG derivatives according to the invention, the immunogenicity of a protein is assayed in presence or absence of PEG derivatives according to the invention. Detection and characterization of binding antibodies (BABs) is performed by solid phase binding immunoassay, e.g., enzyme-linked immunosorbent assay (ELISA), preferably in bridging mode using labeled protein for detection of BABs. Specificity of the

detected antibodies is assessed by immunoblotting, while their neutralizing activity is determined by specific bioassay measuring the bioactivity of the protein.

**Claims:**

1. A stable protein formulation, said formulation comprising a non-covalent combination of an aqueous carrier, a protein and a PEG derivative, wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group.
2. The formulation according to claim 1, wherein the formulation is a pharmaceutical formulation.
3. The formulation according to claims 1 or 2 wherein the protein is at a concentration in the range from about 0.01 ng/ml to about 500 mg/ml.
4. The formulation according to any one of claims 1 to 3 wherein the PEG derivative is at a concentration in the range from about 0.001 ng/ml to 1 g/ml.
5. The formulation according to any one of claims 1 to 4 wherein the PEG derivative is an mPEG derivative.
6. The formulation according to any one of claims 1 to 5, further comprising an excipient.
7. The formulation according to any one of claims 1 to 6, wherein the hydrophobic group is selected from dansylamide, phenylbutylamine, cholesterol and an amino acid.
8. The formulation according to any one of claims 1 to 6, wherein the PEG derivative is of Formula (II):  $R^1-(OCH_2CH_2)_n-R^3$ , wherein  $R^3$  is selected from  $OR^4$  wherein  $R^4$  is selected from substituted heteroaryl, substituted amide and substituted amine; n is selected from 40-120;  $R^1$  is selected from H and optionally substituted  $C_1-C_6$  alkyl.
9. The formulation according to any one of claims 1 to 8, wherein the PEG derivative is selected from:

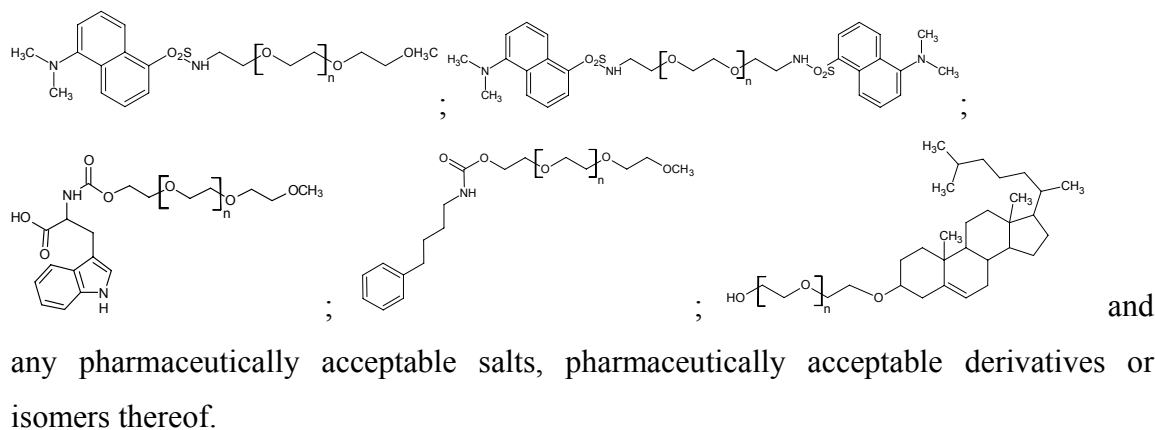


pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof.

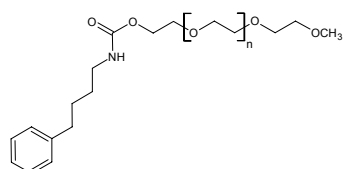
10. The formulation according to any one of claims 1 to 9, wherein the protein is selected from sCT and HEWL.
11. The formulation according to any one of claims 1 to 10, wherein the molar ratio PEG derivative to protein is 1:1.
12. A kit comprising in one or more container a formulation according to any one of claims 1 to 11 together with instruction of use of said formulation.
13. The formulation according to any one of claims 1 to 11 for use as a medicament.
14. A method of stabilizing a protein in aqueous solution by non-covalently combining said protein with a PEG derivative, wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group.
15. A process for the preparation of a protein or a formulation thereof comprising the steps of:
  - (i) non-covalently combining a protein with a PEG derivative into a liquid mixture or forming said protein in a liquid medium containing a PEG derivative, wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group;
  - (ii) collecting the liquid mixture or liquid medium obtained under step (i) containing the stabilized non-covalent protein thereof wherein the percentage of monomers of protein is increased as compared to protein prepared in absence of the said PEG derivative.
16. A method according to claim 14 or a process according to claim 15 wherein the PEG derivative is an mPEG derivative.
17. A method according to claim 14 or a process according to claims 15 or 16 wherein the hydrophobic group is selected from dansylamide, phenylbutylamine, cholesterol and an amino acid.
18. A method according to claim 14 or a process according to claims 15 or 16 wherein the PEG derivative is of Formula (II):  $R^1-(OCH_2CH_2)_n-R^3$ , wherein  $R^3$  is selected from  $OR^4$  wherein  $R^4$  is selected from substituted heteroaryl, substituted amide and substituted

amine; n is selected from 40-120; R<sup>1</sup> is selected from H and optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl.

19. A method according to claim 14 or a process according to claims 15 or 16 wherein the PEG derivative is selected from:



20. A stabilized protein or a formulation thereof obtainable by a method according to claim 14 or a process according to claim 15.
21. A stabilized protein or a formulation thereof according to claim 20 wherein the said PEG derivative is a mPEG derivative.
22. A pharmaceutical formulation comprising a stabilized protein, according to any one of claims 20 to 21.
23. A PEG derivative comprising at least one polyethylene glycol moiety covalently grafted to a hydrophobic group, wherein the hydrophobic group is selected from dansylamide, tryptophan, phenylbutylamine, cholesterol, and an amphipathic peptide.
24. A PEG derivative according to claim 23 of formula:



and any pharmaceutically acceptable salts, pharmaceutically acceptable derivatives or isomers thereof

25. Use of a PEG derivative according to claims 23 or 24 for the preparation of a pharmaceutical formulation.

26. A process for the preparation of a PEG derivative according to the invention comprising the step of reacting an mPEG-p-nitrophenyl carbonate with phenylbutylamine in an anhydrous solvent at a pH between about 9 and 11 at room temperature.
27. A kit for reconstituting a protein in solution comprising in one container a lyophilized protein, notably a therapeutic protein, and a PEG derivative in another container or another part of said container, optionally together with a container containing a sterile buffer for reconstituting the protein and optionally with instruction of use of said kit, wherein the PEG derivative comprises at least one polyethylene glycol moiety covalently grafted to a hydrophobic group.



**Abstract of the invention:**

The present invention is directed to stable protein formulations, related methods and uses thereof. In particular, the invention relates to a method of stabilizing therapeutic proteins in aqueous solution.