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Chemistry of polyethylene glycol conjugates with biologically active molecules

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Abstract

Polyethylene glycol (PEG) is widely used as a covalent modifier of biological macromolecules and particulates as well as a carrier for low molecular weight drugs. In the first two instances proteins and liposomes are of particular importance. Their conjugates with PEG often possess the ability to avoid quick recognition and clearance in vivo, that their unconjugated counterparts are suffering from. In this review (with 133 references) methods for preparation of PEG conjugates with various biologically active compounds are summarized. Since the bulk of the published work in this field involves proteins, drugs, and lipids, an appropriate emphasis is given to the conjugates of these compounds. While the first two types of PEG conjugates are usually intended for a direct use as therapeutics, PEG-lipids are mainly utilized for formation of long-circulating liposomes. Particular attention is paid to the comparative attributes of various reactive PEG derivatives, properties of the linkages formed, and possible side reactions. The relationships between various conjugation strategies and their influence on the relevant biological properties and/or on in vivo performance of the corresponding conjugates is also discussed.

Keywords: Polyethylene glycol (PEG); PEGylation; PEG-proteins; PEG-drugs; PEG-lipids; PEG-liposomes

Contents

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Abbreviations: PEG, polyethylene glycol; mPEG, methoxy-PEG; SC-PEG, succinimidyl carbonate-PEG; SS-PEG, succinimidyl succinate-PEG; RES, reticuloendothelial system; RNase, ribonuclease; PE, phosphatidylethanolamine; DSPE, distearoyl-PE; DOPE, dioleoyl-PE; DPPE, dipalmitoyl-PE; HSPE, hydrogenated soybean-PE; POPE, palmitoyl oleoyl-PE; DMPE, dimiristroyl-PE; PC, phosphatidylcholine; PG, phosphatidylglycerol; DP, degree of polymerization; Su, succinimidyl; TCP, trichlorophenyl; pNP, *p*-nitrophenyl; GPC, gel permeation chromatography; BSA, bovine serum albumin; SOD, superoxide dismutase; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; SMCC, *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; PEA, *Pseudomonas* exotoxin A; 5FU, 5-fluorouracil; DPPE, PA, phosphatidic acid.

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1. Introduction

Covalent attachment of biologically active compounds to polymers became one of the methods for alteration and control of biodistribution, pharmacokinetics and often toxicity of these compounds [1]. The rationale of this approach is based on the assumption that properties of the macromolecular conjugate dominated by the properties of the polymeric carrier determine the above mentioned characteristics. One of the most popular polymeric materials used for this purpose is polyethylene glycol (PEG). It possesses an ideal array of properties: very low toxicity [2], excellent solubility in aqueous solutions [3], extremely low immunogenicity and antigenicity [4]. The polymer is known to be non-biodegradable, yet it is readily excretable after administration into living organisms. Its presence in aqueous solutions has no deleterious effect on protein conformation or activities of enzymes. PEG also exhibits excellent pharmacokinetic and biodistribution behavior [5]. When injected into animals it shows high persistence in blood compartment and low accumulation in reticuloendothelial system (RES) organs, liver and spleen. In addition to its remarkable in vivo behavior, the well-known propensity of PEG to exclude proteins, other macromolecules, and particulates from its surroundings is one of the principal reasons for the use of this polymer for preparation of various bioconjugates. These properties of the polymer have been explained by its chains' high mobility associated with conformational flexibility and water-binding ability [6–9]. Taking advantage of these properties, PEG grafting has been widely used as a method for reduction of various undesirable consequences of biological recognition manifested by immunogenicity and antigenicity in the case of proteins [10–12], rapid clearance from circulation by RES of liposomes [13,14], and thrombogenicity, cell adherence, and protein adsorption in the case of artificial biomaterials [15,16].

PEG is readily available in a variety of molecular weights. With a few exceptions, the molecular weights of PEGs used for synthesis of biologically active conjugates are in the range of 1000–20000 Da. These are homogeneous polymers ($M_w/M_n \leq 1.1$) of the general structure $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$. Monomethyl ether of PEG (mPEG) is also often used for conjugation to biologically relevant materials. It is particularly useful when multiple chains of the polymer have to be linked to the intended substrate. Due to its structural simplicity and possession of only one derivatizable end group, the use of mPEG minimizes crosslinking possibilities and leads to improved homogeneity of conjugates. Thus it is usually the starting material of choice for covalent modification of proteins, biomaterials, and particulates. The polyether backbone of PEG is inert in biological environments as well as in most chemical reaction conditions under which the end groups of PEG can be subjected to chemical modification and/or conjugation reactions. The subject of chemical derivatization of the end groups of PEG, which is often an essential first step in preparation of bioconjugates, was recently reviewed [17]. The syntheses involved in preparation of the various functionalized PEG derivatives mentioned in this review were discussed in detail.

The focus of this review is to identify and summarize attributes of the various methods for preparation of PEG conjugates. Particular attention is paid to the most commonly used methods of conjugation, their scope and drawbacks. Whenever possible the relationship between the conjugation chemistry and activity of conjugates in biological systems is discussed. Although by now representatives of almost every group of biological substances have been modified with PEG, there are only a few classes of compounds

that enjoy routine use as substrates for PEG conjugation. These include proteins, lipids and to a somewhat lesser extent, low molecular weight drugs. This is the reason for concentrating the attention of this review on conjugation methods pertaining to these classes of biologically important molecules. Those readers interested in properties and applications of therapeutic PEG conjugates can refer to some excellent recent reviews dealing with these substances [4,10,11,13,14,18,19]. The secondary aim of this manuscript is to highlight recent major developments in the field since the previous reviews with somewhat overlapping scopes [12,20,21].

2. Conjugates with polypeptides (peptides and proteins)

Judging from the amount of the published work dealing with a single class of PEG conjugates, PEG-polypeptides (peptides and proteins) constitute the single most important group of PEG conjugates. A proportional part of this review is allocated therefore to this class of conjugates. There are three main areas of applications of PEG-polypeptides: (1) therapeutics, (2) enzymatic catalysis in organic solvents, (3) two-aqueous phase partitioning systems for purification and analysis of various biologically derived mixtures. Although the conjugation of PEG to therapeutic proteins is of primary importance in this paper, many of the chemical methods used for protein PEGylation are relevant regardless of the end use of a particular conjugate.

2.1. Common methods for covalent attachment of PEG to polypeptides

Coupling reactions between amino groups of proteins and mPEG equipped with an electrophilic functional group have been used in most cases for preparation of PEG-protein conjugates. Such reactions usually result in conjugates composed of a globular protein in its core to which numerous polymer chains are covalently linked. The composition of such a graft co-polymeric system is dependent on the number of available attachment sites (amino and sometimes other nucleophilic groups) on the starting polypeptide, excess and reactivity of the mPEG reagent, and the conditions of the modification reaction. Fig. 1 illustrates the most com-

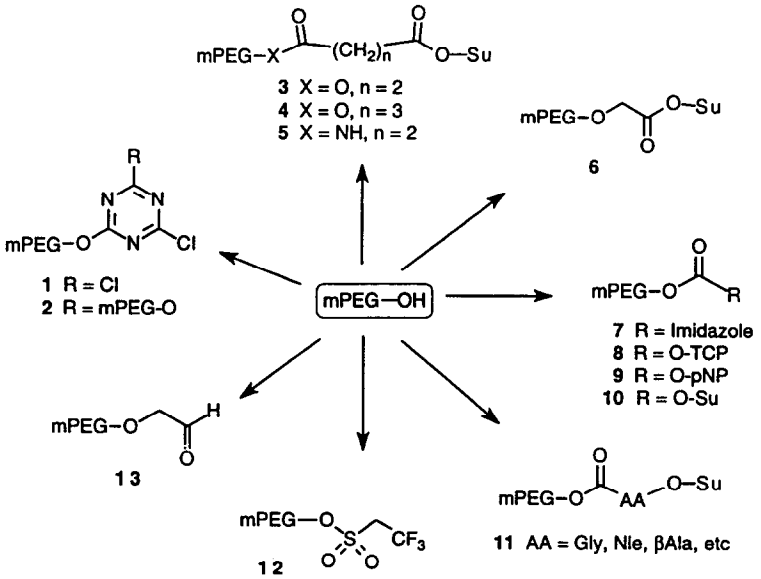


Fig. 1. Commonly used methods for preparation of mPEG-based protein-modifying reagents. For this as well as the following figures mPEG represents $CH_3(OCH_2CH_2)_n$-.

monly used mPEG-based electrophiles, often referred to in the literature as 'activated PEGs'. In most cases commercially available mPEGs of molecular weight 2000 or 5000 were used for preparation of these reagents. The activated PEGs shown in Fig. 1 can be divided into three categories. Derivatives **1** and **2** contain a reactive aryl chloride residue, which is displaced by a nucleophilic amino group upon a reaction with peptides or proteins, as shown in Fig. 2. In this regard **1** and **2** can be called arylating reagents. Since **3–11** contain reactive acyl groups, they are referred to as acylating agents. Protein modification with these agents results in acylated amine-containing linkages: amides, derived from active esters **3–6** and **11**, or carbamates, derived from **7–10**. Alkylating reagents (**12** and **13**) both react with proteins forming secondary amine attachments from amino-containing residues. As shown in Fig. 2, tresylate (**12**) alkylates directly, while acetaldehyde (**13**) is used in reductive alkylation reactions. The numbering (**1–13**) roughly corresponds to the order in which these activated polymers were introduced. The attributes of these derivatives as well as others, seldom-used, PEG derivatives have been described in detail elsewhere [12,17]. Therefore a relatively brief discussion is included in this manuscript.

In the pioneering work of Davis and co-workers [22] PEG-proteins were prepared by modification of bovine serum albumin (BSA) with mPEG-dichlorotriazine (**1**), resulting in a conjugate with dramatically reduced immunogenicity and antigenicity. Despite the fact that cyanuryl halide derivatives are known as some of the least selective protein modifiers [23], this method was gradually adopted by other investigators [24–29]. This lack of selectivity resulted in marked loss of biological activity of some protein conjugates. For example, it was discovered that in the process of modification of phenylalanine-ammonia lyase mPEG-dichlorotriazine reacted not only with amines, as was originally thought, but also with sulfhydryl groups [28]. This led to substantial loss of enzymatic activity. Modification of other nucleophilic residues on proteins with mPEG-dichlorotriazine was suggested in the light of the known similar reactivity of low molecular weight analogs [12,30]. Recently, during the study of silk fibrin modification with **1** Gotoh et al. [31] obtained convincing NMR and amino acid analysis evidence for modification of the tyrosine and histidine residues of the polypeptide.

Inada and co-workers [19] used 2,4-bis-(methoxypolyethylene glycol)-6-chloro-*s*-triazine (**2**) for modification of a variety of proteins. Since this reagent is derived from trichloro-*s*-triazine by replacement of two of the most

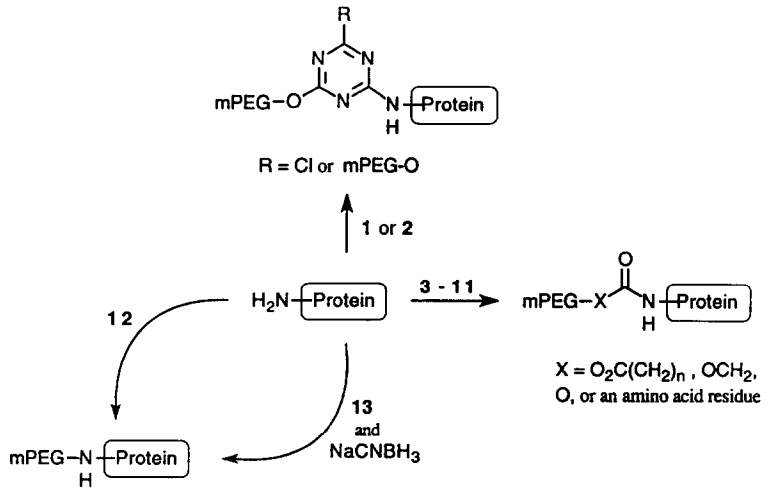


Fig. 2. Use of mPEG-based reagents **1–13** for the preparation of protein conjugates.

reactive chlorides with mPEG-*O* residues, and leaving the least reactive third chloride, protein modification reactions with **2** require somewhat more forceful conditions than with **1** [32]. On the other hand, lower reactivity of a reagent usually translates into higher selectivity. Unfortunately, it was found that the original method for synthesis of **2** was yielding a mixture of reactive derivatives containing comparable amounts of **1** and **2** [33]. One should bear this fact in mind when evaluating the literature published during the 1980s on the use of **2**. An improved new procedure was introduced recently to produce $mPEG_2$-chlorotriazine (**2**) free of the side products as was demonstrated by GPC and NMR spectroscopy [33].

Since many of the beneficial properties of PEG-proteins (extended blood circulation time, reduced immunogenicity, etc.) are directly dependent on the PEG content of conjugates, a two-armed reagent, such as **2**, has an intrinsic advantage over single mPEG chain reagents in its ability to bind double the amount of polymer for the same number of attachment sites. For example, in order to eliminate antigenicity of BSA approximately half the amino groups (30 out of 60 amines) have to be modified with **1** [22], while only 15 residues have to be modified with **2** [32].

Various active ester derivatives of the polymer (**3–6**, **11**) were used for attachment of mPEG residues to proteins via amide bonds [30,34–39]. These reagents generally showed selective reactivity towards amino groups and in many cases resulted in good preservation of biological/enzymatic activity of modified proteins (see Table 1). Protein modification with succinimidyl esters of carboxymethylated PEG (**6**) or mPEG-oxycarbonyl-amino acids (**11**) yields conjugates stable in most biological buffers and physiological conditions. On the other hand, a succinimidyl succinate derivative of mPEG (**3**) was shown to produce hydrolytically labile conjugates [4]. For example, the half-lifetime of mPEG-5000 loss from succinate interlinked PEG-BSA in physiological PBS, at 37°C is approximately 2 days [30]. Formation of succinimide residues with concomitant hydrolytic cleavage of mPEG-OH is considered a likely possibility [17,40]. The main advantage of PEG reagents **11** is in the ease of composition determination of their conjugates. For example, a number of protein attachment sites of **11** containing Nle or β-Ala can be determined by hydrolyzing an aliquot of the conjugate solution and quantitating the relevant component by amino acid analysis [41,42]. Knowing the amino acid composition of the native protein one can determine the protein concentration from the same amino acid analysis chromatogram. This approach to PEG-protein characterization combined with fluorescent detection methods of amino acid chromatography, with detection limits in a single picomole range, is invaluable when quantities of the native protein are limited.

Grafting mPEG chains onto proteins via carbamate (urethane) linkages is achieved by using immidazolylcarbonyloxy [43] derivatives of the polymer (**7**) or various reactive carbonates [44,45] (**8–10**). These derivatives of the polymer mainly differ in their reactivity towards amino groups of proteins. The reactivity, following the order **10** > **9** > **8** > **7**, can affect rate and conditions of the conjugation reactions. It can also influence the extent of protein modifications and thus the number of mPEG chains covalently linked to form a conjugate. These reagents are noted for their ease of preparation and formation of chemically stable attachments upon their reaction with amino groups of proteins. Active carbonates are also used for preparation of single amino acid or dipeptide conjugates, which constitute useful precursors for the preparation of reagents **11** [36,41,42,46].

Tresylate (2,2,2-trifluoroethanesulfonate) of PEG (**12**) was introduced by Nilsson and Mosbach [47] as a reagent for protein modification. Several research groups adopted mPEG-tresylate for modification of proteins [48–50]. The modification reactions take place under mild aqueous buffer (pH 7.5–9.5) conditions and result in alkylation of the amino groups of proteins (thiols, if present, do react as well).

Difficulties were experienced in the introduction of acetaldehyde end groups (**13**) by oxidation of terminal hydroxyls of commercially available mPEGs [17]. Despite the functional group conversion into aldehyde being 60–70%,

Table 1
Influence of conjugation chemistry on biological/enzymatic activities of selected PEG-protein conjugates

Protein	PEG reagent[a]	PEG chains/protein (% modification)	% of native activity (substrate)	Comments	Reference
Gulonolactone oxidase	**1**	15 (38)	67	Conjugates were more stable at 37°C than the native enzyme. They retained immunogenicity and antigenicity. Circulation lifetime of the PEG-enzymes was not extended	38
	3	18 (47)	74		
Peroxidase	**9** (mPEG-750, 1900, 5000; PEG-1450, 10 000, 20 000, 35 000)	4	76–105 (OPD) 39-100 (ODA) 64–106 (4AA)	Good preservation of enzymatic activity was observed. ODA was the most discriminating substrate. No correlation between MW of the PEG and activity of the conjugates	54
	13 (mPEG-350, 1900, 5000)	2–3	88–110 (OPD)	Only conjugate of mPEG-5000 was soluble and active in organic solvents	51
	2	3–4 (60)	70 (OPD)	Benzene-soluble conjugate had 21% of the activity in aqueous solution	58
	bis-hydrazide-PEG-4000/EDC	4	90 (OPD)	Almost fully active conjugate modified on its carboxyl groups. It exhibited binding to ConA column as the native enzyme. PEG chains of the conjugate had terminal hydrazide groups, which were used for attachment to oxidized oligosaccharide residues of antibodies	Zalipsky, unpublished results
	thioesther-PEG-20 000[b]	2	89 (OPD)	Solubility and activity in several organic solvents was observed	60
	$NaIO_4$-oxidized enzyme + bis-amino-PEG-20 000/$NaBH_4$	3	91 (guaiacol)	Oligosaccharide-modified enzyme was soluble and active in toluene, $CHCl_3$	61
SOD (bovine erythrocyte)	**1**	19 (95)	51	PEG-SOD exhibited much longer plasma lifetimes and did not elicit immune response even after repeated injections in mice	53
	1	17 (85)	51	The differences in the linking chemistry did not influence the in vivo persistence of the conjugates in rats	56
	6	16 (80)	75		
	9	16 (80)	70		
	5	3 (15)–18 (90)	90–70	Conjugation reaction was used for evaluation of the PEG reagent	35
	6	3 (13)–18 (90)	90–72		
	7	18 (90)–9 (95)	>95	PEG-SOD conjugates extended plasma half-life from 3.5 min to more than 9 h	43
	8	10 (50)	80	Conjugation reaction was used for evaluation of the PEG reagent	44
Alkaline phosphatase	**1**	19 (88) 14 (62)	33 67	Crosslinking of the enzyme with bis-dichlorotriazine-PEG (M_r 4000, 8000, 20000) produced conjugates with well-preserved activities (70–80%) without correlation to the number of modified amino groups or molecular weight of PEG	27,50
	3	17 (79) 13 (61)	93 98		
	7	17 (78) 12 (56)	77 91		
	12	17 (77) 16 (73)	86 82		

Table 1 (*continued*)

Protein	PEG reagent[a]	PEG chains/protein (% modification)	% of native activity (substrate)	Comments	Reference
Asparaginase (*e. coli*)	**1**	73 (79)	7	In contrast to conjugates of low molecular weight mPEG (750, 1900) enzyme modified with mPEG-5000 showed reduction in antigenicity and resistance to tryptic degradation	55
	2	30 (33)	30		57
		48 (52)	11		
	2	52 (57)	8	Conjugate had the same K_m and optimal pH values as the native enzyme. Half-lives in mice of the conjugate and native enzyme were 56 and 3 h, respectively	59
	3	59 (64)	52	Conjugate was active, non-immunogenic and exhibited extended plasma lifetimes in mice	34
		64 (70)	51		
	10	62 (68)	54	Conjugation reaction was used for evaluation of the PEG reagent	45

[a] If not otherwise indicated, derivative of mPEG-5000.
[b] Polymer obtained by condensation of bis-amino-PEG and dithioester.
OPA, *o*-phenylenediamine; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride; ODA, *o*-dianizidine; 4AA, 4-aminoantypyrene, ConA, concanavalin A.

at best, and the balance of the end groups consumed by side reactions, **13** was successfully used for modification of amino groups of proteins using a reductive alkylation procedure [51,52]. Sodium cyanoborohydrate was used as a reducing agent (Fig. 2). The positive attributes of this conjugation strategy are in its selectivity towards primary amino groups of proteins, and formation of stable attachments of the polymer chains without change in the net charge of the protein. According to Chamow et al. [52] the extent of protein modification with **13**/$NaCNBH_3$ is easily controlled by fine-tuning the reaction conditions and ratio of the reacting species. While this might be true for various other PEG reagents, reductive alkylation with **13** is a relatively slow reaction and thus might be more amenable to control.

2.2. Conjugation chemistry-activity relationship

In order to illustrate the influence of various linking chemistries on biological activities of polypeptide conjugates, several representative examples of PEG-enzymes are summarized in Table 1. Over the last two decades enzymes have constituted the single largest class of proteins which have been subjected to covalent modifications with PEG. Some enzymes, e.g., peroxidase and superoxide dismutase, showed remarkable preservation of activity with very little dependence on the conjugation chemistry. For example, peroxidase was modified not only by some of the common mPEG-based reagents (**2**, **9** and **13**) but also by some of the less conventional methods. These include the reaction of the bis-hydrazide derivative of PEG with carbodiimide-activated carboxyl groups, amino group acylation with thioester-containing polymer and reductive amination of periodate-treated enzyme with amino-PEG. It appears that attachment of two to four PEG chains per glycoprotein causes minimal loss of enzymatic activity, regardless of whether amino, carboxyl or carbohydrate residues were modified.

SOD modified by almost every mPEG reagent discussed in the previous section generally showed good preservation of enzymatic activity, with largest activity loss reported when **1** was used and best preservation of activity when

reagents **5–7** were utilized. Veronese et al. [56] observed no significant differences in pharmacokinetic properties of various PEG-SOD conjugates in rats, regardless of the linking methodology used. Since all three types of linkages, amide, urethane and **1**-derived *sec*-amine are stable in physiological conditions, this result is not surprising. Even when hydrolytically labile PEG conjugates, containing ester attachments derived from reagents **3** and **4**, were compared to very stable urethane-linked counterparts the influence on the pharmacokinetics was often marginal [11]. This is despite the fact that in vitro such conjugates exhibit markedly different chemical stability [30]. In order to make a difference in vivo, the cleavage of PEG from a conjugate has to be significant in the time frame of the experiment (approx. 24 h). For example, in the case of urethane-linked PEG-β-interferon a noticeably longer circulation lifetime was observed as compared to its ester-linked analog [11].

PEG-alkaline phosphatase conjugates were prepared by four different coupling methods [50]. The best preservation of activity was observed when **3** was used as a modifying reagent. Only **1** caused substantial loss of enzymatic activity with increased extent of modification. Interestingly, in contrast to the inactivation pattern observed with the monofunctional reagent (**1**), protein modifications with the bifunctional PEG analogs resulted in better preservation of enzymatic activity which was independent of the extent of the modification or the molecular weight of the polymeric reagent used [27]. This result can probably be explained by the ability of the bifunctional PEG reagent to crosslink the enzyme in its dimeric form, known to be considerably more active than the monomeric form of alkaline phosphatase [62].

Gulonolactone oxidase was PEGylated by two different methods without gaining any improvements in its in vivo properties [38]. The conjugates retained their immunogenicity and did not show any lengthening in plasma lifetime in mice. This example illustrates that, in contrast to some of the previous overly optimistic suggestions [63], the beneficial properties usually conveyed to proteins by their modification with PEG cannot be taken for granted.

It is apparent from the examples listed in Table 1 that enzymes modified with mPEG-dichlorotriazine (**1**) usually possess lower enzymatic activity than their counterparts obtained using the alternative conjugation chemistries. The case of asparaginase, which attracted particular attention due to its utility in treatment of leukemia, provides a clear illustration of this observation. While extended plasma lifetimes and reduced immunogenicity were readily achievable by grafting mPEG chains to the enzyme, catalytic activity preservation has proven to be a challenge. It is pertinent to note that histidine and tyrosine residues are playing an important role in the active site of this enzyme and that it is readily inactivated by a variety of agents reacting with these residues [64]. These facts combined with a known reactivity of cyanuryl halides towards tyrosyl residues [23] was previously offered as an explanation for asparaginase inactivation with **1** [12]. Recently, during studies of silk fibrinoin PEGylation, modification of tyrosine and histidine residues by **1** was unambiguously demonstrated using ^{1}H-NMR and amino acid analysis [31]. As illustrated in Table 1, much better preservation of asparaginase activity was achieved when acylating reagents **3** and **10** were applied. As was mentioned above, although reagent **2** is expected to be more selective than **1**, the earlier preparations of this activated PEG were contaminated by substantial amounts of **1** [33]. This might have been the reason for the poor activity of the **2**-derived asparaginase conjugates.

As can be seen from the above discussion and the examples presented in Table 1, in some cases the preservation of the enzymatic activity of PEG-enzyme conjugates was dependent on the chemistry of PEG activation and subsequent attachment to enzymes, as well as on the extent of modification. Use of PEG-based acylating reagents, which react selectively with amino groups of proteins under very mild conditions, often leads to good preservation of enzymatic activity of the conjugates. This was a particularly general occurrence for enzymes acting on low molecular weight substrates. However, preparation of functionally active, yet extensively modified PEG conjugates derived from proteins hav-

ing large size substrates, proved more difficult (see Table 2). For example, several PEG-tissue plasminogen activator conjugates were prepared using succinimidyl succinate (**3**) and imidazolylcarbonyloxy (**7**) derivatives of mPEG [65]. Regardless of the activated PEG employed, the fibrinolytic activity of the conjugates decreased substantially with the increase in the extent of modification. A similar decrease in fibrinolytic activities was exhibited by PEG-modified acyl-plasmin-streptokinase complexes [66]. Elastase modified with mPEG-dichloro-*s*-triazine (**1**) partially preserved its ability to act on its small synthetic substrate, yet completely lost its elastin hydrolyzing activity [24]. Proteolytic activity of PEG-modified trypsin and chymotrypsin was also lost in contrast to the well preserved and in some cases even enhanced activity towards low molecular weight substrates [30,46,67].

It does not appear that conformational changes as a result of covalent attachment of PEG to proteins occur and thus cannot be used as an explanation for the substrate-size-depen-

Table 2
Substrate-size-dependent activity of selected mPEG-5000 derived protein conjugates

Protein	mPEG reagent	PEG chains/protein (% modification)	% of native activity (substrate)			Comments	Reference
Ribonuclease			(RNA)	(CMP)		Anti-RNase dissociation constants for	37
	6	4 (30)	45	85		RNase and conjugates with 4 and 9	
		9 (80)	10	68		bound mPEG chains were 7.6×10^{-8},	
		11 (90)	3	66		1.3×10^{-7}, and 1.2×10^{-6} M, respectively	
Elastase			(Elastin)	(SANA)		Purified $mPEG_3$-elastase showed 35%	24
	1	1	81	84		of the native activity towards SANA	
		2	0	31		and 17% towards casein. It completely	
		3	0	21		lost anti-elastase binding ability	
		4 (100)	0	3			
Tissue plasminogen activator	**3**	12 (55)	(Fibrin) 36			Disappearance of functional activity in vivo was faster than the clearance of	65
		15 (66)	14			the conjugate. Both clearance and	
		22 (100)	0			inactivation were reduced by increased extent of PEGylation. However, this	
	7	10 (44)	80			increase was accompanied by reduction	
		13 (60)	30			in fibrinolytic activity of the conjugates.	
		17 (75)	20			**7** was preferable to **3** for balancing these properties	
Trypsin			(BEAE)			In contrast to the trypsin PEG_6-trypsin	67
	1	4 (24)	95			exhibited complete lack of	
		9 (59)	150			autodigestion and very slow rate of catalase cleavage	
			(BEAE)	(ZAPA)		PEG conjugates exhibited complete	30
	3	7 (46)	102	241		lack of proteolytic activity as evidenced	
		12 (78)	94	316		by inability to cleave Azocoll	
	10	7 (46)	95	224			
		12 (78)	92	326			
Chymotrypsin			(X-GGF-NA) X = Z	HMPA	mPEG	Ability to digest azoalbumin was 61%	46
	10	10	178	26	26	and 11% for PEG_{10} and PEG_{14}	
		14	102	17	11	conjugates respectively. In contrast to the native enzyme, the conjugates failed to cleave BSA	

NA, *p*-nitroanilide; GGF-NA, Gly-Gly-Phe-NA, ZAPA, N^{α}-benzyloxycarbonyl-L-Agr-NA, BEAE, N^{α}-benzoyl-L-Arg-OEt; CMP, cytidine 2′,3′-cyclic monophosphate; SANA, succinyl-$(L\text{-Ala})_3$-NA, HMPA, *N*-(2-hydroxypropyl)methacrylamide.

dent specificity changes. For example, using far-ultraviolet circular dichroism and intrinsic protein fluorescence Pasta et al. [39] showed that PEG-modified serine protease, subtilisin, maintains its native secondary structure and the integrity of its catalytic site. Similarly, no comformational changes, as judged by circular dichroism spectra, were detected in PEG-ribonuclease conjugates [37], which showed clear dependence of activity on the size of the substrate. While good preservation of activity towards low molecular weight cytidine-2′,3′-cyclic monophosphate was obtained even for extensively modified ribonuclease, large enzymatic activity losses were observed when ribonucleic acid was used as a substrate. Judging from the examples summarized in Table 2, it appears that in many cases when a native enzyme was able to act on both macromolecular and low molecular weight substrates, covalent attachment of multiple chains of mPEG produced conjugates with preferentially boosted activity towards small size substrates. One can look at it as a method for boosting enzymatic activity towards smaller substrates, and thus narrowing the specificity of the enzymes. In most cases this phenomenon was explained by limited access of large substrates to the active sites of PEG-modified proteins, i.e., steric hindrance. Recently, by examining the enzymatic activity of native and PEG-modified chymotrypsin toward several polymeric and low molecular weight substrates derived from tripeptide or tetrapeptide *p*-nitroanilides, and comparing them to protein-based substrates, Chiu et al. [46] demonstrated that it is possible to achieve good chymotryptic activity of PEG-enzymes towards macromolecular substrates, both based on synthetic polymers (HMPA and PEG) and azoalbumin. Furthermore, the quantitative measure of PEG-chymotrypsin activity (k_{cat}/K_m) did not always inversely correlate with the molecular size of the substrates. Thus, the substrate-size-dependent specificity of PEG-enzymes cannot be explained *solely* by steric hindrance considerations. It was suggested that the well-known propensity of grafted PEG chains to exclude proteins from their proximity is also likely to play an important role [46].

Regardless of the explanation, extensive PEG modification of proteins acting on large size substrates, without measurable loss of substrate-binding activity, still constitutes a formidable challenge. In an attempt to overcome this trend of activity loss whenever a large substrate has to interact with a PEG-modified protein, Veronese and coworkers [68,69] suggested a method for protein modification in the presence of a macromolecular inhibitor of the protein. This approach was first explored using the conjugation of trypsin with **11** (AA = Nle) in the presence of Sepharose-immobilized *p*-aminobenzamidine as a reversible inhibitor for active site protection. Comparison of activity of the native enzyme, mPEG-trypsin obtained without any additives, and the conjugates obtained in the presence of benzamidine and in the presence of Sepharose-benzamidine showed that only the last type of conjugate retained almost completely its ability to digest casein and approximately half of the native enzyme activity toward BSA [69]. Application of this method to modification of urokinase resulted in conjugates with markedly better preservation of activity towards plasminogen and fibrin substrates, as compared to mPEG-urokinase obtained by more conventional methods [68].

2.3. Site-specific PEGylation of proteins

In contrast to random protein modification, which reagents **1–13** are designed for, site-specific attachment of a suitably functionalized polymer should be one way to produce PEG conjugates without introducing a steric barrier into an essential binding site on the protein molecule. In the case of glycoproteins it is possible to utilize the reactivity of oligosaccharide residues for attachment of mPEG chains, without affecting the polypeptide portions of the molecule. The hydrazide derivative (**14**) was designed with this goal in mind [42]. It was synthesized, as shown in Fig. 3, by phosgenation generating mPEG-chloroformate first, followed by its coupling with ethyl ester of β-alanine, and finally hydrazinolysis. Transformation of the functional groups after the three-step process was quantitative. Several glycoproteins (ovalbumin, immunoglobulins, glucose oxidase), after pretreatment with periodate to generate alde-

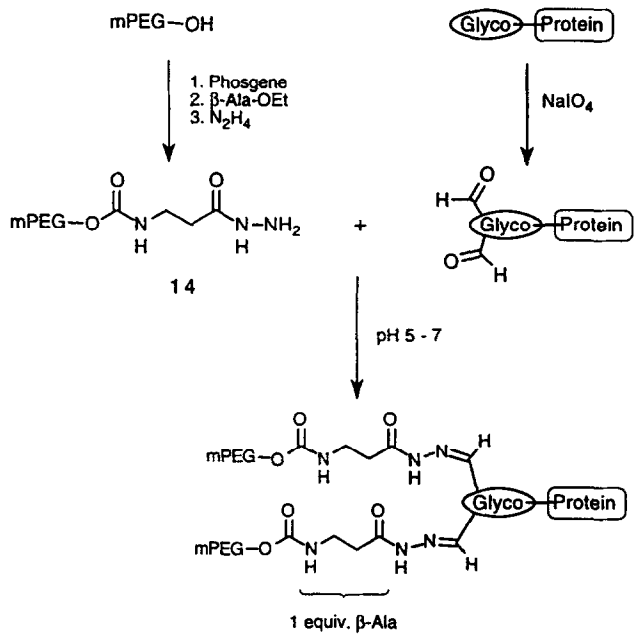


Fig. 3. Preparation and use of a mPEG-hydrazide derivative for modification of glycoproteins using the specific reactivity of their oligosaccharide residue according to Zalipsky et al. [42].

hyde groups on the carbohydrate residue, were reacted with **14** to produce active hydrazone-linked conjugates. While for most purposes hydrazone attachments are sufficiently stable, if necessary they can be reduced with $NaCNBH_3$ to more stable alkyl hydrazides. The presence of one equivalent of β-alanine as part of the linking moiety allows for convenient chemical characterization of **14**-derived conjugates. A hydrolyzed aliquot of the conjugate-containing solution is subjected to amino acid analysis, yielding information on the composition of the conjugate and its concentration.

Reductive alkylation with amino-PEG of periodate-oxidized peroxidase was mentioned above (Table 1) as an example of PEG attachment to the oligosaccharide portion of a glycoprotein [61]. However, amino groups of a protein possess similar reactivity toward aldehydes as amino-PEG and thus are expected to compete with the conjugation reaction. Hydrazides are powerful nucleophiles, yet they are weaker bases (pK_a approx. 3) than primary amines (pK_a approx. 10). Consequently under slightly acidic conditions (pH approx. 5.5), which are preferred for efficient hydrazone formation, they do not encounter competition from protonated amines. Therefore the use of mPEG-hydrazide is preferable for conjugation to aldehyde groups generated on the oligosaccharide portion of glycoproteins [42].

Site-specific mutagenesis is another approach to conjugates with predetermined locations of the PEG attachments. Several attempts have been made to genetically reengineer proteins positioning reactive amino acid residues in the desired locations along the polypeptide sequence, followed by modification of these residues by reactive PEG derivatives. For example, an interleukin-2 mutant produced with cysteine in position 3, which is occupied in the native protein by glycosylated threonine, was selectively modified with maleimide-PEG yielding a fully bioactive conjugate with estimated 4-fold slower plasma clearance rate [70].

Recently, several *Pseudomonas* exotoxin A (PEA) mutants, each containing a cysteine residue replacing one of the most exposed amino acid residues in domain II, were modified covalently with sulfhydryl-specific mPEG reagents carrying 2-pyridyldithiopropionate and maleimide groups [71]. Similar to the above-

mentioned case of PEG-IL2 conjugate preparation, both reagents in this case were prepared by reacting commercially available mPEG-NH_2 (**15**) with commonly used heterobifunctional reagents, SPDP and SMCC. For conjugates derived from mPEG-5000 it was possible to preserve fully the cytotoxic activity of the PEA, regardless of whether the components of the conjugates were interlinked by a stable thioether linkage or a biodegradable disulfide. Since it is known that domain II is responsible for translocation of the 37 kDa fragment of PEA into the cytosol leading to eventual cell death, the preservation of the cytotoxicity meant that attachment of mPEG-5000 does not interfere with this process. In contrast, cytotoxicities of 20 kDa mPEG conjugates were markedly reduced when a thioether linkage but not a disulfide was present. Plasma lifetime in mice of one of the latter conjugates was extended approximately 4-fold from 20 min for the parent mutant PEA. These results demonstrate that careful combination of site-directed mutagenesis with specific reactivity of the appropriate PEG-based reagent provides for a powerful approach to the preparation of active PEG conjugates even when interaction with a large substrate is essential to manifest the activity.

Protein engineering techniques can also be used to increase the total number of modifiable amino groups on a target protein. This approach was successfully applied to the preparation of a purine nucleotide phosphorylase mutant, possessing three arginine to lysine substitutions (of a total of 17 Lys per subunit), which after modification with **3** yielded a fully active yet markedly less immunogenic conjugate than one derived from the native hexameric enzyme [72].

3. PEG conjugates with low molecular weight drugs

There are three main areas of use of PEG conjugates with low molecular weight compounds: (1) PEG ligands in aqueous two-phase partitioning, (2) bioreactor use of PEG cofactors and/or PEG catalysts, (3) PEG-drug conjugates for controlled delivery of the biologically active substances.

The demands placed on synthesis of various conjugates are influenced by their intended end use. For example, for both affinity partitioning and bioreactor applications it is hardly important to achieve complete substitution of the end groups with the ligand molecules. Contamination of the conjugate with unreacted PEG can be usually tolerated as well. On the other hand, the nature of the linkage between the components of conjugates is often critically important. The most stringent requirements on the purity and homogeneity apply to the conjugates intended for use as polymeric drugs. Since demonstration of biological activity and/or efficacy is a very delicate task which can often be influenced by even minute impurities, it is very important to avoid any type of contamination including unreacted or partially reacted PEG derivatives [20,73].

In 1973 Dittmann [74] reported that various properties of PEG-alkyl ethers (surface tension, hemolysis, surface anesthesia, endoanesthesia and lethality in mice), depended largely on the non-PEG part, while the influence of the polyglycol chain was rather small. For the most part this type of behavior was observed for most PEG-drugs reported in the last two decades. The polymer, usually, is acting as an inert carrier dominating the physical properties: solubility, membrane permeability, and biodistribution. The covalently linked substrate is responsible for the biological activity. Also the toxicity of a drug can often be reduced by PEG conjugation [75,76]. Depending on the linkage between the conjugate's components the drug can be released or not in vivo.

Numerous methods for attachment of drug molecules have been reported in the literature, yet there are fewer general methods for preparation of PEG-drug conjugates as compared to the polypeptide-based counterparts. In contrast to the polypeptide modifications with PEG, most low molecular weight drug molecules are hydrophobic enough to allow construction of conjugates in organic solvents. Mild coupling methods developed originally for peptide chemistry found wide application in the synthesis of PEG-drugs [73].

In a few cases, instead of using the appropriate functionalized PEG, conjugates were constructed

by ethylene oxide polymerization onto a drug molecule. This was the method used for the preparation of the biologically active conjugate of cinerubin A aglicon [77], in which the trisaccharide residue present on the natural drug was replaced by a PEG chain. Similarly, boron trifluoride-induced polymerization of ethylene oxide was applied to the synthesis of PEG-cholesterol [78]. Water-soluble polyoxyethylated cholesterol was taken up by cultured fibroblasts and showed an inhibitory effect on lipid synthesis, as evidenced by the suppression of cholesterol and fatty acid synthesis and acyl-CoA:cholesterol acyltransferase, an enzyme mediating intracellular cholesterol esterification and implicated in cholesterol absorption.

The most straightforward approach towards drug-PEG conjugates is relying on utilization of the terminal primary hydroxy groups of the polymer to effect the conjugation reaction. Several PEG esters with various drugs are summarized in Table 3. All these conjugates were prepared from PEG-OH and a free carboxylic acid under relatively mild conditions. The use of dicyclohexylcarbodiimide in the presence of dimethylaminopyridine is a particularly attractive approach. It results in clean transformation of carboxylic acids into PEG esters at room temperature within short periods of time (2–4 h) [73,79]. One has to bear in mind that in aqueous conditions esters of this type can undergo hydrolysis, thus dissociating the conjugate components. The rates of hydrolysis depend on the nature of the carboxylic acid, molecular weight of PEG, pH and temperature. Half-lifetimes of this type of ester hydrolysis can be of the order of magnitude of several hours in phosphate buffer, pH approx. 7.4. Therefore this can be used as an approach for a slow release of an active drug under physiological conditions. However, the approach is limited by relatively few drugs containing carboxylic acid groups. Although the use of appropriate spacers, e.g., succininate, can allow the use of PEG esters of a larger selection of drugs. For example, atropin and quinidine were successfully linked via ester linkages to PEG-succinates of various molecular weights [73].

Direct attachment of drugs to hydroxyl groups of the polymer can also be achieved by forming ether, carbonate and urethane bonds via the appropriate alkylation and acylation reactions. For example, Khue and Galin [86] prepared carbonate-linked conjugates of hydrocortisone and dexamethasone by reacting their chloroformate derivatives, formed selectively on the primary hydroxyl groups, with HO-PEG. The yields of the coupling reactions were low to moderate. Release of the antiinflammatory steroids from the conjugates due to hydrolysis of the

Table 3
Summary of PEG esters with biologically active carboxylic acids

Drug	MW of PEG	Reagent/conditions	Comments	Reference
Ibuprofen	200, 900, 2000	CDI in $CHCl_3$ at 60°C	Longer-lasting antiinflammatory activity of PEG derivatives was observed	80
Ursodeoxycholic acid	1000, 2000	CDI in $CHCl_3$ at 60°C	An efficient method for PEG esterification applicable to bile acids was demonstrated	81
2-Benzoxazolon-3-yl acetic acid	200–1000	DCC in THF	Conjugate of PEG-400 was used as a substrate in penicillin amidase-mediated synthesis of new biologically active cephem	82
Benzoylacrylic acids	mPEGs 550–1900	DCC/pyridine in CH_2Cl_2	Antimicrobial activities were studied in vitro	83
Penicillin V	mPEG-2000, 3000, 4000; poly(PEG-Lys) 50 000	DCC/DMAP	Efficient esterification demonstrated. The conjugate is readily hydrolyzed releasing an active antibiotic	73,84
Aspirin	400, 1000, 3000	DCC/DMAP	Aspirin esterification used as a model reaction for efficient synthesis of PEG-ester conjugates	73,85

CDI, *N,N'*-carbonyl diimidazole; DCC, *N,N'*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; THF, tetrahydrofuran.

carbonate linkage was pH, temperature and steric hindrance dependent [87]. The observed rates of hydrolysis of carbonates of PEG-steroids indicated that the reaction is base-catalyzed and it is tempered enough to be useful for gradual drug release applications. In many instances it is convenient to link various drugs to HO-PEG utilizing the chloroformate intermediate of the polymer. Incubation of PEG-O(C = O)-Cl, easily generated by treatment with phosgene, hydroxyl- or amino-containing drugs at room temperature in the presence of a tertiary amine quickly results in conjugate formation, usually in high yields. Atropine [88], tetrahydrocannabinol [89] and procaine [90] were attached to PEG in this fashion. The conjugates of all three drugs exhibited substantial preservation of biological activity.

Amino-containing drugs, for example doxorubicin, react readily with active carbonate derivatives of the polymer. Caliceti et al. [76] used this approach to synthesize several urethane-linked amino acid (Gly, Phe, Thr) and tripeptide (Gly-Val-Phe)-doxorubicin adducts. The PEG conjugates were considerably less toxic than doxorubicin. The antitumor activity tested in mice, Ehrlich solid tumor model, was dependent on the amino acid/peptide spacer. While the Gly-interlinked conjugate lacked any activity, the Phe and Thr derivatives were 20% and 16% active. The tripeptide-containing conjugate exhibited 50% of the free doxorubicin activity, which was not due to the release of the drug from the conjugate, since all the conjugates showed great stability in plasma.

In contrast to carbonate- and ester-linked PEG drugs, urethane-linked conjugates are very resistant to hydrolysis. Experiments on urethane-linked radioiodinated tyramine and histamine derivatives of PEG showed minimal breakdown under conditions ranging between pH 2 and 11, as well as during incubation in liver homogenate and in disrupted lysosomes [91].

Amino-PEG and its monofunctional mPEG-NH_2 analog (**15**) are useful derivatives for preparation of bioconjugates. Taking advantage of the increased reactivity of amino-PEG over its hydroxy analog, drug molecules can be readily coupled through a variety of reactions forming amide, *sec*-amine, carbamate, urea, and thiourea linkages, all of which are stable in vivo. For example, isothiocyanate derivatives of ion channel blockers, piretanide, disulfonic stilbenes, and phenylantranilates were reacted with amino derivatives of PEG yielding biologically active thiourea-linked macromolecular ion transport inhibitors [92,93].

Further demonstration of the importance of the linkages between components of PEG drug conjugates on their properties is available from a study with 5-fluorouracil (5FU) conjugates. Ouchi et al. [94–96] synthesized a series of 5FU-PEG derivatives. The conjugates of various degrees of polymerization 230-1 were constructed, as shown in Fig. 4, to contain ester, amide and ether linkages. The ester-bound conjugates hydrolyzed easily even under mild conditions approximating physiological and the hydrolysis rates decreased with pH and with increase in molecular weight of the conjugates. The amide conjugates hydrolyzed noticeably slower, while showing similar hydrolysis-molecular weight dependence. The ether-bound conjugates were considerably more stable showing some drug cleavage under acidic pHs, while exhibiting similar dependences on the molecular weights of the carriers. These conjugates were resistant to enzymatic degradation, while the ester conjugates were cleaved slowly by esterase. Amide conjugates were subject to acylase-catalyzed hydrolysis.

The antitumor activity of 5FU-PEGs was evaluated against lymphocytic leukemia in mice. For both ester- and amide-linked conjugates the antitumor activity increased with the increase of molecular weight of the carrier, while the opposite relationship was observed for the ether-bound conjugates. The biological activities of the conjugates were also dependent on the activities of the 5FU derivatives cleaved from the polymer (see dashed arrows in Fig. 4). It was suggested by the authors that larger amounts of the drug were delivered to the tumor cells with increasing degree of polymerization of PEG.

One of the important reasons for the application of PEG as a modifier of biologically active substances lies in its structural simplicity. The polymer has a chemically inert backbone and

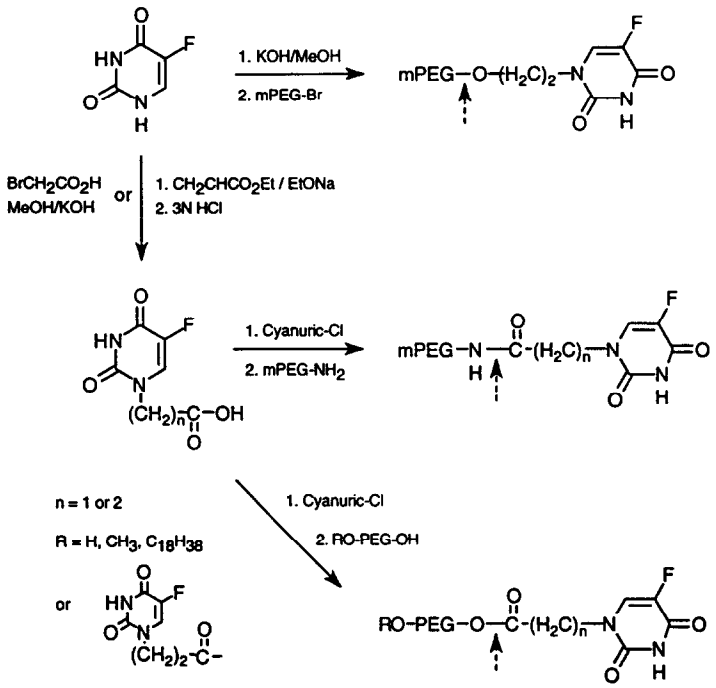


Fig. 4. Preparation of 5FU conjugates of PEG according to Ouchi et al. [94–96].

only two, or in the case of mPEG one, functionalizable end groups. While this simplicity is of clear advantage in situations when it is desirable to link multiple PEG chains to a substrate intended for modification (e.g., protein, liposome), in some situations it might translate into a limitation. For example, for low molecular weight drug-PEG conjugates only a few design options exist. The number of the drug molecules that could be linked to a single PEG chain is also limited. In order to overcome these design and loading drawbacks new drug carriers composed of alternating segments of PEG and lysine bound by urethane linkages, were recently introduced [84,97]. These new polymers were synthesized by polymerization of bis-SC-PEG with lysine, so that both amino groups of the amino acid are incorporated into the backbone-forming urethanes and the carboxyl groups can be chemically manipulated for introduction of various functional groups or conjugation (see Fig. 5). The length of the PEG fragment determines the distance between the functional groups along the backbone. As the molecular weight of the PEG segment is increased the drug-binding capacity of the poly(PEG-Lys) product (equivalents of drug per gram of the carrier) is decreased. Poly(PEG-Lys) of molecular weight 50000, derived from PEG-2000, was studied in some detail as a drug carrier. This material while retaining the beneficial properties of PEG (low toxicity, lack of accumulation in RES organs), possesses high drug attachment capacity through the pendant carboxyl groups of the lysine residues. Various drug molecules were linked in good yields to the suitably functionalized derivatives of this polymer via both stable and biodegradable linkages [84,98]. Poiani et al. [99] demonstrated antifibrotic activity of *cis*-4-hydroxy-L-proline (CHP) conjugates with poly(PEG-Lys). Particularly promising was amide-linked poly(PEG-Lys-CHP), exhibiting over 100-fold higher smooth muscle cell inhibiting activity than the free drug.

Another approach towards PEG-based drug carriers with improved chemical versatility as well as increased drug-loading potential involved polymerization of *N*-carboxyanhydride of β-benzyl esters of aspartic acid onto mPEG-NH_2 (**15**) initiator, followed by removal of the side chain protecting groups from the poly(Asp)

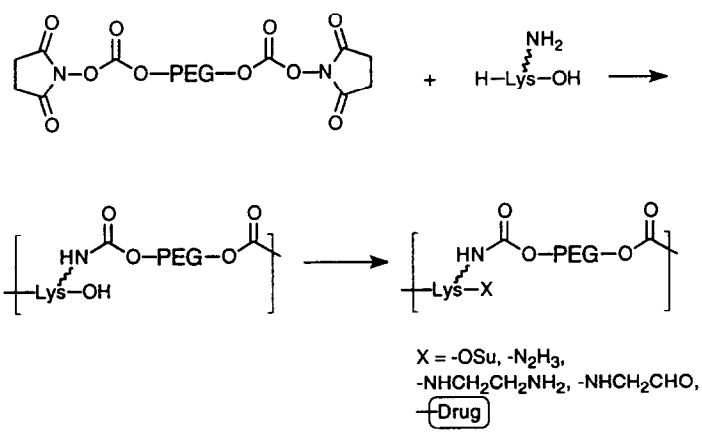


Fig. 5. Preparation and use of alternating copolymer of PEG and lysine according to Nathan et al. [84].

block of the copolymer [100]. The exposed carboxyl groups were used for attachment of doxorubicin via amide bonds. A number of compositions of this block copolymeric system with various mPEG chain lengths, degrees of polymerization of poly(Asp) block, and a number of attached doxorubicin residues were studied. Their chemical, physical and biological (both in vivo and in vitro) properties were described in detail. The doxorubicin conjugates to mPEG-poly(Asp) forming micelles were composed of a drug-containing hydrophobic core with grafted PEG chains on their exterior. This is thought to be responsible for some of the useful properties of these conjugates in vivo: prolonged circulation in the bloodstream, reduced uptake by liver and spleen, accumulation in tumor tissue resulting in enhanced anticancer activity [101].

Other amino acids (Glu, Lys, Phe, etc.) were also successfully copolymerized with amino-PEG by a variety of chemical and enzymatic reactions [102–105]. Thus it appears that amino acids in combination with suitably functionalized PEG derivatives can serve as an arsenal of building blocks for assembly of drug carriers with taylor-made properties, e.g., enzymatic and/or hydrolytic lability [104,105], ability to interact with biological cells [102,103], etc.

4. PEG conjugates with lipids

Great interest in lipid conjugates with PEG was generated recently as a result of the discovery that incorporation of PEG-lipids into liposomes yields preparations with superior performance compared to conventional liposomes. Such liposomes remain in the blood circulation for extended periods of time ($t_{1/2} \geq 48$ h in humans) and distribute through an organism relatively evenly with most of the dose remaining in the blood compartment and only 10–15% of the dose in liver. This constitutes a significant improvement over conventional liposomes (for a comprehensive review of the subject see [14]). Most of the PEG-lipids are prepared by derivatization of phosphatidyl ethanolamines (PEs) with the above-mentioned reagents (**1**, **3**, **6**, **7**, **10**, **12**) originally introduced for modification of amino groups of proteins [21]. Fig. 6 summarizes the synthetic pathways leading to the various mPEG-PE conjugates. Klibanov et al. [106] used SS-PEG (**3**) derived from mPEG-5000 for preparation of mPEG-succinamide-dioleoyl-PE. Urethane linked mPEG-PE conjugates were obtained by reacting mPEG-oxycarbonylimidazole (**7**) or SC-PEG (**10**) with distearoyl-PE [107,108]. The reagent **10**, being the more reactive one, allows preparation of mPEG-DSPE within 10–15 min at 45°C [109]. DSPE conjugates derived from reagents **1** and **12** both of molecular weight 5000, were prepared for formulation of long-circulating liposomes [110,111]. Amide-linked mPEG-DSPE was prepared by coupling **6** to the amino group of PE [112].

Instead of using a PEG-lipid conjugate to form liposomes Senior et al. [113] modified surface amino groups of DSPC-DPPE-cholesterol vesi-

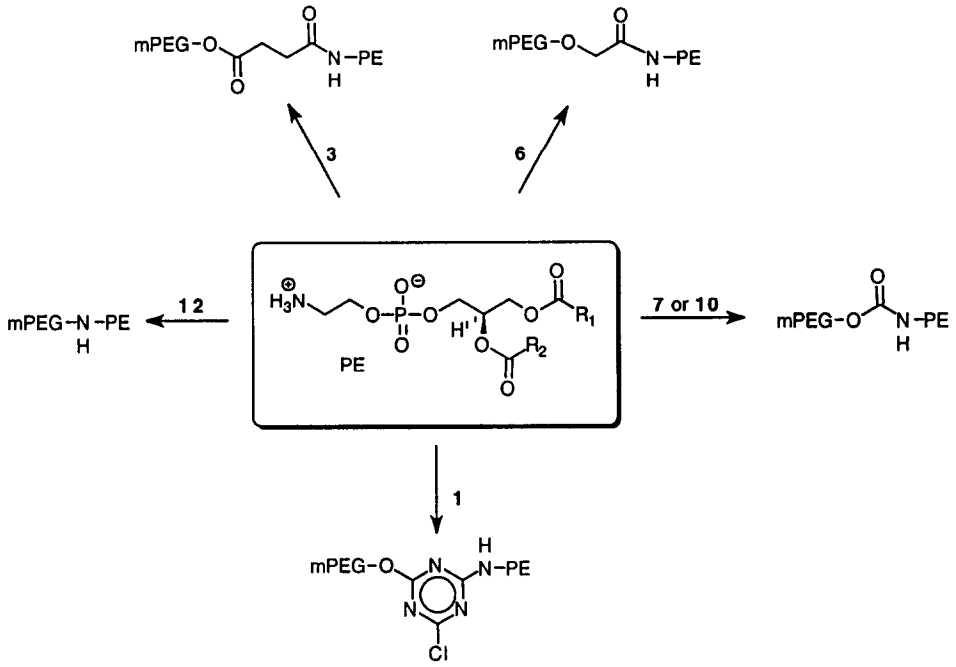


Fig. 6. Methods for the preparation of mPEG-phosphatidylethanolamine conjugates.

cles with mPEG-tresylate (**12**). The attractive feature of this approach is in its selective grafting of the polymer on the exterior of the vesicles. It avoids the presence of mPEG residues inside the liposomes. However, the difficulty in substituting all the available amino groups of PE residues and the formation of an asymmetrical lipid bilayer might be undesirable in some situations. Another method for direct grafting mPEG residues onto liposomes was used by Herron et al. [114]. They prepared maleimido-PE-containing vesicles, which were then reacted with a thiol derivative of the polymer.

Only a couple of examples of mPEG conjugates with other lipids (non-PE) are known. Phosphatidic acid (PA) was attached directly to mPEG-OH using 2,6,6-triisopropylbenzenesulfonyl chloride-mediated coupling in pyridine [112]. Oxidation of the diol residue of DSPG with periodate followed by reaction with mPEG-hydrazide (**14**) led to a hydrazone-linked mPEG-lipid. For some applications it might be useful to have an acid-labile hydrazone linkage connecting the conjugate components [21].

With the data available to this date there is no reason to assume that linkage between lipid residue and mPEG influences the ability of the conjugates to form liposomes. Although there were some differences observed in the properties of liposomes containing various mPEG-PE conjugates, they can be explained by subtle differences in formulations [14]. The chlorotriazine approach to the preparation of the biologically active conjugates is unattractive due to toxicity of the reagent and its degradation products. Structurally similar herbicides are target analytes in the US Enviromental Protection Agency. The presence of slightly reactive chloride in the PE conjugate with **1** is another drawback of this method. Succinate ester linkage present in the conjugate derived from **3** is known to undergo hydrolysis under physiological conditions [30]. Recently Parr et al. [112] convincingly demonstrated that this type of conjugate readily releases free mPEG-OH after a few hours of incubation at 37°C in mouse serum. In this regard amide-, secondary amine- and urethane-interlinked mPEG-PEs, as well as mPEG-PA, all containing stable linkages between the conjugate components, are more appealling.

The PEG-lipid conjugates can be purified from the conjugation reaction mixtures by silica-gel

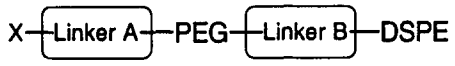
chromatography. Since such conjugates exist in water almost exclusively as micelles, they are readily separated from unreacted PEG reagents by aqueous size exclusion chromatography. Taking advantage of their very low critical micelle concentration, PEG-PEs can be retained in dialysis bags having very large pores (≥300000 MWCO), which allows for free PEG to diffuse freely through the membrane. This method was also used for purification of various PEG-PE derivatives [109,115]. Lipid conjugates of PEG can be conveniently characterized by TLC and NMR [109]. The latter mode of analysis is particularly informative [21].

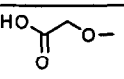
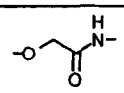
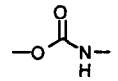
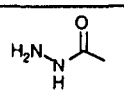
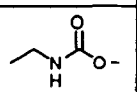
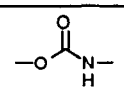
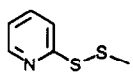
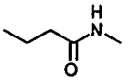
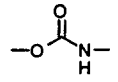
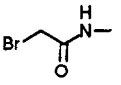
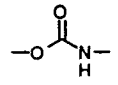
Several urethane-linked mPEG-PEs were tested with various lipid compositions for their ability to form liposomes and for variation in the properties of those liposomes [108]. It was reported that the prolonged plasma presence of PEG-PE-containing liposomes is independent of the degree of saturation of the parent PE or other lipids co-incorporated into the same liposomes. However, better results were obtained with 18 carbon-fatty acid-containing PEs than with shorter chain derivatives. For example, mPEG-DSPE and mPEG-DOPE were superior to mPEG-HSPE conjugates, which contain a significant amount of palmytoyl residues. Conjugates of DSPE derivatives from mPEG of molecular weights 1900 and 5000 incorporated into lecithin/cholesterol liposomes of mean diameter 100 nm, showed comparable blood lifetimes and biodistributions in rats. Maruyama et al. [116] studied the molecular weight dependence on performance of **3**-derived mPEG-DSPE conjugates in large unilameral liposomes (190 nm mean diameter). MPEG-DSPEs of molecular weights 1000 and 2000, in addition to showing the lowest accumulation in liver, extended the circulation time of liposomes more than conjugates derived from mPEG-5000 and -12000.

Reduced non-specific clearance of PEG-grafted liposomes, which translates into their low RES uptake and prolonged circulation lifetimes, is dependent on hydrophobicity and lipid bilayer affinity of mPEG-PE. Silvius and Zuckermann [117] showed that interbilayer transfer of mPEG-linked saturated diacyl lipids decreased exponentially with increasing the fatty acid chain length. Migration of POPE derivatives was 30–40 times slower than that of the corresponding mPEG-DMPE conjugates. These findings are consistent with recent results unambiguously showing superior performance of liposome-containing mPEG-DSPE over their mPEG-POPE-containing counterparts [112]. In general, upon increasing the molecular weight of the polar head group the dissociation of lipid derivatives from the liposomal bilayer becomes easier [117]. Thus the hydrophobic energy required to retain mPEG-PE in lipid vesicles increases with molecular weight of the polymer. This suggests that increasing the molecular weight of the liposome-grafted PEG chains contributes to the departure of PEG-lipids from the vesicles. This perhaps explains why mPEG of molecular weight 2000 and not higher showed optimal performance in liposomes. In contrast, in other applications (e.g., PEG-modified proteins or biocompatible surfaces), where PEG is grafted to reduce undesirable interactions with plasma proteins and particulates, increase of molecular weight of the grafted polymer chains results in increasing the stearic stabilization effect.

A number of PEG-lipid conjugates, containing reactive functional groups at the ends of the polymer chains, were recently prepared. These derivatives, summarized in Table 4, can be viewed as analogs of mPEG-PE conjugates in which the chemically inert methoxy group is replaced with a conjugation-prone functionality [21]. The need for such functionalized PEG-lipids was created by their usefulness in attachment of various biologically relevant ligands to the exterior of PEG-grafted liposomes. The rationale was that, since PEG-liposomes circulate in the bloodstream for extended periods of time, one could use them as platforms for the presentation of various ligands. Such ligand-carrying liposomes can be used for a variety of applications, most notably for receptor-mediated targeting. As indicated in Table 4, some of the end group functionalized PEG-lipids have already been successfully used in various conjugation protocols resulting in attachment of immunoglobulins, peptides and other ligands to the distal ends of liposome-grafted PEG chains.

Table 4
Summary of end group functionalized PEG lipids of general formula:



No	X-	Linker A	Linker B	Applications	Reference
16		None		Used for conjugation of plasminogen to liposomes for targeting to fibrin. Longevity in plasma circulation was well preserved.	[9, 118]
17	H_2N—	None		Used for preparation of other end-group functionalized PEG-DSPE via amino-group modification. Forms long-circulating liposomes, behaving as positively-charged particles.	[21, 119]
18				Forms hydrazone linkages with aldehydes. Used for conjugation of antibodies, oxidized on their carbohydrate residues, to distal ends of PEG chains on surfaces of liposomes. Conjugation of IO_4^--treated N-terminal Ser or Thr peptides.	[109, 120]
19				As a precursor for HS-PEG-DSPE very efficient for attachment of maleimide-containing antibodies to end groups of PEG on liposomal surfaces. Used for binding HS-containing ligands through disulfide linkage.	[21, 121]
20		None		Efficient for thioether-forming reactions with HS-ligands. Utilized for Fab'-SH attachment to end groups of PEG chains on liposomes.	[21]

Carboxyl-PEG-DSPE was prepared by coupling bis-carboxyl-PEG to the amino group of PE followed by isolation of the desired product (**16**) by silica gel chromatography [9]. It is very convenient, however, to synthesize functionalized PEG-lipids from the suitable heterobifunctional derivatives of PEG [17]. This was the principal methodology used for the preparation of conjugates **17–19**. Amino-PEG-DSPE (**17**) has proven useful as starting material for synthesis of other functionalized PEG-lipids, e.g., 2-pyridyldithiopropionamide (**19**) and bromoacetamide (**20**) derivatives. In these experiments **17** was cleanly derivatized with heterobifunctional reagents, SPDP and *p*-nitrophenyl bromoacetate respectively. Placing an amino acid residue as part of Linker A is helpful for the final chemical characterization of the conjugates by amino acid analysis. This was utilized in the case of hydrazide-PEG-DSPE (**18**). Linker B in most cases presented in Table 4 was a stable urethane linker formed by reacting PE with an appropriate SC-PEG-X, where X represents a functional residue or a protected form thereof. It is possible and in some cases might be advantageous, to design a linker with a well defined lability. This might allow further control of the in vivo properties of the relevant conjugated liposomes [21].

5. PEG conjugates with other biological macromolecules

The three classes of PEG conjugates discussed above are subject of the bulk of the work performed in this area. However, in recent years a number of unconventional PEG conjugates have been described in the literature, which were judged important to be mentioned here.

It appears that attachment of PEG to oligo-

nucleotides or their backbone-modified analogs can improve some of the in vivo properties of these materials. Resistance to nucleases, cell membrane permeability, and improved solubilities were some of the attractive features of these types of conjugate. For example, morfolinonucleoside oligomers with carbamate interlinkages, useful non-biodegradable derivatives of DNA suffering from very limited solubility, were modified on their 5′-terminal amino group with PEG-nitrophenyl carbonate (**9**) to produce conjugates of improved water solubility [122]. Recently it was demonstrated that such backbone-modified analogs of AGG and GGAG containing 5′-linked PEG-1000 inhibited colony formation in normal *E. coli*. In contrast, the unconjugated sequences were able to achieve this result only in *E. coli* strains lacking an outer cell wall [123].

Efimov et al. [124] described a solid-phase method of general applicability for preparation of 3′-, 5′- and 3′,5′-PEG-oligonucleotides. It is based on attachment of mono(dimethoxytrityl)-PEG to porous beads (controlled-pore glass) through a phthalate ester residue, followed by removal of the DMT group and assembly of the desired oligonucleotide sequence by the phosphoramidite method of solid-phase oligonucleotide synthesis, and then cleavage of the 3′-PEG conjugate by aminolysis. To obtain 5′- or 3′,5′-conjugates in the last step, prior to cleavage from the support, a phosphoramidite derivative of PEG was used.

A somewhat similar approach was used in conjugation of *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl)-*R*-cysteinyl (Pam_3Cys), the synthetic analog of the N-terminal lipopeptide of *E. coli* [125]. The conjugate was constructed on an amino-PEG-polystyrene graft copolymeric support specifically designed to release the grafted PEG component during treatment with trifluoroacetic acid. Using standard coupling methodology Pam_3Cys was attached to the amino group of the grafted PEG followed by the acidolytic cleavage of the conjugate. Pam_3Cys-PEG retained the biological properties of lipopeptides. It was able to polyclonally activate murine and human B lymphocytes in vitro and proved to be a powerful adjuvant in vivo in mice [125].

A convenient method for grafting mPEG chains onto dextrans was recently described [126]. It involves activation of some of the *sec*-hydroxy groups of the polysaccharide with *p*-nitrophenyl chloroformate followed by aminolysis with mPEG-NH_2 (**15**). The extent of mPEG grafting is controllable by the amount of the reactive *p*-nitrophenyl carbonate groups on dextran. The method appears to be of general applicability to polysaccharides. Another method for dextran-PEG preparation involving alkylation of **15** with epichlorohydrin-activated dextran was described by Duval et al. [127]. The possibility that PEG-dextrans, being conjugates of incompatible polymers, might phase separate at the molecular level and form colloidal aggregates was suggested. The observation of entrapment of free PEG in dextran-PEG, unambiguously demonstrated by analytical GPC [126], was consistent with this hypothesis.

Polysaccharide-PEG conjugates are expected to possess useful properties for their use as drug carriers. For example, Ouchi et al. [128] prepared PEG-chitosan by acylating the amino groups on the polysaccharide with mPEG-carboxylate, and covalently linked 5FU to it. The conjugate showed some activity against lymphocytic leukemia in mice.

6. Concluding remarks

The usefulness of PEG as a material for construction of various bioconjugates is widely recognized. Some of the PEG-modified substrates, e.g., proteins, liposomes, blood-contact materials, already enjoy commercial use. It is expected that the current trend of increasing use of PEG conjugates as well as broadening of their realm of applications will continue. This process should be facilitated by the significant maturity that the art of preparation of various functionalized derivatives of the polymer reached [17].

It is clear that many of the new therapeutic biotechnology products, recombinant proteins, oligosaccharides, oligonucleotides or their analogs can potentially benefit from the protection from enzymatic degradation, extended plasma lifetime, diminished uptake by RES organs, or

from reduction of other unwanted manifestations of biological recognition, that conjugation with PEG is likely to provide. In many instances a fine balance between maintenance of the biological activity and the extent of covalent modification is required. The extensive experience with PEG-modified proteins that act on large size substrates shows that such systems require a particularly great degree of fine tuning. Increasingly sophisticated PEG reagents designed to modify selective sites of biological macromolecules certainly will emerge to address some of these challenges.

The question of 'What is unique about PEG?' has to be addressed for both practical and purely scientific reasons. It is the opinion of this author that PEG enjoyed its popularity as a modifier of biologically active molecules not only because of its excellent physical, chemical and biological properties but also because of its structural simplicity and commercial availability in a variety of molecular weights, high purity and homogeneity. Yet the structural simplicity, particularly in the case of mPEG derivatives, allowed researchers to prepare conjugates containing conformationally free polymer chains bound to a substrate through only one end group. This task was often more difficult with other biocompatible polymers. Recently several attempts were made to prepare reactive derivatives of various water-soluble polymers so that the reactive functionality is positioned either at the initiation or termination ends of the chains. Some functionalized polymers of this type (polyvinyl pyrrolidone, polyvinyl alcohol, polyoxazolines) were applied to the modification of proteins [129–131] and liposomes [132,133]. It will be interesting to see whether such polymers can convey similar properties to their conjugates as does PEG. Already some encouraging results were observed in the case of non-immunogenic, long-circulating polyvinyl alcohol-SOD [129]. Comparison of in vivo properties in rats of liposomes containing DSPE linked to poly(2-methyl-2-oxazoline) or poly(2-ethyl-2-oxazoline) to mPEG-DSPE-containing liposomes [133] showed for the first time the same persistence in circulation and low hepatosplenic uptake for all three types of polymer-grafted vesicles. Further systematic comparison of various polymers suitably functionalized and conjugated is needed to unambiguously answer the question on the 'uniqueness of PEG'.

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