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Poly(ethylene glycol) block copolymers

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Abstract

The ubiquitous use of poly(ethylene glycol) in the biomaterials field has also boosted the research activity in the chemical derivatization of this polymer. We focused our interest on the preparation of tailor-made poly(ethylene glycol)-based structures and on the study of structure–activity relationships for its functionalization, as preliminary steps for the preparation of smart functional materials. More specifically, amphiphilic and cationic block copolymers were prepared for prospective use in the preparation of self-assembled carriers, and Michael-type addition of thiols onto acrylates was studied as a model for end-group reaction leading to hydrogel formation.

Keywords: Poly(ethylene glycol); Block copolymers; Amphiphilic polymers; Polyelectrolytes; Bioconjugation

1. Introduction

Since the beginning of the 1990s, poly(ethylene glycol) (PEG) has been elected as the standard substrate for biocompatible applications (Harris, 1992), because of its ability e.g. to reduce protein adsorption on a variety of hydrophobic substrates (Andrade et al., 1996), such as solid polymers (Bergstrom et al., 1992) or liposomes (Klibanov et al., 1990; Katre, 1993; Lasic, 1995), to minimize platelet deposition onto surfaces exposed to the blood stream (Maechling-Strasser et al., 1989; Lee et al., 1995), and at the same time to be acceptable for in vivo administration as a pharmaceutical adjuvant: PEGylated proteins are used for their increased resistance to enzymatic degradation (Katre, 1993; Brumeanu et al., 1995; Guerra et al., 1998; Ueno et al., 2000), increased thermal stability (Longo and Combes, 1999) and decreased immunogenicity in vivo (Abuchowski et al., 1984; Brumeanu et al., 1995; So et al., 1999).

A number of reactions have been used for PEG derivatization and conjugation to bioactive molecules (Zalipsky, 1995; Veronese, 2001). Besides its biological properties, PEG offers the

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advantage of simultaneously having a backbone with low MW polydispersity, inert to most chemical reactions and soluble in most solvents and in water, and a quantitative termination with well-defined reacting groups, generally alcohols.

Due to these numerous favorable biological and chemical properties, we have chosen to use PEG as a base building block for polymeric structures used in the preparation of biomaterials, such as hydrogels, beads and colloidal carriers.

PEG has been modified in the macromolecular backbone in order to add physical functionality to the final material, and in the terminal reactive groups in order to enhance and/or make more selective its chemical reactivity, e.g. for bioconjugation, but also for materials preparation.

In a general picture, the PEG structure is thus transformed into a reactive and functional block copolymer, which in turn yields the final material through a purely physical process, a chemical reaction, or both (Fig. 1).

In the present paper, we report on the procedures for the preparation of such materials.

It also has to be stressed that considerable effort has been made to implement water-based processes and *one-pot* reactions (reduced organic solvent consumption), while the reaction conditions have generally been optimized by means of parallel synthesis (enhanced scale-up possibilities).

2. PEG block copolymers from backbone modification

We have pinpointed two areas of interest, which could be conceptually summarized as a decrease or increase in the overall polymer polarity, respectively.

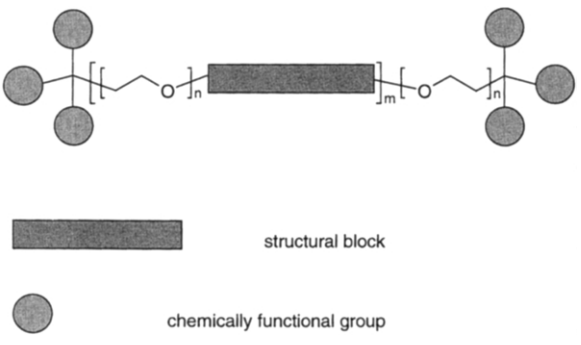

Fig. 1. Structure of PEG block-copolymers.

The first one involved the preparation of amphiphilic structures, where PEG played the role of the hydrophilic component, with self-assembling possibility in water environments and applications as carriers for controlled drug delivery. The second one was based on the generation of PEG-based polyelectrolytes, with potential applications in cell encapsulation (through polyelectrolyte coacervation) and in surface coating.

2.1. PEG sulfamers (Napoli et al., 2001)

The lyotropic behavior in water of amphiphilic synthetic PEG copolymers has been extensively studied on only a fairly restricted number of structures, such as the poloxamer (also known as pluronic) series of PEG-*bl*–poly(propylene glycol) di- and triblock copolymers (Alexandridis, 1997; Schillén et al., 1999; Booth and Attwood, 2000), and more recently on poly(ethylethylene)-*bl*–PEG (Discher et al., 1999), poly(styrene)-*bl*–PEG (Eisenberg and Yu, 1998), and others (Cornelissen et al., 1998; Sommerdijk et al., 2000), as well as on poly(oxazoline)-containing block terpolymers (Nardin et al., 2000a,b). Such block copolymers are generally prepared via ionic polymerization under strictly anhydrous conditions; the synthetic processes make it generally difficult to obtain asymmetric multiblock structures (such as ABC block copolymers), and only a limited number of functional groups can be tolerated.

Aiming at synthetic methodologies that reduce the number of isolation steps (*one-pot* multi-reactions) and of purification procedures of reagents and products, we have developed a versatile method for the preparation of amphiphilic PEG copolymers in which the hydrophobic block is a polysulfide [poly(propylene sulfide), PPS], obtained from the anionic ring-opening polymerization of episulfides.

The episulfide polymerization is supposed to have a living character, tolerates protic groups, such as alcohols (Bonnans-Plaisance et al., 1995; Bonnans-Plaisance and Levesque, 1989) or acids (Bonnans-Plaisance et al., 1992), and due to the

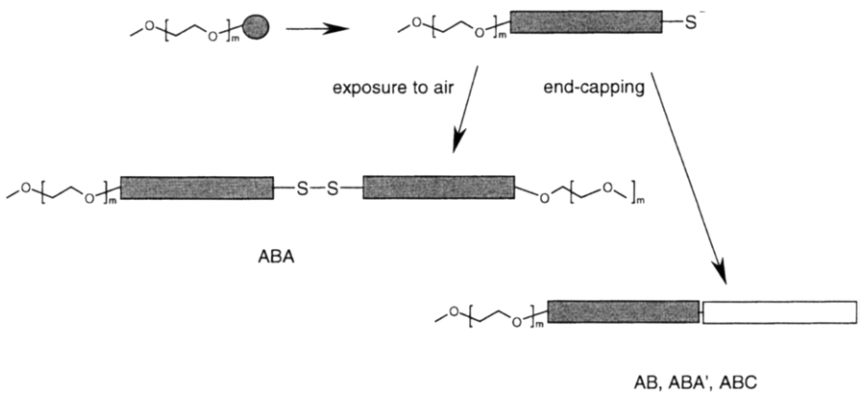

Fig. 2. Synthetic scheme for the preparation of sulfamers. The dark gray box represents the polysulfide block (B block); the white box represents a low MW end-capping agent (AB polymer), a potentially different PEG block (ABA′), or a chemically different hydrophilic block (ABC).

mild character of the propagating species (thiolates), does not require extremely anhydrous conditions. Therefore, it is an ideal candidate for generating well-defined synthetic polymer structures bearing bioactive groups.

Using PEG as an initiator (A block), we have prepared AB, ABA, ABC and ABA′ structures (and potentially higher multiblocks can be prepared), where B represents the PPS block, C and A′ are additional hydrophilic blocked, chemically different and chemically identical, respectively, but with a different MW than A (Fig. 2).

Due to the polysulfide central block and the conceptual similarity to the poloxamers (with an oxygen atom rather than a sulfur atom in the central hydrophobic block), we refer to this class of block copolymers as ‘sulfamers’.

Initiation can be accomplished by a variety of nucleophiles, but sulfur-based moieties (thiolates and the salts of thio- or dithioacids, Bonnans-Plaisance et al., 1994) have shown by far the best results. PEG alcoholates, for example, are not effective initiators, having been demonstrated to be capable of conversion of episulfides to allyl thiolates by proton abstraction, thus generating another initiator species (Boileau and Sigwalt, 1973).

In our scheme, we have avoided the use of thio- or dithioacid derivatives, because of the hydrolytic instability of the corresponding thio- or dithioesters, as well as that of free thiols: their easy oxidation to disulfides makes it difficult to estimate the actual concentration of the active thiolate group, and therefore they should be re-

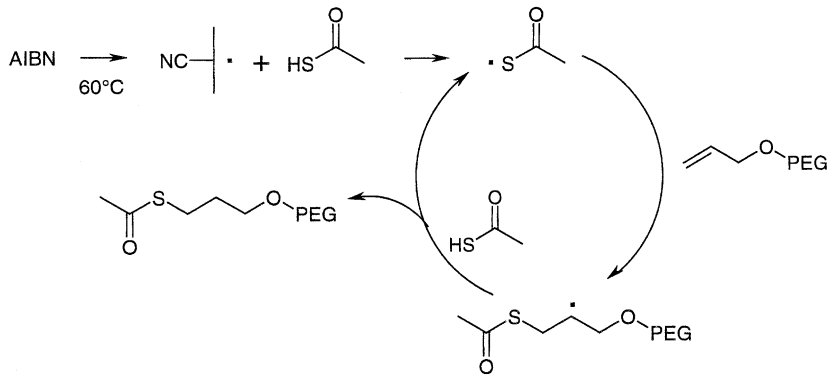

Fig. 3. Synthetic scheme for the preparation of PEG thioacetate.

duced and isolated in oxygen-free environments immediately before their use (Woghiren et al., 1993). In order to reduce the likelihood of dimerization, to enhance the shelf life of the initiators and to determine their concentration in a precise and reproducible way, protected thiols were used and were in situ converted to the initiator species. As protecting groups, thioacetates were successfully employed (Zalipsky, 1995), preparing them through radical addition of thioacetic acid onto PEG allyl ethers (Fig. 3). This reaction has been optimized for PEG with several molecular weights and chain topologies (linear, two-, four- and eight-armed PEGs) to give quantitative conversion of the terminal groups.

The block copolymer preparation consisted of a series of successive reactions performed in a *one-pot* process: the PEG thioacetate was first deprotected to reveal the initiating thiolate; the monomer (propylene sulfide, PS) was then added and promptly polymerized; and finally the polymerization was stopped by the introduction of an end-capping agent or by exposure to air (Fig. 4).

The quantitative deprotection of the thioacetate groups and the living character were demonstrated by analyzing the incorporation of iodoacetamide (a well-known method for thiol quantitative derivatization, Hermanson, 1996, used as an end-capping reaction) at different iodoacetamide/thioacetate ratios (Fig. 5): this showed a 1:1 relationship between the number of reactive end groups and that of original thioacetic esters (Fig. 5). At the same time, the polydispersity values showed the *one-pot* process to be not only living, but also controlled.

The ready dimerization of thiolate-terminated block copolymer upon exposure to air and the negligible interference between this reaction and the end-capping (at least under an inert atmosphere) were proved with the same set of samples: with iodoacetamide in stoichiometric deficit, a mixture of 'monomeric' diblock PEG-*bl*–PPS-amide and disulfide-linked 'dimeric' triblock $(PEG\text{-}bl\text{–}PPS)_2$ was detected after work-up under air (Fig. 6).

As end-capping reaction, the Michael-type addition reaction of thiolates onto acrylates showed the most promising results for attachment of the A′ or C block, because this tolerates a variety of bioactive functional groups (Romanowska et al., 1994; Lutolf et al., 2001) which can be included in a PEG acrylate structure.

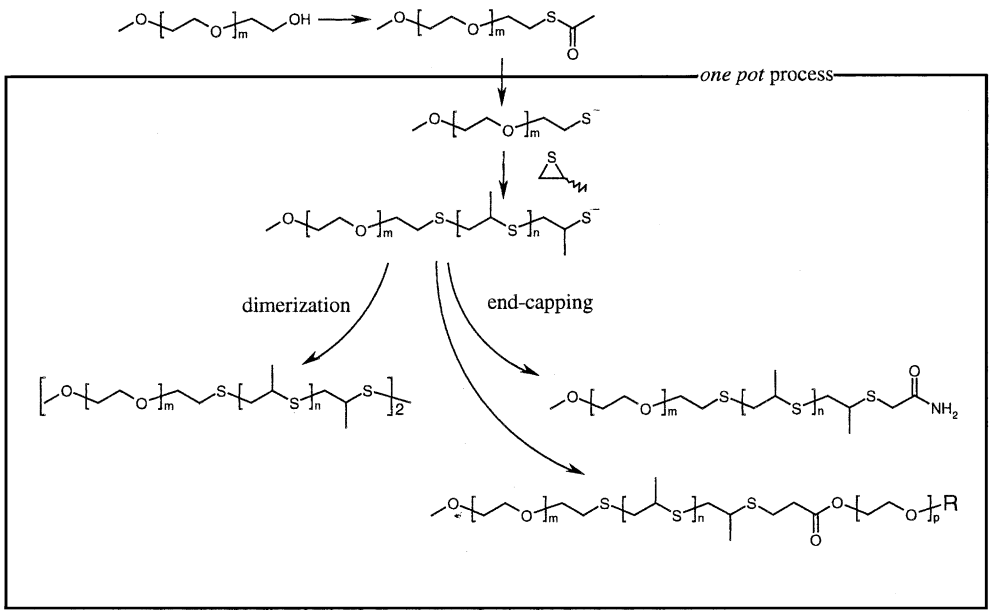

Fig. 4. Sulfamer synthesis.

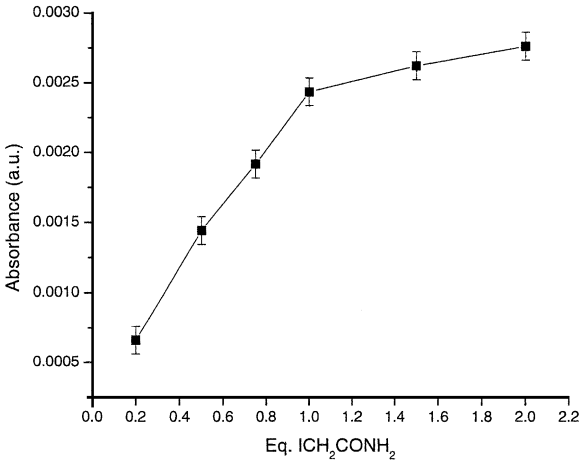

Fig. 5. Amide absorbance vs. equivalents of end-capping agent for the termination reaction with iodoacetamide, demonstrating the living nature of the PS polymerization.

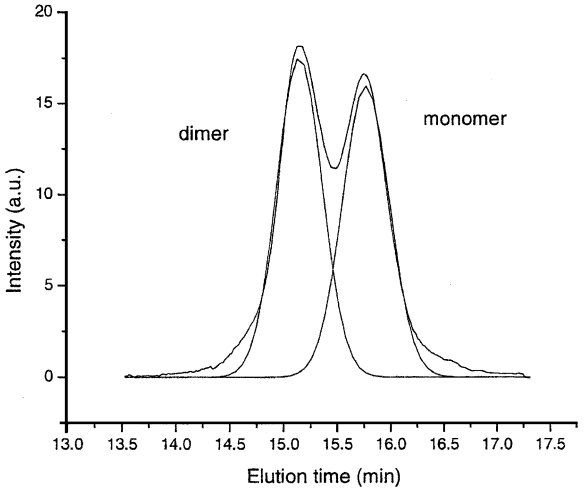

Fig. 6. GPC trace in THF for a polymer mixture constituted by 55:45 dimeric disulfide-linked triblock copolymer and monomeric amide-terminated diblock copolymer.

Sulfamers show extremely promising lyotropic behavior in water, which makes them good candidates for building micellar and vesicular carriers for controlled and targeted drug delivery.

2.2. PEG cationic block copolymers via DADMAC polymerization (Tirelli and Hunkeler, 1999)

The virtual absence of termination reactions through monomer and solvent transfer and disproportionation (Hahn et al., 1984; Hahn and Jaeger, 1992) is the key feature of the *N*,*N*-diallyl-*N*,*N*-dimethylammonium chloride (DADMAC) polymerization, which allows its use in the synthesis of polyelectrolyte structures based on multistep, controlled, radical polymerization: assuming the polymerization to terminate only via recombination, the initiator architecture templates that of the final polymer.

Poly(DADMAC) has not only been used for traditional polyelectrolyte applications (such as colloid flocculation or as paper sizing agent; Ottenbrite and Ryan, 1980), but also in the field of microencapsulation (Dautzenberg et al., 1996). However, the peculiarity of DADMAC polymerization offers the possibility of tailoring its macromolecular architecture, and thus opens new perspective applications.

PEG DADMAC block co- and terpolymers were first synthesized by Jaeger and co-workers (Jaeger et al., 1996; Lieske and Jaeger, 1998), in the form of ABA polymers, where A stands for PEG and B for poly(DADMAC) block. We have enlarged the number of available structures, producing $(AB)_n$ multiblocks or CABAC pentablocks, where C is another radically polymerizable monomer, in this case the cationic 2-(trimethylammonium chloride)ethyl methacrylate (TMAEM) (Fig. 7).

PEG-containing azo initiators derived from bis(3-azo-3-cyanovaleric acid chloride) were synthesized by reaction with a two-fold excess or an equimolar amount of PEG of variable length (Fig. 8). The corresponding macroinitiators were characterized by functional PEG–OH groups at the two ends (**A**), or by a repeating blocky structure (**B**), with a polymerization degree p of approximately 3 for two different PEG molecular weights (600 and 3000). Another macroinitiator (**C**) was produced by Ce(IV)-initiated polymerization of TMAEM from the terminal groups of **A**.

This family of macroinitiators was successfully

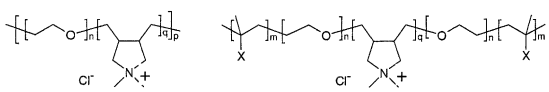

Fig. 7. DADMAC multiblock copolymer structures.

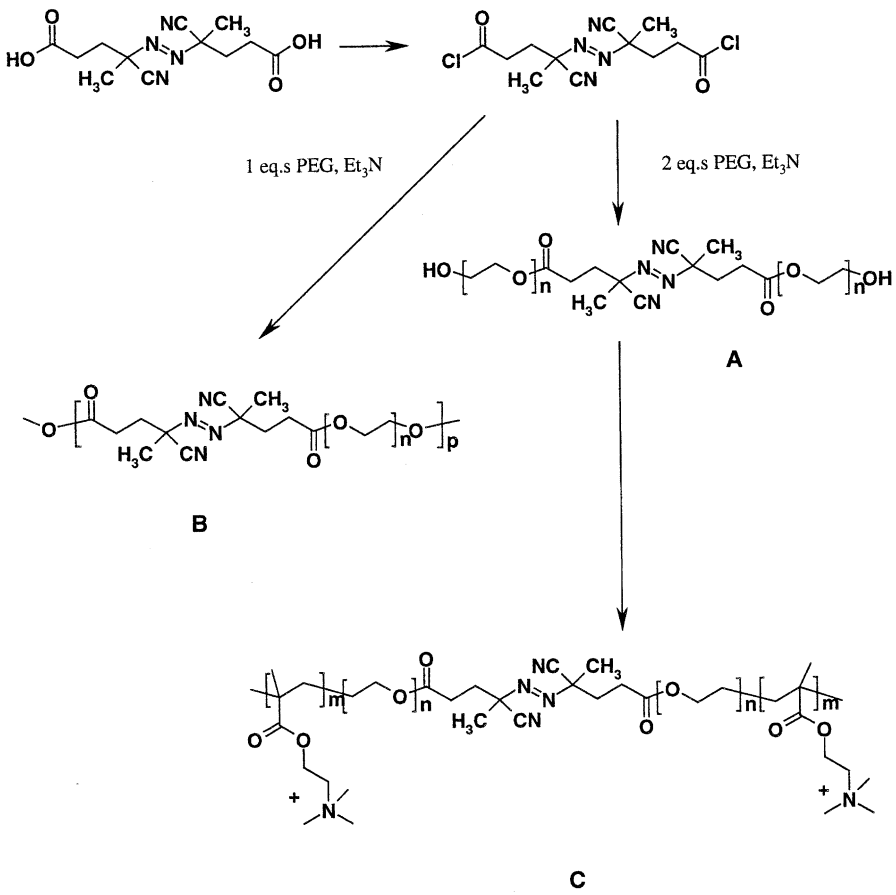

Fig. 8. Synthesis of different PEG-based macroinitiators for DADMAC radical polymerization.

used for DADMAC polymerization (Fig. 9) and the termination by recombination was also basically confirmed in this case.

These polymers could be conveniently used in encapsulation technology: polycations [such as poly(lysine) or poly(ethyleneimine)] are often used to ensure, through surface polyelectrolyte complexation, mechanical long-term stability to Ca-alginate beads and capsules (Sun, 1997). The polycations strengthen the construct by complexing the anionic surface and build a thin but tough membrane; in any case, an additional treatment with a polyanion is often required in order to increase the biocompatibility of the surface (Gaumann et al., 2000). pDADMAC-*bl*–PEG copolymers can offer a significant advantage over conventional polycations, because they preserve the pDADMAC complexing properties (as checked with cellulose sulfate; Tirelli, unpublished results), but can allow PEG molecules to cover the surface with a biocompatible polymer layer (Fig. 10).

3. Structure–activity relationship for a selective and biocompatible PEG end-group chemistry

A crucial step for the preparation of PEG-based materials in the presence of sensitive bioactive groups and/or in a physiological environment, or even in vivo, is the development of a suitable chemistry. The requirements that these reactions should fulfill are: self-selectivity (i.e. negligible interference from biological nucleophiles and electrophiles); quantitative yield; reaction com-

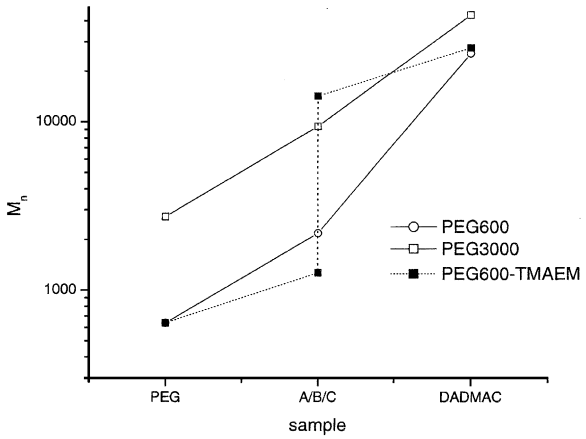

Fig. 9. Molecular weights of PEG precursors, initiators and polymers at different PEG MWs: PEG diol; 'B-type' macroinitiator; $(AB)_n$-type block copolymer with PEG 600 (○) or 3000 (□). PEG diol; 'A-type' macroinitiator; 'C-type' macroinitiator; CABAC-type block copolymer with PEG 600 (■).

pletion in short times (for potential compatibility with surgical techniques); no production of potentially toxic byproducts; and no use of organic solvents. Finally, the synthesis of PEG derivatives must not be exceedingly complicated. The potential applications of such a chemistry are in situ and in vivo preparation of materials via minimally invasive surgery techniques, for applications spanning post-operative adhesion prevention, long- or short-term tissue substitution and controlled release of drugs.

3.1. Michael-type addition (Lutolf et al., 2001)

PEG end-group reactivity has been extensively studied for purposes of bioconjugation (PEGylation). As a result of PEG protein repellence, high hydrophilicity and low degradability by mammalian enzymes, this polymer can dramatically extend the stability of a biomolecule in vivo. One common coupling strategy involves the end-modification with electrophilic functionality, such as the *N*-hydroxysuccinimide esters of PEG carboxylic acids (PEG–NHS) (Belcheva et al., 1998, 1999; Veronese et al., 2001), PEG–oxycarbonylimidazole (CDI–PEG) (Beauchamp et al., 1983) or PEG nitrophenyl carbonate (Demers et al., 2001). However, since most functionalized PEGs react unselectively with both thiols and amines, a distribution of products can be obtained, resulting in grafting at multiple sites of the biomacromolecule. Good coupling selectivity has been achieved with two classes of functional groups: iodo-, bromo- and chloroacetamides and -acetates (Lindley, 1959; Schelté et al., 2000), reacting through a nucleophilic substitution mechanism; maleimides (Schelté et al., 2000) vinylsulfones (Masri and Friedman, 1988; Morpurgo et al., 1996), acrylamides (Romanowska et al., 1994) and acrylates (Jemal and Hawthorne, 1997), reacting through a Michael-type addition reaction.

Among the others, the Michael-type addition of thiols onto acrylates is an ideal candidate for reactions in physiological conditions, due to its high speed, the ease of preparation of the unsaturated compound, the negligible influence of competing reactions with other nucleophiles (at physiological pH, react amines generally at least one order of magnitude more slowly than thiols;

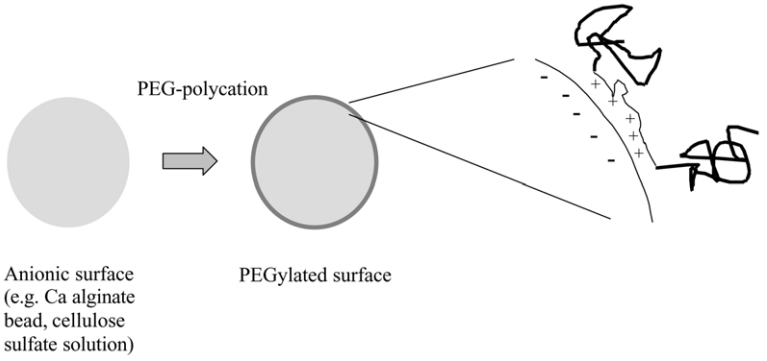

Fig. 10. Surface coverage by PEG in the complexation of an anionic surface by PEG polycations.

Table 1
Series of peptides with charged and/or neutral amino acids close to a cysteine residue

	Peptide				
	GC**RR**G	G**R**C**R**G	GC**R**G	G**R**C$\overline{D}$G	GC**R**$\overline{D}$G
Abbreviation	crr	rcr	cr	rcd	crd
Net charge	+2	+2	+1	0	0
	Peptide				
	GC$\overline{D}$**R**G	GC$\overline{D}$G	G$\overline{D}$C$\overline{D}$G	GC$\overline{DD}$G	
Abbreviation	cdr	cd	dcd	cdd	
Net charge	0	−1	−2	−2	

Friedman et al., 1965) and the absence of any byproduct.

In order to successfully employ this reaction for bioconjugation or biomaterial synthesis, we have tried to elucidate the reaction mechanism and outline a quantitative relationship between the structure of the PEG reaction partner (the thiol) and its activity. Since thiolates rather than thiols are also reported to be the reactive species in Michael-type reactions (Lindley, 1959; Friedman et al., 1965; Bednar, 1990), we have focused our attention on the link between pK_a and the Michael-type reactivity.

Thiol pK_a is known to often influence enzymatic activity (Gitler et al., 1995). For example, thioredoxin (Holmgren, 1990) and thioltransferase (Yang and Wells, 1991) have highly reactive thiols with very low pK_a values, determining their activity towards reducing disulfide bonds. Many examples in the literature reveal that pK_a shifts for thiols can be induced by electrostatic interactions with neighboring ionizible amino acids (Dyson et al., 1997) and/or polar residues (e.g. amides dipoles; Kortemme and Creighton, 1995) or surfactants (Gitler et al., 1995). For instance, changes in enzymatic activity have been observed for thioredoxin (Dyson et al., 1997) and 4-oxalocrotonoate tautomerase (Czerwinski et al., 1999) as a result of pK_a shifts in the active site due to mutations on charged residues close to the active thiol. However, in the literature it has not been clearly stated whether the local charge effect is predominant in determining pK_a shifts, or if there is an effect on the reaction kinetics due to change in the transition state energy (Gitler et al., 1995).

A series of cysteine-containing peptides was synthesized, derived from a positively charged

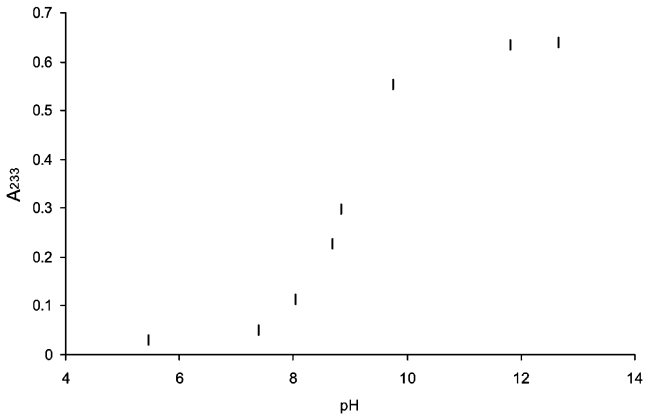

Fig. 11. Absorbance at 233 nm as a function of pH for the peptide GCDDG.

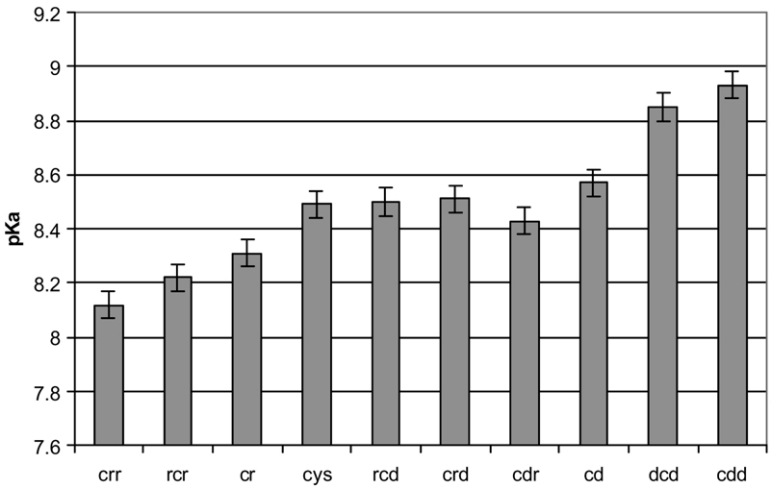

Fig. 12. pK_a values for the peptides.

(arginine, R), a negatively charged (aspartic acid, D), a neutral (glycine, G) and a thiol-bearing amino acid (cysteine, C) (Table 1).

First, the pK_a of all thiols was determined by spectrophotometrically monitoring the absorption at 233 nm at different pH values (Fig. 11). Since thiols are completely deprotonated at high pH, the absorbance A_{max} was assumed equal to $\varepsilon_S^- \times C_{thiol}$, where ε_S^- is the absorption coefficient of the thiolate and C_{thiol} is the peptide concentration.

The presence of charges close to the thiols strongly influenced the pK_a (Fig. 12), hindering the deprotonation in the case of negative charges, and facilitating it for positive ones.

Second, the Michael-type addition kinetics was followed by monitoring the absorbance at 233 nm, related both to thiolates (as shown before) and

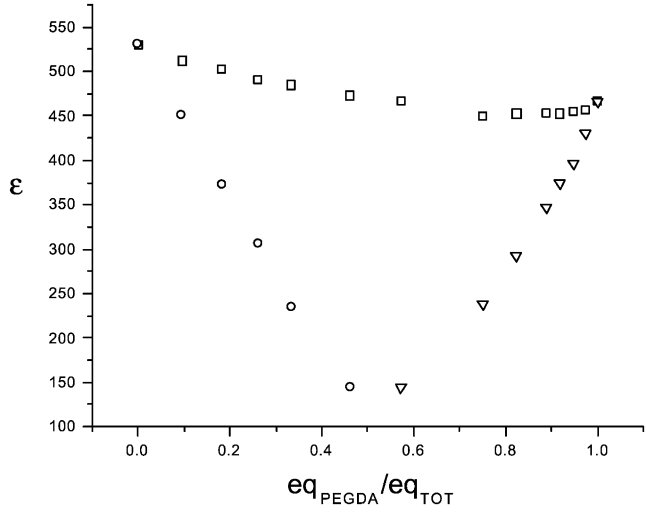

Fig. 13. Reaction diagram for PEGDA and cysteine. The sum of the concentrations of reacting groups was kept constant at $c = 2.5 \times 10^{-3}$ M. The absorbance divided by c is reported on the y-axis as ε. Symbols: ε before reaction (□); ε after reaction in excess of PEGDA (▽) and in excess of Cys (○).

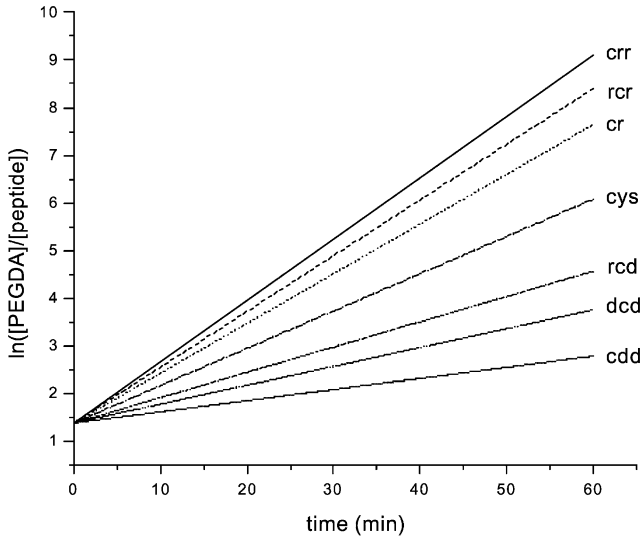

Fig. 14. ln([PEGDA]/[pept]) over time for several peptides reacted with PEGDA (here at 80:20 acrylate/thiol ratio). The linearity indicates true second-order kinetics according to Eq. (1).

unsaturated esters. Whether the active group is a thiol, a thiolate or both, the time evolution of this peak allows on-line monitoring of the extent of every thiol-consuming reaction: the proton transfer is instantaneous, compared to the Michael-addition, and therefore the overall thiol concentration is related to that of thiolates according to the relation $C_{\text{thiol}} = [S^-] + [SH] = [S^-] \times (1 + [H^+]/K_a)$. Saturated esters and thioethers (in the reaction product) have a negligible absorption at the analytical wavelength.

This analytical method was validated for cysteine/PEG diacrylate. If:

- The change in absorption at 233 nm absorption depends only on the reaction extent;
- The partners react with a 1:1 ratio; and
- Disulfide formation plays a negligible role during the reaction (at least for cysteine),

then a graph of ε vs. the equivalent fraction of PEGDA (number of acrylates divided by the

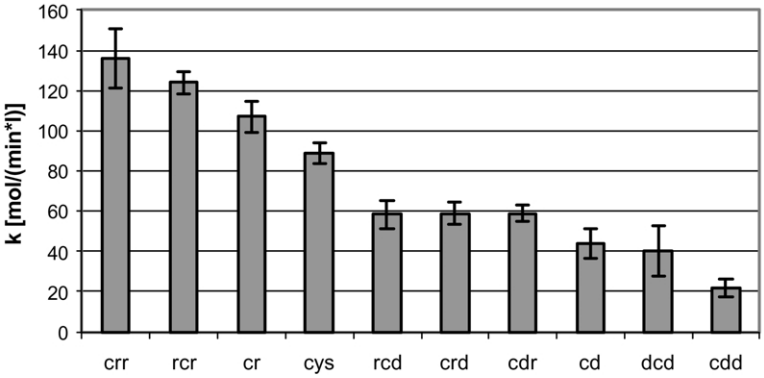

Fig. 15. Second-order kinetic constant for the peptides.

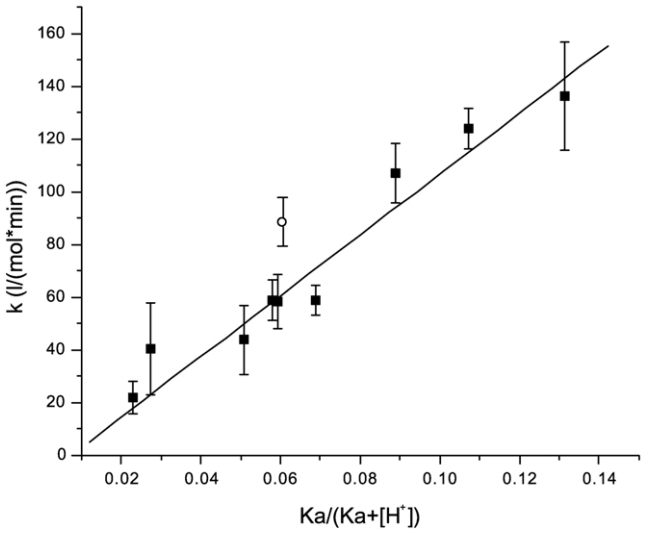

Fig. 16. Correlation of $K_a/(K_a + [H^+])$ and k. Peptides (■), cysteine (○).

number of reacting groups; the same applies to cysteine) at time t_∞ (complete reaction) should show a minimum at 0.5, corresponding to ε of the pure product. These assumptions were all indeed confirmed (Fig. 13).

According to the equation for second-order kinetics:

$$\ln([\text{PEGDA}]/[\text{pept}]) = \ln([\text{PEGDA}_0]/[\text{pept}_0]) + \mathbf{k_{eff}} \times t \quad (1)$$

with $\mathbf{k_{eff}} = \mathbf{k} \times ([\text{PEGDA}_0] - [\text{pept}_0])$, the bimolecular mechanism was confirmed by the linear time dependence for ln([PEGDA]/[pept]) for all the peptides (Fig. 14).

Positive charges close to the SH-groups considerably speeded up the reaction, while the opposite effect was shown by negative charges (Fig. 15). The precise localization did not seem to matter.

The parallel changes in both the reaction rate and pK_a confirmed the active role of thiolates in this reaction. Assuming thiolates to be the only reactive group, the second-order kinetic equation $v = k \times C_{\text{thiol}} \times C_{\text{acrylate}}$ can be expressed as:

$$v = k_{\text{real}} \times [S^-] \times C_{\text{acrylate}} \quad (2)$$

and thus

$$k = k_{\text{real}} \times 1/(1 + [H^+]/K_a) = k_{\text{real}} \times K_a/(K_a + [H^+]) \quad (3)$$

According to Eq. (3), a linear relationship should be observed between $K_a/(K_a + [H^+])$ and k. A good agreement was shown by all peptides (Fig. 16), while cysteine behaved slightly differently, likely due to the shorter distance of the ionic groups from the thiol and to the different structure of the molecule (no peptide bond).

These results provided the basis for the rational design of peptides or proteins involved in conjugation schemes with polymeric structures, where self-selectivity and controlled reaction kinetics is an issue: tailoring the thiol structure to low pK_a values can indeed allow a reduction in polymerization or curing times and minimization of the interference from amines (increasing the pK_a difference between thiols and amines).

This reaction has been used to produce biofunctional gels from thiol-containing peptides and end-functionalized PEG and the resulting materials are currently under study.

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