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Review

Bioconjugation in pharmaceutical chemistry

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Abstract

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drugs of simple structure or complex compounds such peptides, enzymes and oligonucleotides. For long time drugs, mainly with antitumoral activity, have been coupled to natural or synthetic polymers with the purpose of increasing their blood permanence time, taking advantage of the increased mass that reduces kidney ultrafiltration. However only recently complex constructs were devised that exploit the 'enhanced permeability and retention' (EPR) effect for an efficient tumor targeting, the high molecular weight for adsorption or receptor mediated endocytosis and finally a lysosomotropic targeting, taking advantage of acid labile bonds or cathepsin susceptible polypeptide spacers between polymer and drug. New original, very active conjugates of this type, as those based on poly(hydroxyacrylate) polymers, are already in advanced state of development. Labile oligonucleotides, including antisense drugs, were also successfully coupled to polymers in view of an increased cell penetration and stabilization towards nucleases. However, the most active research activity resides in the field of polypeptides and proteins delivery, mainly for the two following reasons: first of all because a great number of therapeutically interesting compounds are now being produced by genetic engineering in large quantity and, secondly, because these products are difficult to administer to patients for several inherent drawbacks. Proteins are in fact easily digested by many endo- and exo-peptidases present in blood or in other body districts; most of them are immunogenic to some extent and, finally, they are rapidly excreted by kidney ultrafiltration. Covalent polymer conjugation at protein surface was demonstrated to reduce or eliminate these problems, since the bound polymer behaves like a shield hindering the approach of proteolytic enzymes, antibodies, or antigen processing cell. Furthermore, the increase of the molecular weight of the conjugate allows to overcome the kidney elimination threshold. Many successful results were already obtained in peptides and proteins, conjugated mainly to water soluble or amphiphilic polymers like poly(ethylene glycol) (PEG), dextrans, or styrene–maleic acid anhydride. Among the most successful are the conjugates of asparaginase, interleukin-2 or -6 and neocarcinostatin, to remind some antitumor agents, adenosine deaminase employed in a genetic desease treatment, superoxide dismutase as scavenger of toxic radicals, hemoglobin as oxygen carrier and urokinase and streptokinase as proteins with antithrombotic activity. In pharmaceutical chemistry the conjugation with polymers is also of great importance for synthetic applications since many enzymes without loss of catalytic activity become soluble in organic solvents where many drug precursors are. The various and often difficult chemical problems encountered in conjugation of so many different products prompted the development of many synthetic procedures, all characterized by high specificity and mild condition of reaction, now known as 'bioconjugation chemistry'. Bioconjugation developed also the design of new tailor-made polymers with the wanted molecular weight, shape, structure and with the functional groups needed for coupling at the wanted positions in the chain.

Keywords: Bioconjugation; Drugs; Pharmaceutical chemistry

Abbreviations: PEG, poly(ethylene glycol); EPR, enhanced permeability and retention; HPMA, *N*-(2-hydroxypropyl)methylacrylamide; PVP, poly(*N*-vinilylpirrolidone); MW, molecular weight; kDa, kilodalton; DIVEMA, poly(divinylethermaleic anhydride); SMA, styrene–maleic anhydride; SOD, superoxide dismutase; Hb, hemoglobin; ADA, adenosine deaminase; PAcM, poly(*N*-acryloyl morpholine).

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1. Introduction

Since the pioneering studies of Ringsdorf [1], bioconjugation has become a central issue in pharmaceutical sciences: it is at the basis of the stabilization of substances in circulation [2], of protection of labile products from enzymatic degradation [3–9], of reduction of immunogenicity of polypeptides [10], and of the exploitation of new pathways in cell penetration [11–14], and also for the development of highly specific diagnostic agents. Indeed, as far as drugs are concerned, bioconjugation has been mostly studied and exploited with proteins as the drug moiety, for the unique advantages that can be obtained with these molecules (Table 1), although relevant examples have also appeared with low molecular weight compounds, some of which have already reached the stage of general application or advanced clinical experimentation.

In the spirit of this journal, it seemed interesting to review the main achievements obtained in the field of bioconjugation for pharmaceutical purposes, together with the advantages and limits of this technology. Therefore, in this paper, a few relevant examples of polymer–drug conjugates (both with low molecular weight drugs and with the more complex peptides and proteins), that have been developed in recent years, will be taken into consideration, together with the merging studies on oligonucleotide conjugation. The main chemical aspects of conjugation will also be reported; however, only the chemistry that yields drug–polymer products will be taken into consideration since, when low molecular weight compounds are involved in drug conjugation the procedure falls into the classic pro-drug approach. To be more precise, despite the many possible definitions of the word 'bioconjugate', in this paper the term will be used to describe any macromolecular complex, synthetically obtained by covalently binding a drug molecule (or more than one) to a polymer molecule. Antibodies directed drugs or toxins, although based on the same chemistry for what the conjugation is concerned, will not be reviewed here because, for the major part, they rely on biologically obtained products (for pertinent references see Refs. [15–17]).

Table 1
Advantages of bioconjugation in pharmaceutical chemistry

Stabilization of labile drugs from chemical degradation
Protection from proteolytic degradation
Reduction of immunogenicity
Decreased antibody recognition
Increased body residence time
Modification of organ disposition
Drug penetration by endocytosis
New possibilities of drug targeting

2. Bioconjugates with low molecular weight drugs

2.1. A successful example of bioconjugation

The greatest achievements in drug conjugates have emerged from the collaboration among the researchers of Kee University, led by R. Duncan (presently at London University), those from Salt Lake City, headed by J. Koocheck, the Prague polymer chemist K. Ulbrich, and industrially developed by Pharmacia. This collaboration aimed at the development of the 'magic bullet', in accordance with an early dream of Erhlich [18], by using the polymer *N*-(2-hydroxypropyl) methylacrylamide (HPMA) for the release of doxorubicin to tumor cells [19–21].

The new concepts developed by these authors represent the basis for any further development of bioconjugates with low molecular weight drugs so they deserve to be described in detail. In these studies, doxorubicin was linked to HPMA in order that the non-toxic and water-soluble polymer could protect the labile drug from degradation and could slow down its renal ultrafiltration. Furthermore, this new construct, due to its specific structure, allowed exploitation of new pathways both in targeting the drug to specific organs (such as cancer tissue) and in the route of penetration inside the cells.

As far as tissue targeting is concerned, the high molecular mass of the polymer was found to promote tumor localization of the conjugates because, at this level, the vascular epithelium is more fenestrated [14].

Besides, tissue localization could also be improved by linking, along the HPMA molecule, antibodies or sugar moieties [22–24] that direct the bioconjugates to the body region with specific ligands for these molecules.

It is also important to note that, in these studies, the molecular mass of the polymer had to be properly tuned in order to avoid rapid kidney ultrafiltration and, in the opposite case, an undesired polymer accumulation in the body [25]; the Second World War's sadly known 'polymeric syndrome', due to poly(*N*-vinylpyrrolidone) transfusions, remains an unforgettable lesson. Regarding the route of cell penetration, a totally new pathway takes place with these high molecular weight conjugates. In fact, the high molecular mass of the polymer forces the conjugates to enter the cells only through the endocytosis process while the diffusion driven by concentration-gradient, typical of small molecules, is only of limited relevance.

In order to improve cell-penetration and cell-disposition of the conjugants, suitable spacers between the polymer and the drug were introduced, consisting, for example, in specific peptide sequences that are not cleaved by blood enzymes but by lysosomal proteases only. By using these spacers, once the macromolecule has entered the cell by the endocytic pathway, the drug

can be liberated from the polymeric matrix only by the lysosomal enzyme machinery. In this respect, the recent knowledge in lysosome composition (for example, regarding the potent cysteine enzymes with unusual specificity or the low pH of the endosomes), has been very important for the design of spacers cleavable only intracellularly [7,20]. One of the best sequences for the spacer was found to be Leu–Gly–Val–Phe, although other tetrapeptides or tripeptides were also found to be effective, differing from each other for the susceptibility to cleavage.

The fact that these bioconjugates were designed for therapeutic applications, therefore with strict requisite of identity, gave a relevant input to the development of new analytical tools since, as only recently faced by Pharmacia, a precise characterization of the constructs is a necessary step in gaining the authorities' approval [26]. Bioconjugate analysis requires the separation and contemporaneous evaluation of each of the components, drug, polymer, spacer and, eventually, targetable moiety, but also of the construct as a whole [27]. The need to characterize the products obtained by bioconjugation is a general and often difficult issue in this field of research, independently of the class of drug molecule that is used for the bioconjugation reaction and will be recalled often throughout this paper.

2.2. Other bioconjugates

The molecular basis of the HPMA–doxorubicin constructs described above inspired several scientists and, since those pioneering studies, many other drugs have been coupled to polymeric moieties to modify their bioavailability and their phamacokinetic profiles. For example, polymer derivatives of other antitumor agents such as melphalan [28] or sarcolysin [29] have recently been developed.

More simple constructs have been used to evaluate the effect of drug binding chemistry, the spacer composition and polymer structure on the delivery of polymer-anticancer drugs. For example, Ohya et al. [30] compared the antitumor activity of doxorubicin, when bound to PEG, either through an amide, an ester or a Schiff base. They demonstrated that the Schiff-base conjugate, stable in physiological conditions but cleaved in the lysosomal environment, maintains the highest activity. The authors also pointed out the formation, in aqueous solution, of self-aggregates of these agents [31] and suggested the possibility that the formation of micelles might influence the endocytic process.

PEG–doxorubicin conjugates having different amino acid or peptide spacers were synthesized in the authors' laboratory. In this case, increased blood stability, decreased toxicity and maintenance of cytotoxic activity were found to be dependent upon the nature of the spacer [32]. Poly(PEG–lysine) [33] a copolymer devised to increase the coupling capacity of drugs, was studied for doxorubicin conjugation. The linkage was obtained by means of an amide bond but also through a Schiff base in order to maintain the positive charge in the drug. More precisely, the Schiff base was obtained either by coupling the amino groups of doxorubicin to an aldehyde PEG or, alternatively, by using an hydrazide on the polymer and the 3-ketogroup in the drug [34].

A thorough investigation on the hydrolytic stability, lysosomal digestion and antitumor activity was carried out by Shacht and co-workers [35] with polymer–drug conjugates using PEG (MW 5 kDa), dextran (MW 38.4 kDa) or poly(2-hydroxyethyl)-L-glutamine (MW 36.4 kDa) and different spacers.

The natural polysaccharide dextran (MW 36.5 kDa) was conjugated to the antimicrobial drug norfloxacin to improve its pharmacokinetics. Here too, the spacers Gly–Phe–Ala–Leu or Gly–Phe–Leu–Gly, linked to the piperazine amino function of the drug, were found to be stable in circulation while allowing the drug release by action of the lysosomal cathepsins [36].

In some cases, bioconjugation with hydrophilic polymers has been used to overcome the problem of low solubility that characterizes certain drugs, this being a relevant drawback to their use. For example, bifunctional PEG has been coupled to taxol, the potent chemotherapeutic agent for breast and ovarian cancer, by using both hydroxylic ends of the polymer as anchoring sites. An increase in solubility of six order of magnitudes of this highly insoluble drug was achieved, without any loss in taxol activity. It is interesting to note that, in these studies, a molecular mass of PEG of 40 kDa, was found to be critical in reaching a $t_{1/2}$ of hydrolysis higher than the $t_{1/2}$ of excretion [37]. Increased solubility without any loss of drug activity was also obtained with methotrexate and doxorubicin coupled to high molecular weight PEG. Polymer conjugation was also studied with the sesquiterpene endoperoxide artemisin, a drug with important antimalarial activity whose use is hampered by low solubility. In this case, the conjugation to a bifunctional PEG yielded a product with solubility and activity even higher than that of the parent drug [38]. Among them other drugs that were successfully coupled to polymers we would like to report camptothecin. Camptothecin is a very active topoisomerase-1 inhibitor, that was succesfully conjugated to PEG yielding longer lasting ester derivatives in blood [33]. A product called Prothecan® is now ready for the market. Polymers with different properties have also been studied for these applications. Among them we remember poly(mPEG–lysine) as well as poly(PEG–aspartic acid) [31] that were used to circumvent the problem of the limited amount of drug molecules loaded by PEG.

Other synthetic or natural polymers have also been studied with different purposes. Among these, poly(divinylethermaleic anhydride) (DIVEMA) of 7 kDa molecular mass, also known as pyran copolymer [39–42], poly(aspartic acid) [43], poly(L-glutamic acid) [44], poly(hydroxyethyl-L-glutamine) [45] albumin [46], all of them used for doxorubicin conjugation [47]; the copolymer *N*-(2-hydroxyethyl) methacrylate-*N*-vinylpyrrolidone was used in daunomicin binding [44]; poly(α-malic acid) for 5-fluoruracil [48] and the block copolymer of poly(ethyleneimine) and palmitic acid were used for cyclophosphamide derivatives conjugation [49]. However, in these cases, a systematic investigation on the role of the conjugation chemistry, the spacer properties, the role of polymer molecular weight or structure was seldom carried out.

Derivatives of α,β-polyaspartic acid were recently proposed as drug carriers by Giammona et al. They are obtained by reaction of polysuccinimide with amine or hydrazine [50–52]. Many drugs were conjugated directly or by a succinic spacer. Increased solubility and bioavailability was observed as in the case of aciclovir or ribovuridine [53,54]. The α,β-polyaspartyl hydrazide was demonstrated useful also as blood expander and drug carrier for its excellent biocompatibility and favourable reactivity [55].

A quite different application of polymers, namely the conjugation with low molecular weight PEG, was also successfully investigated to increase the transdermal penetration of the anti-inflammatory agent, indomethacin [56].

2.3. Advantages in the preparation of bioconjugates with low molecular weight drugs

One of the main achievements obtained by coupling low molecular weight drugs to polymers is the modification of the drugs' pharmacokinetic profile and, as a consequence, of their bioavailability. However, as it is clear from the examples reported above, the methodology described here differs from the one that yields to the best known pro-drug compounds. In fact, in the classic pro-drugs approach (as, for example, with acyl moieties) the modification of the drug pharmacokinetic or metabolic profiles is mainly based on a simple drug's slow release in plasma from the low molecular weight carriers by the action of water only or by enzymes, or on modification in body disposition. On the other hand, in the bioconjugation technology with high molecular weight polymers described here, several factors act together to affect the pharmacokinetics and, therefore, the bioavailability of the drug. Among them, the following should be remembered:

1. the shielding effect of the polymer that conveys a relevant protection from chemical or enzymatic degradation to the conjugated drug. Similarly, polymers are very effective in masking the antigenic sites of the drugs. As a consequence, their antigenic/immunogenic characteristics that, if present, can be responsible for drug depletion, may be greatly reduced. In the case of PEG, both these phenomena have been correlated to the high mobility, associated with conformational flexibility, and water-binding ability of the polymer chain. These characteristics prevent, mainly by thermodynamic effects, the approach of any macromolecule, such as immunoglobulins or degrading enzymes, as well as conjugate adhesion to surfaces;
2. the reduction in renal excretion, due to the large volume of the macromolecular conjugate. This reduction generally occurs when the threshold of serum albumin volume is reached. It is to be noted that, in the case of PEG, the critical point of the albumin hydrodynamic volume is reached at a molecular mass of about one third of the protein with same mass. This is due to the quasi-random coil conformation of the polymer and to its high hydration [57–59];
3. in some cases, polymer coupling was demonstrated to promote a targeted delivery of drugs to body sites characterized by an increased capillary permeability as, for example, inflamed tissues. This phenomenon is also at the basis of the so-called 'enhanced permeability and retention' (EPR) effect. EPR allows the specific localization of a drug at the level of cancer tissue thanks to the higher permeability of blood capillaries in that area, accompanied by a reduced lymphatic drainage. Both these phenomena permit the accumulation of the drug–polymer at the level of the tumor tissue through an ultrafiltration process [60]; and
4. besides the modification of the pharmacokinetic profiles, the macromolecular characteristic of the bioconjugates is responsible for the exploitation of a totally new pathway for the drug's entrance into the cell that can only be based on adsorption- or receptor-mediated endocytosis [61,62]. This new pathway has been exploited even more thoroughly by the design of specific linkages arms between polymer and drug. This linkage has to be stable in blood while cleavable only intracellularly, because of the acidic environment of endosomes or by means of the rich enzymatic machinery of the lysosomes. In this first case, esters or amide conjugates to α-double bonds carrying bicarboxylic acids, labile at low pH while stable at physiological conditions, were found to be a useful approach for targeted drug delivery [63,64], while for the second approach, specific amino acid sequences, described earlier, were found of help.

2.4. Limitations in the conjugation of polymers to low molecular weight drugs

Despite the advantages of bioconjugation, many problems still have to be overcome before an ideal polymeric construct can be obtained. Most of the problems are related to technical tasks that still need to find their optimization. Among the goals to be achieved, we ought to remember:

1. to improve the chemistry of binding in order to find out conditions of activation and conjugation that are mild enough not to affect the stability of the polymer, since chain cleavage with reduction of molecular mass does sometimes occur, and this reaction is not easy to detect;
2. to develop suitable chemical approaches that allow those sites in the drug molecule that are involved in its receptor binding to remain accessible or unmasked. This task, even if not trivial, is more easily achieved in the modification of polypeptides and proteins that carry several different sites which are potentially suitable for polymer anchoring;
3. to develop analytical methods for the characterization of the constructs as a whole and of their individual components, after proper degradation and separation;
4. to obtain well-characterized polymers, possibly with low polydispersivity; and
5. to choose, if possible, polymers which have already been approved by the FDA or, alternatively, with are highly likely to be so.

3. Bioconjugates with protein drugs

3.1. General considerations

The rationale of polymer conjugation to proteins for pharmaceutical applications is somehow similar to that described for low molecular weight drugs. Also in this case, the attachment of soluble and biocompatible polymers aims at the improvement of protein stability and at the modification of their pharmacokinetic profiles. As a matter of fact, one of the major drawbacks in the use of biologically active proteins in therapy is the common short body residence time of these molecules that are either rapidly removed by renal ultrafiltration or inactivated by the immunosystem or by plasma enzymes. After polymer conjugation, the stability and the pharmacokinetic profile of a protein is generally improved because, as in the case of low molecular weight drugs, the polymer increases the drug's volume, protects it from enzymatic and hydrolytic degradation and shields its immunological epitopes. Again, as in the case of low molecular weight drugs, the polymer may also direct the bioconjugate to specific organs or areas in the body.

Many polymers are being studied for these applications, the most popular are dextran and mPEG, the first is poly-functional while the second mono-functional. Other mono-functional polymers with properties similar to mPEG are also being studied. Among these, a new form of poly(*N*-vinyl pyrrolidone) [65] and poly(*N*-acryloyl morpholine) [66], both having a single activable end terminal residue (–COOH or –OH) per polymer chain. These polymers are obtained by radical polymerization of *N*-vinyl pyrrolidone or *N*-acryloyl morholine with isopropoxyethanol in the case of the former monomer, and mercaptoacetic acid or mercaptoethanol in the case of the latter. Poly(oxazolines) and poly(vinylalcohol) are also mono-functional polymers that have been considered for protein modification [67,68]. Poly-functional synthetic polymers that have also gained in popularity due to their intrinsic properties are the co-polymers of divinyl-ether and maleic anhydride (DIVEMA) and of styrene–maleic anhydride (SMA).

In the following sections, we report on some examples of protein conjugates with dextran, PEG and other synthetic polyfunctional polymers that we believe to be among the most relevant.

3.2. Dextran protein conjugates

There are at least 20 polypeptides and proteins conjugated with polysaccharides, mainly dextrans, but only few representative examples will be reported in this review.

Streptokinase was the first therapeutic enzyme to be conjugated to a polymer (dextran of 35–50 kDa molecular mass) with significant therapeutic success. Since 1980, after its approval for clinical use in the treatment of cardio-vascular and ophtalmological pathologies caused by thrombosis, it has been produced in Russia, on a large scale, under the trade name of 'Streptodekase®'. This streptokinase conjugate is characterized by long body permanence in humans where it can last for over three days. As a consequence, streptokinase may be administered in a single bolus instead of by continuous infusion as needed for the non-modified form, whose body permanence is of only a few minutes. Also the overall toxicity of streptokinase is decreased after dextran conjugation, as demonstrated by reduced hemorragic complications, rethromboses and allergic reactions. When the native enzyme is used, these complications are observed in 72, 16 and 27% of the patients, respectively, while the same problems are reduced to 6, 5.5 and 2%, respectively, using Streptodekase® [69].

Another protein, also involved in the coagulation pathway, that has been modified with dextran is plasmin. This proteolytic enzyme was coupled to oxidized dextran yielding a conjugate retaining 85% of the origi-

nal activity and sharing similar pharmacokinetic and immunologic advantages as those described for streptokinase [70].

Hemoglobin was coupled to a bromo–amino or an aldehyde dextran yielding products with oxygen-carrier properties unchanged with respect to the native hemoglobin, but which had a 3–20-fold higher blood-circulation time [71]. With these dextran derivatives, transfused animals with hematocrit values of $< 2\%$, survived and fully recovered without further enrichment with air and oxygen.

Aprotinin, a low molecular weight peptide active as a tripsin–kallicrein inhibitor, was coupled to cyclo-xymethyl dextran in order to solve the problem of its rapid excretion. Significant increase in survival was observed in dogs with experimental acute pancreatitis since the polymer conjugates increased by up to 10-fold the residence time in circulation while the activity in vitro was not reduced. Interestingly, when galactose was contemporarily bound to the polymer, the conjugates were found to accumulate in the liver. This sugar targeted the complex to this organ due to the presence of galactose receptors at its surface [72–74].

3.3. Problems and solutions in polymer–protein conjugation

Despite the important results obtained with Streptodekase®, dextran has not gained general success in polymer derivatization. This is due to the easy occurrence of cross-linking because both dextran and proteins are poly-functional molecules and their coupling usually yields a complex and heterogeneous mixture of products. This problem is generally avoided by using mono-functional polymers [75,76], the only ones that, in the case of poly-functional drug entities, such as peptides or proteins, give rise to chemically homogeneous products. However, micro-heterogeneity may also occur in this case, because of the several amino acid reactive sites present at the protein surface, all potentially reactive, but characterized by different accessibility or reactivity [77].

Only in certain specific cases are poly-functional polymers preferred to the mono-functional ones in protein modification. This happens when an increased conformational stability is needed as, for example, when the protein is to be used as a biocatalyst in organic solvents. In this case the reticulated complex that is formed from the reaction between the two poly-functional entities (the polymer and the protein) often confers a higher stability towards denaturing environments.

A few mono-functional polymers are used nowadays for bioconjugation of proteins although PEG is the most studied. Other mono-functional polymers, as mentioned above, are a new form of poly(*N*-vinyl pyrrolidone) [65], and poly(*N*-acryloyl morpholine) [66], poly(oxazolines) and poly(vinylalcohol) [67,68]. It is important to note that all of these polymers are obtained synthetically which allows one to reach the desired molecular weight and sufficiently low polydispersivity. Besides, when polymers obtained through chemical synthesis are used, the possibility of tailoring their structure according to need is an important advantage.

A further problem in polymer–protein conjugation is the difficulty to fractionate the different entities obtained after coupling due to the presence of the polymer that greatly reduces the resolution in any chromatographic medium. This often impairs the purification of single species of conjugates as well as the establishment of the exact number of polymer chains linked to each protein.

Furthermore, the precise sites of polymer coupling along the protein primary sequence are very difficult to identify.

As a matter of fact, this last problem can be solved using a chemical approach recently devised in the authors' laboratory [78]. This approach relies on the use of a new PEG derivative, terminating with the dipeptide spacer methionine–norleucine (Met–Nle). This dipeptide is easily cleavable by means of BrCN treatment so that, when a protein modified with this PEG derivative is exposed to BrCN, the polymer moiety is removed leaving the norleucine residue on the protein at the site where the polymer was bound [78,79]. Once the polymer has been removed from the protein, any method of digestion, peptide fractionation and amino acid analysis may be used to establish the location of the norleucine reporter groups and, consequently, the sites of polymer attachment.

The long-debated problem of the exact quantification of the number of polymer chains linked to each protein can now be overcome by the use of a single amino acid spacer between polymer and protein. For example, when the unnatural amino acid, norleucine (but also β-alanine) is used as the spacer, amino acid analysis after acidic hydrolysis of the product allows one to calculate the number of bound polymer chains. In fact, this number corresponds to the amount of Nle evaluated by using certain stable amino acids in the protein as internal reference standards [80]. This method has proven to be more accurate than the more common colourimetric [81,82] and fluorimetric ones [83] that measure the amount of unmodified primary amines in the protein to calculate the PEGylated ones by difference. In fact, these methods are subjected to the variability of the primary amines location on the protein molecule that affects both the accessibility of the reagents and the extinction coefficient of the chromophore. On the other hand, all of these methods only give the average value of conjugation. With the more

recent MALDI mass spectrometry, the pattern of the individual conjugated species is obtained. Unfortunately, this technique cannot be considered quantitative, mainly because each mass specie presents in the sample is characterized by a different yield of extraction from the matrix in the ionization process. Therefore, when a mixture of products is analyzed, it is not possible to say whether the pattern which appears in the mass spectrum corresponds quantitatively to the composition present in the sample.

NMR, associated to the UV quantification of the protein, has also been used for polymer evaluation. In this case, the polymer content is evaluated by carrying out NMR analysis of the sample, dissolved in D_2O containing an internal standard (whose signal does not overlap with that of the polymer). The protein concentration in the same sample is evaluated by UV (or by any colourimetric procedure) while a calibration curve, obtained by running NMR spectra of polymer samples, dissolved in D_2O at known concentrations, is used as a reference to evaluate the polymer quantity.

A further problem in enzyme conjugation is the possible loss of activity related to the polymer attachment to amino acids involved in the active site or located in its close environment. This limitation is more relevant for enzymes acting on high molecular weight substrates, because their approach to the active site may be hampered by the hindrance of the close bound polymer. In some enzymes, the problem has been overcome by carrying out the conjugation in the presence of an enzyme substrate or a reversible inhibitor in solution. An evolution of this approach that proved more efficient is to work in a heterogeneous state, in the presence of an active site inhibitor linked to an insoluble resin. Because of steric effects, the resin–inhibitor complex hinders the conjugation also at the active-site surroundings [84].

A further method to protect the active-site area is the use of a more hindered polymer such as the branched PEG. This 'Y' shaped PEG derivative was obtained in the authors' laboratory by linking two PEG chains at both amino groups of lysine while the amino acid carboxylic group is exploited for the coupling reaction [85]. The hindrance of the branched PEG prevents or reduces the entrance into the cleft of the active enzyme site as demonstrated in asparaginase and uricase modifications [86]. Branched PEG also allows one to achieve a higher protection from proteolytic degradation of the conjugated proteins and an increased reduction of immunogenicity and antigenicity as compared to the modification carried out with the linear polymers bound at an equal number of amino acid residues [86]. A similar 'Y' shaped PEG has been also synthesized by Maeda and co-workers using a trichlorotriazine activated polymer [87]. However, the use of this PEG for biopharmaceutical applications is impaired by the potential toxicity of the activating compound.

3.4. Poly(ethylene glycol) protein conjugation

Besides the advantages related to its monofunctionality that prevents the formation of reticulated material, mPEG is non-toxic, non-immunogenic, it can be obtained under GMP conditions and it is FDA-approved [11,88,89]. So far, a large number of proteins and peptides has been conjugated with mPEG and it is difficult to select which one to describe in this review (Table 2). However, bovine adenosine deaminase and asparaginase conjugates cannot be ignored by the authors since they are the first ones whose conjugates have been approved by FDA. Besides, at least part of the work that has been carried out with the enzyme superoxide dismutase (SOD) and with hemoglobin (Hb) deserve special mention. The former protein has been chosen because of the large number of papers dealing with its derivatization while the latter is a significant example of polypeptide devoid of enzymatic activity.

The most impressive clinical results with PEG-enzymes were obtained with adenosine deaminase (ADA). The deficiency of this enzyme causes severe immunodeficiency that is inherited as an autonomal recessive trait. Among other problems, it causes recurrent infections, due to the impaired immune function [90]. When adenosine deaminase was conjugated to PEG of 5 kDa molecular mass, the conjugate was still active and had a longer permanence in the body as compared to the original enzyme [91,92]. Most importantly, its immunogenicity was dramatically reduced, a property that allows the repeated administrations needed in this life-long disease [93]. Up to now, the results obtained with the administration of PEG–ADA to several diseased children (now, still alive after some years of treatment) appear more promising than those obtained

Table 2
Peptides and proteins so far modified by polymers (partial list)

Enzymes
Adenosine deaminase, Alkaline phosphatase, Arginase, Asparaginase, Catalase, Chimotripsin, Cytochrome-C, Elastase, Galactosidase (α and β), Gluconato oxydase, β-Glucuronidase, Glutaminase–asparaginase, Kallicrein, Lipase, Peroxidase, Phenylalanine ammonia-lyase, Purine nucleoside phosphorylase, Streptokinase, Superoxide dismutase, Thermolysin, Tissue plasminogen activator, Trypsin, Tryptophanase, Urokinase, Uricase.
Polypeptides
Albumin, Antigen E, Butroxobina, Erythropoietin, Factor VIII, G-CSF, G-MGSF, Hb, H-Growth hormon, Hirudin, Honeybee venom, IgG, Immunotoxin, Insulin, Interferon (α2A and α2B), Interleukin-6, Interleukin-2, Melanin, α-Proteinase inhibitor, Ragweed allergen, Somatostatin, Tumor necrosis factor.

with the administration of the adenosine deaminase gene, the alternative therapeutic approach for the treatment of this genetic disease [94,95].

PEG–ADA (Adagen®) was approved by the FDA, as was PEG asparaginase (Oncospar®), an antitumor agent which is specific for the treatment of acute lymphocytic leukemia. Asparaginase is commonly used in free form, but some patients develop an immune-based resistance that may, however, be overcome by the PEGylated form. In fact, asparaginase modification with PEG is accompanied by decreased immunogenicity, antigenecity and proteolytic susceptibility, probably all related to the polymer chain characteristics, as mentioned in the previous paragraphs. The positive characteristics of PEG–asparaginase are even enhanced with the branched form of PEG. The 'Y' shaped PEG also seems more convenient in preserving the enzyme activity after conjugation [96]. Most probably this effect is related, as previously reported, to the increased hindrance of the branched PEG that, in this specific case, prevents its own access and coupling at the active site region, located in a cleft between the two subunits of the enzyme.

Superoxide dismutase is an enzyme with scavenger activity towards the toxic superoxide ions which are overproduced in many pathological states such as inflammation or ischemia followed by reperfusion. Certainly, this is the enzyme mostly studied for PEG conjugation, and some derivatives are already at an advanced stage of clinical experimentation even though, to our knowledge, no product has yet been approved for marketing. The main results of the research on superoxide dismutase conjugates are:

1. a dramatic increase in body permanence after i.v. injection that, from the $t_{1/2}$ of 2–3 min of the non-modified enzyme, reaches several days in the case of PEG conjugates [97,98];
2. a slower absorption rate of the conjugate, as compared to the free enzyme, when administered subcutaneously or intramuscularly [96];
3. a higher efficacy of PEG–SOD, as compared to both the native enzyme and the dextran–SOD derivative, in reducing the heart necrosis size after ischemia and reperfusion [99]; and
4. the understanding of the role played by the polymer structure in conjugate behavior. Interestingly, it became clear that it is the overall amount of polymer mass linked to the protein that affects the degree of increased body permanence. More precisely, the same improvement can be achieved by linking several low molecular weight polymer chains or one only single chain with a very high molecular mass [100].

Hemoglobin (Hb) was PEGylated with great success (presently in Phase II clinical trial by Enzon) leading to a new product for blood transfusion that appears to be superior to stroma-free hemoglobin or to the dextran conjugate [101]. The conjugation, performed with PEG of 5 kDa molecular mass activated as succinimidyl carbonate, gave a product with a molecular weight in the range of 120–130 kDa. This conjugate, formulated in bicarbonate or phosphate buffers, at a 6% concentration, was administered for the treatment of hemorragic hypotension. These PEG–Hb solutions, in contrast to the physiological saline or to the dextran–Hb derivatives, restored the mean arterial blood pressure, the cortical oxygen pressure and the extravascular level of striatum dopamine to the same level as did the treatment with whole blood [102]. PEG–Hb was also found to be efficient in the oxygenation of solid tumors in rats even after three days after injection [103]. This property is considered useful for the therapy of many solid tumors, since it was proved that the effectiveness of many antineoplastic agents may depend on the level of cellular oxygenation [104,105]. Indeed, the PEG–Hb therapeutic indication is primarily for radio-sensitization of solid hypoxic tumors.

Other proteins being very much studied at present are citokines, and among them α-interferon, already in Phase III of clinical trial by Shering Plough, either alone or in conjugation with riboflavin (Rebetol®), for malignant melanoma and chronic myelogenous leukemia.

A further field of success in PEGylation is in producing more efficient targetable drug carriers: antibodies based on single chain. These simplified PEGylated antibodies replace the most known monoclonal products and present greater tumor penetration than the previous ones.

Finally, the very easily available albumin was also PEGylated with blood expander indications and it has already been on the market for some years.

3.5. Synthetic poly-functional polymers

In some cases, poly-functional polymers, despite the disadvantages discussed above (see Section 3.3), may have properties that are useful for the biomedical application of their conjugates. For example, this is the case of DIVEMA, the copolymer of divinyl-ether and maleic anhydride (also known as pyran copolymer), characterized by an intrinsic anti-tumor activity [106,107]. Several enzymes were coupled to it in search of a synergic effect, even though the risk of cross-linking is always present. Special care has to be taken in the coupling reaction in order to avoid undesired cross-linked complexes and activity loss. For example, superoxide dismutase was modified with DIVEMA using an original chemistry devised to prevent excessive cross-linking and enzyme inactivation [108]. In this case a reversible protection of part of the protein lysines, using 2,3-dimethylmaleic anhydride, was achieved before polymer

coupling, so that only the unprotected amino groups reacted with the polymer through its anhydride functions. After polymer coupling, the amide bond between the protected amines and 2,3-dimethylmaleic anhydride could be easily hydrolyzed by lowering the pH to 6.0, leaving the enzyme in a very active state. Interesting anti-inflammatory activities were exhibited by this DIVEMA conjugate.

However, a review of the literature demonstrates, without any doubt, that the most successful synthetic polyfunctional polymer is the copolymer of styrene and maleic anhydride (SMA). A very thorough chemical, pharmacological and clinical investigation was carried out in Japan by the group of Maeda that used the copolymer for the conjugation of the 12 kDa mass polypeptide neocarcinostatin, the most powerful (and toxic) anti-tumor polipeptide. Under suitable conditions of coupling, only two SMA chains were linked to the protein, in its α position and at the ε NH_2 of lysine 40. It is thought that each SMA chain has a favorable conformation with a bulky interior formed by the styrene moieties and a negative surface due to the hydrolyzed maleic anhydride COOH groups. Probably, this negatively charged protein conjugate, that also possesses a favorable molecular weight for blood permanence, is entrapped by the cells through EPR mechanism followed by endocytosis. Clinical results are extremely promising with a success in 70–90% of the treated tumor patients [109].

The negative charge of both DIVEMA and SMA polymers were also exploited in targeting anti-inflammatory enzymes to diseased areas, following the same rationale as described for the HPMA conjugates.

4. PEG–enzyme modification for bioconversion in organic solvents

Although not exactly within the scope of this article, we would like to briefly mention another important application of bioconjugation, namely the conjugation of enzymes, with potential usefulness in organic solvent biocatalysis, with amphiphilic polymers.

Bioconjugation is used in the field of biocatalysis to overcome some of the limitations related to the use of enzymes in non-natural organic environment. In fact, one of the most limiting drawbacks in the use of enzymes in bioconversion is their poor solubility in organic solvents where, on the other hand, most of the substances of pharmaceutical interest are soluble. This fact is responsible for a substantial reduction in the catalytic rate which was only partially solved by the use of enzymes in a fine powder or crystalline form, by entrapment in reverse micelles or by their immobilization onto insoluble supports [110,111]. On the other hand, the coupling of an amphiphilic polymer to an enzyme, transfers to the complex many of the properties of the polymer itself and, among these, the organic solvent solubility. It is not a general case but, often, the bioconjugate conformational structure and biological activity in the organic environment are maintained [112,113].

For this application too, PEG is the polymer mostly used since it is highly soluble in water and in most organic solvents, with the exclusion of a few of them like, for example, diethyl ether and *n*-hexane. This last property is indeed very useful because it allows one to carry out the bioconversion in organic solvents, (usually chlorinated or aromatic ones) and, at the end of the reaction, to recover the enzyme by precipitation after ether or *n*-hexane addition. The recovered enzyme may be used for another step of biocatalysis or dried out for later use. Relevant results have been obtained, for example, with the enzyme catalase: when 42% of its 112 amino groups were coupled with PEG, the enzyme activity in benzene was shown to be higher than that of the unmodified starting enzyme [114]. Similarly, PEG–lipase, where half of the seven amino groups were modified, was found to catalyze efficiently both ester synthesis and ester exchange in chlorinated solvents.

As mentioned above, this biocatalytic application of polymers is not an exclusive characteristic of PEG, but it is shared with many amphiphilic polymers. In our laboratory, lipase was modified with poly(*N*-acryloyl morpholine) (see above) and, when four to six chains of polymer were bound to each enzyme molecule this retained, in chlorinated solvent, over 50% of its original activity. The derivative displayed high trans-esterification properties in several solvents and, when compared to PEG–lipase, it showed lower solubility but better catalytic activity in organic media [115].

Promising results have also been obtained by Inada with a poly-functional 'comb-shaped' PEG that, thanks to its multi-point attachment sites, conferred to enzymes a high stability towards denaturation [112].

5. Polymers for oligonucleotide transport

Only recently was the research starting to take advantage of the potentials of oligonucleotides as drugs: oligonucleotides (ODNs) can be used as antigenes exploiting the formation of triple helices with genomic DNA [116], as antisenses hybridizing single stranded mRNA [117] or as ribozymes for sequence-specific cleavage of RNA [118]. However, for an efficient action, similar limitations as those found in protein drugs must be overcome, namely rapid proteolytic digestion, chemical instability and renal excretion. Moreover, the polar and highly charged ODNs suffer from poor cellular uptake so that many approaches are being investigated to overcome this problem. For example,

positively charged polymers interacting with the ODN phosphate charges (polylysine), positively charged lipoidic particles (liposomes) as carriers, or synthetic amphipatic peptides that form specific complexes with plasmid DNA [119] are being studied. Despite some promising data, the tenuous results so far obtained seem to suggest the need of having more defined chemical entities such as ODNs chemically modified with hydrophobic molecules as, for example, cholic acid [120], long chain alcohols [121], steroids [122] or amphiphilic polymers [123]. As a matter of fact, modification with amphiphilic polymers seems to have both advantages of improving the ODNs stability and of masking their charges.

Following the important results obtained in protein conjugation, PEG is the first polymer to have been evaluated for ODN modification, and even though this approach is still at its initial stages, promising biological results, although sometimes contradictory, have already been obtained.

Similarly to what observed with PEG–proteins, the stability to enzymatic digestion is increased. For example, Jaschke et al. demonstrated a great decrease in exonuclease activity towards phosphodiesterase I in the case of PEG–oligo conjugates and complete stability to phosphodiesterase II, as compared to the non-modified oligo [124]. Increased stability towards single-stranded endonuclease S1, that parallels the one in human and mice serum, was also observed in 5′-PEG conjugated products [125]. These authors' data are in agreement with our laboratory experience where a 3′-PEG dodecamer was found to be more stable towards the hydrolytic activity of the snake venom phosphodiesterase [123].

This behavior is reflected in increased residence time in circulation after intravenous administration observed in mice of a PEG-5′–oligodeoxynucleotide, where the naked oligonucleotide disappears rapidly [125].

The role of the molecular weight of PEG in the conjugate body permanence was also investigated and, similarly to what found with proteins, it was demonstrated that a critical mass value also holds for PEG–ODNs [126]. Forty kilodaltons is a convenient molecular weight for PEG to confer a long body retention to the conjugate.

As far as the body absorption and cellular uptake of chemically modified ODNs are concerned, the results reported in the literature so far seem to be quite contradictory and further investigation is probably needed. For example, disappointing results were shown in a single example in which the absorption of the various antisense agents, using human and murine serum as models, has been investigated. In this study, ODNs coupled with PEG, polyamines or cholic acid were compared to unmodified ODNs and lower absorption and activity, together with lower cellular localization, were observed for the conjugated compounds [127]. On the other hand, in another work, higher activity of a PEG-3′–antisense ODN directed towards the HIV virus was reported [128].

6. Chemistry of polymer–drug conjugation

6.1. General considerations

The chemistry of bioconjugation is extensive because it deals with a large variety of molecules, either as ligands or as biologically active moieties. Different approaches may be used depending on the properties of the bioactive molecule to be modified (a small organic compound, a protein, a glycoprotein or a nucleic acid) and of the ones of the ligand to be coupled (a colored, fluorescent or enzymatic probe, a monofunctional polymer such as PEG or a polyfunctional one such as polysaccarides or polyacrylates). Despite the large variability that characterizes this field of chemistry, a common feature shared by the reactions used in bioconjugation is the fact that all of them must be carried out in mild conditions, so that the structure, and therefore the activity, of the biologically active molecules is not disrupted.

In this part of the review, the discussion of the chemical aspects of conjugation will be divided into three sections: in the first, the functional groups most commonly represented in the drug molecules will be described; the second section will regard the functional groups normally present on the polymeric moiety; in the third, the most common strategies for the coupling reactions will be described.

However, due to the large variety of chemical reactions that have been exploited in this field, only the basic aspects of the technology will be reported here, while reading the specialized literature is recommended for a more detailed approach [129,130]. Furthermore, since polypeptides are the most studied compounds considered for conjugation, more space will be deserved to their chemistry.

As a preliminary consideration it may be important to note that, generally, both the drug molecule and the polymer are not reactive by themselves and a preliminary step of activation is usually needed before coupling. Furthermore, since the drug molecule often carries more than one functional group, it is the polymeric moiety that is generally (although not always) transformed into a reactive agent that is then coupled to the drug.

In some cases, it may be necessary to introduce a new functional group in either drug or polymer molecule. This may be achieved either through a specific chemical reaction that transforms a functional group into the desired one [131], through the use of a bifunctional

reagent [132] or, as in the case of proteins, through genetic recombinant techniques that allow the introduction of one amino acid with the desired reactivity, as, for example, the thiol function of cysteine [133] or amino group of lysine [134].

6.2. Functional groups on the drug

6.2.1. Proteins

Among the 21 amino acids of peptides and proteins, up to nine may be derivatized more or less easily at their side chains. All of these amino acids possess functional residues that in the appropriate environment may act, thanks to their ionizable side chains, as nucleophiles. Table 3 summarizes the main characteristics of these amino acid side chains, their structure and their pK_a values. The terminal amino and carboxylic functions are also groups that can be considered as possible sites of conjugation. Due to the nucleophilic properties of these residues, the most common reactions that take place in protein conjugation are nucleophile-to-electrophile attacks. The nucleophilicity of each amino acid residue may be modulated by changing the environmental pH since only when the pH is close or above the residue's pK_a, can nucleophilic attack take place. As a consequence, the order of nucleophilicity for the major groups in proteins, provided that each unprotonated form is more nucleophilic than its protonated parent, can be summarized as follows [125,130]:

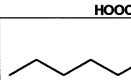
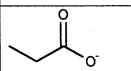
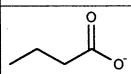
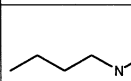
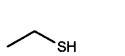
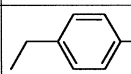
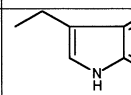
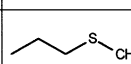
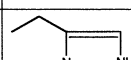
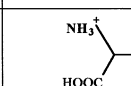
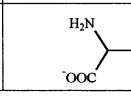
$$R\text{–}S^- > R\text{–}NH_2 > R\text{–}COO^- = R\text{–}O^-$$

Although according to this rank order, cysteine thiols are the most reactive groups in proteins, this amino acid is generally quite rare and when present, it often plays an important role in catalysis or in binding. For these reasons the main target in protein bioconjugation is the α-amino or ε-lysil amino residue. This amino acid is present with high frequency in proteins (up to about 10% of the overall amino acids) and only a few of its residues can be involved in the active site. Nevertheless, in some cases, cysteines have been successfully used as anchoring sites after being introduced into the protein, by recombinant techniques [133], into positions that are known as unimportant for the protein's biological activity.

Carboxyl carrying amino acids are considered as targets of coupling only when the modification of the more reactive lysines is known to impair the biological activity of the protein. The reason why these amino acids are considered as a second choice is because it is difficult to avoid cross-linking with the same protein's amino groups.

In glycoproteins, the sugar moiety can also be used as the target for polymer conjugation. Sugars may carry a few possible sites for modification such as the reducing aldehyde end groups, hydroxyl groups or, in some special cases, primary amines, carboxylates or phosphates. Furthermore, vicinal-hydroxyl groups can be easily oxidized by periodate, leading to two reactive formyl residues.

Table 3
Structure and property of protein amino acid residues

Amino acid	Structure	pKa
Lysine		9.3-9.5
Aspartic acid		3.7-4
Glutamic acid		4.2-4.5
Arginine		>12
Cysteine		8.8-9.1
Tyrosine		9.7-10.1
Tryptophan		
Methionine		
Histidine		6.7-7.1
α-carbonyl; *C*-terminus		2.1-2.4
α-amine; *N*-terminus		7.6-8

It may be interesting to remind also a quite different strategy, useful for a site specific PEGylation, that was used for the conjugation of the growth hormone releasing factor, a polypeptide of 29 amino acids normally obtained by solid phase synthesis. In this case, side chain PEGylated amino acids (lysine or aspartic acid) were directly introduced at the desired level of the sequence during the synthetic preparation of the peptide [135]. This strategy is actually applicable to any low molecular weight peptide that can be obtained by synthesis, and the chemistry used in coupling the polymeric moiety to the starting amino acids involves the same chemical residues (–COOH, $–NH_2$) as in coupling of the whole polypeptides.

6.2.2. Small organic molecules

The main sites of polymer attachment in small organic molecules share similar characteristics with those described for proteins in the sense that they often possess

nucleophilic residues. However, they must be devoid of critical relevance for the molecule's biological activity to be considered as sites of conjugation. Therefore, a polymer is generally attached to a small drug through its –OH, $–NH_2$ or –COOH groups and, as in the case of proteins, these, or other residues, if not available directly in the molecule, may be introduced through a desired spacer by means of an intermediate synthetic step.

6.2.3. Nucleic acids

The strategy for polymer modification of nucleic acids, as with antisense nucleotides, is different from the one used for proteins and small molecules for two main reasons: (a) the nucleic acid molecules do not carry the same reactive groups as those present in proteins and primary $–NH_2$, –COOH, –SH or aromatic –OH functions are not normally present; and (b) the oligonucleotides are obtained by synthesis (chemical or enzyme-driven) so that, if a specific tag has to be linked to the sequence, the synthesis itself may be planned to obtain a final product with the desired properties.

In general, modified nucleic acids can be obtained by two general approaches: by nucleic acid polymerization using nucleotides which have already been modified with the desired tag, or by modification of the oligonucleotide molecule after its synthesis has been achieved.

In many cases, the synthesis can also be planned in such a way that, instead of introducing the desired molecule (polymer, a molecular probe, etc.) directly in the nucleotide structure, a proper spacer arm carrying at its end a desired nucleophilic group (–SH, $–NH_2$, –COOH) can be used. In this case, the final modifying agent will be introduced in a second step by using the same chemistry as that used in protein conjugation.

The chemistry that allows the introduction of any molecule, (spacer arm, polymer, probe) on a nucleotide or on a nucleic acid molecule may be different. Modification can be carried out at the level of the nucleotide bases, of the sugar molecule (ribose or deoxyribose) or also at the 3′ or 5′ end hydroxyl groups of the nucleic acid molecule.

Many reactions can be used to modify specific sites on the nucleic acid bases, but most of this chemistry applied to the introduction of low molecular weight probes, such as biotin, digoxygenin or fluorescent markers. However, when a polymer molecule has to be introduced, the most common anchoring sites are the 5′ and 3′ end groups. In the case of PEGylation, the 3′ modification is achieved either in solid- or liquid-phase synthesis, by using a PEG-modified solid support in the former case [136] or, in the latter case, by using PEG as a soluble polymeric support according to the so-called 'HELP' procedure [137]. PEGylation at the 5′ end has been achieved by coupling the PEG chain to the oligo molecule after its synthesis [124,127,138,139].

The soluble polymer-supported synthesis has also been exploited by using monofunctional amphiphilic polymers others than the most known linear PEG. For example, a branched, more hindered form of PEG [123,140] and poly(*N*-acryloyl morpholine) [141] were used, leading to products which were structurally similar to the linear PEG conjugates, but with different physico-chemical characteristics.

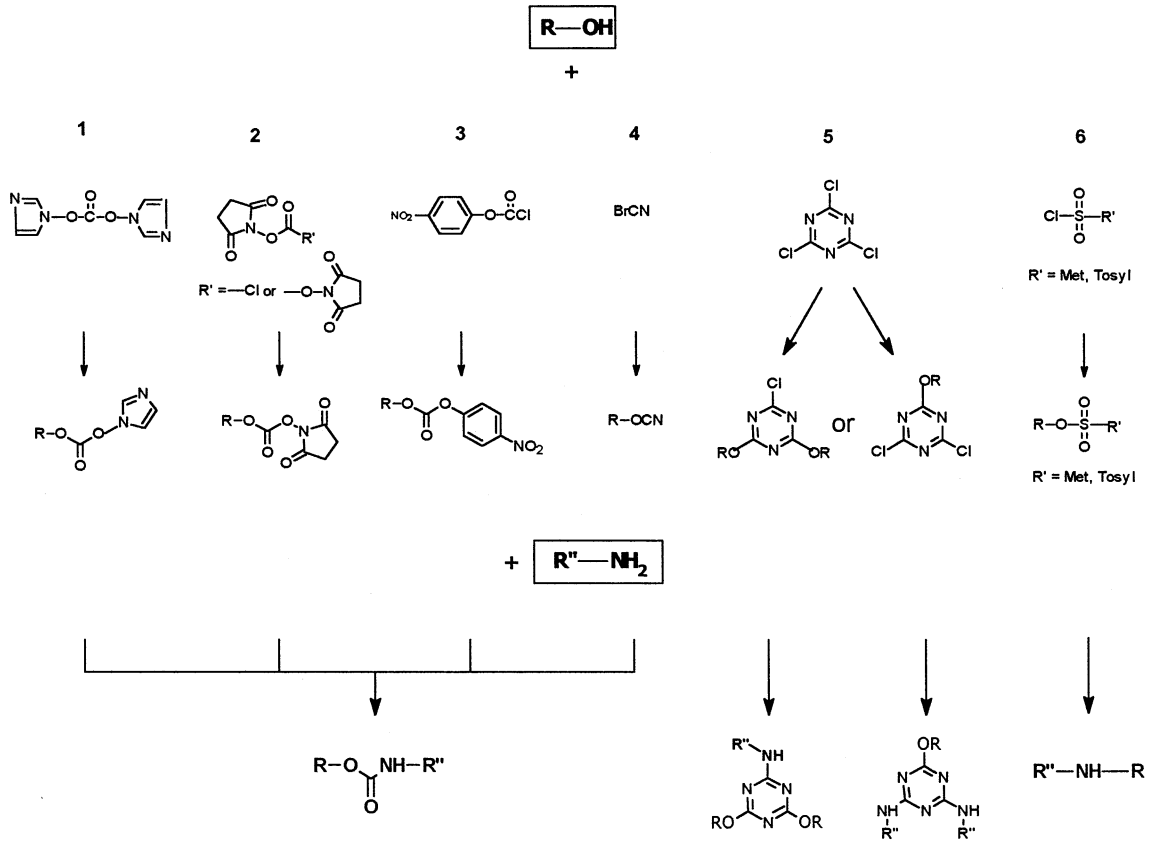
6.3. Polymeric moieties

There are many polymers used in the preparation of bioconjugates for pharmaceutical application. Because of their application in the biomedical field, they all share the common properties of being highly hydrated, non-toxic, non-immunogenic and of having a molecular weight sufficiently low to allow, when they are not biodegradable, filtration (although slow), through the kidney. The two most common polymers presently employed are PEG and dextran but others are also being used, namely, poly(L-lysine), poly(L-aspartic acid) or other poly(amino acids), poly(vinyl alcohols), poly(acrylates), poly(*N*-vinyl pyrrolidine), poly(*N*-acryloyl morpholine), poly(hydroxypropyl acrylate), SMA and DIVEMA or copolymers of the same.

From a general point of view, these polymers can be divided into two main groups, poly- and mono-functional. In bioconjugation, as already mentioned, the choice of a mono- or a poly-functional polymer mainly depends on the type of drug that has to be modified and, generally, mono-functional polymers (mPEG, PVP, PAcM derivatives, etc.) are preferred in the modification of poly-functional or high molecular weight bioactive molecules (such as the proteins). On the other hand, poly-functional polymers are mostly used for the modification of small mono-functional drugs, where the risk of cross-linking does not exist and a more favorable drug/polymer ratio is often desired.

Beside the different structure in their polymeric backbone, the polymers used in bioconjugation have only a limited number of functional residues that are normally exploited in the coupling reaction. Also in this case, the most common anchoring groups are –COOH, –OH and $–NH_2$. These groups must be activated in order to react with the desired drug molecule and many mild activation methods are presently available. Of course, the chemistry of binding can also be planned by the other way round, so that it is the drug molecule to be activated first. The strategy is determined by the best conditions for preserving the drug activity.

Special attention must also be paid to the 3D structure of the polymers, their hydrophobic/hydrophilic balance, their flexibility and biodegradability. All of these factors are important in dictating the fate of the



Scheme 1.

drug in vivo, the rate of elimination from blood and the protection from degradation, both in vivo and in the pharmaceutical formulation. As general rules, it may be important to remember that:

1. C–C backbones are degraded only in rare cases in the body;
2. highly hindered polymers (as, for example, the polysaccharides), greatly protect from chemical and enzymatic degradation;
3. hydrated and flexible polymers (like PEG) are slowly eliminated from the kidney: for a similar reason, they give rise to entanglement of ultrafiltration membranes;
4. hydrophobic polymers are more frequently localized in organs;
5. high molecular weight polymers may localize in specific sites because of the EPR effect, from where they are captured by the cell through an endocytic mechanism;
6. the degradation of polymers inside the cell may occur by means of the action of specific, very active lysosomal enzymes or because of the acidic endosomal environment; and
7. often the polymer, especially when its mass overcomes that of the drug, dictates the in vivo behavior of the conjugate.

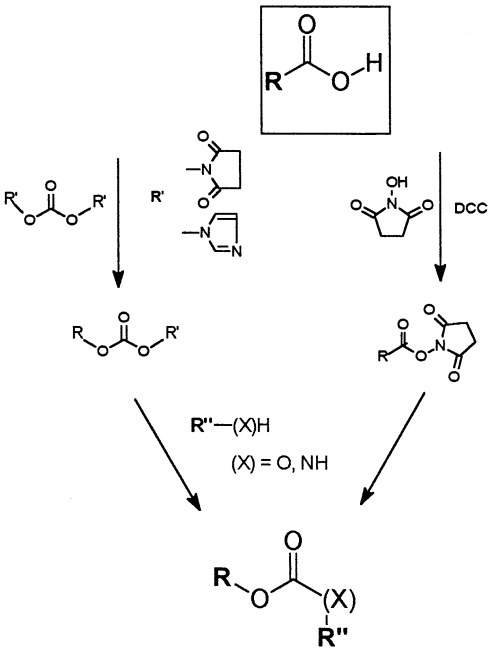
6.4. Functional groups and coupling reactions

6.4.1. Hydroxyl functions

Hydroxyl groups can be transformed into many activated species (see Scheme 1) that are suitable for coupling to nucleophilic residues: for example, they can be activated as mesylates and tosylates but also as succinimido- or imidazolyl-carbonates, or as *p*-nitrophenylformates [5]. All of these activated residues react readily with primary amines, leading to secondary amines in the case of tosylates and mesylates or to stable carbamate bonds in the other cases. Hydroxyl functions can also be oxidized to aldehydes or ketones that, upon reaction with amines, lead to Schiff bases that can in turn be reduced to a stable secondary amine linkage. This latter approach is widely used in coupling of polysaccharides where vicinal diols in sugar molecules are oxidized to aldehydic residues by periodate and then react with amine carrying molecules.

Hydroxyl functions can also be activated into cyanate esters by CNBr, a chemistry widely known for its use in the immobilization of amine containing molecules into hydroxyl-carrying chromatographic supports [142].

Another activation method for the coupling of hydroxyl functions to primary amines is through

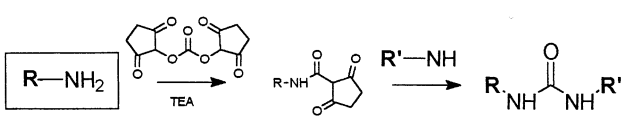


Scheme 2.

trichlorotriazine (cyanuric chloride). This reagent, being tri-functional, can cause the formation of cross-linking and does not always lead to well-defined conjugates [98]. This fact has often been pointed out as the cause for activity loss that has been observed in many enzymes modified with trichlorotriazine activated polymers. Other authors have also reported that this reagent lacks in selectivity [4,53] and can react with nucleophiles others than the lysines or primary amines as for instance tyrosil residues. These facts, together with the potential toxicity of the reagent, are the reasons why it is now used only for the conjugation of enzymes devised for bioconversion in organic solvents, while it is practically ignored for drug modification.

6.4.2. Carboxyl functions

The most common activation method for carboxylic functions (see Scheme 2) is through their *N*-hydroxysuccinimidyl esters, using *N*-hydroxysuccinimide (NHS) and a carbodiimide. These active esters are suitable for coupling primary amines and, less frequently, hydroxyl functions, leading to stable amides in the former case and to hydrolytically unstable esters in the latter. Carboxylic groups can also be coupled directly to amines without NHS, with the use of condensating agents such as carbodiimides. Either water- or organic-soluble carbodiimides can be used, depending on the reaction environment and the solubility of the reagents.



Scheme 3.

In proteins, the activation of carboxylic groups to conjugate amino-carrying polymers cannot be carried out without the risk of cross-linking with the amino groups of the same protein. An original procedure to avoid this problem is through the use of a water soluble carbodiimide to activate the protein carboxylic functions at pH 4–5, in the presence of a hydrazide-containing polymer. In these conditions, only the polymer hydrazide residues, having a very low pK_a, will react, while the primary amines will remain unaffected [54].

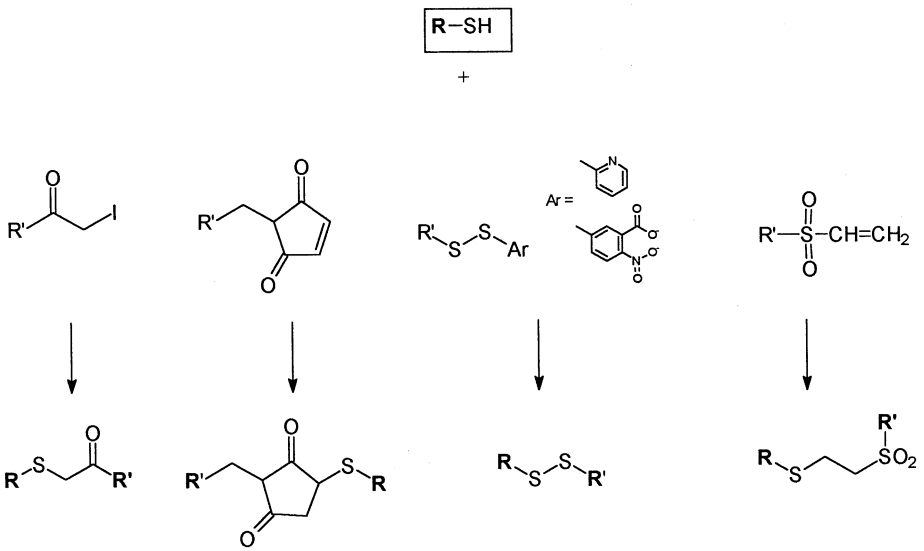
6.4.3. Primary amine functions

Amine-containing polymers are generally coupled to carboxyl-containing drugs with the use of carbodiimide reagents (see Scheme 3), as described in the previous paragraph [143]. Besides, aromatic amines can also be activated as isocyanates or isothiocyanates using phosgene or thiophosgene, respectively. These reagents can then react with primary amines leading to isoureas or isothioureas, respectively, and with hydroxyl-carrying molecules leading to carbamate or thiocarbamate bonds. Isoureas can also be obtained through activation of the primary amine into a succinimidyl carbamate using disuccinimidylcarbonate and triethylamine and through further reaction of this activated reagent with the second amine-carrying molecule [144].

6.4.4. Thiol reactive compounds

As previously mentioned, the thiol group is the most reactive among the nucleophiles present in drug molecules. The chemistry for the modification of this group has been largely developed and many reactive groups, that selectively react with it without affecting the integrity of other nucleophiles, are presently available. Among them, the most popular are those carrying activated double bonds (see Scheme 4), the haloacetyl residues and the thiol–disulfide exchange reagents such as the pyridyl disulfides or the TNB–thiol activated reagents. These residues are not normally available on a polymer but they may be introduced by a simple reaction. For example, they may be introduced by means of bifunctional reagents that carry the thiol reactive part in one side of the molecule, while the other side is activated for binding to the polymer. Several polymers, already functionalized for reaction with thiols are available on the market (Shearwater Polymers, Huntsville, AL, USA), as for example, PEG–pyridyldisulfide, PEG–maleimide and PEG–vinylsulfone [145].

During thiol modification, special care has to be taken in order to avoid the formation of disulfides that may occur through the use of buffers which are not



Scheme 4.

completely devoid of oxygen or in the presence of traces of metals. Furthermore, it is important to remember that for some reagents (as vinyl sulfone or maleimide) the specificity for the thiol residues holds only if the pH is maintained below 6–7, since above this value, some amino groups may also react. Undesired amine reactivity can also occur at unexpected pH if an amine with unusually low pK_a is present, or the reaction is carried out in organic–aqueous conditions [146].

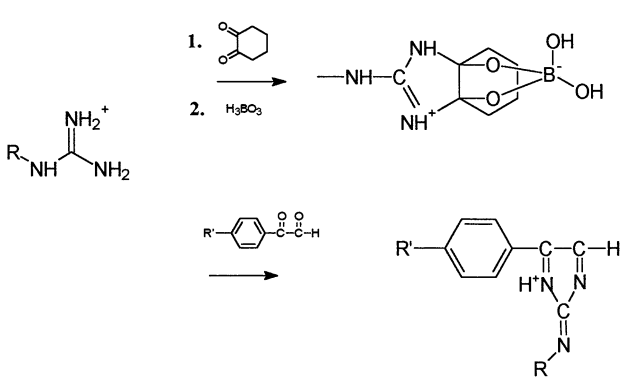
6.4.5. Guanidino groups

Arginine is an amino acid potentially interesting as a target for conjugation because it is not highly represented in peptides and proteins and, consequently, the coupling to its side chain could be selective. Unfortunately, a reproducible and specific chemistry for arginine conjugation is still missing. Linkage to the guanidino group (see Scheme 5) is usually achieved by means of molecules carrying α-diketone residues as, for example, 1,2-cyclohexanedione, in alkaline conditions. Examples of arginine modification is mainly reported in proprietary literature with phenylglyoxal or 1,2-cyclohexanedione linked to PEG [147]. However, to our knowledge, conjugation to arginine has not gained great success, most probably because of the concurrent reactions with other amino acids such as lysines, histidines, cysteines and those containing hydroxyl functions [130].

7. Concluding remarks

In the past, bioconjugation proved to be a very fascinating and useful technology that gave relevant inputs to both basic and applied sciences. Bioconjugates, consisting of biologically active molecules covalently coupled to a second moiety with different, but desired, properties, have been developed in order to obtain new chemical entities with improved characteristics useful for their specific applications. In pharmaceutical sciences, as described in this article, the conjugation of low or high molecular weight drugs with different polymers allowed the development of new and more powerful drugs and, in many cases, solved problems related to the drug's instability or inefficient pharmacokinetics, body-distribution or cell-penetration properties. Besides, in many cases, the immunological drawbacks often related to bioactive peptides and proteins have been overcome by polymer conjugation.

On the other hand, it is worth to noting that, down the years, the study of new bioconjugates and



Scheme 5.

of their interaction with biological moieties has been of relevant importance for unraveling the mechanisms involved in major biological processes, among which, the EPR effect, endocytosis or pinocytosis and immunologic phenomena. It may therefore become clear how important it is for those scientists involved in this field of research to work in strict collaboration, as different scientific backgrounds are needed for basic understanding and optimal results.

In our opinion, the main achievements of bioconjugation in the pharmaceutical field can be summarized as follows:

1. improvement of the pharmacokinetics, biodistribution, targeting efficiency and stability of several powerful drugs, among which low molecular weight antitumor agents, proteins, and the newly introduced oligonucleotides;
2. the unraveling of the mechanisms involved in the cellular uptake and fate of macromolecules; and
3. the masking of antigenic sites and recognition by antigen processing cells.

Some of the points mentioned above have been investigated by several scientists and allowed the development of original drug bioconjugates that are already available in the market or are in advanced clinical experimentation. On the other hand, many questions are still unsolved and a lot of work is needed to develop new and better bioconjugates. As a matter of fact, when conjugation is needed, it must be remembered that every bioactive molecule is characterized by its own chemical, pharmacodynamic and immunological properties, so that any case has to be studied as a unique one.

More specifically, if someone should ask what could be the promising areas of research in this field, few indications may be given:

1. the polymer conjugation of nucleic acids derivatives, since this field is still at its infancy and, although the preliminary results seem very promising, a great deal work still has to be done before any product may gain practical application;
2. the study of more properly designed polymers will be of continuous interest since some may come out with quite new properties useful for the development of original bioconjugates with pharmaceutical applications. As an example, we would like to mention the so-called dendrimers, polymers that, thanks to their well defined structure, size and shape [148] are well suitable as drug carriers;
3. the investigation, taking inspiration from the trafficking of molecules in the body that nature is offering, of new targeting molecules to conjugate to drugs or to drug carrier constructs;
4. furthermore, an application of bioconjugation that we believe will gain importance in the future is the use of polymers for surface modification of drug carrier microparticles. In fact, it has already been shown that, when flexible and highly hydrated polymers are covering a microparticle's surface, this carrier becomes invisible to the fagocytic cells and to RES. PEG, PVP, PAcM and poly(oxazolines) all demonstrated to share this common feature. Thanks to this property, the term 'stealth', referred to polymer-protected liposomes, has recently become commonly accepted also for other systems. The 'stealth' effect is probably based on the same mechanism that is responsible for the repulsion of proteolytic enzymes or antibodies by polymer-conjugated proteins. It is our opinion that, in the future, also thanks to these effects, this aspect of the delivery technology will gain large popularity for the delivery of both high and low molecular weight constructs; and
5. finally, the combination of polymer-modified bioactive molecules and their entrapment into particles, an area now at its infancy [149], should also be considered in order to exploit the best achievements of both technologies.

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References

[1] H. Ringsdorf, Structure and properties of pharmacologically active polymers, J. Polym. Sci., Polym. Symp. 51 (1975) 135–153.
[2] C. Monfardini, F.M. Veronese, Stabilization of substances in circulation, Bioconjug. Chem. 9 (1998) 418–450.
[3] M.L. Nucci, R. Shorr, A. Abuchoski, The therapeutic value of poly(ethylene glycol)-modified proteins, Adv. Drug Deliv. Rev. 6 (1991) 133–151.
[4] C. Delgado, G.E. Francis, F. Derek, The uses and properties of PEG-linked proteins, Crit. Rev. Ther. Drug Carr. Syst. 9 (1992) 249–304.
[5] N.V. Katre, The conjugation of proteins with poly(ethylene glycol) and other polymers. Altering properties of proteins to enhance their therapeutic potential, Adv. Drug Deliv. Rev. 10 (1993) 91–114.
[6] S. Zalipsky, Chemistry of poly(ethylene glycol) conjugates with biologically active molecules, Adv. Drug Deliv. Rev. 16 (1995) 157–182.
[7] R. Duncan, Drug–polymer conjugates: potential for improved chemotherapy, Anti-Cancer Drugs 3 (1992) 175–210.
[8] H. Maeda, SMANCS and polymer conjugated macromolecular drugs: advantages in cancer chemotherapy, Adv. Drug Deliv. Rev. 6 (1991) 181–202.
[9] D. Putnam, J. Kopecek, Polymer conjugates with anticancer activity, Adv. Polym. Sci. 122 (1995) 55–123.

[10] A.H. Sehon, Suppression of antibody responses by conjugates of antigens and monomethoxypoly(ethylene glycol), Adv. Drug Deliv. Rev. 6 (1991) 203–217.
[11] S. Dreborg, E.B. Akerblom, Immunotherapy with monomethoxypoly(ethylene glycol) modified allergens, Crit. Rev. Ther. Drug Carr. Syst. 6 (1990) 315–365.
[12] G.J. Russell-Jones, The potential use of receptor-mediated endocytosis for oral drug delivery, Adv. Drug Deliv. Rev. 20 (1996) 83–97.
[13] C.T. Okamoto, Endocytosis and transcytosis, Adv. Drug Deliv. Rev. 29 (1998) 215–228.
[14] Y. Takakura, R.I. Mahoto, M. Hashida, Extravasation of macromolecules, Adv. Drug Deliv. Rev. 34 (1998) 93–108.
[15] E.J. Wawrzynczak, P.E. Thorpe, Effect of chemical linkage upon the stability and cytotoxic activity of A chain immunotoxins, in: A.E. Frankel (Ed.), Immunotoxins, Kluwer, Boston, 1988, pp. 239–251.
[16] E.J. Wawrzynczak, Rational design of immunotoxins: current progress and future prospects, Anti-Cancer Drug Des. 7 (1992) 427–441.
[17] E.S. Vitetta, P.E. Thorpe, J.W. Uhr, Immunotoxins: magic bullets or misguided missiles?, Immunol. Today 14 (1993) 252–259.
[18] P. Erhlich, A general review of the recent work in immunity, in: Collected Papers of Paul Erhlich, vol. 2, Immunology and Cancer Research, Pergamon, London, 1956, pp. 442–447.
[19] R. Duncan, P. Kopeckova-Rejmanova, J. Strohlam, I.C. Hume, J.B. Lloyd, J. Koyecek, Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. 2. Evaluation of daunomicin conjugates in vivo against L 1210 leukaemia, Br. J. Cancer 57 (1988) 147–156.
[20] R. Duncan, L.W. Seymour, K.B. O'Hare, P.A. Flanagan, S. Wedge, K. Ulbrich, J. Strohalm, V. Subr, F. Spreafico, M. Grandi, M. Ripamonti, M. Farao, A. Suarato, Preclinical evaluation of polymer-bound doxorubicin, J. Controlled Release 18 (1992) 123–132.
[21] R. Duncan, I.C. Hume, P. Kopeckova, K. Ulbrich, J. Strohalm, J. Kopecek, Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. 3. Evaluation of adriamycin conjugates against mouse leukemia L1210 in vivo, J. Controlled Release 10 (1989) 51–63.
[22] K. O'Hare, R. Duncan, J. Strohlam, K. Ulbrich, P. Kopeckova, Polymeric drug carriers containing doxorubicin and melanocyte-stimulating hormone: in vitro and in vivo evaluation against murine melanoma, J. Drug Target. 1 (1993) 217–229.
[23] L.W. Seymour, P.A. Flanagan, A. Al-Shamkhani, V. Subr, K. Ulbrich, J. Cassidy, R. Duncan, Synthetic polymers conjugated to monoclonal antibodies: vehicles for tumor-targeted drug delivery, Sel. Cancer Ther. 7 (1991) 59–73.
[24] R. Duncan, L.W. Seymour, L. Scarlett, J.B. Llyod, P. Rejmanova, J. Kopecek, *N*-(2-Hydroxypropyl)methacrylamide copolymers with pendant galactosamine residues. Fate after intravenous administration in rats, Biochim. Biophys. Acta 880 (1986) 62–71.
[25] L.W. Seymour, K. Ulbrich, J. Strahalm, J. Kopecek, R. Duncan, Pharmacokinetics of polymer-bound adriamycin, Biochem. Pharmacol. 39 (1990) 1125–1131.
[26] V. Rizzo, V. Pinciroli, La caratterizzazione analitica di coniugati polimero-farmaco, Chim. Ind. 80 (1998) 901–905.
[27] E. Configliacchi, G. Razzano, V. Rizzo, A. Vigevani, HPLC methods for the determination of bound and free doxorubicin, and of bound and free galactosamine, in methacrylamide polymer–drug conjugates, J. Pharm. Biomed. Anal. 15 (1996) 123–129.
[28] R. Duncan, I.C. Hume, H.J. Yardley, P.A. Flanagan, K. Ulbrich, V. Subr, J. Strohalm, Macromolecular prodrugs for use in targeted cancer chemotherapy: melphalan covalently coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers, J. Controlled Release 16 (1991) 121–136.
[29] K. Ulbrich, E. Zacharieva, J. Kopecek, I.C. Hume, R. Duncan, Polymer bound derivatives of sarcolysin and their antitumor activity against mouse and human leukaemia in vitro, Makromol. Chem. 188 (1987) 2497–2509.
[30] Y. Ohya, H. Kuroda, K. Hirai, T. Ouchi, Synthesis and cytotoxic activity of conjugates of monomethoxy poly(ethylene glycol) end capped with doxorubicin via ester, amide, or Schiff base bond, J. Bioact. Comp. Polym. 10 (1995) 51–66.
[31] G. Kwon, S. Suwa, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide–aspartate) block copolymers–adryamycin conjugates, J. Controlled Release 29 (1994) 17–23.
[32] P. Caliceti, C. Monfardini, L. Sartore, O. Schiavon, F. Bacchichetti, F. Carlassare, F.M. Veronese, Preparation and properties of monomethoxypoly(ethylene glycol) doxorubicin conjugates linked by an amino acid or peptide as spacer, Farmaco 48 (1993) 919–932.
[33] R.B. Greenwald, A. Pendri, C. Conover, C. Gilbert, R. Yang, J. Xia, Drug delivery system: camptothecin 20-0-poly(ethylene glycol) ester transport forms, J. Med. Chem. 39 (1996) 1938–1940.
[34] A. Nathan, S. Zalipsky, J. Kohn, Strategies for covalent attachment of doxorubicin to poly(PEG–Lys), a new water soluble poly(ether urethane), J. Bioact. Comp. Pol. 9 (1994) 239–251.
[35] M. Nichifor, V. Coessen, E.M. Schacht, Macromolecular prodrugs of 5-fluoruracil. 1. Synthesis and hydrolytic stability, J. Bioac. Comp. Polym. 10 (1995) 199–222.
[36] V. Coessen, E.H. Schacht, D. Domurado, Synthesis and in vitro stability of macromolecular prodrugs of norfloxacin, J. Controlled Release 47 (1997) 283–291.
[37] R.B. Greenwald, C.W. Gilbert, A.D. Pendri, C. Conover, J. Xia, A. Martinez, Drug delivery systems: water soluble taxol 2′-poly(ethylene glycol) ester prodrugs — design and in vivo effectiveness, J. Med. Chem. 39 (1996) 424–431.
[38] M.D. Bentley, B.Y. Chung, W. Ellis, F. Mc Greevy, Polymer Prepr. 38 (1997) 584–585.
[39] T.C. Merigan, W. Regelson, Interferon induction in man by a synthetic polyanion of defined composition, New Engl. J. Med. 277 (1967) 1283–1287.
[40] T. Hirano, S. Ohashi, K. Morimoto, T. Tsuda, Y. Kobayashi, S. Tsuksgoshi, Synthesis of antitumor-active conjugates of adriamicin or daunomycin with the copolymer of divinyl ether and maleic anhydride, Makromol. Chem. 187 (1986) 2815–2824.
[41] T. Hirano, H. Ringsdorf, D. Zaharko, Antitumor activity of monomeric and polymeric cyclophosphamide derivative compound with in vitro hydrolysis, Cancer Res. 40 (1980) 2263–2267.
[42] F. Zunino, G. Pratesi, G. Pezzoni, Increased therapeutic efficacy and reduced toxicity of doxorubicin linked to pyranopolymer via the side-chain of the drug, Cancer Treat. Rep. 71 (1987) 367–373.
[43] G. Bogliolo, C. Muzzulini, R. Lerza, I. Pannacciulli, Activity of doxorubicin linked to poly-L-aspartic acid on normal murine hematopoietic progenitor cells, Cancer Treat. Rep. 70 (1986) 1275–1281.
[44] G. Decher, M. Emmelius, H. Ringsdorf, Synthesis and antitumor activity of daunorubicin-containing polymers, in: 26th Microsymposium on Polymers in Medicine and Biology, Prague, Czechoslovakia, 1984, pp. 40–41.
[45] R. Hrdina, T.A. Bogusova, A. Kunova, J. Kvetina, Changes in the toxicity and therapeutic efficacy of daunorubicin linked with a biodegradable carrier, Neoplasma 38 (1991) 265–273.

[46] A. Trouet, M. Masquelier, R. Baurain, D.D. Campeneere, A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug–carrier conjugate: in vitro and in vivo studies, Proc. Natl. Acad. Sci. USA 79 (1982) 626–629.
[47] K. Fujii, T. Imai, M. Otagiri, Control of pharmacokinetics and nefrotoxicity of *cis*-DDP by alginate, 23rd Proc. Int. Symp. Controlled Release Bioact. Mater., 1996, pp. 5018–5019.
[48] T. Ouchi, A. Fujiino, K. Tanaka, T. Banba, Synthesis and antitumor activity of conjugates of poly(α-malic acid) and fluorouracils bound via esters, amide and carbamoyl bonds, J. Controlled Release 12 (1990) 143–153.
[49] G. Horpel W. Klesse, H. Ringsdorf, Micelle forming co- and block-polymers for sustained drug release, IUPAC Proc. Int. Symp. Macromol., Amherst, MA, 1982, p. 346.
[50] G. Giammona, B. Carlisi, G. Pitarresi, G. Fontana, Hydrophilic and hydrophobic polymeric derivatives of anti-inflammatory agents such as aclofenac, ketoprofaen and ibuprofen, J. Biact. Comp. Polym. 6 (1991) 129–141.
[51] G. Giammona, G. Puglisi, G. Cavallaro, A. Spadaro, G. Pitarresi, Chemical stability and bioavailability of aciclovir coupled to α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide, J. Controlled Release 33 (1995) 261–271.
[52] G. Giammona, G. Cavallaro, G. Fontana, G. Pitarresi, B. Carlisi, Coupling of the antiviral agent ribovuridine to polyaspartamide and in vitro drug release studies, J. Controlled Release 54 (1998) 321–331.
[53] Y. Gotoh, M. Tsukada, N. Minoura, Chemical modification of silk fibroin with cianuric chloride-activated poly(ethylene glycol): analyses of reaction site by ^{1}H NMR spectroscopy and conformation of the conjugates, Bioconjug. Chem. 4 (1993) 554–559.
[54] S. Zalipsky, S. Menon-Rudolph, Hydrazide derivatives of poly(ethylene glycol) and their conjugates, in: J.M. Harris (Ed.), Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications, second ed., Plenum, New York, 1998, pp. 319–341.
[55] G. Giammona, B. Carlisi, G. Cavallaro, G. Pitarresi, S. Spampinato, A new water-soluble synthetic polymer, α,β-polyasparthydrazide, as a potential plasma expander and drug carrier, J. Controlled Release 29 (1994) 63–72.
[56] F.P. Bonina, L. Montenegro, P. DeCaprariis, F. Palagiano, G. Trapani, G. Liso, In vitro and in vivo evaluation of polyoxyethylene indomethacin esters as dermal prodrugs, J. Controlled Release 34 (1995) 223–232.
[57] D. Needham, T.J. McIntosh, D.D. Lasic, Repulsive interactions and mechanical stability of polymer-grafted lipid membranes, Biochim. Biophys. Acta 1108 (1992) 40–48.
[58] V.P. Torchilin, V.G. Omelyanenko, M.I. Papisov, A.A. Bogdanov Jr., V.S. Trubetskoy, J.N. Herron, C.A. Gentry, Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity, Biochim. Biophys. Acta. 1195 (1994) 11–20.
[59] G. Blume, G. Cevc, Molecular mechanism of the lipid vesicle longevity in vivo, Biochim. Biophys. Acta 1146 (1993) 157–168.
[60] H. Maeda, T. Matsumura, Tumoritropic and lymphotropic principles of macromolecular drugs, Crit. Rev. Ther. Drug Carr. Syst. 6 (1989) 193–210.
[61] L.W. Seymour, Passive tumor targeting of soluble macromolecules and drug conjugates, Crit. Rev. Ther. Drug Syst. 9 (1992) 135–187.
[62] C.T. Okamato, Endocytosis and transcytosis, Adv. Drug Deliv. Rev. 29 (1998) 215–228.
[63] Kee University, UK Patent No. 2270920, 1990.
[64] A.J. Kirby, P.W. Lancaster, Structure and efficiency in intramolecular and enzymatic catalysis. Catalysis of amide and hydrolysis by the carboxy group of substituted maleamic acid, J. Chem. Soc., Perkin Trans. II (1972) 1204–1214.
[65] F.M. Veronese, O. Schiavon, P. Caliceti, Protein delivery: PVP as a new polymer for protein conjugation, 24th Proc. Int. Symp. Controlled Release Bioact. Mater., 1997, pp. 507–508.
[66] F.M. Veronese, C. Visco, S. Massarotto, C.A. Benassi, P. Ferruti, New acrylic polymers for surface modification of enzymes of therapeutic interest and for enzyme immobilization, Ann. NY Acad. Sci. 501 (1987) 444–448.
[67] M.C. Woodle, C.M. Engbers, S. Zalipsky, New amphipatic polymer–lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes, Bioconjug. Chem. 5 (1994) 493–496.
[68] Y. Kojima, H. Maeda, Evaluation of poly(vinyl alcohol) for protein tailoring: improvements in pharmacokinetic properties of superoxide dismutase, J. Bioact. Comp. Polym. 8/2 (1993) 115–131.
[69] V.P. Torchilin, J.I. Voronkov, A.V. Mazoev, The use of immobilized streptokinase (Streptodekaza) for the therapy of thromboses, Ter. Ark. (Ther. Arch. Russ.) 54 (1982) 21–25.
[70] E.I. Chazov, V.N. Smirnov, I.M. Torchilin, V.P. Moskivichev, G.M. Grimberg, A.Z. Skuja, G.I. Kleiner, Dextran derivatives of fibrinolysin, US Patent No. 4446316.
[71] S.C. Tam, J.T.F. Wong, Modification of hemoglobin upon covalent coupling to dextran: enhanced stability against acid denaturation and reduced affinity for haptoglobin, Can. J. Biochem. 58 (1980) 732–736.
[72] N.I. Larionova, N.R. Kazanskaya, I.Yu. Sacharov, G.V. Mityushina, Soluble high molecular weight derivatives of pancreatic trypsin inhibitor. Modification of pancreatic trypsin inhibitor by soluble polysaccharides activated by titanium tetrachloride, Biokhimiia 45 (1980) 638–686.
[73] N.I. Larionova, G.V. Mityushina, N.F. Kazanskaya, Y.A. Blidchenko, I.V. Berezin, Carbohydrate-containing derivatives of the trypsin–kallikrein inhibitor aprotinin from bovine organs. I. Modification with lactose, characterization and behaviour of the preparation in vivo, Hoppe Sayler's Z. Physiol. Chem. 365 (1984) 791–797.
[74] N.I. Larionova, G.V. Mityushina, N.F. Kazanskaya, V.A. Blidchenko, I.V. Berezin, Carbohydrate-containing derivatives of the trypsin–kallikrein inhibitor aprotinin from bovine organs. II. Inhibitor coupled to the (carboxymethyl)dextran derivatives of D-galactose, Biol. Chem. Hoppe-Sayler's 366 (1985) 743–748.
[75] A. Abuchowsky, J.R. Mc Coy, N.C. Palczuck, T. Van Es, F.F. Davis, Alteration of immulogical properties of bovine serum albumin by covalent attachment of poly(ethylene glycol), J. Biol. Chem. 252 (1997) 3578–3581.
[76] K.V. Savoca, A. Abuchowski, T. Van. Es, F.F. Davis, N.C. Palczuk, Preparation of a non-immunogenic arginase by the covalent attachment of poly(ethylene glycol), Biochim. Biophys. Acta 578 (1979) 47–53.
[77] J. Snider, C. Neville, L.C. Yuan, J. Bullock, Characterization of the heterogeneity of poly(ethylene glycol)-modified superoxide dismutase by chromatographic and electrophoretic techniques, J. Chromatagr. 599 (1992) 141–155.
[78] F.M. Veronese, B. Saccà, O. Schiavon, P. Caliceti, L. Orsatti, P. Orsolini, PEG–peptide and protein delivery: a procedure to identify the PEGylation site, 26th Proc. Int. Symp. Controlled Release Bioact. Mater., Boston, MA, 1999.
[79] O. Schiavon, F.M. Veronese, P. Caliceti, P. Orsolini, Method for identifying, or analyzing polymer linkage sites on macromolecules using aminoacid reporter binding, Eur. Patent Appl., 1998.

[80] L. Sartore, P. Caliceti, O. Schiavon, C. Monfardini, F.M. Veronese, Accurate evaluation method of the polymer content in monomethoxypoly(ethylene glycol)-modified proteins based on aminoacid analysis, Appl. Biochem. Biotechnol. 31 (1991) 213–221.

[81] S.L. Snyder, P.Z. Sobocinsky, An improved 2,4,6-trinitrobenzenesulphonic acid method for the determination of amines, Anal. Biochem. 64 (1975) 284–288.

[82] A.F.S.A. Habeeb, Determination of free amino groups in proteins by trinitrobenzensulphonic acid, Anal. Biochem. 14 (1966) 328–336.

[83] S. Uderfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, M. Weigele, Fluorescamine: a reagent for assay of aminoacids, peptides, proteins and primary amines in the picomole range, Science 78 (1972) 871–877.

[84] P. Caliceti, O. Schiavon, L. Sartore, C. Monfardini, F.M. Veronese, Active site protection of proteolytic enzymes by poly(ethylene glycol) surface modification, J. Bioact. Biocomp. Polym. 8 (1993) 41–50.

[85] C. Monfardini, O. Schiavon, P. Caliceti, M. Morpurgo, J.M. Harris, F.M. Veronese, Branched monomethoxypoly(ethylene glycol) for protein modification, Bioconjug. Chem. 6 (1995) 62–69.

[86] F.M. Veronese, P. Caliceti, O. Schiavon, Branched and linear poly(ethylene glycol): Influence of the polymer structure on enzymological, pharmacokinetic, and immunological properties of protein conjugates, J. Bioact. Comp. Polym. 12 (1997) 196–207.

[87] K. Ono, Y. Kai, H. Maeda, T. Samizo, K. Sakurai, H. Nishimura, Y. Inada, Selective synthesis of 2,4-bis(*O*-methoxypoly(ethylene glycol))-6-chloro-*s*-triazine as a protein modifier, J. Biomat. Sci., Polym. Ed. 2 (1991) 61–65.

[88] S.N.J. Pang, Final report on the safety of poly(ethylene glycols) (PEGs) 6, 8, 32, 75, 150, 14M, J. Am. Coll. Toxicol. 12 (1993) 429–456.

[89] G.M. Pawel, Poly(ethylene glycol), in: R.L. Davidson (Ed.), Handbook of Water Soluble Gums and Resins, McGraw-Hill, New York, 1980 (Chapter 8).

[90] M.E. Bollinger, D.O.F. Arredondo-Vega, I. Santisteban, K. Schwarz, M.S. Hershfield, H.M. Lederman, Brief report: hepatic dysfunction as a complication of adenosine deaminase deficiency, New Engl. J. Med. 334 (1996) 1367–1371.

[91] R.B. Weiss, Hypersensitivity reactions, Semin. Oncol. 19 (1992) 458–477.

[92] B.G. Peters, B.J. Goeckner, J.J. Ponzillo, W.S. Velasquez, A.L. Wilson, Pegaspargase versus asparaginase in adult ALL: a pharmacoeconomic assessment, Hosp. Formul. 30 (1995) 388–393.

[93] M.S. Hershfield, Biochemistry and immunology of poly(ethylene glycol) modified adenosine deaminase, in: J.M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol) Chemistry and Biological Applications, American Chemical Society, Washington, DC, 1998, p.145–154.

[94] P.M. Hoogerbrugge, V.W. VonBeusechem, L.C.M. Kaptein, M.P.W. Einerhand, D. Valerio, Gene therapy for adenosine deaminase deficiency, Br. Med. Bull. 51 (1995) 72–81.

[95] L.D. Fairbanks, H.A. Simmonds, P.M. Hoogerbrugge, V.W. Von Beusechem, D. Valerio, A. Moseley, R.J. Levinsky, H.B. Gaspar, G. Morgan, Biochemical and immunological status following gene therapy and PEG–ADA therapy for adenosine deaminase (ADA) deficiency, Adv. Exp. Med. Biol. 370 (1994) 391–394.

[96] F.M. Veronese, C. Monfardini, P. Caliceti, O. Schiavon, M.D. Scrawen, D. Beer, Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxy poly(ethylene glycol), J. Controlled Release 40 (1996) 199–209.

[97] R. Somack, M.G.P. Saifer, L.D. Williams, Preparation of long-acting superoxide dismutase using high molecular weight poly(ethylene glycol) (41 000–72 000 Da), Free Rad. Res. Commun. 12–13 (1991) 553–562.

[98] E. Boccu', G.P. Velo, F.M. Veronese, Pharmacokinetic properties of poly(ethylene glycol) derivatized superoxide dismutase, Pharmacol. Res. Commun. 14 (1982) 113–120.

[99] A.V. Maksimenko, A.D. Petrov, P. Caliceti, G.G. Konovalova, E.L. Grigoryeva, O. Schiavon, V.Z. Lankin, F.M. Veronese, Biodistribution of a poly(ethylene glycol) modified superoxide dismutase in mice and its effect on myocardia ischemia treatments, Drug Deliv. 2 (1995) 39–43.

[100] M.G. Saifer, R. Somack, L.D. Williams, Plasma clearance and immunologic properties of long-acting superoxide dismutase prepared using 35 000–120 000 Da poly(ethylene glycol), Adv. Exp. Med. Biol. 366 (1994) 377–387.

[101] C.D. Conover, R. Linberg, C.W. Gilbert, K.L. Sham, R.G.L. Shorr, Effect of poly(ethylene glycol) conjugated bovine hemoglobin in both top-load and exchange transfusion rat models, Artif. Organs 21 (1997) 1066–1075.

[102] D. Song, M. Olano, D.F. Wilson, A. Pastuszko, O. Tammela, K. Nhao, R.G.L. Shorr, Comparison of the efficacy of blood and poly(ethylene glycol)–hemoglobin in recovery of newborn piglets from hemorrhagic hypotension: effect on blood pressure, cortical oxygen, and extracellular dopamine in the brain, Transfusion 35 (1995) 552–558.

[103] P. Vangel, Oxygenation of solid tumors, in: B.A. Techer (Ed.), Drug Resistance in Oncology, Marcel Dekker, New York, 1993, pp. 53–85.

[104] B.A. Teicher, G. Ara, R. Herbst, H. Takeuchi, S. Keyes, D. Northey, PEG–hemoglobulin: effects on tumor oxynegation and responce to chemotherapy, In Vivo 11 (1997) 301–312.

[105] B.A. Teicher, G. Ara, Y. Chen, Y. Emi, Y. Kakeji, M. Ikebe, Y. Maehara, PEG–hemoglobin: effects on tumor oxygenation and radiosensitization, Radiat. Oncol. Inv. 4 (1996) 200–210.

[106] D.S. Breslow, E.I. Edwards, N.R. Newburg, Divinyl ether–maleic anhydride (pyran) copolymer used to demonstrate the effect of molecular weight on biological activity, Nature 246 (1973) 160–162.

[107] D.S. Breslow, Biologically active synthetic polymers, Pure Appl. Chem. 46 (1976) 103–113.

[108] T. Hirano, T. Todoroki, S. Kato, H. Yamamoto, P. Caliceti, F.M. Veronese, H. Maeda, S. Ohashi, Synthesis of conjugate of superoxide dismutase with the copolymer of divinyl ether and maleic anhydride retaining enzymatic activity, J. Controlled Release 28 (1994) 203–209.

[109] H. Maeda, Pharmacological uniqueness and clinical effects, in: H. Maeda, K. Edo, N. Ishida (Eds.), Neocarzinostation: The Past, Present and Future of Anticancer Drugs, Springer, Tokyo, 1997.

[110] A. Zacks, A.M. Klibanov, Enzymatic catalysis in non-aqueous solvents, J. Biol. Chem. 263 (1988) 3194–3201.

[111] A.M. Klibanov, Why are enzymes less active in organic solvents?, Tibtech 15 (1988) 97–101.

[112] Y Inada, K. Takahashi, T. Yoshimoto, A. Ajima, A. Matsushima, Y. Saito, Application of poly(ethylene glycol)modified enzymes in biotechnological processes: organic solvent soluble enzymes, Tibtech 4 (1986) 190–194.

[113] Y. Inada, T. Yoshimoto, A. Matsushima, Y. Saito, Engineering physicochemical and biological properties of proteins by chemical modification, Trends Biotech. 4 (1984) 68–73.

[114] K. Takahashi, T. Yoshimoto, A. Ajima, Y. Inada, Poly(ethylene glycol)-modified catalase exhibits unexpectedly high activity in benzene, Biochem. Biophys. Res. Commun. 125 (1984) 761–766.

[115] R. Bovara, G. Ottolina, G. Carrea, P. Ferruti, F.M. Veronese, Modification of lipase from *Pseudomonas* sp., with poly(acryloyl morpholine) and study of its catalytic properties in organic solvents, Biotechnol. Lett. 16 (1994) 1069–1074.

[116] C. Helene, The anti-gene strategy: control of gene expression by triplex-forming-oligonucleotides, Anti-Cancer Drug Del. 6 (1991) 569–584.
[117] A. De-Mesmaeker, R. Haener, P. Martin, H.E. Moser, Antisense nucleotides, Acc. Chem. Res. 28 (1995) 366–374.
[118] L. Gold, Oligonucleotides as research, diagnostic, and therapeutic agents, J. Biol. Chem. 270 (1995) 13581–13584.
[119] J.G. Duguid, C. Li, M. Shi, M.J. Logan, H. Alila, A. Rolland, E. Tomlimson, J.T. Sparrow, L.C. Smith, A physicochemical approach for predicting the effectiveness of peptide-based gene delivery systems for use in plasmid-based gene therapy, Biophys. J. 74 (1998) 2802–2814.
[120] M. Manoharam, L.K. Johnson, C.F. Bennet, T. Vickers, D.J. Ecker, L.M: Cowsert, S.M. Freier, P. Cook, Cholic acid oligonutide conjugates for antisens application, Bioorg. Med. Chem. Lett. 18 (1994) 3777–3781.
[121] R.G. Shea, J.C. Marsters, N. Bishofberger, Synthesis and hybridazation properties and antiviral activity of lipid–oligodesoxinucleotide conjugates, Nucleic Acids Res. 18 (1990) 3777–3779.
[122] A. Boutorin, L. Guskova, E. Ivanova, N. Kobetz, V. Zarytova, S. Ryte, L. Yurchenko, V. Vlassov, Synthesis of alkylating oligonucleotides deriatives containing cholesterol or phenazinium residues at their 3′-terminus and their interaction with DNA within mammalian cells, FEBS Lett. 254 (1989) 129–131.
[123] G.M. Bonora, E. Ivanova, V. Zarytova, B. Burcovich, F.M. Veronese, Synthesis and characterization of high-molecular mass poly(ethylene glycol)-conjugated oligonucleotides, Bioconjug. Chem. 8 (1997) 793–797.
[124] A. Jaschke, J.P. Furste, E. Nordhoff, F. Hillekamp, D. Cech, V.A. Erdmann, Synthesis and properties of oligodesoxyribonucleotide–poly(ethylene glycol) conjugates, Nucleic Acids Res. 22 (1994) 4810–4814.
[125] T. Kawaguchi, H. Asakana, Y. Tashiro, K. Juni, T. Sueishi, Stability, specific binding activity, and plasma concentration in mice of an oligodesoxynucleotide modified at 5′-terminal with poly(ethylene glycol), Biol. Pharm. Bull. 18 (1995) 474–478.
[126] S.C. Gill, unpublished results.
[127] M. Manoharam, K.L. Tivel, L.K. Andrade, V. Mohan, T.P. Condon, F.C. Bennet, P.D. Cook, Oligonucleotides conjugates: alteration of the pharmacokinetic proerties of antisense agents, Nucleosides Nucleotides 14 (1995) 969–973.
[128] G.M. Bonora, G. Tocco, S. Zaramella, F.M. Veronese, P. Pliasunova, A. Pokrovsky, E. Ivanova, V. Zaryota, Antisense activity of an anti-HIV oligonucleotide conjugated to linear and branched high molecular weight poly(ethylene glycols), Farmaco 53 (1998) 634–637.
[129] G.T. Hermanson (Ed.), Bioconjugate Techniques, Academic Press, San Diego, 1996.
[130] K. Mosbach (Ed.), Methods in Enzymology, vol. XLIV (Immobilized Enzymes), Academic Press, San Diego, 1976.
[131] C. Zioudrou, M. Wilchek, A. Patchornick, Conversion of the L-serine residue to an L-cysteine in peptides, Biochem. J. 4 (1965) 1811–1822.
[132] J. Carlsson, H. Drevin, R. Axen, Protein thiolation and reversible protein–protein conjugation *N*-succinimidyl 3-(2-pyridylthio)propionate, a new heterobifunctional reagent, Biochem. J. 173 (1978) 723–737.
[133] R.J. Goodson, N. Katre, Site-directed pegylation of recombinant interleukin-2 at its glycosilation site, Biotechnology 8 (1990) 343–346.
[134] M.S. Hershfield, S. Chaffee, L. Koro-Johnson, A. Mary, A.A. Smith, S.A. Short, Use of site-directed mutagenesis to enhance the epitope-shielding effect of covalent modification of proteins with poly(ethylene glycol), Proc. Natl. Acad. Sci. USA 88 (1991) 7185–7189.
[135] A.M. Felix, Y. Lu, R.M. Campbell, Pegylated peptides IV: enhanced biological activity of site directed pegylated GRF analogs, Int. J. Peptide Protein Res. 46 (1995) 253–264.
[136] V.A. Efimov, I.N. Pashkova, A.L. Kalinkina, O.G. Chakhmakhceva, Synthesis of conjugates of oligonucleotides with poly(ethylene gycol), Bioorg. Khim. 19 (1993) 800–810.
[137] G.M. Bonora, Poly(ethylene glycol). A high efficiency liquid phase (HELP) for the large-scale synthesis of oligonucleotides, Appl. Biochem. Biophys. 54 (1995) 3–9.
[138] A. Jaschke, R. Bold, E. Nordhoff, F. Hillekamp, D. Cech, V.A. Erdmann, J.P. Furste, Synthesis and analytical characterization of RNA–poly(ethylene glycol) conjugates, Nucleosides Nucleotides 15 (1996) 1519–1522.
[139] T.M. Tarasow, D. Timmermeir, C. Zyrniewski, Characterization of oligodesoxyribonulceotide–poly(ethylene glycol) conjugates by EMS, Bioconjug. Chem. 8 (1997) 89–92.
[140] B. Burcovich, F.M. Veronese, V. Zarytova, G.M. Bonora, Branched poly(ethylene glycol) (bPEG) conjugated antisense oligonucleotides, Nucleosides Nucleotides 17 (1998) 1567–1570.
[141] G.M. Bonora, A. Baldan, O. Schiavon, P. Ferruti, F.M. Veronese, Poly(*N*-acryloylmorpholine) as a new soluble support for the liquid-phase synthesis of oligonucleotides, Tetrahedron Lett. 37 (1996) 4761–4764.
[142] P. Cuatrecases, C.B. Anfinsen, Affinity chromatography, Methods Enzymol. 22 (1971) 345–378.
[143] S. Herman, G. Hooftman, E. Schacht, Poly(ethylene glycol) with reactive endgroups: I. modification of proteins, J. Bioact. Comp. Polym. 10 (1995) 145–187.
[144] M. Morpurgo, E.A. Bayer, M. Wilchek, *N*-hydroxysuccinimide carbonates and carbamates are useful reactive reagents for coupling ligands to lysines on proteins, J. Biochem. Biophys. Methods 38 (1999) 17–28.
[145] M. Morpurgo, F.M. Veronese, D. Kachensky, J.M. Harris, Preparation and characterization of poly(ethylene glycol) vinyl sulfone, Bioconjug. Chem. 7 (1996) 363–368.
[146] PEG–Intron could move Enzon into profit, SCRIP (1999) No. 2424, 9.
[147] Eur. Patent No. 0,340,741 from Sumitomo, Japan.
[148] G.R. Newkome, Z. Yao, G.R. Baker, V.K. Gupta, P.S. Russo, M.J. Saunders, Cascade molecules: synthesis and characterization of a benzene[9]-arboral, J. Am. Chem. Soc. 108 (1986) 849–850.
[149] F.M. Veronese, C. Mammucari, S. Lora, P. Caliceti, O. Schiavon, Influence of PEGylation on the release of low and high molecular weight compounds from PVA matrices, J. Bioact. Comp. Polym. (1999) in press.