REVIEW

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Pegnology: a review of PEG-ylated systems

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Polyethylene glycol conjugation or linking with the system is called PEGylation. Many novel drug systems are used for the delivery of drugs and bioactive substances to particular sites in a controlled or sustained manner, but various side effects or shortcomings restrict their use for the intended purpose. The shortcomings such as RES uptake, drug leakage, immunogenicity, stability, hemolytic toxicity etc. can generally be overcome by PEGylation of novel drug delivery systems such as liposomes, proteins, enzymes, drugs, nanoparticles etc. In this article the whole aspect of PEGylation starting from activation and derivatisation of poly (ethylene glycol) to the linking and designing of systems and their purification and characterization is discussed. The various properties of Pegylated systems are also discussed.

1. Introduction

Polyethylene glycol is a non-toxic water-soluble polymer, which resists recognition by the immune system. The term PEG is used for polyethylene glycol, and often refers to polymer chains with molecular weights below 20000, while polyethylene oxide (PEO) refers to higher molecular weight polymers [1, 2]. These are homogeneous polymers of $Mw/Mn < 1.1$ represented by the formula $HO(CH_2CH_2O)_nH$. and is named poly(oxy-1,2-ethanediyl), α-hydro-ω-hydroxy-polyethylene glycol [3]. It is an addition polymer of ethylene oxide and water, where 'n' represents the average number of oxy-ethylene groups. They are soluble in water, alcohol, acetone, and chloroform,

miscible with glycols but practically insoluble in ether. They are also known as macrogols [4], which are generally of higher molecular weights, such as Macrogols of 20000 Da. The pH of 5% solution is about 4−7.5. Polyethylene glycol available commercially may be named using the molecular weight of the compound as number following the name PEG [5] e.g. PEG 400 has average molecular weight 400 Da and a range of M.Wt. of 380−420 while the value of 'n' is 8 in this case. PEGs having 'n' value up to hundreds are available commercially. Compounds with 'n' up to 15 are liquids at room temperature and their viscosity and boiling point increase with molecular weight. Higher polymers are waxy solids and are termed commercially as Carbowaxes^{TM} (Carbon and Carbide Co.)

The aqueous solutions can be sterilized by filtration, autoclaving etc., and need to be stored in airtight containers. These have relatively low toxicity. Toxicity, if any, appears to be greatest with macrogols of lower molecular weights. On topical administration they may cause stinging, especially to mucous membrane. They may be associated with hypersensitivity reactions such as urticaria, local gastrointestinal discomfort, bloating and nausea, abdominal cramps, vomiting and irritation, when used as bowel cleansing preparation. Macrogols demonstrate oxidizing ability leading to incompatibility with bacitracin or benzyl-penicillin activity, which may become reduced in macrogol bases. Estimated acceptable daily intake of PEG may be up to 10 mg/kg body weight [4]. Its presence in aqueous solution has no deleterious effect on protein conformation or activity of enzymes. It exhibits rapid clearance from the body and has been approved for a wide range of biomedical applications. PEG may transfer its properties to other molecules, to which it is covalently bound. This can result in modification of various properties of molecules as illustrated later on [5].

Polyethylene glycol has a polyether backbone, which is inert in the biological environment, as well as in most chemical reaction conditions such as in chemical modification and/or conjugation reactions. The presence of two terminal hydroxyl groups makes possible the formation of a number of ether and hydroxyl derivatives, which are available commercially. The chemical derivatization of end groups of PEG is an essential first step in the preparation of bioconjugates.

2. Derivatization and activation of polyethylene-glycol

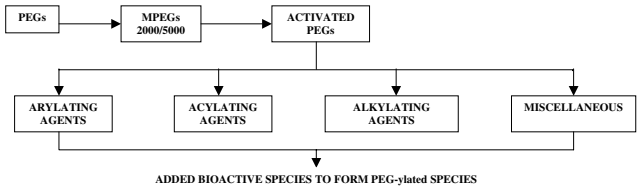
Polyethylene glycol has two equivalent hydroxyl groups, and so could act as a potential cross-linking agent for any system it is attached to. These hydroxyl groups can be attached to various bioactive species by covalent coupling giving a number of simple polyethylene glycol analogues. Some important stable analogues are described in Table 1. These were prepared by reactions with sensitive bioactive systems under mild conditions.

One very important derivative used in a number of derivatization reactions, which has one hydroxyl group blocked, is the monomethyl-ether of polyethylene-glycol or monomethoxy-polyethylene-glycol (MPEG) i.e. considered equivalent in terms of reaction for formation of PEG derivatives. This is generally brought into use for conjugation to bioactive species. It is generally used when multiple chains of polymers have to be linked to the intended substrates. Due to structural simplicity and the possession of only one-derivatizable end group, the use of MPEG minimizes cross-linking possibilities and leads to improved homogeneity of the conjugate. Thus, it is usually a starting material of choice for the covalent modification of proteins, biomaterials, particulates, lipids, drugs etc. [6].

$$MeOH + H(OCH_2CH_2)_nOH \longrightarrow MeO(CH_2CH_2O)_nH$$

Commercially available MPEGs are often contaminated with significant amounts (equivalent to 25%) of dihydroxy terminated polymers. Commercially polymers are purified by chromatography and ethyl ether precipitation. The use of these polymers as starting materials of choice for covalent modification of proteins, biomaterials and particles is dependent mainly on their not forming cross-linked conjugates [8].

Scheme 1: Basic principles of PEGylation of bioactive species



Commercially available MPEGs of molecular weights 2000 and 5000 are used mostly for the preparation of a number of reagents. Tables 1 and 2 discuss MPEG linked and based electrophiles. These are synthesized and used for linking with a number of available attachment sites e.g. amino acids and other nucleophilic groups, as per Scheme 1. These are called 'activated PEGs' [6]. The intermediate linkers are called 'spacers' linking to bioactive groups especially proteins and enzymes. These generally require mild conditions and specific properties of reactants for binding. Another very important PEG adduct of PEG-lysine copolymer has been recently reported with either cysteamine or 1-amino 2-methyl 2-propanethiol. Cysteine − containing peptides could then be disulphide linked to the thiol groups of the polymers in a similar facile manner using up to 8 molecules per molecule of PEG-Lysine copolymer (Mwt.27,000) [9].

In order of reaction and based on ease of introduction these can be arranged roughly as follows:

Arylating agents > Acylating agents > Alkylating agents [6]. These are discussed in Table 2. These derivatives are used mainly for PEGylation of proteins and enzymes.

3. PEG-ylated systems: synthesis and applications

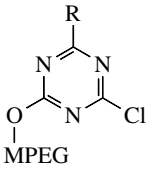
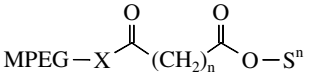
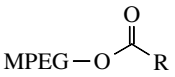
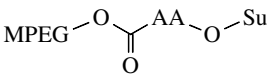
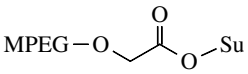
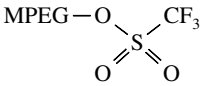
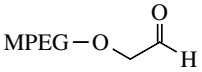
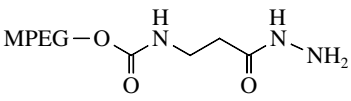
3.1. Controlled, targeted and sustained drug delivery systems

Because of their vapor pressure, solubility, solvent power, hygroscopicity, viscosity and lubricating characteristics, the polyalkylene glycols and their derivatives function in many applications as effective replacements for glycerin and water − insoluble oils [5]. They find considerable applications as plasticizers, lubricating agents, conditioners and finishing agents for processing textiles and rubber. They are also important as emulsifying agents and as dispersants for such diverse substances as dyes, oils, resins, insecticides and various types of pharmaceuticals. In addition, they are frequently employed as ointment bases and in a variety of cosmetic applications. Recently, conjugates of various novel drug delivery systems with PEGs have been used. This, in turn, has launched a whole new range of better drug delivery systems with enhanced properties of sustained and controlled release. The various properties

Table 1: Derivatized PEGs for activation and conjugation [7, 8] showing systems with basic structure $R_1(CH_2CH_2O)_nCH_2R_2$

S.No.	PEG-derivatives	Ingredients, conditions for synthesis and general comments
1	α-(2-Bromoethyl)-ω-bromo poly(oxyethylene glycol) R_1: Br, $-OCH_3$ R_2: Br	• Thionyl bromide in toluene, PEG 6000 or others such as Monomethoxy-PEGs (MPEGs) were used. • Hyflo-supercel diatomaceous earth was used for filtration and washing. • Susceptible to nucleophilic displacement.
2	α-(2-Aminoethyl)-ω-amino poly(oxyethylene glycol) R_1: $-OCH_3/NH_2$ R_2: $-NH_2$	• Ammonia in methanol and bromo-polyethylene glycol derivatives were used as precursors. • PEG aldehyde or PEG-CHO treated with KOH/NH_4Cl in methanol may also be used, which then reacts with $NaCNBH_3$. • Product was gummy in nature; hence centrifugation/HPLC was required for separation in suitable solvents.
3	α-(2-Trimethylammonioethyl)-ω-trimethylammonio poly(oxyethylene glycol) R_1: $-OCH_3$; $-N(CH_3)_3Br$ R_2: $-NH(CH_2)_6-NH_2$	• Trimethylamine in ethanol and bromo derivative of PEG was used. • Glass autoclave was used as a vessel for reaction followed by vacuum desiccation.
4	α-[2-(2-Carboxyethylcarbonyl amino)-ethyl]-ω-(2-carboxyethyl carbonylamino) poly(oxyethylene glycol) R_1: $-NHCO(CH_2)_2COOH/-OCH_3$ R_2: $-NHCO(CH_2)_2COOH$	• Succinic anhydride at pH 9−9.5, with amino PEG derivative was used as precursor. • Vacuum desiccation was generally done.
5	α-Carboxymethyl-ω-carboxy methoxy poly(oxyethylene glycol) R_1: $-CH_2COOH/-OCH_3$ R_2: $-CH_2COOH$	• Sodium naphthalene solution in tetrahydrofuran with bromoethyl acetate and PEG/MPEG-6000 were used for its synthesis.
6	α-[2-(Succinimido-oxy-carbonylamino)-ethylene]-ω-succinimido-oxy-carbonyl-amino poly(oxyethylene glycol)	• Succinic anhydride in ethyl acetate and N-hydroxy succinimide in dicyclohexyl carbadiimide at 30 °C.
7	PEG-Tosylate $PEG-CH_2Ots$ Ts: Toluene sulphonyl	• PEG 3400 with sodium hydride followed by reaction with p-toluene sulphonyl chloride in triethylamine was used for synthesis. This was precipitated by diethyl ether. • A large excess of triethylamine (60%) prevents cleavage, as compared with smaller quantities.
8	PEG-Mesylate. $PEG-CH_2Oms$ Ms: $-CH_3SO_2$	• Methane sulphonylchloride in methylene chloride and triethylamine was used for reaction. • Reaction was carried out at 0 °C for two hours.
9	PEG-aldehyde PEG-CHO	• PEG 3400, potassium tertiary butoxide in benzene and treatment with hydrochloric acid after reaction with bromoacetic acid ethyl ester was used. • Reaction required mild conditions and fourteen to fifteen hours was required for its completion. • Dimethyl sulphoxide in acetic anhydride, when used had additional advantages of ease of reaction, product purity etc. • Pyridinium chlorochromate may also be used for reactions with PEGs. • In all cases, Schiff's test was possible and Beer-Lambert's law follows for analysis. HPLC analysis for detection of chain cleavage.
10	PEG-ocatdecyl amine	• PEG 3680-tosylate, monomethyl ether, octadecyl amine dissolved in benzene with anhydrous sodium carbonate was used for synthesis. • Hydrophilic and hydrophobic groups are present on the same compounds, which was suitable for micellar preparations.
11	PEG-monopalmitate	• PEG 6000 in toluene with triethylamine, palmitic acid, sodium hydroxide in methanol, diethyl ether for precipitation and oxalyl chloride reaction for activation, were used. • Product was gummy hence required centrifugal method of separation.
12	PEG-glucosamine	• By reductive amidation using sodium cyanoborohydrate ($NaCNBH_3$) and PEG-CHO in aqueous solution. • It was isolated by precipitation and solvent evaporation under reduced pressure and dialysis. • Used for cell membrane binding and ligand affinity studies.
13	Porous glass beads (controlled)	• Mono(dimethyl-trityl)-PEG phthalate ester residues, thereby linking aminopropyl derivatized glass with PEG-CHO and sodium cyanoborohydrate ($NaCNBH_3$). • For solid cell separation media and anti-adhesive glass, electro-osmosis etc.
14	Steoroyloxy-PEG (SteO-PEG)	• MPEG using reflux with sodium hydride in tetrahydrofuran and reacting with stearylbromide then isolating mono-substituted derivatives. • Hydrophobic- hydrophilic groups on same molecule, which was able to entrap drug inside as micelles.

Table 2: Various common PEG derivatives used for PEGylation of proteins and enzymes

S.No	Activating agents(references)	Structures	Comments
I	Arylating Agents [6, 10–12]	 R=Cl R=MPEG-O	• Requires some forceful conditions • lower reactivity and higher selectivity, • decreases immunogenicity, • shows good preservation of enzymatic activity, largest activity loss amongst others in case of SOD enzyme, alkaline phosphatase. • Substantial loss of enzymatic activity due to reaction with sulfhydryl groups
II.a	Acylating agents [6, 13]	 i. X = O; n = 2 (succinimidyl succinate derivative) ii. X = O; n = 3. iii. X = −NH; n = 2.	• Hydrolytically labile conjugates. • Selective towards amino groups, preserved bioactvity and enzymatic activity as in case of superoxide dismutase. • Protein modification with bifunctional PEG analogs for better preservation. • React specifically and selectively with amino groups under mild conditions
II.b	Carbamates forming urethane [14–17]	 i. R = imidazolyl carboxyloxy ii. R = O-TCP iii. R = O-pNP iv. R = O-Su iv. > iii. > ii. > i.	• Ease of preparation Formation of stable attachments. • Used for preparation of simple dipeptide or amino acids linking e.g. using succinimidyl ester of MPEG. • Carbonates used or imidazolyl carbonyl oxy derivatives.
II.c	Amidating agents [6]	 (oxy-carboxyl amino acid derivative)	• Same as for II.b. above.
II.d	Amidating agents [6]	 Succinimidyl ester of carboxy methyl PEG.	• Yields stable conjugates, which are stable in biological buffers and physiological conditions.
III.a	Alkylating agents [18]	 Tresylates (2,2,2-trifluoroethane sulphonate)	• Reaction takes place under mild aqueous buffer conditions. • Alkylation of amino groups of proteins and thiols if present.
III.b	Acetaldehyde [6, 19]		• Results in alkylation of amino acids of protein thiols, selective towards primary amino groups, stable attachments without change in net charge of proteins, amenable to control and slower reaction.
IV	Hydrazide derivative [6]		• For linking of glycoproteins after periodate pretreatment and converting to aldehydes.

that are altered due to polyethylene glycol conjugation are discussed in the following sections. Polyethylene glycol conjugation has been tried and found most applicable in the following systems, using activated PEGs with appropriate spacers: Proteins and enzymes, drugs and low molecular weight compounds as prodrugs, lipids and liposomes, nanospheres and polymer micelles, hydrogels, dendrimers and other higher molecular weight polymeric drug delivery systems.

3.1.1. Proteins and enzymes

Enzymes constitute a large class of proteins, which over the last decade, have been subjected to covalent modification with polyethylene glycol. Various linkers or spacers have been used for linking by monomethyl ether called activated PEGs. These spacers include arylating agents, acylating agents and alkylating agents as discussed in Table 2. Using arylating agents, substantial loss of enzymatic activity with increased degree of modification occurred in the case of alkaline phosphatase [20]. Protein modifications with bifunctional PEG analogues have resulted in better preservation of activities independent of the extent of modification and the molecular weight of reagents used [21], especially for enzymes. Polyethylene glycol-tyrosine [6] a bifunctional polyethylene glycol reagent has been used for cross-linking the enzymes in dimeric form. The dimeric form was found to be considerably more active than its monomeric equivalent in the case of alkaline phosphatase [21]. Use of PEG based derivatives reacting selectively with the amino groups of proteins under mild conditions leads to good preservation of activities of conjugates. In particular, this was generally the case for enzymes acting on lower molecular weight

substrates. The preparation of functionally active yet extensively modified PEG conjugates, derived from proteins having large size substrates, has proved more difficult e.g. PEG-tissue plasminogen activators (TPA) conjugates prepared using succinimidyl-succinate (SS) and imidazolyl-carbonyloxy derivatives of methoxy-polyethylene glycol [22]. Some enzymes such as elastase modified by MPEG-Dichlorotriazine, have partially preserved their ability to act on small synthetic substrates, yet completely lost elastin hydrolyzing ability [23]. But by structure determination using ultraviolet circular dichorism and intrinsic fluorescence, proteins show integrity of catalytic sites with a number of proteases and similarly, no conformational changes have been observed. It has been shown that enzymatic activity was lost due to clear dependence of activity on the size of substrates [6], while good preservation of activity was found towards low molecular weight substrates, such as highly modified ribonuclease [24]. Thus the narrowing specificity of enzymes towards smaller substrates is caused in most cases by steric hindrance preventing or limiting access of large substrates to the active site of PEG modified proteins.

The site of covalent conjugation and the specificity of conjugation depend on both the conditions of PEGylation and the activated PEG used [25]. The choice of PEG depends upon the proteins to be PEG-ylated. At higher pH, reaction proceeds faster but hydroxide ion becomes a competitive nucleophile at a lower pH. A higher selectivity of reaction is thus possible. The extent of PEGylation is controlled to some degree by the ratio of activated PEG to protein. In cases where the protein has a number of reactive groups, it is difficult to control the reaction and to obtain a single conjugate. Rather a distribution of conjugated species is obtained, which requires subsequent purification to obtain a homogeneous product. Another variable was the molecular weight of polyethylene glycol for conjugation. The desired degree of modification could be achieved by varying the size, rather than the of number, of PEG molecules.

Reaction conditions depend upon stability of proteins, degree of modification needed to enhance the properties, pH and ionic strength. Site directed modification of proteins could be accomplished by site specific mutagenesis and subsequent chemical modification of mutant proteins, as has been tried for generating free cysteine or lysine on proteins at an accessible location for PEGylation of rIL-2 (recombinant interleukin-2) [26], and purine nucleoside phosphorylase [27]. This was necessary to avoid steric hindrances near the binding sites of proteins and also preservation of activity.

3.1.2. Lipids and liposomes

A numbers of modified lipids are available, which have been conjugated with polyethylene glycol derivatives. These could, in turn, be used to form liposomes. With available data, however, there is no reason to assume that linkage with MPEG influences the ability of the conjugates to form liposomes [28]. Although some differences have been observed in the properties of liposomes containing various MPEG-PE conjugates, they could be explained by subtle differences in formulations. Various mechanisms used for conjugation of proteins have been used for preparation of conjugates of lipids, especially those having $-COOH$ and $-NH_2$ terminal groups using tresylates, succinimidyl succinates, amide linkages, linkages using chlorotriazine approaches, linkages using p-nitrophenyl carbonyl groups etc [29, 30].

The chlorotriazine approach for the preparation of a bioactive conjugate is unattractive, due to toxicity of the reagent and its degradation products. Prolonged plasma presence of PEG-PE containing liposomes is independent of the degree of saturation of the parent PE or other lipids co-incorporated into the same liposomes. However better results have been obtained with ^{18}C-fatty acids containing PEs, than with shorter chain derivatives, e.g. methoxy-polyethylene glycol-disteroyl phosphotidyl ethanolamine (MPEG-DSPE) and methoxy polyethylene glycol-dioleyl phosphotidyl ethanolamines (MPEG-DOPE), which contain significant amounts of palmitoyl residues [31]. Conjugates of DSPE derivatives with polyethylene glycol of molecular weights 1900 and 5000, incorporated into lecithin/cholesterol liposomes of mean diameter 100 nm, showed comparable blood lifetime and bio-distribution in rats. Maruyama et al. [31] studied molecular weight dependence of performance of three derivatives of MPEG-DSPE conjugates in large unilamellated liposomes. MPEG-DSPE of molecular weights 1000 and 2000 shows extended circulation times of liposomes compared with conjugates derived from MPEG 5000 to 12000. Inter-bilayer transfer of MPEG – linked saturated diacyl lipids decreased exponentially with increasing fatty acid chain length [32].

In general, with increasing molecular weights of polar head groups, dissociation of lipid derivatives at the liposomal interface takes place earlier. Thus the hydrophobic energy required to retain MPEG-PE in lipid vesicles, increases with molecular weight of the polymers. This suggests that increasing the molecular weights of liposomal grafted polyethylene glycol chains contributes to loss of PEG-lipids from the vesicles. This explains, why MPEG of molecular weight 2000 but not higher showed optimal performance in liposomes. This is different from other systems, where an increase in molecular weights of PEG results in an increase in steric stabilization effects [32].

A number of PEG-lipid conjugates containing reactive functional groups at the end of polymer chains have been prepared recently. These derivatives could be viewed as analogous to MPEG-PE conjugates, in which the chemically inert methoxy group is replaced with conjugation prone functionality [30, 33, 34]. The need for such functionalized PEG-lipids is due to their usefulness in attaching various biologically relevant ligands to the exterior of PEG grafted liposomes. Some end group functionalized PEG ligands have already been used to attach immunoglobulins, peptides and other ligands to the distal ends of liposomes with grafted PEG chains.

The lipid PEG conjugates could be represented by

X-linker A-PEG-linker B-DSPE

where, *linker A* had in most cases amino acids residues, which was helpful for final chemical characterization of conjugates by amino acid analysis.

Linker B was in most cases a stable urethane linker formed by reacting PE with an appropriate SC-PEG-X, where X, represents a functional residue or protected form thereof.

Common examples of *linker A*, include:

$CH_3-CH_2-NH-COO-$; $CH_3-CH_2-CH_2CONH-$

Common examples of *linker B,* include:

$O-CH_2-CONH-$; $-O-CONH-$ etc.

e.g. a. CH_3O-
b. H_2N-
c. $-H_2N-NH-COCH_3-$
d. $Br-CH_2-CONH-$
e. pyridine-S−S−
f. $HO-CO-CH_2-O-$

X include functional residues or protected forms thereof, for further conjugation of antibodies to protect from cross-linking and linking of proteins. As shown above, 'c' forms hydrazine linkages with aldehydes and has been used for conjugation of antibodies oxidized on their carbohydrate residues, to distal ends of PEG chains on the surface of liposomes.
'd' shown above was effective for thio-ether forming reactions with −SH ligands, used for Fab′-SH attachments to end groups of PEG chains of liposomes.
Linker X, substituted on PEG linked moieties by chemically inert methoxy groups are called conjugation prone functionality, and are needed to attach bioactive ligands to the exterior of PEG-liposomes with long circulation lives, necessary for receptor mediated targeting.
Linkers have generally been designed to have well-defined lability very advantageously. This might allow further control of the *in vivo* properties of the relevant conjugated liposomes [30, 33, 34]. Phosphatidyl ethanolamines linked using succinimidyl succinates (SS)-PEG undergo rapid hydrolysis under physiological conditions. Preparations using succinimidyl carbonates (SC)-PEG with DSPE were easily prepared under mild conditions, due to the greater reactivity of SC-PEG. Other derivatives such as oxy-carboxyl-imidazole and tresylates have also been prepared. Liposomes, could in turn be prepared using DSPC-DPPE-cholesterol-MPEG, which has been separately prepared by MPEG-tresylates earlier. These could also be prepared by selective grafting of polymers on the exterior of vesicles. These avoid the presence of MPEG residues inside the liposome [35]. But there is also difficulty in substituting all the required groups uniformly and properly, resulting in asymmetric lipid bilayer formation. Similarly phosphatidic acid could be attached to MPEG using 2,6,6, tri-isopropyl-benzene sulphonyl chloride in pyridine and used afterwards in liposomes. It has also been shown that the above conjugate readily releases free MPEG-OH after a few hours of incubation at 37 °C in mouse serum. In this regard amide, secondary amine and urethane inter-linked MPEG-PEs and others containing stable linkages are more attractive [36]. Recently, based on the above approaches, sterically stabilized immunoliposomes (SIL) have been described for coupling anti-CD34 My10 mAb to poly(ethylene glycol)-liposomes (PEG-liposomes) containing the anchor pyridyl dithio propionyl amino-PEG-phosphatidyl ethanolamine (PDP-PEG-PE) via a cleavable disulfide bond. Efficient attachment of pyridyl dithio-derivatized mAb took place (equivalent to coupling ca. 70% of total input protein) at 2 mol percent of the functionalized PEG-lipid [37]. Similarly, efficient attachment of cytochrome-C was achieved on triazine or pyridyl thio group activated diamino PEG. The other end of the diamino PEG was linked to water-soluble lysine. Two stearoyl derivatives of lysine were then attached to the lysine end of the diamino-PEG. The whole was later incorporated into liposomes [38].
Liposomes have already been considered as circulatory carriers of a number of drugs, but are highly labile for uptake by the reticular endothelial system (RES) and due to their lipophilicity, they are less available in blood than effective circulatory carriers. But on PEGylation of the systems they were found to have long circulation times and their concentration in blood was found to have increased to a great extent. Also tumour targeting by antibodies has been found to be more possible particularly involving their local macrophage uptake near the tumour site, providing effective tumour targeting. This has been described for cationic PEG-lipids incorporated into preformed vesicles having enhanced binding and uptake into BHK cells [39]. Their clearance and sequestration by the body's defense mechanism, was found to have been decreased to a great extent making them carriers with a long circulatory time.
A novel type of fusogenic liposomes has been prepared with egg yolk phosphatidyl choline (EYPC), dioleoylphosphatidyl ethanolamine (DOPE) and modified with poly(glycidol) having β-alanine residues, which is a poly(ethylene glycol) derivative having positively charged groups. This was prepared by reverse phase evaporation and was found to have better fusion capacity with the cells. This was found to be the case for COS1 and CV1 cells transfection by polymer modified EYPC and DOPE liposomes containing calcein and pSV2cat plasmids. In this case membrane fusion plays an important role in liposome mediated cytoplasmic delivery [40].

3.1.3. Drugs and low molecular weight compounds, monomers and polymers

A number of low molecular weight compounds facilitate intestinal absorption of macromolecular drugs. These compounds, referred to as delivery agents, interact non-covalently with drugs to allow transport of the drugs through biological membranes. Delivery agent-mediated transport of various therapeutics, e.g. interferon and salmon calcitonin has been proposed, and a formulation containing one of these delivery agents with USP heparin is in late stage clinical trials. Most of the delivery agents produced to date have their greatest solubility at basic pH. In an effort to broaden the delivery agents' solubility over a wider pH range, modification by conjugating them to polyethylene glycol (PEG) of different MWs and functionalities has been tried. The goal of this new approach is to combine the interactive capabilities of potent delivery agents with the unique characteristics of PEG [41].
Methods of PEGylation include similar methods to those discussed earlier using activated PEGs. PEGylation of drugs is greatly influenced by their intended end use. The factors involved are a requirement for completion and perfection of synthesis of derivatives e.g. for affinity and bioreactor applications, it is not recessory to achieve complete substitution of end groups with ligand molecules. Contamination of the conjugate is also tolerated. The nature of the linkages between components of conjugates is also not critical. Strength of linkage between components of the conjugates is critically important [6, 42].
Stringent requirements for purity and homogeneity apply, when PEG-adducts are to be used as therapeutic drugs, so any contamination with un-reacted or partially reacted PEG derivative should be avoided. The polymer usually acts as an inert carrier with the dominating physical properties being solubility, membrane permeability and bio-distribution. The covalently linked substrate is responsible for all bioactivity. Although the toxicity of the drug is reduced by PEG conjugation, this depends upon the linkages between the conjugate components and the drug,

Table 3: Common methods of PEGylation of lower molecular weight drugs and compounds

S.No.	Techniques	Mechanisms of linking	Comments
1	Direct polymerization [6, 47]	• Ethylene oxide on to a drug molecule is linked with or without boron trichloride induced method.	• Substituted trisaccharide residues of glycosides • e.g. CynerubinA-aglycone, Cholesterol-PEG conjugates.
2	Dicyclohexyl-carbadiimide(DCC) or dimethyl amino pyridine ester [6, 42, 46]	• Linking PEG-OH groups with free carboxylic acid in milder conditions. • Clear transformation of carboxylic acids onto PEG esters at room temperature in short time.	• Aqueous conditions hydrolyse it. • Rate of hydrolysis depends on nature of acid, molecular weight of PEG, pH and temperature. • Devised for slow drug release.
3	Spacers and esters linkages [6, 43, 48]	• As succinates via ester linkages to PEG succinates of various molecular weight. • Acetic acid, propanoic acid, acrylic acid derivatives may be used. • Activation of drugs by various derivatives and linking to MPEG by using cyanuric chloride.	• Hydrolyzed easily by esterases. • e.g. Atropine, quinidine, 5-fluorouracil etc.
4	Urethane linked conjugates [6]	• For amides containing drugs reacting with carbonate derivatives of the polymers.	• Resistant to hydrolysis. • Toxicity decreases considerably as in case of doxorubicin. • e.g. histamine and radioiodinated tyramine.
5	Carbonate linked conjugates [49, 50]	• Using chloroformate derivatives of drug/PEG. • Formed selectively on primary hydroxyl groups with PEG-OH. • Reaction was base catalyzed. • Incubation of PEG-O-COCl generated by treatment with phosgene, hydroxyl or amino containing drugs at room temperature in presence of tertiary amines.	• Low to moderate yields. • Release by hydrolysis depends upon pH, temperature, and steric hindrances. • Useful for gradual drug release applications. • Conjugation by PEG-chloroformate derivatisation, produces higher yields. • e.g. atropine, tetrahydrocannabional, procaine.
6	Amino-PEG and mono-functional MPEG analogues [43–45]	• Has advantage of amino groups reactivity over hydroxy analogs. • Forms amide, urea, secondary amine, carbamate, thiourea linkages.	• Stable *in vivo* or hydrolyzed noticeably slower. • Amide conjugates by acylase catalyzed hydrolysis. • Antitumour activity increases with increase in molecular weight of carrier. • e.g. 5-fluorouracil-MPEG adducts.
7	Ether bound conjugates [44, 48]	• Using MPEG –Br derivatives directly to hydroxyl groups of drugs.	• More stable showing drug cleavage under acidic pH exhibiting similar dependence on molecular weight of carrier. • Resistant to enzymatic degradation. • Antitumour activity decreases with increase in molecular weight of carrier e.g. 5-fluorouracil.
8	Block copolymer methods [51, 52]	• Polymerization of N-carboxy anhydride of β-benzyl ester of aspartic acid onto MPEG-NH_2 initiator followed by removal of side chains protecting groups from the poly (aspartic acid) block copolymers exposed to carbonyl group linked to doxorubicin via amide bond.	• Doxorubicin conjugates to MPEG-poly(aspartic acids) form micelles. • Composed of drug containing hydrophobic core with grafted PEG chains on the exterior. • Prolonged circulation, reduced liver uptake. • Excessive tumour accumulation leads to enhanced anticancer activity.
9	Alternating segments of copolymers [54, 55]	• Synthesized new polymers after polymerization by SC-PEG with lysine so that both amino groups were incorporated into backbone forming urethane and carbonyl groups could be manipulated for various functional groups introduced or conjugation.	• Length of PEG fragment increases the distance between the functional groups along the backbone. • As molecular weight increases drug binding capacity decreases. • High drug attachment capacity through pendent carbonyl groups of lysine residues.

which may or may not be released *in vivo*. The toxicity of PEG substituted derivatives is influenced by the non-PEG part, while the influence of the polyethylene-glycol chains is relatively small [6, 30, 43, 44, 45].

Fewer coupling methods are generally used, compared with the number of methods available for preparation of PEG-polypeptide conjugates [6]. Drugs of low molecular weights are hydrophobic enough. Formation of conjugates in organic solvents has thus been tried. Mild coupling procedures, as described earlier, were employed and are described in Table 3 [6, 42, 46].

Larger amounts of drugs are delivered to tumor cells as the degree of polymerization of PEGs increases [43, 44]. Important reasons for the use of PEG as a modifier of bioactive substances, lie in its structural simplicity. This polymer has an inert backbone, with only one-functionalizable end group in the case of MPEG. This simplicity is a clear advantage in situations, where it is desirable to

Table 4: Scheme for PEGylation of high molecular weight polymers

S.No.	Macromolecules	Linking mechanisms	Comments
1	Oligonucleotides or backbone modified analogs [56, 57]	• Carbamate inter-linkages, using PEG-nitrophenyl carbamate to modify 5'-terminal amino groups of DNA.	• Conjugates have improved water solubility. • Resistant to nucleases and permeable to cell membrane. • For solid phase oligonucleotide synthesis.
2	N-terminal lipopeptide of E.coli (Pam3-cys) [58]	• Amino-PEG polystyrene graft copolymeric support. • Standard coupling methodology.	• To release the grafted PEG components. • Still retain biological properties.
3	Dextran [59, 60]	• Activation of secondary hydroxyl groups with p-nitrophenyl chloroformate followed by aminolysis with MPEG-NH_2. • p-Nitrophenyl carbonate activation. • Epichlorohydrin activated dextran.	• General applicability to polysaccharides. • PEG dextrans being conjugates of incompatible polymers might separate at the molecular level and form colloidal aggregates.
4	PEG-Chitosan [61, 62]	• By acylating the amino groups of polysaccharides with MPEG carboxylate and covalently linked 5-fluorouracil to it.	• For branched chain water chitosan derivatives. • Rheological studies.
5	Poly lactones [63–65]	• Ring opening polymerization of lactide initiated from the ω-end of α-methoxy-ω-hydroxy-PEG. • Using potassium, 2-methoxyethoxide as initiator by sequential anionic polymerization of ethylene oxide and lactide. • Heating with MPEG with or without stannous octoate at 180ºC in nitrogen atmosphere.	• Block copolymers with PEG had heterogeneity due to side reaction. • Molecular weight could be controlled by monomer/initiator ratio. • Stepwise synthesis and could be used for synthesis of nanospheres of PLGA, PCL etc.
6	Poly-l-lysine [66]	• Using methoxy PEG carbonyl methyl after reaction with thionyl chloride.	• Convenient mild reaction. • Can be used conveniently for delivery of genes and plasmid.
7	Polycyanoacrylate copolymer [67, 68]	• Condensation of methoxy Polyethylene glycol (MPEG) 2000 Da, cyanoacetate with hexadecyl cyanoacetate in ethanol and in formalin and dimethyl amine.	• Method uses toxic intermediates requiring efficient purification techniques such as silica gel column chromatography. • Can be used for preparation of nanoparticles.
8	Acrylates [1, 69, 70]	• Synthesized by γ-irradiation. • Random copolymerization by simply mixing.	• Used for synthesis of star copolymeric hydrogels. • For mucoadhesive controlled drug delivery. • Site specific GIT drug delivery.
9	Bovine serum albumin [71]	• Using activated PEG with p-nitrophenyl chloroformate then reacting with BSA (27 free amino acids).	• For slow releasing hydrogel preparation.
10	Poly amino acids [72, 73]	• Preparing a bis-hydroxylated oligomeric polyester of maleic or fumaric acid with 1,4-butanediol and then condensing it with a PEG-bischloroformate. • by ring opening polymerization of β-benzyl L-aspartate N-carboxyl anhydride using amino MPEG as initiator.	• To prepare a new generation of tumour targeting polymer conjugates of anticancer drugs with improved therapeutic performance. • Used for solubilization of hydrophobic drug either by covalent linking or by entrapment in micelles.
11	Poly butyl terephthalate PBT [74, 75]	• Using pre-prepared PBT-PEG conjugate.	• Used as matrix for protein loaded microspheres.
12	Miscellaneous [76]	• Using N-hydroxy succinimidyl carboxymethyl MPEG.	• For the enteral delivery of proteins like Human Granulocyte Stimulating Factor with retention of biological activity.

link multiple chains of PEG to substrates such as for modifications of proteins and liposomes [6, 43–45], for improving chemical versatility and increasing drug loading potential, respectively. Polymerization of N-carboxy anhydrides of β-benzyl esters of aspartic acid on to MPEG-NH_2 initiator, followed by removal of side chain protecting groups could be possible for amino acid linking. Other amino acids such as lysine, glutamic acid, phenylalanine etc., have been successfully co-polymerized with amino-PEG by a variety of chemical and enzymatic reactions as for poly-aspartic acids and copolymers with doxorubicin. Thus amino acids in combination with suitable functionalizable PEG derivatives could serve as building blocks for the assembly of drug carriers with tailor – made properties e.g. enzymatic and/or hydrolytic lability, ability to interact with biological cells etc [6, 52]. Thus a model compound of the anti-tumour agent, segment B derivative DU-86 conjugated to a tumour specific antibody via PEG and dipeptide has been designed for double targeting [53].

Many PEG-ylated drugs have been studied for their slow release and controlled exposure in the body. Their release depends mainly on ease of freeing the drug. The various types of drug-PEG linkages, existing or studied, are ester, ether, amide types, polymerized form as polymer micelles etc. It was found that the rate of hydrolysis of the ester bond decreased and anti-tumour activity increased as a function of the degree of polymerization (n) of ethylene oxide units. Although hydrolysis of the ester bond of PEG bound 5-fluorouracil, ketoprofen etc. proceeded readily in strong bases or strong acids, hydrolysis barely occurred under milder conditions, such as in physiological saline aqueous solution or in pseudo-humor mixture. The order of the rate of hydrolysis of the ester bond depends upon the ease of breakdown of bonds of linkages and rate of enzymatic attack, both under physiological conditions. Esterase hydrolyzes a hydrophobic substrate preferentially. However ester bonds or linkages of drugs with PEG were found to be hydrolyzed, though slowly, under mild conditions in phosphate buffer, physiological saline aqueous solutions or pseudo-humor mixtures. Hydrolysis in alkaline solution was faster than under acidic conditions. The rate of hydrolysis of the ester bond decreased, with increase in MPEG degree of polymerisation [43–46].

The results of hydrolysis of amide linkage bonds were the same as for ester bonds, but hydrolysis was slower than for esters. The rate of hydrolysis of ether linkages of drugs to MPEG was very much less than under alkaline conditions. In this case also the rate decreased with increasing 'n' in MPEG (i.e. degree of polymerization). The ester bond is hydrolyzed by esterase and α-chymotrypsin, but not by lipase. The amide bond is hydrolyzed by acylase, but not by lipase. The ether linkage is broken by α-amylase in the case of 5-fluorouracil-MPEG linkages *in vitro*.

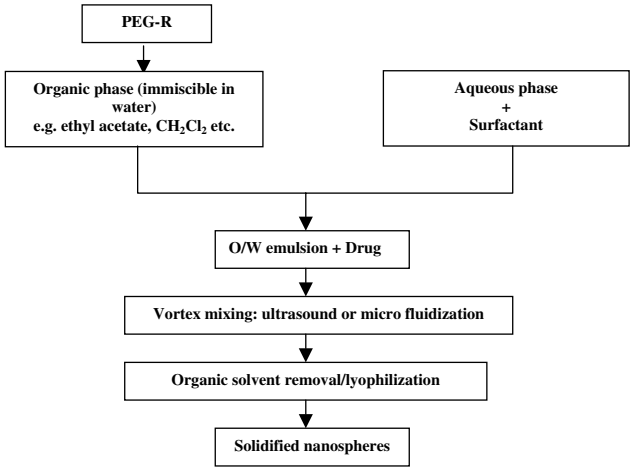
Transport of the drug artelinic acid, an anti-malarial drug follows a passive transcellular route. When artelinic acid was coupled to both hydroxyl ends of a PEG molecule, permeability of the conjugates was significantly reduced in both directions, regardless of the size. In the presence of EGTA, m-to-s permeability of these conjugates was significantly enhanced (Table 2), suggesting that, unlike artelinic acid, transport of these PEG di-artelinamides took place primarily via the passive paracellular route. When artelinic acid was coupled to the single hydroxyl end of a methoxy-PEG (compounds **4** and **5**), the conjugate having PEG with a mw = 1000 had low permeability [78]. This provides a potential prodrug approach for drug delivery across the GIT membrane. Similarly new insulin derivatives with improved physico-chemical and biopharmaceutical properties can be obtained by conjugation of high molecular weight PEGs. Interestingly polymer conjugation achieved derivatives maintaining high level of activity. However, both maximum activity and pharmacological bioavailability were found to depend on the mass of polymer bound to the protein. Surprisingly the site of conjugation was found to play a minor role in comparison with polymer weight in dictating the new insulin properties [79–81].

Conjugation of multi-arm PEG (8-arm PEG butanoic acid) to drugs such as quinidine [82] offers a way to increase drug loading in a water-soluble delivery system. Use of carboxylic acid derivatives of alcohols leads to a prodrug ester formulation. Doubly activated 40KDa PEG has been used to conjugate taxol and camptothecin [82]. Some control over hydrolytic release rate is possible through variation of the linking group. The carboxylic acid derivatives can be useful intermediates for forming other types of linkages. The use of high molecular weight PEG, however, results in a conjugate having a lower weight percentage of the drug and this can pose problems unless the drug has very high activity. The use of PEG derivatives having a greater number of termini for drug attachment can improve the loading capacity of conjugates while retaining advantages of solubility and circulating life. Conjugation of PEG to low MW hydrophobic delivery agents increases the ability of the delivery agents to transport rhGH into circulation, when administered intracolonically to rats. The favorable combination of the high potency of PEG-delivery agent conjugates and the low toxicity inherent to PEG suggests that they will represent a useful class of agents for the delivery of protein drugs [41, 83].

3.1.4. Nanoparticles, nanospheres and microspheres

These have PEG chains attached to one end in a brush configuration, which should avoid or reduce interactions with blood proteins and therefore impart RES avoiding properties. To achieve the core shell structure described above, block amphiphilic polymers of the types PEG-R were synthesized. R was chosen from bioerodible polymers such as PLA (poly D,L-lactide), PLGA (polylactide-co-glycolide), PCL(poly-caprolactone), Poly(butylene terephthalate) (PBT), poly(ortho ethers) (POE) etc (Table 4). The two blocks have a tendency to easy phase separation in the presence of water and have different solubility in water and organic solvents. This property was used to obtain the core-shell structure by an o/w emulsification procedure as described in Scheme 2 [65, 67, 74, 84–88].

Scheme 2: The process of preparation of PEGylated nanoparticles



This happens due to the tendency of PEG chains to migrate towards the aqueous phase to form sterically stabilized particles with a core composed of R-chains. After complete evaporation the nanosphere core solidifies, thus entrapping hydrophobic active molecules. Various drugs such as lidocaine, prednisolone, carmustine, ibuprofen, lysozyme and oligonucleotides have been entrapped successfully [67, 74, 75, 89–91]. Regardless of the R-chains (PLA, PLGA, PCL, PSA, PBT, POE etc.) used, high drug loading and entrapment was obtained. The mean hydrodynamic diameter and entrapment efficiency of nanospheres depend strongly upon the polymer's physicochemical

character. The hydrodynamic diameter increases with increasing PEG and R molecular weights and is independent of the drug loading. In the case of diblock PEG-PLGA polymers the entrapment efficiency was practically independent of PEG molecular weight. Conversely, in the case of PEG-PLA polymers, a slight decrease in entrapment efficiency was observed with increasing PEG molecular weights [67, 84]. The final particle size of microspheres made by the double emulsification solvent evaporation technique with varying degree of PEGylation of poly(ortho ethers) (POE) was found to be in the order: POE-PEG20% > POE-PEG10% > POE-PEG30% > POE-PEG40%. This is the same order as the viscosity of the polymer solution since it is more difficult to break up a more viscous polymer solution into smaller droplets. However BSA entrapment efficiency increases with increase in PEG content, which is up to 90% [92]. Moreover, POE-PEG30% yields a fairly constant and higher BSA release rate compared with non-PEGylated types. POE-PEG-POE triblock copolymers are more hydrophilic than POE due to the addition of PEG. Also PEG-poly-dl-lactide (PELA) microspheres carrying glucose oxidase (GOD) as a model protein, prepared by an extraction/evaporation method based on the formation of a double emulsion of polymer PELA have been reported recently [106].

Similarly, HSA-MPEG nanoparticles have been prepared by using a modified phase-separation coacervation technique, and could be used as circulatory carrier particles [93]. Polymer micelles (made by using hydrophilic and hydrophobic polymers) also behave as a nanoparticular drug delivery system. Direct solvent extraction from w/o emulsions in ethanol or methanol resulted in the formation of microspheres, which were not of better types [75]. Using w/o/w emulsions with PVA (polyvinyl alcohol) as stabilizer in the external phase followed by extraction of solvent ethanol and methanol resulted in the formation of microspheres having a uniformly prolonged 28 day lysozyme release rate [74]. A new method of free radical dispersion polymerization has also been tried to prepare nanoparticles of PAA-PEG [94], which are otherwise prepared by inverse emulsion polymerization.

The analysis of plasma protein adsorption on nanoparticles was studied by two dimensional-PAGE to separate nanoparticles after incubation with plasma proteins, after which they were washed and studied by electrophoresis. The adsorption of fibrinogen, immunoglobulin G, apolipoproteins J, CIII on PEG-PLGA particles was drastically reduced depending upon the PEG content as compared to PLGA particles alone by In^{111} radioactivity studies. It was found that liver accumulation of PEG 20,000 coated PLGA particles decreases from 66% to 17% on coating and the also increases blood level [84].

The release kinetics and entrapment efficiency of the drug are governed by the polymer's physicochemical characteristics, such as chemical structure, molecular structure and crystallinity. Thus an optimal polymer could be tailored for different therapeutic applications. GOD release from PELA microspheres exhibited a triphasic profile. It showed an one initial burst release during the first release on the first day then gradual release over one month and then a second burst release. This all depends upon the balance between hydrophilic components and matrix polymers. Here the initial burst release is release of the surface bound drug while the intermediate release is slow and sustained [106]. The drug release profile also depends largely on drug solubility characteristics rather than on polymer matrix degradation e.g. with lidocaine. The nanospheres function as long circulating depots releasing active compound slowly in blood. The drug plasma concentration profile could be altered by slow release techniques. They also reduce the systemic toxicity of the drug. The drug release kinetics were found to be influenced by nanosphere drug loading, but this may lead to crystallization inside drug carriers. Drug release properties were influenced by nanosphere drug loading. There is a possibility of lidocaine crystallization inside nanospheres at high drug loading. Part of the drug homogeneously mixed with chain and parts co-exist as crystallites as verified by freeze fractured microscopy [67]. A biphasic release was observed, due to non-homogeneity of particles made from PEG-PCL. The initial fast release was observed for drug molecules entrapped in PEG-PLGA nanoparticles in the first few hours followed by constant slow release over long time. This was also found in the case of particles with a PSA core. However the presence of PEG and its molecular weight do not affect the cumulative amount of drug released. A higher PEG chain density as well as a high molecular weight of PEG increased the particle size of nanoparticles made of multi-block brush copolymers. PEG chains were generally believed to be involved in release processes. *In vitro* drug release requires only partial hydrolysis of the macromolecular carrier.

Release was also influenced by the wettability of the nanoparticle surface and the water permeability of the nanoparticles. An additional lag phase was found during the initial drug release phase. It was found that higher PEG content and lower total molecular weight enhance drug release. The total molecular weight of the polymer was a factor in controlling drug release. The initial release of progesterone was found to be greater, when the molecular weight of PEG was greater, thus removal of oligomer PLA-PEG-PLA nanoparticles may restrain the burst. Water penetration rate through the POE-PEG-POE microsphere matrix may be much higher, resulting in a more rapid BSA diffusion rate. Another possible reason is that the POE-PEG-POE polymer erosion rate increases with increased PEG content [74, 92]. Thus, monodispersity, in other words the existence of the oligomer, and total molecular weight are likely to be important factors. It was found that molecular weight loss reflecting polymer decomposition does not contribute during release, since only slight molecular weight loss was observed during the initial main release period. Drug release was due to water permeation and drug diffusion through the PLA matrix so it was faster for homopolymeric nanoparticles with more than 10% PEG, due to enhancement of water permeation and drug diffusion by PEG segments. Release also depends on the hydrophilicity and erosion of particles. Long circulation times and lower RES uptake made them a long circulating depot, that liberated the necessary amount of drugs such as lidocaine, blood anticoagulants, adriamycin, progesterone etc. at the diseased tissue, thus minimizing the toxic effects. So these are also known as 'magic-missiles'. They have longer shelf life and also avoid destabilization.

3.1.5. Polymer micelles

The unique application of PEG-PLA block copolymers in the medicinal field involves their use as carriers for drug targeting. This is based on the micelle forming propensity of PEG-PLA block copolymers in aqueous media through multi-molecular association. Polymer micelles, prepared from various types of amphiphilic block copolymers, in-

cluding PEG-PLA are characterized by core-shell architecture in which a segregated core of associated hydrophobic segments (PLA) is surrounded by a dense palisade of tethered hydrophilic chains (i.e. PEG) [95]. Diverse types of drugs with a hydrophobic nature could be loaded with high efficiency into the core of the micelles, allowing them to be solubilised in the aqueous compartment. Furthermore the hydrophilic and sterically stabilized shell of the micelles has a long half-life in the blood compartment, due to reduced interactions with biological components. A remarkable improvement in tumour targeting properties of anticancer agents is demonstrated by polymer micelles composed of block copolymers of PEG with hydrophobic poly(amino acids), poly(lactic acids), diacyllipid (lipid) etc, which are safe and biodegradable. One of the advantages of using PLA as core forming segments in the micelles was that a variety of procedures for drug loading into plasma micro- and nanospheres have already been established in the pharmaceutical field, so drug loaded micelles may be formed in a similar systematic manner. Size and distribution of micelles have crucial effects on the *in vivo* characteristics. Thermodynamic parameters such as critical association concentration is also taken into consideration when designing long circulating micelles which are used in very dilute conditions [63].

The micelles of the PEG-PLA block copolymer are dissolved in dimethylacetamide. The solution is transferred through 0.5 μm filters, preswollen semi-permeable membrane of molecular cut-off weight 35000 and dialyzed against water to form the micelles. The dialysate is changed regularly after a certain period of time. Besides the surface properties and size of the carrier particulate, the size distribution may also crucially affect the particulate distribution process in the body. This effect of size sieving becomes crucial for so-called long circulating carriers, where RES disposition is no longer a major route of their removal from the blood compartment. Micelles of pure block copolymer have a narrow distribution property. Provided that micelles have a core shell structure due to phase separation of PLA and PEG segments, the junction of two segments in the block copolymer should align at the core-shell interface. On the other hand based on the lattice theory, PLA segments are uniformly distributed in the core. The molecular weight distribution of the block copolymers may be one of the key factors in deciding the size and distribution of the micelles and nanoparticles derived from PEG-PLA block copolymers. It has been shown that CAC values decrease with increase in molecular weight of PLA/PEG [58]. Monodispersed polymer micelles with extremely low CAC have been prepared from PEG/PLA block copolymers with long segments. The size of micelles is in the range of sub-50 nm, for high extravascular efficacy.

The polymer micelles prepared may be sonicated and centrifuged to give uniform sized and lower particle sized micelles. This also removes low molecular weight drugs such as indomethacin and aggregated particles. The supernatant micellar solution thus obtained is frozen and lyophilized by a freeze dryer system to obtain dried products [96]. Another novel macromolecular association system, "polyion complex (PIC) micelles", having a core-shell structure from a pair of oppositely charged block copolymers with poly (ethyleneglycol) (PEG) segments has also been developed. These PIC micelles are also formed from mixtures of charged block copolymer with the other kinds of polyelectrolytes including poly (amino acid), enzyme, oligonucleotide and DNA. It is considered that enzyme trapped in PIC micelles in the core should be useful as a vehicle for enzymes and a nanometric bioreactor such as that described as having chicken egg lysozyme and PEG-p (asp) complex micelles [97].

Another type of self-assembling unimolecular polymer micelles of core (laur) PEG 5000 having an interior core of lauroyl ester (laur) of mucic acid and exterior shell of MPEG 5000 has been formulated entrapping the drug lidocaine. It demonstrated sustained release characteristics for 24 h due to hydrophobic-hydrophobic interactions between the drug and the hydrophobic core of the polymer. This type of micelles, unlike block polymer micelles, has no CMC values by definition [98]. Another type of micelles based on MTX esters of PEG-b-PHEA has diameters ranging from 10–30 nm, depending on the level of drug conjugation. TEM revealed spherical micelle formation of size and shape similar to that of serum lipoproteins, a biological transport for water insoluble lipids. The polymer micelles may act as synthetic analogs of serum lipoproteins for drug delivery [99].

Block copolymers in selective solvents, thermodynamically favorable for one block, but not for others, resemble in many ways low molecular weight amphiphiles but the critical micelle concentration (CMC) is much lower than for low molecular mass amphiphiles. Nevertheless conformational behavior is similar in both cases. At concentrations below the CMC, all the block copolymers are in single chain form. At the stage of CMC all the collapsed blocks begin to associate to form loose aggregates and the insoluble blocks remain in their individual collapsed states in order to maintain equilibrium with single chains. Micelle formation requires two opposite forces. One is an attractive force between the amphiphiles leading to aggregation. The other is a repulsive force that prevents unlimited growth of micelles into a distinctive macroscopic phase. As the concentration increases above the CMC [100], the equilibrium state shifts to the micellar form. Insoluble blocks in the micellar core rearrange to find the low energy conformation and the solvent molecules are gradually driven out of the micellar cores. At higher polymer concentrations large collapsed micelles, consisting of insoluble blocks are present, surrounded by a diffuse outer shell (coronas) formed from the insoluble blocks. These micelles have hydrophilic MPEG 5000 of fixed chain length and hydrophobic ε-caprolactone of varying chain length in a diblock copolymeric system with solubilized indomethacin, as a model drug. As solubilized hydrophobic components of the copolymer were increased critical micelle concentration values decreased. Nature and length of the hydrophobic block determined the onset of micellisation, where the nature of the soluble hydrophilic blocks had only slight dependence on the onset of micellisation. Other factors such as co-polymeric structure, solvent composition and temperature influence free chain micelle equilibrium, micelle structure and dynamics of unimolecular micelle exchange. It has been shown that size of micelle increases with the molecular weight of block copolymer and drug loading, but size distribution remains mono-dispersed and unimodal. Solvent effects have also been studied. Solvent dissolved the copolymer significantly but size and size distribution of the micelles were affected. To form micelles by dialysis against water a solvent should be miscible with water and it could be expected that the miscibility of a polymer and solvent or water and a solvent would affect micelle formation [96].

The release rate of a drug from polymer micelles or drugs linked with PEG-polymer diblock copolymers viz. ε-ca-

prolactone (ε-CL), poly(lactide), poly(l-lactide-co-glycolide), polyaspartate etc. as the amphiphilic moiety, which forms polymer micelles, seems to decrease with increase in molecular weight of MPEG-polymer diblock copolymerase as in the case of MPEG-ε-CL block copolymers. In the release patterns of all samples, no initial burst effect is observed. However at a later stage the drug release rate is reduced. It showed significant sustained release characteristics. The drug binding affinity and polymer degradation are similar phenomenon. The drug is released by several processes such as diffusion through polymer matrix, release by polymer degradation, solubilization and diffusion through micro-channels in the polymer matrix formed by erosion etc. Drugs with moderate lipophilic character, which are physically entrapped or chemically bound into the core, such as doxorubicin, adriamycin-polymer conjugates, show *in vitro* release behavior corresponding to lipophilic components of these polymer micellar systems [65]. The inner core with hydrophobic properties largely affects these polymer micelles. If its hydrophobicity increases, it causes binding affinity, as between indomethacin and ε-caprolactone inside the core. The other factor affecting release behavior is polymer degradation, where the polymer degradation rate increases with increasing molecular weight of the polymer. However, it is not greatly affected because poly(ε-caprolactone) degrades quite slowly. It has been found that the influence of binding affinity on release profile is greater than that of polymer degradation. Consequently for a micellar system with amphiphilic block copolymer, the release rate of the drug is inversely proportional to the molecular weight of the block copolymers [96]. However in some cases PLGA-PEG micelles containing free doxorubicin have been found to show initial burst release at the initial stage of incubation as compared to those containing conjugated doxorubicin which showed sustained release properties for over two weeks [65].

All the above suggests the potential use of PEG-ylated drugs of high/low molecular weight for sustained release of proteins and drugs with minimal side effects [101]. This provides a convenient method of drug delivery, with minimal side effects and maximal effectiveness, due to optimum blood level or bioavailability at the sites. In addition conjugates exhibit increased resistance to proteolytic digestion while minor variations in the inhibitory constant, optimal pH, heat stability, affinity for substrates etc. [102, 103] have been observed. This has been found or studied for recombinant interleukin-2 [26], atropine [42], 5-fluorouracil [43, 44, 48], adriamycin [65], indomethacin, blood anticoagulants, l-asparaginases, penicillin V, esters such as aspirin, amphetamine, methotrexate [99], uricases [103], doxorubicin [65, 104], interferon α-2b [105], glucose oxidase [106], urease [107], mouse nerve growth factor [108], lignan and podophyllotoxin [109], recombinant tumour necrosis factor-α [110], tricosanthin [111], tamoxifen [112], superoxide-dismutase [147], etc. These compounds are very attractive from a practical point of view since they easily micellize yielding extremely stable micelles with high loading capacity, and accumulate in areas with leaky vasculature such as tumours and infarcts etc. The micelles can be loaded or labeled with various agents for gamma, magnetic resonance and computed tomography imaging [183, 88]. Many other such systems are under clinical trials, showing promising results for controlled and sustained delivery of proteins, enzymes and low molecular weight drugs e.g. rIL-2 (recombinant interleukin-2) [113], PEG asparaginase for treatment of cancer [114], pyridoxalated hemoglobin polyoxyethylene (PHP) conjugates for treatment of shock associated with systemic inflammatory response syndrome (SIRS), ischemia, reperfusion injury and hemorrhagic shock [115–118] etc.

3.1.6. Hydrogels

These are hydrophilic polymers of synthetic or natural origin, which have the ability to swell in the presence of water. Hydrogels are used for controlled release of drugs. These are polymers, copolymer or blend polymers made with poly (hydroxy-ethyl methacrylate), polyvinyl alcohol, polyurethane [1, 69], polyethylene oxide, poly(vinyl pyrollidine), diacrylate [119], β-methylvinylsiloxane [120] etc. Most of the matrices are molded or synthesized in the presence of drugs and are glassy. It is easier to incorporate a drug during the synthesis process or molding step of the hydrogel. The use of preformed swollen hydrogels is useful for applications such as wound dressing, topical application etc. New types of hydrogels prepared using polyethylene with co-polymerization with bovine serum albumin or methacrylic acids have high water contents, good mechanical and optical properties and biocompatibilty [71]. Due to PEG mediated reduced adsorption and cell adhesion to the surface, there is reduced immunogenicity and an increase in the plasma life time.

Because of the high water contents of these PEG-ylated hydrogels, they have good adhesive properties, so are used as a matrix for controlled release of drugs such as theophylline, steroids and others [1, 69]. Fick's law of diffusion, giving in a linear relationship to square root of time governs the release of dissolved species. The high water contents allow release to occur even for smaller proteins whether hydrophilic and hydrophobic such as lysozyme. It has been shown that diffusion coefficients are slightly increased with increase in the molecular weight of PEGs, which controls the porosity and EWC (Equilibrium Water Contents). The half-life of release of the drug increases with the thickness of the slab [1, 71]. The rate of release is also found to be dependent on the method of preparation, cross-linking density, molecular weight of PEO chains and drug solubility.

It has been shown to have muco-adhesive properties and could be used for delivery of proteins to the upper part of the small intestine. An important contributor to good adhesion is the presence of molecular adhesion promoters such as polymer tethered structure e.g. polyethylene glycol grafted to cross-linked networks or even linear chains, which are free to diffuse across the gel/gel interface. The ideas of bio-adhesion have led to the development of copolymers of poly(methacrylic acid) grafted poly(ethylene glycol) which exhibit pH dependent swelling due to reversible formation and dissociation of inter-polymeric complexes by a complexation/decomplexation mechanism [121]. These hydrogels swell from three to twenty times more in neutral or basic media than in acidic solutions due to complexation [1, 69, 122, 123].

As a relatively small amount of liquid PEG is added to glassy PVP, the H-bonded network complex of a stoichiometric composition is formed, and following mixing represents, in essence, gradual swelling and dissolution of the complex in excess solvent (PEG). Molecular mobility of the PVP-PEG complex is of great importance not only as a rate-controlling factor of drug release from the matrix, but also as dictating the adhesive behavior. Molecular mo-

tion in PVP-PEG blends can be studied using the pulsed field gradient nuclear magnetic resonance (PFG NMR) technique [124].

PEG hydrogels can be prepared by exact chemical cross-linking techniques or by radiation cross-linking techniques in order to control the gel structures. The hydrogels in combination with other polymers produce the corresponding PEG-adduct, forming the basis of a commercial product, Vigilon™ (formed by radiation cross-linking of high molecular weight PEO chains) [125]. PEG star polymeric gels are prepared using various molecular weights, numbers of star arms, concentrations and radiation doses of Co^{60} γ-irradiation. Hydrogel spheres are formed by mixing PEG-DA, the photoinitiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) and differing amounts of the cross-linker, pentaerythritol triacrylate. This solution is added dropwise to mineral oil and polymerized with UV light, creating a spherical hydrogel [119]. Cross-linked/grafted PEG rich networks are also reported to have been prepared by free radical solution copolymerization of macromonomers PEGDMA (polyethylene glycol dimethacrylate) and PEGMA (PEG monomethylacrylate) or could be prepared in the form of microspheres by free radical bulk suspension polymerization of methacrylic acid and PEGMA [1, 69, 126]. Synthesis of a NO-nucleophile complex hydrogel has been reported using polyethylene glycol N-hydroxysuccinimide monoacrylate (ACRL-PEG-NHS; MW 3.4 kDa). It was reacted with poly-L-lysine (DP = 5). The resultant copolymer was dissolved in ethanol, reacted with NO gas, and lyophilized. This PEG-Lys-5-NO polymer was then incorporated into photopolymerizable hydrogels by mixing with PEG-diacrylate in aqueous solution with 2,2-dimethoxy-2-phenyl acetophenone (900 ppm) as a long wavelength ultraviolet initiator (365 nm, 10 mW/cm^2, 1 min). To form a NO donor with a shorter half-life, diethylenetriamine (DETA) was reacted with ACRL-PEG-NHS. The copolymer was then dissolved in water and exposed to NO gas to form NO-nucleophile complexes. The PEG-DETA-NO was lyophilized and then photopolymerized as described above to form hydrogels [127].

The diffusion coefficient increases with the mesh size, due to the larger area available for diffusion. Since in networks, mesh size varies widely over quite small pH changes, these hydrogels could be used to alter the diffusional character of various sized solutes, based on the pH of the swelling media. The diffusion of a protein drug through swollen polymer is hindered by cross-linking as well as by physical obstruction such as entanglements, crystallites or polymer complexes. Insulin release from p(MAA-g-EG) gels containing PEG grafts of molecular weight 1000, indicates a significant release of it as the gel decomplexes and insulin diffuses through the structure. Thus, PEG-g-MAA gels are able to release protein drugs at a rate controlled by complexation/decomplexation mechanisms associated with the interaction of etheric groups of the ethylene glycol units and carbonyl groups of the acid [122]. However, studies show that in the case of diltiazem (a fairly small molecular weight drug of effective hydrodynamic radius of 4.24 Å), the solute release takes place by diffusion through the swollen networks [1]. At least 90% of drug release occurred in the first 90 min., the rate of diltiazem release decreasing with increasing molecular fractions of PEG-DMA. Release profiles of different drugs from BSA-PEG hydrogels, as a function of incubation time in the release media, have been found for controlled release of the drug [71]. Acetaminophen and tetracycline have similar release patterns. The release-kinetics of cortisone are typically similar to those of hydrocortisone, prednisone and methylene-blue. Release occurred by Fickian mechanisms in the cases of theophylline, hydrocortisone and lysozyme. The range of linearity was little beyond the Higuchi approximation for early time release.

Erosion of the gel through de-threading of the PEG chains from the PEG-cyclodextrin gel cavity contributes to drug release. A potential advantage of this feature is that release kinetics would be less dependent on the properties of the drug, and hence the gel would be more widely applicable to different drugs. Hydrogels formed by PEG block copolymers have previously been proposed as a sustained release matrix. This delivery system differs in that gelation relies on the formation of a tubular polymer complex induced by the PEG-threaded cyclodextrins. The properties of the supramolecular hydrogel can be fine-tuned with the composition, molecular weight and chemical structure of the polymer. Various block copolymers with PEG flanking the two ends can also form hydrogels, opening up a wide choice of structures in the mid block segments to control the release properties. The rate decreases sharply with an increase in the molecular weight of PEG up to 35,000, presumably because of chain entanglement effects and different complex stability [128].

Another type of pH dependent release pattern has been reported in the case of diethylaminoethyl methacrylate (DEAEM) grafted with PEG and 2-hydroxyethyl methacrylate (HEMA). DEAEM-containing gels show swelling transition behavior that is dependent on the pH in the gel microenvironment. Glucose oxidase (GOD) and insulin have been incorporated into such hydrogels [126]. GOD catalyzes the reaction between glucose and oxygen to produce gluconic acid (GluAc). As glucose diffuses into the gel, its conversion to GluAc lowers the pH within the microenvironment. The reduced pH results in increased ionization of the pendent amine groups, and hence electrostatic repulsion between ionized groups, increased osmotic pressure from counterions in the gel, and consequentially increased swelling [126]. At the higher degree of swelling the hydrogel becomes more permeable to insulin. The membrane permeability to insulin is a function of glucose concentration external to the membrane and insulin delivery is accelerated by an increase in glucose level. Peppas studied the dependence of glucose concentration on the swelling behavior of the polymer [129]. Also simultaneous catalase entrapment significantly increased swelling of p (DEAEM-g-EG). This results in reversible swelling in response to pulsatile variations in glucose [126].

For theophylline and hydrocortisone loaded in pastilles, there is a slight increase in diffusion coefficients as the molecular weight of PEG is increased. Variations in coefficients of diffusion are only significant between slabs with PEG of molecular weights of 3350 or 4600 and slabs with PEG of molecular weights of 10000 or 20000. The effect on diffusion coefficients of reagent ratio i.e. moles of activated hydroxyl groups of PEG vs. moles of available amino groups of BSA is due to reticulation of hydrogel networks at higher OH/NH_2 ratios and hence reduced porosity, which limits the rate of diffusion. For each loading concentration a slight increase in diffusion coefficient is observed when increasing the molecular weight of PEG, as in the case of theophylline. Diffusion coefficients have no dependency on the amount of drug present in hydrogels, when a Fickian diffusion mechanism is involved. Thus this new family of hydrogels could be used in controlled release devices for peptides and smaller proteins.

These hydrogels could also find applications as drug release devices for wound dressings, treatment of infections or as therapeutic contact lenses [1, 69, 70].

3.1.7. Polyethylene glycol modified dendrimers

Water soluble dendritic unimolecular micelles have recently been explored as potential drug delivery agents [130], with a hydrophobic core surrounded by a hydrophilic shell, prepared by coupling dendritic hypercores with PEG-mesylates. The monomeric core selected to build it was 4,4-bis(4′-hydroxy phenyl)pentanol as this larger monomeric unit provides flexibility to the dendritic structures while contributing to the container capacity of the overall structure. Four generations of dendritic hypercores with six, twelve, twenty-four and forty-eight phenolic end groups were prepared. Subsequent coupling reactions with PEG-mesylates yielded four generations of dendritic unimolecular micelles [131]. The micelles were characterized by matrix assisted laser desorption ionization time of flight mass spectrophotometry (MALD-TOF-MS), ^{1}H NMR and gel permeation chromatography (GPC). The container property was characterised by pyrene solubilisation in aqueous solution, and gives indomethacin entrapment upto 11% and sustained release characteristics. In general copolymers containing low generation dendrons especially G1, tend to form unimolecular micelles, whereas G2 and G3 copolymers form multimolecular micelles, presumably driven by hydrophobic effects and π-π interaction between dendritic blocks. Coexistence of two well-separated peaks in the SEC indicate a slow exchange process between these two species of different sizes.

Frechet et al. have also synthesized a novel class of amphiphilic star copolymers. To a four-armed PEG star, with scaffolds derived from a pentaerythritol core were attached four polyether dendrons. Results of SEC/VISC and ^{1}H NMR studies indicated the formation of unimolecular micelles in chloroform, tetrahydrofuran or methanol, but with strikingly different structures. Thus the star copolymers could self-organize into different micellar structures as a function of their environment. A potential application of these 'stimuli responsive copolymers' involves their uses as solvent specific encapsulation agents [132].

Chapman et al. have prepared amphiphilic copolymers derived from linear PEO and tBoC-terminated poly-α,ε-lysine dendrimers. The use of PEO as a platform for the synthesis of the dendrimers greatly facilitated separation because product up to fourth generation could be precipitated from reaction mixtures by ether. A surface tension method was employed to determine Critical Micelle Concentration and micelle formation behavior. The surface of the aggregates appeared to be highly compact. The existence of a transition concentration for the solubility of dye orange OT by G4 hydramphiphiles in water supports the micellar behavior [133]. A type of polyamidoamine (PAMAM) starburst or polypropyleneimine with either diaminobutane or diaminoethane as core and polyethyleneoxide (PEO) grafted carbosilane (Csi-PEO) dendrimers have been reported and studied systematically for the effect of dendrimer generations and surface functionality on biological properties *in vitro* [134].

One type of Poly Ion Complex (PIC) micelles involves micellization behavior between positively or negatively charged third generation dendrimer, Zinc porphyrins and respectively negatively or positively charged block copolymers of PEG viz. PEG-asp (-ve) and PEG-lys (+ve) charged block copolymers. The dendrimers are of interest as highly efficient photosensitizers for photodynamic therapy. The dendrimer porphyrins have been found to have photosensitizing properties and have potential for passive targeting of dendrimer porphyrins, due to its linking with PEG [135].

3.2. Enzymatic catalysis in organic solvents

Catalase, peroxidase, cholesterol-oxidase etc. conjugated with PEG are all active in organic solvents such as benzene, toluene, chloroform and trichloroethane etc. The solubility of PEG-catalase is enhanced by increasing the degree of modification and the PEG-ylated enzymes exhibit very high activity in benzene. PEG-hydrolytic enzymes such as lipase, chymotrypsin, and papain, catalyze reverse reactions of formation such as amide formation, ester synthesis etc, instead of hydrolysis breakdown [136].

3.3. Liquid phase synthesis

This could be used as a soluble matrix for peptide synthesis in aqueous systems in which coupling and deblocking are carried out at pH 6 and pH 8.5, respectively [137]. Water-soluble carbodiimide and amino ethyl ester are used to elongate the chain from the carbonyl end. The removal of ethyl ester also elongates the chain from the carbonyl end. The removal of ethyl ester blocking group is achieved by treatment with immobilized carboxy-peptidasesY (CPY) at pH 8.5 and room temperature. The bound enzyme is removed as filtrate, which is used directly for the next amino acid linking.

Similarly synthesis of oligonucleotides is done by conjugation with high molecular weight polyethylene glycol. The success of this strategy has increased the demand for large scale synthesis of oligonucleotides [138], and promoted their use as new therapeutic substances because of their ability to interact with specific single stranded RNA messengers and double stranded DNA, as anti-sense agents. Solid phase processes permit rapid synthesis of even long sequences, but they result in lower yields because of diffusion problems, inside resin beads. Hence a large excess of high cost monomer is required to get a higher yield, due to heterogeneity. So the liquid phase process has been proposed as a method to overcome these limitations. In this procedure synthesis is carried out in homogeneous media by linking the growing chain to a soluble supporting polymer. The polymer product is recovered from the reaction mixture by precipitation, thus allowing the rapid elimination of excess reagents and soluble by-products. In this newer method called HELP (High Efficiency Liquid Phase) synthesis, PEG of molecular weight ranging between 5 and 20 KDa is used, since it is highly soluble in large variety of organic solvents and is easily precipitated by addition of ether as co-solvent. An automated synthesis has been developed to carry out the overall process.

The introduction of higher molecular weight PEG at the 3′ and 5′ end of oligonucleotides has recently been achieved. Modifications of the 3′ position are performed by starting from a PEG modified solid phase support. The PEG chain is introduced at the 5′ end as a post-synthetic modification or when oligonucleotides are bound to the solid support or after its release. However PEGs of higher molecular weight display a lower reactivity, because of their viscosity and solid phase process due to scale-up difficulty. The HELP method [138] has been used as a soluble polymeric support for large-scale synthesis of oligonucleotides of up

to twenty monomers. This method has been modified by a stable bond between the polymer and the growing chains. PEG is used as a synthetic helper as well as a biological carrier of oligonucleotides [138, 139, 144].

3.4. Cell-fusion agents

Transfection is done using PEG-poly-l-lysine as a water soluble polymeric gene carrier of plasmid psV-β-gal into an *E. coli* strain, with moderate transfection ability, but with lower cytotoxicity and longer expression of gene such as to lipofectin® [66, 140] (Table 4). Inclusion of short PEG-chains containing amphiphiles by inclusion in the liposome prior to complex formation can enhance cationic liposome mediated gene transfer *in vivo* while long PEG-chains inhibit it. Inclusion of PEG-chains containing amphiphiles does not change the organ distribution of cationic liposome-mediated gene transfer [141].

3.5. Bioreactor use of PEG cofactors and/or PEG catalysis

These have been used as immobilized enzyme systems due to enhanced stability of PEG-enzymes. It increases their usefulness in industrial processes as magnetized PEG-enzymes could be recovered with full activity using magnetic fields, allowing recycling of industrial enzymes [142]. Similarly PEG-modified choline and acetylcholinesterase physically immobilized in poly (vinyl alcohol) cryogel membrane can be applied to a platinum electrode to form an amperometric sensor for choline and acetylcholine based on electrochemical detection of enzymatically generated hydrogen peroxide [143].

3.6. Miscellaneous uses

Epitopes selected from immunogenic Bb proteins by epitope mapping, e.g. synthesized by solid phase peptide synthesis and conjugated to a multifunctional PEG carrier are used as antigens in ELISA for diagnosis of Lyme disease with improved sensitivity and specificity [144].
For photodynamic therapy (PDT) of malignancies pegylated photosensitizer tetrakis-(m-methoxy PEG) derivatives of 7,8-dihydro-5,10,15,20-tetrakis(3-hydroxyphenyl)-2]-23-[H]-porphyrin (PEG-m-THPC) are described as a safe photosensitizer with promising properties as determined by laser irradiation [83].

4. Purification and characterization of PEG-ylated systems

There are various properties of conjugates, which differentiate them from normal PEGs and half-reacted intermediates and other compounds. PEG-lipid conjugates can be purified from conjugate reaction mixtures by silica gel chromatography. Since such conjugates exist in water almost exclusively as micelles, they are readily separated from unreacted PEG reagents by aqueous size-exclusion chromatography. Taking advantages of the very low critical micelle concentration (CMC) values of PEG-PEs, these can be retained in dialysis bags having very large pore size ($\geq$300,000 MWCO), which allows for free PEG to diffuse freely through the membrane. This method has been used for purification of various PEG-phosphotidylethanolamine derivatives. Lipid conjugates can be conveniently characterized by TLC and NMR [6].

PEG-ylated nanoparticles can be purified by washing twice with demineralized water and ultra-centrifugation, then all are filtered with a Millex-Millipore 1.2 μm filter to remove unwanted toxic solvents and intermediates before being administered [68]. Copolymers of poly-l-lysine methoxy polyethylene glycol can be purified by dialyzing against water in tubing with MWCO 25000 and these are lyophilized [68]. Separation of PEG from derivatized PEG and also other intermediates and reactants such as fatty acids is done by TLC on silica gel, which clearly separates polyethylene glycol and PEG-ester from fatty acids and in turn reduces fatty acid contamination (to 0.3% w/w). In the second stage, remaining fatty acid impurities are removed by chromatography on Sephadex LH20 gel, with 5 : 1 of methanol : water eluent. Recovery is greater than 95% in all cases provided the sample concentration or viscosity is fairly low. In the third stage the monoester and any diester are separated from unsubstituted PEG by chromatography on Octadecyl sepharose CL-4B [48]. Purification of the bromo-derivative is performed by diatomaceous earth 80%–100%, when terminal hydroxyl groups can usually be substituted by bromine.
The various characterization techniques developed are explained as follows.

4.1. Direct chemical analysis

4.1.1. TNBS titration and colorimetry

This is used to determine modification of SOD (superoxide dismutase) by the amino groups located in the enzyme molecule and subsequent molecular weight increase. It involves titration of amino groups by trinitro benzene sulphonate (TNBS). This then gives a color, which is then measured by spectrophotometer [145] as is done in the cases of trypsin [146] and SOD [147].

4.1.2. Biuret test

This is used to determine the amino groups initially in the bioactive species such as enzyme trypsin and the number of amino groups linked with PEG, so as to determine the extent of modification by PEG [146, 148].

4.2. Chromatographic techniques

4.2.1. FPLC elution profile

This is done on a superose 12 TM column of SOD-MPEG. There is a decrease in retention of anion exchange mono-Q-column operated on a FPLC instrument, due to modification of the SOD enzyme surface by polymer in ion exchange chromatography [147].

4.2.2. Gel permeation chromatography

This is conducted by JASCO liquid chromatography equipped with TSK gel chromatography columns and an internal refractive index (RI) detector. Molecular weight calibration is done using a series of standard PEGs with varying molecular weight. This could be used to determine molecular weight and propensity of reaction of the mixture after various times. The consumption of monomer lactide and glycolide is observed from a decrease in peaks of lactide and glycolide with time as peaks disappear simultaneously and only one peak of PLA–PEG remains and is observed [63, 84].

4.2.3. Hydrophobic interaction chromatography

It is used to determine surface chemical composition and molecular weights of the adducts of higher molecular weights and block copolymers [84].

4.3. Size determination

Various recent instrumental techniques have been brought into use to determine and confirm actual structures as detailed below.

4.3.1. Photon correlation spectroscopy (PCS)

This is done with a Malvern 4700 sub-micron particle size analyzer system. Size distribution could be characterized by Polydispersity Index (PI) measurement [93].

4.3.2. Microscopic determination

SEM needs a previous coating of the sample with gold or palladium, and then observing the particle size in photos [84, 93]. Atomic Force Microscopy (AFM), allows higher resolution, without the need to coat the system, and is a non-destructive technique [84]. TEM of freeze fractured nanospheres of PEG-PCL particles, has shown islands of guest drug crystals in the particle core of the host polyester matrix [67].

4.3.3. Dynamic light scattering (DLS)

This is used to confirm the spherical shape of the formed micelles. Angle trace measurements are performed at 30°, 60°, 90°, 120° and 150° detection angles. For spherical particles the characteristic line width Γ/K^2 should be independent of detection angle, due to the undetectable rotational motion. If Γ/K^2 has no angular dependence, it suggests that the formed associates may be spherical in shape. DLS measurement is done at 25 °C using a light scattering spectrophotometer with a vertical polarized incident beam at 488 nm supplied by an argon ion laser. A scattering angle of 90° is used. The normalized coefficient of Γ^2, μ/Γ^2 is called the Poly-Dispersity (PD) and is used as an indication of degree of polydispersity. A histogram analysis is used to show the size distribution pattern, showing a unimodal profile. For mono-dispersity, values should be always below 0.1 [63, 154].

4.3.4. Quasi elastic laser light scattering (QELS)

This is used to determine the particle size of nanometric-sized micelles or block copolymers. Hydrodynamic diameter and size distribution are obtained using a Coulter N_4MD nanosizer [68].

4.3.5. Laser doppler anemometry (LDA)

It has been used to determine the surface charge of PEG-PLGA samples. Amplitude weighted phase structurization can also achieve this [67, 84].

4.4. Spectroscopic analysis

4.4.1. ^{1}H NMR

Compositional analysis is performed using ^{1}H NMR of the PEG-PLA block copolymer [63, 66] in DMSO-d_6 at 80 °C with a JEOL EX-400 spectrophotometer at 400 MHz, giving peaks of PEG at δ values of 3–4 ppm.

4.4.2. FT-IR spectroscopy

Using a Nicolet model Magna, FT-IR550 of MPEG-εCL, shows a strong carbonyl band at 1722 cm^{-1}, attributed to the formation of di-block copolymers of εCL and MPEG. An absorption band at 3436 cm^{-1} is assigned to terminal hydroxyl groups in MPEG-εCL block copolymers and absorption at 1187 cm^{-1}–1085 cm^{-1}, to C–O stretch. By shifting of bands, it is possible to characterize the reaction and its completion [149]. Another method of near infrared analysis is used and studied on pulverulent samples (after lyophilization) of nanoparticle suspensions, crude nanoparticles and their dispersion medium (obtained after ultracentrifugation). Near infrared spectra are pretreated by first derivative before principal component analysis (PCA). The objective of PCA was to modelize the variability of each class of compounds and to represent each class as a unique cluster [150].

4.4.3. X-ray photoelectron spectroscopy (XPS)

This method is used to determine stability and for surface characterization and determines the relative amount of PEG in the first 5–10 nm surface layers of a PEG-adduct. It is used to study stability during degradation at 37 °C in water. The coating is stable during a 24 hr period on the basis of the level of released PEG in the supernatant [84].

4.4.4. Secondary ion mass spectroscopy (SIMS)

This is used to determine surface chemical composition and also the molecular weights of the adducts of higher molecular weight and block copolymers [84].

4.4.5. Electron spectroscopy for chemical analysis (ESCA)

This method is based on the emission of electrons from materials, in response to irradiation by photons of sufficient energy, to cause ionization of core level electrons. These electrons are emitted at an energy characteristic of the atoms from which they were emitted. Since photons have low ability to penetrate matter, only those electrons pertaining to atoms at or near the surface (down to 100 Å) can escape and could be counted, hence the name. These can be thus used to determine the increased PEG density on the surface of nanosphere, due to the brush configuration. Also, when a broad spectrum is observed due to peak overlap, the individual peaks are analyzed using appropriate spectrum, deconvolution, algorithm etc., as in the case of higher PEGs-PLA copolymers. This method can also be used to determine drugs on nanoparticle surfaces [67].

4.5. Fluorescent probe measurements (critical association concentration (CAC))

The dissociation of micelles on dilution has an important influence on the drug delivery capacity of polymer micelles, composed of amphiphilic block copolymers. A lower CAC is favorable for drug retention in micelle under considerable dilution *in vivo* [63, 151]. The CAC is determined by use of the fluorescent probe molecule pyrene to monitor change in polarity of micro-environment, by the change in polarity of the micro-environment in micelles based on Ham effect. A change in the polarity of vibrational structures, with local polarity is used in this study to determine CAC by determining pyrene monomer emission, especially between the intensity ratio I of 0.0

band and III of 0.2 band. The I/III ratio correlates with the polarity of the pyrene surroundings, which is known as Pyrene scales [152]. Preferential partition of pyrene into hydrophobic micro-domains (e.g. interior of micelles) with concurrent changes in molecular phyto-physical properties is demonstrated through monitoring of the pyrene micelle peak in GPC, by tandemly aligned fluorescent detectors. The CAC values are extremely low and have a good inverse correlation with the weight ratio of polylactide segment to PEG segments in the block copolymer. Low CAC values for block copolymeric micelles gave been reported [63].

4.6. Intrinsic viscosity measurement and diffusion coefficients

Molecular radius (r) of a PEG modified trypsin like macromolecule-PEG complex, can be determined by sedimentation velocities using ultra-centrifugation (Hitachi Analytical Centrifuge Model II). The following formulas can be used to determine various parameters of PEG-ylated systems [153, 155].

$$D = SKT/(1\text{-}Vd)M \quad \text{and} \quad r = KT/6\pi\eta_0 D$$

$$\eta l = 2 + 0.024\ M^{0.73}\ (\text{ml/g})$$

where, D is diffusion constant; M is molecular weight of MPEG-trypsin adduct; V is specific volume of polymer; S is sedimentation constant; d is density of the solution; T is absolute temperature and K is Boltzman constant. Cyanuric chloride activated polyethylene glycol (PEG)-5000 is covalently coupled to murine and human red blood cells (pegylated RBC) leading to modification of their rheological properties, which are documented by evaluation of cell deformability. This is based on cell transit time through calibrated micropores and cell aggregation, which is measured by ultrasonic interferometry. This parameter is used to evaluate rheological properties of the systems [156].

4.7. Equilibrium swelling studies

These are used to investigate the effects of molecular weight and number of star arms in PEG star polymeric hydrogels, synthesized using γ-irradiation. The swelling and release behavior are studied as a function of cross-linking structures and nature of PEG-acrylate studies [1]. The equilibrium water content and swelling are determined by keeping hydrogel pastilles of definite size and shape in water (deionised) or borate buffer [71] and weighing pastilles before and after vacuum drying using the equation:

Equilibrium water content (EWC) = (Weight of swollen gel-Weight of dry gel)/weight of swollen gel.

A newer method, isothermal microcalorimetry, can also be used to investigate the swelling characteristics of pharmaceutical hydrogels. The instrument offers the unique ability to study either single or multi-phase reaction processes in real-time and allows continuous monitoring of the swelling process [157].

4.8. Wide angle X-ray diffraction patterns (WAXD) and X-ray studies

WAXD is used to determine crystallinity, by recording, using Nickel Cu : Kα radiation produced by a Rigaku Denki model RAD-C diffractometer. X-ray studies confirmed that only PEG crystallizes in the samples. This is confirmed by the diffracto-chromatograms, showing only specific peaks corresponding to PEG crystallites, as the PCL polymers are amorphous [149].

4.9. HLB values

HLB values of polymer micelles can be calculated for a block copolymer using Griffith's relation for poly(oxyethylene) compounds as $HLB = 20\ M_H/(M_L + M_H)$, where M_H and M_L are the molecular weights of the hydrophilic and lipophilic moieties, respectively [149].

4.10. Differential scanning calorimetry (DSC)

This study is based on the results of thermograms of a quenched sample prepared by rapid freezing after high temperature annealing. From the lower temperature a curve of a endotherm and exotherm are observed, corresponding to the glass transition temperature (Tg), the crystalline temperature (Tc) and the melting temperature (T_m) of PEG segments, respectively. The Tg of PLA segments is not clearly observed in the thermogram, because it may overlap with the endotherm, accompanying the melting of crystallized PEG segments. It has been shown that the Tg and Tc of the PEG segments, clearly increased with an increase in the weight fraction of PLA segments in block copolymers, reflecting more restricted motion of the PEG segments. Melting of crystallized PEG occurred in a similar temperature region regardless of composition, because of the highly phase separated nature of the crystalline domains. Nevertheless the heat capacity of crystallized PEG in melting decreased with increase in the molecular weight fraction of PLA segments, which is in line with trends of Tg and Tc. This can be used to quantify crystallinity by the ratio of heat of fusion of copolymers at Tm, with heat of fusion of 100% crystalline PCL. This has also been reported to negate drug-carrier interactions in PEG-PLL nanoparticles [63, 84].

4.11. Electrophoresis techniques

This is used for characterization of enzymes such as superoxide-dismutase linked with PEG by their migration patterns, as with SDS-PAGE. This can also be used as a purification method. Micro-heterogeneity could also be determined by this method using iso-electric focussing on the same fraction [147]. Similarly, cell electrophoretic mobility analysis (CEM) of pegylated RBC has been used to distinguish a new population of cells bearing a characteristic CEM [156].

4.12. Zeta-potential measurements

This has been performed for albumin nanoparticles in a phosphate buffer at pH 7.0 (ionic strength 0.05 M), the nanoparticles having a negative zeta-potential on their own. Electrophoretic laser Doppler anemometry using a Malvern Zetasizer-4 has been used to measure this for PEG-HSA nanoparticles according to degree of PEG-ylation and type of MPEG modification. HSA-MPEG had a lower zeta-potential than simple human serum albumin (HSA)-nanoparticles. The extent of modification however was not so clearly indicated as the type of MPEG modification by zeta-potential changes. The reason for this is still very uncertain; it may be due to highly efficient movement of the shearing plane of the electric double

layer by the higher molecular weight of PEG. The sterically hydrated PEO layer on HSA-MPEG nanoparticles is responsible for their stabilization even when the zeta-potential is near zero as with simple HSA-nanoparticles [93].

4.13. Enzyme activity determination

On PEG modification, there is somewhat of a decrease in enzyme activity, which is used to determine the degree of modification of enzymes. Binding activity studies of the substrate are thus involved. This also differentiates enzymes from the bound enzymes, by the loss of enzyme activity, which is undesirable as in the case of SOD. This is done by Cu_2; CO_2 derivatisation of SOD-MPEG and subsequent structural investigation by ^1H NMR spectroscopy or by spectroscopic titration at 370 nm of the Cu_2, Zn_2,-SOD derivative to determine the binding affinity of the enzymes. To gain further insight into the properties of the active site on modification of SOD by MPEG derivatisation, the binding affinity of the N_3^- ion, a competitive inhibitor of O_2^-, which binds active Cu in a similar way to the superoxide ion has been determined indirectly [147].

4.14. pH induced flocculation and turbidity ratio test

The degree of cross-linking increases with an increase in the concentration of glutaraldehyde in HSA-MPEG. There is a decrease in the availability of amino groups on the HSA-MPEG surface, because of MPEG linking, so a steric barrier exists for cross-linker interactions. This change could be determined by turbidimetric measurements. Thus a decrease in the isoelectric point of HSA-MPEG nanoparticles, due to decrease in free amino groups in conjugates could be determined only by this test. Hydrated MPEG can be determined using electrolyte by $Na_2SO_4^-$ induced flocculation. This undergoes flocculation and alteration i.e. stabilization by a barrier in the colloidal system, even if the electrostatic double layer is destroyed, due to MPEG linking. Hence flocculate is formed and degree of flocculation increases [93].

4.15. Stability studies of bio-active species

These are performed to determine the stability of the enzyme against denaturation by guanidium-chloride, *in vitro*, as modified by PEGylation. It is generally seen to have increased in the case of SOD (superoxide dismutase)-PEG adducts upon derivatisation. Here thermostability is reduced with respect to non-derivatized enzyme. This is used as an evaluation parameter for MPEG modification of the SOD enzyme [147].

4.16. Protein adsorption studies (in vitro MPEG effect determination)

Due to the hydrated MPEG layer on particle surfaces, there is a sufficient steric barrier to serum protein adsorption, so there should be least change in size (i.e. increase in hydrodynamic diameter), of the system on incubation with serum proteins. Thus using the relative increase in hydrodynamic diameter, by a method mentioned above, PEGylation can be established. The degree or effects of MPEG modification can be determined from these data. But the chain length of MPEG modification cannot not be determined by this method, due to hydrolysis during preparation [93].

4.17. Miscellaneous methods

Recently an ELISA method for determination of levels of PEG modified proteins has been reported using an IgM monoclonal antibody (AGP3) against PEG modified beta-glucuronidase at concentrations as low as 15 mg/ml corresponding to 750 pg (1.8 fmol) of conjugate irrespective of linkers used [158].

A combined technique which is similarly applicable and has been reported to have been used for determination of the molecular mass of PEG conjugated compounds such as temoporfin, a photodynamic therapeutic agent, is HPLC linked to electrospray ionisation mass spectrometry (HPLC-ESI-MS). The use of ESI-MS alone could not achieve this because of elimination of the detail of the mass spectrum due to the presence of four PEG 2000 side chains with a wide molecular mass distribution [187].

5. Properties of polyethylene-glycol conjugates

5.1. Effects on bio-distribution and pharmacokinetics

PEG-adducts have remarkable *in vivo* properties. They have been shown to decrease the RES uptake of liposomes, nanoparticles, proteins etc. [25, 84, 159]. They increases the concentration of bioactive compounds in blood compartments. Hence, there is an increase in *in vivo* circulation i.e. systemic exposure. All these effects are due to the steric hindrance of heavily hydrated chains of PEG molecules. Several enzymes with therapeutic potential give adducts with dramatically increased circulatory life on modification by PEG. Oral absorption of PEG adducts has been found to be improved in the case of PEG-uricase entrapped in liposomes. It decreased urate levels in chicken, after oral absorption. Similar effects are found in the cases of PEG-streptokinase, PEG-SOD (super oxide dismutase) and insulin entrapped in PEGylated liposomes [77]. The effects are due to higher chain length of PEG or dense PEG substitution or uniform protein modification. In some cases e.g. PEG-gluconolactone-oxidase however, there is an increase in systemic clearance due to hydrolysis of PEG-enzyme linkage or insufficient degree of protein modification. The increased systemic exposure due to PEG-ylation leads to an increase in potency, which has improved therapeutic indexes in tumour bearing mice.

Higher systemic bioavailability e.g. of the SOD-MPEG adduct is available by any route of administration. This behavior is important, since it demonstrates stability and diffusibility of this high molecular weight derivative in the body [147]. Similarly tetra PEGylation of meta-tetra (hydroxyphenyl) chlorin (mTHPC), results in a two-fold increase in the plasma half life time, a five-fold decrease in liver uptake and an increase in tumour selectivity shortly after drug administration. However although mTHPC concentration in liver decreases rapidly with time, mTHPC-PEG liver concentration increases as a function of time. This leads to a loss of tumour selectivity at all but the earliest time points, whereas with mTHPC tumour selectivity increases with time. It has been found that on balance mTHPC-PEG does not appear to show any benefits over native mTHP for treatment of liver tumours as normal liver tissue accumulates the compound [160]. Coupling of polyethylene glycol (PEG) to the enzymes and encapsulating them in liposomes increases the bioavailability and enhances the protective effects of enzymes such as superoxide dismutase and catalase against vascular oxidative stresses. Hence chemical modifications and genetic manipulations of SOD and catalase have been pro-

posed to provide more effective delivery to the endothelium for augmentation of endothelium defenses [161]. In the case of liposomes also, reduction in uptake by bone marrow derived murine macrophages, CV1 cells, primate cell-lines of epithelium origin, and resident macrophages in liver and spleen has been found. This is due to the lack of recognition by high affinity saturated binding sites of the above on PEGylation. At the same time there was an increase in the blood level of PEGylated liposomes [159]. Similar results have been found in the case of the biodistribution of the PLGA-mPEG nanoparticles between blood and MPS, but the nanoparticle levels in other animal tissues vary with the variation of the proportion of PEG in the PLGA-mPEG copolymer, which was significantly affected. An optimum range of PEG proportions appears to exist which results in long blood circulation times of the nanoparticles [162]. Substitution of PHEG with PEG side chains similarly slightly alters the degradability of the carrier. Blood clearance and body distribution studies have been performed on female BALB/c mice. PEG-modified polymers with a high hydrodynamic volume stay longer in the blood stream compared with the non-modified polymers. As far as the body distribution is concerned, the PEG-conjugates are not excreted very fast and are not taken up by any particular organ [163]. I^{125}-OMP labeled *Leptospira interrogans* antigen loaded poly(DL-lactide)-PEG (PELA) microspheres prepared by double emulsion solvent extraction methods, have been found after i.v. administration to be located within immunization related tissues (56.7%) and less than half the amount (40.1%) in liver and spleen. But there was limited uptake (8.33%) after oral administration [164]. A higher tumor [165] accumulation and accumulation in inflamed tissues occurs by extravasation. The distribution of particles by extravasation in the above sites depends mainly on their size distribution. Only the smaller particle sizes of PEG liposomes were found to accumulate [166]. Liposomes containing 2′-MPEG(5000)-paclitaxel showed the best performance in terms of stability, entrapment efficiency and drug concentration. The pharmacokinetic parameters for both free and liposomal prodrugs fitted a biexponential plasma disposition. The most important change in pharmacokinetic values of prodrug vs. free drug liposomal formulations is in the plasma half-life $t_{1/2}$, which was longer in liposomes containing 2′-mPEG (5000)-paclitaxel [167].

5.2. Alteration of solubility of the systems

PEG enhances the solubility of proteins and a number of bio-active moieties at physiological pH and permits the design of stable formulations. These are suitable for clinical uses e.g. interleukin 2(IL-2), interferon β (IFN-β) etc. on covalent conjugation. The enhancement of solubility may be due to hydrated chains of PEG molecules. It may be due to supramolecular micellar structure formation with mono-block or diblock copolymers [66, 96]. These supramolecular micellar structures have a high solubilization capacity and rather low CMC value that makes them very stable *in vivo* and suitable for long-circulating soluble drug delivery systems [101,168].

5.3. Alteration of toxicity

5.3.1. Reduced release and contact

PEGylation prolongs the action of proteins and enzymes in the body. Steric hindrance [146], creates a great difficulty for enzyme-substrate interactions and slows down a number of such interactions, in such a way that there is a decrease in toxicity mediated by their excessive interaction.

Bulk release and availability of lipophilic drugs in the body is generally decreased. This leads to reduced systemic toxicity of the drug by burst release or greater absorption of some drugs than is required at a particular time and site [84]. As an example release of anti-coagulants from nanoparticles is reduced hence less bleeding is seen on intramuscular administration. Reduced release of adriamycin, similarly reduces cardiotoxicity, quite markedly, on PEGylation. Similarly release of the drug MTX from self-assembling micellar systems after hydrolysis of ester bonds in the hydrophobic environment at the core of micelles in unimers is spread over several weeks and is related to the level of conjugated drugs [99].

PEG has been shown to enhance the rate of drug release from delivery systems when added to a semipermeable membrane as a coating composition. However it can decrease the rate of drug release when used as a pretreatment agent. The results from one study have shown that the type of polymer used as a coating agent is also a factor in determining the role of PEG in drug release [169]. This may happen because PEG can produce a separate layer and act as a barrier to decrease drug release. Also PEG absorbs water and prevents its influx, hence decreasing drug release. However PEG in coating materials as a composition inside the coating, increases the hydrophilicity of the coating composition, which facilitates diffusion of water inside the coating in the case of microcapsules [169]. Interpolymer complexes (IPC) have been designed to act as delayed release or pulsatile drug delivery systems (DDS). IPCs are products of interaction between two complementary macromolecules through secondary forces, such as electrostatic interaction, hydrogen bonding, charge-transfer interaction or hydrophobic interaction. Formation of hydrogen-bonded complexes between proton-donating and proton-accepting macromolecules such as polyacrylic acid (PAA) and polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP) is used to coat the tablets [170, 171].

PEG-DA hydrogels have been prepared and have been shown to release a protein, BSA, and an active protein, horse radish peroxidase (HRP), for several days from arrays and for hundreds of days from spheres. The release of angiogenic factors from hydrogel arrays could then be used to increase the efficacy of implanted biosensors and other devices [119].

The permeability of PEG-PBT block copolymers for lysozyme, a hydrophilic moiety, is strongly dependent on the degree of swelling of the copolymers. An increase in swelling in case of polymers with long PEG chains or segments which display a relatively high degree of swelling ranging from 1.48 to as much as 3.66 causes an almost 50,000-fold increase in the lysozyme diffusion coefficient. It can be expected that this will influence the characteristics of lysozyme-containing microspheres to a large extent. Also microspheres having relatively hydrophobic block copolymers are found to have smooth surfaces with smaller pores, whereas hydrophilic particles are much rougher [74]. A similar trend is found with the release of I^{125}OMP (*Leptospira interrogans*) antigens from PELA microspheres as determined by decrease in radioactivity in tissues. A burst effect is found in such cases [164].

5.3.2. Decrease of immunogenicity, cell-adherence, thrombogenicity and protein adsorption

There is a drastic alteration in allergic manifestations, due to steric hindrance of the PEG-ylated moieties. This is due to a decrease in direct contact between a protein drug and immuno-competetent cells, hence PEGylation protects the drug or system from the attack of proteases, antibodies or inhibitors [146]. Similarly there is a reduction in fibrinogen adsorption to negatively charged liposomes as studied by radiolabelling techniques [172]. Hen egg white lysosome linked with modified succinyl ester derivative of monomethoxyPEG (mPEG-COONSu) has been described as having increased thermostability and minimal reactivity with anti-lysozyme antibody from BALB/c mice or human lymphocytes due to modifications with mPEG. Hence it has been suggested as suitable method of protein tailoring [173]. The hydrophilic and flexible polymers can prevent opsonin-nanoparticle or system interactions, which are the first step in recognition by the immune system [68]. Reduction in adsorption of opsonin and selective adsorption of dysopsonins, a certain plasma component, prevents recognition and uptake of nanoparticles by macrophages; the competition between mechanisms of opsonins and dysopsonins controls particle uptake by macrophages and hence biodistribution *in vivo* [174]. Linking of mPEG to endothelial cells (EC) or coating by mPEG prevents antibody-antigen interactions. Furthermore binding of TNF and LPS to EC is blocked, since mPEG treatment of EC inhibits subsequent up-regulation of E-selectin by these stimuli [175].
PEG-ylation of systems such as nanoparticles, liposomes, enzymes, proteins etc, reduces adhesion of cells and proteins [176]. This is due to sterically hindered hydrophilic coating, chain flexibility, and the transient and rapidly changing structure of PEG. The immune system has difficulty in modeling an antibody around it [28] due to the protective layer of long PEG chains, which is sometimes at one end in a brush configuration [177]. This has high surface density and is considered as a cloud of long chains. The density is high enough to prevent the interaction of opsonins with the surface of the particles. This condition leads to protein repulsion from the hydrophobic plane surface to which PEG chains are attached [175]. Recently autoreactive T-cells have been found to be clonally deleted at the immature double positive stage in the thymus against PEG-protein viz. OVA specific T-cell receptor genes (OVA-TCR-genes) antigens. This effect is partly mediated by central tolerance in the thymus and is probably caused by markedly enhanced stability of PEG-protein conjugates in the circulatory system due to PEGylation [178]. Similarly experimental auto-immune Myasthenia gravis (EMAG) induced by *Torpedo californica* acetyl choline receptor (tAchR) immunization has been found to be suppressed by a mPEG conjugate of Mysthenia gravis tAchR α-chain peptide (α-125–148) in C57BL/6CB6 mice. This is not due to any inability or defect in antigen presenting cells (APC) function but to T-cell involvement [179].

5.4. Stabilization of systems

Improved biological half-life and reduced biodegradation of the system occurs, either directly or indirectly, due to steric hindrance by long chains of PEG, leading to decreased interactions or adsorption of plasma proteins or serum components on the bio-materials. This also reduces RES uptake. It stabilizes the system by direct suppression of enzymatic activity on the system, depending on the degree of branching of the substrates. Steric hindrance or excluded volume effects of long chain containing high molecular weight PEG derivatives [68, 93] randomly coiled on the substrates are responsible for such interactions. As a result both casein hydrolysis by PEG modified trypsin and autolysis of PEG adducts of trypsin are reduced by this polymer effect. But effects of this type disappear, if the substrate is a lower molecular weight molecule or does not contain coiled chains. This is seen in hydrolysis by trypsin inhibitors or pepsin digestion of various PEG-trypsin adducts. This depends upon the degree of branching of the PEG derivatives and the extent of modification of trypsin [146].
Retention, by prevention or suppression of glomerular filtration of PEG-adducts occurs, due to increase in the molecular weight of the systems, hence prolonging the bioavailability indirectly [146].

5.5. Improved utilization as a drug delivery system

5.5.1. Increasing drug loading per system

Improved utilization of PEG is also made possible in some cases by a star polymer structure. Star polymers are three-dimensional hyper-branched structures in which linear arms of the same or different molecular weights emanate from a central core. Star polymers are used in variety of bio-medical and pharmaceutical applications. They provide a high density of functional groups in a small volume. This can result in star polymers that can act as drug delivery carriers by covalent linkages. Rempp and colleagues [180, 181] reported various such systems. PEG star molecules from dendrimeric cores have also been described, being well characterized, monodispersed star polymers [182].

5.5.2. Improving drug targeting

Various ligands could be attached directly in order to target liposomes to a site using PEG as spacers e.g. antibodies could be linked to lipids as

X-linker A-PEG-linker B-DSPE

where DSPE is disteaoryl phosphatidylethnolamine, and the X group on linker A, could be used for chemical modification of the system such as maleimide containing PE, maleimido-phenyl butyrate (MPB-PE) and N-pyridyl dithiopropionyl-PEPE (PDP-PE). The thiol groups can be introduced on to Ab using succinimidyl, 3-(2-pyridyldithio)propionate (SPDP) reagents. The coupling reaction between the thiolated-IgG and MPEG-2000 grafted liposomes is very efficient. A model compound anti tumour agent comprising segment B of duocarmycin(DU-86) conjugated to a tumour specific antibody via a cleavable linker consisting of PEG and dipeptide l-alanyl-l-valine as immunoconjugate with murine monoclonal antibody KMb41 has the possibility of tumour targeting [53]. This had also has the possibility of double targeting due to the specific release of drug DU-86 near tumour tissues by selective cleaving of the peptide bond by thermolysin protease [53]. Furthermore p-nitrophenyl carbonyl-PEG-PE long circulating liposomes permit binding of several dozen proteins such as monoclonal antimyosin antibody 2G4 (mon2G4), and monoclonal antinucleosome antibody 2C5 (mon2C5) and ligands like concavalin A (Con-A), wheat germ agglutin (WGA), avidin etc having preserved activities [183].

Similarly PEG-ylated hydrogels and star co-polymeric drug dosage forms remain in a particular site of the GIT for drug release, by virtue of muco-adhesion. This is an efficient method of upper intestinal targeting of drugs having local action or absorption from sites such as the colon, buccal cavity etc [184]. The encapsulation of antigens into PEG-coated PLA nanoparticles has been discovered to be a useful strategy for mucosal antigen delivery. More specifically, recent work aimed at examining the *in vivo* behaviour of TT-loaded PEG-PLA nanoparticles revealed that the presence of PEG around the nanoparticles helped to improve the transport of the encapsulated TT across the nasal and intestinal mucosae. These nanoparticles can be obtained under exceptionally mild conditions, have a great capacity for the association of proteins and provide a continuous delivery of TT in the active form. In addition, recent *in vivo* experiments have revealed their ability to enhance significantly the transport of peptides through the nasal mucosa [185].
PEG-enzyme conjugates coupled to magnetite, Fe_3O_4, form magnetite enzyme, that could then be recovered without loss of enzymatic activity, by applying magnetic fields e.g. magnetic PEG-Lipase, which catalyses ester synthesis, urokinase and plasmin. This could be guided to the sites, where clots are present [186].

5.5.3. Diffusion controlling measurements

These are accomplished by mucoadhesion properties. PEG could be used for diffusion controlled delivery of proteins from such hydrogels containing PEG. This is controlled by the three dimensional structures. These hydrogel carriers are muco-adhesive and could be used for protein delivery. Understanding PEG containing carrier/mucosal adhesion is of the utmost importance in local protein delivery. In the presence of molecular adhesion local protein delivery is possible, especially in the upper small intestine. An important contributor to good adhesion is the presence of molecular adhesion promoters, such as polymer tethered structures e.g. PEG chains grafted to cross-linked networks or even linear chains, which are free to diffuse across the gel/gel interfaces [129].
Also PEGs linked to poly(methacrylate) exhibit pH-dependent swelling behavior due to reversible formation/dissociation of inter-polymer complexes. In are acidic medium these behave in a different manner, due to a complexation/decomplexation phenomenon (pH dependent swelling mechanisms). These gels exhibit large changes in their structures and can deliver proteins at varying rates. This depends on the pH of environmental fluids, which changes the mesh size on expansion and contraction by hydrogen bonding and complexation. The average network mesh is twenty times larger and more swollen in neutral or basic media, than in acidic solutions, in which complexation occurs [129]. A newly synthesized NO-nucleophile complex polyethylene glycol N-hydroxysuccinimide monoacrylate (ACRL-PEG-synthesis) hydrogel to coat tissues produces NO for prolonged periods of time. Results of initial experiments involving platelet adhesion and smooth muscle cell proliferation indicate that these NO-releasing materials may be useful in the prevention of thrombosis and re-stenosis following angioplasty [127].

5.5.4. Micellar delivery system

PEG-PLA [58] and PEG-ε-caprolactone [96, 149] block copolymers are used in the field of medicine as carriers for drug targeting, based on the micelle-forming propensity of PEG-PLA block copolymers, through multi-molecular association in aqueous milieu. Polymer-micelles prepared from various types of amphiphilic block copolymers, including PEG-PLA are characterized by a core shell architecture in which a segregated core of associated hydrophobic segments, (i.e. PLA) is surrounded by a dense palisade of tethered hydrophilic chains i.e. PEG. Various drugs with hydrophobic natures can be loaded with high efficacy into the core of the micelles allowing solubility in aqueous media. Furthermore the hydrophilic and sterically stabilized shells cause the micelles to have a long half-life in the blood compartment. Size and distribution of micelles have a crucial effect on *in vivo* disposition characteristics. Thermodynamic parameters, such as critical association concentration (CAC), should be taken in to consideration in designing long circulating life micelles, which is important for delivery in diluted conditions [63].

5.5.5. Sustained and controlled drug delivery

This can safely and appropriately be achieved by decreasing burst effects and decreasing drug leakage.

- Decreasing burst effects

Release patterns of all samples of MPEG-ε-CL block copolymers show significant sustained release characteristics, but no initial burst effects for drug release. This system also has minimal side effects and provides a convenient method of drug delivery, while maximizing drug effectiveness [96]. However some burst release is shown with PELA microparticles of entrapped GOD in earlier and later phases of drug release [106] and entrapped doxorubicin from PLGA-PEG micelles [65]. This is all due to some drug being entrapped on the surface layers of particles and to the eventual breakdown and metabolism of particles by enzymes.

- Decreasing drug leakage

PEG-ylated liposomes, nanoparticles etc., due to shielding and coating effects in the nanometric particles, prevent release of drug by leakage from the system. It is superior to other polymers in showing significant pharmacokinetic effects. However the amount of PEG that can be included in lipid bilayers, decreases with increased PEG molecular weights [84].

5.6. Improved transfection capability

PEG block copolymers with PLL (poly-l-lysine) at the poly-cationic polymers region can form complexes with DNA. This maintains a low cytotoxicity comparable to the cations above, but with higher solubility. It increases transfection efficiency in 293 cells (human primary embryonic kidney cells) [66, 188, 189]. Choi et al. described synthetic comb like polymers of mPEG-g-PLL, with increased transfection capacity as compared to PLL on Hepg2 cells, but with lower cytotoxicity, early gene expressions and good maintenance of gene expression up to 96 h [66].
An optimized nanoparticulate gene carrier is formed with two structural units of DMAEMA and NVP after free radical copolymerization using a carboxyl group terminated azo-initiator. The terminal carboxylic group is conjugated with bisamine-PEG to yield poly(DMAEMA)-NVPb-PEG. For specific gene targeting to the asialo glycoprotein re-

ceptor of hepatocytes, a galactose moiety is incorporated into the amino terminal end of PEG by reductive coupling using lactose and sodium cyanoborohydride. This cationic poly(DMAEMA-NVP)-b-PEG with a targeting moiety, galactose at the end of PEG block and coating polymer/DNA complex with pH dependent, endosomal disruptive peptide, KALA (WEAK-LAKA-LAKA-LAKH-LAKA-LAKA-LKAC-EA) shows adequate transfection efficiency, as high as a commercial agent [190].

5.7. Higher tumour uptake

Liposomes can be sterically stabilized by PEG headgroups of synthetic phospholipids (PEG-DSPE) that are included in formulations (PEG-liposomes). New microscopic evidence indicates, that accumulation of liposomes in tumour involves extravasation, presumably due to increased permeability of the capillary endothelial membrane, unlike conventional liposomes, which show dose-dependent decrease in drug concentration in blood. Most importantly, this produces a marked enhancement of antitumour activity, with concomitant decrease in toxicity. This indicates an increase in therapeutic index to a value much higher than that observed with conventional liposome formulations [159] on conjugation with PEG.

PEG-liposome conjugates and their accumulation in the tumor, prove to be of considerable advantage in future clinical applications. PEG-liposomes from synthetic phospholipid derivatives can be prepared at higher purity and in larger quantities and also at considerably lower cost compared to GM1 gangliosides for improving tumour uptake properties. Fluid and solid bi-layer compositions give similar blood circulation time adding considerable flexibility, needed for various clinically relevant formulations. Additionally, negatively charged PEG in liposomal bilayers provides better encapsulation of a variety of positively charged drugs, macromolecules and gives a reduced likelihood of segregation.

Microscopic studies of PEG-DSPE liposomes indicate the presence of PEG-liposomes in the interstitial space around tumour cells, as a result of their long-circulation time in blood and increased permeability of the endothelial barriers of newly vascularised tumours. Although diffusion and deep penetration within the tumour mass is not expected for particles of liposomal size, it is possible that free drug could diffuse locally from extravagated liposomes to the surrounding tumour cells. The presence of intact extra-vascular liposomes within tumour cells provides a local depot for drug release. Enhanced localization of liposomes in other anatomically distinct regions is beneficial for other diseases involving increased capillary permeability [159, 191]. Extravagated liposomes can be engulfed exclusively by tumor associated macrophages and are not taken up by tumour cells, depending upon local blood flow and micro-vascular permeability. PEG-chains are cleavable in physiological conditions in the reduced blood flow in the interstitial spaces between tumour cells. The chains are gradually removed and cleaved from extravagated-PEG liposomes, resulting in increased uptake by tumour associated macrophages. However, the size of liposomes associated with PEG is important or critical for extravagation [165].

Similarly free doxorubicin (Dox) is cleared from blood and from all the organs more rapidly than Dox bound to PEG as a conjugate. The liver is an organ of high accumulation but the highest accumulation takes place in tumours and this depends upon the size and weight of the conjugate [192]. There is enhanced uptake of DOX-PLGA-PEG micelles, due to their easier fluid-phase endocytic transport compared with the passive diffusion of free doxorubicin through the cell membrane. This in turn is able to circumvent the multidrug resistance effect, where free doxorubicin entering into cells is pumped out of cytosol by p-glycoprotein expressed in the membranes of cancerous cells. An enhanced uptake of doxorubicin by HepG2 cells is observed as indicated by the intense fluoroscence observed at the nucleus and cytosol of HepG2 cells when conjugated with PLGA-PEG micelles as compared to the uptake when incubated with free doxorubicin. This in turn also leads to increased or comparable cytotoxicity of doxorubicin when administered by micelles compared with free doxorubicin [65, 193]. Polymer conjugation can be used to concentrate a drug in solid tumour tissue simply by improving its pharmacokinetics. Conjugation reduces loss to the endocytic route, thus preventing the drug randomly entering all tissues and causing toxicity [194]. Relatively high blood concentrations of polymer conjugate allow selective uptake by tumours due to the increased leakiness of tumour blood vessels. Most normal vasculature has a tight endothelium, which precludes extravasation, and lymphatic drainage. It quickly recirculates any macromolecules that escape. The reverse is true for tumour vasculature, leading to preferential concentration of macromolecular compounds in tumours. This phenomenon is called the enhanced permeability and retention effect (EPR). For the above purpose, multiblock copolymers are used in which polyester residues, containing maleate or fumarate units, are coupled with residues of polyethyleneglycol (PEG) [73]. Similarly folate coated PEGylated nanoparticles are successfully used to target folate binding receptors expressed on tumour surfaces [195].

6. Conclusion

It is well established that PEGylation of systems greatly improves the stability, efficacy, and efficiency of various systems and makes them suitable for delivery of bioactive species in the body for much longer periods and targeted to the active sites, with reduced immunogenicity, antigenicity, and toxicity in the body. This is a relatively new emerging technology for appropriate delivery of bioactive species and drug delivery systems in a much more advantageous way than using common conjugates such as dextran for the same purpose [196]. It simultaneously hides the system from recognition by the detection and destruction mechanisms of the body, while preventing the sequestration and uptake of the system by reticular endothelial systems, such as liver, spleen etc. Many PEG-ylated systems are under clinical trials for delivery into the body, and may soon be available to serve mankind as proper and effective therapeutic systems. PEGylation technology is simpler, cheaper and therapeutically advantageous.

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