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Engineering of Amphiphilic Block Copolymers for Drug and Gene Delivery

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Introduction

Amphiphilic block copolymers have been used for diverse applications in pharmaceutical industry for decades (Croy and Kwon, 2006; Alexandridis and Lindman, 2000). They have been used as safer replacements for low molecular weight surfactants in the solubilization of poorly soluble drugs (Kwon, 2003), as stabilizing agents in the formulation of coarse and colloidal dispersions (Tadros, 2006; Shenoy and Amiji, 2005), as gels providing depot or formulations (Vinogradov et al., 2002), and, more recently, as core/shell self-assembled colloids for nanoscale drug and gene delivery (Nishiyama and Kataoka, 2006).

Modern pharmaceutics rely heavily on the design and development of nanoscale dosage forms that can incorporate therapeutic agents effectively, change the normal fate of drugs in a biological system, and direct them toward their cellular or sub-cellular targets. Polymeric micelles are important dosage forms in this regard, since segregation of core/shell structure along with versatility of block copolymer chemistry provides infinite opportunities for the manipulation of their structure. This can lead to the development of optimum delivery system for challenging therapeutic agents, e.g., poorly soluble drugs, proteins, and genes. Block copolymers and polymeric micelles have been the subject of several excellent and extensive review articles, book chapters, and books in recent years (Croy and Kwon, 2006; Alexandridis and Lindman, 2000; Xiong et al., 2006; Aliabadi and Lavasanifar, 2006; Osada and Kataoka, 2006; Lavasanifar et al., 2002a). To our knowledge, six polymeric micellar formulations, all developed for the solubilization and delivery of anticancer drugs, are currently in different stages of clinical trials in Japan, Canada, Europe, and South Korea.

Perhaps one of the most widely used types of block copolymers, especially in traditional pharmaceutics, are di- or triblocks of ethylene oxide (EO) and propylene oxide (PO), namely, Pluronics® or Polaxamers. The water-soluble EO-*b*-PO block copolymers are stable over a wide range of pH and compatible with biological tissue. It is possible to change the size of EO and PO blocks with or without change in the hydrophilic lipophilic

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balance (HLB) of amphiphile and vary the molecular weight of the whole polymer. As a result of such structural changes, different properties and functions may be achieved from the amphiphilic macromolecules at the interfaces (Newman et al., 1998a,b). Pluronics have also shown unique biological activities themselves, such as reducing the activity of membrane efflux pumps in drug-resistant tumor cells (Kabanov et al., 2002; Minko et al., 2005) and enhancing the transfection efficiency of viral vectors in gene delivery (Kabanov et al., 2005; Kabanov et al., 2002; Wang et al., 2005).

Amphiphilic block copolymers with poly(*l*-amino acid) (PLAA), poly(esters), and poly(amine) as their hydrophobic block are the most extensively researched micelle-forming polymers for nanoscale delivery (Aliabadi and Lavasanifar, 2006; Osada and Kataoka, 2006; Arnida et al., 2006). The aim of this chapter is to review the general synthesis of amphiphilic block copolymers and provide an overview of chemical modifications performed to enhance the biological performance of micelle-forming PLAA, poly(esters), and poly(amine) block copolymers in drug and gene delivery. Such polymers have been an integral part of first-generation polymeric micelles, whose function relies on prolonged residence of drugs in systemic circulation. For each category, development of second generation polymeric micelles, i.e., polymeric micelles that can specifically seek and actively deliver their therapeutic cargo to diseased cells, will be briefly discussed. A condensed overview on third-generation polymeric micelles, i.e., multifunctional carriers designed to respond to more than one internal/external stimulus at the same time, will be provided, as well. Most recent research in the field is highlighted, without an extensive overview on single polymeric micellar system. It is noteworthy to mention that this chapter does not provide a critical review, but rather a survey of different chemical strategies for generating polymeric micellar delivery systems.

PEO-*b*-poly(amino acid) Block Copolymers

General Synthesis

Synthesis of PLAAs is usually achieved though ring-opening polymerization (ROP) of α-amino acid-*N*-carboxyanhydrides (NCAs) (Figure 13.1) (Smeenk, 2005), using different nucleophiles or bases (e.g., primary amines and alkoxide anions) (Harwood, 1984; Kricheldorf and Mulhaupt, 1979; Kricheldorf et al., 2005; Sekiguchi, 1981). NCAs can be prepared from

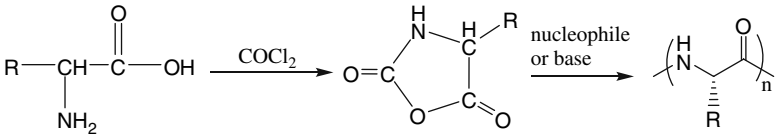


Figure 13.1 General scheme for the synthesis of PEO-*b*-PLAA-based block copolymers by ring-opening polymerization of α-amino acid-*N*-carboxyanhydrides (NCAs).

α-amino acids using a solution of phosgene in THF by the Fuchs–Farthing method (Fuller et al., 1976; Bikram et al., 2004).

PEO-*b*-PLAA block copolymers are synthesized using α-methoxy-ω-amino PEO as the initiator (Harada and Kataoka, 1995). When primary amines are used as initiators, the polymerization of NCAs may proceed by two mechanisms: amine mechanism and activated monomer (AM) mechanism (Figure 13.2) (Sekiguchi, 1981; Deming, 2002). The amine mechanism is a nucleophilic ring-opening chain-growth process in which the polymer grows linearly with monomer conversion (Figure 13.2A) and the initiator becomes ($R'NH_2$) part of the final product, leading to a copolymer. In AM mechanism, NCA will be deprotonated forming a nucleophile that initiates chain growth (Figure 13.2B). The initiator is not included in the final product and only homopolymer will be expected in this mechanism. In a given

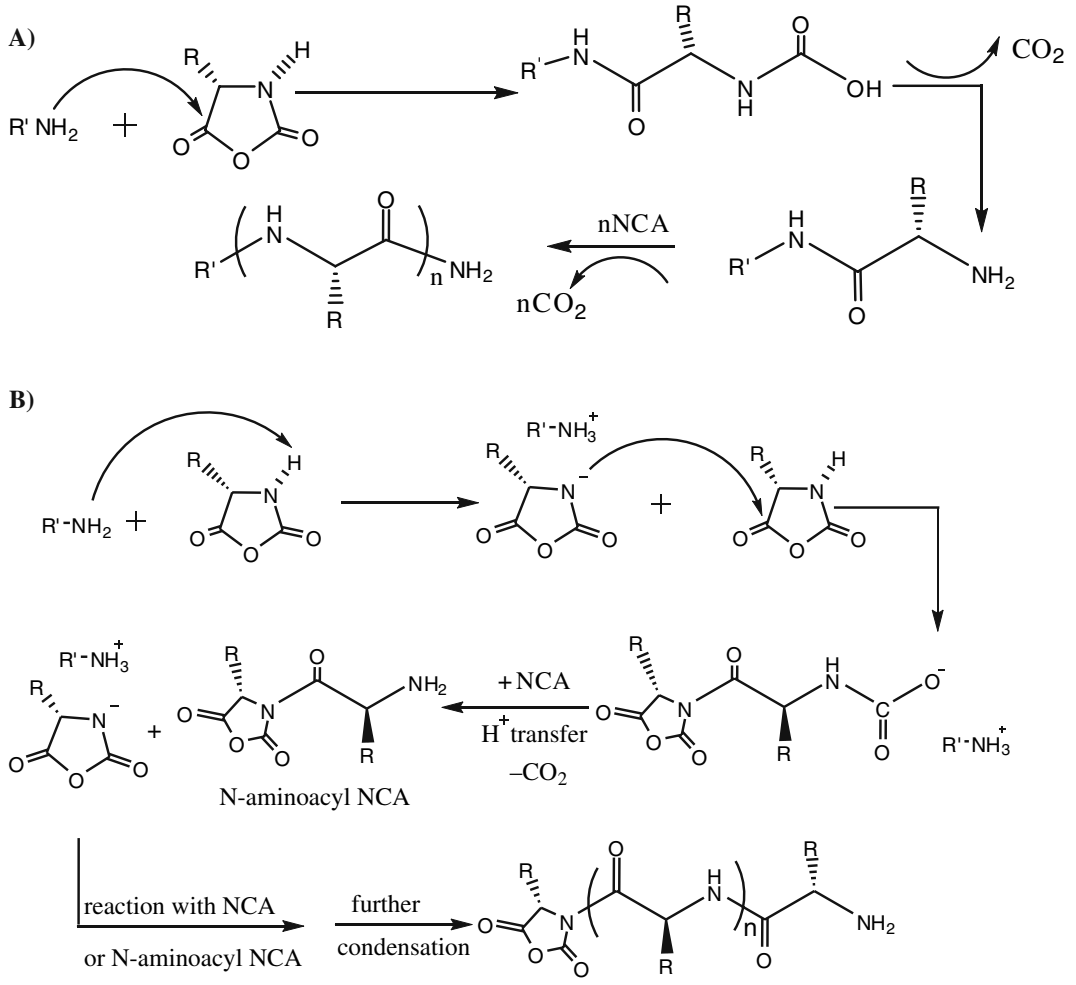


Figure 13.2 **(A)** Amine mechanism and **(B)** activated monomer (AM) mechanism of NCA polymerization (reproduced from Deming, 2002 with permission).

polymerization process, the system can switch back and forth between the amine and AM mechanisms. Since a propagation step for one mechanism is a side reaction for the other and vice versa, block polymers prepared from NCA method using amine initiators have structures different from those predicted by monomer feed compositions (Tsuruta et al., 1965) and most likely have considerable homopolymer contamination. Therefore, efforts to control the polymerization in the way of amine mechanism but to avoid the way of AM mechanism are expected to increase the product yield and efficiency to produce copolymers.

Transition metal initiators (Tsuruta et al., 1965; Deming, 1997; Freireic et al., 1974; Yamashit and Tani, 1974; Yamashit et al., 1974) have been developed to control the addition of each NCA monomer to the polymer chain-ends to reduce the side reactions in the NCA polymerization. However, this method is not suitable for the preparation of high molecular weight PLAAs due to the presence of chain-transfer reactions.

Living polymerization has been developed to overcome the limitations in the preparation of PLAAs (Brzezinska and Deming, 2004; Dvorak and Rypacek, 1995; Vayaboury et al., 2004). In this method, the transition metal initiator activates the monomers and forms covalent active species, which permit the formation of polypeptides via the living polymerization of NCAs (Figure 13.3). The metals react identically with NCA monomers to form metallacyclic complexes by oxidative addition across the anhydride bond of NCA (Deming, 2002; Brzezinska and Deming, 2004; Deming, 2000).

Alternatively, PEO-*b*-PLAA can be synthesized by coupling PEO to PLAA after the polymerization of PLAA. For instance, poly(L-Histidine) (p(L-His)) has been synthesized by base-initiated ring-opening polymerization of protected NCA of L-His and then coupled to carboxylated PEO to form PEO-*b*-p(L-His) via an amide linkage using dicyclohexyl carbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) as catalysts (Lee et al., 2003b). Poly(L-aspartic acid-co-PEO) (p(L-Asp)-co-PEO) bearing amine side groups on the p(L-Asp) block has been synthesized by the melt polycondensation (post-polymerization) of the prepolymer prepared from *N*-CBz-L-aspartic acid anhydride and low molecular weight PEO using acid catalysts. The product was an alternating copolymer containing reactive amine groups on the p(L-Asp) residue (Won et al., 1998). Finally, solid and liquid phase peptide synthesis has also been used to prepare PEO-*b*-PLAAs (Van Domeselaar et al., 2003; Choi et al., 1999).

Chemical Modification of the Core in PEO-*b*-PLAA Micelles for Physical Drug Encapsulation

Existence of several functional side groups on a PLAA block is the primary advantage of the PEO-*b*-PLAAs over other micelle-forming block copolymers. Free functional side groups on the PLAA backbone provide several sites for the conjugation of different molecules. Besides, the systemic alterations in the chemical structure of the PLAA block and its side chains may lead to the customized optimization of polymeric micelles for the physical encapsulation and controlled delivery of individual therapeutic agents.

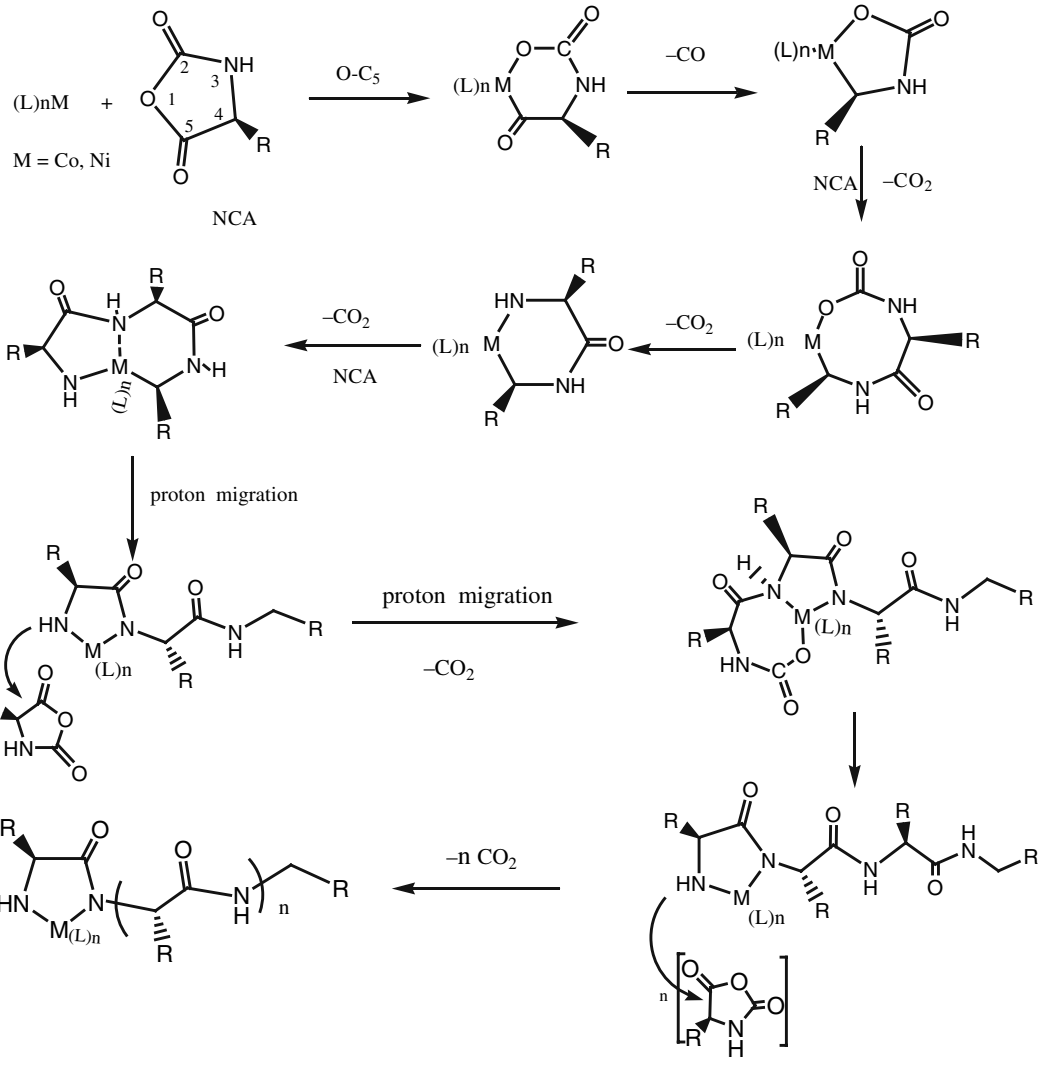


Figure 13.3 Living polymerization of NCA (reproduced from Xiong et al., 2006 with permission).

The most attention for the production of chemically modified cores in PEO-*b*-PLAA micelles for physical encapsulation of drugs has been focused on p(L-Asp) and poly(L-glutamic acid) (p(L-Glu)) (Osada and Kataoka, 2006; Lavasanifar et al., 2002a; Bae and Kataoka, 2006; Nishiyama and Kataoka, 2003; Kakizawa and Kataoka, 2002). Synthesis of PEO-*b*-poly(β-benzyl L-aspartate) (PEO-*b*-PBLA) and PEO-*b*-poly[ε-(benzyloxycarbonyl)-L-lysine] (PEO-*b*-PBLL) from polymerization of β-benzyl L-aspartate-*N*-carboxyanhydride (BLA-NCA) and *N*-carboxyanhydride of ε-(benzyloxycarbonyl)-L-lysine (BLL-NCA), respectively, using α-methoxy-ω-amino PEO as initiator has been reported (Harada and Kataoka, 1995). PEO-*b*-PBLA has shown extremely low critical micelle concentration (CMC) and such micelles were found to be highly rigid in core (Kwon et al., 1993). Micelles assembled from PEO-*b*-PBLA block

copolymers of different molecular weight and composition have been applied for the solubilization and delivery of DOX by Kataoka's group (Kataoka et al., 2000; Kwon et al., 1995). PEO-*b*-PBLA efficiently encapsulated DOX and sustained its release. The degree of polymerization of PBLA in the block copolymer seems to have little effect on the loading capacity of DOX in the micelles. Compared to free drug, the maximum tolerable dose (MTD) of DOX was raised 2.3-fold by PEO-*b*-PBLA in C26-bearing mice (Kataoka et al., 2000), which was possibly due to a change in the normal distribution of DOX by its polymeric micellar formulation away from heart (site of DOX toxicity).

PEO-*b*-PBLA micelles were also used to encapsulate Camptothecin (CPT) (Opanasopit et al., 2004; Yokoyama et al., 2004). The PBLA chain was modified by alkaline hydrolysis of its benzyl group followed by esterification with benzyl, *n*-butyl, or lauryl groups. Camptothecin has been physically encapsulated in micelles of PEO-*b*-p(L-Asp) having various side chains in the hydrophobic section. The presence of aromatic structures, e.g., benzyl or methylnaphthyl groups, was shown to be more efficient, resulting in the better stabilization of encapsulated CPT in the micellar structures. Release of CPT from the micelles was dependent on the benzyl contents and chain lengths. Sustained release, a property that relies on efficient drug diffusion from the core, was obtained when the benzyl content was high.

Micelles of PEO-*b*-PBLA have been used to solubilize indomethacin, resulting in a sustained release for the encapsulated drug. The release rate of the drug from PEO-*b*-PBLA micelles was found to be dependent on the ionization state of indomethacin, where maximum control on drug release was observed at pH values below the p*K*a of indomethacin (4.5). At this condition, indomethacin was unionized and favored the nonpolar environment of PBLA core in polymeric micelles (La et al., 1996a).

Star block copolymers and 'flower' micelles composed of PEO and poly (γ-benzyl-L-glutamate) (PBLG) have also been developed and used for DOX encapsulation (Jeong et al., 1999). Inhibition of DOX crystallization by the polymer was observed in micellar core. The PEO-*b*-PBLG-based micelles can remarkably sustain and prolong DOX release, resulting in threefold longer mean residence time for encapsulated versus free DOX in vivo.

Chemical conjugation of DOX to the p(L-Asp) block of PEO-*b*-p(L-Asp) was pursued to increase the physical entrapment of DOX inside the hydrophobic core of PEO-*b*-p(L-Asp)-DOX micelles (Nakanishi et al., 2001; Yokoyama et al., 1998; Yokoyama et al., 1994). In this system a 40–50% of DOX substitution on p(L-Asp) and a decrease in proportion of p(L-Asp)-DOX to PEO segment were necessary to achieve stable micelles. Polymeric micelles developed based on this design showed superior properties in terms of DOX release and targeting in animal models in comparison to PEO-*b*-PBLA micelles. This formulation is in clinical trials in Japan under the name of NK911.

By hydrolysis/ester exchange, or aminolysis of PBLA, benzyl ester has been replaced by various aliphatic chains to produce a variety of poly(aspartamide) derivatives that can potentially enhance the delivery of aliphatic drugs (Figure 13.4A). During the process of hydrolysis of PEO-*b*-PBLA under alkaline conditions, racemization of the aspartic acid units takes

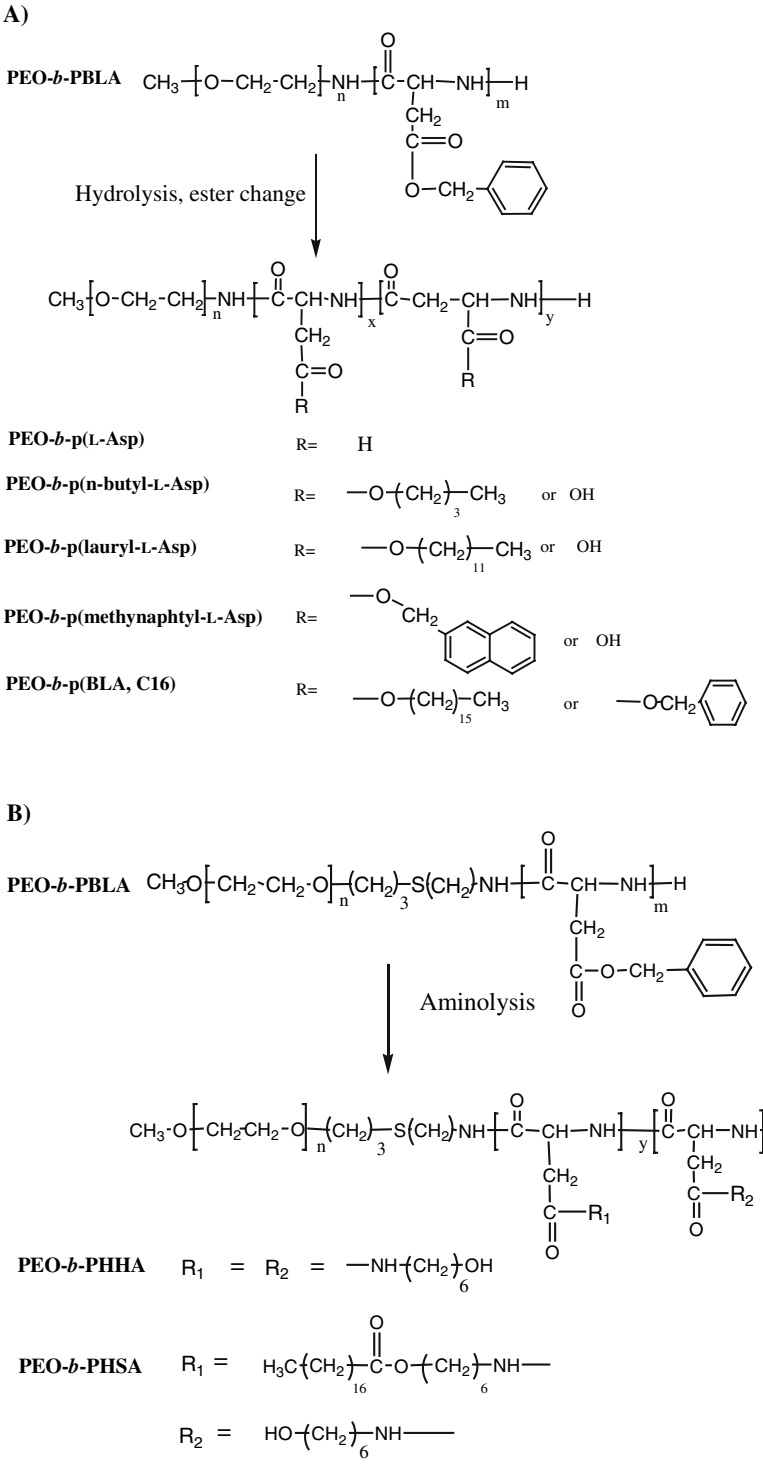


Figure 13.4 Chemical structure of PEO-*b*-PBLA bearing different substituted groups by (**A**) hydrolysis/ester change or (**B**) aminolysis.

place to form α- and β-aspartic units (Wolk et al., 1994). Notably, aminolysis reaction of PBLA with various amino compounds quantitatively proceeds even under mild conditions of room temperature (Figure 13.4B). This quantitative aminolysis reaction is quite unique for PBLA, and almost no reaction occurs for PBLG under the same conditions. Presumably, the ester group in the side chain of PBLA may be in the activated form due to the interaction with amide moieties in the main chain (Osada and Kataoka, 2006).

Block copolymers produced from hydrolysis/ester exchange (Figure 13.4A) were used to physically incorporate an aliphatic anticancer drug, KRN5500. Using DMSO as the solvent to prepare KRN5500-encapsulated micelles by the dialysis method, PEO-*b*-p(BLA, C16) (Figure 13.4A) micelles showed significantly higher KRN5500 encapsulation leading to a homogenous KRN5500 micellar solution, while precipitate was observed with KRN5500 in PEO-*b*-PBLA micelles (Yokoyama et al., 1998). Polymeric micellar KRN5500 and free drug were found to be similar in terms of anti-tumor activity against HT-29 (human colonic cancer) in vitro and MKN-45 xenografts in a mouse model, but polymeric micellar formulation was less toxic (Matsumura et al., 1999), possibly due to a reduction in non-specific interaction with healthy organs.

Micelles based on PEO-*b*-p(*l*-Asp)-bearing fatty acid esters as a side chain (Figure 13.4B) have been developed and used for the solubilization of amphotericin B (AmB) (Lavasanifar et al., 2002a; Adams et al., 2003; Adams and Kwon, 2003; Aramwit et al., 2000; Lavasanifar et al., 2001; Lavasanifar et al., 2002b). This system has shown clear advantage over PEO-*b*-PBLA in the solubilization of AmB due to the more lipophilic core. Besides, higher levels of fatty acid substitution on the p(L-Asp) backbone were shown to enhance AmB encapsulation and to lower the rate of drug release. As a result, hemolytic activity of AmB was reduced in its polymeric micellar formulation in comparison to free AmB or the common formulation Fungizone®. Polymeric micellar AmB has shown equal anti-fungal efficacy to that of Fungizone® both in vitro and in vivo (Adams et al., 2003).

Synthesis of Self-Associating PEO-b-PLAA-Drug Conjugates

PEO-*b*-PLAA-based block copolymers have also been extensively used for chemical conjugation of anticancer drugs to the core-forming block. Drugs were conjugated to the PLAA section via ester, amide, hydrazone, or disulfide bonds. The bond between the drug and polymeric carrier may be either hydrolyzable at acidic pH of the prelysosomal or lysosomal compartments, or cleaved by enzymatic hydrolysis in the lysosomal compartment (specific for amide or ester bonds).

The first example of this type of polymeric micelle has been developed by Ringsdorf et al. who reported on the preparation of micelle-forming conjugates of cyclophosphamide (CP) sulfide and PEO-*block*-poly(L-lysine) (PEO-*b*-PLL) (Hirano et al., 1979). Simultaneous conjugation of fatty acids to the polymeric backbone was used to reduce CMC and increase thermodynamic stability of this system. This formulation was found to be efficient in the stabilization of active CP metabolite in vivo, and caused a fivefold increase in the life span of L1210 tumor-bearing mice even at a reduced CP-equivalent dose.

Block copolymer drug conjugates of PEO-*b*-P(L-Asp)-DOX have been developed by Kataoka's group (Yokoyama et al., 1991; Kataoka et al., 1993). DOX was covalently conjugated to the side chain of the P(L-Asp) segment by an amide bond between the carboxylic group in P(L-Asp) and the primary amine group of the glycosidyl residue in DOX with a substitution ratio of 50% (Figure 13.5A). However, the amide bond was found to be too stable for efficient drug release in vivo. Therefore, in further studies, DOX was conjugated to p(L-Asp) through a hydrazone linker that is stable under physiological conditions but cleavable under the acidic intracellular environments of endosomes and lysosomes (Figure 13.5B) (Bae et al., 2003). When the micelles were incubated under various pH conditions from 7.4 to 3.0 in vitro, DOX was released in a time-dependent manner as external pH decreased, while no DOX release was observed under the physiological condition of pH 7.4 for over 48 h of incubation. The animal tests revealed that the pH-sensitive micelles showed an effective anti-tumor activity over a broad range of injection dose to suppress the tumor growth in mice, whereas the toxicity remained extremely low.

Development of Polyion Complex Micelles from PEO-b-PLAA for Drug Delivery

An electrostatic interaction between the ionizable groups on the PLAA block of PEO-*b*-PLAA block copolymers and charged drugs/genes has been used to produce polyion complex micelles. Incorporation of cisplatin (CDDP) into the PEO-*b*-p(L-Asp) and PEO-*b*-p(L-Glu) micelles uses this approach where an electrostatic complex between positively charged drugs/moieties and carboxyl ions on p(L-Asp) or p(L-Glu) block neutralized polymer and self-assembled it into micelles (Yokoyama et al., 1996; Nishiyama and Kataoka, 2001; Nishiyama et al., 2003). The PEO-*b*-PLAA-CDDP micelles showed an environment-responsive drug release behavior, long circulation, and passive tumor targeting (Figure 13.6) (Nishiyama et al., 2001; Nishiyama et al., 1999). The PEO-*b*-p(L-Glu) formulation of CDDP is currently in clinical trials in Japan under the name of NC-6004.

Development of Polyion Complex Micelles from PEO-b-PLAA for the Delivery of Nucleic Acid-Based Therapeutics

An exciting area of research involving micelles is the efforts to deliver nucleic acid-based therapeutics systemically. For a successful micelle-based gene delivery via systemic administration, four main concerns need to be addressed: (a) the core-forming block of the micelles should be able to combine and condense nucleic acid-based therapeutics in the core; (b) the micelles should maintain integrity under physiological conditions before they reaching the target cells; (c) the micelles should interact with the target cell specifically; and (d) the delivery system should dissociate in a predictable manner in the intracellular compartment of the target cell, facilitating the release of the entrapped nucleic acid-based moiety and making the therapeutic agent available for interaction with its intracellular targets. One can argue that the same properties are also required for an efficient

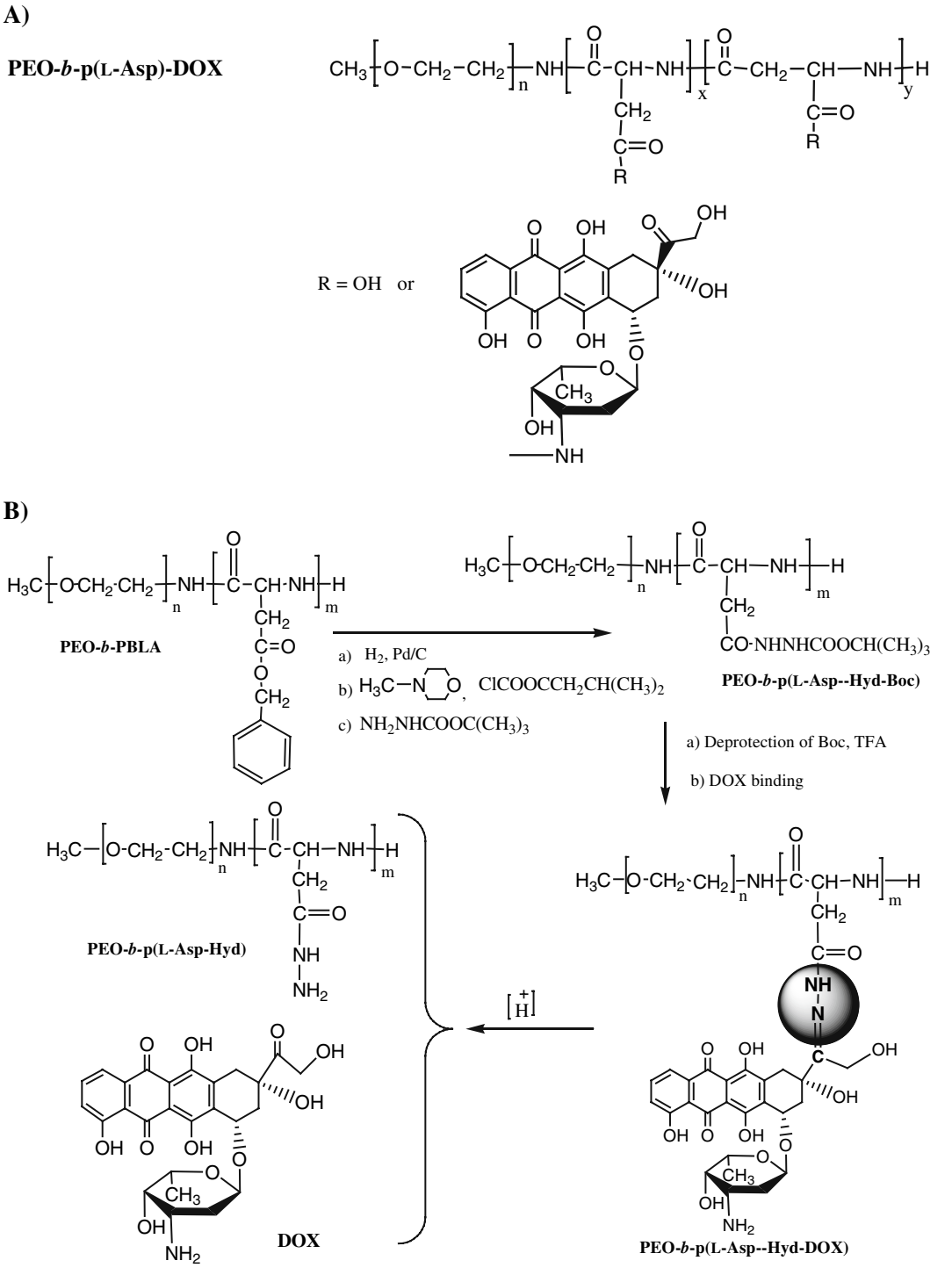


Figure 13.5 Chemical structure of PEO-*b*-PLAA-drug conjugate: (**A**) PEO-*b*-p(L-Asp-DOX) prepared by formation of amide bond, (**B**) PEO-*b*-p(L-Asp-DOX) prepared by the formation of hydrozone bound.

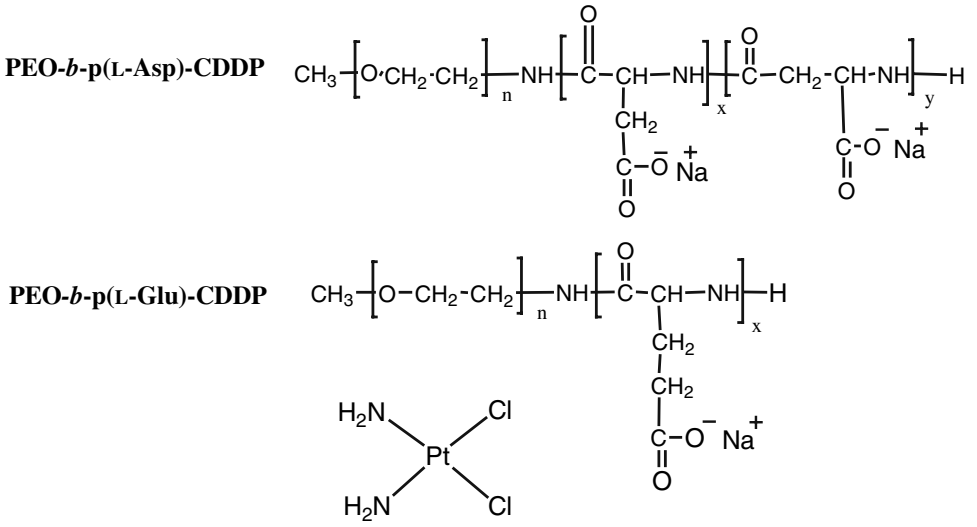


Figure 13.6 PEO-*b*-PLAA/CDDP complex (reproduced from Xiong et al., 2006 with permission).

polymeric micellar system when delivering small drugs. Given the larger molecular weight, higher charge density and toxicity of nucleic acid-based agents achieving success in targeted delivery for this new class of therapeutics is not only more crucial but rather challenging.

PEO-*b*-PLAA-based gene vectors can be engineered to meet these requirements for effective gene delivery. PEO-*b*-PLL have been used for application in gene delivery because the amine-contained core-forming block can form a stable polyion complex with macromolecular (plasmid) DNA through electrostatic interaction (Ward et al., 2002; Katayose and Kataoka, 1998; Katayose and Kataoka, 1997). PEO-*b*-PLL micelles were injected via supramesenteric vein and showed gene expression in the liver. The gene expression was sustained for 3 days. To increase the stability of PEO-*b*-PLL micelles against dissociation, a fraction of the lysine residues in the PLL core was substituted with thiol groups, that can readily form disulfide cross-links with other thiol groups on PEO-*b*-PLL and develop a network structure in the micelle core after DNA complexation (Figure 13.7, scheme 1) (Kakizawa et al., 1999; Kakizawa et al., 2001; Harada et al., 2001). The cross-linked core of the micelles were cleavable inside the cell due to the increased concentration of glutathione, which is a reducing agent abundant in the cytoplasm but not in blood compartment (Meister and Anderson, 1983). However, introduction of thiol groups was found to decrease the electrostatic association sites for the interaction of PLL and DNA. To deal with this problem, Traut's reagent was used to introduce the cross-linking thiol groups to the PLL and, meanwhile, avoid the loss of charge density of the block copolymer segment (Figure 13.7, scheme 2) (Miyata et al., 2004).

In most cases, gene/vector complexes entry into cells is dependent on endocytosis. For enhancing the transfection, the delivered gene needs to be

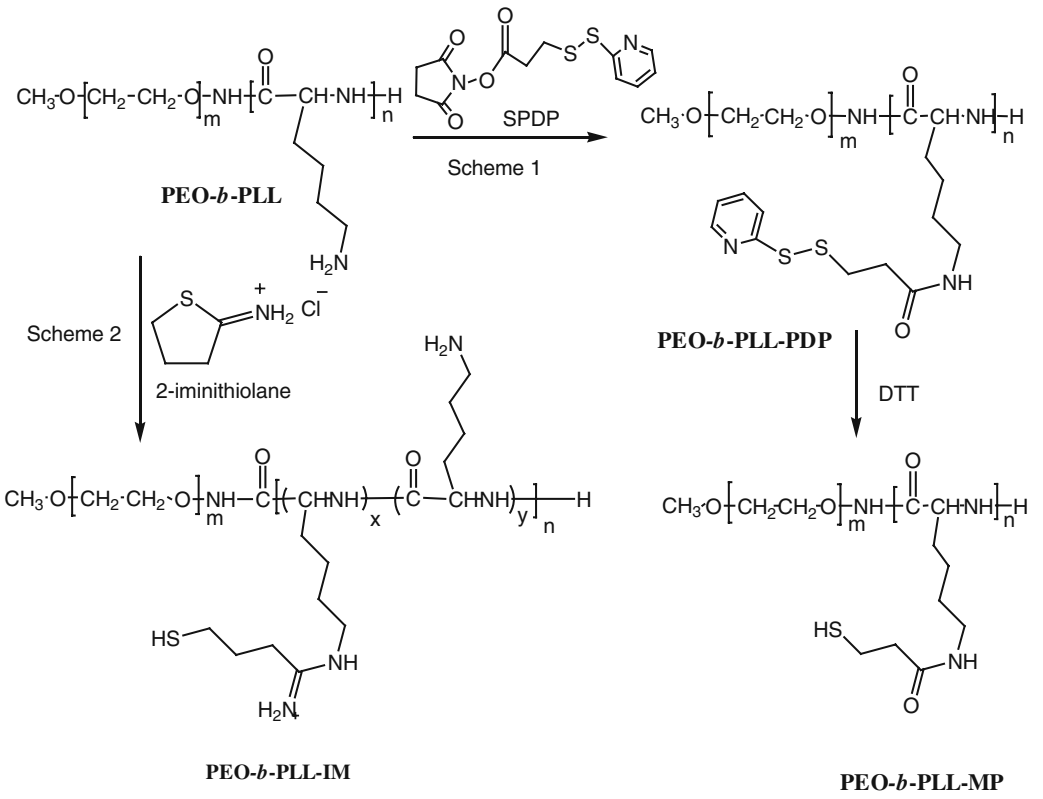


Figure 13.7 Synthesis of PEO-*b*-PLL copolymers with crosslinking and DNA binding groups in the core-forming block. Scheme 1: thiolation by *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) proceeds through the substitution reaction of the ε-amino groups, leading to decreased charge density (PEO-*b*-PLL-MP); Scheme 2: thiolation with 2-iminothiolane proceeds through the introduction of cationic imino groups so that the charge density of the PLL segments remained constant (PEO-*b*-PLL-IM).

released from the endosome into cytosol before endosomes fuse with lysosomes, where the vector and the encapsulated DNA will be destroyed (Erbacher et al., 1996). Certain polycations, such as polyethyleneimine (PEI), are believed to increase osmotic pressure in the endosome by the so-called 'proton sponge effect' resulting in endosomal disruption and DNA release (Behr, 1997; Boussif et al., 1995), although some studies debate the involvement of this mechanism as the main reason for endosomal escape for cationic polymers (Funhoff et al., 2004).

Triblock copolymer of PEO-*b*-PEI-*b*-PBLG has been synthesized where positively charged PEI and hydrophobic PBLG have been used to condense DNA and induce micellarization, respectively. The prepared micelle was shown to incorporate plasmid DNA effectively (Tian et al., 2005). High transfection efficiency and low cytotoxicity may be expected from this gene carrier because of the nature of micellarization and PEI-containing core.

Kataoka et al. designed new types of PEO-*b*-PLAA-based block copolymers that contain two or more amino groups in the side chain (Figure 13.8, scheme 1) (Kanayama et al., 2006; Itaka et al., 2004). The terminal primary amine group displayed a high p*K*a that was suitable for complexation with phosphate groups of siRNA or DNA. The secondary amine of the side

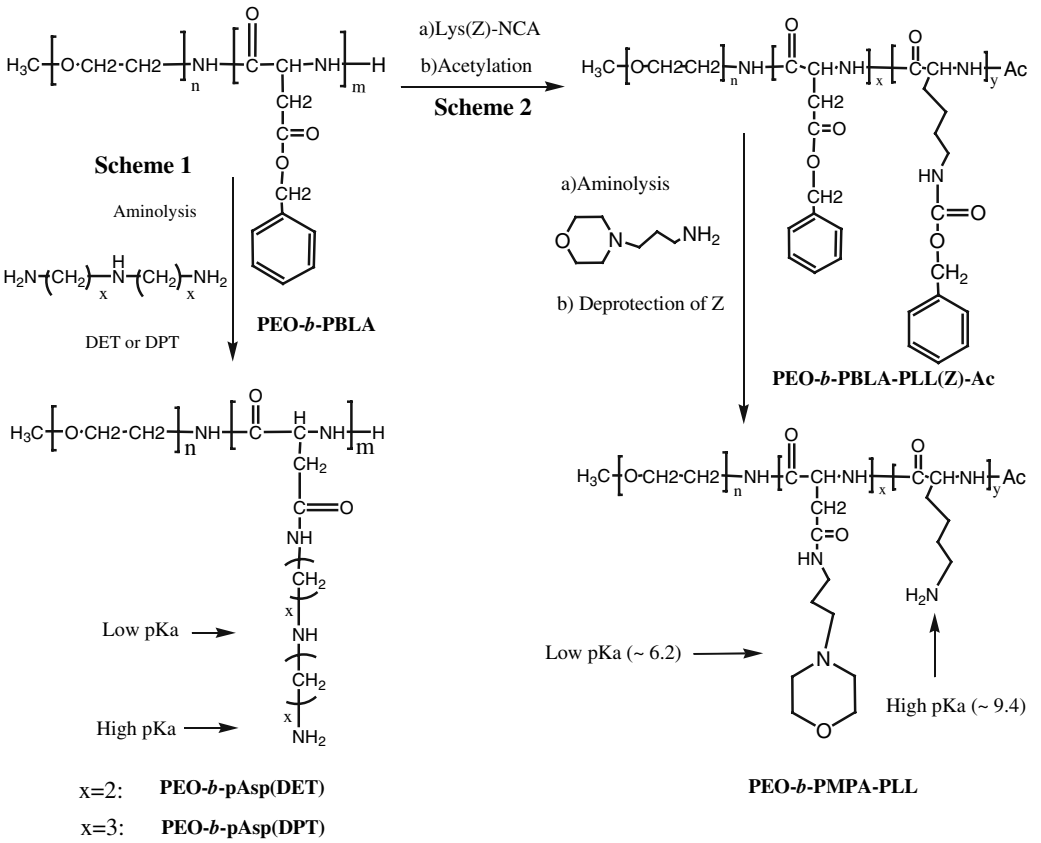


Figure 13.8 Synthesis of PEO-*b*-PLAA copolymers which possess the function of buffering capacity and high DNA affinity in the core-forming block. Scheme 1: synthesis of the diblock polymer PEO-PAsp(DPT) by aminolysis of PEO-*b*-PBLA with dipropylenetriamine; Scheme 2: synthesis of the triblock polymer PEO-*b*-PMPA-PLL by ring-opening polymerization of Lys(Z)-NCA using PEO-*b*-PBLA as the initiator followed by aminolysis and deprotection of benzyl group.

chain which was located closer to the polymeric backbone showed a lower p*K*a and was expected to provide buffering capacity for proton sponge effect. These block catiomers were prepared by the aminolysis of PEO-*b*-PBLA with either dipropylenetriamine (DPT), diethylenetriamine (DET), 4-methyldiethylenetriamine (MDET), or *N*,*N*-diethyldiethylenetriamine (DEDET). Both the PEO-*b*-p[Asp(MDET)] and PEO-*b*-p[Asp(DEDET)] polyplex micelles showed an appreciably lower transfection than the PEO-*b*-p[Asp(DET)] polyplex micelles (Kanayama et al., 2006). Especially, the polyion complex of PEO-*b*-p[Asp(DPT)] with siRNA has shown superior transfection efficiency over lipid-based commercial vector for siRNA delivery, i.e., RNAiFect (Itaka et al., 2004).

Free polycations substantially contribute to efficient transfection but mediate toxic effects as well. Hence, polyplex systems useful for in vivo gene delivery should achieve efficient transfection without free polycations. In order to achieve such delivery system, Kataoka et al. have designed a triblock copolymer consisting of PEO, as the hydrophilic segment, poly[(3-morpholinopropyl)aspartamide] (PMPA) as the low p*K*a

segment with buffering capacity, and PLL as the high p*K*a segment to condense DNA (Figure 13.8, scheme 2) (La et al., 1996b). Notably, when plasmid DNA was encapsulated in PEO-*b*-PMPA-*b*-PLL it revealed one order of magnitude higher transfection efficiency than PEO-*b*-PLL, which was comparable to the transfection efficiency of plasmid DNA-encapsulated PEI at the corresponding negative to positive (N/P) ratio, without showing appreciable cytotoxicity.

Synthesis of PEO-*b*-poly(ester) Block Copolymers

General Synthesis

Ring-opening polymerization of lactones or lactides was frequently used to synthesize poly(ester)s (Albertsson and Varma, 2003). Depending on the initiator, the polymerization can proceed by three different reaction mechanisms: cationic, anionic, or 'coordination-insertion' mechanisms (Stridsberg et al., 2002). The cationic ROP involves the formation of positively charged species which are subsequently attacked by a monomer (Figure 13.9A). Anionic ROP of cyclic ester monomers takes place by the nucleophilic attach of a negatively charged initiator on the carbonyl or on the carbon atom adjacent to the acyl oxygen, resulting in linear polyester (Figure 13.9B). Coordination-insertion ROP, also called pseudo-anionic ROP, is thought to proceed by coordination of the monomer to the active species, followed by insertion of the monomer into the metal–oxygen bond by rearrangement of the electrons (Figure 13.9C).

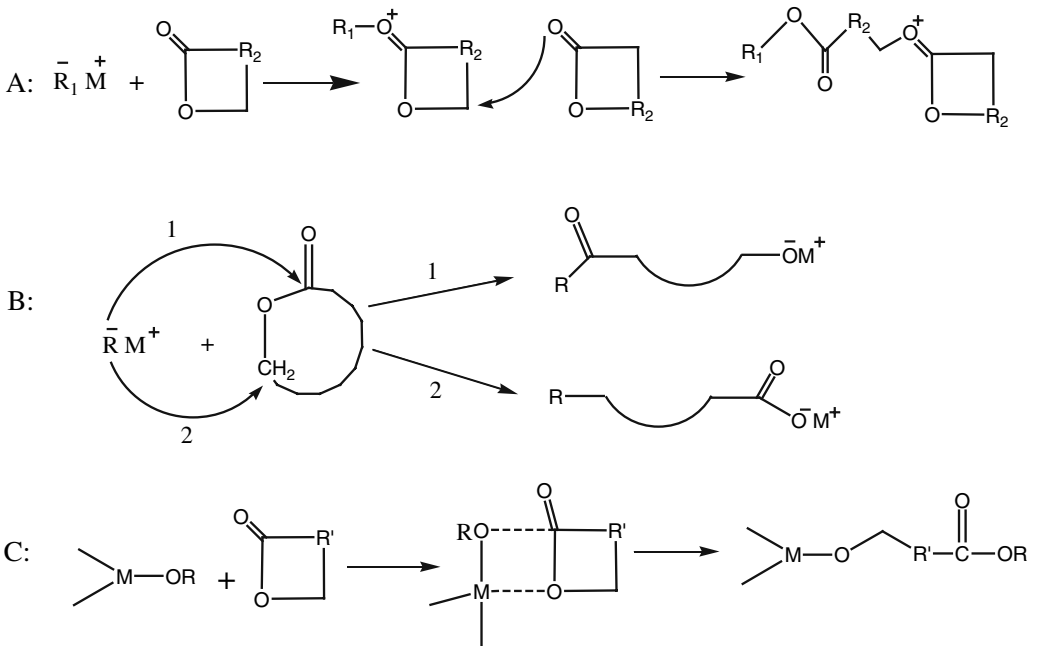


Figure 13.9 The ROP of a cyclic ester by **(A)** cationic, **(B)** anionic, and **(C)** coordination-insertion mechanism (reproduced from Stridsberg et al., 2002 with permission).

An extensive research effort has been made in the past few years to refine the technique of ring-opening polymerization so that polyesters with controlled architecture and properties could be prepared. Various monomers, such as glycolide (GA), lactide (LA), β-butyrolactone (β-BL), ε-caprolactone (ε-CL), 1,5-dioxepan-2-one (DXO), or their derivatives have been used to prepare poly(ester)s. The representative structures of these monomers and polymers are given in Figure 13.10. The polymerization is generally carried out in bulk or in solution (using THF, dioxane, toluene, etc.), emulsion, or dispersion (Scholz et al., 1995; Gadzinowski et al., 1996). The temperature of bulk polymerization is generally in the range of 100–150°C, whereas low temperature has been used (0–25°C) in solution polymerization. For example, PEO-*b*-PLA or PEO-*b*-PCL has been synthesized by solution polymerization using potassium naphthalene as the catalyst, or by bulk polymerization using stannous octoate as the catalyst (Iijima et al., 1999; Nguyen et al., 2003; Aliabadi et al., 2005; Deng et al., 2004). Studies by our research group and others have shown polymerization in solution generally leads to PLA or PCL chains with a lower molecular weight than expected values calculated from the initial monomer/initiator feeding ratios (Stridsberg et al., 2002; Ito et al., 1977; Xiong et al., 2007a). This observation can be explained by higher probability of inter- or intra-molecular transesterification in solution polymerization.

Chemical Modification of the Core in PEO-*b*-poly(ester) Micelles for Physical Drug Encapsulation

Synthesis of PEO-*b*-PLA block copolymers containing a small quantity of carboxylic acid side groups on the PLA block through ROP of 3(s)[(benzyloxycarbonyl)methyl]-1,4-dioxane-2,5-dione (BMD) monomer has been reported by Lee et al (Figure 13.11A) (Lee et al., 2004). The prepared block copolymer was observed to form micelles at a relatively low CMC. An increase in the drug loading was seen in polymeric micelles bearing higher carboxyl groups in their core structure. The observation was attributed to an increased interaction via hydrogen bonding between the micellar core and encapsulated drug.

Our research group has synthesized α-benzylcarboxylate-ε-caprolactone (BCL) (Mahmud et al., 2006) and α-cholesteryl carboxylate-ε-caprolactone (ChCL) (unpublished data). The functionalized monomers were then used in ROP reactions with methoxy PEO to produce poly(ethylene oxide)-*block*-poly(α-benzylcarboxylate-ε-caprolactone) (PEO-*b*-PBCL) and poly(ethylene oxide)-*block*-poly(α-cholesteryl carboxylate-ε-caprolactone) (PEO-*b*-PChCL) block copolymers (Figure 13.11B). PEO-*b*-PBCL was then reduced in the presence of H_2 to produce poly(ethylene oxide)-*block*-poly(α-carboxylic-ε-caprolactone) (PEO-*b*-PCCL). The average CMC for PEO-*b*-PBCL and PEO-*b*-PCCL block copolymers was estimated at 0.098 and 12.20 μM, respectively. By adjusting the molar ratio of unsubstituted and substituted monomers, e.g., ε-CL and BCL, block copolymers with a random copolymer structure in the core, e.g., PEO-*b*-poly(CL-co-BCL), can be obtained to meet different requirements for the delivery of individual drugs.

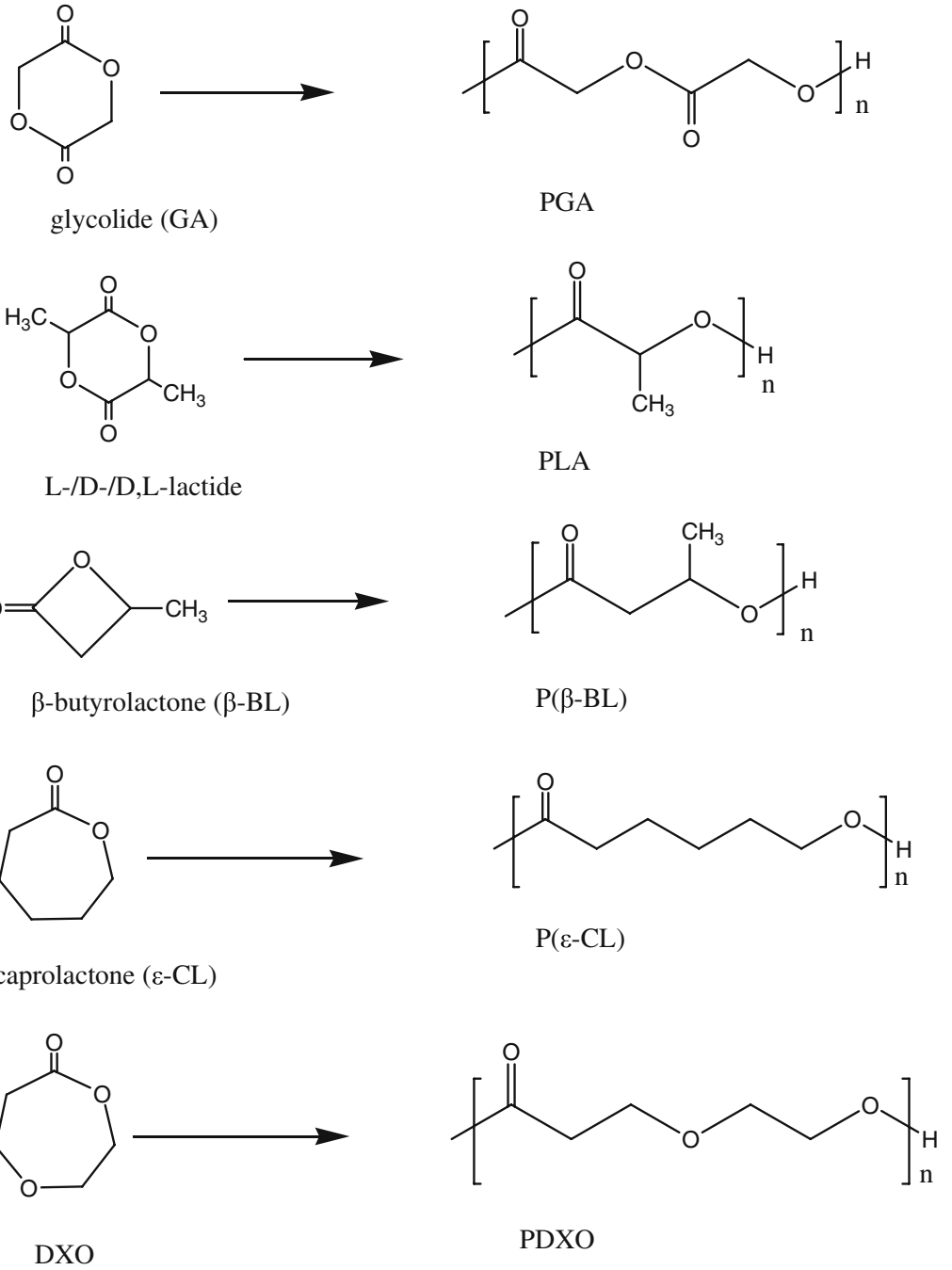


Figure 13.10 Chemical structure of common cyclic lactones used in the ROP reactions for the synthesis of poly(ester)s.

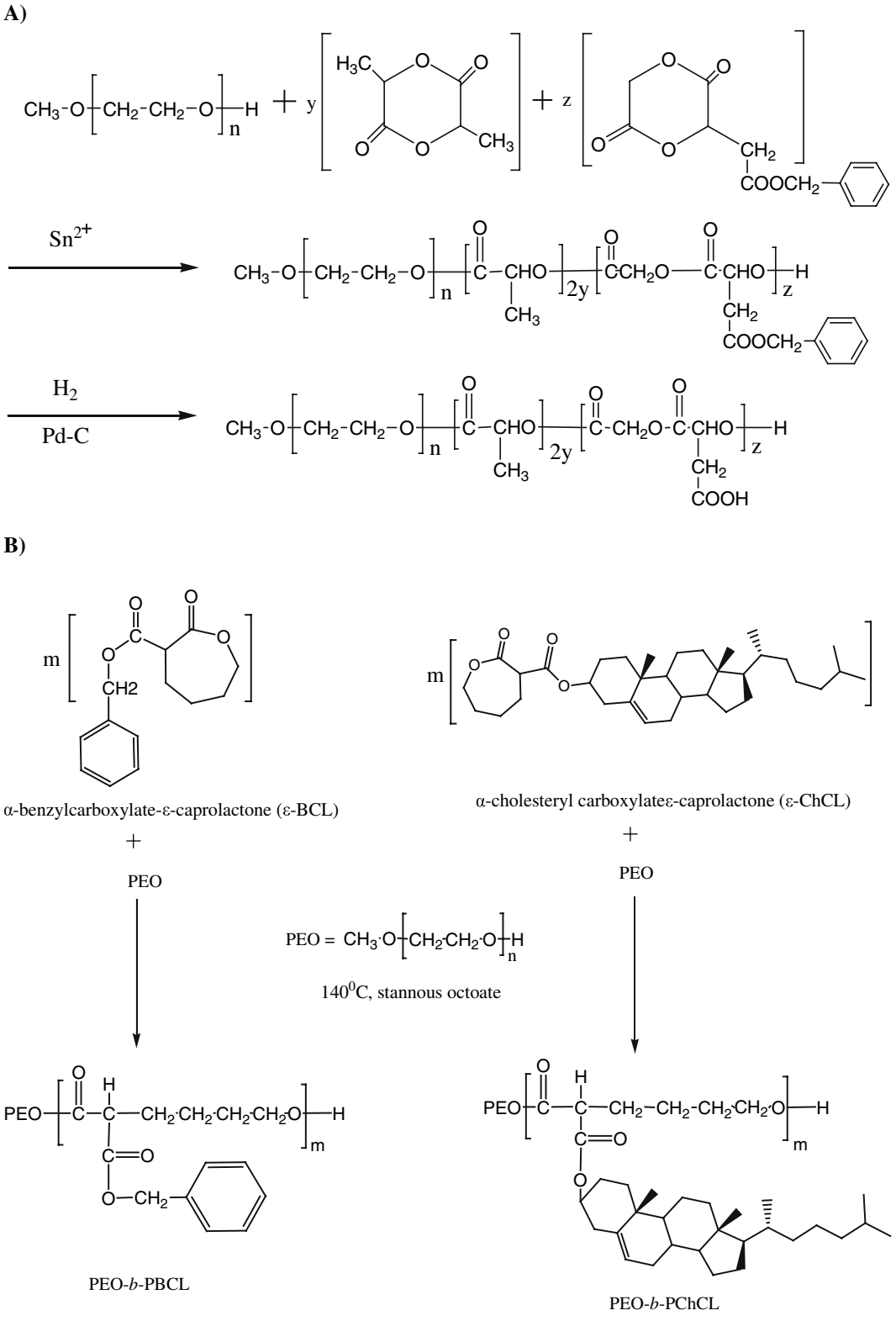


Figure 13.11 Synthetic scheme of polyester-substituted **(A)** PEO-*b*-PLA and **(B)** PEO-*b*-PCL block copolymers.

Synthesis of PEO-*b*-poly(ester)s-Drug Conjugates

Conjugation of drugs to PEO-*b*-poly(ester)s has usually been accomplished by functionalization of the terminal poly(ester) end which is followed by reaction with drugs. Zhang et al. have attached the water-insoluble anticancer drug, paclitaxel, to the PLA section of the PEO-*b*-PLA to increase its solubility (Figure 13.12A) (Zhang et al., 2005). Toward this goal, hydroxyl-terminated diblock copolymer of monomethoxy-poly(ethylene oxide)-*b*-poly(lactide) (MPEO-*b*-PLA) was first synthesized by ring-opening polymerization of L-lactide using MPEO as a macroinitiator. The terminal hydroxyl group of the PLA block was then converted to carboxyl group by reacting PEO-*b*-PLA with mono-*t*-butyl ester of diglycolic acid and subsequent deprotection of the *t*-butyl group with trifluoroacetic acid (TFA). Paclitaxel was then conjugated to the copolymer through formation of ester bonds between the terminal carboxylic groups of the copolymer and the hydroxyl group of paclitaxel in the presence of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP). Because of the spatial hindrance in paclitaxel, 2′-hydroxyl is more active than the 7-hydroxyl for esterification and preferentially used for paclitaxel conjugation. Paclitaxel was released from the conjugate upon hydrolysis without loss of cytotoxicity.

Conjugation of DOX to the PEO-*b*-PLGA after activation of the PLGA terminus by *p*-nitrophenyl chloroformate has also been reported (Yoo and

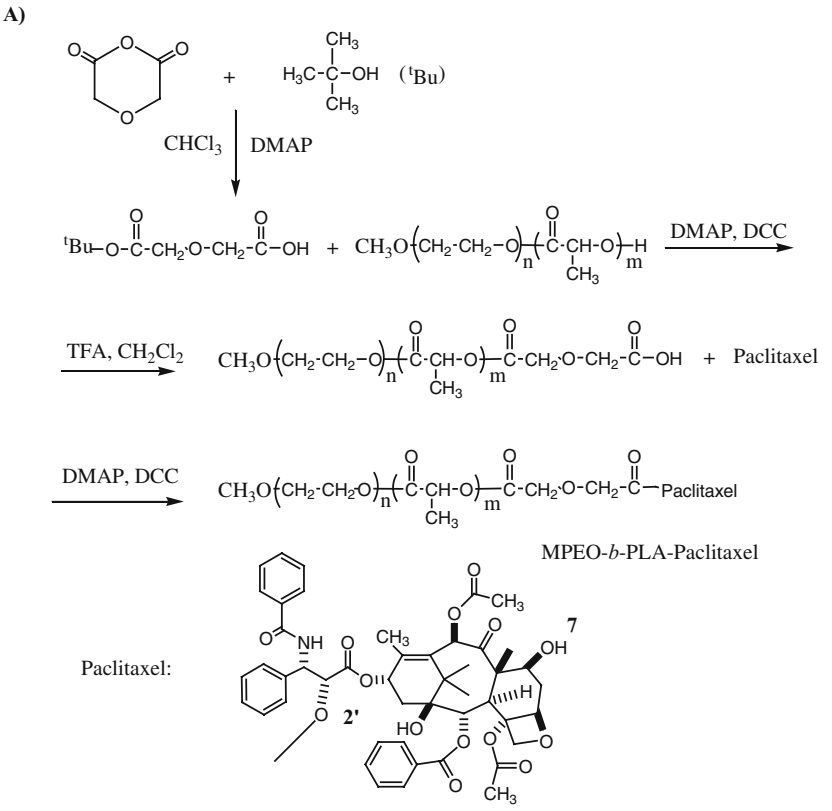


Figure 13.12 (*Continued*).

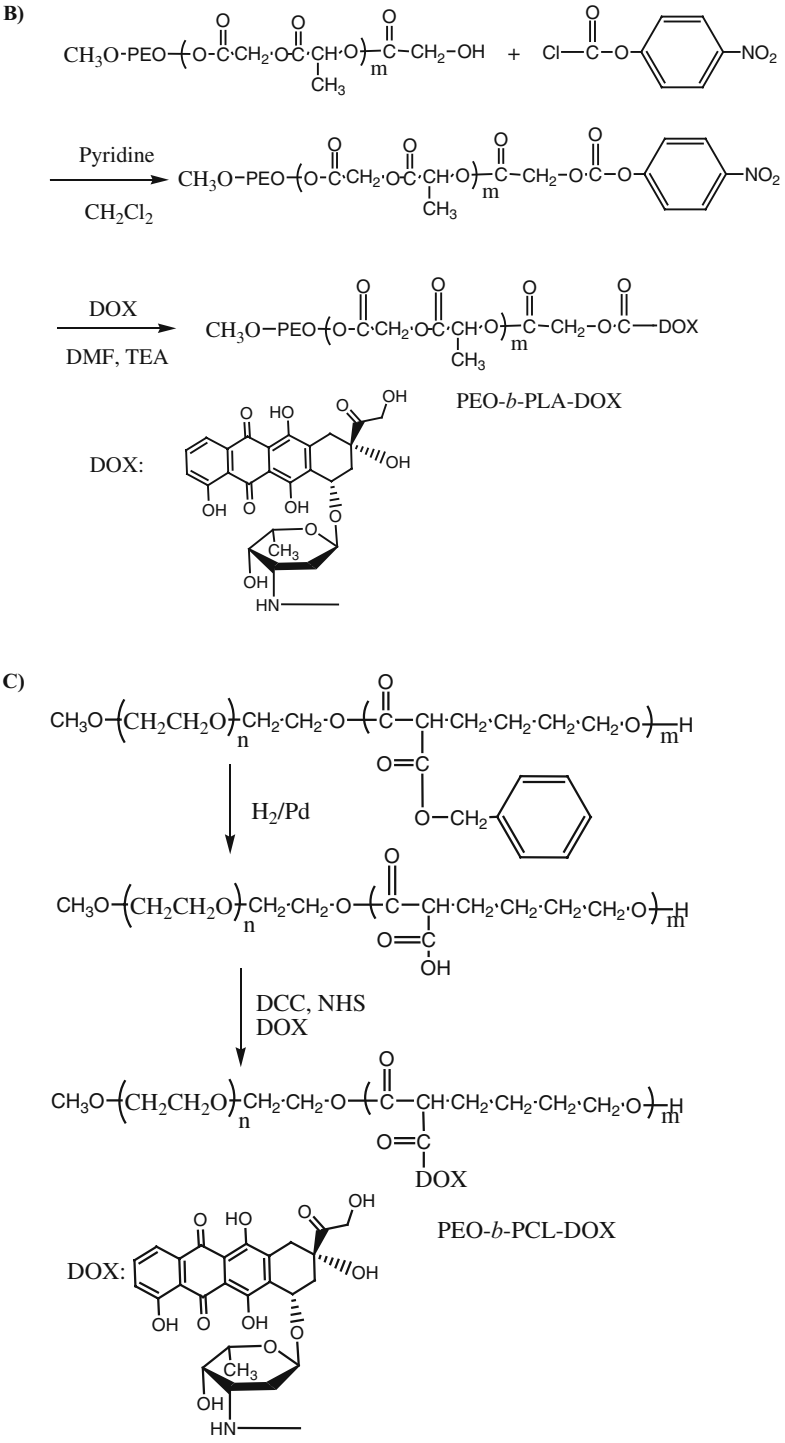


Figure 13.12 Synthesis of polymer-drug conjugates: (**A)** PEO-*b*-PLA-paclitaxel, (**B)** PEO-*b*-PLA-DOX, and (**C)** PEO-*b*-PCL-DOX.

Park, 2001) (Figure 13.12B). The micelles containing chemically conjugated DOX exhibited a more sustained release profile than PEG–*b*-PLGA micelles containing physically entrapped DOX. Interestingly, the cellular uptake of the DOX-conjugated micelles was more efficient than free DOX against HepG2 cells, leading to higher cytotoxic activity than free DOX.

By utilizing the single functional group at the end of the poly(ester) chain, the chemical drug-loading efficiency can only achieve 1:1 molar ratio (drug: polymer) at most. By attaching multiple functional side groups, however, one can achieve multiple copies of the drug per polymer chain and achieve higher drug:polymer molar ratios. In our group, DOX was conjugated to the PCL section of the PEO-*b*-PCCL copolymers by the reaction between the –COOH side group on the PCCL and amine group of DOX (Figure 13.12C). The degree of DOX conjugation to single polymer chains reached 2:1 molar ratio in our preliminary study (Mahmud et al., 2007).

Synthesis of PEO-*b*-poly(amine) Block Copolymers

The cationic polymer polyethylenimine (PEI), which is widely used for non-viral transfection, is advantageous over other polycations because it combines strong DNA compaction capacity with an intrinsic endosomolytic activity (Demeneix and Behr, 2005; Kircheis et al., 2001). PEI can be prepared in the form of branched or linear architectures.

Linear PEI has been synthesized via cationic ring-opening polymerization of either *N*(2-tetrahydropyranyl)azidirine or unsubstituted and two-substituted 2-oxazolines followed by acid- or base-catalyzed hydrolysis of the corresponding N-substituted polymer (Figure 13.13A) (Brissault et al., 2003). Branched PEI is synthesized by polymerization of aziridine either in aqueous or alcoholic solutions, where the reaction is controlled by adjusting the temperature and initiator concentration, or in a rather vigorous bulk polymerization of anhydrous aziridine at a lower temperature (Figure 13.13B) (von Harpe et al., 2000).

Incorporation of PEO into DNA complexes has been achieved either by condensing DNA with PEO-*b*-PEI copolymers (pre-PEGylation) or coupling a PEO layer onto the surface of preformed PEI/DNA polyplexes (post-PEGylation). Several strategies have been used to prepare PEO-*b*-PEI copolymers, most of them using homobifunctional or heterobifunctional PEO for conjugation onto branched or linear PEI (Figure 13.14) (Lungwitz et al., 2005). For post-PEGylation, the PEI/DNA polyplexes are usually formed in a solution with low ionic strength to generate small particles and the PEO chains are only allowed to react with the particle surface to create the protective PEO shield.

Although one may desire to utilize PEO-*b*-PEI conjugates for precise control over the complexation, since PEO-b-PEI can be separately prepared and characterized in a controlled fashion, attachment of PEO to PEI through pre-PEGylation approaches compromises the binding ability of PEI to the DNA, as well as the cells (Clements et al., 2006). Grafting PEO after the formation of nanoparticles may obviate this problem, at the expenses of a mere controlled chemistry. Post-PEGylation also places PEO molecules on the surface where they can exert a maximal impact on the micelle biodistribution.

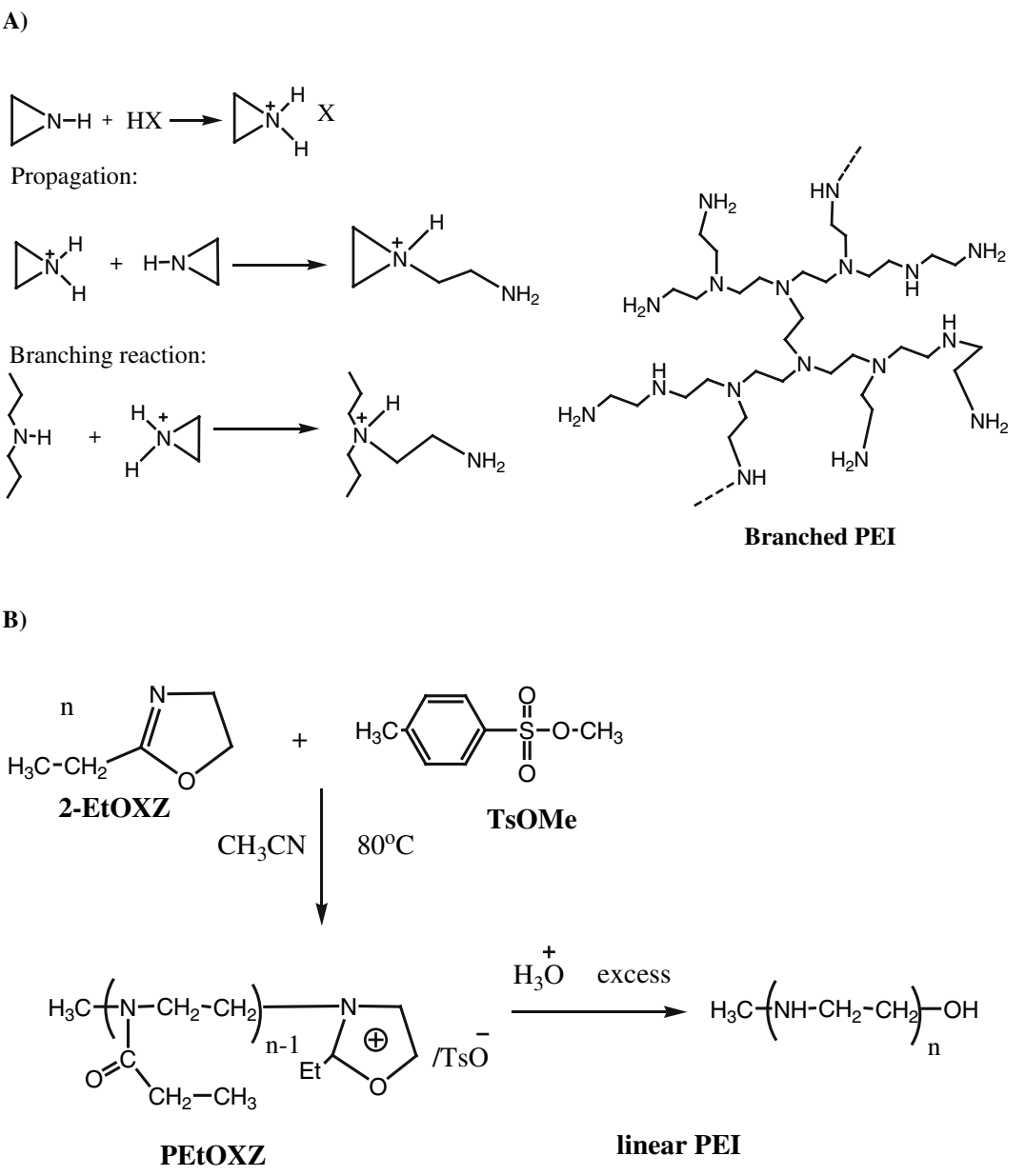


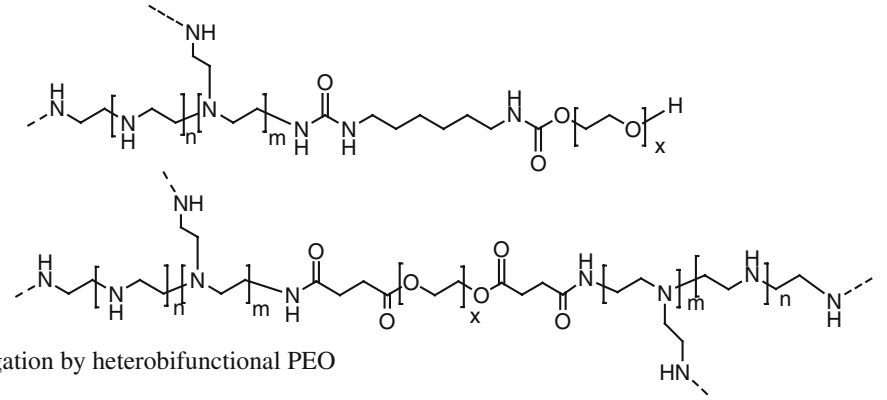
Figure 13.13 Synthesis scheme for **(A)** branched and **(B)** linear PEI (reproduced from Demeneix and Behr, 2005 with permission).

Chemical Modification of the Shell-Forming Block for Drug/Gene Targeting

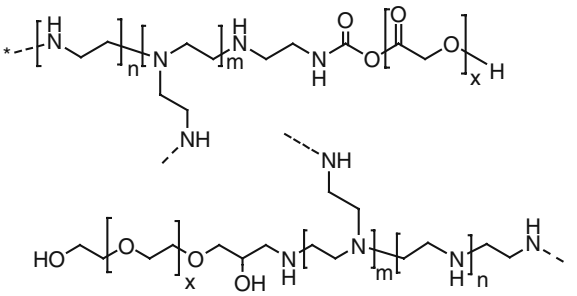
Separation of the core and shell domains in polymeric micelles is a useful property. This architecture permits conjugation of ligands to the shell domain that can enhance tissue specificity and modify cellular, intracellular, or molecular interactions without affecting core-related properties,

Pre-PEGylation

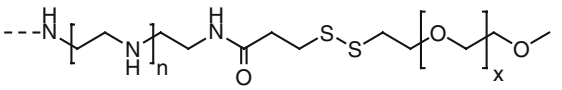
Conjugation by homobifunctional linker



Conjugation by heterobifunctional PEO

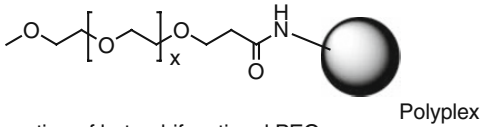


Copolymers cleavable by intracellular reduction



Post-PEGylation

Conjugation of monofunctional PEO



Conjugation of heterobifunctional PEO

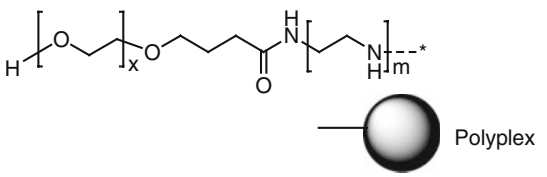


Figure 13.14 Various strategies for the production of PEO-*b*-PEI/DNA polyplexes (reproduced from Demeneix and Behr, 2005 with permission).

such as micellar stability and encapsulation efficiency. Various ligands including small organic molecules, carbohydrates, peptides, and antibodies (or their fragments) have been coupled to polymeric micelles to achieve active targeting. Ligands can be coupled to the shell-forming polymers before or after polymerization of the core-forming block. Conjugation of ligands to preformed polymers (or micelles) via ester, amide, disulfide, thioether, carbon-nitrogen bonds are more common. Otherwise, the ligands generally need to be derivatized to become suitable as initiator of the polymerization reaction.

Conjugation of folic acid to PEO-*b*-PLL, PEO-*b*-poly(*l*-Histidine) (Figure 13.15A) (Kim et al., 2005; Lee et al., 2005), and PEO-*b*-PLGA by amide bond (Figure 13.15B) (Lee et al., 2003a) has been pursued to enhance the interaction of polymeric micelles with cancer cells that overexpress folate receptor. In these studies, PEO with functional groups on both ends was used to couple folate and core-forming block at each end of the PEO. To synthesize the folate-PEO-*b*-PLL, the amine groups on the core-forming block have to be protected to avoid undesired side reactions.

Carbohydrate-mediated drug targeting via glycol receptors on hepatocytes have been pursued by several researchers (Goto et al., 1994; Omelyanenko et al., 1998; Wu et al., 2002; Murao et al., 2002; Opanasopit et al., 2002). Carbohydrate molecules such as galactose and mannose act as specific ligands for the glycol receptors. Kataoka et al. used both pre- and post-polymerization approaches to synthesize carbohydrate-decorated PEO-*b*-poly(D,L-lactide) (carbohydrate-PEO-*b*-PDLA) block copolymers (Nagasaki et al., 2001; Jule et al., 2003). In the pre-polymerization approach, the copolymers were synthesized through sequential anionic ROP of EO by chemically modified sugars like glucose and galactose. Four hydroxyl groups out of five were specifically protected by the acetal substituents in the glucose structure (Figure 13.16A). This step was followed by ROP of lactide and deprotection of initiator to achieve carbohydrate-PEO-*b*-PDLLA. In the post-polymerization method, PEO-*b*-PDLA micelles with aldehyde group at the distal end of the PEO chains, which is derived from the acetal-ended PEO-*b*-PLA block copolymers, were prepared. Carbohydrate conjugation was then accomplished by forming Schiff base between the amine group on the sugar and aldehyde group on the PEO chains (Figure 13.16B). Using the heterofunctional PEO (vinyl sulfone-PEO-hydroxysuccinimidyl, VS-PEO-NHS), galactose has also been conjugated to the PEO-*b*-PEI to form galactose-PEO-*b*-PEI for gene delivery to hepatocytes (Sagara and Kim, 2002).

Targeting tumor cells or tumor vasculature by small peptides is another promising strategy for delivering cytotoxic drugs for cancer therapy. Small size of the ligand, diversity of functional groups on the peptide (thiol, amine, and carboxyl groups) as well as possibility for engineering high-affinity peptides make them preferential ligands for active drug targeting.

Peptides containing the RGD sequence can recognize integrins that are overexpressed on the angiogenic endothelial cells of the tumor vasculature or on metastatic tumor cells. Gao et al. developed polymeric micelles that can selectively deliver hydrophobic drugs to angiogenic tumor endothelial cells that overexpressed $\alpha v\beta 3$ integrins (Figure 13.17A) (Nasongkla et al., 2004). To couple the cyclic pentapeptide C(Arg-Gly-Asp-d-Phe-Lys)

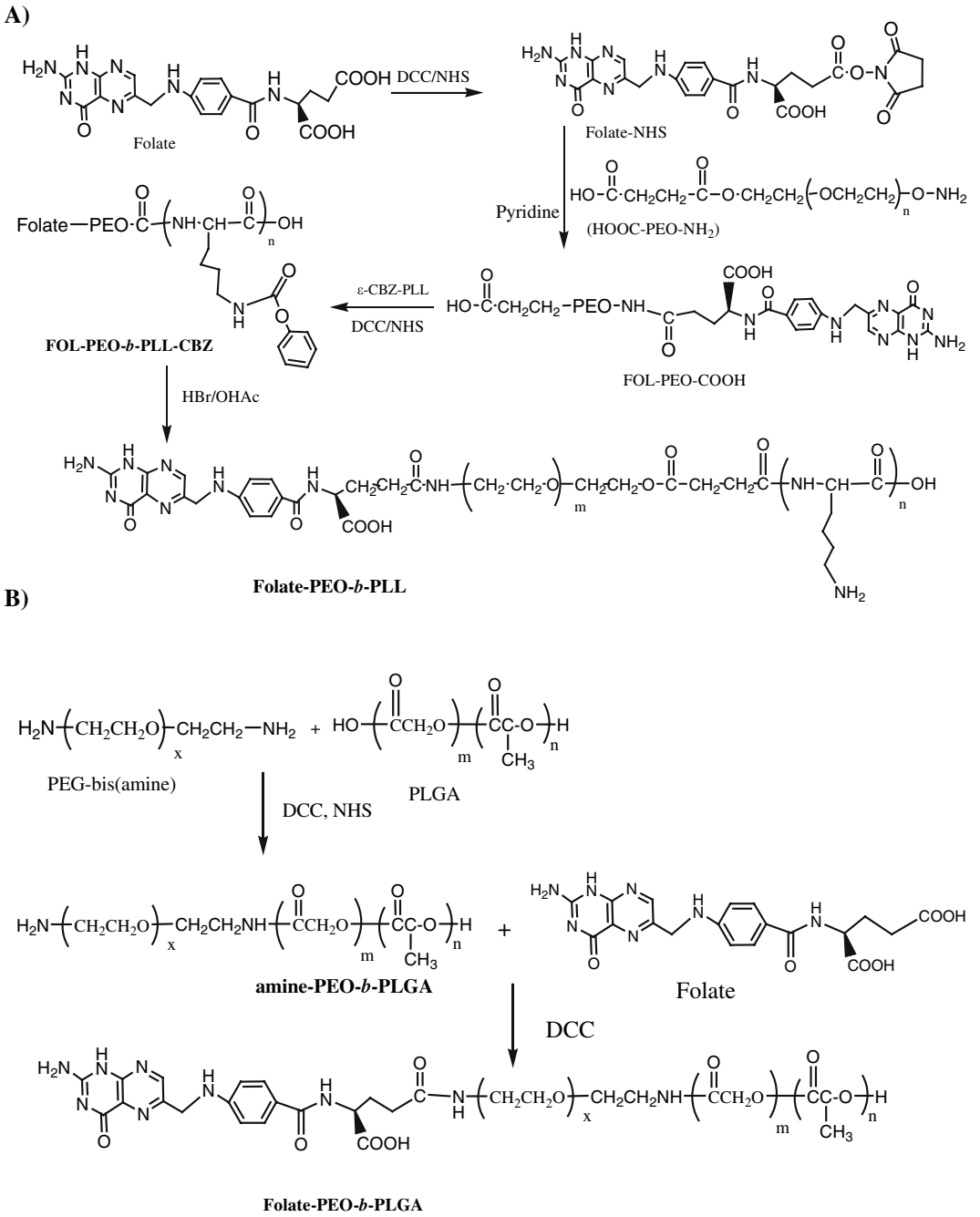


Figure 13.15 Synthesis of **(A)** folate-conjugated PEO-*b*-PLL and **(B)** folate-conjugated PEO-*b*-PLGA.

(cRGDfK) containing thiol in the structure, Gao et al. synthesized maleimide-terminated PEO-*b*-PCL (MAL-PEO-*b*-PCL). After micellarization, cRGDfK was coupled onto the micelle surface by electrophilic addition to form thioether bond between the thiol group on the peptide and ethylenic bond on the maleimide. Conjugation of epidermal growth

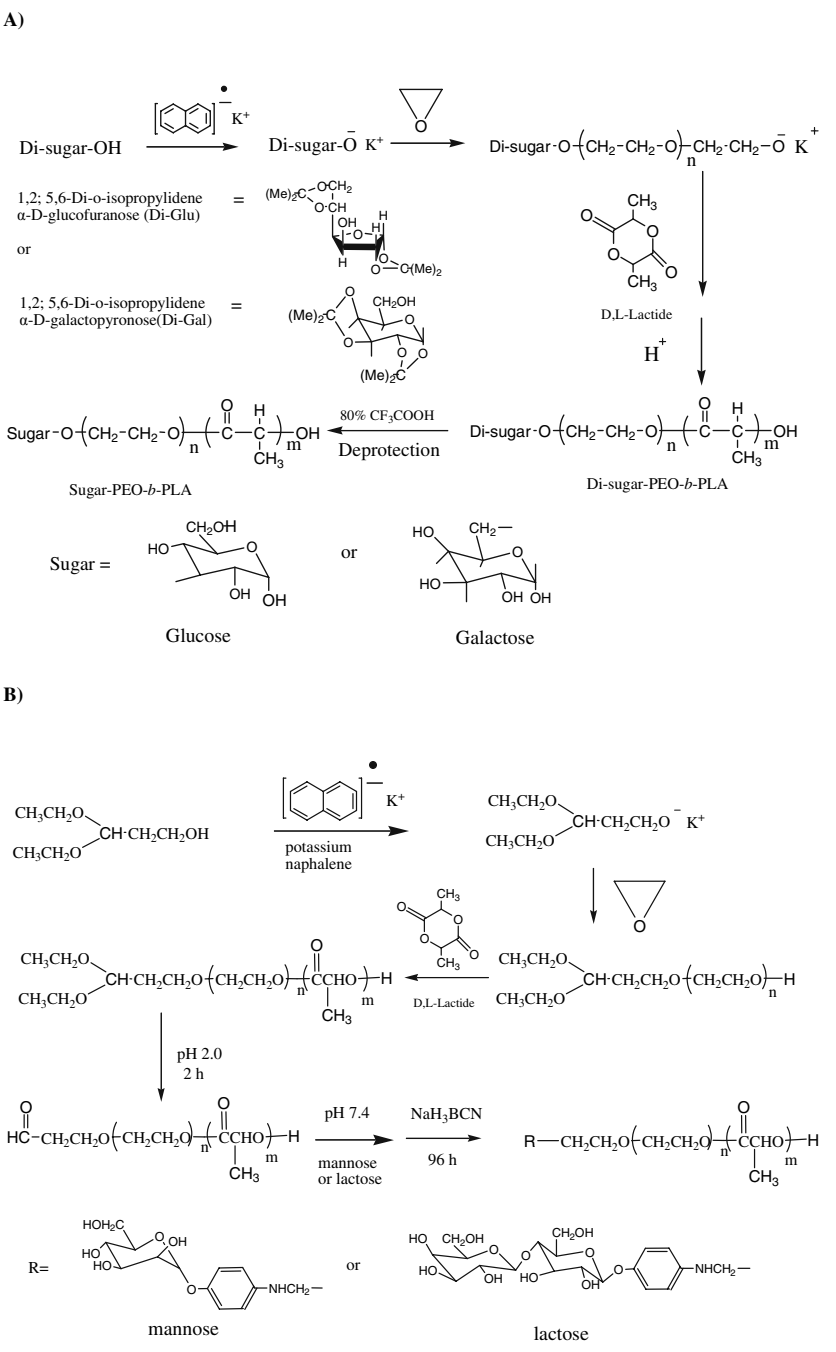


Figure 13.16 Synthesis of carbohydrate-conjugated PEO-*b*-PDLA through (**A**) pre- and (**B**) post-polymerization approaches.

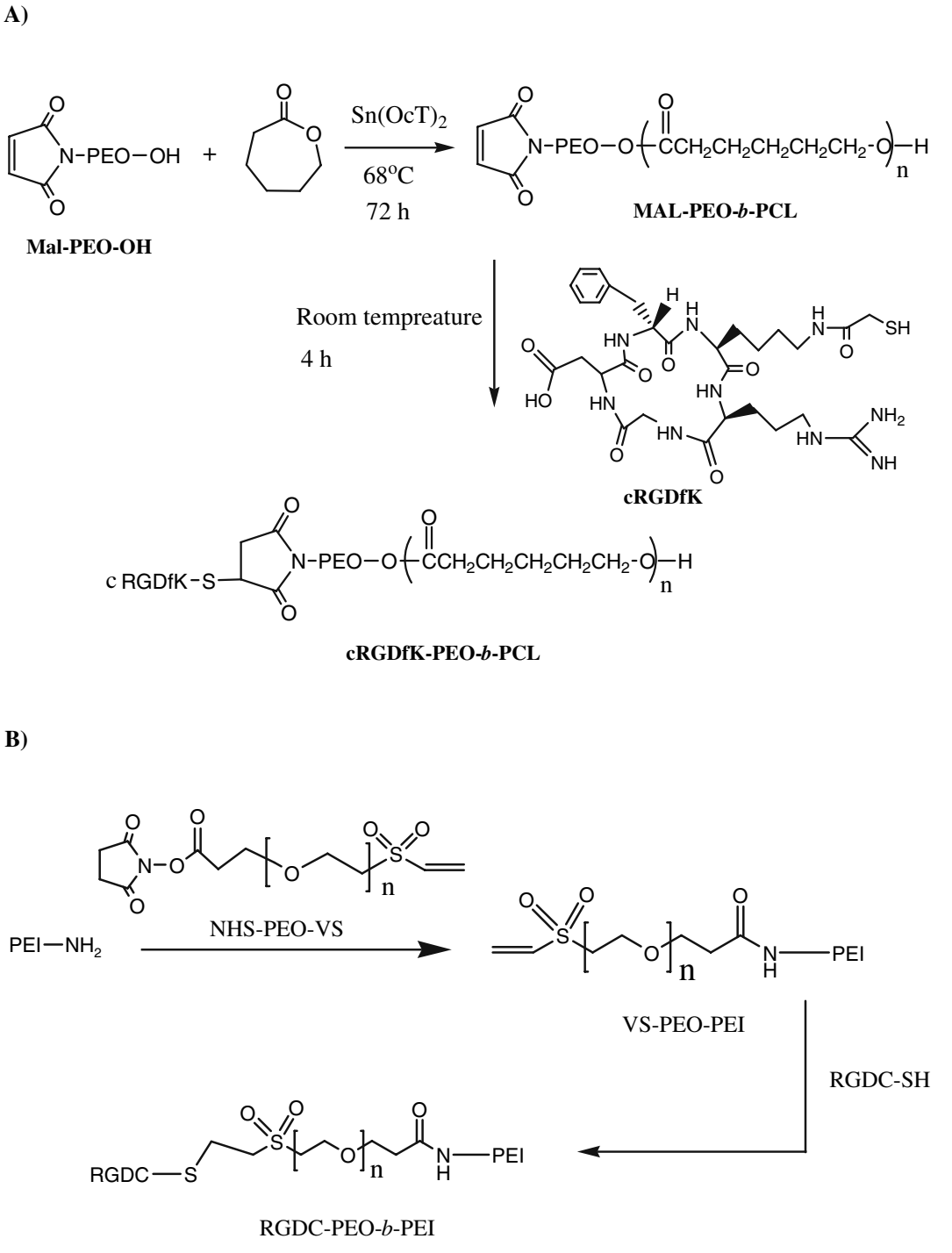


Figure 13.17 Synthesis of (**A**) cRGDfK-PEO-*b*-PCL and (**B**) RGDC-PEO-*b*-PEI copolymers.

factor (EGF) to the PEO end of PEO-*b*-PEI by the reaction of thiol group on the EGF and maleimide on the PEO section for targeted gene delivery has also been reported (Ogris et al., 2003). Using the heterofunctional PEO, i.e., VS-PEO-NHS, RGDC-conjugated PEO-*b*-PEI was synthesized for gene delivery (Figure 13.17B) (Kunath et al., 2003).

Amine group in the N-terminus of peptide has also been used for peptide conjugation to block copolymer micelles. Small peptides were coupled to acetal-terminated PEO-*b*-PDLA micelles to modify their surface charge by Kataoka et al. (Yamamoto et al., 1999). Our group has synthesized acetal-terminated PEO-*b*-PCL through anionic ROP of γ-caprolactone by acetal-PEO (Figure 13.18A) (Xiong et al., 2007a). After formation of micelles, the acetal group was converted to aldehyde at acidic pH and used for the conjugation of GRGDS to micellar surface by Schiff base reaction between the aldehyde group on the PEO chain and the amine group on the peptide. To extend the research, the more versatile polymer, acetal-PEO-*b*-PBCL block copolymer, was synthesized and then reduced in the presence of H_2 to produce acetal-PEO-*b*-PCCL. This novel polymer was aimed to covalently couple ligands to the shell-forming block and anticancer drugs to the core-forming block. The anticancer drug, DOX, was then covalently conjugated to the free-side COOH groups on the PCCL block by an amide bond to form acetal-PEO-*b*-PCL-DOX. After conversion of the acetal group into aldehyde, RGD-containing peptides such as GRGDS and ACDCRGDCFCG (RGD4C) (unpublished data) were attached to the surface of aldehyde-PEO-*b*-PCL-DOX micelles (Figure 13.18B). RGD peptide-modified micelles bearing conjugated DOX demonstrated higher cytotoxicity on B16-F10 cells than the control conjugate due to better uptake by the cells (Xiong et al., 2007b).

Preparation of shell-modified micelles for gene delivery has also been reported. For the conjugation of transferrin (Tf), a ligand that binds ubiquitously to a wide variety of cells, to PEO-*b*-PEI, the branched PEI was preactivated by succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to introduce thiol groups. Amino groups of Tf was then reacted with heterobifunctional PEO (α-maleimide-ω-*N*-hydroxysuccinimide ester, MAL-PEO-NHS) to form Tf-PEO conjugate. The maleimide group at the distal end of the PEO chain in the Tf-PEO conjugate was then reacted with the thiol-functionalized PEI. The resulting Tf-PEO-*b*-PEI polymer after purification contained approximately one Tf ligand per two PEI molecules (Figure 13.19A). In the same study, a separate conjugation method was used to synthesize Tf-PEO-*b*-PEI polymers with linear PEI. In this method, both PEI and Tf were modified separately with iminothiolane. The modified transferrin was reacted with bifunctional PEO derivative PEO-bis-orthopyridyl-disulfide. The product was subsequently reacted with the sulfhydryl groups of the iminothiolane-modified PEI. The purified conjugate contained approximately one Tf ligand to one PEI molecule (Figure 13.19B) (Kursa et al., 2003).

Monoclonal antibodies are another promising class of tumor-targeting ligands. Similar to peptide conjugation, thiol, amine, or the carboxyl in the antibodies may be used for conjugation reaction. One typical example is the attachment of C225, i.e., the antibody against epidermal growth factor (EGF) receptors, to the PEO terminus of a PEO-*b*-p(L-Glu)-DOX using the reaction between thiol and vinylsulfone (Figure 13.20) (Vega et al., 2003).

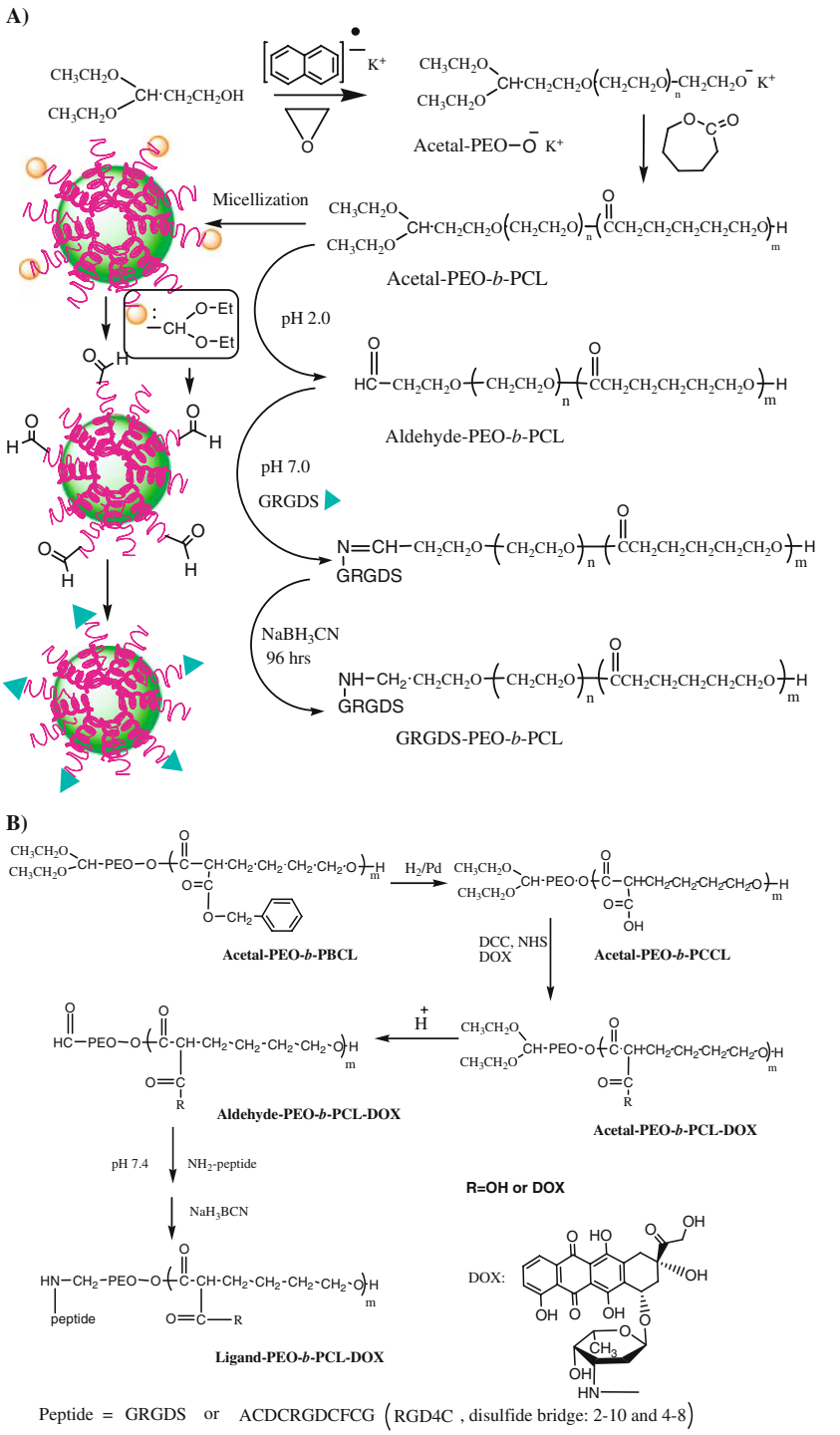


Figure 13.18 Preparation of (**A**) GRGDS-*b*-PEO-PCL (reproduced from Xiong et al., 2007 with permission) and (**B**) GRGDS-PEO-*b*-PCL-DOX block copolymers and micelles. (*See* Color Plate 18)

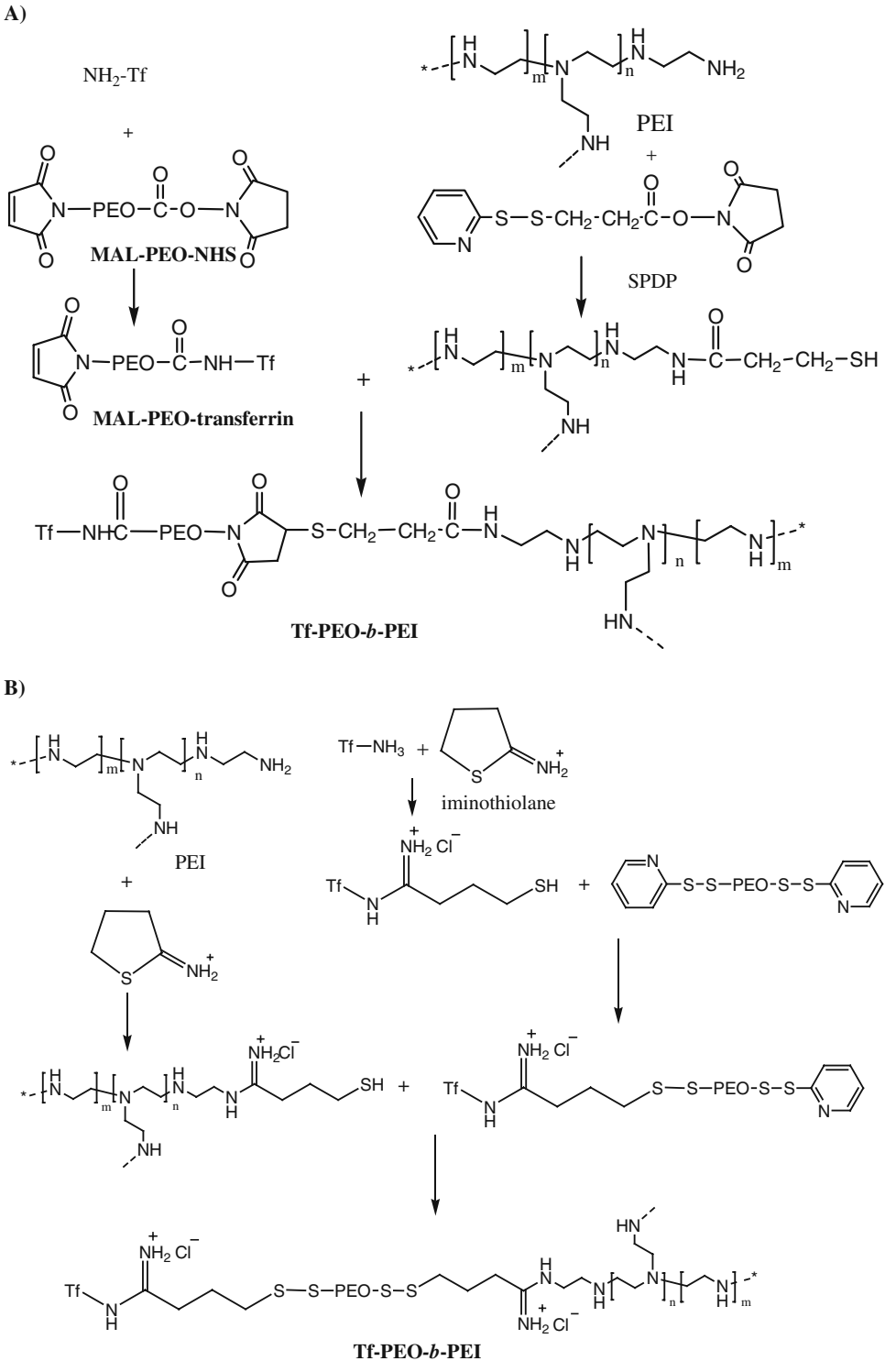


Figure 13.19 Conjugation of Tf to PEO-*b*-PEI.

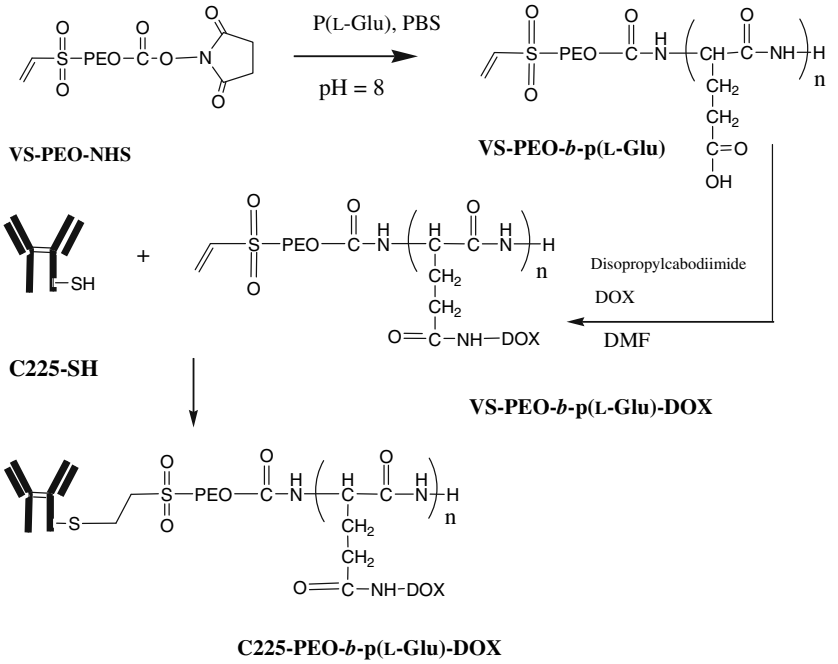


Figure 13.20 Synthesis of C225-PEO-*b*-p(L-Glu)-DOX and micelles with chemically loaded anticancer drugs (reproduced from Vega et al., 2003 with permission).

Conclusion

In this chapter, we provided an update on several chemical strategies used to enhance the properties of nanoscopic core/shell structures formed from self-assembly of amphiphilic block copolymers, namely polymeric micelles. Clearly, versatility of polymer chemistry in amphiphilic block copolymers provides unique opportunities for tailoring polymeric micelles for optimal properties in gene and drug delivery. Chemical modification of the polymer structure in the micellar core through introduction of hydrophobic or charged moieties, conjugation of drug compatible groups, core cross-linking has led to enhanced stability for the micellar structure and sustained or pH-sensitive drug release. The modification of polymeric micellar surface with specific ligands (carbohydrates, peptides, antibodies) has shown benefit in enhancing the recognition of carrier by selective cells leading to improved drug and gene delivery to the desired targets. Research in drug delivery by polymeric vesicles is still in its infancy, but a similar principle on the importance and benefit of chemical flexibility of block copolymers in improving the delivery properties of polymeric vesicles can also be envisioned. The demanding challenge of the future research in this field is to find the right carrier architecture and optimum polymer chemistry that can improve the delivery of sophisticated and complex therapeutic agents (e.g., poorly soluble drugs, proteins, and genes) to their cellular and intracellular targets.

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