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REVIEW

Polymeric micelles from poly(ethylene glycol)–poly(amino acid) block copolymer for drug and gene delivery

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Dramatic advances in biological research have revealed the mechanisms underlying many diseases at the molecular level. However, conventional techniques may be inadequate for direct application of this new knowledge to medical treatments. Nanobiotechnology, which integrates biology with the rapidly growing field of nanotechnology, has great potential to overcome many technical problems and lead to the development of effective therapies. The use of nanobiotechnology in drug delivery systems (DDS) is attractive for advanced treatment of conditions such as cancer and genetic diseases. In this review paper for a special issue on biomaterial research in Japan, we discuss the development of DDS based on polymeric micelles mainly in our group for anti-cancer drug and gene delivery, and also address our challenges associated with developing polymeric micelles as super-functionalized nanodevices with intelligent performance.

Keywords: nanomedicine; drug delivery systems; polymeric micelle; non-viral gene delivery; block copolymer; cancer therapy

1. INTRODUCTION

Biomaterials have changed medicine in both qualitative and quantitative manners through application to medical technologies such as artificial organs, which partially replace the functions of the original organs. Biomaterials have also received much attention in the field of pharmacy for the formulation of drug delivery systems (DDS). Recently, the trend in materials research in the DDS field has been shifting towards the development of materials with 'active' functionality exerting the ability to change their properties responding to specific stimuli. In this regard, materials design based on the nanometre-scaled control of the alignment and assembly of atoms and molecules, termed 'nanotechnology', becomes an important concept for constructing effective devices suitable for smart active functions. Application of nanotechnology-based biomaterials to DDS has been strenuously promoted in a global manner through research projects such as the National Nanotechnology Initiative and the Cancer Nanotechnology Plan in the USA, the Nanomedicine Project in Europe and the Nano-DDS project in Japan. It should be noted that a new class of drugs namely 'biopharmaceutics' consisting of peptides, proteins and nucleic acids has emerged. To apply such biologically fragile compounds as medicine, development of proper carrier systems is necessary. In this review for the special issue of *J. R. Soc. Interface* focusing on biomaterials research in Japan, we mainly overview our long-term achievements in the study on polymeric micelles from poly(ethylene glycol) (PEG)–poly(amino acid) block copolymers for drug and gene delivery.

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One contribution of 10 to a Theme Supplement 'Japanese biomaterials'.

2. DRUG DELIVERY SYSTEMS

Once a drug is administered into the body by injection, it circulates throughout the body; but it is then quickly eliminated by hepatic metabolism and renal excretion. Owing to such an elimination, only a limited portion of the dose reaches the target sites and thus the medicinal effect is reduced. Moreover, the portion of the dose that does not reach the target can be toxic to off-target tissues, leading to undesired side effects. For optimal medicinal effect, the drug concentration must be maintained at an effective dose at the target sites for a certain duration, with minimal dose accumulation at off-target sites; thus, pinpoint therapies allowing control of drug distribution while suppressing side effects are desired in advanced medicine. For these issues, careful design of multifunctional drug carriers with nanoscale

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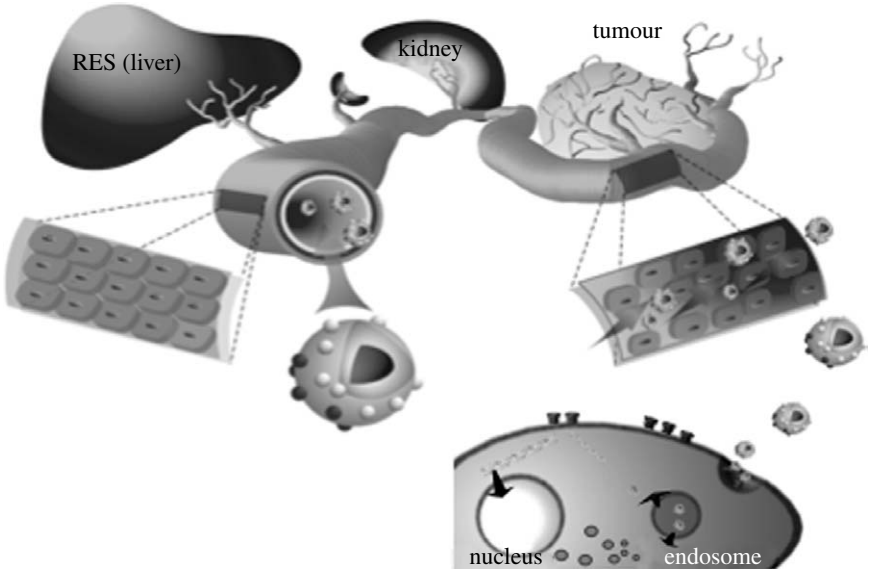

Figure 1. Schematic of the biological distribution for carriers administered intravenously. The carriers are required to circulate in the bloodstream by avoiding RES recognition and glomerular excretion by the kidney, and finally must extravasate to the target tissue through the hyperpermeable region of the capillaries (EPR effect). Carrier uptake occurs by non-specific or receptor-mediated endocytosis, followed by transport into the endosome. The delivered drugs and genes should be released into the cytosol before lysosomal degradation.

dimension has become a popular research subject. Such advanced drug carriers can be intelligently designed based on the inherent properties of the target site such as pH, presence of specific enzymes and tissue-specific markers. In this regard, studies on DDS designed to control drug action in terms of timing, location and dose have been progressively demonstrated (Jones & Leroux 1999; Torchilin 2001; Kabanov *et al.* 2002; Lavasanifar *et al.* 2002; Duncan 2003; Haag 2004). For example, in a typical DDS with controlled drug-releasing function, drugs are released slowly and passively from a certain substrate to maintain a desired blood concentration, or are actively released from prodrugs, in which drugs are activated only at target sites by site-specific catabolization. Studies on such 'nano-DDS' in Japan have been integrated into the field of materials engineering, which is one of Japan's strongest technologies. Recently, nano-DDS has been assigned as a subject of cooperative research by several Japanese ministries and consists of a number of ongoing projects.

Several obstacles pose a challenge for selective delivery of drugs to desired sites such as solid tumours, as shown in figure 1. The first important issue for the carrier is stability during the course of blood circulation. Drugs must be stably loaded into the carrier without leakage or catabolism by enzymes in the blood. Carriers must avoid glomerular excretion by the kidney and avoid uptake by the reticuloendothelial systems (RES) in the liver, spleen and lung. Since there is a molecular weight threshold for glomerular filtration (42 000–50 000 for water-soluble synthetic polymers), elimination by this mechanism can be avoided by increasing the molecular weights of the carriers. Carriers in the blood circulation may also induce non-specific complement activation and opsonization, resulting in the drug's elimination from the blood compartment due to RES recognition. In this regard, it is crucial to modify the carrier surface with biocompatible materials such as PEG to provide a 'stealth' characteristic. The second challenge for DDS is that the carriers must permeate tumour blood vessels to access the target tissue. One of the most important advantages of macromolecular carriers is their preferential accumulation in solid tumours. Such an elevated tumour accumulation is facilitated by microvascular hyperpermeability and impaired lymphatic drainage in tumour tissues, known as the 'enhanced permeability and retention (EPR) effect' (Matsumura & Maeda 1986). In this case, the carriers should be small enough (less than 100 nm) to allow effective extravasation from the blood compartment into solid tumours. The EPR effect found by Matsumura and Maeda has become the rule of thumb in 'passive targeting'. The third challenge is selective uptake into the target cell. In this regard, an attractive strategy is 'active targeting', which exploits cell surface receptors by installing moieties onto carriers that specifically recognize target cells. Finally, controlled intracellular trafficking or organelle targeting is important to enhance medicinal effects, such as nuclear targeting in the case of gene delivery. Carriers ranging from 5 to 100 nm in size are internalized into cells by the endocytotic pathway, where endosomes and encapsulated carriers are separated from the cell membrane by a process of inward folding. These endosomes have an acidic pH value (pH$\sim$5.5) and eventually fuse with lysosomes, where drugs can be degraded by a host of lysosomal enzymes. Therefore, careful modulation of both size and surface properties is required for functionality and pinpoint targeting, which allow for the maximum medicinal effect to be realized. As a result, nanobiotechnology-based carriers integrating specific performance enhancing functions have been demonstrated, resulting in creative and innovative supramolecular nanodevices (Nishiyama & Kataoka 2006; Osada & Kataoka 2006).

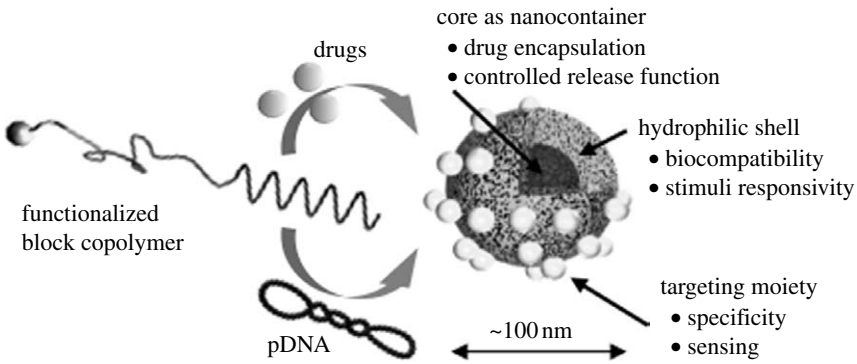

Figure 2. Design of a multifunctional delivery system based on polymeric micelles formed with block copolymers capable of encapsulating drugs and pDNA.

3. POLYMERIC MICELLES FOR DRUG DELIVERY SYSTEMS

In aqueous media, amphiphilic block copolymers, which have a large solubility difference between the hydrophilic and hydrophobic segments, spontaneously form polymeric micelles with a distinct core–shell structure in which a hydrophobic inner core is surrounded by a hydrophilic shell (figure 2; Kataoka *et al.* 1993). PEG is often used as the hydrophilic segment by virtue of its biocompatibility. A polar PEG shell of the micelle facilitates solubility in water through steric stabilization and also provides biocompatibility and a stealth effect on the polymeric micelles, since PEG prevents the adsorption of proteins and is generally believed to be non-interactive with biological components (Jeon *et al.* 1991; Otsuka *et al.* 2001).

Systematic control of the core-forming block structure provides micelle stability and a wide variety of materials for drug loading, release and activation. Thermodynamic and kinetic stability of the polymeric micelles is an important issue in their application to systemic administration, because their fast dissociation in the blood compartment results in the burst release of loaded drugs, inducing systemic toxicity. In contrast to micelles from small surfactant molecules, polymeric micelles are generally more stable and can retain the loaded drug for a prolonged period of time even in a very diluted condition in the body due to appreciably lowered critical micelle concentration, particularly for polymeric micelles from highly regulated block copolymers with distinct core–shell structure (Kwon *et al.* 1993). Functional groups, such as amines and carboxylic acids in the core-forming segments, are useful for introducing drugs into the micelle core. In this sense, PEG–polypeptide block copolymers, prepared by the polymerization of the appropriate *N*-carboxyanhydrides from a terminal amine on PEG, are advantageous because a series of block copolymers with different functional groups in the side chain can be prepared from the same platform. To facilitate micelle formation, a combination of intermolecular forces is available, such as hydrophobic interactions (Bader *et al.* 1984; Yokoyama *et al.* 1989; Gref *et al.* 1994), electrostatic interactions (Harada & Kataoka 1995, 1999; Kabanov *et al.* 1996; Kataoka *et al.* 1996; Harada & Kataoka 1999), metal complexation (Yokoyama *et al.* 1996; Nishiyama *et al.* 1999) and hydrogen bonding (Kataoka *et al.* 1998). The variation of the intermolecular force of core-forming segments enables one to regulate micelle stability for prolonged circulation as well as for controlled drug release property.

The sizes of these micelles are determined by thermodynamic parameters and are typically in the size range of several tens of nanometres with a relatively narrow size distribution, similar to the size range of viruses and lipoproteins. Owing to the combination of the high molecular weight and biocompatibility of polymeric micelles, avoidance of renal glomerular filtration and RES uptake is expected, thus providing longevity in blood circulation. This characteristic of polymeric micelles may facilitate tumour accumulation by the EPR effect, namely passive targeting (Matsumura 2008). Furthermore, functionalization of the distal end of PEG chains allows for incorporation of targeting ligands or other biomarkers into the micelle shell, providing control of biodistribution and site-specific cellular uptake, namely active targeting. These polymeric micelle systems may produce highly effective therapeutics, and are also expected to provide a framework for the design of multifunctional and intelligent nanodevices having combined ability for detection, diagnosis, analysis and therapy in a single platform (Pack *et al.* 2005; Mastrobattista *et al.* 2006; Sutton *et al.* 2007; Torchilin 2007; Davis *et al.* 2008).

3.1. Polymeric micelles delivering anti-cancer drugs

The first polymeric micelle developed in our group was one loaded with the anti-cancer drug doxorubicin (Dox). It was found that Dox-conjugated PEG-*b*-poly(aspartate) (PEG–PAsp) block copolymers spontaneously formed polymeric micelles in aqueous media (figure 3*a*; Yokoyama *et al.* 1987, 1989, 1990). Here, Dox was covalently conjugated to side chains of the poly(aspartate) (PAsp) segment by an amide bond between the carboxylic group in PAsp and the primary amino group of the glycosidyl residue in Dox. This conjugation provided sufficient hydrophobicity in the PAsp segment due to the hydrophobicity of Dox, thus forming a stable inner core of polymeric micelles with diameters of several tens of nanometres.

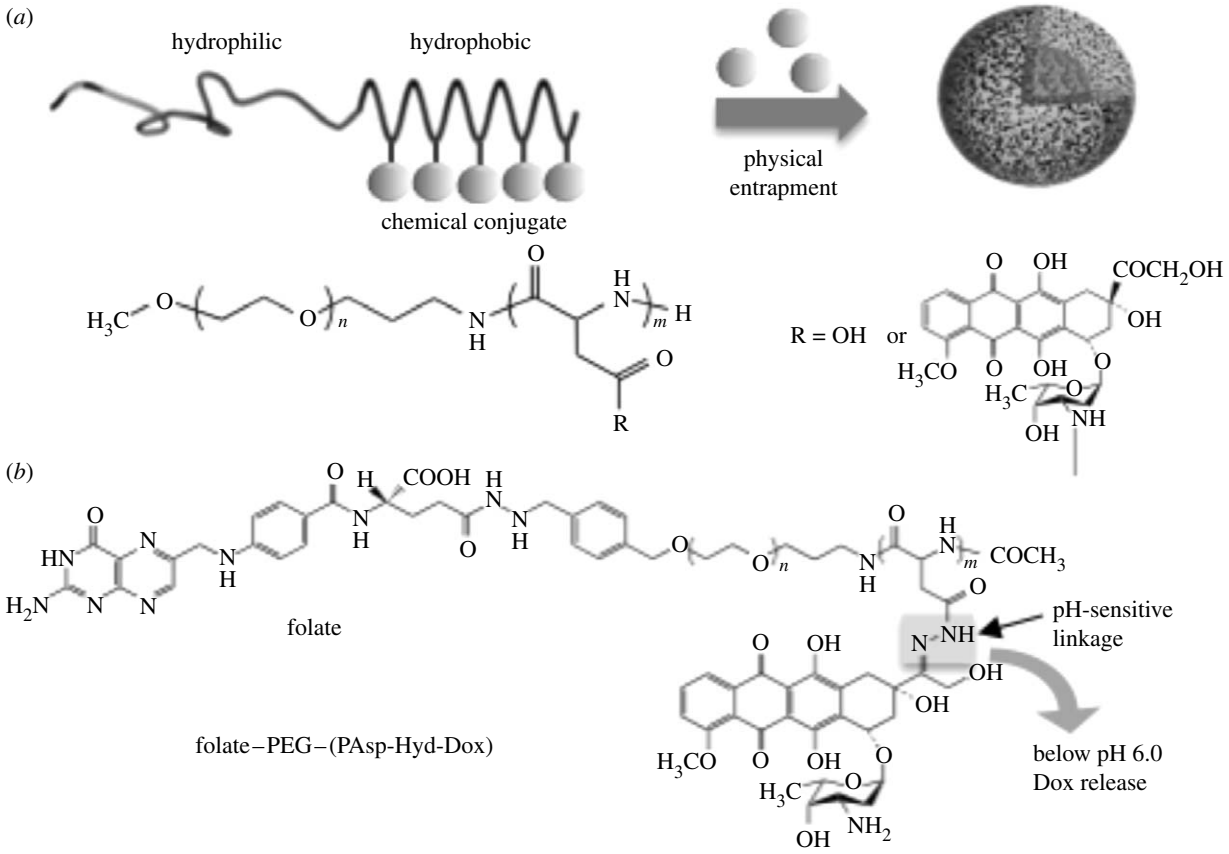

Figure 3. Dox-loaded polymeric micelles prepared by hydrophobic interaction. (*a*) 'First-generation' Dox-loaded micelle comprising PEG–PAsp block copolymer with chemically conjugated Dox. Micelle formation occurs spontaneously and additional Dox is physically entrapped within the hydrophobic core. (*b*) 'Second-generation polymeric micelle' prepared with multifunctionalized block copolymer containing a folate ligand and a pH-sensitive drug linkage for active targeting and selective intracellular Dox release.

The dissociation rate of the micelle in phosphate-buffered saline was estimated to be of the order of days and was quite slow even in the presence of 50 per cent rabbit serum (Yokoyama *et al.* 1993). A Dox molecule can be physically entrapped in the micellar core by hydrophobic interaction with conjugated Dox in a PAsp segment. It should be noted that physically entrapped Dox exerts the major cytotoxic function, while the conjugated Dox works mainly to increase the micelle stability (Yokoyama *et al.* 1993). The biodistribution of the PEG–PAsp(Dox) micelle was investigated by radiolabelling the PAsp(Dox) segment of the block copolymer. The micelle showed remarkably prolonged blood circulation, such that a quarter of the injected dose remained in the blood at 24 hours, whereas free Dox disappeared immediately from the blood in a few minutes (Kwon *et al.* 1994; Yokoyama *et al.* 1999). The PEG–PAsp(Dox) micelle effectively accumulated into a subcutaneously inoculated tumour (murine colon adenocarcinoma (C-26)), and eventually Dox in the micelle (total of conjugated and physically entrapped Dox) exhibited a 7.4-fold higher tumour accumulation than free Dox at 24 hours due to the EPR effect, achieving significantly higher *in vivo* anti-tumour activity against C-26 than free Dox (Yokoyama *et al.* 1991). Furthermore, the efficacy of the PEG–PAsp(Dox) micelle was further improved by controlling block copolymer composition and the dose of Dox, providing a complete cure of the C-26 tumours. The optimized PEG–PAsp(Dox) micelle is currently under phase II clinical trial as NK911 in Japan. Similarly, paclitaxel was also incorporated into the micelle core by hydrophobic interaction. NK105, a paclitaxel-incorporating micelle, proved to have higher efficacy with fewer side effects than free paclitaxel (Hamaguchi *et al.* 2005). The phase I clinical trial showed that NK105 can be administrated safely as a short infusion without the support of anti-allergic agents (Hamaguchi *et al.* 2007). A phase II study is now underway in Japan in patients with advanced stomach cancer. 7-Ethyl-10-hydroxy-camptothecin (SN-38), a water-insoluble anti-cancer drug that is a biologically active metabolite of irinotecan hydrochloride (CPT-11), was also loaded into a polymeric micelle of PEG–poly(glutamate) (PEG–PGlu) by hydrophobic interaction (Koizumi *et al.* 2006). The SN-38-loaded micelle showed markedly enhanced anti-tumour activity compared with free drug, and is thus a promising SN-38-based formulation. Note that the SN-38-loaded polymeric micelle was also effective against metastasis (Sumitomo *et al.* 2008). This micelle system is currently being tested in a phase I clinical trial as NK012 in Japan and in the USA.

Micelle formation is also driven by metal complexation as in the case of cisplatin (CDDP)-containing micelles. CDDP is a well-known platinum metal complex exhibiting a wide range of anti-tumour

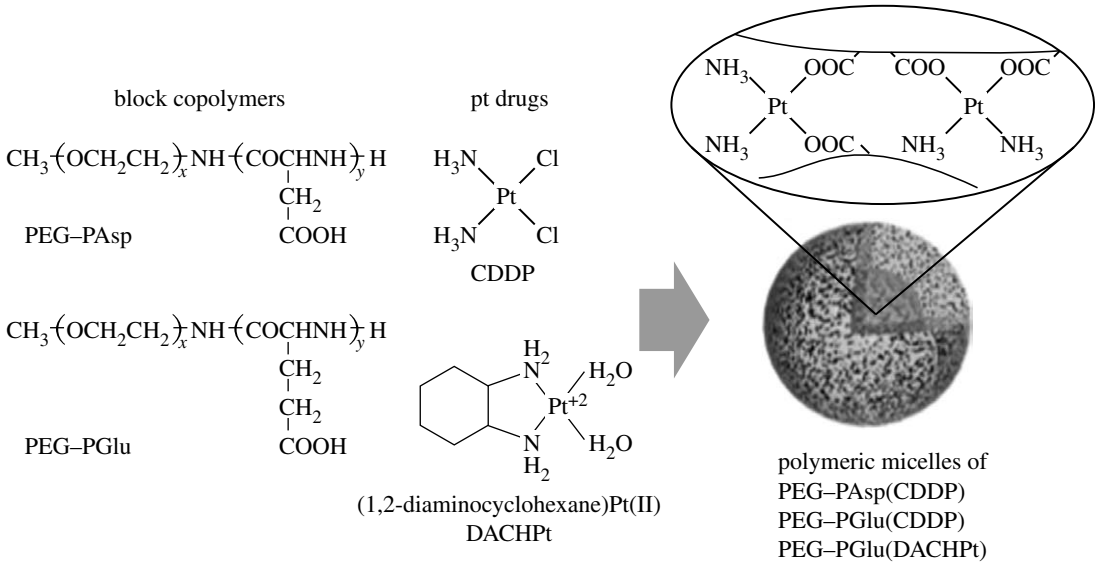

Figure 4. Platinum drug-loaded polymeric micelles formed by metal complexation. Carboxylic groups of PEG–PAsp or PEG–PGlu block copolymers are linked with the platinum of CDDP or DACHPt through coordination bonds.

activities (Rosenber *et al.* 1969). However, its clinical use is limited due to its significant toxic side effects, such as acute nephrotoxicity and chronic neurotoxicity. CDDP shows a rapid distribution over the whole body and high glomerular clearance within 15 min after intravenous injection. Therefore, many efforts have been devoted to develop a DDS aimed at increasing the blood circulation period and accumulation in solid tumours (Bogdanov *et al.* 1997; Gianasi *et al.* 1999). We introduced CDDP into a micelle system by metal complexation between platinums of CDDP and carboxyl groups of PEG–PAsp (figure 4). The complex spontaneously formed a polymeric micelle with a very narrow size distribution (Yokoyama *et al.* 1996). The PEG–PAsp(CDDP) micelles showed environmentally responsive drug-release behaviour in response to salt concentration; they were stable in distilled water at room temperature, yet the loaded drug was sustainably released for over 50 hours as a result of exchange of chloride ion with CDDP in 150 mM NaCl (Nishiyama *et al.* 2003). The PEG–PAsp(CDDP) micelle exhibited a sixfold higher accumulation in tumour sites compared with free CDDP at 8 hours. Nevertheless, the *in vivo* anti-tumour activity was only slightly higher than that of the free CDDP for the same dose (Nishiyama *et al.* 2001). The micelle composition was then further modified to regulate CDDP release and extend the blood circulation time by using PEG–PGlu instead of PEG–PAsp. This modification was aimed at increasing micelle stability with more hydrophobic PGlu side chains, which contain an additional CH_2 (figure 4). The PEG–PGlu(CDDP) micelle showed more sustained release of CDDP (half-value period: more than 90 hours) than the PEG–PAsp(CDDP) micelle (half-value period: approx. 30 hours) with a longer induction period (PEG–PGlu(CDDP): more than 20 hours; and PEG–PAsp(CDDP): approx. 10 hours) under physiological conditions (Nishiyama *et al.* 2003). Biodistribution of the PEG–PGlu(CDDP) micelle revealed a high plasma platinum level with a longer persistent time (11% of the injected dose at 24 hours) than the PEG–PAsp(CDDP) micelle (1.5% at 24 hours) with decreased accumulation in the liver and spleen. As a consequence of the longer circulation period, the tumour accumulation of the PEG–PGlu(CDDP) micelle was 20-fold higher than that of the free CDDP, indicating tumour-selective targeting due to the EPR effect. Treatment of tumour-bearing mice with the PEG–PGlu(CDDP) micelle by intravenous injection achieved complete tumour regression for five out of six mice, with only minimal body weight loss (within 5% of the initial weight). By contrast, treatment with free CDDP at the same drug dose exhibited tumour regression for only one mouse out of six and significant body weight loss (20% of the initial weight). The PEG–PGlu(CDDP) micelle (Uchino *et al.* 2005) is currently undergoing a phase I clinical trial as NC-6004 in the UK.

3.2. Polymeric micelles delivering therapeutic genes

Recent progress in the understanding of the biological mechanisms driving life processes at the molecular level has led to the development of novel nucleic acid-based therapies such as plasmid DNA and siRNA as innovative medicines. Their clinical application, however, is hindered particularly by their instability under physiological conditions as well as low cellular uptake efficiency due to their large molecular weight and anionic nature. When DNA or RNA is directly administered into the blood stream, it is rapidly eliminated, mainly by DNase and RNase attacks. Thus incorporating DNA or RNA into appropriate nanocarriers is necessary for their practical use. For gene therapy, intracellular delivery to the nucleus is required in addition to accumulation within desired tissues. Owing to these crucial requirements, viral vectors such as retroviruses, adenoviruses and adeno-associated vectors have been commonly used as gene carriers in clinical trials for gene therapy. However, problems associated with immune response (Lehrman 1999; Marshall 1999) as well as the possibility of recombination with endogenous genes leading to

oncogene effects (Cavazzana-Calvo *et al.* 2000; Aiuti *et al.* 2002; Hacein-Bey-Abina *et al.* 2003) prevent their use in clinical treatments. From the standpoint of preparation, viral vectors are not well suited for efficient mass production and thus are limited in clinical applications.

On the other hand, non-viral vectors composed of polymers or lipids offer a superior alternative in terms of safety, mass preparation and cost. With these incentives in mind, the research and development of safe and highly functionalized non-viral vectors are being undertaken worldwide (Gebhart & Kabanov 2001; Merdan *et al.* 2002; Niidome & Huang 2002; Glover *et al.* 2005; Wagner & Kloeckner 2006) and also in Japan (Kawano *et al.* 2004; Kogure *et al.* 2008). In this regard, a polymeric micelle system would be a promising formulation even for delivering nucleic acids due to its advanced and tunable characteristics. A polymeric micelle comprising nucleic acids is formed by polyion complexation between anionically charged DNA or RNA and a block copolymer having a hydrophilic segment and a cationic segment (figure 2; Kataoka *et al.* 1996; Katayose & Kataoka 1997; Itaka *et al.* 2004; Matsumoto *et al.* 2009). Complexation of plasmid DNA (pDNA) with a PEG–polycation such as PEG–poly(lysine) (PEG–PLys) occurs spontaneously, resulting in a polyion complex micelle (PIC micelle or polyplex micelle) with a size of approximately 100 nm (Katayose & Kataoka 1997, 1998; Oupicky *et al.* 2000). The polyplex micelles exhibit a near neutral zeta potential due to the PEG shell even in the presence of an excess amount of PEG–polycations (Itaka *et al.* 2003). Therefore, non-specific interaction with serum proteins or cells in the blood compartment is expected to be suppressed. Eventually, long-circulation property and thus tumour accumulation by the EPR effect will be readily expected. Polyplex micelles containing pDNA indeed exhibited efficient gene introduction in cultured cells, and also showed gene expression in the liver following intravenous injection into a mouse tail vein (Harada-Shiba *et al.* 2002). By packaging DNA into a polymeric micelle, prolonged blood circulation was achieved in which pDNA remained intact after 3 hours, whereas naked pDNA was immediately degraded by nucleases in a few minutes. These results demonstrate that polymeric micelles exhibit great feasibility as gene carriers as well.

4. EVOLUTION OF POLYMERIC MICELLES INTO SUPRAMOLECULAR MULTIFUNCTIONAL NANODEVICES

The polymeric micelles described above may be considered 'first generation' in the sense that they are monofunctional for the most part. A delivery system with multifunctions is not just a carrier, but rather an innovative supramolecular 'nanodevice' in which loaded materials and the carrier are integrated structurally and functionally in a nanometre scale. In this regard, smart polymeric micelles designed with stimuli-responsive properties may improve the therapeutic index towards solid tumours and to reduce side effects. For example, differences in the concentrations of reductive agents such as glutathione between the outside and the inside of a cell and pH reduction in endosomes are feasible targets for chemical differences. Nanocarriers taken into cells via endocytosis are compartmentalized in endosomes (pH$\sim$5.5) where the proton concentration increases to approximately 100-fold relative to the extracellular environment (pH 7.4). Thus, drug carriers with pH-sensitive linkages based on hydrazone, *cis*-aconityl or acetal groups that degrade at low pH have been prepared (Christie & Grainger 2003). Glutathione is the most abundant reducing agent in the cytoplasm present in mM concentrations inside the cell and only in μM concentrations in the blood compartment (Meister & Anderson 1983). The disulphide bond is known to be stable in the extracellular environment, yet is readily cleaved inside the cell due to the increased concentration of glutathione, thus providing another chemical target allowing for site-specific release.

In contrast to the concept of passive targeting mainly by the EPR effect, the concept of active targeting using specific ligands is an attractive strategy to increase delivery efficiency and to decrease side effects. For such specific ligands, antibodies or their fragments, transferrin, folate, sugars and peptides are often considered (Davis *et al.* 2008). Cancer-specific antibody is a promising class of tumour-targeting ligands due to its high binding affinity (Torchilin *et al.* 2003). Transferrin, an iron-binding glycoprotein, is a well-studied ligand for tumour targeting (Schmidt *et al.* 1986; Sorokin *et al.* 1989). In rapidly dividing cells, such as malignant cells, transferrin receptor expression on their surfaces is elevated due to an increased cellular demand for iron. For cell-specific delivery towards liver parenchymal cells, a galactose moiety, which is recognized by the asialoglycoprotein (ASGP) receptors, may be introduced onto the surfaces of the carriers (Wu & Wu 1988; Yasugi *et al.* 1999; Nagasaki *et al.* 2001; Jeong *et al.* 2005), since a large number of cell surface receptors that bind and subsequently internalize ASGP are extensively expressed in hepatocytes. Folate is also a promising candidate (Yoo & Park 2004), because the folate-binding receptor is overexpressed in a large fraction of human tumours, but is only minimally distributed in normal tissue (Weitman *et al.* 1992). Macrophages can be a target for gene therapy in diseases such as Gaucher's disease and human immunodeficiency virus infection. In this case, mannose ligands are used because of the large numbers of mannose receptors expressed on the surfaces of macrophages. Dendritic cells also express a large number of mannose receptors, a feature exploited for the delivery of mannosylated carriers containing antigen-coded pDNA (Wickham 2003). Small peptides have also been used as ligands for cancer targeting. cRGD peptide has a high affinity to the $\alpha_v\beta_3$ integrin receptor, a cellular transmembrane protein overexpressing in angiogenic vessels, and is often used as such a ligand (Wermuth *et al.* 1997; Nasongkla *et al.* 2004). Artery wall-binding peptide is of interest for effective targeting of gene into artery wall cells (Nah *et al.* 2002). Note that modulation of the surface charge of nanocarriers by peptide conjugation is feasible for regulating their biodistribution after systemic administration (Yamamoto *et al.* 1999).

4.1. Polymeric micelles delivering anti-cancer drugs

Versatile design and engineering of block copolymers enable preparation of polymeric micelles with targetability to specific tissues. Targeted anti-cancer micelles were prepared by conjugating folate to the distal end of PEG–PAsp block copolymer aimed at increasing tumour accumulation (figure 3*b*). The folate-conjugated polymeric micelle loaded with Dox indeed showed significantly increased cellular uptake. Cytotoxicity analysis *in vitro* indicated that the cell growth-inhibitory activity of the folate-conjugated micelle was enhanced, suggesting that this could be an effective approach for ligand-mediated uptake for cancer treatment (Bae *et al.* 2005*a*).

The folate-conjugated polymeric micelle was also equipped with additional functionality, allowing for site-specific drug release, exploiting the difference in proton concentration between the extracellular environment and endosomal environments. Dox was conjugated to the core-forming PAsp segment of the PEG–PAsp through a hydrazone bond, which is stable under physiological conditions but cleavable under acidic intracellular environments of endosomes and lysosomes (figure 3*b*; Bae *et al.* 2003*a*,*b*). Indeed, the intracellular release of conjugated Dox from the micelle was confirmed by confocal laser scanning microscopy using an *in vitro* tumour model of multicellular tumour spheroids of a C26 cell line (Bae *et al.* 2005*b*). In animal tests, pH-sensitive micelles showed effective anti-tumour activity to suppress tumour growth in mice over a broad range of injection doses, whereas toxicity remained extremely low. The micelles were safe when injected at doses up to 40 mg kg^{-1} with three out of six mice completely cured and no toxic deaths among the treated mice having occurred. This is in sharp contrast to the case of free Dox, in which tumour growth was suppressed by a 10 mg kg^{-1} dose, but body weight fell substantially due to toxicity. Moreover, all of the mice treated with a 15 mg kg^{-1} dose of Dox experienced toxic death. The therapeutic efficacy of the micelles designed for site-specific targeting and environment-responsive drug release was significantly improved over that of free Dox.

Notably, the PEG–PAsp(Dox) micelle was even able to accumulate in pancreatic cancer cells (which are well known for their malignancy and difficulty for drug access), by co-administration of TGF-β type I receptor (TβR-I) inhibitor at a low dose (Kano *et al.* 2007). Administration of the TβR-I inhibitor at a low dose probably plays a role in enhancing the EPR effect in the intractable tumour. The use of the TβR-I inhibitor combined with long-circulation nanocarriers such as polymeric micelles may be of significant clinical and practical importance in treating intractable solid cancers.

Another approach using pH-triggered drug release was reported where an accelerated release of physically incorporated Dox from the micelles was achieved with a decrease in pH (Lee *et al.* 2003). That study investigated a pH-sensitive polymeric micelle composed of a mixture of PEG–poly(L-histidine) as a pH-sensitive polybase possessing pK_a values around the physiological pH and biodegradable PEG–poly(L-lactic acid) block copolymers. The Dox-loaded mixed micelles were stable under physiological pH condition, but were unstable in the pH ranges of the tumour sites. When the mixed micelles were conjugated with folic acid as a targeting moiety, the micelles were more effective in killing tumour cells due to accelerated drug release in the tumour region and folate receptor-mediated tumour uptake. Furthermore, the fusogenic activity of poly(L-histidine) in the endosomes facilitated the cytosolic delivery of Dox to achieve improved cytotoxicity. This approach is also expected to be useful for the treatment of solid tumours *in vivo*.

Improvements of polymeric micelle systems have been demonstrated from the standpoint of both block copolymer structure and drug-loading strategies. Owing to the difficulty of treatment with CDDP due to acute dose-related side effects (such as nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting and myelosuppression) and the appearance of intrinsic and acquired resistance, an improved platinum drug, dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt), was developed by Kidani *et al.* (1977). DACHPt has shown a wide and markedly different spectrum of activities than CDDP, such as lower toxicity than CDDP and no cross-resistance to it in many CDDP-resistant cancers (Rixe *et al.* 1996; Zhang & Lippard 2003). However, DACHPt is significantly less soluble in water than CDDP (Kidani *et al.* 1978). To enhance the water solubility of DACHPt as well as pinpoint delivery, DACHPt-loaded micelles were developed by metal complexation between platinum and carboxylic acids contained in a PEG–PGlu block copolymer (figure 4; Cabral *et al.* 2005). The DACHPt-loaded (PEG–PGlu(DACHPt)) micelles showed prolonged blood circulation and increased tumour accumulation (20-fold greater accumulation of PEG–PGlu(DACHPt) micelles at the tumour site compared with free oxaliplatin, which is a related free drug and a third-generation platinum drug with improved water solubility). Moreover, the optimized micelles exhibited reduced non-specific accumulation in the liver and spleen, resulting in higher specificity to solid tumours. These marked results could be correlated to the extended blood circulation and preferential tumour accumulation of the polymeric micelles against primary and metastatic tumours. The *in vivo* results suggest that PEG–PGlu(DACHPt) could be an outstanding DDS for platinum-based drugs in the treatment of not only primary tumours but also metastatic tumours (Cabral *et al.* 2007).

4.2. Polymeric micelles delivering therapeutic genes

For gene delivery directed to clinical application, further developments in carrier design are needed to increase both transgene efficiency and reduce cytotoxicity while maintaining homeostasis. In addition to efficient passive accumulation to target tissues by improving the stealth effect, it will also be necessary to develop new designs for responsive functionality by sensing spatio-temporal signals that promote enhanced cellular uptake

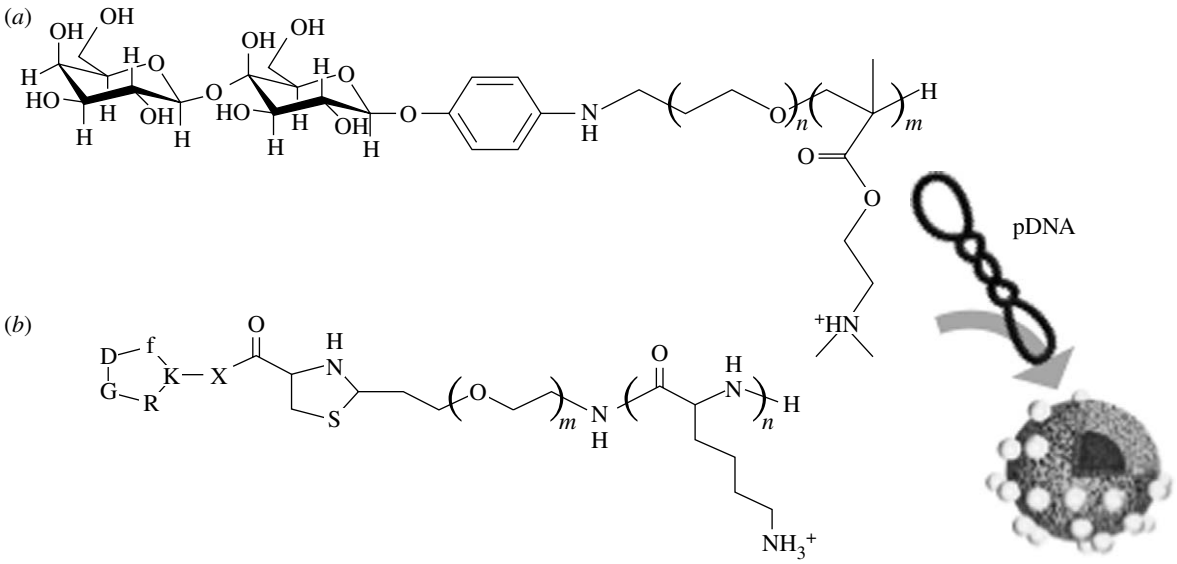

Figure 5. Block copolymers with ligand installed for targeted gene delivery. (*a*) Lactose for ASGP receptor and (*b*) cyclic RGD peptides for $\alpha_v\beta_3$ integrin receptor.

and prompt endosome escape and efficient intracellular trafficking to the nucleus (Khalil *et al.* 2006).

Active targeting is also attractive for site-selective gene delivery. We have developed a lactose-equipped polymeric micelle encapsulating pDNA targeted to hepatocytes, which possess abundant ASGP receptors that recognize lactose moieties (figure 5*a*; Kataoka *et al.* 1999). The transfection efficiency of these micelles in HepG2 cells (hepatoma) showed that the lactose micelle was significantly more efficient at transfection than the micelle-lacking lactose (Wakebayashi *et al.* 2004). A competitive assay using asialofetuin (ASF), a natural ligand against ASGP receptors, which would inhibit uptake of the lactose-installed micelle, suggested that ASGP receptor-mediated endocytosis is a major pathway for the cellular uptake of the lactosylated micelle.

For the treatment of cancer by gene therapy, it is difficult to deliver lethal genes to all malignant cells to extinguish tumour tissues. Therefore, anti-angiogenesis treatment is an attractive strategy (Folkman *et al.* 1971; Schiffelers *et al.* 2004; Kim *et al.* 2005, 2006). This strategy inhibits the formation of new blood vessels and thus cuts off the supply of nutrition to cancer cells. Here, the target is not cancer cells themselves but vascular cells around the tumour. Thereby, a delivery system specifically targeting vascular endothelial cells would be of great benefit. We focused on $\alpha_v\beta_3$ integrin receptors for carrier design (Hood *et al.* 2002) because it is generally known that they are expressed on various cell types such as endothelial cells, osteoclasts, macrophages, platelets and melanomas, and that they play a significant role in angiogenesis, vascular intima thickening and the proliferation of malignant tumours (Brooks *et al.* 1994). Cyclic RGD peptide (c(RGDfK)), which specifically recognizes these receptors, was conjugated at the end of PEG–PLys block copolymer (figure 5*b*). The c(RGDfK)–PEG–PLys/pDNA polyplex micelle showed a remarkably increased transfection efficiency compared with ligand-free polyplex micelles against HeLa cells expressing $\alpha_v\beta_3$ integrins (Oba *et al.* 2007). Flow cytometric analysis revealed a higher uptake of the c(RGDfK)–PEG–PLys/pDNA micelle than the PEG–PLys/pDNA micelle in HeLa cells, consistent with the transfection results. Furthermore, confocal laser scanning microscopic observation revealed that the pDNA in the c(RGDfK)–PEG–PLys micelle preferentially accumulated in the perinuclear region of the HeLa cells within 3 hours of incubation, whereas no such fast and directed accumulation of pDNA to the perinuclear region was observed for the micelles without c(RGDfK) ligands. These results indicate that the increased transfection efficiency induced by the introduction of the c(RGDfK) peptide ligand was attributed to an increase in cellular uptake and also enhanced intracellular trafficking of micelles towards the perinuclear region via $\alpha_v\beta_3$ integrin receptor-mediated endocytosis. This, in turn, suggested that the cyclic RGD peptide-conjugated polyplex micelle has promising feasibility as a site-specific targetable gene delivery system.

For delivery of nucleic acids such as DNA and siRNA, loaded material should be stably packaged in the carrier but also be released in the targeted cells. In this regard, bioresponsive smart polymeric micelles that dissociate inside of cells in response to chemical stimuli present in the intracellular compartment were designed (Miyata *et al.* 2004). The inner core of the micelle was cross-linked through disulphide bonds, which are expected to be cleaved inside the cell (figure 6). Owing to disulphide cross-linking of the core, the stability of the micelle was increased. At the same time, the efficient release of packaged pDNA was demonstrated in response to reducing reagent (dithiothreitol), mimicking the intracellular environment (Kakizawa *et al.* 1999). These distinctive environmental sensitivities were well reflected in the transfection efficiency, as gene transfection was higher, by an order of magnitude, than that of the non-cross-linked system. The cross-linked micelle exhibited appreciable gene expression in parenchymal

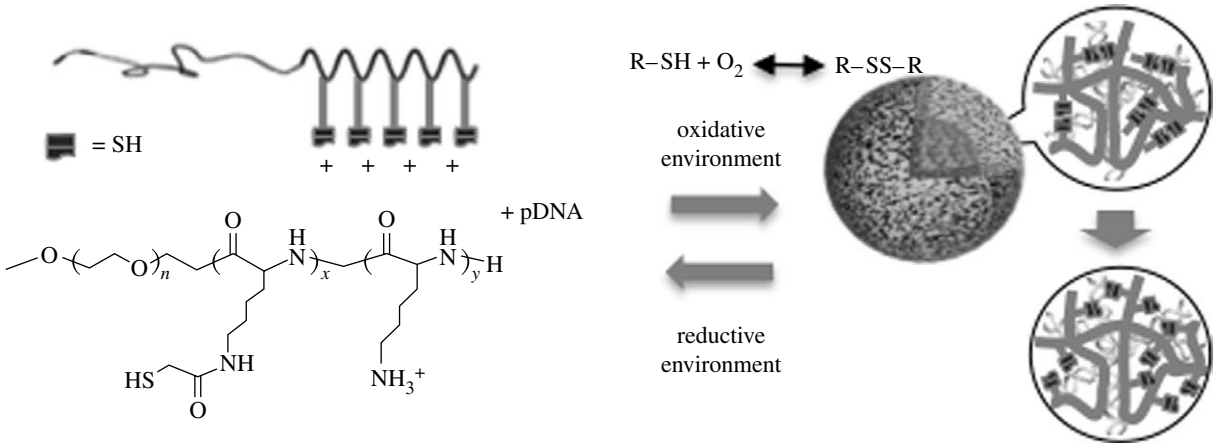

Figure 6. Cross-linked micelles responsive to the intracellular environment. Disulphide cross-linking imparts stability in the extracellular environment, which is readily reversed in the intracellular reductive environment.

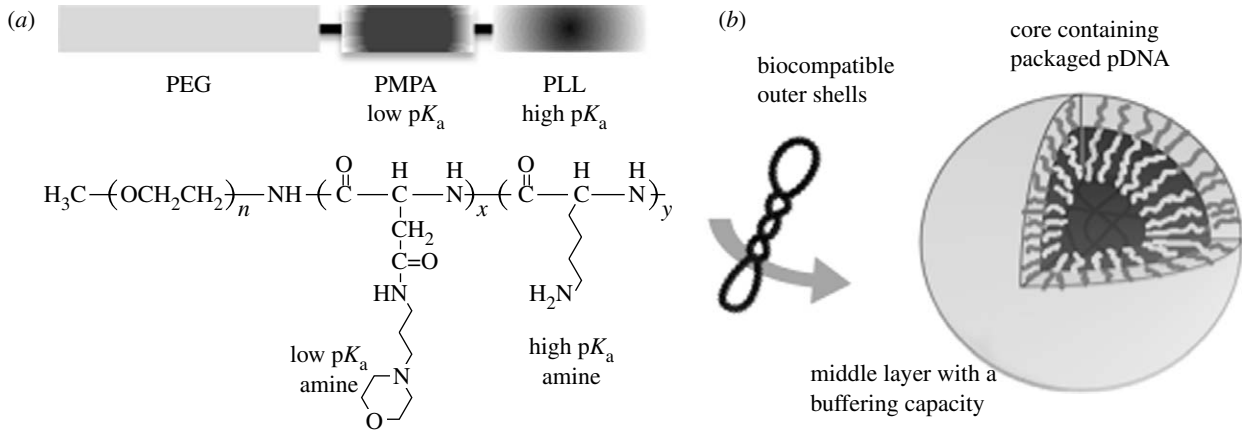

Figure 7. Design of a triblock copolymer for promotion of endosome escape. (*a*) The triblock copolymer PEG–PMPA–PLys system performs specific functions; the PEG segment improves biocompatibility, the PMPA segment contains inherent buffering capacity to promote early endosome escape and the PLys segment facilitates packaging pDNA in the micelle core. (*b*) Schematic of the hypothesized three-layered micelle formed from the triblock copolymer and pDNA with a spatially regulated structure.

cells of the mouse liver through intravenous injection (Miyata *et al.* 2005). From a practical viewpoint, the long-term storage of gene carriers is a critical issue. The disulphide cross-linking micelle maintained the original transfection capacity even after freeze–thaw treatment without the use of any protective reagents, while the non-cross-linked micelles showed significantly lower transfection efficiency after the same treatment.

Prompt endosome escape of therapeutic genes into the cytosol is a major factor for achieving effective transgene expression. When carriers are internalized by endocytosis, they are transferred to lysosomes via endosomes where nucleases digest foreign nucleic acids at lowered pH. Therefore, quick escape from the endosomes into the cytosol is a critical issue. It is believed that polycations with a low apparent pK_a, such as polyethyleneimine (PEI), promote endosome escape by the so-called proton sponge effect (Boussif *et al.* 1995). However, the binding of polyamines with low pK_a to DNA is weaker that that of polyamines with high pK_a, thus their complexes are not sufficiently stable in the biological entity. In regard to these issues, we designed a new type of A-B-C triblock copolymer for micelle formation by tandemly aligning two cationic segments in a single polymer strand (Fukushima *et al.* 2005). In this triblock copolymer, each segment has its own distinctive role: a PEG segment for biocompatibility, a second segment composed of low pK_a amines (poly[(3-morpholinopropyl) aspartamide] (PMPA) segments) for buffering capacity and a third segment composed of high pK_a amines (PLys segment) for DNA binding (figure 7*a*). Detailed NMR studies suggested that the micelle formed with the triblock copolymers has a spatially regulated three-layered structure (figure 7*b*), in which DNA associates only with the PLys segment and the PMPA segments remain free. The transfection activity of the triblock system exhibited one order of magnitude higher transfection compared with the PEG–PLys block copolymer with similar PLys unit length. This transfection efficiency is comparable with that of the PEI/pDNA polyplex, but with a remarkably lower cytotoxicity. These results suggest that well-designed tandem alignment of two cationic segments allows for efficient endosome escape, as initially expected.

In the search for the most suitable chemical structure for a cationic segment, PEG block copolymers with a series of cationic side chains were synthesized through the aminolysis of PEG–poly(β-benzyl L-aspartate) (PBLA), namely an ester–amide exchange reaction. The reaction proceeds via the formation of a succinimide intermediate in the polymer backbone,

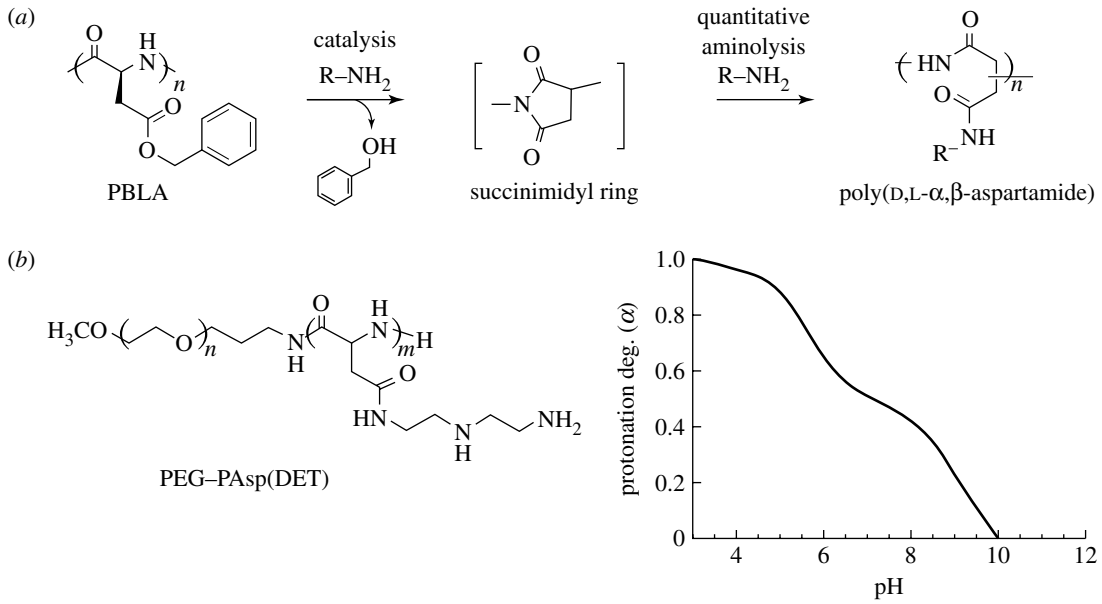

Figure 8. (*a*) Quantitative aminolysis reaction of PBLA via a succinimidyl ring intermediate, by which appropriate compounds can be installed into the polymer backbone by displacement of benzyl groups in PBLA side chains. (*b*) Chemical structure of PEG–PAsp(DET) where an ethylenediamine unit is installed by aminolysis and its corresponding pH–α (α = [protonated amino groups]/[whole amino groups]) curve of PEG–PAsp(DET) at 37°C in 150 mM NaCl, showing that protonation takes place in two distinct steps.

which is efficiently converted to a polyaspartamide in which quantitative introduction of a desired side chain is demonstrated without undesired side reactions (figure 8*a*; Nakanishi *et al.* 2007). The reaction, which enables side chains to be introduced as aspartamides, is crucial in the design of functionalized polymers for biomaterials. A polymer library was prepared using the above-mentioned reaction, from which we have found that PEG–polyaspartamide with an ethylenediamine unit as a side chain (PEG–PAsp(DET); figure 8*b*) had high transfection efficiency as well as low cytotoxicity (Kanayama *et al.* 2006). The side chain of the block copolymer exhibits single protonation in physiological pH at 7.4 while exhibiting double protonation at the endosomal pH of 5.5 (figure 8*b*; Han *et al.* 2007). Therefore, it is expected that the block copolymer exhibits low cytotoxicity in extracellular conditions due to single protonation. On the other hand, it becomes able to interact with the endosomal membrane due to increased positive charges by double protonation, which eventually results in the promotion of endosome escape. This polymeric micelle provided gene expression in primary cells (Kanayama *et al.* 2006) as well as in *in vivo* disease models with low toxicity. The polyplex micelle prepared with PEG–PAsp(DET) and pDNA was also applied to vascular lesions with good results (Akagi *et al.* 2007). The micelle was instilled intravascularly into rabbit carotid arteries with neointima, and it exhibited appreciable reporter gene transfer into vascular lesions without any vessel occlusion by thrombus. These findings indicate the feasibility of the polyplex system for treating vascular diseases. The PEG–PAsp(DET) polyplex system was also applied for *in vivo* gene transfer in a bone-defect model of mouse skull bone (Itaka *et al.* 2007). The polyplex micelle was mixed with a calcium phosphate cement scaffold and installed into the bone defect. Sustained release of PEG–PAsp(DET) from the scaffold and transfection of surrounding cells were observed. By the transfection of constitutively active form of activin receptor-like kinase 6 (caALK6) and runt-related transcription factor 2 (Runx2), osteogenic differentiation was induced in mouse calvarial cells with no sign of inflammation. This result demonstrated the first successful *in vivo* gene transfer with therapeutic potential using polyplex micelles, and showed that the system holds promise for constructing a practical gene-activated matrix for tissue engineering and also for bone-regenerative medicine. It is worth pointing out the biocompatibility of the PEG–PAsp(DET) polyplex system in addition to efficient *in vivo* gene transfer. It was demonstrated that the PAsp(DET) polyplex system exhibited no interference of endogenous housekeeping gene expression; thus, cellular homeostasis is maintained (Masago *et al.* 2007). Biocompatibility with regard to cellular homeostasis is a crucial issue for practical use, especially when gene transfer is applied to primary cells to regulate cell functions such as differentiation.

4.3. Regulation of plasmid DNA packaging in polyplex micelles

Various types of block copolymers have been designed to improve gene expression efficacy by exploring chemical modifications for desirable functions, as described in previous sections. The properties of polyplex micelles must differ from site to site during the delivery process; thus, it is essential to optimize the packaging of DNA into the micelle according to the requirements at each site for improved gene expression efficacy. Packaging of pDNA into a polyplex micelle, pDNA condensation in other

words, induced by block catiomer is not so simple, because pDNA has the topological characteristic of supercoiled closed-circular form and its condensation occurs with topological constraint. It was shown that pDNA packaging is affected by both polymer structure and charge ratio between pDNA and PLys. Change in PLys chain length and the charge ratio between pDNA and PLys resulted in the formation of rod-like, toroidal and collapsed sphere structures (Mann *et al.* 2008). We have found an interesting activity of S1 nuclease, a selective enzyme that is known to cleave single-stranded DNA in condensed pDNA. When pDNA was condensed into rod-like or toroidal structures, it was cleaved into highly ordered distinct fragments, each being 10/12, 9/12, 8/12, 6/12, 4/12, 3/12, 2/12 in length with respect to the original pDNA (Osada *et al.* 2005). This S1 nuclease activity suggests that double-strand dissociation might be induced in micelle-packaged pDNA. Presumably, the specific cleavage may relate to DNA folding in pDNA condensation, which proceeds under the constraints of helical double-stranded DNA rigidity and the characteristic supercoiled closed-circular topology.

5. SUMMARY

Biomaterials have developed from passive materials that support the functions of target tissues or organs to active and smart materials that themselves act by sensing appropriate signals and exerting their function. Polymeric micelles for DDS have also developed from simple carrier systems that only deliver therapeutic materials to intelligent and multifunctional nanodevices by integrating bioresponsive functions. These advances are mediated by nanobiotechnology, designing the biological activity of engineered materials in terms of physicochemical viewpoints. Polymeric micelles are being developed as total delivery systems integrating multifunctionality to improve their efficacy. Although this review focuses on anti-cancer drugs and pDNA as deliverable materials, we have also developed DDS for other therapeutic materials such as proteins (Jaturanpinyo *et al.* 2004; Yuan *et al.* 2005; Kawamura *et al.* 2007), siRNA (Itaka *et al.* 2004; Kakizawa *et al.* 2006; Matsumoto *et al.* 2009), imaging agents for diagnosis (Imai *et al.* 2007; Kumagai *et al.* 2007) and photosensitizers for photodynamic therapy (Ideta *et al.* 2005; Jang *et al.* 2005, 2006, 2007) or co-delivery with pDNA for photochemical gene delivery (Nishiyama *et al.* 2005, 2006, 2007; Arnida *et al.* 2006). The development of polymeric micelles with smart functions such as environment sensitivity and tissue selectivity may enhance the desired activity of potent bioactive compounds, facilitating their clinical application. Polymeric micelle-based DDS, which can perform versatile functions based on innovative nanobiotechnologies, will lead the way for development of future advanced medicines.

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