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Paclitaxel loaded nano-aggregates based on pH sensitive polyaspartamide amphiphilic graft copolymers

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ABSTRACT

Polyaspartamide (PASPAM) derivatives grafted with 1-(3-aminopropyl)imidazole (API), *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol (MPEG), and octadecylamine (C18) groups were synthesized and their pH-sensitive structure and Paclitaxel (PTX) load/release properties were investigated. C18/MPEG/API-g-PASPAMs systems synthesized showed a strong pH-dependent phase transition behavior near pH 6.7. Large amount of PTX up to 60–75%, depending on polymer composition, was possibly loaded into the C18/MPEG/API-g-PASPAMs nano-aggregates using a solvent-free protocol. Its pH dependent release pattern was affected correspondingly by the phase transition behavior associated with the composition of graft substituents. The pure C18/MPEG/API-g-PASPAMs systems did not show cell toxicity but the PTX-loaded copolymer systems showed a similar cell toxicity to a Taxol-type PTX. From the in vivo animal study, PTX-loaded nano-aggregates showed the much improved inhibition effect on tumor growth compared to the conventional PTX formulation.

1. Introduction

Polymeric micelles or aggregates have been attracting great interest in their application as nano- to microscaled carriers for bioactive materials, including drugs, genes, cells, peptides, and enzymes since nanotechnology was embodied into biomedical (diagnotherapic) technology. While a variety of amphiphilic block copolymer systems have been employed towards formation of micelles or aggregates by self-assembly in aqueous media (Bae et al., 2005; Cho et al., 2010; Nishiyama and Kataoka, 2006; Savic et al., 2006; Zhang and Zhang, 2005), the graft copolymer systems have become more emphasized in their synthesis, functionalization, and phase transition behavior. In the application of an anticancer drug delivery, the pH sensitive sol–gel transition or swelling/de-swelling behaviors of polymeric micelles or self-assembled aggregates are of importance, as the drug delivery carriers are specifically located at cancer cells by the enhanced permeability and retention (EPR) effect (Campbell, 2006), along with a triggered release allowed at the relatively low pH environment of cancer (Borisov and Zhulina, 2005; Rodriguez-Hermandez and Lecommandoux, 2005; Shim et al., 2006, 2007). The pH sensitivity of the polymer micelles or aggregates also plays an important role in endosomal lysis for effective intracellular delivery (Park et al., 2006c). Recently, several cationic or anionic polymeric systems such as poly(ethyleneimine) (PEI), cationic peptide, polyhistidine, modified chitosan, polyamidoamine, polyamidoamine dendrimer, and poly(propylacrylic acid) have focused on this objective (Berna et al., 2006; Lavignac et al., 2005; Park et al., 2006a,b; Swami et al., 2007; Wu et al., 2005). Although each system shows promising results in part, it still needs development of a new polymeric system that satisfies a simpler synthetic process, lower material toxicity, and more utilizable pH range and strength.

Polypeptides and their related synthetic poly(amino acid)s have attracted a great deal of attention as they are biocompatible and biodegradable with less toxicity (Kricheldorf, 2006; Kulkarni et al., 2005). Polyamino acids with refined molecular weights can be synthesized via a well-known *N*-carboxylanhydride (NCA) method, but its synthetic route is quite complex. Poly(L-aspartic acid) has garnered much attention since its simple synthetic route via thermal condensation was first reported. Poly(succinimide) (PSI), an intermediate in the synthesis of poly(amino acid), has an advantageous molecular structure, enabling easy chemical modification through alkylation, hydrolysis, and aminolysis. This produces biodegradable derivatives such as poly(aspartic acid), poly(asparagine), and poly(hydroxyethylaspartamide). Moreover, it can be easily functionalized by linkage of hydrophilic/hydrophobic ligands, peptides, and even drug (Caliceti et al., 2001; Cavallaro et al., 2003; Chen Xu et al., 2005; Licciardi et al., 2006; Jeong et al., 2005; Moon et al., 2006; Takeuchi et al., 2006).

Paclitaxel (PTX) is one of the most effective and commonly used drugs for ovarian, esophageal, breast, and lung cancer treatment. However, its poor water-solubility (<0.1 $\mu L\,mL^{-1}$) is a serious

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problem in drug formulation. In clinics, it is usually injected into the human body as a Cremophor EL (polyethoxylated castor oil) and ethanol (1:1) solution. It is sometimes reported to bring about hypersensitivity reactions such as breathing difficulty, rash, flushing, and nephrotoxicity. Solutions to those problems require new types of drug release methods or structures, and one of them is the nano- to microscaled micelle or aggregate structures prepared from amphiphilic graft copolymers (Elkharraz et al., 2006; Hu et al., 2006; He et al., 2007; Liu et al., 2005; Seow et al., 2007).

In this lab's previous report (Seo et al., 2009, 2010; Seo and Kim, 2010), the synthesis and pH-sensitive properties of a new class of polyaspartamide (PASPAM) derivatives, C18/MPEG/API-g-PASPAMs, possessing 1-(3-aminopropyl)imidazole (API), *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol (MPEG), and octadecylamine (C18) groups, were discussed. As they showed a strong pH-dependent phase transition and aggregation behavior near pH 6.7, it was noted that they possess high potential in the application as pH-sensitive anti-cancer drug carriers. In this study, the PTX was loaded into the polyaspartamide derivatives using a solvent-free protocol, and their reversible aggregation behavior and inhibition effect on tumor growth was investigated both in vitro and in vivo.

2. Materials and methods

2.1. Materials

L-Aspartic acid (>98%) was purchased from Aldrich (Milwaukee, WI, USA) and used as a monomer; octadecylamine (Aldrich), 1-(3-aminopropyl)imidazole and *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol (M_w: 5000, Fluka, Buchs, Switzerland) were used as graft reagents. *O*-phosphoric acid (98%) and phosphoric acid (85%) were used as catalysts. Phosphate buffered saline (PBS 7.4), *N*,*N*-dimethylformamide (DMF), mesitylene, sulforane, dimethyl sulfoxide-d_6 (DMSO-d_6), octadecylamine, 1-(3-aminopropyl)imidazole, *N*,*N*-diethylnicotinamide, ethanol, acetonitrile, and Cremophor EL were used as solvents. All chemicals were purchased from Sigma–Aldrich except for *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol. Paclitaxel (99%, Genexol®; Samyang Genex, Korea) was used as received. All other chemicals purchased were of sufficient quality for use without purification.

2.2. Methods

2.2.1. Synthesis of C18/MPEG/API-g-PASPAMs

The PSI was first synthesized from L-aspartic acid. Octadecylamine (C18) (0.27–1.08 g, 0.001–0.004 mol) was added to a solution of PSI (1.0 g, 0.01 mol) in dry DMF (50 mL) under a N_2 atmosphere. After stirring for 24 h at 60 °C, the reaction mixture was poured into methanol for purification. The MPEG and API were grafted onto the prepared C18-g-PSI, sequentially. The MPEG and C18-g-PSI were each dissolved in DMF. The MPEG solution was then added dropwise into the C18-g-PSI solution for its graft reaction at 70 °C for 48 h. After the MPEG graft reaction, the API was added for its graft reaction for 24 h. Finally, the prepared C18-conjugated MPEG/API-g-PASPAM was dialyzed using a dialysis membrane (molecular weight cut-off = 10,000–12,000 g/mol) for 3 days and then freeze-dried for storage. The schematic synthetic procedure is illustrated in Fig. 1.

Chemical structure was confirmed using Fourier transform nuclear magnetic resonance spectroscopy (Varian Unity Inova 500 MHz HMR, Agilent Technologies, Santa Clara, CA, USA). Samples were dissolved in DMSO-d_6. The degree of substitution of the MPEG, C18, and API was determined from comparison of the NMR characteristic peak intensities associated with each component. The average molecular weights of the synthesized copolymers were measured using a gel permeation chromatography (GPC, model 410, Waters, USA) system equipped with KF-803L and KF-802.5 columns (Shodex, Showa Denko KK, Japan) in series. For GPC measurements, samples were dissolved in *N*,*N*-dimethylformamide (DMF) containing lithium bromide (50 mM), and the solution was injected into the column at the rate of 1.0 mL min^{-1} at 50 °C. The data was analyzed by means of an RI detector (RI-101, Shodex). Polystyrene standards (Waters) were used to determine the molecular weight (M_w).

2.2.2. Phase transition and buffering behavior

The pH-dependence of the light transmission intensity was measured for the polymer solution using the UV/vis spectroscopy (Hitachi, U-3210, Japan) at a 500 nm wavelength. The transmission % is given by the ratio of light transmission intensity for the sample to that of pure PBS solution at pH 7.4. Samples were prepared by dissolving each polymer in pH 7.4 PBS solution at 1.0 mg mL^{-1}; the pH was adjusted using 0.1 N HCl and 0.1 N NaOH. The buffering

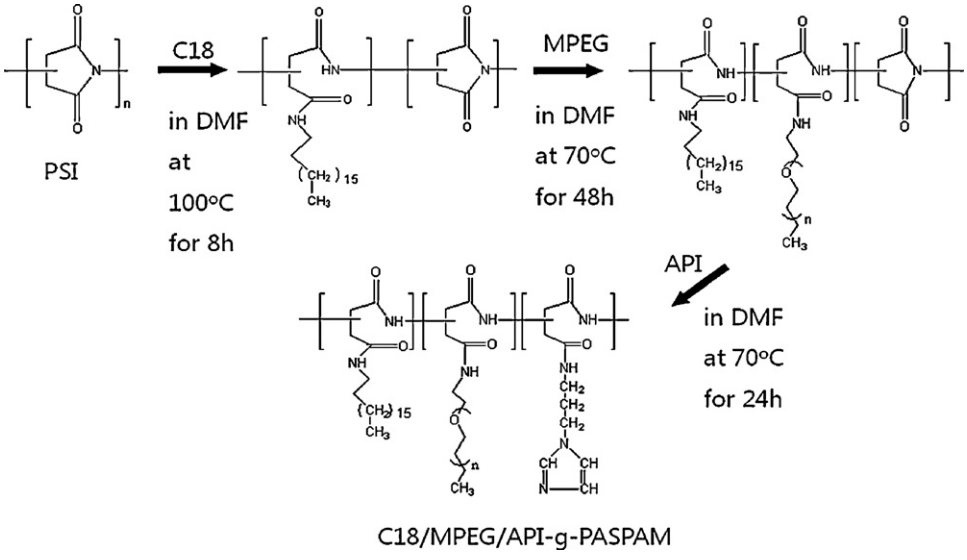

Fig. 1. Synthetic pathway of C18/MPEG/API-g-PASPAM.

effect of the synthesized graft copolymers was investigated using an acid–base titration method. The sample (20 mg) was dissolved in 35 mL of aqueous 150 mM NaCl. After addition of 100 μL of aqueous 1.0 N NaOH, the polymer solution was titrated using aqueous 0.1 N HCl.

Dynamic light scattering (DLS) (Brookhaven, BI-200SM, USA) was employed to measure the size and distribution of the polymer nanoaggregates. The polymers were dispersed in PBS 7.4 solution at 1.0 mg mL^{-1} and the pH controlled using NaOH and HCl. All DLS measurements were performed at scattering angles from 30° to 150°, after filtration with a 0.45 μm filter.

2.2.3. In vitro Paclitaxel loading and release experiment

Paclitaxel-encapsulated pH-sensitive polyaspartamide nanoparticles were prepared using a pH-changing method. Briefly, a polymer powder was dissolved in PBS solution (pH 7.4) at a concentration of 2.0 mg mL^{-1}, and the pH of solution lowered to 5 with aqueous 1.0 N HCl. Then, 0.2 mL of PTX dissolved in ethanol was added to this polymer solution. The resulting solutions contained 5, 10, 15, 20, or 25 mg of PTX per 20 mg of C18/API/MPEG-g-PASPAM. The pH of the mixtures was immediately adjusted to 7.4 using aqueous NaOH and sonicated in ice bath for 20 min. Finally, the PTX-loaded nanoparticles were filtered through a 0.45 μm pore-sized syringe filter disc to remove free PTX.

The amount of PTX in the filtrate was analyzed by reversed-phase HPLC (NS-2004 series, Futechs, Korea), composed of a quaternary gradient pump, variable UV detector, and data management software (Multichro 2000). The amount of PTX was determined by HPLC with a C-18 reversed-phase column (Agilent, 4.6 mm × 250 mm, 5 μm) and UV detection at 227 nm using a 50:50 (v/v) mixture of acetonitrile and water as the mobile phase at a flow rate of 0.5 mL min^{-1}. Calibration curves were obtained for different PTX concentrations ranging from 0.1 to 0.001 mg mL^{-1} in acetonitrile. The calibration equation was described as follows: $y = 41{,}954{,}712x + 18{,}425$, where y is the area of the peak and x the concentration of the PTX. The mean correlation coefficient was 0.9999. The PTX loading content was calculated using the following formula:

$$\text{Drug loading content } (\%) = \frac{\text{weight of loaded PTX}}{\text{weight of copolymer}} \times 100 \quad (1)$$

The in vitro release profiles of PTX from pH-sensitive nanoparticles were investigated at different pH as a function of time. The release media was prepared using 1.0 M *N*,*N*-diethylnicotinamide PBS at various pH values. The PTX-loaded nanoparticles (5.0 mL) were added to membrane tubes (MWCO 6000–8000), and the dialysis tube immersed in a vial containing 50 mL of release medium. The vial was thermostated in a shaking water bath at 37 °C. At regular time intervals, 2.0 mL of the medium was taken and replaced with 2.0 mL of fresh medium. The cumulative amount of the drug released was quantified by HPLC, as described above.

2.2.4. In vitro viability test

The cell viability was tested and compared to the pure C10/MPEG3/API87-g-PASPAM system, the PTX-loaded C10/MPEG3/API87-g-PASPAM (PTX:polymer = 1:4 in wt) system, and the PTX solution against L929 mouse fibroblasts. Cells were incubated in a cultivating medium under a 5% CO_2 environment at 37 °C. Dulbecco's modification of Eagle's Medium (DMEM) badge containing 10% FBS and 1% penicillin/streptomycin in 100 unit mL^{-1} was used as the cultivating medium. The PTX solution was prepared by dissolving PTX in a mixture of Cremophor Eland ethanol (1:1 in volume), followed by dilution in PBS 7.4. Cell viability was measured using the well-known 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The L929 cells (2.0×10^5) were dispensed and cultivated per well in the 24-well cultivating dish for 24 h. In the cases of the PTX solution and PTX-loaded polymer solution, the PTX concentration in the badge was adjusted to 100, 10, and 1.0 μg μL^{-1}. After cultivation, the MTT solution in 5.0 mg mL^{-1} (100 μg) was added per 1.0 mL badge. After 4 h, the cultivated solution was discarded; formazan crystal was then dissolved by introducing DMSO (200 μg) into each well. Absorption light intensity was measured using an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 570 nm wavelength. The absorption intensity of the control sample (pure PBS solution) was set to 100%.

2.2.5. In vivo animal studies

SCC7 carcinoma was obtained from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 media (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) FBS, 1% (v/v) penicillin, and streptomycin (Invitrogen, Carlsbad, CA, USA). Carcinomas were cultured at 37 °C and 5% CO_2 in a humidified atmosphere. The animal model was established by dorsal flank subcutaneous inoculation of 1×10^6 cells per mouse (7 weeks old male C3H/HeN mice (Orient, Korea)). When the tumor volume was reached 50–70 mm^3, drug was continuously administered. There were five mice groups as follows; one group was served normal saline as the control and the others were received one of the followings by daily intravenous administration: 2 mg kg^{-1} Paclitaxel; 1 mg kg^{-1}, 2 mg kg^{-1} and 5 mg kg^{-1} of PTX loaded C10/MPEG3/API87-g-PASPAM. After 15 days, mice were sacrificed and the tumors were isolated and weighed.

3. Results and discussion

3.1. Chemical structure and molecular weight

C18/MPEG/API-g-PASPAMs copolymer systems with different graft composition were synthesized according to the synthetic pathway shown in Fig. 1. Table 1 shows the degree of substitution of the C18, API and MPEG segments on the PSI backbone from the NMR measurement (Seo et al., 2009). The number and weight average molecular weights, $\overline{M_n}$ and $\overline{M_w}$, and polydispersive index (PDI) were determined from GPC and the results are shown in Table 1.

3.2. Phase transition behavior

pH dependence of size and structure of self aggregates of C18/API/MPEG-g-PASPAM copolymer systems in aqueous media is shown in Fig. 2. The C10 polymer system dissolved in an aqueous solution below pH 6.7 as the hydrophobicity was not strong enough to maintain the aggregate structure at this low alkyl content. However, the C20 and C40 systems maintain aggregate structure, even at pHs below 6.7. The lower C18 content resulted in smaller particle size as the hydrophobic core size is mostly governed by the content of the alkyl group. Instead, de-aggregation particles were swollen slightly in the aqueous medium for the C20 and C40 systems due to the presence of the pH-sensitive API group. It is ionized at low pHs below 6.7.

Fig. 3 shows the pH buffering effect of the C18/API/MPEG-g-PASPAM copolymer systems. The strength of the buffering effect is dependent on the concentration of the pH-sensitive moiety. Thus, the C10 polymer system shows the strongest buffering ability among the three systems. This buffering effect implies their utilization for endosomal escapable delivery. The mean particle size was in the range from 70 to 160 nm, suitable for intracellular delivery.

Table 1
Feed molar ratio and degree of substitution of C18, MPEG, and API of C18/MPEG/API-g-PASPAM copolymers synthesized and molecular weight.

Sample name	Feed molar ratio of C18[a]	DS of C18[b]	Feed molar ratio of MPEG[c]	DS of MPEG[b]	DS of API[b]	$\overline{M_n}$[d]	$\overline{M_w}$[d]	PDI[d]
C10-MPEG3-API87	0.1/1.0	8%	0.03/1.0	2.5%	89.8%	36,000	53,000	1.47
C20-MPEG3-API77	0.2/1.0	15%	0.03/1.0	2.2%	82.9%	33,000	48,000	1.45
C40-MPEG3-API57	0.4/1.0	32%	0.03/1.0	2.0%	66.1%	23,000	34,000	1.48

[a] Feed C18 mole/succinimide unit mole.
[b] DS was determined by ^{1}H NMR (mol%).
[c] Feed MPEG-NH_2 mole/succinimide unit mole.
[d] $\overline{M_n}$, $\overline{M_w}$, and PDI were determined by GPC.

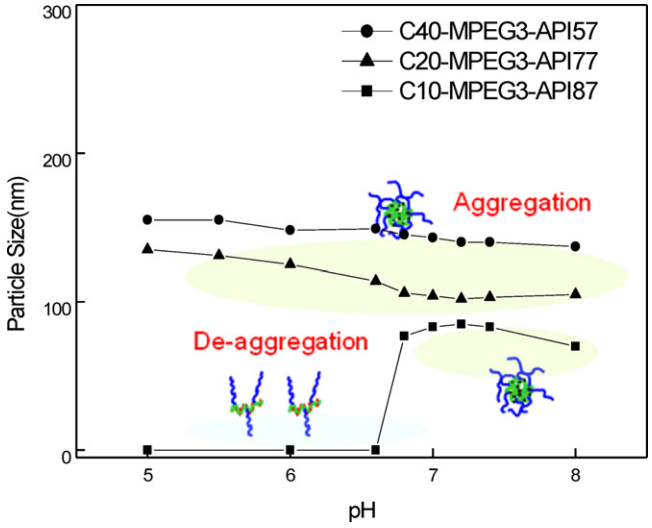

Fig. 2. Mean particle diameter of a series of MPEG/API-g-PASPAM systems as a function of pH.

3.3. *In vitro* PTX loading and release behavior

The loading capacity and releasing behavior of C18/MPEG/API-g-PASPM aggregates for PTX were investigated. In general, a hydrophobic drug is loaded into amphiphilic copolymers via direct sonication, W/O/W emulsion or dialysis method in the presence of appropriate solvents. However, in case of C10-MPEG3-API87 copolymer, PTX was loaded into the polymer matrix by applying the reversible sol gel transition behavior of the polymer material without using any solvent. The PTX was dissolved in a small amount of ethanol, and the resulting solution mixed with a polymer solution until homogeneous dispersion. Increase of pH above the sol-to-gel phase transition (pH 6.7) resulted in aggregation of polymeric molecules. Being different from C10-MPEG3-API87 copolymer system, C20-MPEG3-API77 and C40-MPEG3-API57 systems were structurally stable in physiological pH range. For those C20 and C40 systems, the PTX was possibly loaded into polymers by application of ultra-sonication. During ultra-sonication process, nano-aggregates are dissociated to combine surrounding PTX molecules by hydrophobic interaction. The subsequent shut-down of the sonication leads to re-self-assembled polymer nano-aggregate containing PTX in the cores. In Fig. 4(1) and (2) are the pictures before and after filtration of the PTX/ethanol system in water, while Fig. 4(3) and (4) show those after filtration of the PTX/C10-MPEG3-API87 and PTX/C20-MPEG3-API77 in water, respectively. The PTX was completely removed by filtration in the PTX/ethanol system because it was not easily dissolved in aqueous medium, whereas the PTX was still present for the PTX-loaded polymer system as nanoaggregates, because the polymeric materials used in this study are highly compatible with the PTX. Thus, this drug loading method was much simpler and safer compared to the conventional loading methods, including the solvent removal step.

Table 2 shows the PTX loading capacity of a series of C18/MPEG/API-g-PASPAM systems at different C18 concentrations. The PTX at different amounts (5.0, 10, 15, 20, and 25 mg) was initially mixed with 20 mg polymer each, and then the actual loading amount measured. The loading capacity increased with the PTX amount initially added into mixture. The polymer systems with higher C18 concentrations possess more PTX because of larger hydrophobic cores interacting with hydrophobic PTX. The maximum loading capacity reached upwards of 60–75%, depending on polymer composition. Such a high loading capacity is attributed to the unique drug loading method associated with the sol–gel phase transition of the polymer without any solvent. In this method, nearly all the PTX is incorporated in then polymer in the absence of solvent. Fig. 5 shows the PTX-loaded particle size according to its loading amount. The PTX-loaded particles showed a minor increase in mean diameter compared to the pure polymer particle.

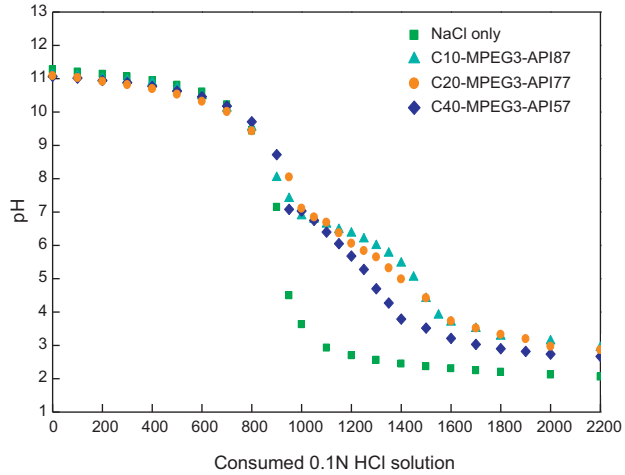

Fig. 3. Acid–base titration curves for a series of C18/MPEG/API-g-PASPAM systems.

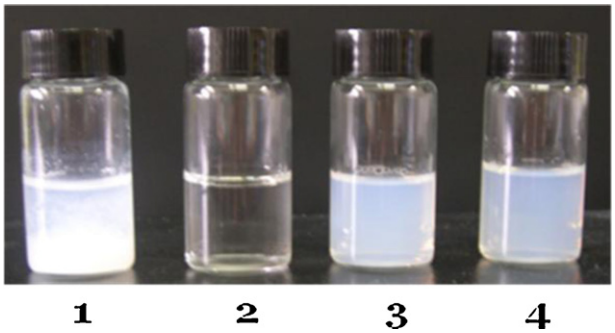

Fig. 4. PTX dispersed ethanol aqueous solution (2.5 vol%) before filtration (1) and after filtration (2), PTX-loaded C10-MPEG3-API87 dispersed aqueous solution after filtration (3), and PTX-loaded C20-MPEG3-API77 aqueous solution after filtration (4).

Table 2
Amount of PTX added and loaded in unit mass of polymer.

	Added PTX (g) per polymer(g)	Loaded PTX (g) per polymer (g)
C10-MPEG3-API87	0.250	0.159
	0.500	0.314
	0.750	0.518
	1.00	0.612
	1.250	0.638
C20-MPEG3-API77	0.250	0.197
	0.500	0.370
	0.750	0.499
	1.00	0.611
	1.250	0.628
C40-MPEG3-API57	0.250	0.223
	0.500	0.388
	0.750	0.557
	1.00	0.742
	1.250	0.743

An in vitro release experiment for a hydrophobic drug is usually complex as the drug is insoluble in the aqueous phase. Because the solubility of PTX is very low in PBS (0.45 μg mL^{-1}) the in vitro drug release from the micro/nanoparticles is usually tested in the presence of surfactants or hydrotropic agents such as *N*,*N*-diethylnicotinamide (DENA). However, those cases have some drawbacks: the external solvent should be exchanged frequently; the PTX load/release content measurement requires complete removal of external solvents by condensation; drying or extraction, thus large amounts of samples and external solvents are required. While it has been reported that a DENA aqueous solution has a few times higher PTX solubility than acetonitrile or ethanol, this study employed 1.0 M DENA PBS solution as a release medium. In this release experiment C10-MPEG3-API87 and C40-MPEG3-API57 samples with a drug-adding ratio of 0.5, corresponding to loading ratios 0.314 and 0.388, respectively, were used (see Table 1). The release experiments were conducted in two pH PBS solutions, pH 7.4 and 5 at 37 °C. The PTX release content was periodically measured using HPLC and the results illustrated in Fig. 6. For the C10-MPEG3-API87 sample, approximately 30% of the loaded drug was released within 5 h at pH 7.4, whereas almost all drug was released in the same time at pH 5.0 and 6.0. At pH 7.4, the drug was released solely via diffusion through nanoaggregates, but at pH 5.0 and 6.0, the release was triggered by dissociation of the polymer

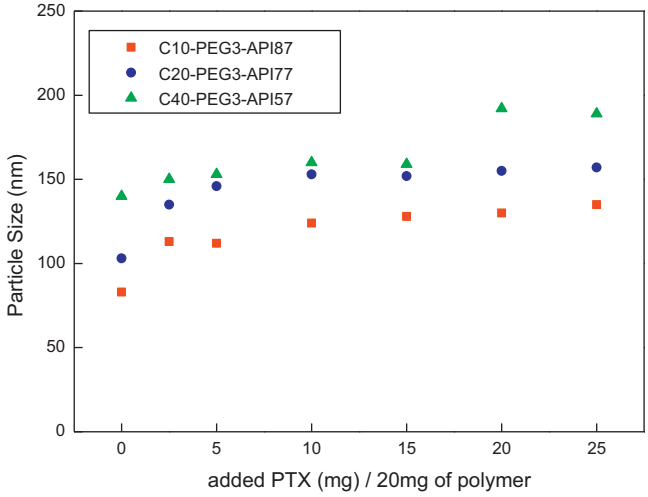

Fig. 5. Particle size of C18/MPEG/API-PASPAMs as a function of the PTX amount added per unit mass of polymer.

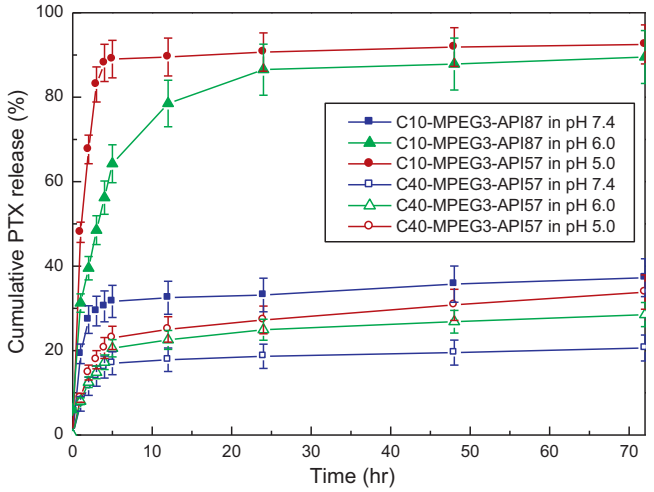

Fig. 6. pH dependence of cumulative PTX release kinetics for C10-MPEG3-API87 and C40-MPEG3-API57 nano-aggregates.

aggregates associated with ionization of the imidazole group. This pH dependence is more significant at lower pH.

The pH dependence of drug release kinetics is not noticeable for C40-MPEG3-API57 systems, compared to C10-MPEG3-API87, as the structure of C40-MPEG3-API57 aggregates is stable without any dissociation at all experimental pHs. Although the overall release patterns are similar to one another, a slight increase in release kinetics is still observed at lower pH due to ionization of the imidazole groups contained in the alkyl-dominating hydrophobic cores.

The pH triggering effect is clearly observed in Fig. 7, where the release pattern is illustrated according to the variation of pH from 7.4 to 5.0. Only approximately 30% of the drug is released in the initial 8 h at pH 7.4. When the pH is, however, reduced to 5.0, nearly all drugs are released within 5 h. Those pH-sensitive aggregates are not only structurally very stable in circulation of blood at pH 7, but also very effective in a targeted drug release for cancer at low pH environments.

3.4. Cell viability

The viability of L929 cells in the presence of C10-MPEG3-API87 self-aggregates is shown in Fig. 8(a). While the pure PEI

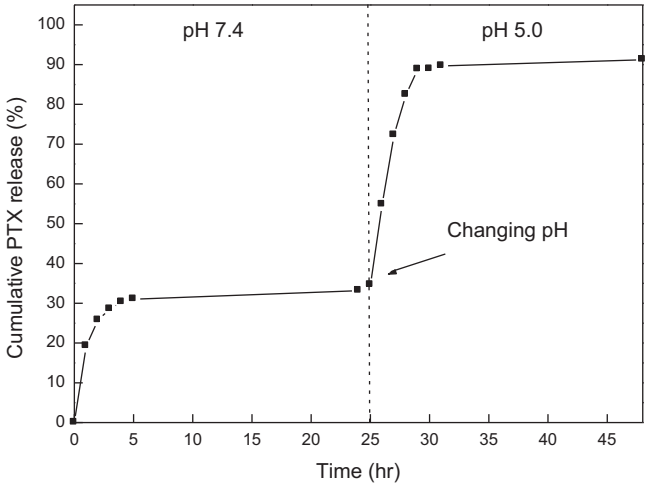

Fig. 7. pH triggered PTX release from nano-aggregates composed of C10-MPEG3-API87.

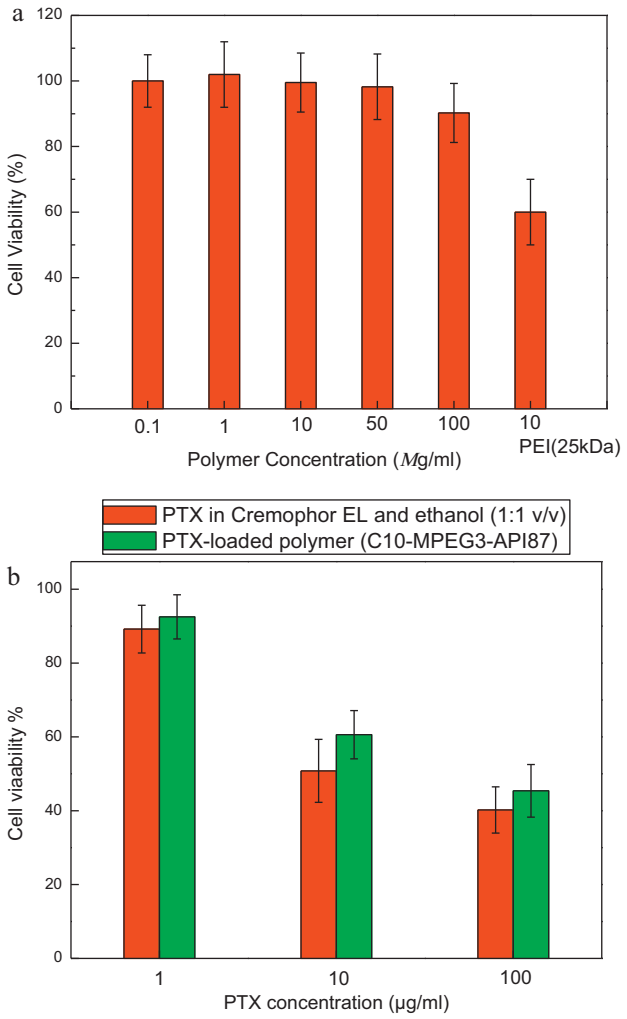

Fig. 8. In vitro cytotoxicity of (a) C10-MPEG3-API97 system in different polymer concentration and (b) PTX in cremophor EL/ethanol solution (1:1, v/v) and PTX-loaded C10-MPEG3-API97 at different PTX concentration.

(M_w 25,000 Da) sample shows only about 60% cell viability at 10 μg mL^{-1}, the pure C10-MPEG3-API87 aggregates are almost 100% at concentrations up to 50 μg mL^{-1}. Fig. 8(b) compares the cell viability for the two drug-loaded systems, the PTX-loaded C10-MPEG3-API87 system and PTX in the Cremophor EL/ethanol solution (1:1 volume). The increased toxic effect illustrated in Fig. 8(b) in comparison with Fig. 8(a) is caused by the inherent toxicity of the PTX released from the polymer carriers. The cell toxicity increases with increasing PTX content, but the PTX-loaded C10-MPEG3-API87 system shows comparable to or slightly higher cell viability than the Taxol-type PTX system. This indicates that the toxicity of PTX cannot be totally eliminated by the C10-MPEG3-API87 system, but can be reduced by it.

3.5. *In vivo animal studies*

The potential to suppress tumor growth by C10/MPEG3/API87-g-PASPAM aggregates at three different PTX was investigated in SCC7 carcinoma grafted animal model as shown in Fig. 9. SCC7 carcinomas were subcutaneously grafted into the dorsal flank of C3H/HeN mice and subsequent growth of tumors was observed for 15 days after the initial treatment of drugs. The tumor growth was potently suppressed by 2 mg kg^{-1} of PTX in a conventional formulation, inhibited by 48% as compared to the control mice. However, when PTX was loaded in C10/MPEG3/API87-g-PASPAM aggregates,

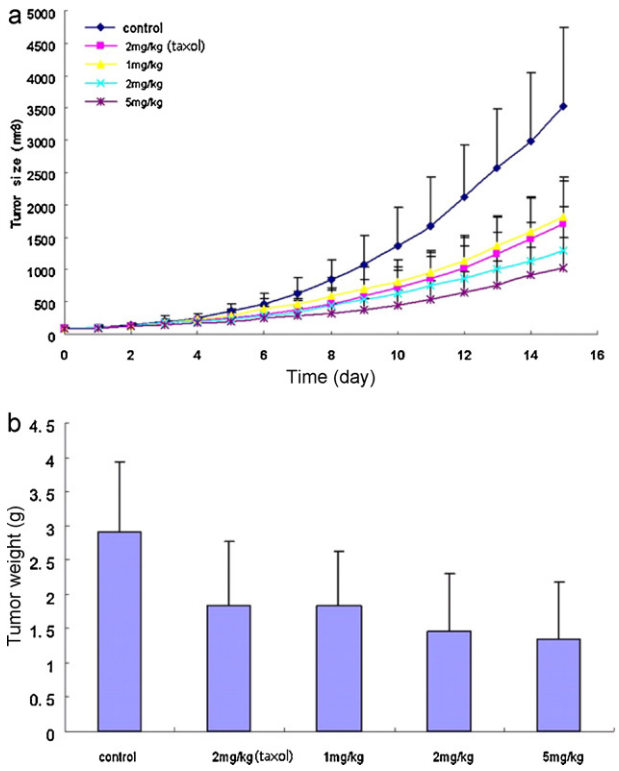

Fig. 9. Tumor suppression by PTX-loaded C10/MPEG3/API87-g-PASPAM aggregates on SCC7 grafted animal model. (a) Tumor growth tendency for each control and treatment groups and (b) average weight of the isolated tumors from each groups.

it showed equally potent anti-cancer effect with only half a dose of PTX (1 mg kg^{-1}). This positive dosage effect is related with the effective cancer targeting and release pattern provided by a few tens to hundreds nanometer-scaled pH sensitive polymeric drug carriers. When PTX was loaded more in the nano-aggregates, tumor growth was inhibited by 73% and 57% as compared to PTX treated group for 2 mg kg^{-1} and 5 mg kg^{-1} of PTX-loaded nano-aggregates, respectively, giving further regression of tumor growth. Therefore, PTX-loaded nano-aggregates showed much improved inhibition effect on tumor growth compared to the conventional PTX formulation. This improved anti cancer growth effect was mostly attributed to the pH sensitive drug release characteristics of the C10/MPEG3/API87-g-PASPAM aggregates mentioned above.

4. Conclusions

A series of poly(amino acid)-based amphiphilic graft copolymers, C18/MPEG/API-g-PASPAMs, were synthesized. The C18/MPEG/API-g-PASPAMs systems show self-aggregation/deaggregation behaviors depending on environmental pH and graft composition. The C18/MPEG/API-g-PASPAMs aggregates were spherical in size from 80 to 150 nm and show a pH buffering effect. While the C10 polymer system dissolves in aqueous solutions below pH 6.7, the C20 and C40 systems maintain the aggregate structure at lower pHs. The PTX could be effectively loaded into C10/MPEG/API-g-PASPAM cores employing this pH-dependent phase transition behavior in the absence of any solvent. The PTX loading content increased with increasing C18 graft substitution. The C10/MPEG/API-g-PASPAM system showed a rapid and abrupt release when the pH was reduced below 6.7, caused by deaggregation of micelles, while the C20 and C40 showed slightly increased but still sustained release kinetics. This release pattern enables an efficient intracellular delivery associated with endosomal pH change. The pure C18/MPEG/API-g-PASPAMs systems did

not show cell toxicity and the PTX-loaded copolymer systems showed a similar cell toxicity corresponding to a Taxol-type PTX. As C18/MPEG/API-g-PASPAMs carrier systems have sensitive pH triggered release ability, they can be efficiently applied towards a targeted delivery of PTX for cancer cells.

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References

Bae, Y., Nishiyama, N., Fukushima, S., Koyama, H., Yasuhiro, M., Kataoka, K., 2005. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. Bioconjug. Chem. 16, 122–130.

Berna, M., Dalzoppo, D., Pasut, G., Manunta, M., Izzo, L., Jones, A.T., Duncan, R., Veronese, F.M., 2006. Novel monodisperse PEG-Dendrons as new tools for targeted drug delivery: synthesis, characterization and cellular uptake. Biomacromolecules 7, 146–153.

Borisov, O.V., Zhulina, E.B., 2005. Reentrant morphological transitions in copolymer micelles with pH-sensitive corona. Langmuir 21, 3229–3231.

Caliceti, P., Quarta, S.M., Veronese, F.M., Cavallaro, G., Pedone, E., Giammona, G., 2001. Synthesis and biopharmaceutical characterisation of new poly(hydroxyethylaspartamide) copolymers as drug carriers. Biochim. Biophys. Acta 1528, 177–186.

Campbell, R., 2006. Anti-cancer agents mediate tumor physiology and delivery of nanopharmaceuticals. Chemistry 6, 503–512.

Cavallaro, G., Licciardi, M., Giammona, G., Caliceti, P., Semenzato, Salmaso, S., 2003. Poly(hydroxyethylaspartamide) derivatives as colloidal drug carrier systems. J. Control. Release 89, 285–295.

Chen Xu, W., Chen, T., Yang, W., Hu, J., Wang, C., 2005. Aggregation of biodegradable amphiphilic poly(succinimide-co-N-propyl aspartamide) and poly(N-dodecyl aspartamide-co-N-propyl aspartamide) in aqueous medium and its preliminary drug released properties. Polymer 46, 1821–1827.

Cho, H.K., Cheong, I.W., Lee, J.M., Kim, J.H., 2010. Polymeric nanoparticles, micelles and polymersomes from amphiphilic block copolymer. Korean J. Chem. Eng. 27, 731–740.

Elkharraz, K., Faisant, N., Guse, C., Siepmann, F., Arica-Yegin, B., Oger, J.M., Gust, R., Goepferich, A., Benoit, J.P., Siepmann, J., 2006. Paclitaxel-loaded microparticles and implants for the treatment of brain cancer: preparation and physicochemical characterization. Int. J. Pharm. 314, 127–136.

Hu, F.-Q., Ren, G.-F., Yuan, H., 2006. Shell cross-linked stearic acid grafted chitosan oligosaccharide self-aggregated micelles for controlled release of paclitaxel. Colloid. Surf. B: Biointerfaces 50, 97–103.

He, G., Ma, L.L., Pan, J., Venkatraman, S., 2007. ABA and BAB type triblock copolymers of PEG and PLA: a comparative study of drug release properties and stealth particle characteristics. Int. J. Pharm. 334, 48–55.

Jeong, J.H., Kang, H.S., Yang, S.R., Park, K., Kim, J.-D., 2005. Biodegradable poly(asparagine) grafted with poly(caprolactone) and the effect of substitution on self-aggregation. Colloid. Surf. A: Physicochem. Eng. Aspects 264, 187–194.

Kricheldorf, H.R., 2006. Polypeptides and 100 years of chemistry of α-amino acid *n* carboxyanhydrides. Angew. Chem. Int. Ed. 45, 5752–5784.

Kulkarni, R.P., Mishra, S., Fraser, S.E., Davis, M.E., 2005. Single cell kinetics of intracellular, nonviral, nucleic acid delivery vehicle acidification and trafficking. Bioconjug. Chem. 16, 986–994.

Lavignac, N., Lazenby, M., Franchini, J., Ferruti, P., Duncan, R., 2005. Synthesis and preliminary evaluation of poly(amidoamine)–melittin conjugates as endosomolytic polymers and/or potential anticancer therapeutics. Int. J. Pharm. 300, 102–112.

Licciardi, M., Campisi, M., Cavallaro, G., Cervello, M., Azzolina, A., Giammona, G., 2006. Synthesis and characterization of polyaminoacidic polycations for gene delivery. Biomaterials 27, 2066–2075.

Liu, S.Q., Tong, Y.W., Yang, Y.-Y., 2005. Thermally sensitive micelles self-assembled from poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)-b-poly(D,L-lactide co-glycolide) for controlled delivers of paclitaxel. Mol. Biosyst. 26, 158–168.

Moon, J.R., Kim, B.S., Kim, J.-H., 2006. Preparation and properties of novel biodegradable hydrogel based on cationic polyaspartamide derivative. Bull. Korean Chem. Soc. 27, 981–985.

Nishiyama, N., Kataoka, K., 2006. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol. Ther. 112, 630–648.

Park, J.H., Kwon, S., Lee, M., Chung, H., Kim, J.-H., Kim, Y.-S., Park, R.-W., Kim, I.-S., Seo, S.B., Kwon, I.C., Jeong, S.Y., 2006a. Self-assembled nanoparticles based on glycol chitosan bearing hydrophobic moieties as carriers for doxorubicin: in vivo biodistribution and anti-tumor activity. Biomaterials 27, 119–126.

Park, J.S., Han, T.H., Lee, K.Y., Han, S.S., Hwang, J.J., Moon, D.H., Kim, S.Y., Cho, Y.W., 2006b. N-acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: endocytosis, exocytosis and drug release. J. Control. Release 115, 37–45.

Park, T.G., Jeong, J.H., Kim, S.W., 2006c. Current status of polymeric gene delivery systems. Adv. Drug Deliv. Rev. 58, 467–486.

Rodriguez-Hermandez, J., Lecommandoux, S., 2005. Reversible inside-out micellization of pH-responsive and water-soluble vesicles based on polypeptide diblock copolymers. J. Am. Chem. Soc. 127, 2026–2027.

Savic, R., Eisenberg, A., Maysinger, D., 2006. Block copolymer micelles as delivery vehicles of hydrophobic drugs: micelle–cell interactions. J. Drug Target. 14, 343–355.

Seo, K., Kim, J.-D., Kim, D., 2009. pH-dependent self-assembling behavior of imidazole containing polyaspartamide derivatives. J. Biomed. Mater. Res. A, 478–486.

Seo, K., Kim, D., 2010. pH-dependent hemolysis of biocompatible imidazole-grafted polyaspartamide derivatives. Acta Biomater. 6, 2157–2164.

Seo, K., Lee, D.S., Kim, S.C., Kim, D., 2010. Effect of hydrophobic octadecyl groups on pH-sensitive aggregation behavior of imidazole-containing polyaspartamide derivatives. J. Nanosci. Nanotechnol. 10, 6986–6991.

Seow, W.Y., Xue, J.M., Yang, Y.-Y., 2007. Targeted and intracellular delivery of paclitaxel using multi-functional polymeric micelles. Biomaterials 28, 1730–1740.

Shim, W.S., Kim, S.W., Choi, E.K., Park, H.J., Kim, J.S., Lee, D.S., 2006. Novel pH sensitive block copolymer micelles for solvent free drug loading. Macromol. Biosci. 6, 179–186.

Shim, W.S., Kim, J., Kim, K., Kim, Y., Park, R., Kim, I., Kwon, I.C., Lee, D.S., 2007. pH and temperature-sensitive, injectable, biodegradable block copolymer hydrogels as carriers for paclitaxel. Int. J. Pharm. 331, 11–18.

Swami, A., Aggarwal, A., Pathak, A., Patnaik, S., Kumar, P., Singh, Y., Gupta, K.C., 2007. Imidazolyl-PEI modified nanoparticles for enhanced gene delivery. Int. J. Pharm. 335, 180–192.

Takeuchi, Y., Uyama, H., Tomoshige, N., Watanabe, E., Tachibana, Y., Kobayashi, S., 2006. Injectable thermoreversible hydrogels based on amphiphilic poly(amino acid)s. J. Polym. Sci. Polym. Chem. 44, 671–675.

Wu, Y., Zheng, Y., Yang, W., Wang, C., Hu, J., Fu, S., 2005. Synthesis and characterization of a novel amphiphilic chitosan–polylactide graft copolymer. Carbohydr. Polym. 59, 165.

Zhang, Y., Zhang, J., 2005. Surface modification of monodisperse magnetite nanoparticles for improved intracellular uptake to breast cancer cells. J. Colloid Interface Sci. 283, 352–357.