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ORIGINAL CONTRIBUTION

Polymeric nanoparticles prepared using salt bridges between [(dimethylamino)propyl]-octadecanamide and poly (*N*-isopropylacrylamide-co-methacrylic acid)

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Abstract Polymeric lipid nanoparticles were prepared in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 8.0, 10 mM) by taking advantage of salt bridges formed between poly(*N*-isopropylacrylamide-co-methacrylic acid) (P(NIPAM-co-MAA)) and *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA). The homogeneous nanoparticles of 200–250 nm were obtained when the copolymer was included in the preparation so that the relative mass of copolymer to lipid was more than 5. However, when the relative amount of copolymer was less than 5, large agglomerates more than 10 μm were observed together with nanoparticles. The protonated amino groups of DMAPODA will attach to the ionized carboxyl groups of P(NIPAM-co-MAA), and they would act as polymeric amphiphiles. It is believed that the hydrophilic copolymer can stabilize the hydrophobic core of the lipid. The critical association concentrations were determined to be 32, 112, and 158 mg/l, when the lipid/copolymer ratios were 1:5, 1:23, and 1:50, respectively.

Keywords Polymeric lipid nanoparticles · Salt bridges · Poly(*N*-isopropylacrylamide-co-methacrylic acid) · *N*-[3-(Dimethylamino)propyl]-octadecanamide

Introduction

Nanoparticles have extensively been used in drug and gene delivery systems. Owing to their small size, they readily escape from the vascular system, leading to favorable delivery of anti-cancer drug to solid tumors [1]. Hydrophilic modification of nanoparticles can minimize the clearance by reticuloendothelial system because the hydrophilic shells prevent the adsorption of plasma protein and the adhesion of cells [2, 3]. Surface functionalization of nanoparticles, which are modified with hydrophilic polymers such as poly (ethylene glycol) (PEG), also can prolong the residence time of nanoparticles in the systemic circulation after intravenous administration [4, 5]. Self-assembled nanoparticles prepared from amphiphilic polymers also have been investigated, especially as biodegradable stimuli-sensitive anti-cancer drug carrier [6]. Various kinds of amphiphilic copolymers, for example, poly(3-hydroxybutyrate)–poly (ethylene glycol)–poly(3-hydroxybutyrate) and poly(ethylene glycol)-poly(ε-caprolactone)-poly(2-aminoethyl ethylene phosphate), have been used for the preparation of nanoparticles as biodegradable carriers [7, 8]. On the other hand, polymeric nanoparticles, having the structure of lipophilic core and hydrophilic shell, also can be obtained by the conjugating of hydrophilic polymers (e.g., PEG) and hydrophobic lipids (e.g., fatty acid) [9]. Hydrophilic segments constituting the shell prevent core-to-core interaction between nanoparticles and they act as a stabilizer [10].

Recently, hydrophobically modified poly(*N*-isopropylacrylamide) (PNIPAM) was used for the preparation of thermo responsive polymeric nanoparticles and micelles, such as poly(*N*-isopropylacrylamice)-*b*-poly(ε-caprolactone) (PN-PCL) nanoparticles which were composed of different PCL block lengths, poly(γ-benzyl-L-glutamate)-poly(*N*-isopropylacrylamide) (PBLG-PNIPAM) nanoparticles, and poly (*N*-isopropylacrylamide-*b*-methyl methacrylate) (PNIPAM-*b*-PMMA) micelles [11–13]. In addition, poly(*N*-isopropylacrylamide-co-methacrylic acid) P(NIPAM-co-MAA) are also used for the preparation of pH-sensitive polymeric

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nanoparticles and micelles where several kinds of hydrophobic compounds were covalently attached to MAA residues [14].

In this study, a novel polymeric nanoparticle was developed by taking advantage of salt bridge formed between copolymer of P(NIPAM-co-MAA) and *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA). DMAPODA was reported to form vesicles with fatty acid by salt bridge [15]. The carboxyl groups in MAA residue of the copolymer are points of negative charge, and the amino groups in the heads of DMAPODAs are points of positive charges. As a result, DMAPODAs will be electrostatically attached to MAA residues, which are randomly distributed along the copolymer chain. The polymeric nanoparticles are prepared by a dialysis method. Critical association concentrations (CACs) were determined by a fluorescence technique using pyrene. The shape of the nanoparticle was investigated on transmission electron microscope (TEM) using a negative staining technique.

Experimental

Materials

DMAPODA (M.W. 369) was gifted from Inolex Chemical Co. (Philadelphia, PA, USA). Monomer of NIPAM (M.W. 113.16) was purchased from Tokyo Chemical Industry Co. (TCI, Tokyo, Japan), and MAA (M.W. 86.09) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). α, α′-Azobis(isobutyronitrile) (M.W. 164.21) was purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M.W. 238) and pyrene (M.W. 202.26) were purchased from Sigma (St. Louis, MO, USA). All other reagents were in analytical grade.

Preparation and characterization of NIPAM/MAA copolymers

Copolymer of NIPAM/MAA (P(NIPAM-co-MAA)) were prepared by a free radical reaction [16]. The molar ratio of NIPAM/MAA monomer was 95/5. The molecular weight was measured by gel permeation chromatography [17]. The content of MAA in the copolymer was determined by titrating MAA residue [18].

Preparation of DMAPODA/P(NIPAM-co-MAA) suspension

Twenty milligrams of DMAPODA and variable amounts of P(NIPAM-co-MAA) were dissolved in 1 ml of ethanol so that the mass ratios of lipid to copolymer were 1:5, 1:23, and 1:50. The solution was put into 24 ml of HEPES buffer (pH 8.0, 10 mM), and then the mixture was stirred for 5 min. The mixture was contained in a dialysis bag (molecular weight cut-off, 10,000), and it was dialyzed against 1,000 ml of HEPES buffer (pH 8.0, 10 mM) for 24 h with six times exchanges of the buffer.

Characterization of DMAPODA/P(NIPAM-co-MAA) particles

The shapes of DMAPODA/P(NIPAM-co-MAA) particles suspended in HEPES buffer (pH 8.0, 10 mM) were investigated on TEM (Leo-912AB Omega, Leo, Germany) using negative staining method [19]. The particle size and the zeta potentials were measured at various pHs (3.0–9.0) on a particle size analyzer (Plus 90, Brookhaven, NY, USA).

Determination of critical association concentration

One milliliter of pyrene (6×10^{-6}M in acetone) was put in a 20-ml glass vial, and acetone was allowed to evaporate at room temperature. The suspensions of lipid/copolymer mixture were diluted with HEPES buffer (pH 8.0, 10 mM) so that the concentrations of lipid were 0.00045, 0.0022, 0.0045, 0.022, 0.045, 0.22, and 0.45 mg/ml. Ten milliliter of each diluted suspension was put into the vial containing pyrene, and it was kept preventing the light at room temperature for 24 h. The intensity of fluorescence was measured at the first emission peak (373 nm, I_3) and the third emission peak (393.5 nm, I_3) with excitation of 338 nm. The intensity ratio (I_3/I_1) of the third peak to the first peak was plotted against the logarithmic concentration of lipid [20].

pH-sensitivity of DMAPODA/P(NIPAM-co-MAA) particles

The pH-sensitivity of DMAPODA/P(NIPAM-co-MAA) particles was investigated using pyrene, as the above method. The suspensions of lipid/copolymer mixture were diluted with HEPES buffer at different pH (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). The concentration of lipid was fixed to 0.2 mg/ml. The intensity ratio (I_3/I_1) of the third peak to the first peak was plotted against pH values.

Results and discussion

Characterization of NIPAM/MAA copolymers

The molar ratio of NIPAM to MAA residues was 94.6/5.4. The number average molecular weight (Mn) and the weight average molecular weight (Mw) were 6,645 and 14,202, respectively.

Transmission electron microscopy

Figure 1 shows TEM photos of DMAPODA particles and DMAPODA/P(NIPAM-co-MAA) particles. Without the copolymer, large clusters were obtained, and there was no evidence that micro- or nanoparticles were formed (Fig. 1a). When the ratio of lipid/copolymer was 1:3 (*w*/*w*), nanoparticles were formed together with large agglomerates (Fig. 1b). When the ratio increased to 1:5 (*w*/*w*), large agglomerates disappeared, and homogeneous nanoparticles were observed (Fig. 1c). In fact, visible clusters were formed during dialysis when the lipid/copolymer ratio was less than 1:5. The DMAPODA will attach to P(NIPAM-co-MAA) through salt bridges formed between the amino groups and the carboxylic acids. Hence, a polymeric amphiphiles having a comb-like structure, where the polymer is a hydrophilic backbone and the lipid is a pendent group, could be obtained. The lipid/copolymer mass ratio of 1:5 corresponds to the amino group/carboxylic group molar ratio of 1:1. Accordingly, if the mass ratio is less than 1:5, the excess amount of DMAPODA will hardly participate in the formation of salt bridges, and it will aggregate into large clusters. On the other hand, when the ratio is greater than 1:5, the copolymer could accommodate all the amount of DMAPODA since the amount of acrylic acid is in excess compared with that of the amino group. As a result, the hydrophobic lipid will be readily dispersed in an aqueous phase with the aid of the hydrophilic copolymers. Due to the hydrophobic interaction among the hydrocarbon chains, the lipid would form the core of nanoparticles, as shown on TEM photos, and the copolymers would be the shell of the particles and act as a stabilizer.

Measurements of zeta potentials

Figure 2 shows pH-dependent zeta potentials of DMAPODA particles and DMAPODA/P(NIPAM-co-MAA) particles. Without the copolymer, the surface potentials were strongly positive at acidic pHs, and they were weakly positive at alkali pHs. With the copolymer, the surface potentials were markedly reduced in the full range of pH tested. This is because that the negative charges of the carboxylic groups of the copolymers neutralize the positive charges of the amino groups of the lipids by forming salt bridges. When the lipid/copolymer ratio was 1:5, the zeta potential decreased with pH in a saturation manner. The potential was +30 mV at pH 3.0, about zero at pH 5.0, and it was almost constant in the range of pH 7.0–9.0 (−18 mV). The potential would mainly depend on the number of ionizable groups and the degree of ionization. When the lipid/copolymer ratio was 1:5, the molar ratio of amino group to carboxyl group was calculated to be almost 1:1. Thus, the degree of ionization would be a major factor to determine the zeta potential. The pK value is a measure of the degree of ionization. The pK value of MAA is 5.4 and that of DMAPODA is 10.5 [15, 21]. Assuming that carboxylic acid forms a salt bridge with amino group in equal molar ratio, the point of zero charge should be observed around the midpoint of pH 5.4 and pH 10.5. According to the data, however, the point of zero charge was observed around pH 5.0, where the number of negative charge would be less than that of positive charge. How is this possible? As described previously, the copolymer would act as a stabilizer for the lipid particles. Therefore, the electrostatic potential of the anionic copolymer could have a greater effect on the surface potential than that of the cationic lipid. This may account for why the point of zero charge occurred around pH 5.0. On the other hand, the zeta potential was almost constant in the range of pH 7.0–9.0. Above pH 7.0, the number of negative charge would be greater than that of positive charge. As a result, in the lipid/copolymer particles, the number of ionized carboxylic acid would be the same as that of protonated amino group. Excess amount of ionized copolymers would have little effect on the surface potentials of the particles, since they will be in a bulk aqueous phase. Accordingly, the surface potential could be constant in the pH 7.0–9.0. When the mass ratio further increased to 1:23 and 1:50, the point of zero charge was

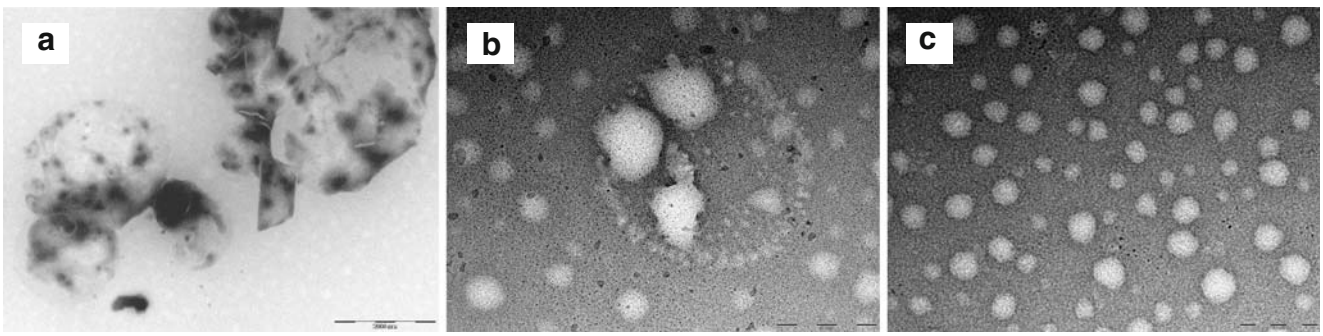

Fig. 1 TEM photos of DMAPODA agglomerate (**a**) and DMAPODA-attached P(NIPAM-co-MAA) (1:3 *w*/*w* (**b**), 1:5 *w*/*w* (**c**)) aggregates. The *bars* were 2,000, 2,000, and 500 nm, respectively

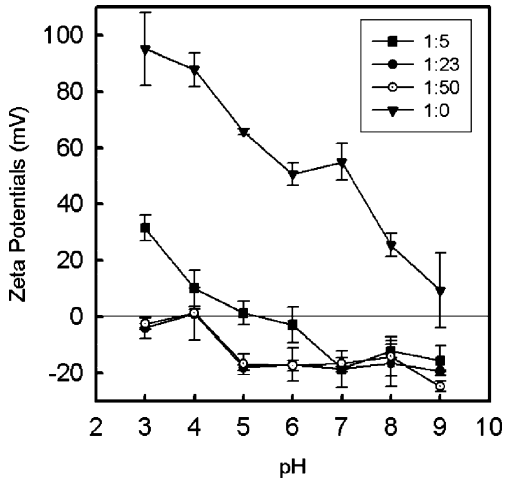

Fig. 2 pH-Dependent zeta potentials of DMAPODA agglomerate (*inverted triangle*) and DMAPODA-attached P(NIPAM-co-MAA) aggregates (1:5 *w/w* (*square*), 1:23 *w/w* (*filled circle*), 1:50 *w/w* (*open circle*))

obtained at pH 3.0–4.0, and the surface potential was almost constant in the range of pH 5.0–9.0. That is, the point of zero charge and the saturation point shifted to lower pHs. This is possibly because the relative amount of the anionic polymer increased, leading to the increased number of negative charges.

Measurements of size

Figure 3 shows pH-dependent size variations of DMAPODA particles (Fig. 3a) and of DMAPODA/P(NIPAM-co-MAA) particles (Fig. 3b). Without copolymers, the size was 350–390 nm at pH 3.0–4.0, and it dramatically increased to tens of microns as pH increased up to 9.0. In strong acidic conditions, most of amino groups will be protonated, and they may act as hydrophilic heads. Accordingly, the stable nanoparticles could be obtained in strong acidic conditions without the aid of amphiphilic dispersants. As pH increased, the degree of the protonation will decrease, and the hydrophilicity of the amino acid will also decrease. Thus, the lipid in neutral and alkali conditions would hardly be dispersed into small particles. In fact, large and visible clusters were observed at pH 8.0 and pH 9.0. When the copolymer was included, however, the size was hundreds of nanometer (Fig. 3b). This means that the copolymer acts as a dispersant for the formation of nanoparticles. The copolymer is hydrophilic and water-soluble at room temperature, and thus, it is surface-inactive. Nevertheless, it turned to be a good stabilizing agent. It is believed that the hydrophilic copolymer could accommodate hydrophobic DMAPODA on its chain through salt bridges, and the lipid/copolymer conjugates could be surface-active and act as a dispersant. When the lipid/copolymer ratio was 1:5, the size almost linearly increased from 110 to 320 nm as pH increased from 3.0 to 9.0. When the medium was alkali, the lipid tended to be unionized, and most of the lipid would be in a unionized form. Since unionized lipid would be surface-inactive and it would hardly be electrostatically attached to the ionized polymer, the unionized lipid is likely to form the matrix of nanoparticles. This is in account for why the particles were relatively large at alkali pHs. On the contrary, when the medium was acidic, most of lipid would be in an ionized form. Since ionized lipid would be surface-active and it would readily be electrostatically attached to the ionized polymer, the ionized lipid is likely to form either lipid micelle by itself or polymeric micelles together with ionized polymer. This may explain why the particles were relatively small at acidic pHs. When the lipid/polymer ratio increased to 1:23 and 1:50, the particles at pH 5.0–6.0 was less than 100 nm, much smaller than in case the lipid/polymer ratio was 1:5. Because the amount of ionized carboxylic acids will increase at the increased ratio of lipid/polymer, the cationic lipids would have more chance to attach to the anionic copolymer. In this circumstance, polymeric micelles are likely to be formed rather than lipid nanoparticles. This could explain why nanoparticles less than 100 nm was obtained at pH 5.0–6.0. On the other hand, the size increased again at

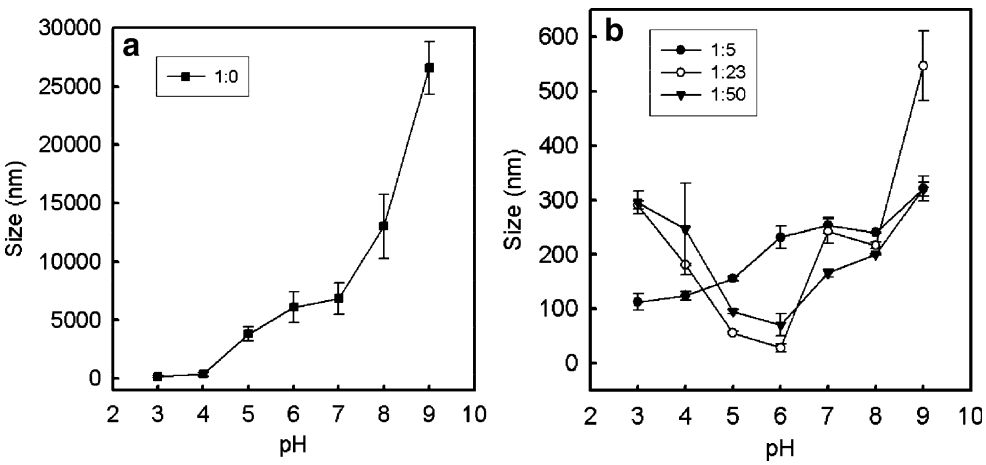

Fig. 3 pH-Dependent size variations of DMAPODA agglomerate (**a**) and DMAPODA-attached P (NIPAM-co-MAA) aggregates ((**b**) 1:5 *w/w* (*filled circle*), 1:23 *w/w* (*open circle*), 1:50 *w/w* (*inverted triangle*))

pH 3.0–4.0. Either polymeric micelles or lipid micelles are believed to be formed in strong acidic condition. The increased size is possibly due to the agglomeration of the micelles. Yin H. et al. found that the micelles might have a chance to associate together and form a secondary structure [22]. In fact, the surface potentials were almost zero at those pHs (Fig. 2), and there would be no repulsion force between the micelles, leading to the extensive agglomeration.

Determination of critical association concentration

Figure 4 shows change in the ratio of the fluorescence intensities (I_3/I_1) of pyrene with logarithmic concentration of lipid. When only DMAPODA was dispersed in HEPES buffer (pH 8.0, 10 mM), the fluorescence intensity ratio was relatively high, and there was no significant inflection in the ratio. It means that DMAPODA is so hydrophobic, and the lipid assembles into large aggregates in the full range of the concentration tested. In fact, without copolymer, the size of the particles in HEPES buffer (pH 8.0, 10 mM) was greater than 10 μm (Fig. 2). The large particles were believed to provide strong hydrophobic environments, resulting in a high fluorescence intensity ratio. When P(NIPAM-co-MAA) was included, significant inflection points were observed. The inflection point occurs due to the formation of a hydrophobic domain [23]. The copolymer is hydrophilic, and it would hardly provide hydrophobic environments. In contrast, DMAPODA is lipidic, and it could aggregate into particles having hydrophobic domains. Therefore, the inflection point corresponds to the CAC. According to the inflection points, the CACs were estimated to be 32, 112, and 158 mg/l, when the lipid/copolymer ratios were 1:5, 1:23, and 1:50, respectively. If the relative amount of the copolymer to lipid increases, the hydrophilicity of lipids-attached polymers will increase and, accordingly, they will associate at a higher concentration. The higher the hydrophilic lipophilic balance number of an amphiphile is, the higher the CAC is [24].

pH-sensitivity of DMAPODA/P(NIPAM-co-MAA) particles

Figure 5 shows the change in the ratio of the fluorescence intensities (I_3/I_1) of pyrene with varying pH values. When only DMAPODA was dispersed in HEPES buffer, the fluorescence intensity ratio was relatively high at the range of pH 6.0–8.0, and the intensity ratio decreased sharply from pH 5.0 to 3.0 for all the preparations containing DMAPODA. This is possibly because DMAPODA was ionized at acidic pHs to provide a more hydrophilic environment. When only P(NIPAM-co-MAA) was dissolved in HEPES buffer, the intensity ratios in the range of pH 6.0–8.0 were almost the same as the control sample (HEPES buffer at different pH with the same concentration of pyrene), indicating that a hydrophilic environment was obtained in the present of P(NIPAM-co-MAA) in the pH range. The slight increase of intensity ratio in the range of pH 3.0–5.0 may be ascribed to the deionization of MAA (pKa=5.4 [21]), which will slightly decrease the hydrophilicity of the copolymer. The particles with the lipid/copolymer ratios of 1:5, 1:23, and 1:50 showed higher fluorescence intensity ratios at pH 5.0 and 6.0. As mentioned in the section of "Measurements of size", polymeric micelles are the most likely to be formed at those pHs. Therefore, the number of hydrophobic domain spots would be higher, leading to the higher fluorescence intensity ratios. When the pH increased to 7.0 and 8.0,

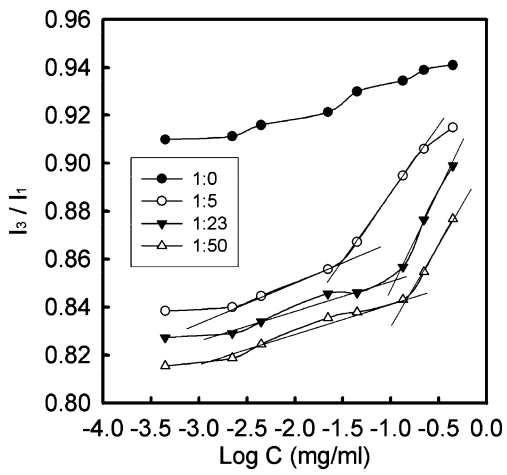

Fig. 4 Fluorescence intensity ratios of pyrene with logarithmic concentration of lipid. The DMAPODA/P(NIPAM-co-MAA) (*w*/*w*) ratios were 1:0 (*filled circle*), 1:5 (*open circle*), 1:23 (*inverted filled triangle*), and 1:50 (*open triangle*)

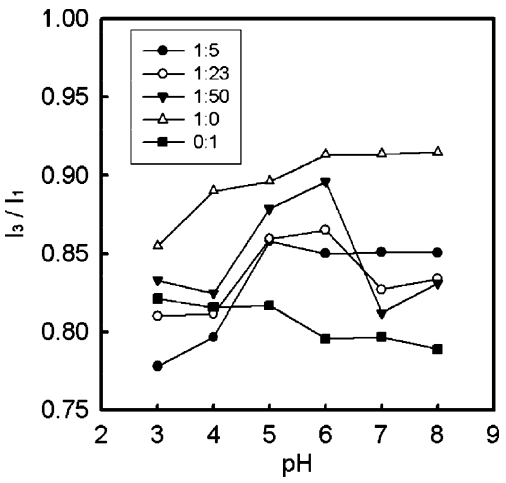

Fig. 5 Fluorescence intensity ratios of pyrene with pH. The concentration of lipid was 0.2 mg/ml. The DMAPODA/P(NIPAM-co-MAA) (*w*/*w*) ratios were 1:0 (*open triangle*), 1:5 (*filled circle*), 1:23 (*open circle*), 1:50 (*inverted filled triangle*), and 0:1 (*square*)

there was a decrease in the fluorescence intensity ratio, especially at the lipid/copolymer ratio of 1:50. This is possibly due to the excess amount of ionized copolymer, which will provide a hydrophilic environment. On the other hand, NIPAM segments in P(NIPAM-co-MAA) will not participate in the salt bridging process, and they would form outer shells of nanoparticles. Accordingly, the nanoparticles are expected to show a temperature-dependent aggregation/re-dispersion. The thermo-sensitivity will be covered in a future work.

Conclusions

Homogeneous nanoparticles could be formed by dispersing a cationic lipid (DMAPODA) and an anionic water-soluble copolymer (P(NIPAM-co-MAA)). It is believed that the cationic lipids attach to the anionic polymers through salt bridges between the amino groups and the carboxylic groups. Due to the pH-sensitivity of the salt bridges, the nanoparticles were pH-sensitive in terms of change in their size. The nanoparticles might be applicable to the delivery of therapeutic agents or genes.

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