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pH-Sensitive Cationic Vesicles Prepared Using *N*-[3-(Dimethylamino)propyl]-Octadecanamide and Stearic Acid

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Vesicles composed of N-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA) and stearic acid (SA) were prepared through melting and stirring after hydrating with HEPES buffer (10 mM, pH 7.4). On micrographs, DMAPODA/SA molar ratios of 2:3, 3:2, 1:2, and 2:1 led to the formation of multilamellar vesicles. However, the degree of vesicle formation and the size of vesicles decreased as the ratio deviated from 1:1. The mean size after sonication was 529 to 2318 nm, depending on the molar ratio of DMAPODA/SA. The Zeta potential of vesicles of 2:1, 3:2 and 1:1 were greater than +25 mV at physiological pH, and thus they are expected to show their colloidal stability at the same pH. In calorimetric study, the vesicles of 3:2 prepared in HEPES buffer, and the vesicles of 1:1 prepared either in the buffer or in distilled water exhibited endothermic peaks around 51°C to 52°C. The endothermic peaks are due to the phase transition of the vesicle membranes, indicating that vesicles were successfully formed even when DMAPODA was present in excess. Finally, pH-dependent changes in shape and integrity were investigated on a microscope. The disintegration of vesicles was observed in acidic and alkali medium. This is due to that salt bridging was destroyed at those conditions which caused the vesicles to be unstable. The vesicles might be used as pH-sensitive delivery carriers for therapeutic agents or genes.

Keywords: cationic; liquid crystal; pH-sensitive; vesicles

1. INTRODUCTION

Vesicles that are sensitive to temperature [1], light [2], pH [3,4] and other stimuli [5] are widely used in gene and drug delivery due to their

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stability, low toxicity, ease of preparation and active respond to stimuli. In most cases, sensitization of vesicles was achieved by modification of the surfaces of vesicles with pH-sensitive polymers [6] or temperature-sensitive polymers [7] or inclusion of ionizable lipids in the bilayer of vesicles [8]. Cationic vesicles have been used for gene delivery for more than 20 years since the vesicles have positive charges which allow them to complex with DNA better than any other types of vesicles [9]. In previous studies, cationic vesicles were successfully bonded to negatively charged DNA for gene delivery [10–12]. On the other hand, the drugs such as rifampicin were also entrapped in cationic vesicles for drug delivery [13]. And cationic vesicles were also reported to be used as a carrier for chemotherapy agents such as doxorubicin since they have been showed to selectively target tumor endothelial cells [14]. *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA) and stearic acids (SA) are reported to form lipid bilayer of vesicles by a salt bridging between NH_3^+ of DMAPODA and COO^- of SA, and either the positive charges of NH_3^+ or negative charges of COO^- will disappear if the pH value is lower than 4.5 or higher than 10.5 [15–17]. Thus, the vesicles should change their stabilities in respond to various pH values and they would be destabilized either in the acidic or in the alkali conditions. DMAPODA/SA vesicles containing triclosan were prepared in distilled water by Kim *et al.* [16], and they also modified DMAPODA/SA vesicles with *N*-methylol urea-dodecylamine in order to prevent the vesicles from destabilization [17]. However, the reports which are relevant to DMAPODA/SA vesicles are still seldom, especially about their pH-sensitivity. In this study, cationic vesicles composed of DMAPODA and SA were prepared by sonication method [16,17]. The pH-sensitivity of vesicles was observed in terms of the integrity. In parallel, the physical properties such as particle size, zeta potentials, and thermal phase transition were investigated.

2. EXPERIMENTAL

2.1. Materials

N-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA, M.W. 369) was gifted from Inolex Chemical Co. (Philadelphia, PA, USA). Stearic acid (SA, M.W. 284) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, M.W. 238) were purchased from Sigma (St. Louis, MO). DMAPODA and SA were used together as bilayer-forming materials for vesicle formation. All other regents were in analytical grade.

2.2. Preparation of Vesicles

DMAPODA and SA were put together in a 50 ml beaker so that the molar ratios of DMAPODA/SA were 1:3, 1:2, 2:3, 1:1, 3:2, 2:1, 3:1 and the total weight of lipid was 2 g. The mixture was then heated in a water bath at 60∼70°C until it melted to be clear. Distilled water or HEPES buffer (10 mM, pH 7.4) of 20 ml which was pre-heated to the same temperature was added to the melt so that the concentration of vesicles was 10 mg/ml. The dispersion was stirred at 500 rpm for 1 h until it cooled down to room temperature. Finally, the dispersion was processed by a bath type sonicator (VCX500, Sonics, USA) at 50∼55°C for 20 min.

2.3. Inverted Microscopy

The inverted microscopy was used to confirm the vesicles formation before sonication. The vesicles with different molar ratios of DMAPODA/SA were observed under an inverted microscope (400X, CKX41, Olympus, Japan).

2.4. Transmission Electron Microscopy

To make sure the vesicles formation after sonication, the vesicles were investigated on a transmission electron microscope (TEM, LEO-912AB OMEGA, LEO, Germany) using negative staining method. A drop of vesicles suspension was applied on a 300-mesh formvar copper grid and the suspension was allowed to adhere to the formvar film for 10 min. After the excess of suspension was removed with filter paper (Whatman no. 2), a drop of staining solution (2% phosphotungstic acid, pH 6.8) was added on the film. This solution was left for 10 min and then drawn off by a filter paper. The stained vesicle on the grid was dried in air overnight.

2.5. Measurement of Particle Size and Zeta Potentials

The particle size and the zeta potentials of vesicles were measured using a diluted suspension on a particle size analyzer (Plus 90, Brookhaven, USA). 50 μl of a vesicle suspension (10 mg/ml) was put into a cuvette and then it was filled up with HEPES buffer (10 mM, pH 7.4) to 1.5 ml. The suspension was equilibrated for 4 min at 22°C. The measurement was done with three runs where each run consists of 10 single gauging. And the suspensions for zeta potentials were diluted with HEPES buffer (10 mM) with different pH.

2.6. Differential Scanning Calorimetry

The vesicles were centrifuged at 15000 rpm for 1 h and the HEPES buffer was removed from the bottom since the density of vesicles were less than that of buffer solution. The concentrated vesicles were scanned on a differential scanning calorimeter (TA instruments DSC 2010) from 30 to 90°C at a heating rate of 2°C/min. In parallel, pure DMAPODA and SA were also investigated by DSC.

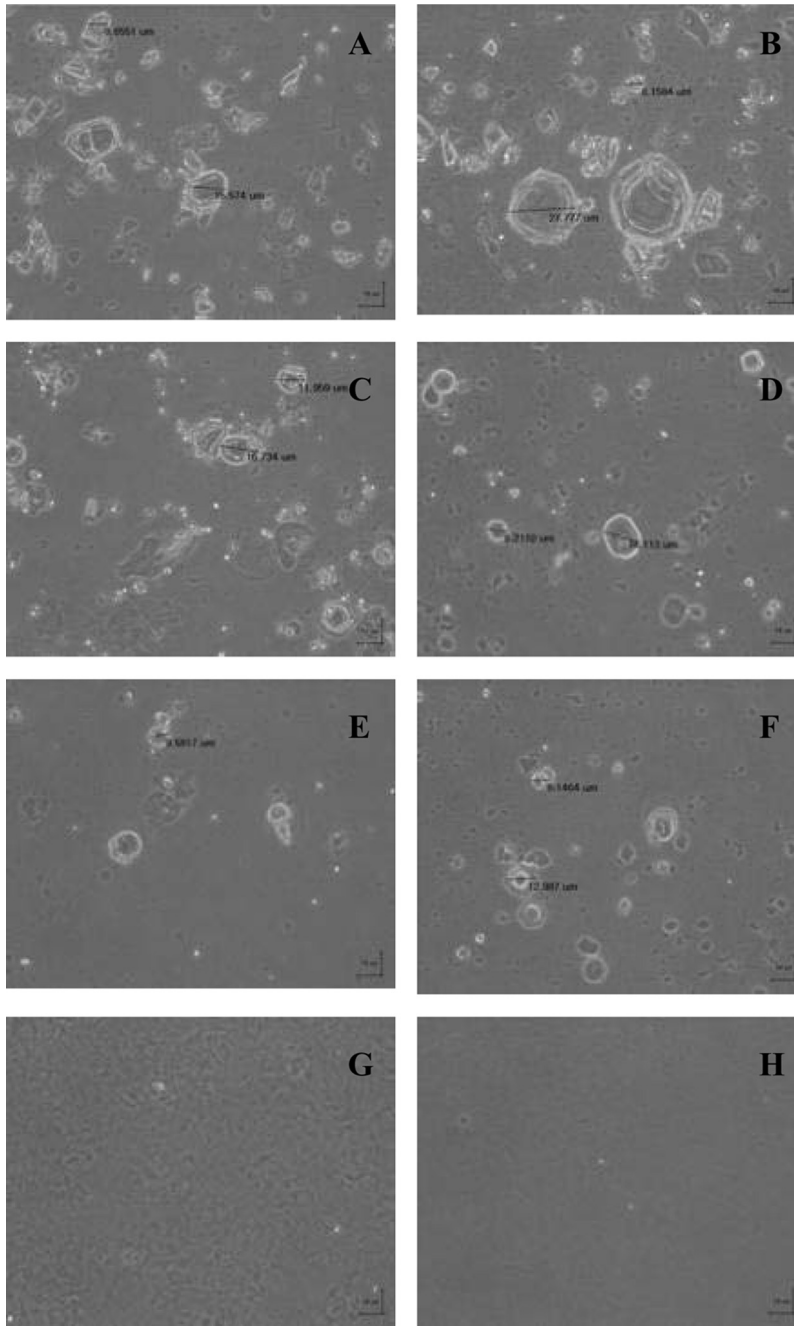
2.7. pH-Dependent Stability

0.1 ml of vesicles was injected to 0.9 ml of HEPES buffer (10 mM) which had been pre-adjusted to pH 3, 4, 7, 10 and 11. The suspensions were stood at room temperature for 24 h, and then the vesicles were observed on an inverted microscope (400X, CKX41, Olympus, Japan).

3. RESULTS AND DISCUSSION

3.1. Inverted Microscopy

Figure 1 shows the inverted micrographs of before-sonication vesicles, prepared with various molar ratios of DMAPODA/SA. When DMAPODA/SA molar ratios of 1:1, relatively large multi-lamellar vesicles with diameters ranging from 5 to 30 μm were clearly observed in both distilled water (Fig. 1A) and HEPES buffer (Fig. 1B). If ionic strength of the preparation medium is higher than 1 M, the vesicle may not be formed [15]. The vesicles are formed through the salt bridging between NH_3^+ of DMAPODA and COO^- of SA. The charge intensities of head groups would be lower in electrolyte solution, due to the bindings of counter ions to the charged heads. As a result, the salt bridging would hardly occur. Under our experimental condition, vesicles have been successfully prepared in HEPES buffer as in the distilled water. In fact, the ionic strength of the buffer solution is 10 mM, and according to the previous data, the value is not high enough to hinder the salt bridging. Hence, other vesicles having various molar ratios were prepared in HEPES buffer. As shown in Figures 1C, D, E and F, DMAPODA/SA molar ratios of 2:3, 3:2, 1:2, and 2:1 led to the formation of vesicles. However, the degree of vesicle formation and the size of vesicles decreased as the ratio deviated more from 1:1. Furthermore, when the molar ratios became to 1:3 and 3:1 (Figs. 1G and H), vesicles were hardly observed. It was reported that the vesicles are formed through the salt bridging between NH_3^+ of DMAPODA and COO^- of stearic acids [15–17]. When DMAPODA or SA was present in excess (This is the cases of DMAPODA/SA molar ratios of 3:1, 2:1, 3:2, 2:3, 1:2 and 1:3), the excess



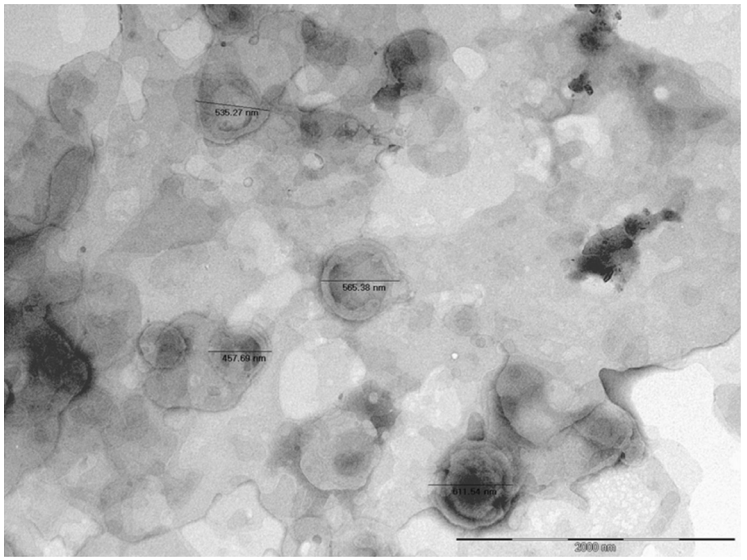


FIGURE 2 TEM images of vesicles prepared in HEPES buffer (10 mM, pH 7.4) with DMAPODA/SA molar ratio of 1:1 after sonication. Bar represents 2000 nm.

amount of the amphiphiles may intercalate into the vesicle membranes, leading to the reductions in the degree of vesicle formation and the size. Therefore, the optimal ratio for the vesicle formation is thought to be 1:1.

3.2. Transmission Electron Microscopy

Figure 2 shows the transmission electron micrographs of after-sonication vesicles, of which DMAPODA/SA molar ratios were 1:1. According to the result, the vesicles were obviously formed and the sized markedly decreased from a few microns to sub-micron by

FIGURE 1 Inverted micrographs of vesicles prepared with various molar ratios of DMAPODA/SA before sonication. Vesicle was prepared in distilled water with DMAPODA/SA molar ratio of 1:1 (A). Other vesicles were prepared in HEPES buffer (10 mM, pH 7.4) with DMAPODA/SA molar ratio of 1:1 (B); 2:3 (C); 3:2 (D); 1:2 (E); 2:1(F); 1:3 (G) and 3:1 (H). Bars represent 10 μm.

sonication. In parallel, vesicles prepared with DMAPODA/SA molar ratios of 2:1, 3:2, 2:3 and 1:2 exhibited no significant difference in shape but the number of vesicles was much less on the electron micrographs. In case DMAPODA/SA molar ratios of 3:1 and 1:3, the vesicles were hardly found. These results are in a good agreement with those of the microphotographs. The deviation from the ratio of 1:1 would lead to the reduction in the degree of vesicle formation, since excess amount of DMAPODA or SA could be incorporated into the vesicle membrane and disintegrate the vesicles.

3.3. Measurement of Particle Size and Zeta Potentials

Figure 3 shows the size distributions of vesicles with various molar ratios of DMAPODA/SA and the mean diameter of each vesicle was summarized in Table 1. As shown in Figure 3, the diameters exhibited bimodal curves before sonication. After sonication, the mean size decreased markedly but the mean size was still large, ranging 529 to 2318 nm, depending on the molar ratio of DMAPODA/SA (Table 1). In addition, the bimodal distribution was still observed after sonication (Fig. 3). In general, it was reported that a sonication method for the preparation of phospholipid bilayer vesicle (liposome) gives a rise to homogeneous small unilamellar vesicles [18]. The inhomogeniety in size and the large size in this study is possibly because mild sonic energy was applied during the vesicle preparations. Furthermore, the vesicle was prepared by taking advantage of the salt bridging between NH_3^+ of DMAPODA and COO^- of SA. By associating with DMAPODA, SA may play pseudo tails of the cationic amphiphile [16,17]. As a result, the associated two amphiphiles behaved like one molecule having two tails. In this circumstance, the cross section area of hydrophobic tails is much larger than that of phospholipids. Therefore, the curvature of the vesicle would be greater than that of liposomes. This may be another reason for why the size is relatively large even after sonication. On the other hand, when vesicles were prepared with the ratio of 1:1, they were dispersed either in distilled water or in HEPES buffer. The mean diameter of vesicles in the buffer was much less than that of vesicle in distilled water (Table 1). In the presence of ions, the charge intensities of head groups of DMAPODA and SA would decrease, resulting in the reduction in the head size. Thus, the effective volume of the associate of two charged amphiphiles would decrease. As a result, vesicles having lager curvature could be formed. This might explain why the mean diameter of vesicles in the buffer was much smaller than that of vesicle in distilled water.

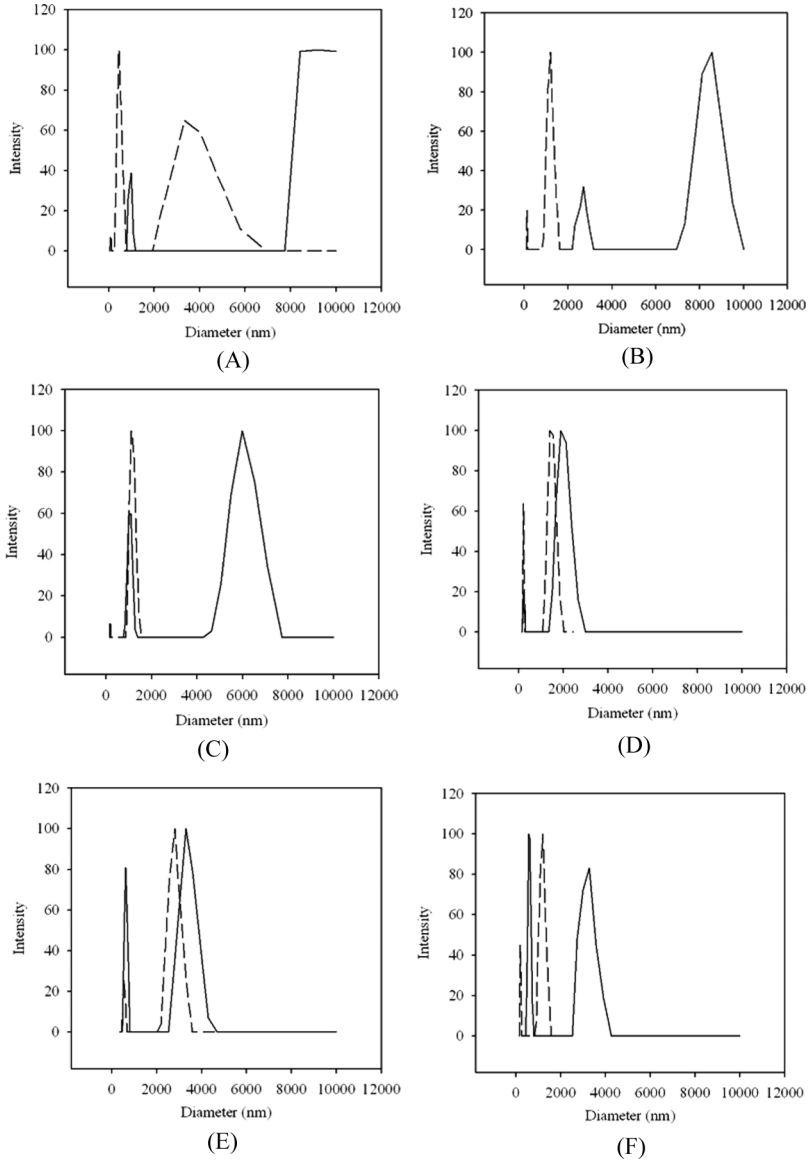


FIGURE 3 Size distributions of vesicles before sonication and after sonication. Vesicle was prepared in distilled water with DMAPODA/SA molar ratio of 1:1 (A). Other vesicles were prepared in HEPES buffer (10 mM, pH 7.4) with DMAPODA/SA molar ratio of 1:1 (B); 2:3 (C); 3:2 (D); 1:2 (E) and 2:1(F). Solid line: Before sonication; Dotted line: After sonication.

TABLE 1 The Mean Diameters of Vesicles Prepared with Various DMAPODA/SA Molar Ratios

DMAPODA/SA (molar ratio)	1:1 Distilled water	1:1 HEPES buffer	2:3 HEPES buffer	3:2 HEPES buffer	1:2 HEPES buffer	2:1 HEPES buffer
Diameter (nm) before sonication	8459.7 ± 2212.1	5808.1 ± 2114.5	2200.8 ± 402.0	1118.1 ± 212.7	1870.4 ± 83.3	881.8 ± 12.8
Diameter (nm) after sonication	2318.2 ± 310.6	825.0 ± 150.8	944.0 ± 196.0	591.4 ± 26.3	1198.7 ± 103.3	529.3 ± 11.6

Figure 4 shows the pH-dependent zeta potentials of vesicles prepared with various molar ratios in pH ranging from 4 to 8. In pH region of pH 4.0–6.0, the potentials were almost constant with respect to pH and the values were between +50 mV and +110 mV at all DMAPODA/SA molar ratios except 1:3. And then the values started to decrease around pH 6.0. This is because that with increasing pH, the amino group of DMAPODA is likely to be deprotonated and the carboxylic group of SA tends to be ionized. The values of zeta potential were greater as the content of DMAPODA increased. That is, the values were in the order of 3:1 > 2:1 > 3:2 > 1:1 > 2:3 > 1:2 > 1:3 (DMAPODA: SA ratio). One of criteria for colloidal stability is that the absolute value of zeta potential should be greater than 25 mV [19]. Thus, the vesicles of 2:1, 3:2 and 1:1 are expected to show their colloidal stability in physiological pH. Compared with the zeta potentials measured by Kim *et al.* [16], the zeta potentials of vesicles prepared in HEPES buffer were a little smaller than that prepared in distilled water. This is because the surface charges of the vesicles were suppressed by ions contained in the buffer.

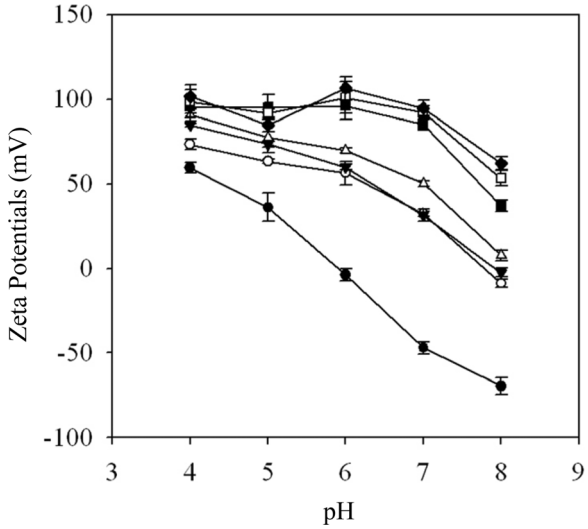


FIGURE 4 pH-dependent zeta potentials of vesicles prepared in HEPES buffer (10 mM, pH 7.4). The molar ratios of DMAPODA/SA were 1:3 (●); 1:2 (◯); 2:3 (▼); 1:1 (▽); 3:2 (■); 2:1 (□) and 3:1 (◆).

3.4. Differential Scanning Calorimetry

Figure 5 shows the thermograms of DMAPODA/SA vesicles, of which molar ratios are 3:2 and 1:1, along with those of DMAPODA and SA. The vesicles of molar ratios of 3:2 (Fig. 5A) and 1:1 (Fig. 5B) prepared in HEPES buffer, and vesicles molar ratios of 1:1 (Fig. 5C) prepared in distilled water exhibited endothermic peaks around 51.3°C, 52.0°C and 51.3°C, respectively. The endothermic peaks are due to the phase transition of the vesicle membranes from a solid gel to a liquid crystal [16,17]. This means that vesicles were successfully formed when DMAPODA was present in excess (Fig. 5A) and when prepared in HEPES buffer (Fig. 5B). On the other hand, for pure solid DMAPODA and SA, the endothermic peak appeared around 67.4°C (Fig. 5D) and 69.5°C (Fig. 5E), respectively. The peaks are due to the melting of the compounds. Since no endothermic peak of DMAPODA and SA was observed in the thermograms of the vesicles, it is believed that all of DMAPODA and SA participated in constituting the vesicles and all of them exist as constituents of the vesicles. Unfortunately, at the ratios deviated much from 1:1, (for example, 3:1, 2:1, 1:2 and 1:3), the suspensions were too viscous to concentrate lipid particles by centrifugation, which is required for the calorimetric study.

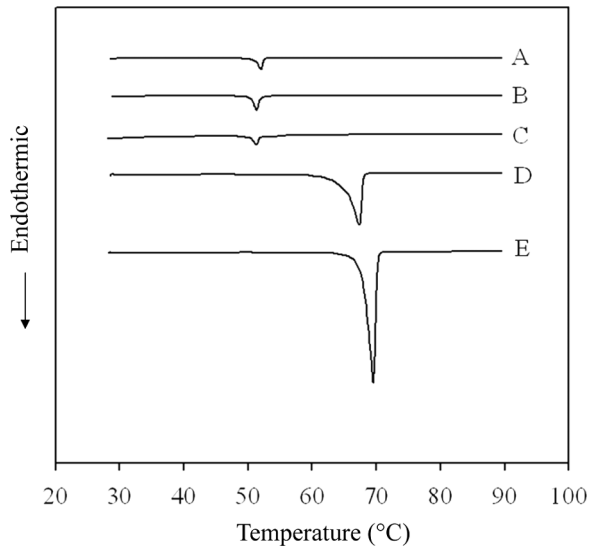


FIGURE 5 Thermograms of vesicles prepared with DMAPODA/SA molar ratios of 3:2 in HEPES buffer (10 mM, pH 7.4) (A); 1:1 in HEPES buffer (B); 1:1 in distilled water (C); pure DMAPODA (D) and pure SA (E).

3.5. pH-Dependent Stability

Figure 6 shows the integrities of vesicles (molar ratio of 1:1) after 24 h standing at various pHs. The vesicles were found obviously at pH 7.4 (Fig. 6C) and pH 7 (Fig. 6D). But at pH 3 (Fig. 6A) and pH 4 (Fig. 6B), vesicles were hardly observed under inverted microscopy. At pH 10

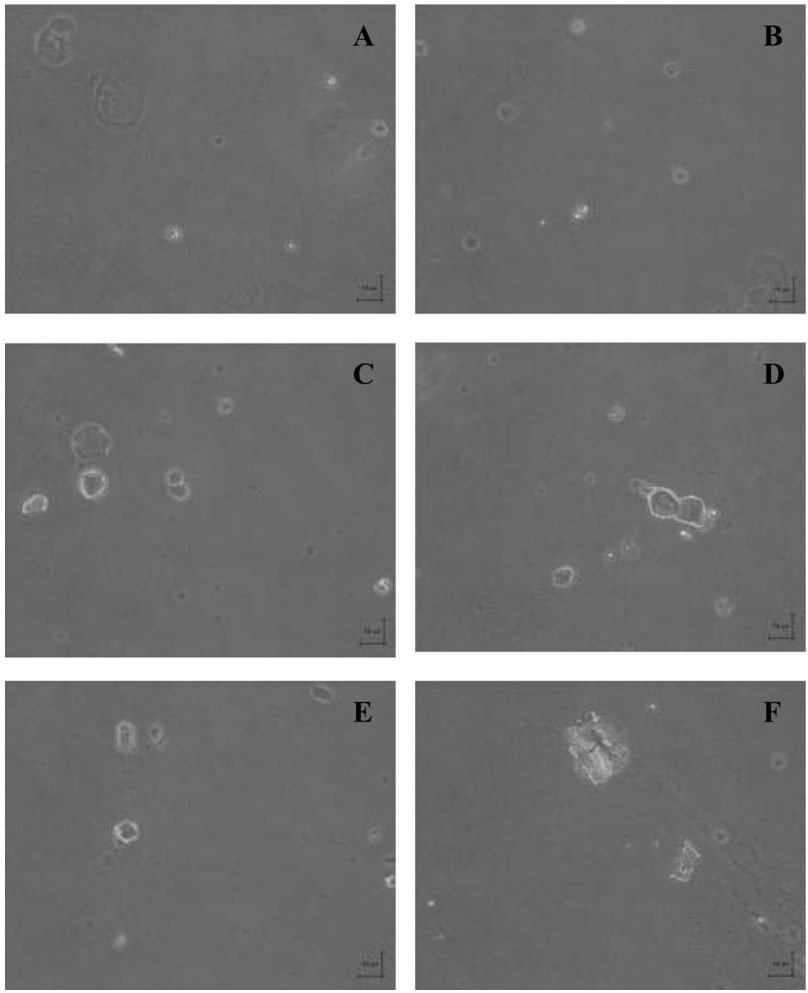


FIGURE 6 Inverted micrographs of non-sonicated vesicles prepared with DMAPODA/SA molar ratio of 1:1 in HEPES buffer (10 mM). pHs of medium were 3 (A); 4 (B); 7 (C); 7.4 (D); 10 (E) and 11(F). The photos were taken after standing each suspension for 24 h. Bars represent 10 μm.

(Fig. 6E) and pH 11 (Fig. 6F), aggregates rather than vesicles were observed. The phenomena may be due to that NH_3^+ of DMAPODA and COO^- of SA can hold their charges only in the pH range of 4.5 to 10.5. When pH is out of the range ($pH < 4.5$ or $pH > 10.5$), the salt bridging between NH_3^+ and COO^- would be destroyed, leading to the disintegration of vesicles.

4. CONCLUSIONS

The preparations of vesicles in HEPES buffer or distilled water were both successful at DMAPODA/SA molar ratios of 1:2, 2:3, 1:1, 3:2 and 2:1, while molar ratios of 1:3 and 3:1 were hard to form vesicles. The vesicles were multi lamellar and the size was markedly reduced by sonication from a few microns to sub-microns. Following the results of zeta potential measurements, vesicles of which DMAPODA/SA ratio was 2:1, 3:2 and 1:1 were expected to show their colloidal stability at physiological pH. In calorimetric study, vesicles were found to be successfully formed, even when DMAPODA was present in excess. Finally, it was observed that the vesicles lost their integrities at acidic and alkali conditions. Thus, they might be used as pH-sensitive delivery carriers for therapeutic agents or genes.

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