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Interaction of cationic vesicle with ribonucleotides (AMP, ADP, and ATP) and physicochemical characterization of DODAB/ribonucleotides complexes

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Abstract

The interaction between ribonucleotides (AMP, ADP, and ATP) and cationic vesicles prepared from dioctadecyldimethylammonium bromide (DODAB) were investigated in detail. The physicochemical properties of ribonucleotides/cationic lipid complexes were present. Gel exclusion-UV spectroscopic results showed that all the charge ratios of DODAB/ribonucleotides (AMP, ADP, and ATP) are 2:1 when the maximal ribonucleotides were adsorbed onto DODAB, while the molar ratios were different, e.g., 2:1 for DODAB/AMP, 4:1 for DODAB/ADP and 6:1 for DODAB/ATP. These differences may be attributed to the different anion charges of AMP, ADP and ATP. The results demonstrated that ribonucleotides combined with DODAB vesicles with the electrostatic attraction in the complexation of DODAB and ribonucleotides. Transmission electron microscopic results revealed the different extents of aggregation of cationic vesicles in the complexation process of ribonucleotides with cationic lipid. The variation dependence of ζ-potentials or electrophoretic mobilities on vesicle size was also different. The ζ-potentials and electrophoretic mobilities of the DODAB vesicles (0.01 and 0.02 mM) gradually decreased when the ribonucleotide concentration increased. However, the mean diameters of the DODAB vesicles (0.1 and 0.5 mM) gradually increased when the ribonucleotide concentration increased.

Keywords: Ribonucleotides (AMP, ADP, and ATP); DODAB; Transmission electron microscopy; Gel exclusion and UV spectroscopy; Zeta-potentials and electrophoretic mobilities; Dynamic light scattering

1. Introduction

Cationic vesicles have attracted great interests of the biomedical sciences for a long time [1–3] because of their potential as a core technology in the emerging field of gene therapy [2]. In this field, cationic vesicles function as vectors to deliver therapeutic genes and/or nucleic acids into cells [4–6]. The process of nucleic acid/cationic lipid complexation is primarily induced by electrostatic interaction of nucleic acids with cationic vesicles [7] and it is closely related to the efficacy of gene therapy [8,9]. Therefore, it is necessary to understand more about the general physicochemical properties of cationic vesicles in the process of nucleic acid/cationic lipid complexation that resulted from electrostatic interaction. Many studies on the formation and structural characterization of the complexes of cationic vesicles with plasmid DNA [10–12], SiRNA [13], and/or antisense oligonucleotides [14,15] have been reported. However, limited information is known about the details of the more general physicochemical properties of such nucleic acid/cationic lipid complexes.

It is well known that the monomeric unit of nucleic acid, such as adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP), which can unite to give macromolecular chains, plays a central role in many biological processes. In the present work, with the aim at extending and enriching a quantitative physicochemical description of nucleic acid/cationic lipid complexes, we

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investigate the interaction between a monomeric unit of nucleic acid (namely AMP, ADP, and ATP) and DODAB vesicles and the physicochemical properties of DODAB/ribonucleotides complexes using more straightforward experimental methods. Although the complexation process induced by ribonucleotides differs from that induced by nucleic acidic polyanions, the electrostatic interaction plays a primary role in the promotion of the formation of complexes because the two systems have similarities [16,17].

In this article, we used gel exclusion–UV spectroscopy (GE–UV) as a more straightforward method to detect the ribonucleotides adsorbed onto cationic vesicles. The GE–UV results demonstrated the interaction and determined the respective adsorption curves in the complexation of AMP, ADP, and ATP with DODAB vesicles. Transmission electron microscopic (TEM) observations revealed the different extents of the aggregation of cationic vesicles in the complexation process that resulted from the electrostatic interaction. With the addition of ribonucleotides, ζ-potentials and electrophoretic mobilities of the DODAB vesicles gradually decrease as a function of the ribonucleotide concentrations at a millimolar range of DODAB concentrations, but the mean zeta-average diameters of the DODAB vesicles exhibit a reverse trend of change. These results can provide new information on the mode of the interaction between nucleic acid and cationic lipid bilayers and may enhance our understanding of the nucleic acid-cationic lipid complexation process from the important physicochemical viewpoint.

2. Materials and methods

2.1. Materials

Dioctadecyldimethylammonium bromide (DODAB) with purity of more than 99% was purchased from Acros (Belgium). Adenosine 5′-monophosphate (AMP, 99.7%), adenosine 5′-diphosphate (ADP, 99%) and adenosine 5′-triphosphate, disodium salt (ATP, 99%) were purchased from Sigma. Chloroform and ethanol from Tianjin Chemicals Co., China, were of analytical grade. All the above chemicals were used without further purification.

2.2. Preparation of cationic vesicles

Cationic vesicle suspension composed of DODAB was prepared by lipid film hydration and sonication method as previously described [18,19]. For the preparation of a vesicle sample, the required mass of DODAB was dissolved in a methanol-chloroform solution (1:1, v/v) and deposited as a film on a round-bottomed flask by blowing purified nitrogen followed by overnight vacuum drying. The dried lipid film was hydrated to the desired concentration of 5.0 mM lipid using an aliquot of pure water at 60 °C, which was well above the phase transition temperature of this amphiphile (above Tm=45 °C) [20,21], for 30 min. The obtained aqueous suspension was placed in a sealed conical flask at 60 °C in an ultrasonic bath for about 30 min to give a clear dispersion [22]. Vesicle suspensions of lower concentrations were prepared by the dilution of these 5.0 mM preparations.

2.3. Gel exclusion and UV spectroscopy measurements

The DODAB/ribonucleotides complexes were prepared by mixing 0.3 ml of 2.0 mM DODAB dispersion with 0.3 ml of ribonucleotide solution ranging from 0 to 5.0 mM AMP, 0 to 2.5 mM ADP, and 0 to 1.7 mM ATP in polypropylene microcentrifuge tubes, respectively. Thereafter, all the above mixtures were incubated at 40 °C for 30 min and subjected to column chromatography respectively to separate vesicles from free AMP, ADP and ATP in solution using a Sephadex G-50 column (fine, Pharmacia Biotech, Sweden, typically 17×270 mm).

Elution were carried out at a flow-rate of 0.5 ml min^{-1} controlled by a peristaltic pump (HL-2S, Shanghai, China) using water as the mobile phase and 1.5 ml fractions were collected. The elution of vesicles adsorbed or non-adsorbed ribonucleotides and free ribonucleotides was monitored by measuring their UV absorptions at 259 nm on a HP UV–vis photodiode array spectrophotometer (HP 8453) with a quartz cuvette of 1 cm optical path length. Separation was considered complete since no UV absorption signal was detected. The collected vesicular dispersions were studied by dynamic light scattering (DLS) to assure that the separation procedure had no effect on their size. All the collected free ribonucleotides elutions were then mixed together to determine the free ribonucleotides in solution. For the determination of AMP, ADP and ATP, the standard UV absorption curves ranging from 0 to 40 μM AMP, 0 to 60 μM ADP, and 0 to 70 μM ATP in water were prepared, respectively. The adsorbed ribonucleotide concentration percentages were calculated from the formula:

$$EE\% = (C_t - C_f)/C_t \times 100\%$$

Where EE% is the entrapment efficiency, C_t is the total amount of the mixed ribonucleotides, and C_f is the amount of free ribonucleotides detected in solution.

2.4. TEM measurements

Samples for TEM were prepared at room temperature by conventional negative staining method. A drop of the sample

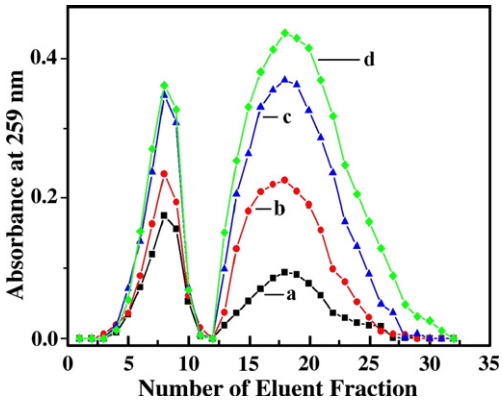

Fig. 1. Typical gel exclusion-UV spectroscopy for DODAB-AMP mixtures of different molar ratios: (a) 2.0 mM DODAB+1.0 mM AMP (-■-); (b) 2.0 mM DODAB+2.0 mM AMP (-●-); (c) 2.0 mM DODAB+4.0 mM AMP (-▲-); (d) 2.0 mM DODAB+5.0 mM AMP (-◆-).

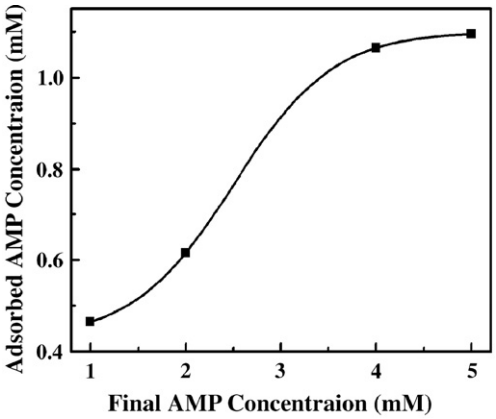

Fig. 2. Adsorbed AMP concentration curve as a function of incubated AMP concentrations in the mixtures at the fixed DODAB concentration of 2.0 mM.

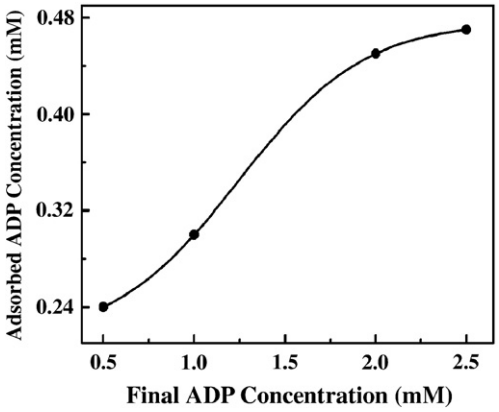

Fig. 3. Adsorbed ADP concentration curve as a function of incubated ADP concentrations in the mixtures at the fixed DODAB concentration of 2.0 mM.

solution was placed on a G-400 mesh carbon-coated copper grid and after 1 min the excess material was removed with a filter paper. A 4% (w/v) uranyl acetate solution was dropped onto the grid and the excess of the staining solution was removed with a filter paper in 60 s. Finally, the grids were examined on a JEM 1200-EX transmission electron microscope at 75–100 kV.

2.5. Vesicle zeta-potential (ζ-potential) and electrophoretic mobility (EM) measurements

At the fixed DODAB concentration, the electrophoretic behavior of the samples was determined at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, U.K.) apparatus. DODAB dispersions in water obtained as previously described were successively diluted to obtain the two different DODAB concentrations (0.01 and 0.02 mM), mixed v/v with AMP, ADP, and ATP solutions respectively over a range of ribonucleotide concentrations and incubated at 40 °C for 1 h before performing the electrophoretic measurements as a function of mixed ribonucleotide concentrations. All the above samples prepared in a syringe were injected through the folded capillary cell. Each result is an average of six measurements at the given values of DODAB and ribonucleotide concentrations. Standard electrophoretic mobility deviation amounted to about 2% of the mean. All experiments were carried out for three times at least.

Table 1
Amounts of ribonucleotides (AMP, ADP, and ATP) adsorption onto 2.0 mM DODAB vesicles obtained from GE–UV, respectively

Ribonucleotides	Final ribonucleotides concentrations/mM	EE%	Adsorbed ribonucleotides concentrations/mM
AMP	1.00	46.5	0.465
	2.00	30.8	0.616
	4.00	26.6	1.064
	5.00	21.9	1.095
ADP	0.50	48.0	0.240
	1.00	30.0	0.300
	2.00	22.5	0.450
	2.50	18.8	0.470
ATP	0.30	51.7	0.155
	0.70	36.7	0.257
	1.30	24.3	0.310
	1.70	18.8	0.320

EE% is the entrapment efficiency.

2.6. Determination of mean zeta-average diameters (Dz) and polydispersity index (PDI) for the dispersions

Determination of the vesicle size and polydispersity index was conducted with dynamic light scattering using a Zetasizer Nano ZS at 25 °C. The samples to be determined were prepared in the same manner as described in Section 2.4. DODAB vesicles were diluted to obtain 0.1 and 0.5 mM, respectively. The DLS used in this study is capable of analyzing the fluctuations arising from the Brownian motion of submicron particles using photon correlation spectroscopy to give mean zeta-average diameter and polydispersity index, which illuminated the particles with the laser and analyzed the intensity fluctuations in the scattered light [23]. The sample to be measured was placed into a standard plastic cuvette, then the cell was placed in a cell holder and a helium-neon laser was focused on the sample with a

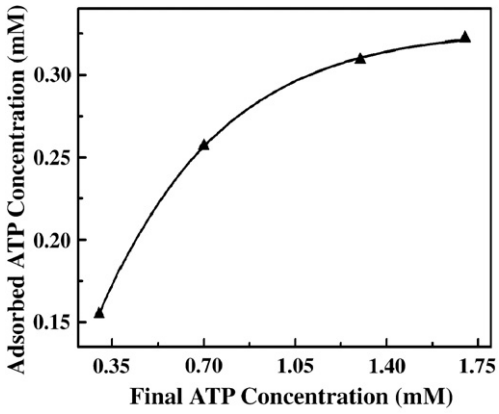

Fig. 4. Adsorbed ATP concentration curve as a function of incubated ATP concentrations in the mixtures at the fixed DODAB concentration of 2.0 mM.

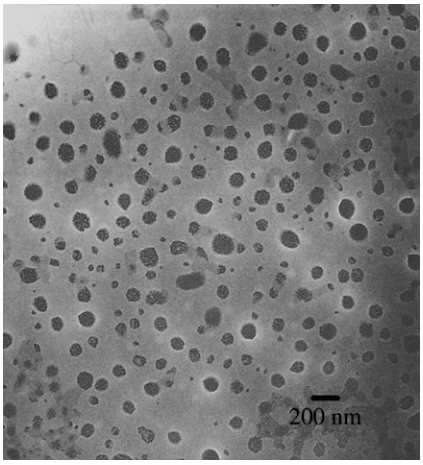

Fig. 5. Typical transmission electron micrograph of 5.0 mM DODAB vesicle.

scattering angle of 173°. The size quoted throughout is the mean harmonic zeta-average diameter of at least six independent measurements at the given concentration of DODAB vesicle suspension. Standard zeta-average diameter deviation amounted to about 3% of the mean. All experiments were carried out for three times at least. The mean PDI values of six independent measurements for each sample are listed in Table 2.

3. Results and discussion

3.1. GE–UV methodology for separating free and adsorbed ribonucleotides, and determination of ribonucleotides adsorption onto DODAB vesicles

Gel exclusion has often been used for separating small objects from vesicles [24,25]. In this work, the gel exclusion and UV measurements have been performed to detect the interaction between ribonucleotides and DODAB vesicles and to quantify ribonucleotides adsorption onto DODAB vesicles. Fig. 1 displays the typical elution profiles for the mixtures of DODAB vesicles with AMP of different concentrations at the fixed DODAB concentration (2.0 mM). The DODAB vesicles complexed with AMP are eluted in the first peak in the four elution curves [26] and the amounts of AMP adsorption onto DODAB vesicles increase with the increasing of incubated AMP concentration. This result shows that AMP appears to strongly adsorb onto DODAB vesicles. In addition, the elution profiles in Fig. 1c and d display nearly the same amount of AMP (ca.

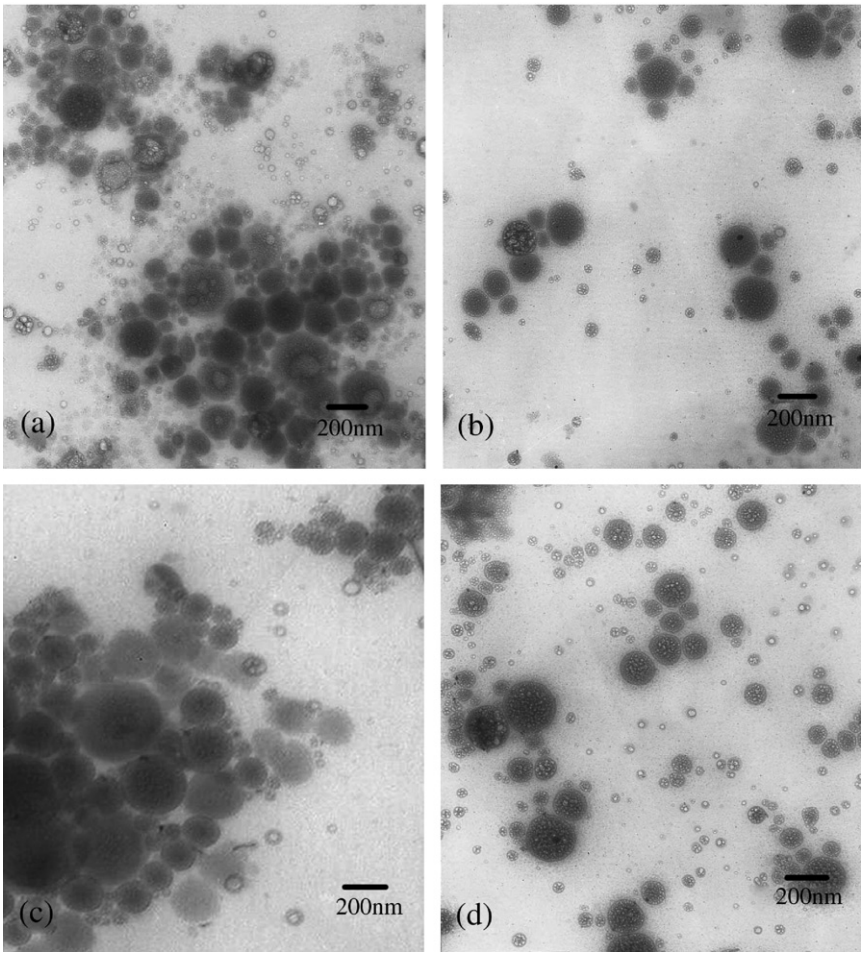

Fig. 6. Typical transmission electron micrographs of 5.0 mM DODAB vesicles after incubating with ribonucleotides: (a) 5.0 mM DODAB+10.0 mM AMP; (b) 5.0 mM DODAB+2.0 mM AMP; (c) 5.0 mM DODAB+5.0 mM ADP; (d) 5.0 mM DODAB+1.0 mM ATP.

1.0 mM) at the maximal adsorptions, which illustrate that all the molar ratio and charge ratio for DODAB/AMP complexes are equal to 2:1 under the conditions that AMP is in excess. This conclusion is further deduced both from the adsorption curve (Fig. 2) and from the calculation values of adsorption amounts (Table 1). The phenomenon of the 2:1 DODAB/AMP molar proportion is in fairly good qualitative agreement with the turbidity analysis for DODAB/DMP (2′-deoxyadenosine 5′-monophosphate) mixtures by Kikuchi et al. [16].

To further characterize the strong adsorption of ribonucleotides onto DODAB vesicles, similar experiments were performed using ADP and ATP with DODAB vesicles, respectively. When ADP or ATP within different concentrations region was incubated with DODAB vesicles and then subjected to the same fractionation procedure, the elution patterns were essentially analogous with those of AMP (not shown in this paper). Figs. 3 and 4 display the adsorption curves for ADP and ATP adsorption onto DODAB vesicles and the adsorption amounts are also shown in Table 1. Not only the adsorption curves (Figs. 4 and 5) but also the calculation of adsorption amounts (Table 1) for ADP and ATP show the same trend of change with that of AMP and all the charge ratios are equal to 2:1 (Table 1).

Nevertheless, the adsorption curves and the adsorption amounts for ADP and ATP indicate that at the maximal

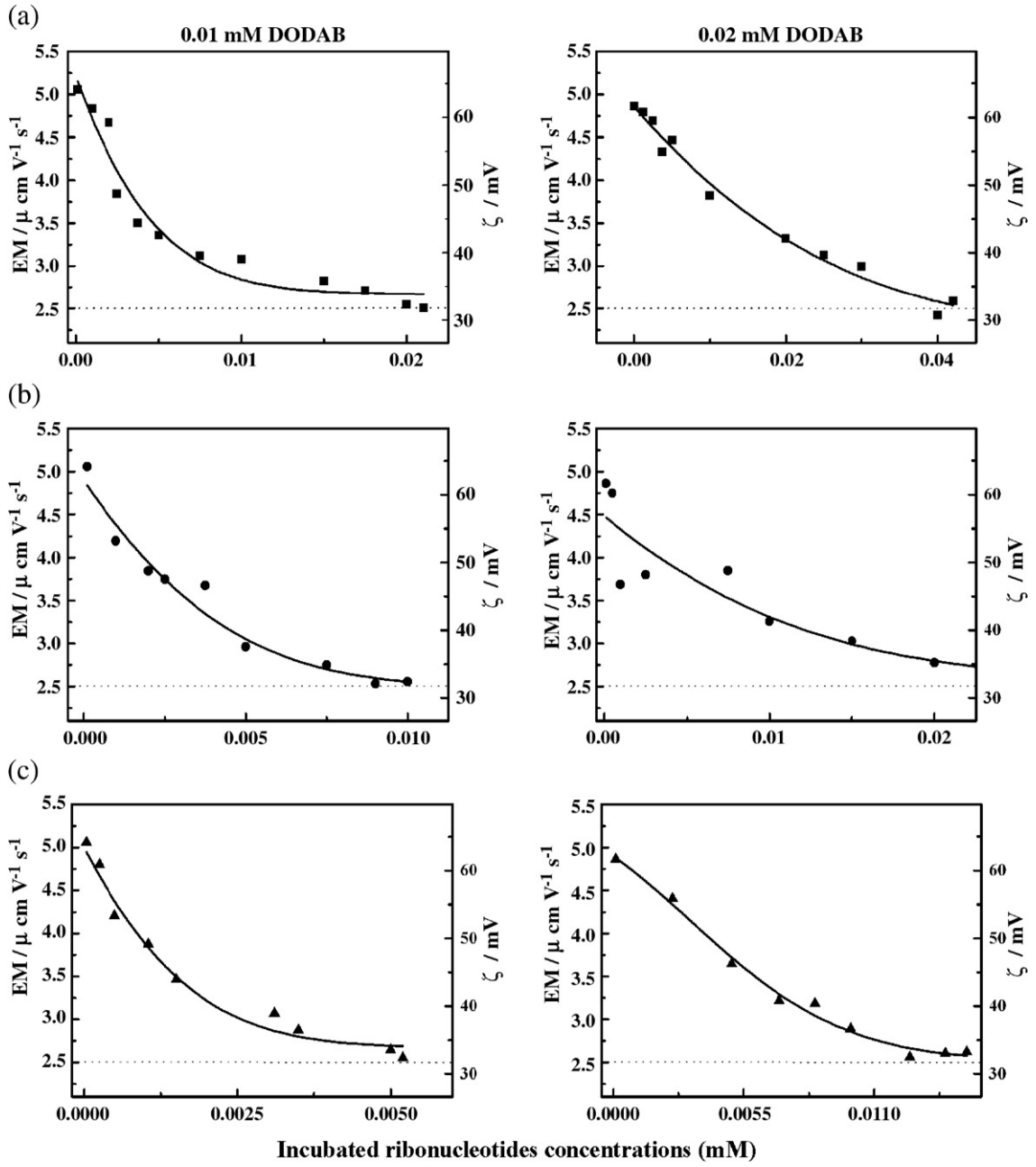

Fig. 7. Electrophoretic mobilities (EM) and zeta-potentials (ζ-potentials) of DODAB–ribonucleotide mixtures as a function of incubated ribonucleotide concentrations for two different DODAB concentrations (0.01 and 0.02 mM). (A) AMP (-■-), (B) ADP (-●-), (C) ATP (-▲-). Dashed lines: leveling-off of EM (at ca. 2.5) and ζ-potentials (at ca. 30 mV) at a final incubated ribonucleotide concentration, approximately twice the DODAB concentrations in each figures.

ribonucleotides adsorption the respective molar ratio of DODAB/ADP is 4:1 and of DODAB/ATP is 6:1, which are different from the molar ratio of DODAB/AMP 2:1. The difference of the respective molar ratios may be resulted from the different anion charges of −1, −2 and −3 for AMP, ADP and ATP. This result demonstrates that the electrostatic attraction essentially facilitates the DODAB/ribonucleotides (AMP, ADP, and ATP) complexation. The conclusion is further proved by the followed ζ-potentials and electrophoretic mobility measurements. Therefore, all the adsorbed amounts onto DODAB vesicles for AMP, ADP, and ATP are relative to the respective anion charge of ribonucleotides.

3.2. TEM observations

It has been previously demonstrated that the TEM technique applied to negatively stained vesicles is an appropriate method to study the formation and morphology of vesicular structures and their interaction with biological tissues [27–30]. The TEM micrographs of 5.0 mM DODAB vesicles before and after incubating with ribonucleotides of different concentrations are shown in Figs. 5 and 6, from which it can be seen that all display typical vesicular morphology.

As expected, the referred test performed on the DODAB vesicles before incubating with ribonucleotides is shown in Fig. 5 where no aggregation or fusion is seen. However, in the TEM micrographs for samples after incubating with ribonucleotides of different concentrations, for which aggregation is expected [31,32], surprisingly, amorphous aggregations are observed. Fig 6a and b show images of such amorphous vesicle aggregations for DODAB-AMP mixtures. Similarly, Fig 6c and d also show images of such amorphous vesicle aggregations for DODAB-ADP and DODAB-ATP mixtures, respectively. In conclusion, all these TEM observations indicate the effects of ribonucleo-

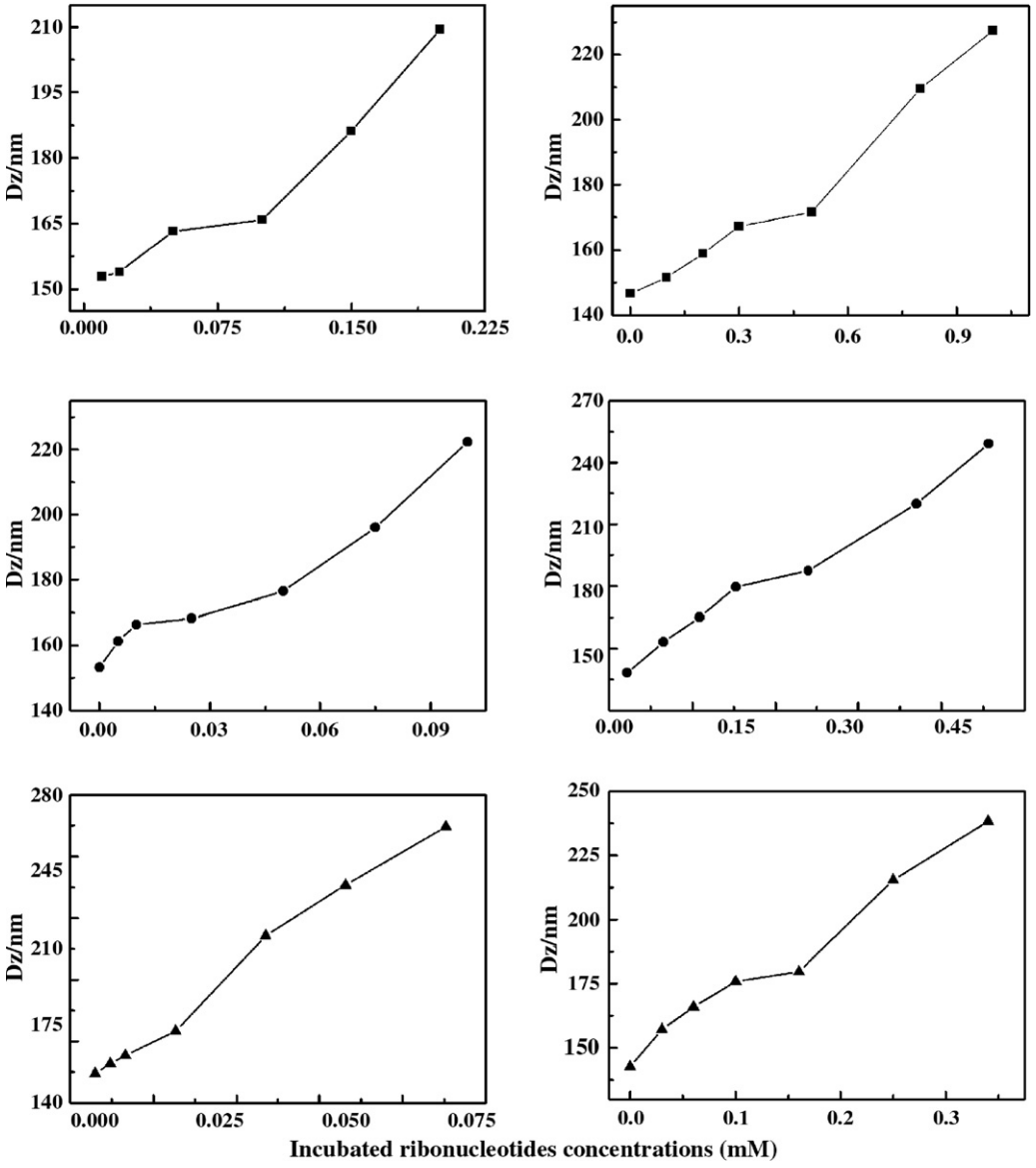

Fig. 8. The mean zeta-average diameters (Dz) of DODAB–ribonucleotide mixtures as a function of incubated ribonucleotide concentrations for two different DODAB concentrations: (A) AMP (-■-); (B) ADP (-●-); (C) ATP (-▲-). Left, the fixed DODAB concentration is 0.1 mM; right, 0.5 mM.

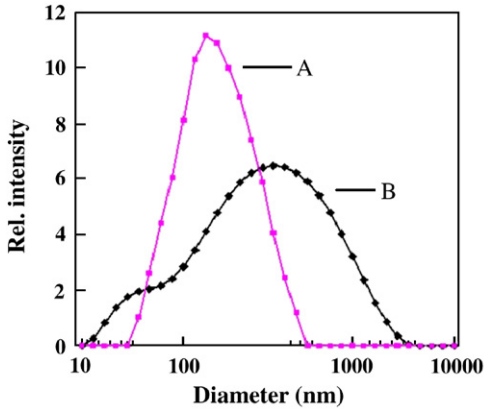

Fig. 9. Size distributions of 0.5 mM DODAB vesicles followed by DLS measurements (A) before incubation of ADP (-■-) and (B) after incubation of 0.25 mM ADP, with formed aggregates (-◆-). The difference in mean zeta-average diameters ((A) 146.6±2.2 nm, (B) 255.8±3.4 nm) is within the standard deviation of the measurements.

tides on DODAB vesicles after incubation. Therefore, it is not difficult to understand that the DODAB/ribonucleotides (AMP, ADP, and ATP) complexation may result in the aggregation of DODAB vesicles.

However, in the TEM micrographs for samples after incubating with ribonucleotides of different concentrations, images analogous with that of the referred test were also displayed. These resemblances suggest the existence of non-aggregated DODAB vesicles after incubation. It is worth mentioning that, to our knowledge, this work is the first TEM evidence of the DODAB/ribonucleotides complexation and the first TEM observation of the existence of cationic lipid-ribonucleotides aggregation.

3.3. ζ-potentials and electrophoretic mobilities

The ζ-potential of a charged particle suspended in a fluid arises from its electrokinetic motion when an electric field is applied. For comparison, the electrophoretic behavior of DODAB vesicles with two different concentrations (0.01 and 0.02 mM) is detected by measuring the ζ-potentials and EM before first incubating with ribonucleotides. For the empty DODAB vesicles the ζ-potentials are 57 mV and 42.6 mV for the 0.01 mM and 0.02 mM concentrations, respectively, and EM is 5.06 mobility and 4.86 mobility for the 0.01 mM and 0.02 mM concentrations, respectively. Subsequently, the variations of ζ-potentials and EM for DODAB-ribonucleotides mixtures with different molar ratios of incubated ribonucleotides to DODAB vesicles have been investigated. For the two different DODAB concentrations the ζ-potentials and EM display a degressive trend with the increasing incubated ribonucleotides (Fig. 7). Based on the result of Bordi et al. [17] referred, we consider that aggregation can be induced in cationic liposome suspensions when electrostatic repulsions between particles are reduced. This electrophoretic behavior for DODAB-ribonucleotides mixtures can in verse verify the TEM results. A similar observation has been made by Carmona-Ribeiro and co-works [33] with differently sized dioctadecyldimethylammonium acetate, chloride, or bromide vesicles. As is known to all that ζ-potentials and electrophoretic mobilities are complex variables and do not directly represent the electrophoretic behavior of the particle surface. Nevertheless, the decrease in the ζ-potentials and EM still indicates that the electrostatic interaction must be the

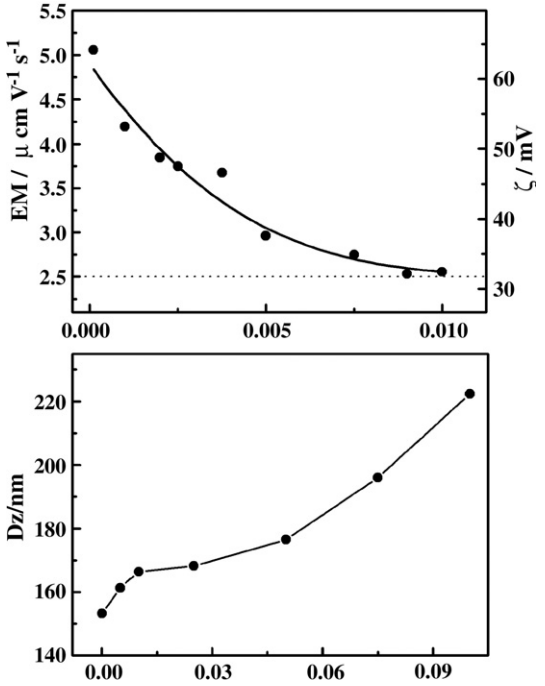

Fig. 10. Inverse relationship of the trend of change between zeta potentials or electrophoretic mobilities and particle size for DODAB–ADP mixtures Above, the fixed DODAB concentration is 0.01 mM; below, 0.1 mM.

Table 2
Mean zeta-average diameters (Dz) and polydispersity index (PDI) of two different DODAB concentrations (0.1 and 0.5 mM) corresponding to different incubated ribonucleotide concentrations

DODAB vesicle	Ribonucleotides	Final ribonucleotides concentration/mM	Dz/nm	PDI
0.1 mM	AMP	0.025	163.3	0.205
		0.075	186.2	0.209
		0.10	209.5	0.192
	ADP	0.003	161.3	0.214
		0.038	196.0	0.220
		0.05	222.3	0.254
	ATP	0.002	216.0	0.218
		0.025	239.0	0.237
		0.035	265.4	0.247
0.5 mM	AMP	0.15	167.4	0.286
		0.40	209.5	0.302
		0.50	227.4	0.289
	ADP	0.05	168.7	0.174
		0.13	190.1	0.203
		0.25	249.9	0.307
	ATP	0.03	165.9	0.227
		0.08	179.6	0.259
		0.17	238.3	0.316

Dz is the mean harmonic zeta-average diameter of at least six independent measurements at the given concentration of DODAB vesicle suspension. PDI is directly related to the standard deviation of each distribution curve and is the mean value of six independent measurements.

important reason for this decrease. On the contrary, the DODAB/ribonucleotides complexation resulted from the electrostatic interaction can cause the decrease of ζ-potentials and EM.

Due to the 2:1 DODAB/ribonucleotides charge proportion obtained by gel exclusion and UV measurements, the sign of the DODAB/ribonucleotides complexes should be positive. This prediction is truly confirmed from the decrease to ca. 30 mV of ζ-potentials and ca. 2.5 mobility of EM. Furthermore, it is well known that the preservation of positive electrophoretic behavior is in favor of the stability of vesicles [16]. Fig. 7 shows ζ-potentials and electrophoretic mobilities for particles in DODAB–ribonucleotide mixtures as a function of incubated concentrations of ribonucleotides for two different DODAB concentrations.

3.4. Effect of ribonucleotide concentrations on mean zeta-average diameters and reverse relationship between ζ-potentials or electrophoretic mobilities and mean zeta-average diameters

Size and size distribution of vesicles are essential parameters for evaluating the physicochemical properties of vesicles. It has been previously demonstrated that the relationship between size and ζ-potential or electrophoretic mobility of vesicles is on the contrary [13,34]. Namely, the decrease of ζ-potential or electrophoretic mobility of vesicles exists with the increase of the mean zeta-average diameter. Therefore, the mean zeta-average diameters in this work should be dependent on ζ-potentials or electrophoretic mobilities. Since ζ-potentials and electrophoretic mobilities of DODAB dispersions have regularly changed after incubation with ribonucleotides, we consequently assume that the mean zeta-average diameters of the DODAB dispersions should be affected after incubation of the different concentrations of ribonucleotides. This prediction is fully confirmed from the determination of the average diameters of DODAB-ribonucleotides mixtures as a function of ribonucleotide concentrations (Fig. 8).

From the six figures in Fig. 8, the Dz of DODAB vesicles of the two different concentrations (0.1 and 0.5 mM) before incubation with ribonucleotides are 153.23 nm and 146.62 nm, respectively. The size distributions of 0.5 mM DODAB vesicles before and after incubation with ADP obtained by DLS are shown in Fig. 9. It is obvious that the DLS size distribution of the DODAB vesicle before incubation with ADP is relatively narrow. After incubation, this mixture gives a very broad peak in the DLS size distribution with an obviously increased Dz. Thus we hold that broad peak in the DLS size distribution and increasing Dz of all the mixture populations exist with increasing ribonucleotide concentrations. Therefore, the DODAB/ribonucleotide (AMP, ADP, and ATP) complexations in the DODAB-ribonucleotides mixtures induce the changes of sizes and size distributions. On the other hand, in reverse the fractions of aggregated vesicles in TEM micrographs reflect the changes of sizes and size distributions. Thus, this is agreed not only with the TEM results but also with what is described by the well-known DLVO theory [35]. Finally, it is demonstrated that the trends of change in sizes and ζ-potentials or EM display an inverse relationship (Fig. 10), which are in fairly good qualitative agreement with the DLVO theory [34]. The mean PDI values of six independent measurements for each sample are listed in Table 2.

4. Conclusions

In the present work, with the purpose of extending and enriching a quantitative physicochemical description of nucleic acid-charged lipid complexes, the interaction between a monomeric unit of nucleic acid (AMP, ADP, and ATP) and DODAB vesicles and the physicochemical properties of ribonucleotides/cationic lipid complexes have been studied in detail.

Interactions between ribonucleotides and DODAB vesicles driven by the electrostatic attraction and the molar ratios as well as the charge ratios of cationic lipid/ribonucleotides complexes at the maximal adsorption of ribonucleotides have been studied by GE–UV spectroscopy. TEM observations indicate that incubations of AMP, ADP or ATP with DODAB vesicles have induced fractions of aggregated DODAB vesicles in the DODAB-ribonucleotides mixtures. This result is in agreement with the particle size analysis by DLS. In addition, the measurements of the ζ-potentials, electrophoretic mobilities and the mean zeta-average diameters as a function of the incubated ribonucleotide concentrations further confirm the results obtained by TEM and GE–UV. It is also demonstrated that the trends of ζ-potentials or electrophoretic mobilities and particle sizes display an inverse relationship that is in fairly good qualitative agreement with the DLVO theory [34].

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