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Title
Influence of Preparation Method on Polynucleotide Conformation and Pharmacological Activity of Lipoplex

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Abstract

Conformations of polyinosinic acid [poly(I)] and polycytidylic acid [poly(C)] in liposomes (lipoplex) were investigated by both circular dichroism (CD) spectroscopy and fluorescence resonance energy transfer (FRET) measurements, and compared with those in aqueous solution. The results indicate that poly(I) and poly(C) take double-stranded structure in aqueous solution at pH 6.5 to 7.5 in the presence of NaCl at higher concentration than 50 mM. Although lipoplex was prepared without NaCl to avoid aggregation of lipoplex particles, poly(I) and poly(C) were double-stranded in pre-mixed poly(I)/poly(C) lipoplex (pre-mixed LIC), prepared by adding a mixed solution of poly(I) and poly(C) to the cationic liposomes. However, poly(I) and poly(C) did not take double-stranded structure in separately mixed LIC, prepared by separate addition of poly(I) solution and poly(C) solution to the cationic liposomes. The physicochemical properties (particle diameter and zeta potential) of pre-mixed LIC and separately mixed LIC were not different, but the anti-proliferative effect of pre-mixed LIC on human epidermoid carcinoma A431 cells was about 8 times greater than that of separately mixed LIC. Our results indicate that polynucleotide conformation in lipoplex is markedly influenced by the preparation method, and the polynucleotide conformation in lipoplex has a substantial effect on pharmacological activity.

Keywords
lipoplex, polynucleotide, liposome, conformation, poly(I), poly(C)
Main Text

1 Introduction

Cationic liposomes are used as a non-viral vector for gene therapy, because they can function as a carrier of polynucleotides (Felgner et al., 1987). Such lipoplexes are widely used because they are safe and can easily be manufactured on a large scale (Feber, 2001).

The ability of cationic liposomes to transfer polynucleotides is dependent upon the nature of the cationic lipids used to prepare the liposomes. Many cationic lipids have been studied in attempts to improve the delivery characteristics and physical stability of lipoplex. The lipids of lipoplex can condense polynucleotides into chemically and physically diverse aggregates (Akao et al., 1996), and the structure and/or transfection activity depend upon not only the nature of the lipid, but also the method of lipid dispersion, the buffer strength and the order of mixing of polynucleotides and liposomes (Gershon et al., 1993; Sternberg et al., 1994; Yang and Huang 1997; Zuhorn et al., 2002). Although various structures have been observed macroscopically, the most commonly found microscopic structure, as revealed by X-ray diffraction and Cryo-TEM (Rädler et al., 1997; Dan et al., 1998; Koltover et al., 1998; May et al., 2000; Lin et al., 2000; Lima et al., 2001), is a multilamellar array of polynucleotide strands intercalated between lipid bilayers (Boukhnikachvili et al., 1997; Lasic et al., 1997; Rädler et al., 1997; Bell et al., 2003). Hexagonal arrays (Koltover et al., 1998; Rakhmanova et al., 2000; Bell et al., 2003) and bilayer-coated strands (Sternberg et al., 1994) have also been observed. However, the conformation of polynucleotides in lipoplex is still not clearly understood, because the polynucleotides and cationic lipids were not the same in all of the above reports.

Synthetic double-stranded RNA, polyinosinic-polycytidylic acid [poly(I:C)], is an effective inducer of interferon. Poly(I:C) has been the subject of several clinical trials for treatment of tumors (Robinson et al., 1976; Levine and Levy, 1978; Levine et al., 1979), but polyinosinic acid [poly(I)] or polycytidylic acid [poly(C)] alone is unable to cross the plasma membrane of tumor cells and is easily hydrolyzed by RNase (Nordlund et al., 1970). To protect poly(I) and poly(C) from hydrolysis and improve delivery into tumor cells, Hirabayashi et al used a complex of poly(I), poly(C) and cationic liposomes (Hirabayashi et al., 1999).

Poly(I) and poly(C) become double-stranded in aqueous solution. The three-dimensional structures of polynucleotides in aqueous solution are generally estimated by means of circular dichroism (CD) measurements. However, the heterogeneous particle size distribution of
lipoplex has the potential to introduce significant artifacts into the CD spectra, because the heterogeneous particle of the lipoplex can significantly influence the absorption (Braun et al, 2003). On the other hand, fluorescence resonance energy transfer (FRET) is useful for estimation of the distance between fluorescence-labeled acceptor and donor molecules.

In this study, we focused on poly(I) and poly(C) as complementary polynucleotides to establish an evaluation method for the conformation of poly(I) and poly(C) in lipoplex by FRET. Moreover, we examined the effects of poly(I) and poly(C) conformation on the pharmacological activity of lipoplex prepared by different methods.
2 Materials and Methods

2.1 Materials
Poly(I) and poly(C) were purchased from Yamasa Corporation (Chiba, Japan). Their average chain lengths were confirmed within range of 200 to 400 bases by size exclusion chromatography. The cationic lipid 2-O-(2-DEAE)-carbamoyl-1,3-O-dioleoylglycerol (CLZ-42) was synthesized at Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Alexa Fluor 546 and 568 were purchased from Molecular Probes (Eugene, OR, USA). Egg yolk lecithin was purchased from Q.P. Corporation (Tokyo, Japan), and 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny1tetrazolium bromide (MTT) was from Molecular Probes. All other excipients were of pharmaceutical grade, and solvents and chemicals were of analytical grade.

2.2 CD Spectral Measurement
Aqueous solutions containing from 0 to 150 mM NaCl in deionized water were prepared and adjusted in the range of pH 4.5 to 6.5 with 10 mM MES or to pH 7.0 to 7.5 with 10 mM HEPES. Poly(I), poly(C) and poly(I)/poly(C) mixed solution were obtained by dissolving poly(I) and poly(C) at 5 mg/mL in the above solutions or distilled water. CD spectra were obtained with a circular dichroism spectrophotometer J-720 (JASCO Corporation, Tokyo, Japan) at 25°C in a thermostatically controlled 1-cm cuvette. Samples were scanned three times from 200 to 350 nm with a 1 nm step size at 50 nm/min. CD ratio was calculated from the following equation,

\[
\text{CD ratio} = \frac{\text{peak height at 240 nm}}{\text{peak height at 280 nm}} \quad \text{Eq (1)}
\]

2.3 Fluorescence Measurement
Poly(I) and poly(C) were labeled with the fluorescence probes Alexa Fluor 568 and Alexa Fluor 546, by the use of ULYSIS® nucleic acid labeling kits (Molecular Probes, Eugene, OR, USA). The labeled poly(I) and poly(C) were purified with a Micro Bio-Spin P-30 (BioRad, Hercules, CA). The labeling efficiency of poly(I) and poly(C) was about 80 bases/dye, as determined with a DU-640 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

Steady-state fluorescence measurements were carried out with a fluorescence recording spectrophotometer, F-3100 (Hitachi Co., Ltd., Tokyo, Japan), at \(\lambda_{\text{exc}} = 555\ \text{nm}\) and \(\lambda_{\text{em}} = 570\ \text{nm}\). FRET efficiency was calculated from the following equation,
\[ E = 1 - \frac{I_{DA}}{I_D} \quad \text{Eq (2)} \]

where \( I_{DA} \) and \( I_D \) are the measured donor (Alexa Fluor 546) fluorescence intensities in the absence and the presence of acceptor (Alexa Fluor 568), respectively.

The separation distance, \( R \) (nm), was calculated from the following equation,

\[ R = R_0 \times \left( \frac{1}{E} - 1 \right)^{\frac{1}{5}} \times \frac{1}{10} \quad \text{Eq (3)} \]

where \( R_0 \), the Förster distance of Alexa Fluor 546 and 568, is 7 nm, and \( E \) is estimated from Eq (2).

2.4 Preparation and Characterization of Lipoplex

The preparation methods of lipoplex are illustrated in Fig. 1. Cationic liposomes with egg yolk lecithin/CLZ-42 = 10:6 (w/w) were emulsified using a probe-type sonicator (Branson Sonifier Model 250D; Branson Ultrasonics, Danbury, CT, USA). To prepare lipoplex, poly(I), poly(C) and cationic liposome were emulsified in 10% (w/v) maltose (tonicity agent) with a probe-type sonicator during injecting poly(I) and/or poly(C) dissolved in distilled water into cationic liposome solution. The solution pH of lipoplex was adjusted to 4.5 by 1 mol/L HCl (final HCl concentration 4μM). The particle size distributions of lipoplexes were determined by laser dynamic light scattering with a Nicomp 370 particle sizer, and their zeta-potentials were determined by laser Doppler electrophoresis with a Zetasizer 2000 (Malvern Instruments, Worcestershire, UK).

2.5 Assessment of Cell Growth

A431 cells (American Type Cell Collection, Manassas, VA, USA) were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\) in air. A431 cells from monolayer cultures were placed in 96-well microplates (Corning Costar, Corning, NY, USA) at a density of \( 10^5 \) cells/well (90 μL/well). After 4 - 6 hours, compounds were added to each well at concentrations of 10 - 1000 ng/mL, and incubation was continued at 37°C for 3 days. The number of cells surviving after 3 days was determined by MTT assay (Mosmann, 1983). \( IC_{50} \) was defined as the drug concentration that induced 50% cell death in comparison with untreated controls, and was calculated by nonlinear regression analysis.
3 Results and Discussion

3.1 Interaction of Poly(I) and Poly(C) Estimated by CD Spectroscopy

To gain insight into the properties of nucleotides in lipoplex, a novel analytical approach is required, because conventional methods of analysis usually require dyeing or freezing treatment. First, the conformation of poly(I) and poly(C) in aqueous solution was estimated by CD without any treatment. Fig. 2 shows CD spectra of poly(I) and poly(C) mixed solution with (150 mM) or without NaCl at pH 7.5. A strong positive peak in the vicinity of 240 nm was displayed in 150 mM NaCl, but not in NaCl-free solution. Since it has been reported that the conformation of poly(C) is pH-dependent (Imahori and Watanabe, 1970), we measured the CD spectra of poly(I) and poly(C) mixed solution in several solutions at various pH values and calculated the CD ratios (Fig. 3). When the NaCl concentration was higher than 50 mM, the CD ratio was 1.2 at pH 6.5 through 7.5. On the other hand, the CD ratio was very low at pH 4.5 through 5.0 in all solutions tested. The above results suggest that poly(I) and poly(C) interact with each other in aqueous solution containing more than 50 mM NaCl at pH 6.5 through 7.5. Homopolynucleotide is composed of not only double-stranded structure, but also triplex or quadruplex chains. Wang and Keiderling reported that triplex of poly(CH⁺) · poly(I) : poly(C) was produced via the formation of poly(CH⁺) from poly(C) (Wang and Keiderling, 1994). A decrease of UV absorption was caused by the association of two homopolynucleotides (Ikehara and Inaba, 1974). In the present study, we also observed a decrease in the UV absorption of poly(I) and poly(C) aqueous solutions, and the association ratio of poly(I) and poly(C) was 1:1 (w/w) (data not shown).
3.2 Interaction of Poly(I) and Poly(C) Estimated by FRET

When complementary polynucleotides consisting of DNA or RNA take double-stranded structure, the distance between complementary nucleotides is about 4 nm (Choosakoonkriang et al., 2001). FRET occurs when an acceptor emits fluorescence as a result of resonance energy transfer from a donor, and can occur when the distance between the donor and acceptor is 1 - 10 nm (Stryer and Haugland, 1967). Hence, measurements of FRET between fluorescence-labeled poly(I) and poly(C) as donor and acceptor could be useful to estimate conformation.

Thus, poly(I) and poly(C) were labeled with the fluorescence probes Alexa Fluor 568 and 546, and the FRET efficiency of the mixed solution was estimated at various pH values (Fig. 4). The FRET efficiency profiles of mixed solutions of labeled poly(I) and poly(C) were in good agreement with the CD ratio profiles obtained from non-labeled poly(I) and poly(C) mixed solution, as shown in Fig. 3. The labeling with fluorescence probes appeared to have no effect on the interaction of poly(I) and poly(C) in solution. Both the CD and FRET results suggested that the conformation of poly(I) and poly(C) is dependent on pH and NaCl concentration. Poly(I) and poly(C) became double-stranded at pH 6.5 through 7.5 in the presence of NaCl at a concentration higher than 50 mM, but not in the absence of NaCl at any pH examined. Moreover, we demonstrated that the conformation of poly(I) and poly(C) can be estimated by means of FRET measurements as well as by the CD method.
3.3 Particle Size and Zeta Potential of Lipoplexes

Poly(I) and poly(C) became double-stranded in the presence of NaCl, as shown in Fig. 3 and Fig. 4. It has been reported that an increase of ionic strength causes aggregation of liposome particles and increases their diameter (Kennedy et al., 2000). Lipoplex diameter is known to influence tissue distribution. For example, large particles (> 500 nm) accumulate in the lungs, which are the first-pass organ after intravenous injection (Litzinger et al., 1996, Li et al., 1998, Sternberg et al., 1998). We prepared lipoplex with a particle size smaller than 200 nm in diameter in the absence of NaCl to avoid the aggregation of lipoplex particles, since the mean diameter of lipoplex prepared in 50 mM NaCl solution achieved above 500 nm (data not shown).

To establish whether or not the preparation method influences the conformation of polynucleotides in lipoplex, we examined two different methods of preparing lipoplex without NaCl. Pre-mixed LIC was prepared by the addition of a mixed solution of poly(I) and poly(C) to cationic liposomes. Separately mixed LIC was prepared by the separate addition of poly(I) solution and poly(C) solution to cationic liposomes.

As shown in Table 1, the diameters and zeta potentials of lipoplex prepared by the two methods were similar, at about 150 nm and +50 mV, respectively. Moreover, since their particle distributions were homogenous by the estimation of the weight average diameter/number average diameter value (data not shown), the lipoplex in the present study had no impact on CD spectra. The morphology of lipoplex is influenced by the excipient (cationic lipid, helper lipid) and lipid-to-polynucleotide ratio, in addition to the preparation method (Ma et al., 2007). It is known that lipoplex with the excipient used in the present study forms multilamellar vesicles (Sonoke et al., 2008). Further, the particle diameter and zeta potential of our preparations were similar to those reported by Sonoke et al. Therefore, it seems likely that our pre-mixed LIC and separately mixed LIC formed multilamellar vesicles.
3.4 Interaction of Poly(I) and Poly(C) in Lipoplex

As shown in Table 1, lipoplexes prepared by means of the two methods without NaCl had similar physicochemical characteristics, while poly(I) and poly(C) in aqueous solution without NaCl could not interact with each other. The interaction of poly(I) and poly(C) in the lipoplexes was estimated from CD spectral and FRET measurements. Fig. 5 shows CD spectra of a mixed solution of poly(I) and poly(C) in the presence of 150 mM NaCl at pH 7.4 [poly(I)/poly(C) mixed solution], as well as pre-mixed LIC and separately mixed LIC. The CD spectrum of pre-mixed LIC is characterized by a strong positive peak in the vicinity of 240 nm and 280 nm, and this was also the case for poly(I)/poly(C) mixed solution, but the 240 nm peak was not observed in the CD spectra of separately mixed LIC. Although two strong positive peaks were observed in the CD spectrum of pre-mixed LIC, the CD ratio of pre-mixed LIC was lower than that of poly(I)/poly(C) mixed solution. The lower CD ratio of pre-mixed LIC was thought to be caused by the following two reasons. The first reason was the absence of NaCl, because pre-mixed LIC was prepared without NaCl to avoid the aggregation of lipoplex particle. The second reason was the reduction of CD intensity, because base pair of polynucleotides was disturbed by stacking with cationic lipid (Braun et al., 2003).

In accordance with the CD findings, the FRET efficiency of pre-mixed LIC was similar to that of poly(I)/poly(C) mixed solution. However, the FRET efficiency of separately mixed LIC was significantly lower than that of poly(I)/poly(C) mixed solution. Since FRET efficiency depends on the distance between probes, the distance in each mixture can be calculated. The results are shown in Table 2. The calculated distances in poly(I)/poly(C) mixed solution and pre-mixed LIC were 5.38 nm and 5.19 nm, respectively, which are closer to the distance for double strands (4 nm) (Choosakoonkriang et al., 2001) than in the case of separately mixed LIC (7.73 nm), suggesting that poly(I) and poly(C) take a double-stranded structure in pre-mixed LIC, as in aqueous solution, and coexist in the same interlayer space of the multilamellar lipoplex particles.

The calculated distance for separately mixed LIC (7 nm) is similar to the reported thickness of the lipid lamellar layer measured by small XRD and Cryo-TEM (Schmutz et al., 1999). These results suggest that poly(I) and poly(C) in separately mixed LIC exist in different interlayer spaces of the multilamellar lipoplex.

As shown in Fig. 3 and Fig. 4, poly(I) and poly(C) in aqueous solution were double-stranded at neutral pH in the presence of NaCl. The results in Fig. 5 and Table 2,
indicate that, although poly(I) and poly(C) in pre-mixed LIC formed double-stranded structure in the absence of NaCl, this was not the case in separately mixed LIC. The difference of the polynucleotide conformation between pre-mixed LIC and separately mixed LIC may be caused by the adhesion of polynucleotide to cationic liposome. Poly(I) and poly(C) in pre-mixed LIC were sandwiched in the same space of cationic liposome particles, because mixed solution of poly(I) and poly(C) were added to liposome solution. On the other hand, poly(I) and poly(C) in separately mixed LIC were sandwiched between different spaces of cationic liposome particles, because poly(I) solution and poly(C) solution were separately added to liposome solution. In short, the conformation of poly(I) and poly(C) in lipoplex was influenced by the method used to prepare the lipoplex, as well as by solution conditions such as pH and NaCl concentration.
3.5 Pharmacological Activity of Poly(I) and Poly(C) in Lipoplex

As shown in Table 1, no significant differences were observed in the physicochemical properties (diameter and zeta potential), but the conformation of poly(I) and poly(C) was different in pre-mixed LIC and separately mixed LIC (Fig. 5). To estimate the effect of poly(I) and poly(C) conformation on pharmacological activity, the anti-proliferative effect of pre-mixed LIC and separately mixed LIC on A431 cells was evaluated by MTT assay.

The IC<sub>50</sub> of pre-mixed LIC was about 8 times lower than that of separately mixed LIC, while poly(I) and poly(C) mixed solution had no effect on proliferation of A431 cells (Table 3). This result indicates that LIC more potently inhibited the growth of A431 cells when poly(I) and poly(C) were present in double-stranded form, coexisting in the same interlayer space of the multilamellar lipoplex particles. This is consistent with a report that poly(I) and poly(C) bind to a double-stranded RNA-binding protein to activate interferon regulatory factor 3 (IRF3) in cytoplasm, leading to induction of apoptosis (Uno et al., 2005). On the other hand, since poly(I) and poly(C) in separately mixed LIC exist in different interlayer spaces of the multilamellar lipoplex particles, poly(I) and poly(C) may not readily bind to double-stranded RNA-binding protein, so that IRF3 is not efficiently activated and cell growth of A431 is not potently inhibited, compared to pre-mixed LIC.

In summary, we have demonstrated that the conformation of poly(I) and poly(C) in lipoplex can be estimated by means of FRET measurements, as well as by the CD method. The results of the two methods were in good agreement. Moreover, we found that the conformation of poly(I) and poly(C) in lipoplex is influenced by the preparation method, and that double-stranded poly(I) and poly(C) structure in the lipoplex is required for potent anti-proliferative activity against cancer cells. This study suggests that consideration of the polynucleotide conformation in lipoplex is essential to optimize the pharmacological activity.
Abbreviations

CLZ-42: 2-O-(2-DEAE)-carbamoyl-1,3-O-dioleoylglycerol; IRF3: interferon regulatory factor 3; LIC: poly(I)/poly(C) lipoplex; MTT: (3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide; poly(C): polycytidylic acid; poly(I): polyinosinic acid.
References


Legends

**Fig. 1 Preparation of Lipoplex**
Pre-mixed LIC; after dissolving poly(I) and poly(C) in distilled water and mixing, poly(I), poly(C) and the cationic liposome were emulsified during injecting the mixed solution into cationic liposome solution. Separately mixed LIC; after dissolving poly(I) and poly(C) in distilled water separately, poly(I), poly(C) and the cationic liposome were emulsified during injecting the two polynucleotide solutions into cationic liposome solution.

**Fig. 2 CD Spectra of Poly(I) and Poly(C) in Aqueous Solution.**
Poly(I) and poly(C) were dissolved in solution at pH 7.4, containing 0 mM (dashed line) or 150 mM NaCl (solid line).

**Fig. 3 CD Ratio Profile of Poly(I) and Poly(C) in Aqueous Solution**
Poly(I) and poly(C) were dissolved in solution containing 0 mM (circle), 50 mM (triangle), 100 mM (diamond) or 150 mM NaCl (square). The CD ratio was measured at various pH values from 4.5 to 7.5.

**Fig. 4 FRET Efficiency between Fluorescent Poly(I) and Fluorescent Poly(C) in Aqueous Solution.**
Poly(I) and poly(C) were dissolved in solution at 0 mM (circle), 50 mM (triangle), 100 mM (diamond) or 150 mM NaCl (square). FRET efficiency was measured at various pH values from 4.5 to 7.5.

**Fig. 5 CD Spectra of Pre-mixed LIC, Separately Mixed LIC and Poly(I)/poly(C) Mixed Solution**
Poly(I)/poly(C) mixed solution; poly(I) and poly(C) were dissolved in 150 mM NaCl solution at pH 7.5. Dash-dotted, dashed and solid lines represent the results for pre-mixed LIC, separately mixed LIC, and poly(I)/poly(C) mixed solution, respectively.
Tables

Table 1  Mean Diameter and Zeta Potential of Lipoplex Prepared by Different Mixing Methods

<table>
<thead>
<tr>
<th>Lipoplex</th>
<th>Mean diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mixed LIC</td>
<td>143.1 ± 4.58</td>
<td>49.9 ± 4.88</td>
</tr>
<tr>
<td>Separately mixed LIC</td>
<td>146.6 ± 12.91</td>
<td>45.7 ± 1.59</td>
</tr>
</tbody>
</table>

Mean diameter was determined by laser light scattering measurement. Zeta potential was determined by laser Doppler electrophoresis. Data are presented as mean ± S.D. of three independent experiments.
Table 2 CD Ratio, FRET Efficiency and Calculated Distance for Poly(I) and Poly(C) in Lipoplex and Aqueous Solution.

<table>
<thead>
<tr>
<th></th>
<th>CD ratio</th>
<th>FRET efficiency</th>
<th>Calculated distance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mixed LIC</td>
<td>0.68 ± 0.04</td>
<td>0.83 ± 0.00</td>
<td>5.38 ± 0.01</td>
</tr>
<tr>
<td>Separately mixed LIC</td>
<td>0.07 ± 0.05</td>
<td>0.36 ± 0.06</td>
<td>7.73 ± 0.34</td>
</tr>
<tr>
<td>Poly(I)/poly(C) mixed solution</td>
<td>1.15 ± 0.07</td>
<td>0.86 ± 0.02</td>
<td>5.19 ± 0.10</td>
</tr>
</tbody>
</table>

Poly(I)/poly(C) mixed solution; poly(I) and poly(C) were dissolved in 150 mM NaCl solution at pH 7.5. Calculated distance was estimated from the FRET efficiency and the Förster distance of Alexa Fluor 546 and 568. Data are presented as mean ± S.D. of three independent experiments.
Table 3 Inhibition of A431 Cell Growth by Pre-mixed LIC, Separately Mixed LIC and Poly(I)/poly(C) Mixed Solution

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mixed LIC</td>
<td>3.1 ± 0.21</td>
</tr>
<tr>
<td>Separately mixed LIC</td>
<td>22.0 ± 1.67</td>
</tr>
<tr>
<td>Poly(I)/poly(C) mixed solution</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Poly(I)/poly(C) mixed solution: poly(I) and poly(C) were dissolved in 150 mM NaCl solution at pH 7.5. Data are presented as mean ± S.D. of three independent experiments.
Fig. 1

Pre-mixed LIC

Poly(I)  Poly(C)

Cationic liposome

Emulsify

Separately mixed LIC

Poly(I)

Cationic liposome

Emulsify

Poly(C)
Fig. 2

![Graph showing the relationship between observed angle (θ) and wavelength (λ) with and without 0 mM NaCl and 150 mM NaCl concentrations.]
Fig. 3

Graph showing the relationship between pH and CD ratio at different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM). The graph illustrates how the CD ratio changes with pH at each NaCl concentration level.
Fig. 4

![Graph showing FRET efficiency vs. pH with NaCl concentrations of 0 mM, 50 mM, 100 mM, and 150 mM.](image-url)