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Double-Stranded RNA Homopolymer Poly(rC)·Poly(rG) for a New pH-Sensitive Drug Carrier

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Abstract: Double-stranded RNA homopolymer poly(rC)·poly(rG) has been used as a new pH-sensitive drug carrier. The poly(rC)·poly(rG) had proton buffering capacity around pH 6, owing to protonation of cytosine, as determined by acid–base titration. By circular dichroism measurement, the protonation caused conformational change of the RNA. The poly(rC)·poly(rG) and doxorubicin (Dox), as an anticancer drug, formed the complexes which released the drugs at endosomal pH. The resulting complex exhibited higher anticancer activity than the Dox alone. These results result suggest that the poly(rC)·poly(rG) is a promising biopolymer for a new class of pH-sensitive drug carriers.

Keywords: poly(rC)·poly(rG); double-stranded RNA homopolymer; pH-sensitive drug carrier; endosomal pH; anticancer drug

Here we have utilized double-stranded RNA homopolymer poly(rC)·poly(rG), as shown in Figure 1, as a new pH-sensitive drug carrier. Drug delivery systems that use polymeric drug carriers have been studied from a human health perspective to improve the efficient delivery of drugs to target sites in the body.[1,2] However, the major shortcoming of these delivery systems is that most internalized carrier/drug (hydrophilic one) complexes remain entrapped in endosomes and are unable to reach cytoplasmic space. These complexes internalized via endocytic pathway are delivered into acidic endosomes where they are subjected to a pH change from pH 7 to 5.[3] Such a change of pH has led us to design a new pH-sensitive drug carrier at endosomal pH.

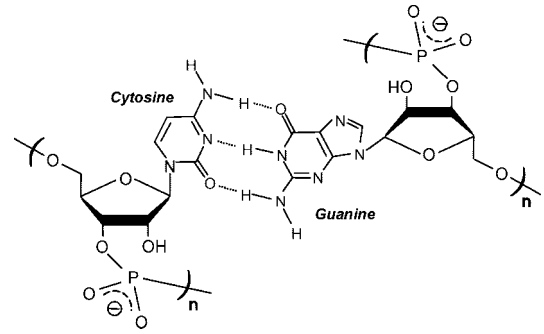

Figure 1. Chemical structure of double-stranded RNA homopolymer poly(rC)·poly(rG).

pH-sensitive polymers such as polyethylenimine, which is able to capture protons entering an endosome, have been used to achieve efficient release of the delivered material from endosomes.[4,5] The polyethylenimine induces swelling of the endosomes that leads to membrane disruption.[6] If a polymer has H^+ buffering capacity at endosomal pH as well as drug-loading sites, the design of polymeric drug carriers would be promising. In this study, we have examined whether the double-stranded RNA homopolymer poly(rC)·poly(rG) functioned as a drug carrier with pH-sensitivity at endosomal pH. This paper describes the pH-dependent properties of the poly(rC)·poly(rG) in the context of its acid–base titration, circular dichroism (CD) spectrum, the

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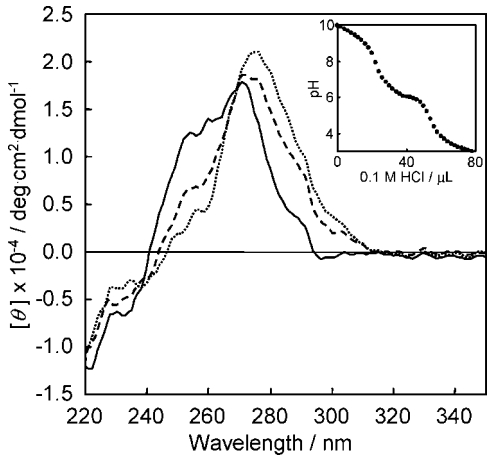

Figure 2. Effect of pH on the CD spectra of poly(rC)·poly(rG): pH 7 (solid line), pH 5 (dashed line), and pH 4 (dotted line). The molecular ellipticities based on nucleotide units (7.9×10^{-5} M) are represented. Inset: acid–base titration curve of poly(rC)·poly(rG). Basic polymer solution was titrated with the stepwise addition of 0.1 M HCl.

release of loaded drugs, and the cytotoxicity by the loaded drug; detailed studies on the mechanism of the drug release and the anticancer activity are outside the scope of the present study.

To examine the ionic properties of cytosine−guanine base pairs, we carried out the acid–base titration of the poly(rC)·poly(rG) solution.[7] As shown in the inset of Figure 2, it should be noted that H^+ buffering around pH 6.0 occurred. Based on the fact that cytosine possesses the highest pK_a among the four bases,[8] the H^+ buffering suggests that the N3 of cytosine was the protonation site at endosomal pH. To examine further the pH-dependent properties of the poly(rC)·poly(rG), we studied the structural change of the double-stranded RNA. Figure 2 shows the effect of pH on the CD spectra of the poly(rC)·poly(rG).[9] At pH 7, the A-form structure of the RNA was characterized by a typical CD spectrum that exhibited a maximum around 270 nm followed by a characteristic less intense signal at 255 nm.[10] After the protonation at pH 5, the positive peak ellipticity enhanced and exhibited red shift, while the signal at 255 nm slowly disappeared. The protonated structure of the RNA at pH 4 had a CD spectrum with higher ellipticity for the positive peak. These results suggest that two conformations depended on the pH of the solution: the A-form at physiological pH and the protonated form at endosomal pH.

Subsequently, we examined whether the resulting conformational change drove the poly(rC)·poly(rG) to release loaded drugs. As a drug, doxorubicin (Dox, called adriamycin or hydroxydaunorubicin) was chosen because Dox is a DNA-interacting drug used in chemotherapy for cancer.[11] Furthermore, Dox is amphiphilic and has both amino and hydroxyl functional groups, which are expected to form both electrostatic and hydrogen bond with the poly(rC)·poly(rG). The poly(rC)·poly(rG) and the anticancer drug Dox were mixed, followed by dialysis against isotonic phosphate buffer at pH 7 or pH 5.[12] The fluorescence intensity of the Dox in the resulting buffer was measured to determine the released Dox concentration.[13] Figure 3 shows the plot of the amount of net released Dox at pH 5 against incubation time. The amount of the released Dox increased and reached a plateau after 24 h incubation at endosomal pH. The drug release profile is considered consistent with the persistence time of an endosome.[6] Moreover, the effect of the concentration of the poly(rC)·poly(rG) at pH 7 and pH 5 on the absorption spectra of Dox is evaluated (see the Supporting Information). As the poly(rC)·poly(rG) concentration increased, the absorbance at λ_{max} decreased (Figure S1, Supporting Information). The resulting hypochromism at pH 7 was larger than that at pH 5, exhibiting the relatively stable association of the Dox with the poly(rC)·poly(rG) at pH 7. These results suggest that the poly(rC)·poly(rG) formed the complex with the Dox at physiological pH and that the resulting complex released the Dox in response to endosomal pH. The H^+ buffering around pH 6.0, assessed by the acid–base titration

(7) A typical procedure is as follows: Poly(rC)·poly(rG) (the average degree of polymerization of 2400) was purchased from Sigma Chemical Co. (St. Louis, MO). To 1.5 mL of an aqueous solution (containing 150 mM NaCl) of the poly(rC)·poly(rG) (1.4 mg/mL) was added a 0.1 M NaOH solution, and the resulting basic poly(rC)·poly(rG) solution (pH 10) was titrated with a 0.1 M HCl solution. The titration was carried out by the stepwise addition of 0.1 M HCl and stopped at pH 3. The pH value was checked with a pH meter (model F-52T, Horiba, Kyoto, Japan).

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(9) A typical procedure is as follows: The concentration of an aqueous solution (containing 150 mM NaCl) of the poly(rC)·poly(rG) was adjusted to 26 μg/mL (7.9×10^{-5} M based on the nucleotide units). The pH of the solution was varied from pH 7 to 4 by adding HCl solution. The CD was measured at each pH point and with a spectropolarimeter (J-700, JASCO, Tokyo, Japan). The values of CD in Figure 2 are expressed as molecular ellipticity, calculated for one nucleotide residue in the polynucleotide.

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(12) A typical procedure is as follows: The poly(rC)·poly(rG) (2.9 mg) and Dox (0.2 mg) were mixed in 1 mL of PBS (−). The resulting mixture was incubated for 1 day, followed by dialysis against isotonic phosphate buffer (pH 7 or pH 5) using a Spectra/Por 7 membrane (molecular weight cutoff = 10^3). During the dialysis, the released DOX concentration was determined by measuring the emission intensity at 562 nm (excitation at 490 nm) of each phosphate buffer using the calibration curve.

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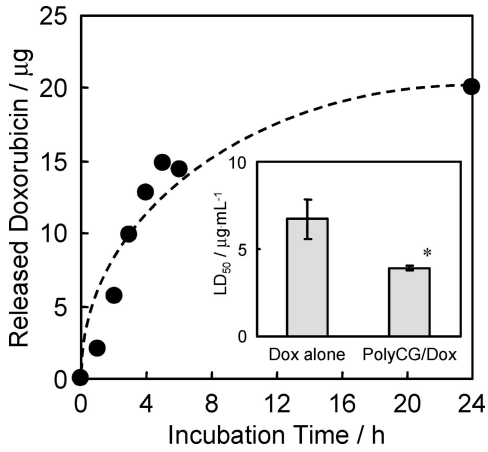

Figure 3. Release of Dox from the poly(rC)·poly(rG)/Dox complex at pH 5. The result is represented by the plot of the amount of net released Dox against incubation time. Inset: effect of the poly(rC)·poly(rG)/Dox complex on the viability of HepG2 cells. After 48 h incubation with Dox in the presence (PolyCG/Dox) or absence (Dox alone) of the poly(rC)·poly(rG), the cell viability was measured by Alamar Blue assay. The result is represented by LD_{50} values. Symbols and error bars represent the mean and standard deviation of the measurements made in quadruplicate wells. *, statistically significant ($p < 0.002$) when compared to the value in the absence of the poly(rC)·poly(rG).

of the resulting poly(rC)·poly(rG)/Dox complex (Figure S2, Supporting Information), supports the Dox release at endosomal pH. The formation of the poly(rC)·poly(rG)/Dox complex was presumably mediated by the intercalation between CG base pairs. Furthermore, electrostatic interaction and hydrogen bond formation also seemed to occur because only about 10% of bound drug was released; even so, the pH-dependent conformational change attributed to cytosine protonation is mainly considered to weaken the poly(rC)·poly(rG)/Dox interaction and to release the Dox at endosomal pH.

Finally, we examined whether the resulting poly(rC)·poly(rG)/Dox complex enhanced the anticancer activity of the Dox.[14] HepG2 cells, human hepatoma cell line, were treated with the Dox in the presence or absence of the poly(rC)·poly(rG), and the percentage of the cell viability was determined. An alamar blue assay[15] was used to detect the cytotoxicity induced by the poly(rC)·poly(rG)/Dox complex. The cell death of the HepG2 cells increased in a dose-dependent manner (Figure S3, Supporting Information), whereas the treatment with poly(rC)·poly(rG) alone exhibited no apparent cytotoxicity such as interferon response (Figure S4, Supporting Information). The LD_{50} values of the carrier/drug complex are shown in the inset of Figure 3. The LD_{50} value of the poly(rC)·poly(rG)/Dox complex (3.9 ± 0.1 μg/mL) was significantly ($p < 0.002$) smaller than that of the Dox alone (6.7 ± 1.1 μg/mL), meaning that the Dox in the presence of the poly(rC)·poly(rG) showed higher cytotoxicity for the human hepatoma cell. These results suggest that the duplex conformational change released the drug diffusing out of the endosome. The released Dox is therefore considered to be delivered from endosome to cytoplasmic space where the drug exhibited pharmaceutical activity.

In conclusion, we have exploited proton buffering capacity of poly(rC)·poly(rG) around pH 6 for a new pH-sensitive drug carrier. When poly(rC)·poly(rG) is protonated, it undergoes a conformational change that results in release of bound drug (Dox). The pH-change driven release of drug is achieved at endosomal pH values. Cell culture studies showed that the poly(rC)·poly(rG)/Dox complex possessed higher anticancer activity than the Dox alone. This is the first demonstration of the potential of poly(rC)·poly(rG) to act as a pH-sensitive drug carrier, and our future investigation will be focused on the mechanism of delivery and release as well as structure optimization of the delivery agent.

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Supporting Information Available: Figures S-1−S-4. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) A typical procedure is as follows: HepG2 cells (a gift from the Japan Health Sciences Foundation), human hepatoma cell line, were cultured in tissue culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS. The cells were seeded at 2×10^4 cells/well in a 96-well plate and incubated overnight at 37 °C in a 5% CO_2 incubator. The poly(rC)·poly(rG)/Dox mixtures incubated for 1 day were added to the cells; namely, the cells were treated with Dox (0–100 μg/mL) in the presence or absence of the poly(rC)·poly(rG) (0–1.4 mg/mL) and incubated for 48 h at 37 °C. The cell viability was measured using the Alamar Blue assay in quadruplicate.

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