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Self-Assembled Nanoparticles of Ribozymes with Poly(ethylene glycol)-*b*-Poly(L-lysine) Block Copolymers

Jae Hyun JEONG, Yong Woo CHO[1], Bokyung JUNG, Kinam PARK[2] and Jong-Duk KIM*

Department of Chemical and Biomolecular Engineering, and Center for Ultramicrochemical Process Systems, Korea Advanced Institute of Science and Technology, Kusong-dong, Yusong-gu, Daejon 305-701, Republic of Korea
[1]*Asan Institute for Life Sciences, University of Ulsan College of Medicine, 388-1 Pungnap-2 dong, Songpa-gu, Seoul 138-736, Republic of Korea*
[2]*Department of Pharmaceutics and Biomedical Engineering, Purdue University, West Layfayette, IN 47906, U.S.A.*

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Complex nanoparticle formation of poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-*b*-PLL) block copolymers with ribozyme (*Rz*s) in an aqueous solution was studied. Nanoparticles formed from PEG-*b*-PLL and *Rz*s have about 100 nm in an effective diameter with the hydrophilic PEG segments stabilizing and surrounding the core of complexes formed between the PLL segments and *Rz*s. The block copolymer having a higher positive charge formed relatively smaller complex nanoparticles. The profile of the ethidum bromide (EtBr) displacement assay and images of agarose gel electrophoresis revealed the condensation of *Rz*s to form complex nanoparticles above a critical weight ratio, *r* (*r*: 4.0 for PEG_{5000}-*b*-PLL_{1920} and 3.0 for PEG_{5000}-*b*-PLL_{3840}). Furthermore, the PEG-*b*-PLL/*Rz* complex nanoparticle showed high resistance against RNase attack in biological fluids. [DOI: 10.1143/JJAP.45.591]

KEYWORDS: nanoparticle, self-assembly, poly(L-lysine), ribozyme (*Rz*)

1. Introduction

Nanoparticles formed from DNA and cationic lipids, cationic polymers (or polycations) have been considered as a powerful delivery vehicle for introducing foreign genes into recipient cells.[1,2] Complex nanoparicles can be formed spontaneously by the electrostatic interaction between negatively charged nucleic acids and cationic polymers. A large number of cationic polymers have been studied as nonviral vectors, such as poly(L-lysine), poly(ethylenimine), poly(amidoamine) dendrimers, poly(2-dimethylaminoethyl methacrylate), and chitosan.[3–6] In particular, cationic polymers have the flexibility in designing a carrier with well-defined structural and chemical properties on a large scale as well as the ability to introduce functional moieties.

Ribozymes (*Rz*s) are catalytically competent RNAs that can be used to reduce the expression of a pathogenic gene by binding and cleaving specific regions of target RNA with endoribonuclease activity.[7,8] After transcription of a gene in the nucleus, the mRNA transcript is transported to a ribosome, which translates mRNA into proteins. *Rz*s inhibit gene expression by blocking the translation of mRNA into proteins. However, one problem of using these *Rz*s is their low stability against ubiquitous RNases in most biological environments.

Aiming to develop polymeric delivery vehicles for *Rz*s, we synthesized poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-*b*-PLL) block copolymers. Poly(L-lysine) has been widely used as a nonviral gene delivery carrier because of its excellent DNA condensation property.[9] Poly(ethylene glycol) (PEG) was chosen as a hydrophilic block for its well-known biocompatibility and unique aqueous properties. The cationic block copolymer could protect *Rz*s from RNases in biological fluids by forming complex nanoparticles through self-assembly. Techniques such as light scattering, ethidium bromide (EtBr) displacement assay, and electrophoretic mobility shift assay were used to characterize the stability of nanoparticles formed using cationic polymers of varying weight ratio.

2. Experiments

2.1 Materials

ε-(Benzyloxycarbonyl)-L-lysine was purchased from Sigma and used without further purification. α-Methoxy-ω-amino-PEG ($M_w = 5000$, $M_w/M_n = 1.05$, functionality of amino group, 0.98) was purchased from Nektar Therapeutics. Tetrahydrofuran (THF), *N*,*N*-dimethylformide (DMF), and hexane were dried and doubly distilled before use. Triphosgene, trifluoroacetic acid, anisole, and methanesulfonic acid were purchased from Adrich. Agarose, ethidum bromide (EtBr), bromophenol blue, and xylene cyanole FF were purchased from Bio-Rad. A 32mer oligonucleotide *Rz* composed of deoxy-, ribo-, and 2′-*o*-methyl ribo-nucleotides linked by a phosphodiester backbone was used.

2.2 Synthesis of PEG-b-poly(L-lysine)

The block copolymer, PEG_{5000}-*b*-poly(L-lysine)$_{1920}$ as a representative example, was synthesized by a ring-opening polymerization of Lys(z)-NCA which was previously synthesized by the Fuchs–Farthing method[8] using triphosgene. Lys(z)-NCA (0.37 g) in DMF (3 ml) was added to a solution (DMF, 3 ml) of α-methoxy-ω-amino PEG (0.15 g), which was stirred and purged with nitrogen at 35°C for 10 min. The reaction was maintained at 40°C under a dry nitrogen atmosphere for 2 days. After filtration, the resulting solution was evaporated under reduced pressure and precipitated with an excess of diethyl ether. PEG-*b*-P(Lys(z)) was then deprotected using TFA, anisole and methanesulfonic acid to obtain PEG-*b*-PLL. The composition of PEG-*b*-PLL was determined by 300 MHz ^{1}H-NMR (Bruker ARX 300, Billerica, MA) in DMSO-d_6 or D_2O.

2.3 Nanoparticle formation and measurements

A ribozyme (*Rz*) (20 μg/ml) was added in ultrapure water to an Ependorf tube and mixed thoroughly. PEG-*b*-PLL (2.0 mg/ml stock in water) was added to *Rz* solutions with different weight ratios, *r* (= [polymer]/[ribozyme]) and

*To whom corresponding should be addressed.
E-mail address: jdkim@kaist.ac.kr

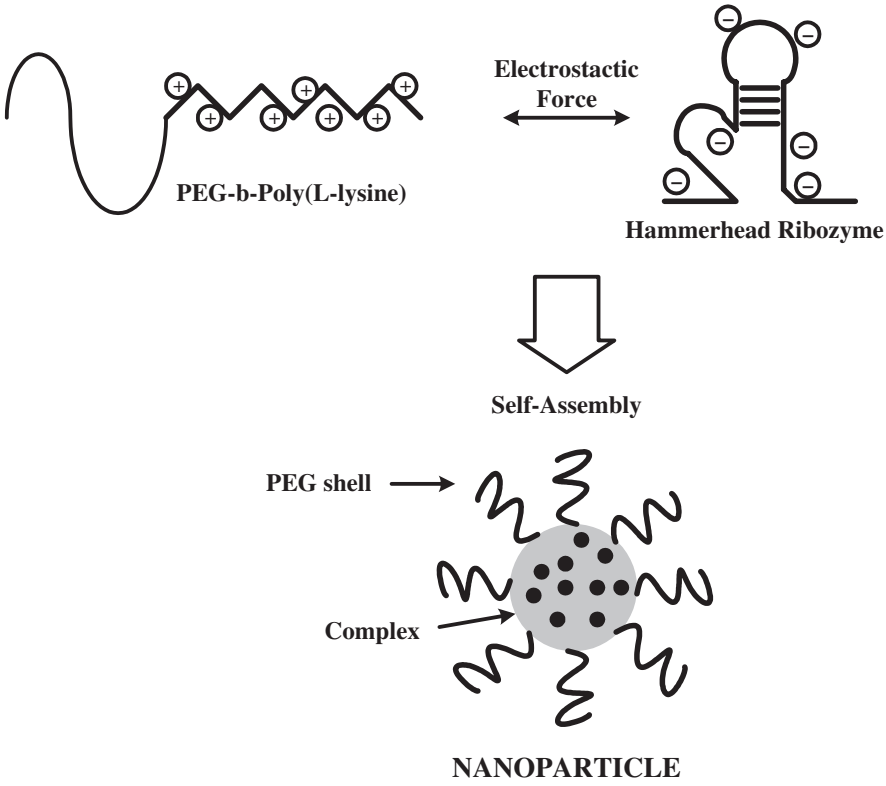
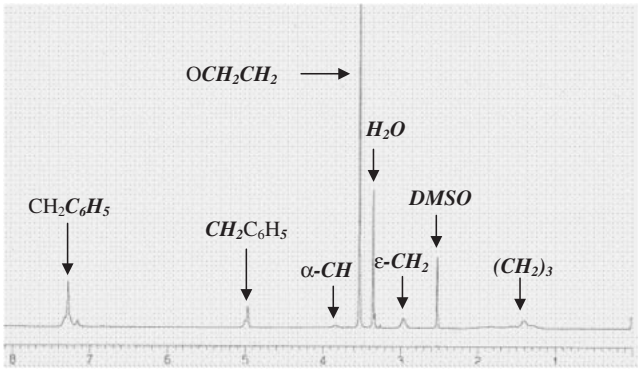


Fig. 1. Schematic diagram of designed cationic polymer complex for *Rz* delivery.

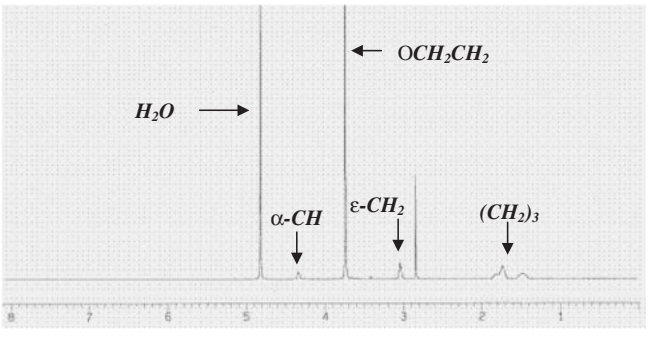
gently mixed together. Complexes were allowed to form for 30 min at room temperature before use. The size and size distribution of nanoparticles were measured by dynamic light scattering (DLS; Brookhaven Instruments) equipped with a He–Ne laser at a scattering angle of 90° (by the Stokes–Einstein relationship). The particle size and size distribution were calculated using non-negative least squares (NNLS) algorithms. The morphology of complexes was observed using transmission electron microscopy (TEM). A drop of complex solution was placed on a copper grid coated with carbon film. The grid was held horizontally for 30 s to allow the complexes to settle down and then vertically to allow excess fluid to drain. Observation was carried out at 80 kV with a Philips CM200. The degree of *Rz* condensation by polycations was determined by EtBr displacement assay using a spectrofluorometer (Spex FluoroMax-2). The complex nanoparticle formation and biological stability were observed by electrophoretic mobility shift assay using a Mini-PROTEAN 3 electophoresis cell system (Bio-Rad).

3. Results and Discussion

PEG-*b*-PLL block copolymers were successfully synthesized by a ring-opening polymerization of Lys(z)-NCA with α-methoxy-ω-aminopoly(ethylene glycol) (PEG) as a macroinitiator. Figures 2(a) and 2(b) respectively show ^{1}H-NMR spectra of PEG-*b*-P(Lys(z)) in DMSO-d_6 as well as of PEG-*b*-PLL in D_2O, which was prepared from PEG-*b*-P(Lys(z)) by the deprotection of ε-(benzylocycarbonyl) groups. For PEG-*b*-PLL, the peak intensity ratio of protons ($OC\mathbf{H}_2C\mathbf{H}_2$) (3.7 ppm) of PEG and protons [$(CH_2)_3C\mathbf{H}_2NH_3$] (3.0 ppm) of poly(L-lysine) in Fig. 2(b) was measured to calculate the DP value. As shown in Table I, we successfully synthesized the polymers as the DP values of 15 and 30. We used the



(a)



(b)

Fig. 2. ^{1}H-NMR spectra of (a) PEG-*b*-P(Lys(z)) in DMSO-d_6 and (b) PEG-*b*-PLL in D_2O.

PEG-*b*-PLL copolymers as the counterpart of *Rz* to form nanoparticles through electrostatic interaction (Fig. 1). The *Rz* and PEG-*b*-PLL were separately dissolved in pure water

Table I. Characteristics of polymers and nanoparticles.

Polymer	Block length[a] (g/mol) Composition [EG]:[Lys]	Mass per charge (Da)	Weight ratio[b] (*r*)	Diameter[c] (nm)
PEG-*b*-poly(L-lysine)1	5000/1920 [113]:[15]	461	4.0	128
PEG-*b*-poly(L-lysine)2	5000/3840 [113]:[30]	295	3.0	89

a) Calculated from the peak integration of ^{1}H-NMR spectra
b) Weight ratio (*r*) to achieve stable nanoparticle formation by EtBr displacement assay
c) Effective diameter of complex nanoparticles at $r = 10$

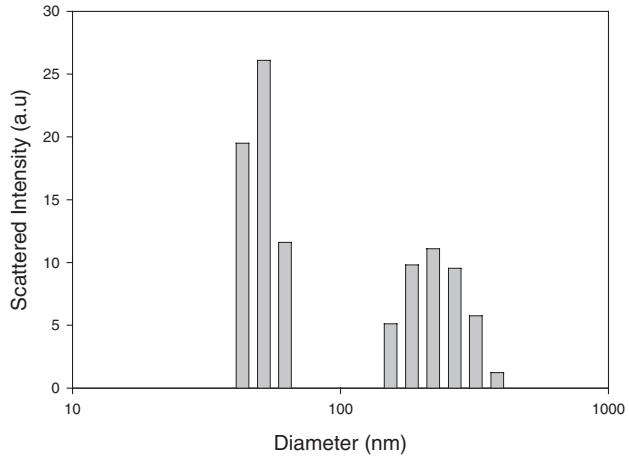


Fig. 3. Size distribution of nanoparticles (PEG-*b*-PLL1) at weight ratio of 10 (*r*).

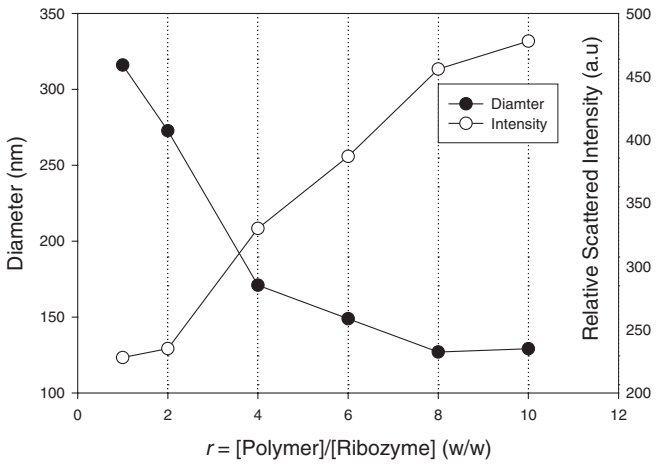


Fig. 4. Effective diameter and scattered intensity of complex nanoparticle with varied weight ratios ($r = 0$–10).

and then mixed gently varying weight ratio (*r*). The DLS measurements showed a biomodal size distribution with 128 nm in an effective diameter as shown in Fig. 3. The small ones are located near 40 nm, while the large ones vary between 200 and 300 nm. In spite of the transparent appearance of the complex solution, the light scattering intensity of the solution with $r = 10$ was increased by about two fold compared with that of $r = 1$ as shown in Fig. 4. Also, the effective diameter of the complex nanoparticles decreased as *r* increased. The degree of charge neutralization of *Rz*s increases as the PLL homopolymer condensation is

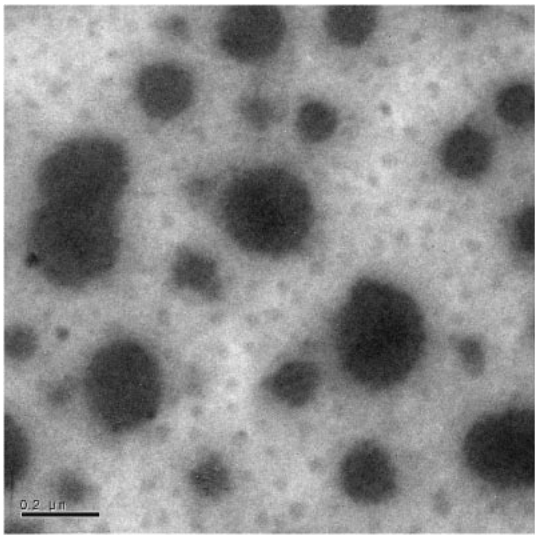


Fig. 5. TEM photographs of complex nanoparticles with PEG-*b*-PLL1 and *Rz* at $r = 3$.

increased, and *Rz*s above the critical polycation concentration condenses into a compactly dense structure thereby precipitating from the solution. However, the PEG-*b*-PLL copolymer complexes did not precipitate even above $r = 10$ unlike that of the PLL homopolymer. The effective diameters were reduced as the PLL part increased apparently (Table I, $r = 10$). The block copolymer having a higher positive charge forms smaller complex nanoparticles with *Rz*s. The results of DLS indicate that nanoparticles were formed, requiring the theoretical amount of the cationic polymer with the hydrophilic PEG segments stabilizing and surrounding the core of complexes that formed between the PLL segments and *Rz*s. Also, the result presented in Fig. 5 clearly indicates that the nanoparticles formed between PEG-*b*-PLL and the *Rz* have a spherical shape.

The degree of *Rz* condensation by PEG-*b*-PLL coppolymers was determined by an EtBr displacement assay using a spectrofluorometer.[10] The spectrofluorometer was operated with an excitation wavelength (λ_{ex}) of 510 nm and an emission wavelength (λ_{em}) of 590 nm. The *Rz* stock solution (5 mg/ml) was diluted to a final concentration of 20 μg/ml including 10 μg/ml EtBr in a test curvet, and the fluorescence was calibrated to 100%. Aliquots of PEG-*b*-PLL were added sequentially, and the fluorescence was measured after each addition. At a low weight ratio, there is little exclusion of EtBr demonstrating minimal complexation. However, EtBr intercalates in between stacked base pairs of *Rz*s to give

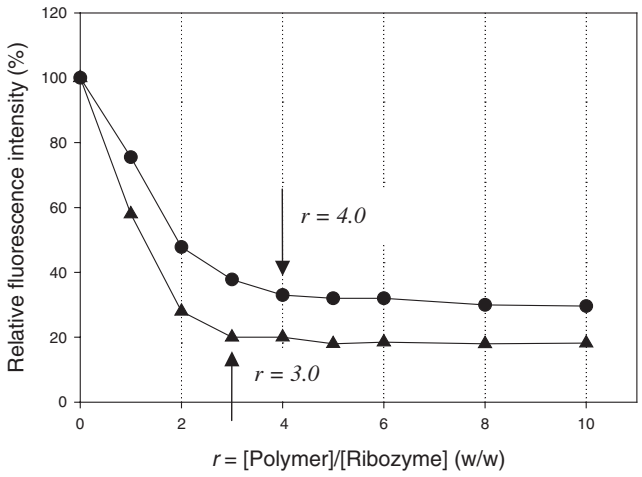


Fig. 6. Ethidium bromide displacement by PEG-*b*-PLL1 (circle) and 2 (triangle) interacting with ribozyme with varied weight ratios ($r = 0$–10).

a significant increase in fluorescence intensity. The addition of PEG-*b*-PLL causes a large fall in fluorescence intensity (Fig. 6) due to the displacement of EtBr molecules from *Rz*s, which indicates the condensation of *Rz*s to form complex nanoparticles. The complexes were formed with stability for weight ratios above $r = 4.0$ for PEG-*b*-PLL1 and 3.0 for PEG-*b*-PLL2, respectively. As shown in Table I, *r* required to achieve stable nanoparticle formation by EtBr displacement assay is decreased as the molecular weight of PLL is increased. This result indicates that the self-assembled nanoparticles were formed with stability, requiring the theoretical amount of the cationic polymer.

Images of agarose gel electrophoresis with a series of weight ratios of PEG-*b*-PLL1/*Rz*s are shown in Fig. 7(a). Complexes were prepared with varied weight ratios (*r*) and incubated at R.T. for 30 min to allow the complexes to form properly. The complexes were loaded onto the wells of the agarose gel containing EtBr (1.0 μg/ml) and were electrophoresed in a loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanole FF, 20% glycerol) at 150 V. For each gel, lane 1 contains ribozyme only, and lanes 2 to 5 contain complexes with *r* values of 0.5, 1.0, 3.0 and 5.0, respectively. The migration of *Rz*s in the gel was apparently retarded as the weight ratio of the polymer was increased above $r = 3$, indicating that PEG-*b*-PLL1 was able to bind *Rz*s tightly. Therefore, it is consistent with the EtBr displacement assay results in which the complexes were formed with stability for weight ratios above $r = 4.0$ and 3.0 for PEG-*b*-PLL1 and 2, respectively. Generally, the N:P ratio is defined as the molar ratio of amino acids groups in polycations to phosphate groups in DNA. In the case of the PEG-*b*-PLL/*Rz* complex, the N:P ratio corresponds to 2.0 ($r = 3$) and 2.7 ($r = 4$) above stoichiometric conditions in PEG-*b*-PLL1 copolymer.

The stability of *Rz*s entrapped within nanoparticles was studied from the viewpoint of RNase resistance. The complex nanoparticles were incubated with RNases at 37°C under gentle shaking for 2 h. After incubation, *Rz*s were extracted from the nanoparticles at 70°C for 30 min and analyzed by electrophoresis at 150 V. Physical entrapment of ribozymes within complex nanoparticles significantly increases their biological stability, as shown in Fig. 7(b). For each gel, lane 1 contains *Rz* only, and lane 2 contains *Rz* and RNase, lanes 3 to 5 contain polymers, *Rz*, and RNase with *r* values of 1.0, 3.0 and 5.0, respectively. As expected, the incubation of *Rz* with RNase led to a complete loss of *Rz* within minutes, as indicated by the absence of the full-length *Rz* band (lane 2). After complexation with PEG-*b*-PLL, however, *Rz* was stable and completely protected against degradation by RNase (lanes 4 and 5). No second band appeared when the weight ratio was above $r = 3$, lane 4. It is proved that the PEG-*b*-PLL/*Rz*s complex has a high resistance against RNase in biological fluids, suggesting *Rz* protection through the segregation into the core of the nanoparticle surrounded with PEG palisade.

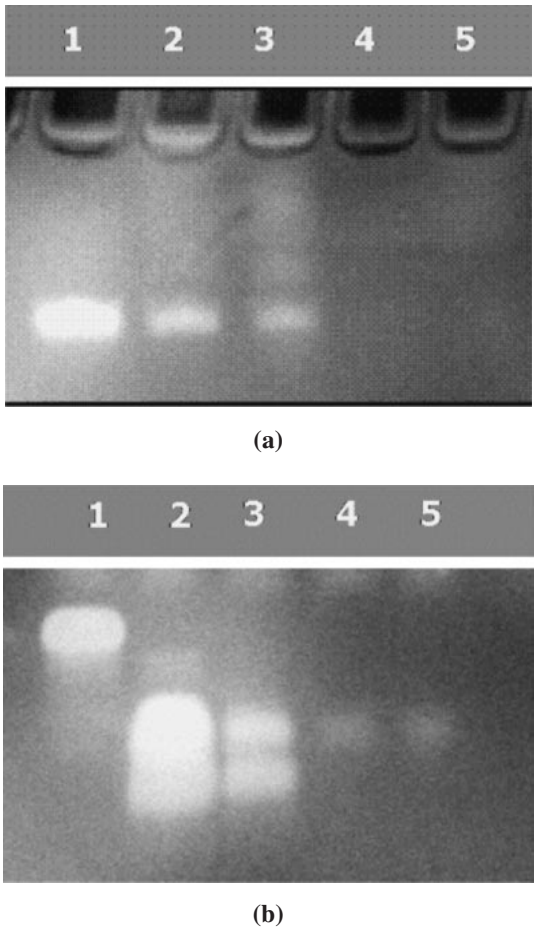


Fig. 7. (a) Gel Electrophoresis results for PEG-*b*-PLL1/*Rz* complexes. For each gel, lane 1 contains ribozyme only, lanes 2 to 5 contain complexes with 0.5, 1.0, 3.0 and 5.0, respectively. (b) Biological stability of *Rz*. For each gel, lane 1 contains *Rz* only, and lane 2 contains *Rz* and RNase, lanes 3 to 5 contain polymers, *Rz* and RNase with *r* values of 1.0, 3.0 and 5.0, respectively.

4. Conclusions

PEG-*b*-PLL block copolymers were successfully synthesized for *Rz* delivery. PEG-*b*-PLL copolymer formed nanoparticles with *Rz* through the electrostatic force between PLL segments and nucleic acids. The complexes were formed with stability for weight ratios above $r = 4.0$ for PEG-*b*-PLL1 and 3.0 for PEG-*b*-PLL2. Also, PEG-*b*-PLL protected *Rz*s from RNase in biological fluids by forming complex nanoparticles.

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