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Core-Corona Structure Formed by Hyaluronic Acid and Poly(L-lysine) via Kinetic Path

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Electronic Supplementary Information

Abstract The structure and kinetics of the complex formed by hyaluronic acid (HA) and poly(L-lysine) (PLL) were studied by time-resolved laser light scattering, TEM, and AFM. Because HA has a hydrophilic backbone, the complexes formed by HA and PLL are different from those formed by oppositely charged polyelectrolytes both having hydrophobic backbones. Instead of forming strong aggregates and even precipitates, the complex in the presence of excess HA is stable in the studied time period. More importantly, the complex spontaneously forms core-corona structure by the rearrangement of HA chains. The core is composed of complex rich of PLL and the corona is mainly HA. Further analysis shows that the hydrogen bond formed by HA creates a barrier hindering the further relaxation of HA chains. The automatic formation of core-corona structure by PLL/HA is helpful not only to understand the relaxation of polyelectrolyte in complex, but also to develop drug carriers with desirable properties.

Keywords Polyelectrolyte complex; Core-corona structure; Hyaluronic acid; Poly(L-lysine); Laser light scattering

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INTRODUCTION

The polyelectrolyte complexes (PECs) have been studied for decades due to their potential applications in protein separation,^[1] wastewater treatment,^[2,3] catalyst support,^[4] drug (gene) delivery,^[5–8] and controlled release^[9–13]. A variety of synthetic polyelectrolytes and biopolymers, such as DNA^[14,15] and proteins,^[16] have been applied to form PECs for the above mentioned purposes. It is generally accepted that the major driving force for PEC is the entropic gain caused by the release of counter-ions,^[17] as well as the short range ion-correlation force.^[18] Since electrostatic interaction is strong and long-ranged, the formation of PEC is affected by multiple parameters, including charge density,^[19] chain length,^[20] mixing ratio,^[21,22] ionic strength,^[22] and so on.

Little attention has been paid to the structure of PECs. It was firstly suggested that the oppositely charged polyelectrolytes, which adopted extended chain conformation, could zipper together to form a ladder network.^[23,24] However, rheology experiments on PEC supported the “scrambled egg” model, in which the interlinked chains were completely random.^[24] Hubbe and coworkers proposed “segment entrapment” model and “surface excess” model to describe the dis-

tribution of the charges in PEC.^[25,26] In the “segment entrapment” model, the complex core contained unneutralized segments, while in the “surface excess” model, the unneutralized segments mainly stayed on the surface of PEC. These models do not specify whether the structures are at equilibrium conditions or just kinetically trapped state.

The study on kinetics has demonstrated that the formation of PECs consists of two or more steps: formation of preliminary complex, followed by aggregation or even precipitation.^[8] The rearrangement of polyions inside the preliminary complex, also named as disproportionation,^[27] is a key process for the PEC to reach equilibrium state. It has been demonstrated that chain length, charge density, hydrophobicity, and ionic strength are major factors affecting the disproportionation rate.^[27–30] Our previous study on the complex formed by DNA and peptides showed that the kinetics of PEC was closely related to the water content inside the complex.^[30] Water molecules served as “lubricant” to the relaxation of polyions. Expulsion of water molecule upon forming complex would freeze the PEC structure in a kinetically trapped state.^[30]

Inspired by the enhanced relaxation rate of polyions in the complex with higher water content, we speculate that the polyions possessing hydrophilic backbone, which have an affinity for water, should exhibit fast kinetics when forming PECs with oppositely charged polyelectrolyte. Since the PEC generally has a lower dielectric constant than that of the

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aqueous environment, the hydrophilic polyions, if the amount is in excess, should move to the periphery of the PEC during the rearrangement. A core-corona structure thus should be formed. This structure would prevent the phase separation of the PECs. To test these hypotheses, we chose hyaluronic acid (HA) as the hydrophilic polyions, and studied its complex with poly(L-lysine) (PLL). The molecular formulas of HA and PLL are shown in Fig. 1. HA is a major structural component of the extracellular matrix.^[31] It has specific interaction with many receptors, such as CD44,^[32] providing targeting ability for drug delivery if coated on the surface of a drug carrier. Therefore, the study on the relaxation behavior of HA in PEC also helps to design drug carriers with desirable properties. PLL is a polycation with hydrophobic backbone. The PEC with PLL in excess should exhibit a different behavior from that with HA in excess. Laser light scattering (LLS) and transmission electron microscopy (TEM) were applied to monitor the complexation process and to determine the structure of the complex.

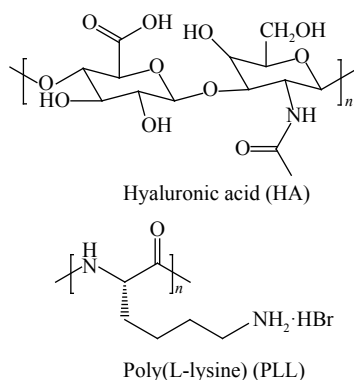


Fig. 1 Molecular formulas of HA and PLL

EXPERIMENTAL

Materials and Sample Preparation

PLL (molecular weight: 3.0×10^4 – 7.0×10^4) was purchased from Sigma-Aldrich (St Louis, US) and used as received. HA (molecular weight: 1.0×10^6 – 1.5×10^6) was purchased from Bloomage Freda Biopharm Co. Ltd. (Jinan, P. R. China) and used as received. Dulbecco's phosphate-buffered saline (DPBS) buffer (2.67 mmol/L KCl, 1.47 mmol/L KH_2PO_4 , 138 mmol/L NaCl, and 8.06 mmol/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) was purchased from Invitrogen (Shanghai, P. R. China). 2,2,2-Trifluoroethanol (99.5%) was purchased from Aladdin (Shanghai, China). Stock solutions of HA and PLL were obtained by dissolving the sample in DPBS buffer. The concentrations of PLL and HA were fixed at 2.0×10^{-5} g/mL.

Laser Light Scattering (LLS)

A commercialized spectrometer from Brookhaven Instrument Corporation (BI-200SM Goniometer, Holtsville, NY) was used to conduct both static light scattering (SLS) and dynamic light scattering (DLS) over a scattering angular range of 20° – 150° . A vertically polarized, 17 mW He-Ne laser (Newport, USA) operating at 633 nm was used as the light source. In static light scattering (SLS), the angular

dependence of the excess absolute time-averaged scattered intensity, known as the Rayleigh ratio $R_{90}(\theta)$, was measured. For a very dilute solution, the weight-averaged molar mass (M_w) and the root mean-square radius of gyration (R_g) can be obtained on the basis of

$$HC/R_{90}(\theta) = (1/M_w) \left[1 + (1/3)R_g^2 q^2 \right] + 2A_2c \quad (1)$$

where $H = 4\pi^2 n^2 (dn/dc)^2 / (N_A \lambda^4)$ and $q = 4\pi n / \lambda \sin(\theta/2)$ with N_A , n , dn/dc , and λ being the Avogadro's number, the solvent refractive index, the specific refractive index increment, and the wavelength of light in a vacuum, respectively.

In dynamic light scattering (DLS), the intensity-intensity time correlation function $G^{(2)}(\tau)$ in the self-beating mode was measured. A Laplace inversion program, CONTIN, was applied to obtain the line width distribution and the diffusion coefficient D . The diffusion coefficient can be further converted into the hydrodynamic radius R_h by using the Stokes-Einstein equation:

$$D = k_B T / 6\pi\eta R_h \quad (2)$$

where k_B , T , η are the Boltzmann constant, the absolute temperature, and the viscosity of the solvent, respectively.

For LLS study, HA and PLL solutions were filtered through sterile-EO membranes (0.45 μm Sartorius Stedim Minisart®, Germany) to remove the dust. The complex was formed by adding PLL to HA or adding HA to PLL. The point of mixing was set as the starting time. The mixture was quickly swirled at ~ 1200 r/min for 30 s before the measurement.

Transmission Electron Microscopy (TEM)

The complexes were prepared by the same procedure in LLS measurement. 20 μL of the complex solution was deposited onto a freshly glow discharged carbon-covered copper grid. The solution was left for 30 s and the extra solution was removed by filter paper. About 10 μL 2% uranyl acetate was then placed on the grid, and the excess stain was wicked away 30 s later. TEM image was recorded using a Technai T20 microscope operated at 120 kV.

Atomic Force Microscopy (AFM)

AFM measurements were conducted in tapping mode on Nanoscope III (Veeco instrument Inc., USA) equipped with a 110 μm scanner. The data were collected in air by a commercial silicon tip (SI-DF3, Seiko instruments Inc.). The resonant frequency of the cantilever is ~ 70 kHz. The complex was prepared by following the same procedure in light scattering measurement. At each time point, 20 μL of sample solution was collected at a position about 5 mm to the bottom of the vial. To minimize the morphology change during drying, the sample was deposited on a mica surface for 30 s and the extra solution was removed by a filter paper. The sample was dried at room temperature for 1 day before the AFM measurement.

RESULTS AND DISCUSSION

Complexes of PLL/HA at Different +/- Molar Ratios

Firstly, the complexes at different +/- ratios are prepared by adding a known amount of PLL to HA in DPBS buffer. The zero time point is set exactly at the moment of adding PLL to

HA. LLS is then applied to monitor the kinetics of the complexes. Fig. 2 shows a snapshot of laser beam passing through the sample vial. Only one diffusive mode is observed at all the studied conditions (Fig. S1 in electronic supplementary information, ESI). Fig. 3 shows the time-dependence of the apparent hydrodynamic radii ($R_{h,app}$) of the complex at different $+/-$ ratios. At $+/- = 0.20$ or 0.50 , no apparent change in $R_{h,app}$ is observed with time. The stability is also verified by the excess scattered intensity at 30° in Fig. 4(a). At $+/- = 1.0$, the size of the complex slightly increases (Fig. 3) and the scattered intensity exhibits fluctuation with time. This suggests that the complex at $+/- = 1.0$ starts to form secondary aggregate. This tendency is stronger with increasing $+/-$ ratios. As shown in Fig. 3, the size of the complexes at $+/- > 1.0$ is even larger, and increases with time. The diameter reaches micron level in hours. On the contrary, the excess scattered intensity drops with time (Fig. 4b). Such opposite changes in size and scattered intensity indicate that the complexes form strong aggregates and even precipitates, in agreement with the findings in other PECs consisting of polyelectrolytes with hydrophobic backbones.^[8] The results in Figs. 3 and 4 imply



Fig. 2 Laser beam passing through a solution of PLL/HA complex

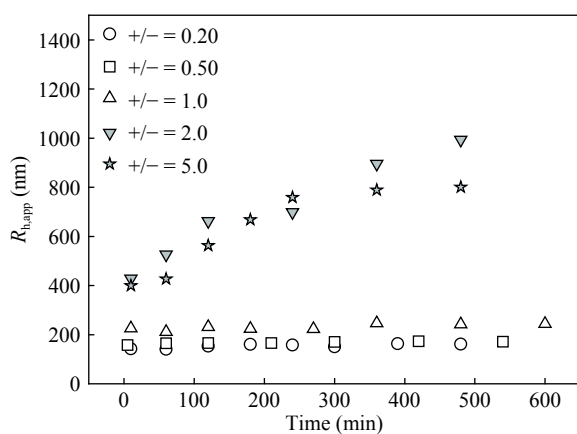


Fig. 3 Time dependence of $R_{h,app}$ of the complexes formed by HA and PLL at different $+/-$ ratios in DPBS buffer. The complexes are prepared by adding a known amount of PLL to HA.

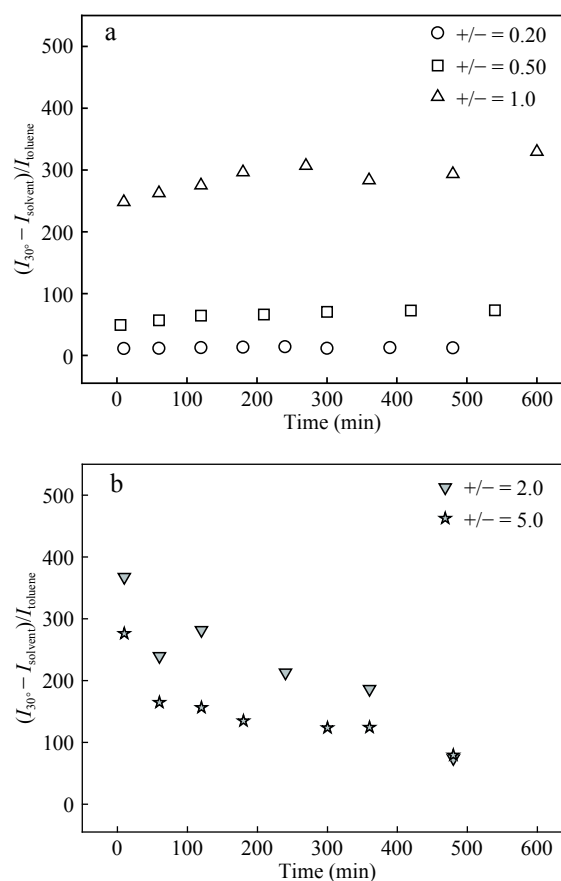


Fig. 4 Time dependence of the excess scattered intensity at 30° in DPBS buffer: (a) $+/- = 0.20$, 0.50 , and 1.0 . (b) $+/- = 2.0$ and 5.0 . The complexes are prepared by adding PLL to HA.

that the complex is stable when HA is in excess, while it is not stable when PLL is in excess.

Effects of Mixing Order

Mixing order determines whether PLL is wrapped by HA or HA is wrapped by PLL right after mixing. Fig. 5 compares the evolutionary change of the sizes of the complexes prepared by two different mixing orders. Since the complexes form precipitates at $+/- > 1.0$, only the results on the complexes at $+/- \leq 1.0$ are shown. As shown in Fig. 5, no prominent changes in either $R_{g,app}$ or $R_{h,app}$ are observed for the complexes prepared by different mixing orders at studied $+/-$ ratios. Since the dn/dc values of PLL and HA are large and close (about 0.15 mL/g)^[33] and only the peaks corresponding to the complex are observed (Fig. S1, in ESI), the apparent weight-average molecular weight ($M_{w,app}$) of the complex is obtained based on Eq. (1). The aggregation numbers of HA and PLL in each complex are calculated and listed in Table 1. Even at $+/- = 0.20$, each complex contains 40–60 HA molecules. The number of PLL is larger because its chain length is short. The aggregation number of PLL increases by two orders with the charge ratio close to unity. Fig. 6 and Table 1 show that the $M_{w,app}$ and aggregation numbers of the complexes formed by adding HA to PLL are larger than those of the complexes formed by the reverse order. Kabanov *et al.* studied the effect of mixing order on the complexation formed by synthetic polyelectrolytes both

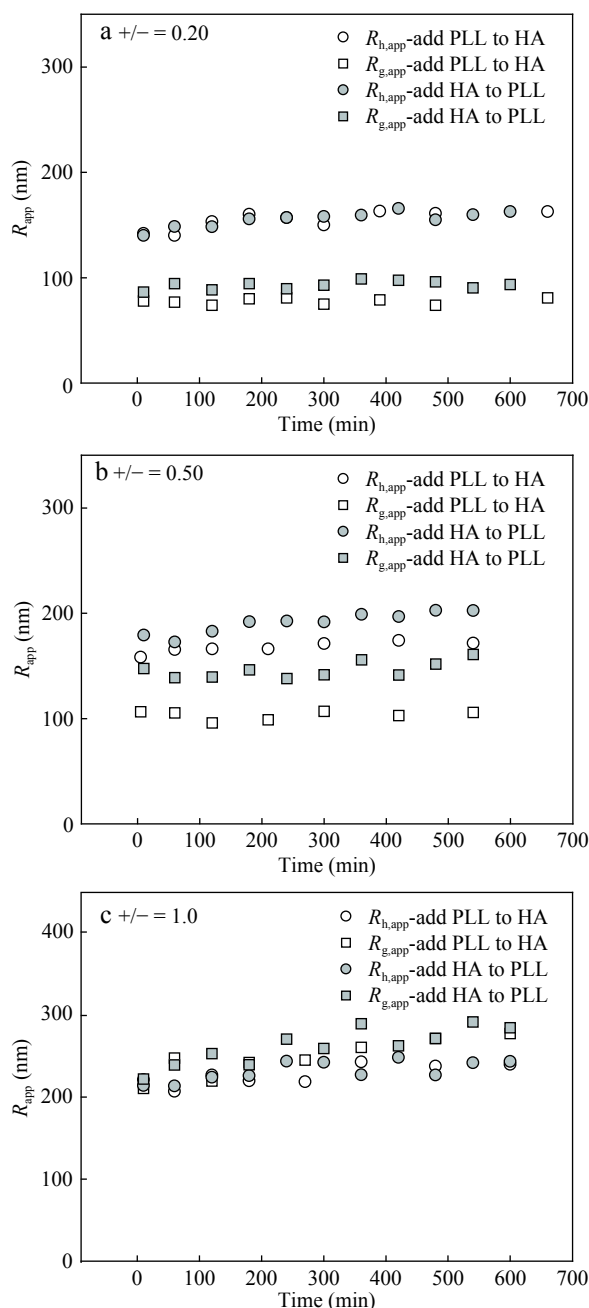


Fig. 5 Time dependence of $R_{h,app}$ and $R_{g,app}$ of the complexes formed by adding PLL to HA and adding HA to PLL at different $+/-$ ratios in DPBS buffer: (a) $+/- = 0.20$, (b) $+/- = 0.50$, (c) $+/- = 1.0$

Table 1 Numbers of HA and PLL in the complex at different charge ratios

$+/-$	Adding PLL to HA		Adding HA to PLL	
	$N_{HA} \times 10^{-2}$	$N_{PLL} \times 10^{-2}$	$N_{HA} \times 10^{-2}$	$N_{PLL} \times 10^{-2}$
0.20	0.41	0.80	0.60	1.2
0.50	2.0	9.8	3.2	16
1.0	9.2	92	14	137

having hydrophobic backbones.^[34] They found that the complex prepared by adding longer chain polyions (LPE) to shorter chain oppositely charged polyions (BPE) was water soluble at $[LPE]/[BPE] < 1$, while the complex prepared by

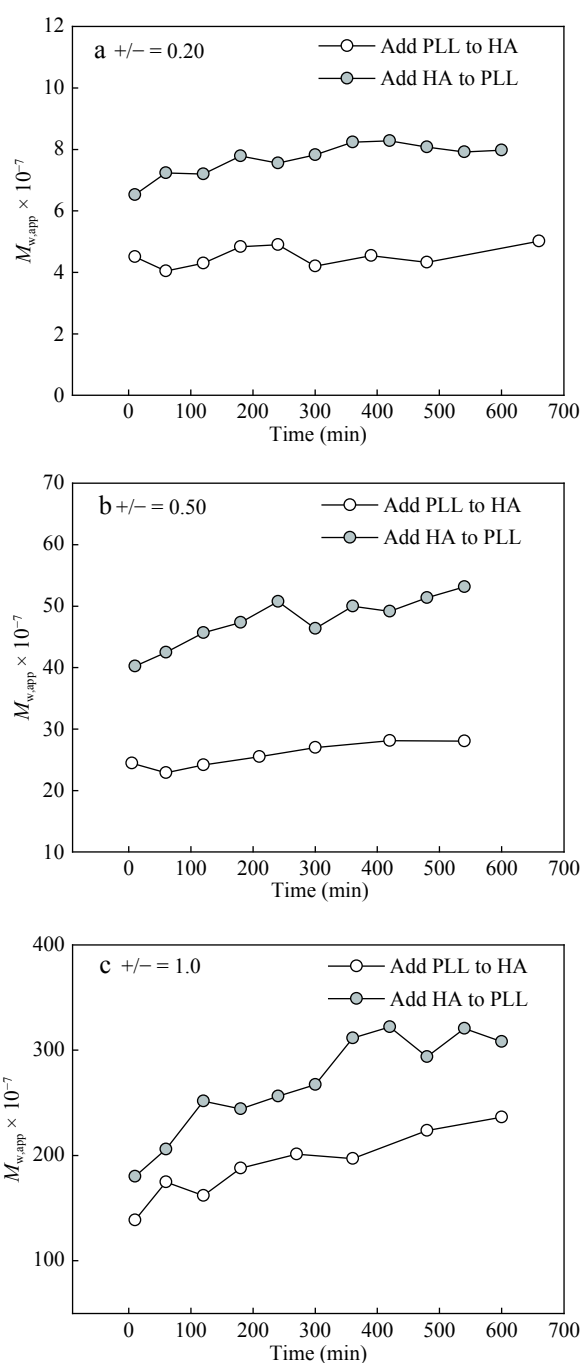


Fig. 6 Time dependence of apparent weight-average molecular weight ($M_{w,app}$) of the complexes formed by adding PLL to HA and adding HA to PLL at different $+/-$ ratios in DPBS buffer: (a) $+/- = 0.20$, (b) $+/- = 0.50$, (c) $+/- = 1.0$

inversed order formed precipitates at the same conditions. This trend is different from the complex formed by HA and PLL. We attribute it to the hydrophobicity of PLL after charge neutralization. The complex formed by adding HA to PLL leads to a heavier aggregation. However, the complex does not result in precipitation, which is different from the complexes formed by PLL with other polyelectrolytes, such as DNA,^[8] or by polyelectrolyte both having hydrophobic backbones.

The conformation of the complex can be derived from the

R_g/R_h ratios. It is established in literature that the R_g/R_h ratios for random coil and hard sphere are 1.5 and 0.775, respectively.^[35] Shown in Fig. 7 are the $R_{g,app}/R_{h,app}$ ratios of the HA/PLL complexes calculated using the data in Fig. 5. Interestingly, the $R_{g,app}/R_{h,app}$ ratios increase with $+/-$ ratio in both cases. For the complexes prepared by adding PLL to HA, the size ratios are 0.6, 0.7, and 1.1 at $+/- = 0.20$, 0.50, and 1.0, respectively. The $R_{g,app}/R_{h,app}$ ratios at $+/- = 0.20$ and 0.50 are lower than 0.775, indicating that the structures contain a dense core and fluffy periphery.^[36,37] Since HA has a hydrophilic backbone and it is also excess in amount, the

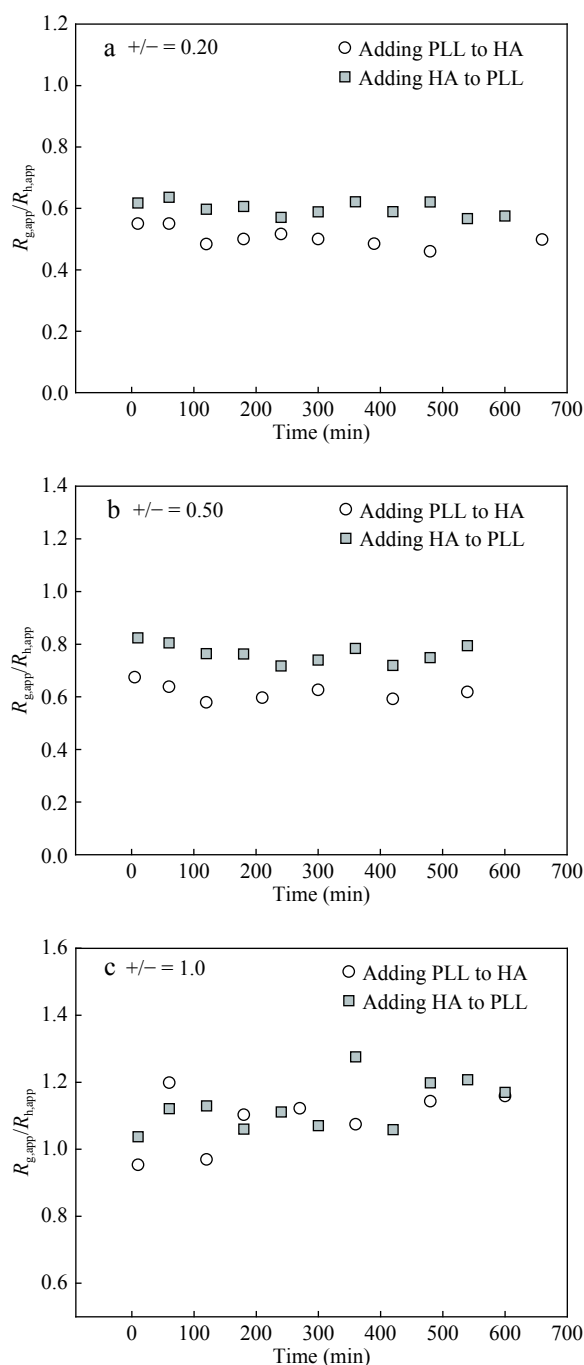


Fig. 7 Time dependence of $R_{g,app}/R_{h,app}$ of the complexes formed by adding PLL to HA and adding HA to PLL at different $+/-$ ratios in DPBS buffer: (a) $+/- = 0.20$, (b) $+/- = 0.50$, (c) $+/- = 1.0$

fluffy periphery should consist of main HA chains. For the complex prepared by the reversed order, the $R_{g,app}/R_{h,app}$ ratios are correspondingly larger at $+/- = 0.20$ and 0.50. However, it is still lower than 0.775 at $+/- = 0.20$, suggesting that the complex forms fluffy structure independent of the mixing order when HA is a large amount in excess.

Role of Hydrogen Bond in HA Rearrangement

HA has a strong capacity to form hydrogen bonds due to the large amount of hydroxyl groups. The formation of hydrogen bonds may create a barrier for the arrangement of HA in the complex. To test this hypothesis, we monitored the stability of the complex after the addition of 2,2,2-trifluoroethanol (TFE), which can effectively destroy hydrogen bonds.^[38] The complexes are formed by adding PLL to HA at $+/- = 0.20$. The amount of HA is much more than that of PLL at this charge ratio. The effect of hydrogen bonds should be more obvious if they exist. The final concentration of TFE is 3%. Firstly, TFE was added before complexation. The stability of the complexes is not changed after the addition of TFE (Fig. S2, in ESI), indicating the formation of hydrogen bonds is not the source of stability. To further confirm the effects of hydrogen bonds, we added TFE to the complexes about 3 h after the complexation. As shown in Fig. 8, $R_{h,app}$ slightly increases, while $M_{w,app}$ drops about 5% after the addition of TFE. No apparent change in $R_{g,app}$ is observed. The different

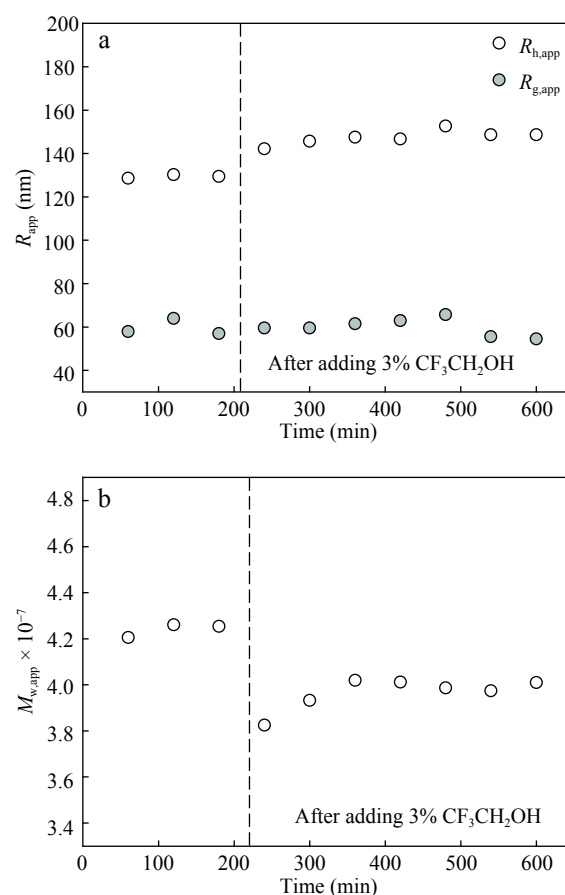


Fig. 8 (a) Size and (b) $M_{w,app}$ of the complex at $+/- = 0.20$ in the presence of 3% TFE. The complex is prepared by adding PLL to HA in DPBS buffer. The dotted line shows the moment of adding TFE.

changes in $R_{g,app}$ and $R_{h,app}$ suggest that the conformation of the complex varies after the hydrogen bonds break down. One possible explanation is that more HA molecules redistribute to the surface of the particle, leading to a smaller $R_{g,app}/R_{h,app}$ ratio. The drop in $M_{w,app}$ is caused either by the change in particle conformation or by the release of molecules to the solution, or by both. All these results suggest that the breakdown of the hydrogen bonds facilitates the rearrangement of HA chains.

Core-Corona Structure Determined by TEM

To confirm the formation of core-corona (fluffy) structures by HA/PLL complex, we investigated the morphology of the complexes by TEM and AFM. The complexes are prepared by adding PLL to HA at $\pm$ ratios of 0.20, 1.0, and 5.0, separately, following the same procedure used in LLS measurements. As shown in Fig. 9, only spherical structures are observed from TEM at $\pm = 0.20$ and 1.0, while an aggregate of complexes is observed at $\pm = 5.0$. Similar results are obtained from AFM experiments (Fig. S3, in ESI). These variations at different $\pm$ ratios are in agreement with LLS data. Further analysis shows that the spheres in Fig. 9 contain detailed structure. As shown in the inset in each panel, the complex at $\pm = 0.20$ and 1.0 possesses an irregular dense core and a more uniform corona. The core has a smaller size and lower density with decreasing $\pm$ ratio. We attribute it to the complex rich of PLL. The corona is mainly composed of long chain HA. The heavy aggregate in Fig. 9(c) is composed of small size complexes, which

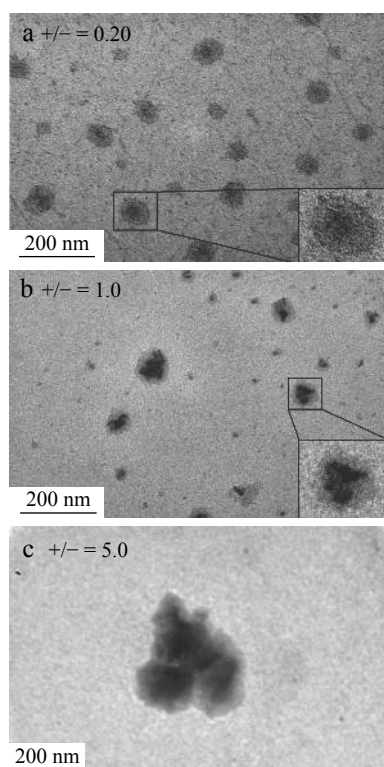


Fig. 9 TEM morphology of the complexes (10 min after complexation) formed at different $\pm$ ratios by adding PLL to HA: (a) $\pm = 0.20$, (b) $\pm = 1.0$, (c) $\pm = 5.0$ (The inset in each panel magnifies the structures.)

possess the core-corona structure too. The difference is that the size of the hydrophilic corona is too thin to stabilize the structures.

CONCLUSIONS

The complexes formed by oppositely charged polyelectrolytes with hydrophobic backbones generally resulted in heavy aggregation and even precipitation when the long-range electrostatic repulsion is screened.^[8] However, the complex formed by PLL and HA is much more stable when the amount of HA is in excess. No prominent kinetics is observed in the studied time periods. We attribute the fast kinetics of complexation to the higher water content in the complex, which serves as a “lubricant” for the redistribution of polyelectrolytes.^[30] Because of the fast kinetics and the hydrophilicity of HA, the complex forms a core-corona structure, with PLL/HA dense complex being the core and HA chain or loose PLL/HA complex being the corona. The property of the complex and the thickness of the corona can be easily tuned by varying the charge ratios. Even though the conclusions are based on HA, other biopolymers with hydrophilic backbone should yield similar results. The tunable core-corona complex structure has great potential in drug or gene delivery considering that the hydrophilic corona can be modified to fulfil targeting, increasing the circulation time, and releasing the cargo in a controlled manner. The core-corona structure formed by PLL/HA is different from those formed by block copolymers containing a charged segment and PEG.^[39,40] Even though PEG increases the circulation time of the particles in blood stream, it effectively screens the surface charge of the particle and deteriorates the targeting ability of the ligand if it exists.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-018-2166-z>.

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