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Hyaluronan/Ru(II)-cyclodextrin supramolecular assemblies for colorimetric sensor of hyaluronidase activity†

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A hyaluronidase (HAase)-induced colorimetric change was found in a solution of a supramolecular assembly constructed from hyaluronan (HA) and a cationic Ru(II)-cyclodextrin complex under laser (532 nm) irradiation. The colorimetric change would be due to the relative intensities of scattered light from the assembly and the fluorescence of the Ru(II) complex.

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Enzymes, which are a series of biomacromolecules with highly efficient catalytic activities in physiological conditions, play essential roles in the metabolism of organisms and the activities and abnormalities in the expression of enzymes are correlated with diseases.¹ Recently, new enzyme assay methods, such as supramolecular tandem assays² and colorimetric detection based on aggregation of gold nanoparticles,³ have been developed and applied in qualitative and quantitative detection. However, applications of enzyme-responsive supramolecular assembly (ERSA) in the detection of enzyme activity are still rare despite the fact that ERSA has found significant applications in drug and gene delivery.⁴

Because the level of expression of hyaluronidase (HAase) could be used to diagnose or predict the progression of cancer,⁵ chemists have tried to investigate the activity of HAase employing ERSA constructed through the macrocycle-induced aggregation of charged biomolecules (*e.g.* choline, polysaccharide, and nucleic acid).⁶ To construct an ERSA that is responsive to HAase, hyaluronan (HA), which is the native substrate of HAase⁷ and widely used to construct drug⁸ and gene⁹ delivery systems, and a Ru(II)/tris-(phenanthroline)

complex modified with cationic cyclodextrin (CD) pendants (\mathbf{RuL}_3) were employed (see Scheme 1).

Significantly, the HAase-induced disassembly of ERSA could affect the relative intensities of scattered light from ERSA⁶ and the fluorescence emission of Ru complexes,¹⁰ leading to an obvious color change in a HAase assay. It is our special interest to provide a convenient and visible sensor for the activity of enzymes in degrading polyanionic substrates based on the concept of supramolecular assembly.

The syntheses of **L** and the **RuL**₃ complex are described in ESI.[†] As shown in Fig. S6 (ESI[†]), the UV-vis spectra of **RuL**₃ exhibit obvious absorbance peaks at 266 nm and 450 nm, which are respectively assigned to ligand-to-ligand charge transfer (LLCT) and metal-to-ligand charge transfer (MLCT).¹¹ Moreover, the fluorescence spectra of **RuL**₃ (Fig. 1) display two excitation peaks at 266 nm and 450 nm, as well as an emission maximum at 600 nm. The fluorescence lifetime (see Fig. S7 and S8[†]) of **RuL**₃ ($E_x = 450$ nm and $E_m = 600$ nm) was found to be 0.22 ± 0.02 µs ($\chi^2 = 1.009$).



 $\mbox{Scheme 1}$ Structures of L and \mbox{RuL}_3 complex with imidazolium groups and CD pendants.

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Fig. 1 Excitation (red) and emission (blue and purple) fluorescence spectra of RuL_3 (2 μ M) in redistilled water at 298 K. The half-frequency peak of the excitation spectrum is marked.

The aggregation of HA induced by RuL₃ was investigated by UV-vis spectroscopy following the reported methods.^{4,6,12} On the addition of RuL₃, a solution of HA displayed an obvious Tyndall effect at a concentration lower than its critical aggregation concentration (CAC). As shown in Fig. 2, the optical transmittance of HA at 650 nm (T_{650} %) gradually decreased with the addition of RuL_3 (Fig. 2a). An inflection point at 22 μ M was observed on a plot of T_{650} % versus the concentration of RuL₃ (Fig. 2b), which referred to a CAC value induced by RuL₃. Moreover, the suitable mixing ratio between HA and RuL₃ was also investigated. On the addition of HA to a RuL₃ solution ([\mathbf{RuL}_3] = 40 μ M), T_{650} % of the HA- \mathbf{RuL}_3 solution first decreased rapidly and then increased gradually. The minimum value was found at $[HA] = 0.5 \mu M$. Therefore, the solution of the assembly was prepared with such a composition $(H_{0.5}R_{40}:[RuL_3] = 40 \ \mu M)$, $[HA] = 0.5 \mu M$ in the subsequent experiments.

To investigate the structural details of the assembly, transmission electron microscopy (TEM) and dynamic light



Fig. 2 (a) Optical transmittance of aqueous solutions containing HA (0.5 μ M) and RuL₃ (1–40 μ M) at 25 °C. (b) Dependence of T_{650} % versus the concentration of RuL₃. (c) Optical transmittance of aqueous solutions of HA at different concentrations (0.1–1.5 μ M) in the presence of RuL₃ (40 μ M) at 25 °C. (d) Dependence of T_{650} % on the concentration of HA in the presence of RuL₃ (40 μ M).

scattering (DLS) were carried out. As shown in Fig. 3, the $H_{0.5}R_{40}$ assembly existed as solid spherical particles with an average diameter of *ca*. 400 nm and the diameter measured by DLS was *ca*. 600 nm. This difference could be due to dryness in preparing the TEM sample. The zeta (ζ) potential of $H_{0.5}R_{40}$ (-11.40 mV) showed that the surface of the assembly was mainly covered by HA. According to previous reports about assemblies constructed from charged macrocycles and macromolecules,^{4,6,12} we proposed that $H_{0.5}R_{40}$ might have a "plum pudding model" structure, where cationic CDs were buried in the chains of HA to form multiple layers.

Before the investigation of the response to HAase of $H_{0.5}R_{40}$, the stability of $H_{0.5}R_{40}$ was investigated by UV-vis spectroscopy and DLS (Fig. 3c and d). The transmittances and diameters of $H_{0.5}R_{40}$ were nearly unchanged for at least 5 h, which indicates that such assemblies were sufficiently stable in an aqueous solution. The response to HAase was investigated by detecting the transmittance of $H_{0.5}R_{40}$ solution on the addition of the enzyme (see ESI, Fig. S9†). In the presence of HAase, the transmittance of $H_{0.5}R_{40}$ (*e.g.*, at 650 nm) increased gradually, which might be due to the degradation of large assemblies.⁴ In addition, a positive correlation between the concentration of HAase and the rate of increase in transmittance was also observed (see Fig. 4).

To obtain more visual information on the formation and degradation of the assembly, a green laser (532 nm) was used to investigate the HAase-induced degradation of the $H_{0.5}R_{40}$ assembly.¹³ Samples containing $H_{0.5}R_{40}$, $H_{0.5}R_{40}$ + HAase (mixed for 7 h) and RuL_3 were irradiated with the green laser. As shown in Fig. 5, an obvious color distinction was found under irradiation with the 532 nm laser: that is, $H_{0.5}R_{40}$ was green, $H_{0.5}R_{40}$ + HAase was orange-yellow, and RuL_3 was red.

To understand this color change, the fluorescence spectra of $H_{0.5}R_{40}$ in the absence/presence of HAase were obtained, and the wavelengths and intensities of the emission peaks were almost the same when the excitation wavelength was set to 532 nm (see ESI, Fig. S10†). This result implied that such a color change was not related to a change in emission intensity.



Fig. 3 TEM image (a) and DLS results (b) of $H_{0.5}R_{40}$ and time-dependent UV-vis transmittance (c) and particle diameter from DLS (d). The black bar in (a) represents 200 nm. [RuL₃] = 40 μ M, [HA] = 0.5 μ M.



Fig. 4 Time-dependent changes in transmittance of $H_{0.5}R_{40}$ in the absence (control) and presence of HAase. $[RuL_3]=40~\mu$ M, $[HA]=0.5~\mu$ M, $T=37~^\circ\text{C}.$



Fig. 5 $H_{0.5}R_{40}$ assemblies irradiated with 532 nm laser. Lane 1: $H_{0.5}R_{40}$; Lane 2: $H_{0.5}R_{40}$ + HAase (7 h); Lane 3: RuL_3 only. Conditions: $[RuL_3] = 40 \ \mu$ M, $[HA] = 0.5 \ \mu$ M, $[HAase] = 10 \ U \ mL^{-1}$.

Another possible explanation is a change in the intensity of scattered light. Without HAase, $H_{0.5}R_{40}$ displayed an intense green light, which was due to the strong Tyndall effect of $H_{0.5}R_{40}$ nanoparticles. In the presence of HAase, the degradation of HA led to the disassembly of $H_{0.5}R_{40}$ and weakening of the Tyndall effect. This proposition could be supported by the transmittance change shown in Fig. 4.

To investigate the relationship between the color change and HAase-induced degradation of $H_{0.5}R_{40}$, the dependence of the color change on time and HAase concentration was investigated. On placing a $H_{0.5}R_{40}$ solution with HAase (10 U mL⁻¹) in



Fig. 6 Time-dependent color changes of $H_{0.5}R_{40}$ solution with a green laser. Conditions: $[RuL_3] = 40~\mu$ M, $[HA] = 0.5~\mu$ M, $[HAase] = 10~U~mL^{-1}$.



Fig. 7 HAase concentration-dependent color changes of $H_{0.5}R_{40}$ solution with a green laser. Lane 1: [HAase] = 0.1 U mL⁻¹; Lane 2: [HAase] = 1 U mL⁻¹; Lane 3: [HAase] = 10 U mL⁻¹; Lane 4: RuL₃ only. Conditions: [RuL₃] = 40 μ M, [HA] = 0.5 μ M, time = 7 h.

a sealed glass bottle in a 37 °C water bath, a clear color change of a light beam was observed. As shown in Fig. 6, the color of the light beam gradually changed from green to orange-yellow. On the other hand, a comparison of $H_{0.5}R_{40}$ solutions containing HAase at different concentrations (0.1, 1, and 10 U mL⁻¹) showed that a higher concentration of HAase induced a more significant color change under the same conditions (Fig. 7). Therefore, we deduced that HAase activity could be detected by a laser colorimetric assay with a $H_{0.5}R_{40}$ assembly.

Conclusions

In summary, a supramolecular assembly that was responsive to HAase was constructed from a cationic Ru(II)/CD complex and HA. The addition of HAase could induce detectable color changes in the solution of the assembly under irradiation of a green laser, which could be due to the relative intensity of scattered light and fluorescence emission. Therefore, the assembly could be used as a colorimetric sensor of HAase activity. Furthermore, other anionic macromolecules, such as specific peptides and nucleic acids, would also form assemblies with RuL_3 , and the resultant assemblies could be used as colorimetric sensors for specific enzymes.

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