

Enzymes



Construction, Enzyme Response, and Substrate Capacity of a Hyaluronan–Cyclodextrin Supramolecular Assembly

Ping Hu,^[a] Yong Chen,^[a, b] Jing-Jing Li,^[a] and Yu Liu^{*[a, b]}

Abstract: A supramolecular assembly was constructed with a cationic cyclodextrin (EICD) and native hyaluronan (HA). The cationic carboxylic ester pendants on HA support hyaluronidase (HAase)-responsive sites and the EICD supports artificial carboxylic esterase responsive sites. Substrate-binding models were investigated by using environment-sensi-

tive fluorescence probes 2-*p*-toluidino-6-naphthalenesulfonate sodium (2,6-TNS) and thioflavin T (ThT). On a HA/EICD assembly, EICD was able to bind an anionic substrate and HA and EICD constructed the cationic substrate binding site together. This assembly could be used as a sequential dual-substrate carrier.

Introduction

Responsive assemblies have gained more and more attention for their potential application in many fields including signal transduction, drug delivery systems, and bio-inspired materials.^[1] Among them, enzyme-responsive assemblies (ERAs) are regarded as a hot topic since enzymes play essential roles in biochemical processes and diseases.^[2] To construct such an assembly, the selection of enzyme–substrate pairs is an essential step. Recently, several typical enzyme–substrate pairs,^[3] including cholinesterase–alkylcholine^[4] and hyaluronidase (HAase)–hyaluronan (HA),^[5] were successfully applied in the construction of an enzyme-responsive assembly. The HAase–HA pair attracted significant attention because 1) HA, an anionic glycosaminoglycan, exists in tissues of the human body and shows low toxicity to living cells;^[6] 2) in many cancer cells, HA acceptors such as CD44 are overexpressed on the surface of cancer cells;^[7] and 3) HAase plays an essential role in the movement of cancer cells to degrade HA.^[8]

Cyclodextrin (CD), a class of cyclic oligosaccharides with seven D-glucose units linked by 1,4-glucose bonds, has the ability to bind various biological molecules.^[9] Recently, the interaction between CD derivatives and biomacromolecules was investigated widely and cationic CD derivatives such as side-chain-modified polymers,^[10] star polymers,^[11] dendrimers,^[12]

and nanoparticles^[13] were found to have significant potential for applications in the design of DNA-based artificial non-viral vectors.^[14] In a preliminary study, we prepared a series of cationic β -cyclodextrin (β -CD) DNA condensers bearing seven ester groups as artificial carboxylic ester responsive sites and they were responsive to the base or esterase in the experiment.^[15]

Since hyaluronans also form a series of anionic biomacromolecules in neutral aqueous environment like DNA, which could be condensed by cationic CD derivatives, we tried to design a series of supramolecular assemblies constructed from the polycationic β -CD derivative with seven ethyl imidazolium–acetyl pendants (EICD) bearing cleavable ester linkages, which are stable in acidic or neutral environments but could be hydrolyzed in the presence of NaOH and the negatively charged HA (Scheme 1). Through formation of a hybrid of native HA response sites and artificial esterase response sites the resultant supramolecular assemblies exhibited multiple enzyme-responsive assembly/disassembly behaviors. Through the combination of CD cavities and anionic charge surface of assemblies these assemblies performed the capability of uploading cationic/anionic substrates.

Results and Discussion

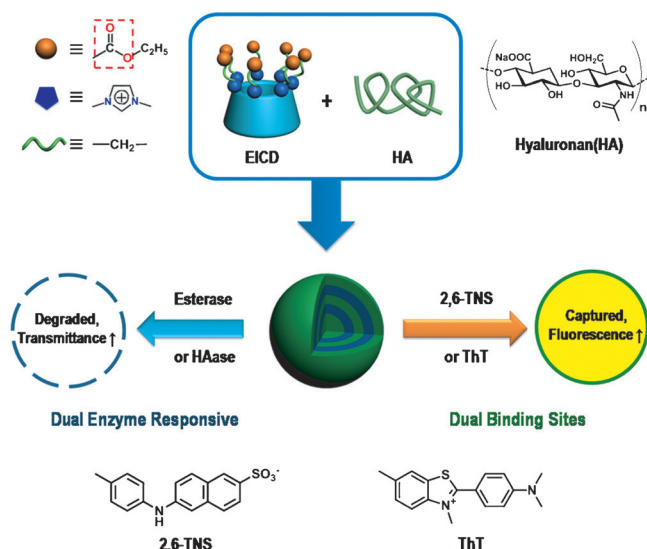
Construction of HA/EICD Assemblies

Since HA may have the tendency to self-aggregate in water, the critical aggregation concentration (CAC) of HA was measured by detecting the transmittance at various concentrations (see the Supporting Information). When the concentration of HA was increased, the optical transmittance of HA gradually decreased, which indicates the formation of aggregates in solution. An inflection point at 15 μM , assigned to the CAC, was observed on the plot of the optical transmittance of HA at 400 nm ($T_{400}\%$) versus [HA]. In addition, the transmittance of

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Scheme 1. Molecular structures of EICD and HA employed to construct the assemblies. 2,6-TNS and ThT were employed as model substrates.

EICD at the same concentration was detected, and no significant absorbance was found. Therefore, we deduced that either free EICD or free HA should exist as monomers under our experimental conditions.

At a concentration lower than the CAC of HA, a significant Tyndall effect was observed when adding EICD to a solution of HA ($0.5 \mu\text{M}$). The $T_{400}\%$ decreased gradually following the addition of EICD (Figure 1a). An inflection point at $11 \mu\text{M}$ was observed on the plot of $T_{400}\%$ versus [EICD] (Figure 1b), which was assigned to the EICD-induced CAC value. Moreover, the preferable mixing ratio between HA and EICD was also determined by gradually adding HA to a solution of EICD ($20 \mu\text{M}$). Following the addition of HA, the transmittance of the system decreased and then gradually increased as shown in Figure 1c

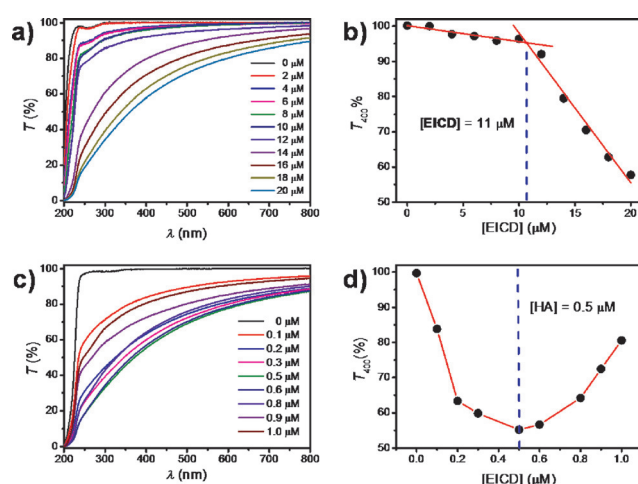


Figure 1. a) Optical transmittance of aqueous solutions containing HA ($0.5 \mu\text{M}$) and EICD ($0\text{--}20 \mu\text{M}$) at 25°C . b) Dependence of $T_{400}\%$ versus [EICD]. c) Optical transmittance of aqueous solutions of HA at different concentrations ($0\text{--}1 \mu\text{M}$) in the presence of EICD ($20 \mu\text{M}$) at 25°C . d) Dependence of $T_{400}\%$ on HA concentration in the presence of EICD ($20 \mu\text{M}$).

and d. The minimum was reached at $[\text{HA}] = 0.5 \mu\text{M}$. The decrease of the optical transmittance may indicate the formation of a large aggregate. Then the addition of an excess amount of HA led to disassembly of a large aggregate, which resulted in a decrease of the turbidity. According to these results, the preferable mixing ratio for the formation of the HA/EICD aggregate was $[\text{EICD}] = 20 \mu\text{M}$ and $[\text{HA}] = 0.5 \mu\text{M}$ ($\text{H}_{0.5}\text{E}_{20}$). Further investigations were based on this ratio.

Characterization of HA/EICD Assemblies

The conformation of $\text{H}_{0.5}\text{E}_{20}$ was investigated following the determination of the preferable mixing ratio. Since the construction of the assemblies was in an aqueous environment, dynamic light scattering (DLS) and confocal laser scanning microscopy (CLSM) were employed to investigate the behavior of $\text{H}_{0.5}\text{E}_{20}$ in the solution state. As is shown in Figure 2a, the diameter of $\text{H}_{0.5}\text{E}_{20}$ was approximately 630 nm from DLS and such a diameter value was supported by images from CLSM (Figure 2b) with an oil immersion lens and 2-*p*-toluidinylnaphthalene-6-sulfonate (2,6-TNS) was used as the fluorescence probe ($\lambda_{\text{ex}} = 405 \text{ nm}$).

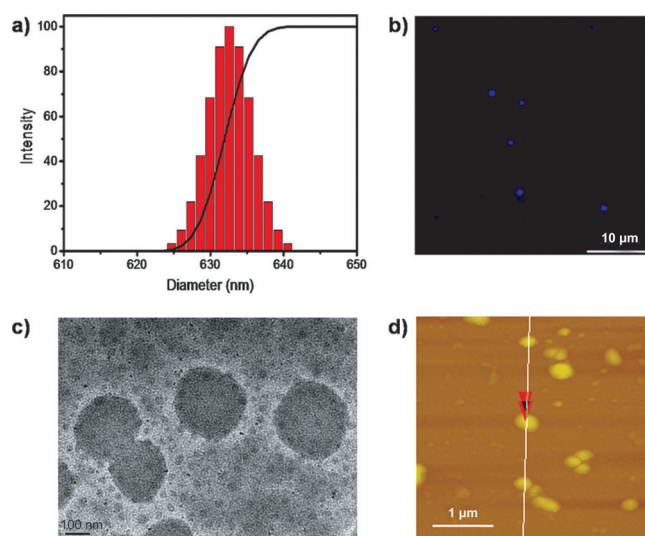


Figure 2. a) DLS and b) CLSM, c) TEM, and d) AFM images of HA/EICD.

Moreover, the $\text{H}_{0.5}\text{E}_{20}$ assemblies could be spherical solid particles. This was supported by the transmission electron microscopy (TEM) and atomic force microscopy (AFM) images. They had a smaller diameter (ca. 300 nm), which could be due to the drying process in preparing the samples. In addition, such spherical particles could be found on the combined images from CLSM (see Figure 3). $\text{H}_{0.5}\text{E}_{20}$ assemblies would have a soft core since the width/height ratio was found to be near $10:1$ from the AFM analysis (see the Supporting Information). However, there was no critical evidence to prove that such assemblies were micelles or vesicles,^[16] and thus we classified them as a kind of nanoparticle. Unexpectedly, although the $\text{H}_{0.5}\text{E}_{20}$ assemblies were constructed by cationic EICD and anionic HA,

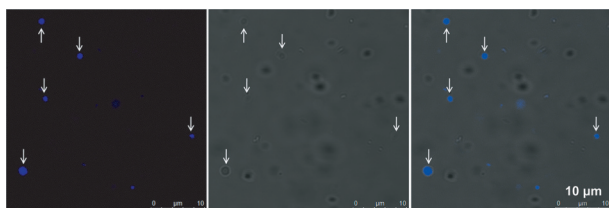


Figure 3. Fluorescence field (left), bright field (middle), and combined images (right) of HA/EICD assemblies.

their surfaces had a negative charge (-5.55 mV). Based on this and previous results, we proposed that the surface of $H_{0.5}E_{20}$ was dominated by HA and that parts of EICD would be captured inside the assemblies according to an investigation of assemblies constructed by charged macrocycles and polymers.^[7,9]

Stability of HA/EICD Assemblies

Investigations about the stability of $H_{0.5}E_{20}$ were focused on their tolerance to time and temperature. At physiological temperature (37°C), the transmittance spectra of $H_{0.5}E_{20}$ were recorded every 30 minutes. As shown in Figure 4a, no appreciable change was found over at least four hours. By using the temperature controller on the DLS equipment, the diameters of the $H_{0.5}E_{20}$ assemblies were recorded. The assembly solution in a glass cell was heated from 25 to 75°C and then cooled to 25°C again (10°C per step). Unexpectedly, the diameters were stable in the range of 600 – 700 nm in both channels (Figure 4b). According to these results, the $H_{0.5}E_{20}$ assemblies had sufficient stability for further investigation.

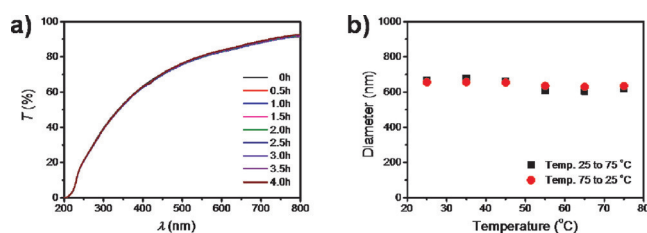


Figure 4. a) Time-dependence transmittance spectra at 37°C and b) temperature dependence (25 – 75°C) of the DLS diameter of $H_{0.5}E_{20}$ assemblies.

Enzyme Responses of HA/EICD

After verifying the structure and morphology, we started to investigate the HAase response of $H_{0.5}E_{20}$. For this, the samples with and without HAase were heated in a 37°C (310 K) water bath for six hours. As shown in Figure 5a, the addition of HAase significantly increased the transmittance of $H_{0.5}E_{20}$, which indicated the disappearance of the large aggregate. Moreover, $H_{0.5}E_{20}$ showed no appreciable Tyndall effect after the addition of HAase (Figure 5b). When HAase was replaced by inactivated HAase under the same conditions, no appreciable changes of optical transmittance, turbidity, or Tyndall

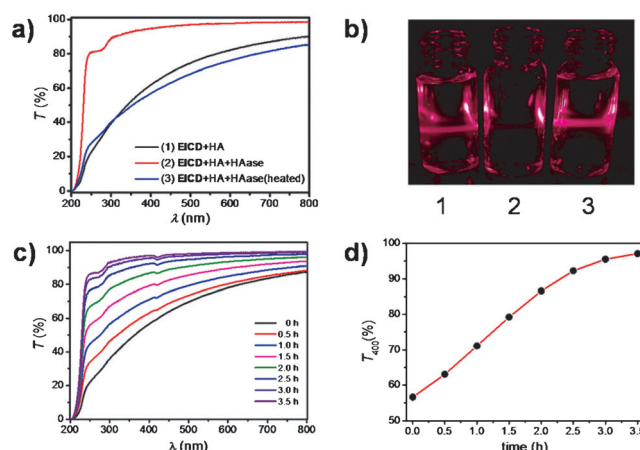


Figure 5. a) The transmittance and b) Tyndall effect of $H_{0.5}E_{20}$ with/without HAase at 37°C after 6 h. c) Transmittance spectra and d) transmittance at 400 nm of $H_{0.5}E_{20}$ with HAase from 0 to 3.5 h. Conditions: $[\text{EICD}] = 20 \mu\text{M}$, $[\text{HA}] = 0.5 \mu\text{M}$, $[\text{HAase}] = 10 \text{ U ml}^{-1}$ in water, $T = 37^\circ\text{C}$.

effect were found. In addition, no spherical aggregates were observed in TEM images after treating $H_{0.5}E_{20}$ with HAase. Furthermore, the time-dependence transmittance spectra were employed to observe the HAase degradation of $H_{0.5}E_{20}$. (Figure 5c and d). In four hours, the $T\%$ of the solution rose gradually then reached a quasi-plateau when no appreciable change of $T\%$ was found in the absence of HAase. After treatment with HAase, the zeta potential of the $H_{0.5}E_{20}$ solution changed from -5.55 to $+15.8$ mV. (In the case of inactivated HAase, the zeta potential was -5.72 mV.) Such a charge reversal could be due to the HAase-induced degradation of HA and the exposure of more EICD.

In addition to HAase, $H_{0.5}E_{20}$ also showed a detectable esterase response owing to the presence of artificial esterase response sites, the ester linkages on EICD. Acetylcholinesterase (AChE) was selected because its catalytic triad (Ser-His-Asp) was similar to that of many carboxylic esterases. As shown in Figure 6, the addition of AChE significantly, while the inactivated AChE only slightly, increased the transmittance of $H_{0.5}E_{20}$, accompanied by a greatly weakened Tyndall effect when the samples were kept in a 37°C (310 K) water bath for 24 hours. Such a less effective response could be due to the structural

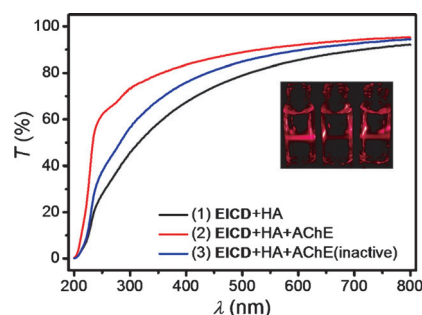


Figure 6. The transmittance and Tyndall effect (inset) of the $H_{0.5}E_{20}$ assembly with and without AChE at 37°C after 24 h. Conditions: $[\text{EICD}] = 20 \mu\text{M}$, $[\text{HA}] = 0.5 \mu\text{M}$, $[\text{AChE}] = 10 \text{ U ml}^{-1}$ in water.

difference between EICD and acetylcholine (ACh, the natural specific substrate of AChE) or the block of outer HA chains.

Model Drug Loading of HA/EICD

Since the CD cavity was able to bind various charged or neutral substrates,^[17] the ability of $H_{0.5}E_{20}$ to associate anionic guest molecules was investigated. For this, 2,6-TNS was selected as a model substrate because it barely fluoresces in water but emits strong fluorescence in a hydrophobic environment such as the CD cavity.^[18] As shown in Figure 7a, the emission

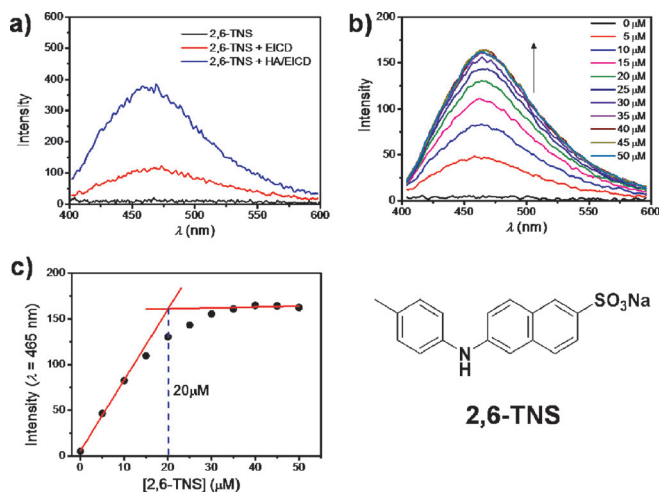


Figure 7. a) Fluorescence spectra of 2,6-TNS (10 μM) in the absence/presence of EICD and $H_{0.5}E_{20}$. b) The fluorescence spectra and c) intensity at 465 nm of the addition of 2,6-TNS (0–50 μM) to $H_{0.5}E_{20}$ solution. Conditions: [EICD] = 20 μM, [HA] = 0.5 μM, in Tris-HCl buffer (pH 7.5) 10 mM, l = 10 mm with NaCl, T = 298 K.

intensity of 2,6-TNS at 465 nm (λ_{ex} = 350 nm) significantly enhanced with the addition of $H_{0.5}E_{20}$. Moreover, the intensity of 2,6-TNS with $H_{0.5}E_{20}$ was higher than that with EICD and the maximum emission peaks did not shift significantly. These results implied that 1) the binding state between EICD and 2,6-TNS did not change significantly on addition of HA; 2) many EICD molecules were located inside the assemblies where the polarity was lower than that of water. To investigate the capacity of $H_{0.5}E_{20}$ for 2,6-TNS, a titration in which 2,6-TNS was added to $H_{0.5}E_{20}$ was carried out and monitored by using fluorescence spectra (Figure 7b,c). The emission intensity increased following the addition of ThT and then reached a quasi-plateau. The inflection point was at [2,6-TNS] = 20 μM, which supported the theory that EICD was the binding site of 2,6-TNS on $H_{0.5}E_{20}$ assemblies.

Thioflavin T (ThT), a cationic cell-permeable benzothiazole dye, was used as a model substrate for $H_{0.5}E_{20}$ assemblies. It gives a slightly negative zeta potential in water (−5.55 mV), which indicated that this assembly may have the ability to associate cationic substrates. ThT could be used as a probe to detect the existence of a hydrophilic pocket.^[19] As shown in Figure 8a, the fluorescence of ThT near 485 nm (λ_{ex} = 410 nm), which is assigned to the twisted intramolecular charge-transfer

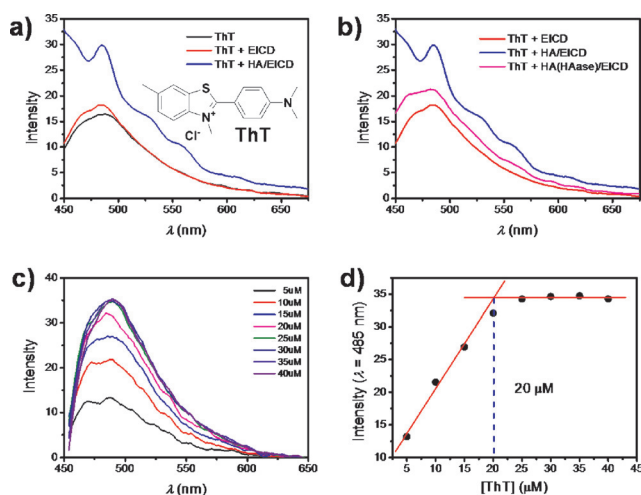


Figure 8. a) Fluorescence of ThT (5 μM) in the absence/presence of EICD, HA, and HA/EICD and b) the HAase-induced fluorescence decrease. Conditions: [EICD] = 20 μM, [HA] = 0.5 μM, [ThT] = 5 μM, [HAase] = 10 U ml^{−1}, T = 298 K in water. c) Fluorescence spectra and d) intensity at 485 nm of the addition of ThT (5–40 μM) to $H_{0.5}E_{20}$ solution.

(TICT) emission,^[20] clearly enhanced with the addition of $H_{0.5}E_{20}$, but was nearly unchanged on addition of EICD. This phenomenon indicated that ThT could be captured by the assemblies mainly through electrostatic interactions. To investigate the HAase-controlled release of ThT, two kinds of mixtures, 1) HA + EICD + ThT and 2) HA (mixed with HAase for 12 h before experiment) + EICD + ThT, were prepared to mimic the case before/after HAase degradation. As shown in Figure 8b, the TICT emission intensity of ThT decreased significantly in the case of HA, which was premixed with HAase. Such a decrease could be due to the release of ThT from the assemblies into a less crowded environment.

To further investigate the capacity of $H_{0.5}E_{20}$ for ThT, a titration in which ThT was added to $H_{0.5}E_{20}$ was carried out and monitored by using fluorescence spectra (Figure 8c,d). The fluorescence intensity increased following the addition of 2,6-TNS and then reached a quasi-plateau. Unexpectedly, the inflection point was at [ThT] = 20 μM, which was similar to the concentration of EICD in the system. Since the TICT intensity of ThT hardly increased in the presence of EICD alone, a probable explanation was that the HA and EICD constructed the ThT binding site together and the negative charge from HA plays an essential role in the capture of ThT.

Conclusion

In summary, a supramolecular assembly ($H_{0.5}E_{20}$) was constructed from biocompatible polysaccharide hyaluronan (HA) and cyclodextrin derivate EICD. Although such an assembly had a negative charge, it had the ability to associate not only the cationic substrates by means of the cooperation of HA and EICD, but also the anionic substrates by means of the EICD cavity. Moreover, this assembly could be recognized by HAase or carboxylic esterase though the combination of HA (native

substrate of HAase) and ester bonds on EICD (artificial substrate of esterase).

Interestingly, the surface charge of $H_{0.5}E_{20}$ was negative and could be reversed to positive in the presence of HAase. Since anionic particles tend to stay in the circulatory system and cationic particles tend to bind with tissues, $H_{0.5}E_{20}$ could be used as a sequential multiple-drug carrier to locate the HAase-over-expressed area and then release cationic drug and cationic EICD–drug complexes. The cationic EICD–drug complex would be absorbed by the tissue and then release the other drug. In our further investigations, such assemblies would be used as drug carriers in cell or animal experiments. Improvement of the esterase-responsive site is another direction in our investigation.

Experimental Section

Materials

All solvents and reagents were commercially available and used without further purification unless otherwise noted. Anhydrous *N,N*-dimethylformamide (DMF) was dried and distilled over CaH_2 under reduced pressure. All aqueous solutions were prepared with distilled water. EICD was synthesized according to a reported method.^[15] β -CD of reagent grade (Shanghai Reagent Factory) was recrystallized twice from water and dried under vacuum at 95 °C for 24 h prior to use. I_2 , PPh_3 , imidazole, and ethyl bromoacetate were purchased from Tianjin FuChen Chemical Reagents Factory. 2-*p*-Toluidino-6-naphthalenesulfonate sodium (2,6-TNS) was purchased from Sigma–Aldrich. Thioflavin T chloride (ThT) was purchased from Acros. Hyaluronan (MW = 100 kDa) was purchased from Shandong Freda Biopharm Co., Ltd, and the concentration of HA could be calculated based on this value. Hyaluronidase (HAase) from bovine tests (Type I-S, lyophilized powder, 400–1000 units/mg solid) was purchased from Sigma–Aldrich. Acetylcholinesterase (AChE) from *Electrophorus electricus* (Type V-S, lyophilized powder, ≥ 1000 units/mg protein) was purchased from Sigma–Aldrich.

Instruments

UV/Vis spectra were recorded in a quartz cell (light path 10 mm) on a Shimadzu UV-3600 spectrophotometer equipped with a PTC-384WI temperature controller. Fluorescence spectra were recorded in standard quartz cell and micro cells ($V = 1$ mL, 3 mm \times 10 mm) on a Cary Eclipse fluorescence spectrophotometer (Agilent). Confocal laser scanning microscopy (CLSM) images were recorded with a TCS SP8 confocal laser scanning microscope (Leica, Germany); dynamic light scattering (DLS) and zeta potential were detected on Nanobrook 173plus (Brookhaven Co. USA); atomic force microscopy (AFM) images were examined with a Nanoscope IIIa Multimode 8 AFM (Bruker); TEM images were examined with a Tecnai G2 F20 field-emission transmission electron microscope (FEI).

Determination of the critical aggregate concentration (CAC)

Stock solutions of HA (0.2 mM) and EICD (20 mM) were prepared with redistilled water separately. The original stock solutions were diluted to the needed concentration. In a general method, a series of samples (e.g., HA or EICD) with sequential concentrations were prepared, added to standard quartz cells, and stabilized for about 10 min before the transmittance spectra (200–800 nm) were recorded. The transmittance [%] of these samples at $\lambda = 400$ nm was

used to calculate the CAC following the reported method.^[4b] Unless mentioned otherwise, the temperature of the experiments was 25 °C.

DLS Measurement

The HA/EICD sample for DLS contained $[HA] = 0.5 \mu M$, $[EICD] = 20 \mu M$. The water used in this experiment was filtered by using a 0.22 μm membrane. These samples were prepared fresh with re-distilled water and stabilized for 5 min. In the experiment of the temperature response of $H_{0.5}E_{20}$, the sample in the standard glass cell was heated from 25 to 75 °C then cooled to 25 °C. (The heat/cool rate was 10 °C per step and the stabilizing time was 5 min following the recommendation of the software).

CLSM Measurement

For the convenience of observation using CLSM, the HA/EICD sample for DLS contained $[HA] = 5 \mu M$, $[EICD] = 200 \mu M$ to maintain the molar ratio of $[HA]/[EICD]$. 2,6-TNS was used as the probe (50 μM in the sample). This sample solution was dropped on a microscope glass slide and then covered by another smaller glass slide. An excess amount of liquid was absorbed by paper from the margin. An oil immersion lens on a TCS SP8 confocal laser scanning microscope was used. A purple-blue laser ($\lambda = 405$ nm) was used as the excitation light source. For significant light quenching, the time of observation must be limited to 5–10 min.

TEM Measurement

The HA/EICD sample for TEM contained $[HA] = 0.5 \mu M$, $[EICD] = 20 \mu M$. Carbon membranes for TEM were put on small pieces of filter paper separately. About 5–6 μL of solution was dropped on the carbon membrane to form half-sphere liquid drops. These samples were dried at room temperature and under moisture conditions before the TEM experiment.

AFM Measurement

The HA/EICD sample for AFM contained $[HA] = 0.5 \mu M$, $[EICD] = 20 \mu M$. The mica pieces were fixed on iron plates and the old surfaces were removed by sticky tape to expose the surface. The sample solution was dropped onto the mica piece and the excess amount of liquid was carefully removed from it by using the paper from the margin. These samples were dried at room temperature and under moisture conditions before the AFM experiment.

Stability of HA/EICD

The HA/EICD sample contained $[HA] = 0.5 \mu M$, $[EICD] = 20 \mu M$. In the stability over time experiment, this sample was added to a standard quartz cell at 37 °C. The transmittance spectra were recorded every 30 min and the total time was 4 h. The diameters of the $H_{0.5}E_{20}$ assemblies at different temperature were recorded on DLS equipment. The assembly solution in a glass cell was heated from 25 to 75 °C and then cooled to 25 °C again (10 °C per step)

HAase Response of HA/EICD Assemblies

Samples with no HAase, native HAase, and inactivated HAase were prepared. Stock solution of HAase (4000 U mL^{-1}) was prepared and the inactivated HAase solution was prepared from this stock solution. The process of inactivating HAase is described below: The stock solution of HAase was sealed in an Eppendorf tube by using polytetrafluoroethylene tape and was then put in a 90 °C water bath

for 2 h. In the experiments, three samples were prepared: 1) [EICD] = 20 μM , [HA] = 0.5 μM ; 2) [EICD] = 20 μM , [HA] = 0.5 μM , [HAase] = 10 U ml^{-1} ; 3) [EICD] = 20 μM , [HA] = 0.5 μM , [HAase] (inactivated) = 10 U ml^{-1} (this value is not the true activity of the sample here). These samples were stored in a 37 °C water bath for 6 h and were then investigated by UV/Vis spectroscopy.

The time-dependence transmittance spectra for the degradation of $\text{H}_{0.5}\text{E}_{20}$ were also recorded. The sample contained [EICD] = 20 μM , [HA] = 0.5 μM , [HAase] = 10 U ml^{-1} and it was added to a standard quartz cell at 37 °C. Transmittance spectra were recorded every 30 min and the total time was 3.5 h.

Esterase Response of the HA/EICD Assemblies

Samples with no AChE, native AChE, and inactivated AChE were prepared. Stock solution of AChE (1000 U ml^{-1}) was prepared and the inactivated AChE solution was prepared from this stock solution. The process of inactivating AChE is described below: The stock solution of HAase was sealed in an Eppendorf tube by using polytetrafluoroethylene tape and was then put in a 90 °C water bath for 2 h. In the experiments, three samples were prepared: 1) [EICD] = 20 μM , [HA] = 0.5 μM ; 2) [EICD] = 20 μM , [HA] = 0.5 μM , [AChE] = 10 U ml^{-1} ; 3) [EICD] = 20 μM , [HA] = 0.5 μM , [AChE] (inactivated) = 10 U ml^{-1} (this value is not the true activity of the sample here). These samples were stored at 37 °C in a water bath for 24 h and were then investigated by UV/Vis spectroscopy.

2,6-TNS Capacity of HA/EICD Assemblies

Since the fluorescence of 2,6-TNS can be affected by the pH of the solution, tris(hydroxymethyl)aminomethane (Tris) buffer was used in the experiment. 2,6-TNS was dissolved in redistilled water to prepare the stock solution with [2,6-TNS] = 5 mM. The fluorescence spectra of 2,6-TNS in the absence/presence of EICD and HA/EICD were recorded in 1 mL micro quartz cells at 25 °C (λ_{ex} = 350 nm, λ_{em} slit = 10 nm, λ_{em} slit = 10 nm) in Tris-HCl buffer ([Tris] = 10 mM, ion strength = 10 mM with NaCl, pH 7.5 at 25 °C). The samples are listed below: 1) [2,6-TNS] = 10 μM ; 2) [2,6-TNS] = 10 μM , [EICD] = 20 μM ; 3) [2,6-TNS] = 10 μM , [EICD] = 20 μM , [HA] = 0.5 μM .

2,6-TNS stock solution was added to the $\text{H}_{0.5}\text{E}_{20}$ buffer (3 mL) gradually (3 μL per step). In the final solution the [2,6-TNS] rose from 0 to 50 μM and the volume change of the system was not over 1%. A standard quartz cell was used (λ_{ex} = 350 nm, λ_{em} slit = 5 nm, λ_{em} slit = 5 nm).

ThT Capacity of HA/EICD Assemblies

ThT was dissolved in redistilled water to prepare a stock solution of [ThT] = 5 mM. The fluorescence spectra of ThT in the absence/presence of EICD and HA/EICD were detected in standard quartz cells (3 mL) at 25 °C (λ_{ex} = 410 nm, λ_{em} slit = 10 nm, λ_{em} slit = 10 nm). All these samples were prepared with redistilled water. The samples are listed below: 1) [ThT] = 5 μM ; 2) [ThT] = 5 μM , [EICD] = 20 μM ; 3) [ThT] = 5 μM , [EICD] = 20 μM , [HA] = 0.5 μM . In the HAase response experiment, the stock solution of HAase (4000 U ml^{-1}) was mixed with the stock solution of HA (0.2 mM) in a 1:1 volume ratio. This mixture was sealed in an Eppendorf tube by using polytetrafluoroethylene tape and stored at 37 °C in a water bath for 12 h. Then this solution was used to prepare the sample in the experiment. [ThT] = 5 μM , [EICD] = 20 μM , [HA] (premixed with HAase) = 0.5 μM . The concentration of HA here was calculated from the original concentration before adding HAase.

ThT stock solution was added to a solution of $\text{H}_{0.5}\text{E}_{20}$ (3 mL) gradually (3 μL per step). In the final solution, the [ThT] rose from 0 to

40 μM and the volume change of the system was less than 1%. Standard quartz cells were used. λ_{ex} = 410 nm, λ_{em} slit = 10 nm, λ_{em} slit = 10 nm. All of the spectra in titration were deduced from the spectrum of $\text{H}_{0.5}\text{E}_{20}$ alone.

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