A label-free and sensitive fluorescence assay for hyaluronidase activity through electrostatic-controlled quantum dots self-assembly

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Abstract
A label-free fluorescence assay for hyaluronidase (HAase) activity based on self-assembly of quantum dots is developed. A cationic polymer (polycation) can induce aggregation of the negatively charged quantum dots through electrostatic interactions and the fluorescence of the quantum dots is quenched. When the polycation is mixed with hyaluronic acid (HA), intense binding of HA to the polycation makes the quantum dots free and recovery of the fluorescence of the quantum dots is observed. However, in the presence of HAase, HA is hydrolyzed into small fragments and the polycation induces reaggregation of the quantum dots. A simple and rapid fluorescence sensor with high sensitivity and selectivity for HAase activity detection is therefore successfully established with a detection limit of 0.01 U/mL. Moreover, we have demonstrated an assay that can be applied to detect HAase activity in a complex mixture sample including 1% human serum.

Keywords
fluorescence, hyaluronic acid, hyaluronidase activity, quantum dots, self-assembly

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Introduction
Hyaluronic acid (HA) with repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units is a negatively charged linear glycosaminoglycan. It exists widely in the extracellular matrix and is closely related to various biological processes, such as cell proliferation, differentiation, and migration. As its specific enzyme, hyaluronidase (HAase) is involved in many important physiological and pathological processes. The overexpression of HAase has a relationship with many malignant tumors, such as bladder, brain, neck, and colorectal cancers. HAase is considered as a type of tumor marker. Therefore, the development of simple and sensitive detection techniques or methods for the analysis of HAase activity is of great importance and valuable for diagnosis and therapy of cancer at its early stages.

A number of techniques for HAase activity detection have been developed based on viscosimetry, turbidimetry, colorimetry, zymography, and fluorometry. Among them, fluorescence methods are more popular owing to their high sensitivity, convenience, and rapidity. Many advanced materials have been successfully used for fluorescence analysis of hyaluronidase activity, including gold nanoparticles, organic fluorescent dyes, conjugated polyelectrolytes (CPEs), quantum dots (QDs), and an upconversion luminescence material. However, these assays based on covalent labeling can be complicated, time-consuming and might affect the activity of HAase. Recently, some label-free fluorescent methods have been developed to monitor the bioactivity of HAase. For instance, Yang et al. developed a modification-free fluorescence assay for HAase detection via the electrostatic interaction between HA and positively charged fluorescent components. Liu et al. have established a label-free fluorescence-sensing platform for HAase activity detection based on the IFE (inner filter effect) between AuNPs and CS-Au/AgNCs. Compared with these HAase detection methods using fluorophore-labeled HA, the label-free assay...
is simple and flexible. Thus, the development of simple and
label-free fluorescent HAase assays with high stability,
selectivity, and in particular, sensitivity, is still needed for
early diagnosis of diseases.

Quasi-zero-dimensional nano-sized particles with quantum
confinement effects are well known as quantum dots.
Due to their high fluorescence quantum yields, high photostability, and excellent color tunability, QDs have been
widely used for sensing various biomarkers such as DNA,
aptamers, antibodies, and specific binding proteins.44
However, QD-based methods, especially methods based on
FRET ( Förster resonance energy transfer), require complicated and expensive labeled peptides or antibodies and a
modification of the QDs. Recently, some label-free fluorescent sensors based on QDs were developed. For instance,
Xu et al.45 developed the label-free fluorescent detection of
protein kinase activity based on the aggregation behavior of
unmodified quantum dots. Hu et al.46 established a label-
free fluorescence method for the detection of alkaline phos-
phatase (ALP) based on nucleic acid controlled aggregation
of QDs.

Here, we report a label-free hyaluronidase activity assay
based on electrostatic-controlled self-assembly of CdTe
QDs. Our method is simple, fast, and highly sensitive. Our
design principle is illustrated in Scheme 1. It can be
explained as follows: (1) the polycation 1 (Figure 1) can
induce aggregation of the negatively charged CdTe QDs
through electrostatic attractions, which results in an obvi-
ous fluorescence quenching of the CdTe QDs. (2) When
HA was mixed with polycation 1, intense binding of the HA
to the polycation 1 releases the CdTe QDs. Recovery of the
fluorescence of the CdTe QDs is then observed. (3) In the
presence of HAase, HA is degraded into fragments and the
polycation 1 can induce CdTe QDs reaggregation. The flu-
oforescence signal change of the CdTe QDs was found to be
related to the concentration of the HAase. A simple, label-
free, and sensitive fluorometric method for HAase activity
was therefore established.

**Results and discussion**

**Polycation-induced CdTe QDs fluorescence quenching**

The quantum dots were prepared according to the literature
description.47 In a buffer solution (5 mM Tris-HCl, pH = 7.4),
the CdTe QDs emit intense blue luminescence and the maxi-
mum fluorescence emission is at 534 nm (Figure 2).

Polycation 1 induced aggregation of CdTe QDs. Figure
3(a) shows that the CdTe QDs emission intensity decreased
gradually on increasing the polycation 1 concentration. As
reported previously,48,49 the aggregation of QDs caused the
red-shift and quenching of the QDs’ fluorescence by elec-
tronic energy transfer between neighboring dots in the QDs.
aggregate. Figure 3(b) shows that the quenching efficiency (QE) of the QDs increased with the concentration of polycation 1 (0–0.4 µM). Further increasing the polycation 1 concentration caused hardly any change of fluorescence QE, which indicated that the binding of the QDs to the polycation 1 was gradually saturated. This result indicates that polycation 1 could induce aggregation of the QDs and the monomer fluorescence of the QDs was quenched.

Disaggregation of the QDs in the presence of HA and fluorescence recovery

Figure 4 shows how the fluorescence spectrum and emission intensities of the CdTe QDs change with increasing amounts of HA, indicating that competitive binding of the negatively charged HA to polycation 1 leads to the liberation of the CdTe QDs. When 1 nM of HA was introduced, the fluorescence recovery of the CdTe QDs reached its maximum. A further increase in HA concentration caused no further increase in the fluorescence intensity, indicating that all of polycation 1 had bound to HA. The fluorescence recovery of the CdTe QDs is directly related to the amount of HA added, which forms the basis for HAase activity monitoring.

Hyaluronidase activity assay

Figure 6 shows that the CdTe QDs emission intensity gradually decreases with increasing the hyaluronidase enzymatic reaction time (0–50 min) in the presence of 0.25 U/mL HAase. This result indicates that HA is gradually degraded into fragments at an enzymatic reaction time of 50 min and the released polycation 1 could induce the reaggregation of the CdTe QDs.
with HAase concentration added to the samples at 0–0.8 U/mL. The inset shows a linear relationship between the fluorescence emission intensity and HAase concentration in the range of 0–0.1 U/mL, with a calibration curve of \( y = 136.510 - 491.32x \). The detection limit is as low as 0.01 U/mL (3σ/slope, correlation coefficient \( R^2 = 0.989 \)).

Compared with previously reported methods, our assay method is highly sensitive.

**Selectivity studies**

The selectivity of our method was studied. Several potential interfering enzymes such as collagenase, ALP, trypsin, and lysozyme were selected to perform the control experiment. Each enzyme was tested under the same experimental conditions as mentioned above. From Figure 8, none of these enzymes brought about the obvious fluorescence signal change of the QDs, which indicated that these enzymes did not interfere with our assay. Thus, our assay is highly selective for HAase.

**Assay in biological fluid**

Our assay was also tested in a complex mixture sample including 1% human serum. The assay was carried out under the same experimental conditions. Figure 9 shows that the QDs emission intensity decreased gradually with HAase concentration (0, 0.03, 0.15, 0.25, 0.4, and 0.6 U/mL, respectively). This result shows that our method could be applied in a complex biological fluid.

Figure 5. SEM images of (a) CdTe QDs, (b) CdTe QDs + polycation I, and (c) CdTe QDs + polycation I + HA.

Figure 7. (a) Emission spectra of the QDs against hyaluronidase concentration (0, 1, 5, 10, 15, 25, 50, and 80 mU/mL, respectively). (b) Plot of the QDs emission intensity at 534 nm versus hyaluronidase concentration at a reaction time of 40 min. Inset: the expanded linear region.

![Figure 5](image_url)

![Figure 6](image_url)

![Figure 7](image_url)
Conclusion

In summary, we successfully developed a sensitive, simple, and rapid fluorescence method for HAase activity detection based on the self-assembly of QDs. Polycation 1 could induce CdTe QDs aggregation and the fluorescence of the CdTe QDs was quenched. In the presence of HA, electrostatic interactions between polycation 1 and HA weakened the binding of CdTe QDs to the polycation 1 and fluorescence recovery of the QDs was observed. When HAase was added, HA was hydrolyzed into small fragments and the CdTe QDs reaggregated in the presence of polycation 1. The CdTe QD emission intensity decreased with HAase concentration. In comparison with other HAase activity assays, our assay is highly sensitive and selective. The detection limit of HAase was as low as 0.01 U/mL. The experimental procedures do not need complicated or time-consuming covalent labeling, with a simple “mix-and-detect” mode being needed. We anticipate that our assay could potentially be used in early tumor diagnosis and in biomedical fields.

Experimental section

Apparatus

A Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA) was used for the fluorescence emission spectra measurements. The excitation wavelength was 350 nm. Excitation and emission slit widths of 3 nm were selected. Quartz cuvettes with a path length of 10 mm were used for fluorescence emission measurements. A Sigma 300 field-emission electron microscope (Carl Zeiss, Jena) was used to acquire SEM images. Unless otherwise specified, all spectra were taken in 5 mM Tris-HCl buffer solution (pH = 7.4).

Materials

Cadmium(II) chloride (CdCl₂·5H₂O) was provided by Beijing Chemical Works (Beijing, China). Tellurium powder and sodium borohydride (NaBH₄) were purchased from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 3-Mercaptaoactic acid was purchased from the Sinopharm Chemical Reagent Co., Ltd. Poly(diallyldimethylammonium chloride) (polycation 1; 35 wt% in H₂O; MW < 100,000) was purchased from Sigma Aldrich (St. Louis, MO, USA). The polycation 1 concentration is defined as the concentration of the repeating unit. HA, hyaluronidase, lysozyme, and collagenase were supplied by the Sangon Biological Engineering Technology & Service Co., Ltd (Shanghai, China). Trypsin was purchased from the Beijing Dingguo Changsheng Biotech Co., Ltd (Beijing, China). ALP was supplied by the TaKaRa Biotechnology Co., Ltd (Dalian, China). Enzyme solutions were kept at 4 °C before use. The other reagents were used with no further purification and were of analytical grade. Ultrapure water (18.2 MΩ·cm) in all the experiments was prepared with a Milli-Q AE10 filtration system (Millipore, Billerica, MA, USA).

Polycation 1-induced CdTe QDs aggregation and fluorescence quenching

Different amounts of polycation 1 (0, 0.1, 0.2, 0.4, 0.7, 1, and 1.2 µM, respectively) were mixed with buffer solutions containing 4 µL of CdTe QDs (5 µM). The sample solutions were mixed briefly and fluorescence emission spectra and emission intensities at 534 nm were obtained. Total sample volume is 400 µL, buffer is 5 mM Tris-HCl, and pH is 7.4. The QE is defined as: $QE = (I_0 - I)/I_0$, in which $I$ and $I_0$ are the emission intensities of the QDs at 534 nm in the presence or absence of polycation 1, respectively.

Fluorescence recovery of the CdTe QDs in the presence of HA

Different amounts of HA and 4 µL of polycation 1 (40 µM) were left standing at room temperature for 5 min and 4 µL of CdTe QDs (5 µM) was introduced. Then, the sample solutions were mixed briefly and the fluorescence emission spectra and emission intensities of the CdTe QDs at 534 nm were obtained. Total sample volume is 400 µL, buffer is 5 mM Tris-HCl, and pH is 7.4.
Assay for hyaluronidase activity

A total volume of 200 µL of buffer solutions containing different concentrations of HAase (0, 0.01, 0.05, 0.1, 0.15, 0.25, 0.5, and 0.8 U/mL) and 4 µL of HA (100 nM) were left standing at 37 °C for 40 min and 4 µL of polycation 1 (40 µM) was introduced. Next, the solutions were left standing at room temperature for 5 min and 4 µL of the CdTe QD (40 µM) was introduced. Next, the solution was left standing at 37 °C for 40 min and 4 µL of polycation 1 (5 µM) was introduced. Finally, the sample solution was mixed briefly and the fluorescence emission spectra and emission intensities of the CdTe QDs at 534 nm were obtained. Total sample volume is 400 µL, buffer is 5 mM Tris-HCl, and pH is 7.4.

Selectivity studies

Collagenase, ALP, trypsin, lysozyme, and HAase (0.25 U/mL, each) were added to the buffer solutions containing 4 µL of HA (100 nM), respectively. The sample solutions were kept at 37 °C for 40 min and 4 µL of polycation 1 (40 µM) was introduced. Next, the solutions were left standing at room temperature for 5 min and 4 µL of CdTe QD (5 µM) was introduced. Finally, the sample solution was mixed briefly and the fluorescence emission spectra and emission intensities at 534 nm were obtained. Total sample volume is 400 µL, buffer is 5 mM Tris-HCl, and pH is 7.4.

Assay in biological fluid

A total volume of 200 µL of the buffer solutions containing different concentrations of HAase (0, 0.03, 0.15, 0.25, 0.4, and 0.6 U/mL), 4 µL of HA (100 nM), and 1% human serum were prepared. The solutions were kept at 37 °C for 40 min and 4 µL of polycation 1 (40 µM) was introduced. Next, the sample solution was left standing at room temperature for 5 min and 4 µL of CdTe QD (5 µM) was introduced. Finally, the sample solution was mixed briefly and the fluorescence emission spectra and emission intensities at 534 nm were obtained. Total sample volume is 400 µL, buffer is 5 mM Tris-HCl, and pH is 7.4.

Declaration of conflicting interests

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