Biosensors and Bioelectronics 64 (2015) 333–337

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Off–on phosphorescence assay of heparin via gold nanoclusters modulated with protamine

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ARTICLE INFO

Article history:
Received 19 July 2014
Received in revised form
9 September 2014
Accepted 11 September 2014
Available online 16 September 2014

Keywords:
Phosphorescence
Gold nanoclusters
Heparin
Protamine

ABSTRACT

A sensitive and selective phosphorescence "turn-off–on" nanosensor to detect heparin by gold nanoclusters (Au-NCs) modulated with protamine was developed. The carboxyl groups on the surface of Au-NCs can interact with amino groups in protamine via electrostatic interactions and hydrogen bonding to induce the aggregation of Au-NCs, which results in the photoluminescence to "turn-off". However, in the presence of heparin, protamine prefers to combine with heparin to release Au-NCs, which results in the photoluminescence to "turn-on". The photoluminescence lifetime measurements demonstrate that the photoluminescence belongs to phosphorescence, and that the quenching progress is dynamic. The concentration of heparin can be determined by measuring the Au-NCs phosphorescence recovery. The linear response range was obtained from 0.006 to 25.0 μg mL^{-1} with the detection limit of 6 ng mL^{-1}. Moreover, the proposed phosphorescence nanosensor was used to detect heparin in fetal bovine serum samples with satisfactory results.

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1. Introduction

Heparin discovered in 1916 (Whitelock and Iozzo, 2005), the most negatively charged linear polysaccharide formed predominantly (>70%) by a repeating unit of trisulfated disaccharide, has been widely used as a major anticoagulant with about half a billion doses applied annually (Yan and Wang, 2011). In the surgical intervention, additional heparin dosing bears the risk of hemorrhagic complications (Maurer et al., 2011). Therefore, monitoring the amount of heparin used during the surgery and the anticoagulant therapy is of crucial significance.

Many approaches have been used for the determination of heparin, such as the activated clotting time method (ACT), the electrochemical methods (Ding et al., 2010), the light scattering method (Maurer et al., 2011), the colorimetric and fluorescence methods (Hu et al., 2009; Pu and Liu, 2008; Sommers et al., 2011). To develop fluorescence method with high sensitivity, researchers have utilized different materials, such as polyadenosine–coralyne (Hung and Tseng, 2014), gold nanoparticles (Kalita et al., 2014; Vasimalai and John, 2013), $CuInS_2$ quantum dots (Liu et al., 2014), polyethyleneimine–Mn–ZnS quantum dots (Shao and Wang, 2013), organic molecules (anthracene derivatives and tetraphenylethene derivatives) (Gu et al., 2012). Although many materials have been used and many methods have been established, the development of sensitive, selective, facilely operated and reliable detection method for heparin is still great challenge.

Gold nanoclusters (Au-NCs), a new type of photoluminescent nanomaterials, have recently attracted great interest. Au-NCs are nanoparticles with a core size below 2 nm and typically contain several to tens of gold atoms. This size is comparable to the Fermi wavelength of conduction electrons. Owing to the strong quantum confinement of free electrons in this size regime, Au-NCs possess discrete electronic states and exhibit interesting molecule-like properties, including quantized charging and size-dependent fluorescence (Hu et al., 2014). Owing to their strong photoluminescence, ultrasmall size, excellent biocompatibility and photostability, and the availability of multifunctional groups for covalent linkage of diverse bioactive molecules, Au-NCs have emerged as powerful and multifunctional biomaterials for molecular biosensing and bioimaging (Liu et al., 2013).

Recently, the facile one-pot synthesis strategies of Au-NCs have been paid great attention, because this method is green and straightforward and can improve the stability, functionality and solubility of Au-NCs (Mu et al., 2013). Especially, in the one-pot method, one can control the properties of Au-NCs by using different reducing agents. The usual reducing agents used contains bovine serum albumin (BSA) (Xie et al., 2009), trypsin (Liu et al., 2013), L-proline (Mu et al., 2013), human serum (Hussain et al., 2011), glutathione (GSH) (Hu et al., 2014), polymer (Tsunoyama et al., 2005), dendrimer templates (Gröhn et al., 2000), the benzene rings

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http://dx.doi.org/10.1016/j.bios.2014.09.024
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of polystyrene derivatives (Miyamura et al., 2007), thiolates (Li and Jin, 2013), and etc. Among them, the Au-NCs capped with 11-mercaptoundecanoic acid (11-MUA) shows very stable and strong photoluminescence (Sun et al., 2013).

Protamine containing about 32 amino acid can combine with heparin by a strong non-covalent interaction (Whitelock and Iozzo, 2005), so it is used as the standard antagonist for heparin to restore normal blood coagulation. The molecular weight of a protamine is approximately 4.3 kDa (Taylor and Folkman, 1982). In its primary polypeptide sequence, the content of arginine can be up to 67%, thereby leading to a highly positively charged surface with an isoelectric point of 12–13 (Koichi SUZUKI, 1972).

Herein, a novel off–on photoluminescence assay of heparin on the basis of 11-MUA-Au-NCs modulated with protamine is presented. The nanosensor for the determine heparin is simple, sensitive and rapid. The detection process is illustrated in Scheme S1 in the supplemental information and explained as follows: (1) the Au-NCs with a large number of carboxyl groups on their surface are stable in hydroxyethyl-piperazineethane-sulfonic acid (HEPES) buffer solutions, and emit orange–red light at the exciting light of 285 nm; (2) after the addition of protamine, the photoluminescence of Au-NCs is quenched due to the aggregation, which is known as aggregation-caused quenched emission. In detail, the photoluminescence of Au-NCs capped with negative carboxyl groups becomes very weak after mixing with protamine charged positively due to the formation of aggregation via the electrostatic interactions; and (3) when heparin is introduced into the Au-NCs/protamine mixed solution, the emission of Au-NCs can be recovered. The reason is that protamine can combine with heparin by non-covalent interaction, and this interaction is much stronger than electrostatic interaction between Au-NCs and protamine. Therefore, Au-NCs are released and the photoluminescence can be recovered. According to the luminescence lifetime measurement, the quenching progress is dynamic.

2. Experimental

2.1. Reagents

Heparin (sodium salt from porcine intestinal mucosa) was obtained from Shanghai Solarbio Co. Ltd. Protamine (from salmon) and 11-MUA was purchased from Sigma-Aldrich. Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 4H_2O$) was of analytical grade and obtained from Shanghai Chemical Plant. Fetal bovine serum (FBS) was obtained from Sangon Biotech (Shanghai) Co., Ltd. All other reagents were of analytical grade and were used without further purification. All aqueous solutions and HEPES buffer were prepared with doubly distilled water.

2.2. Instruments

The fluorescence spectra were performed on a Fluoromax-4 fluorescence spectrofluorometer (Horiba, USA). The UV–vis absorption spectra were recorded by a Shimadzu UV-2450 spectrophotometer. Photographs were obtained from the Apple iPhone 4S. The photoluminescence lifetime measurements were performed on an FL-TCSPC fluorescence spectrophotometer (Horiba Jobin Yvon Inc., France).

2.3. Synthesis of Au-NCs capped by 11-MUA

The Au-NCs were synthesized according to the previous literature (Sun et al., 2013). The final concentration of Au-NCs prepared was about 30 μM.

2.4. Optimizing experimental c onditions

In order to obtain a sensitive response for the detection of heparin, the optimization of pH values of HEPES solutions and concentrations of protamine were carried out in our experiment, respectively. To optimize buffer pH values, 50 μL of Au-NCs (30 μM) was added in HEPES buffer solution (10 mM) with different pH value. The final volume of the Au-NCs was adjusted to 500 μL. The diluted Au-NCs were incubated for 15 min. Similarly, the mixture of Au-NCs (50.0 μL, 30 μM) and protamine (100 μL, 60 μg mL^{-1}), and the mixture of Au-NCs (50.0 μL, 30 μM), protamine (100 μL, 60 μg mL^{-1}) and heparin (50 μL, 120 μ g mL^{-1}) were also diluted in different pH values of HEPES buffer solutions and incubated.

To optimize the concentration of protamine, the mixture containing Au-NCs (50.0 μL, 30 μM), heparin (50 μL, 120 μ g mL^{-1}) and different concentrations of protamine was diluted with HEPES buffer solution (10 mM, pH=9) till the final volume of 500 μL and incubated for 15 min. The resulting solutions were studied by fluorescence spectra at room temperature with excitation at 285 nm, both the excitation and emission slit widths were 5 nm.

2.5. Measurement of luminescence lifetimes

The luminescence lifetimes of 11-MUA-Au-NCs, 11-MUA-Au-NCs/protamine were measured using the luminescence decay mode. The complex was excited with a flash of the xenon lamp at 265 nm, and the luminescence decay was subsequently recorded. Luminescence lifetime was calculated from the decay curves by using exponential decay function with the Origin 8.0. exe.

2.6. The detection of heparin in HEPES buffer solution

Solutions of Au-NCs (50.0 μL, 30 μM), protamine (100 μL, 60 μ g mL^{-1}), and different amounts of heparin solution were added in a microtube (1.5 mL) successively. Then the mixed solution was diluted with HEPES buffer (10 mM, pH=9) to a final volume of 500 μL and vortexed thoroughly. After incubation for 15 min, the resulting solutions were studied by fluorescence spectra at room temperature with excitation at 285 nm, both the excitation and emission slit widths were 5 nm.

2.7. The detection of heparin in FBS

The FBS samples were diluted 50 times with HEPES buffer solution before detection. The concentrations of heparin spiked in the diluted FBS samples were detected by the standard addition method.

3. Results and discussion

3.1. Characterization of Au-NCs

The Au-NCs were prepared by "one-pot" method with $HAuCl_4 \cdot 4H_2O$ and 11-MUA, thus the surface of the obtained Au-NCs is covered with carboxylate groups (Sun et al., 2013). The as-prepared Au-NCs are pale yellow in visible light, and an orange–red luminescence emission was observed under a UV light source with wavelength of 254 nm.

Absorption and emission features of the as-prepared Au-NCs were measured. There exists a series of absorption peaks centered at 231 nm, 285 nm, 356 nm, 395 nm, respectively, in the UV–vis absorption spectrum due to molecular-like properties of the Au-NCs

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