Analytical Biochemistry 393 (2009) 149–154

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Surface-enhanced Raman spectroscopy combined with atomic force microscopy for ultrasensitive detection of thrombin

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ARTICLE INFO

Article history:
Received 7 January 2009
Available online 27 June 2009

Keywords:
Thrombin
Surface-enhanced Raman spectroscopy (SERS)
Atomic force microscopy (AFM)
Gold nanoparticles

ABSTRACT

We have developed an ultrasensitive analytical method based on surface-enhanced Raman spectroscopy (SERS) exploiting a Raman probe covalently bound to gold nanoparticles. The biological marker to be detected was adsorbed on functionalized gold nanoparticles. The capture of these nanoparticles via a biorecognition process between the marker and the immobilized receptor was demonstrated by atomic force microscopy (AFM) imaging. The vibrational fingerprints of the Raman probe on the capture substrate were followed to reveal the presence of the biological marker. The method, which was applied to reveal thrombin captured on a substrate containing antithrombin and heparin, resulted in the ability to detect marker concentrations down to the picomolar (pM) level.

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Specific recognition between two biological partners is widely exploited in biosensors nowadays such as for detection of environmental pollutants, in early and clinical diagnostics, and for screening of pharmaceutical chemicals [1,2]. Once the complex between the two partners is formed, different readout tools can be used for detection; examples of commonly applied techniques include fluorescence, surface-enhanced Raman spectroscopy (SERS)[1], surface plasmon resonance, electrochemistry, and quartz crystal microbalance [3–5].

SERS, which can reach single molecule detection with a rewarding chemical specificity [6–9], offers high potentialities in early biodiagnostics if coupled with biorecognition events, as witnessed by recent pioneering research [10–14]. SERS is based on the huge enhancement of the Raman cross section of molecules when they are placed in the proximity of a nanostructured metal surface as due to the contribution of an electromagnetic (EM) and chemical effect [15–18]. In general, SERS spectra arise from a superposition of signals from a few molecules at active "hot spots" characterized by a highly enhanced Raman cross section, with the preponderant molecules at the other sites ("cold sites") giving a much lower contribution to the final spectrum [19]. Moreover, the SERS phenomenon is regulated by several factors that are difficult to control and reproduce such as the roughness of the metal surface, the distance and orientation of the Raman probe with respect to the surface, and the electronic coupling between the molecule and the metal [15]. In particular, the distance of the probe to the metal surface has been found to play a dominant role in determining the final enhancement [20] that, on the other hand, can be maximized when coupled with a charge transfer process that requires direct contact of the molecules with the metal surface [21]. Thus, it would be crucial to develop approaches able to control these factors.

By keeping these aspects in mind, we have developed an SERS-based method for ultrasensitive detection of a biomolecular marker by exercising care in controlling and optimizing the geometry of the SERS probe with respect to the metal surface, also in connection with the possibility of having a contribution from a charge transfer mechanism. In this respect, we exploited a molecule derived from fluorescein called Samsa and containing an activable thiol group able to form a covalent bond with the gold nanoparticles (NPs). Indeed, we recently showed that Samsa bound to gold NPs undergoes an electron transfer process under light illumination as well as a drastic quenching of its intrinsic high fluorescence signal at the level of a single molecule [22]. Accordingly, here Samsa was stably bound to gold NPs by exploiting the thiol group, reaching a controlled short distance between the molecule and the metal surface, to achieve a significantly enhanced Raman signal.

As a biological marker, we chose thrombin, a serine protease whose concentration level in blood has high relevance in some pathologies [23,24]. Whereas thrombin is nearly absent in blood in normal conditions, its presence—even at a very low concentration—could be indicative of coagulation abnormalities [25]. Moreover, because a variety of sensors for thrombin, using different techniques and approaches and covering a wide concentration

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[1] *Abbreviations used:* SERS, surface-enhanced Raman spectroscopy; EM, electromagnetic; NP, nanoparticle; AFM, atomic force microscopy; APTMS, (3-aminopropyl)triethoxy-silane; PBS, phosphate-buffered saline; CCD, charge-coupled device; NA, numerical aperture; NC, noncontact; S/N ratio, signal-to-noise ratio.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.06.034

range, have been developed [26–31], including our previous work [32], this protein may represent a suitable benchmark to test the capabilities of a new approach for ultrasensitive detection.

The capability of thrombin to form a complex with antithrombin, through a biorecognition process, was exploited to promote the capture of thrombin–Samsa-labeled NPs from a substrate previously functionalized with antithrombin biomolecules. Heparin was added to the substrate to stably anchor the NPs to the substrate [33]. Indeed, heparin, an anionic polysaccharide with high affinity for antithrombin, is able to induce the formation of an irreversible complex between thrombin and antithrombin. The capture of the functionalized gold NPs from the substrate was investigated by atomic force microscopy (AFM) imaging; with the percentage of gold NPs stably deposited on the substrate also being estimated.

The applied strategy, based on the enhanced SERS signal of Samsa and the biorecognition-assisted capture, allowed us to detect thrombin at a concentration in the picomolar (pM) range. Such a detection level is comparable to that we obtained recently by the same biorecognition system but a different SERS probe [32]. Indeed, in the previous work, we used a bifunctional molecule having, on one end, a tail able to bind to a gold NP and, on the other end, a diazonium moiety capable of targeting aromatic lateral chains of protein. However, the method presented here can be more easily extended to the detection of other kinds of biomolecules and does not require preliminary treatment of the Raman probe to promote a reaction with protein molecules. In this respect, it represents a good starting point for the development of ultrasensitive biosensors in early biodiagnostics. Furthermore, the combined approach, exploiting SERS and AFM imaging, might deserve some interest from the perspective of implementing innovative multisensing detection.

Materials and methods

Gold NP colloidal solution ($HAuClO_4?3H_2O$), 50 nm diameter (distribution ~ 20%), with 4.5×10^{10} particles/ml (corresponding to a concentration of 75 pM) were purchased from Ted Pella. Thrombin (34 kDa), antithrombin III (58 kDa) from human plasma, heparin sodium salt, and all other chemicals were purchased from Sigma–Aldrich.

Samsa (10 mg, A-685, Molecular Probes) was dissolved in 1 ml of 0.1 M sodium hydroxide and incubated for 15 min to remove the acetyl group protecting the thiol. The solution was then neutralized with 6 M chloridric acid and buffered with sodium phosphate at pH 7.0. The activated Samsa was then incubated with a suspension of gold NPs to promote the attachment to gold via the thiol group [34]. The formation of the S–Au bond between Samsa and gold NPs was witnessed by the disappearance of the band centered at 2550 cm^{-1} inherent in the stretching vibration of S–H when Samsa is bound to gold NPs (see the inset of Fig. 1C) [35]. Then Samsa-labeled NPs were incubated for approximately 2 h with a thrombin solution. The concentration of thrombin in the final samples was in the range of 1 pM to 10 nM, corresponding to approximately 1 to 10^4 ng/cm^2. At the lowest concentration of thrombin, a 1:1 ratio between biomolecules and gold NPs was reached. The resulting sample was then deposited on the capture substrate.

Glass substrates, previously cleaned with piranha solution (30% H_2O_2/70% H_2SO_4), were immersed in 7% (3-amino-propyl)triethoxy-silane (APTMS) to form a self-assembled monolayer [36]. Subsequently, glasses were reacted with 1% glutaraldehyde solution for 1 h at room temperature and washed thoroughly with Milli-Q water. These glutaraldehyde-treated glasses were then reacted with 60 µl of antithrombin III (10^{-6} M) in phosphate-buffered saline (PBS, 46 mM, pH 7.0) for 24 h at 0 °C. Afterward, 30 µl of heparin sodium salt (3.2 mM) in PBS (46 mM, pH 7.5) was added.

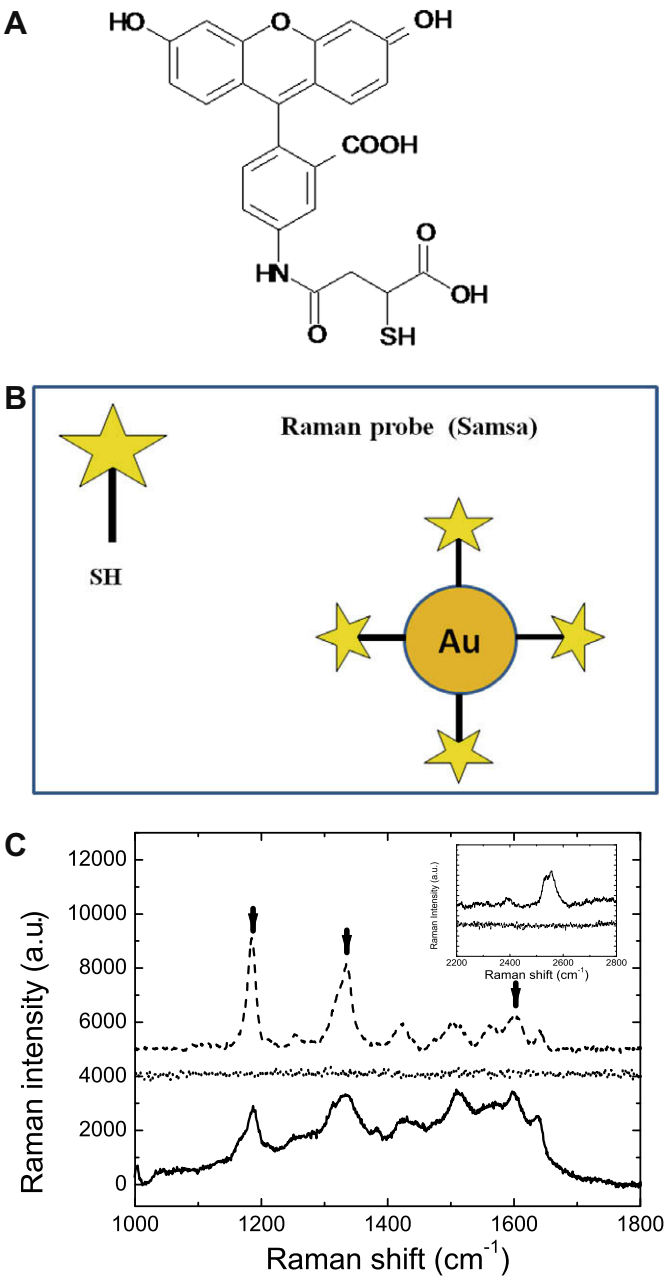

Fig. 1. (A) Chemical structure of Samsa. (B) Sketch of the labeling of gold NPs with Samsa. (C) Raman spectrum of Samsa at 10 mM (dashed line) and SERS spectrum of gold NPs labeled with Samsa at a concentration of 30 nm (continuous line). Both of the spectra were obtained from a dried drop of solution deposited on a glass with an integration time of 60 s. The arrows mark the frequencies at 1184, 1330, and 1585 cm^{-1}. a.u., arbitrary units.

The functionalized glasses were incubated overnight with 70 µl of gold NPs marked with thrombin and Samsa at 4 °C. Then the glasses were washed several times with Milli-Q water to remove excess NPs. As a control system, functionalized glasses were incubated with 70 µl of NPs marked only with Samsa and then rinsed.

SERS spectra were recorded by a Labram confocal setup (Jobin–Yvon) equipped with a charge-coupled device (CCD) Peltier-cooled detector and a single-grating spectrograph with an 1800-g/mm grating allowing a resolution of 5 cm^{-1}. The microscope objective was 100× with a numerical aperture (NA) of 0.9 producing a laser

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