Virology Nanomedicine

SERS detection of thrombin by protein recognition using functionalized gold nanoparticles

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Abstract

We present a method based on surface-enhanced Raman spectroscopy and exploiting a protein-protein recognition process able to detect thrombin at subpicomolar concentrations. Gold nanoparticles (NPs) were capped with a bifunctional molecule capable of forming a covalent link with the aromatic residues of the protein moiety. The typical vibrations of the diazo bond established between the bifunctional molecule and the target protein are found to be selectively enhanced by the conjugated gold NPs, and therefore constitutes the Raman marker. After the interaction of functionalized NPs with antithrombin as a sensitive recognition element, immobilized on a capture substrate, we have detected thrombin at a concentration of about 10^{-13} M.
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Introduction

The ability of advanced spectroscopic methods to detect extremely low concentrations of analytes, even at the single-molecule level, is opening new perspectives for early diagnostics and screening of disease treatments [1,2]. Actually, these techniques are much more promising for applications in nanomedicine than are current detection methods, which are able to reveal specific markers only above a concentration threshold, at which diseases are often significantly advanced.

Surface-enhanced Raman spectroscopy (SERS), occurring when molecules are adsorbed on nanostructured surfaces-nanoparticles (NPs) or rough electrodes-of noble metals [3,4] is one of the most powerful microanalytical techniques with single-molecule capabilities, due also to its rewarding chemical specificity [5-10]. Recently SERS has been exploited in nanodiagnostics to detect biological molecules (protein, DNA) by using Raman labels conjugated to nanoparticles [11-13].

Here we present a SERS-based approach to detect a protein (target molecule) at very low concentration by exploiting a protein-protein recognition process. We have used a bifunctional molecule having, on one side, a thiol group able to bind to a gold NP [14], and on the other side a diazonium moiety capable of reacting with electron-rich aromatic lateral chains of proteins [15,16]. The typical vibrations of the diazo bond between the bifunctional molecule and the protein were seen to be strongly enhanced by the NPs. The target protein conjugated to a NP was transferred on the capture substrate previously charged with a protein recognition element. The diazo bond vibrations, constituting the Raman marker, were followed to demonstrate the presence of a low concentration of target protein upon its interaction with the corresponding recognition element.

The performance of the method was tested by using thrombin as target protein and antithrombin III as recognition element. Thrombin is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin [17]. Antithrombin III is an essential protease that forms an irreversible complex with thrombin, in the presence of the sulfated glycosaminoglycan heparin [18].
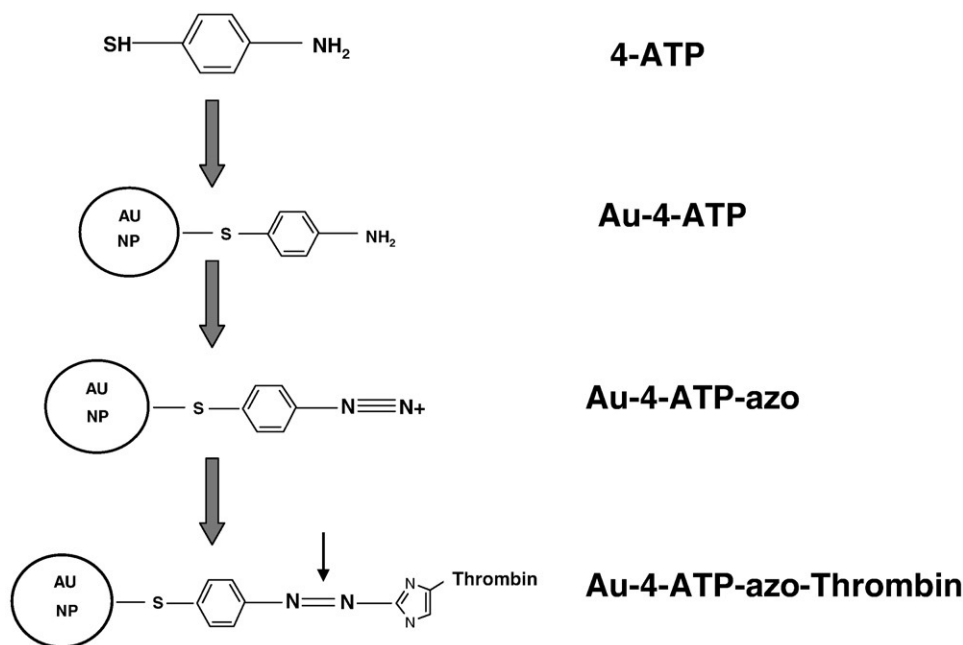

Fig 1. Principal chemical reactions in the preparation of functionalized gold NPs. The bond corresponding to the Raman marker is indicated by an arrow.

Our SERS-based approach has been demonstrated to reveal thrombin initially present in a solution at 0.5 pM. This method has the advantage when compared with others of combining a very high sensitivity with a considerable versatility and rapidity. Moreover, it can be easily extended to detect other proteins by both exploiting the suitability of the diazonium-functionalized gold NP to react with the protein aromatic lateral chains and using an appropriate recognition element. Furthermore, the method can be easily implemented into a multiplexing approach upon preparing capture substrates with the different recognition elements organized in arrays.

Materials and methods

Gold tetrachloro acid trihydride (HAuClO$_4$·3H$_2$O), with 4.5×10^{10} particles/mL of 50 nm diameter, was purchased from Ted-Pella (Reading, CA). 4-aminothiophenol (4-ATP), thrombin (34 kilodaltons) and antithrombin III (58 kilodaltons) from human plasma, heparin, sodium salt, and all other chemicals products were purchased from Sigma-Aldrich (St. Louis, MO). Water used for these experiments was purified by MilliQ Reagent water system (Millipore, Billerica, MA).

The steps followed in the preparation of the functionalized gold NPs are briefly described in Figure 1. Gold NPs coated with the bifunctional molecule 4-ATP (Au-4-ATP) were prepared by the dropwise addition of 0.01 M aqueous NaBH$_4$ solution in an equal volume of ammoniac aqueous 0.5 mM HAuClO$_4$ (pH ~7.8) and 1 mM 4-ATP in toluene under vigorous stirring [19,20]. The as-prepared Au-4-ATP NPs were separated and washed thoroughly several times with water and toluene and dried under nitrogen, followed by dispersion in dimethyl formamide. The formation of a covalent bond between 4-ATP and gold NPs was followed by Raman spectroscopy (see the next section). We have estimated that about 4×10^4 4-ATP molecules can fully cover a single gold NP [14].

To obtain the diazonium salt of Au-4-ATP (Au-4-ATP-azo), 1 mL of Au-4-ATP solution was put to react with 10^{-3} M acid solution of sodium nitrite (NaNO$_2$) at pH 4 at 0°C for 30 minutes [21,22]. The mixture was then neutralized by addition of phosphate-buffered saline (PBS 46 mM, pH 7.5). Successively, 200 µL of Au-4-ATP-azo 1 µM in a buffer solution at pH 7.5 was reacted with a thrombin solution at the required concentration (from 1 µM to 0.5 µM), and kept at 0°C for 4 hours, giving the Au-4-ATP-azo-thrombin; the formation of a diazo bond between Au-4-ATP-azo and thrombin was followed by SERS (see the next section).

To prepare the capture substrates, glasses, previously cleaned with piranha solution (30% H$_2$O$_2$/70% H$_2$SO$_4$), were immersed in 7% (3-amino-propyl)triethoxy-silane to form a self-assembled monolayer [23]. Subsequently, these glasses were reacted with 1% glutaraldehyde solution for 1 hour at room temperature (22-24°C) and washed thoroughly with MilliQ water. These treated glasses were then reacted with 60 µL of antithrombin III 10^{-6} M (PBS 46 mM, pH 7.0) for 24 hours at 0°C. Afterwards, 30 µL of heparin sodium salt 3.2 mM in PBS (46 mM, pH 7.5) were added.

To prepare the final detection system the capture substrates were incubated with 60 µL of Au-4-ATP-azo-thrombin overnight at 4°C. Then they were washed several times with MilliQ water to remove unreacted Au-4-ATP-azo-thrombin. As a control system the capture substrates were also incubated with 60 µL of Au-4-ATP for 24 hours at 0°C and then rinsed.
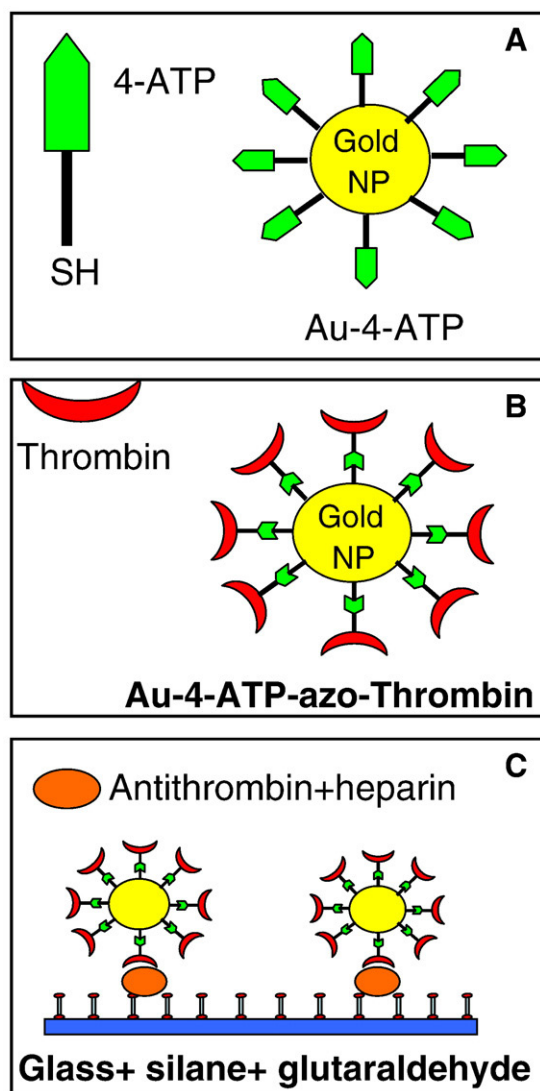
Fig 2. Schematic representation of the procedure followed for detection of thrombin by a protein-protein recognition process combined with SERS. **A**, Formation of Au-4-ATP by linking the thiol group of 4-ATP with gold NP. **B**, Conjugation of Thrombin with Au-4-ATP via a diazo-coupling reaction, **C**, Deposition of Au-4-ATP-azo-Thrombin on the capture substrate, previously incubated with antithrombin.

SERS spectra were recorded by Labram confocal setup (Jobin-Yvon, Longjumeau, Cedex, France) equipped with a charge-coupled device 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 a 100× with a numerical aperture of 0.9, producing a laser spot size of about 1 μm in diameter. The source was a HeNe ion laser (MellesGriot, Carlsbad, CA) providing a 632.8-nm radiation, with power kept below 5 mW.

Results and discussion

The approach designed for SERS detection of thrombin follows the procedure sketched in Figure 2. In the first step
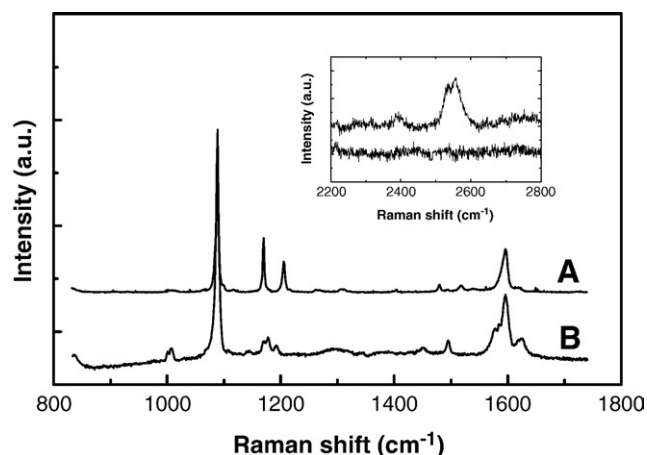

Fig 3. Raman spectrum of 4-ATP **A,** and 4-ATP conjugated to gold NPs (Au-4-ATP). **B,** Inset: high-frequency region of the spectra. All the spectra were recorded with a 30-second integration time.

the 4-ATP molecules are covalently bound to gold NPs through the thiol group to form Au-4-ATP (Figure 2, *A*). In the second step, Au-4-ATP, after the formation of the diazonium salt (Au-4-ATP-azo), is conjugated with thrombin, via a diazo-coupling reaction, by obtaining Au-4-ATP-azo-thrombin (Figure 2, *B*). In the last step the capture substrate, previously coated with silane and glutaraldehyde, and reacted with antithrombin and heparin, is incubated with the Au-4-ATP-azo-thrombin sample (Figure 2, *C*).

The formation of the different compounds formed during the outlined procedure was checked by Raman spectroscopy. The spectra of 4-ATP, before (curve *A*) and after (curve *B*) conjugation with gold NPs (Au-4-ATP), are shown in Figure 3. The formation of the S-Au covalent bond between 4-ATP and gold NP is witnessed by the disappearance of the band centered at 2550 cm^{-1}, inherent in the stretching vibration of S-H (see inset of Figure 3) [24]; for a complete assignment of the 4-ATP lines see Jiao et al [21]. Most of the peaks appearing in the 1000-1700 cm^{-1} region of 4-ATP do not show any appreciable shift in frequency when the molecule is bound to the NPs; however, some changes in their relative intensity are evident. In particular, the apparent enhancement of the modes at 1090 and 1590 cm^{-1} can be ascribed to a charge transfer between the metal and the 4-ATP molecule [24,25]. Notably, such a process is one of the mechanisms responsible for the enhancement of the Raman cross section (SERS effect) for molecules put in the proximity of a metal surface [3,4].

The Raman spectra of the sample obtained upon reacting Au-4-ATP-azo with thrombin (Au-4-ATP-azo-thrombin), through a diazo coupling (see Figure 1), are shown in Figure 4 for two different concentrations of thrombin. Both the spectra show two bands at about 1390 and 1430 cm^{-1}, which can be specifically attributed to N = N stretching vibration modes (see the arrows in Figure 4) [21,25,26]. The appearance of these vibrational features constitutes a spectroscopic fingerprint for the diazo-coupling product between
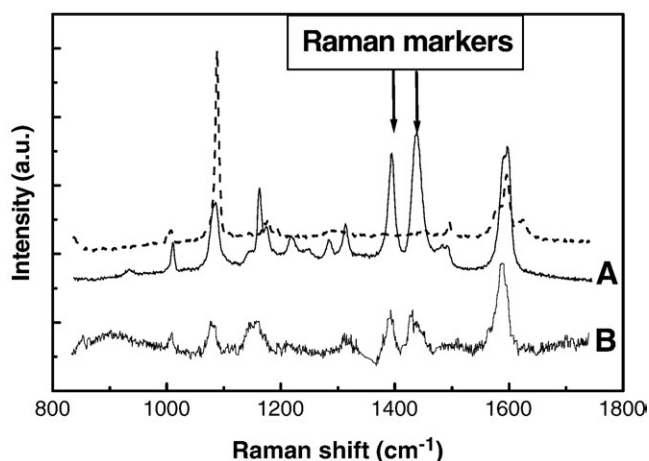
Fig 4. SERS spectra of Au-4-ATP-azo conjugated with thrombin (Au-4-ATP-azo-thrombin) at two different concentrations: **A,** 5×10^{-11} M and **B,** 5×10^{-13} M. The arrows indicate the position of the vibrational fingerprints corresponding to the diazo bond (Raman marker). The other peaks in the spectra correspond to the vibrational modes of 4-ATP, whose Raman spectrum is shown as a dashed line, and of the imidazole ring (see Jiao et al [21] and Zheng et al [25]). All the spectra were recorded with a 300-second integration time.

Fig 5. SERS spectra of the capture substrate incubated with Au-4-ATP-azo-thrombin at concentrations of **A,** 50 pM and **B,** 0.5 pM. **C,** Spectrum of the control system obtained from the substrate incubated with Au-4-ATP and then rinsed. The arrows indicate the vibrational frequencies corresponding to the diazo bond (Raman marker). For the other peaks see the legend of Figure 4. All the spectra were recorded using a 300-second integration time.

Au-4-ATP-azo and the imidazole ring of the accessible histidine residues of thrombin [15,21,22]. We assume that these bands constitute the Raman marker, and we have followed them to detect the conjugation of thrombin with the gold NPs. The other lines appearing in the spectrum of Au-4-ATP-azo-thrombin are characteristic of 4-ATP (see for comparison the dashed line in Figure 4) as well as of the imidazole ring [21,25], thus providing further evidence for the thrombin-NP conjugation.

We have also analyzed the Raman spectrum of 4-ATP-azo upon reacting with thrombin without gold NPs at the same concentration of reagents; no signal was found over the noise (not shown). This indicates that an enhancement of the Raman signals occurs for the Au-4ATP-azo-thrombin, as a result of the presence of the gold NPs.

Figure 4 also shows that the signal-to-noise (S/N) ratio, evaluated for the peaks corresponding to the Raman marker, passes from about 10 for thrombin at 5×10^{-13} M to 125 for thrombin at 5×10^{-11} M. This also suggests that the Au-4-ATP-NP-azo system can react with a greater number of thrombin molecules as long as a higher thrombin concentration is used. In this respect we note that each 4-ATP molecule on the gold NP provides a potential binding site for available histidine residues of thrombin. However, the steric factor is expected to limit the effective number of protein molecules that can be put onto a single NP. We have estimated that more than 500 protein molecules can be put onto a single 4-ATP-NP-azo particle. This means that a maximum concentration of thrombin bound to NP of about 1.5×10^{-11} M can be reached by using our functionalized gold NPs.

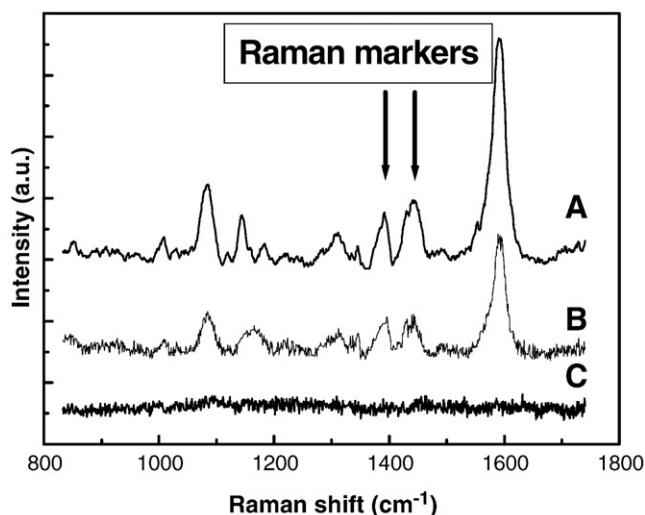For detection of thrombin, the Au-4-ATP-azo-thrombin solution was flowed onto the capture substrate prepared by

antithrombin III and heparin, and then washed several times with MilliQ water. Thus, the formation is promoted of an irreversible complex between thrombin and antithrombin III with the assistance of heparin and mediated by electrostatic bonds [27]. The substrate sample was then analyzed by scanning it under the microscope objective.

Representative SERS spectra from Au-4-ATP-azo-thrombin samples as obtained at two different concentrations of thrombin (50 pM and 0.5 pM) are shown in Figure 5. Among the Raman peaks of the Au-4-ATP-azo-thrombin sample, the characteristic vibrational fingerprints of the Raman marker (diazo bond) can be clearly detected in both the spectra of Figure 5, indicated by the arrows. The spectrum corresponding to the sample obtained by depositing Au-4-ATP molecules that have not been marked with thrombin does not reveal any Raman signal over the noise (see curve C in Figure 5). These results indicate that the method is able to detect thrombin present only on the capture substrate.

From Figure 5 we note that lowering the thrombin concentration from 50 pM to 0.5 pM decreases the S/N ratio of the spectra from about 70 to 9. This is in agreement with the observation that, at the lowest concentration of thrombin a minor number of thrombin molecules are available for interaction with the recognition partner. We note that 0.5 pM represents the lowest thrombin concentration at which a S/N ratio for the Raman marker higher than a threshold of 3 has been obtained. We have therefore assumed that such a concentration provides the detection limit of thrombin by our method.

The presented method, providing the first SERS-based approach for thrombin, has allowed us to detect such a protein at a very low concentration ($\sim 10^{-13}$ M). A variety of biosensors

have been developed to reveal thrombin in a wide concentration range, from nanomolar to picomolar, by using fluorescence, quartz microbalance, electrochemical approach, surface plasmon resonance, nanotube-based structures, and atomic force spectroscopy [28-31]; our detection value is comparable with the best results obtained by these biosensors [32].

Notably, our approach combines high sensitivity with a considerable versatility. Indeed, it can be easily adapted to reveal other proteins by using the same bifunctional molecule bound with gold NPs in connection with a capture substrate charged by the related recognition element (protein or aptamer). Furthermore, this method can be easily extended to multiplexing for simultaneous detection of different target proteins upon preparing a capture substrate functionalized with the corresponding recognition elements organized in arrays.

Acknowledgments

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