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Stereoselective and Chiroselective Surface Plasmon Resonance (SPR) Analysis of Amino Acids by Molecularly Imprinted Au-Nanoparticle Composites

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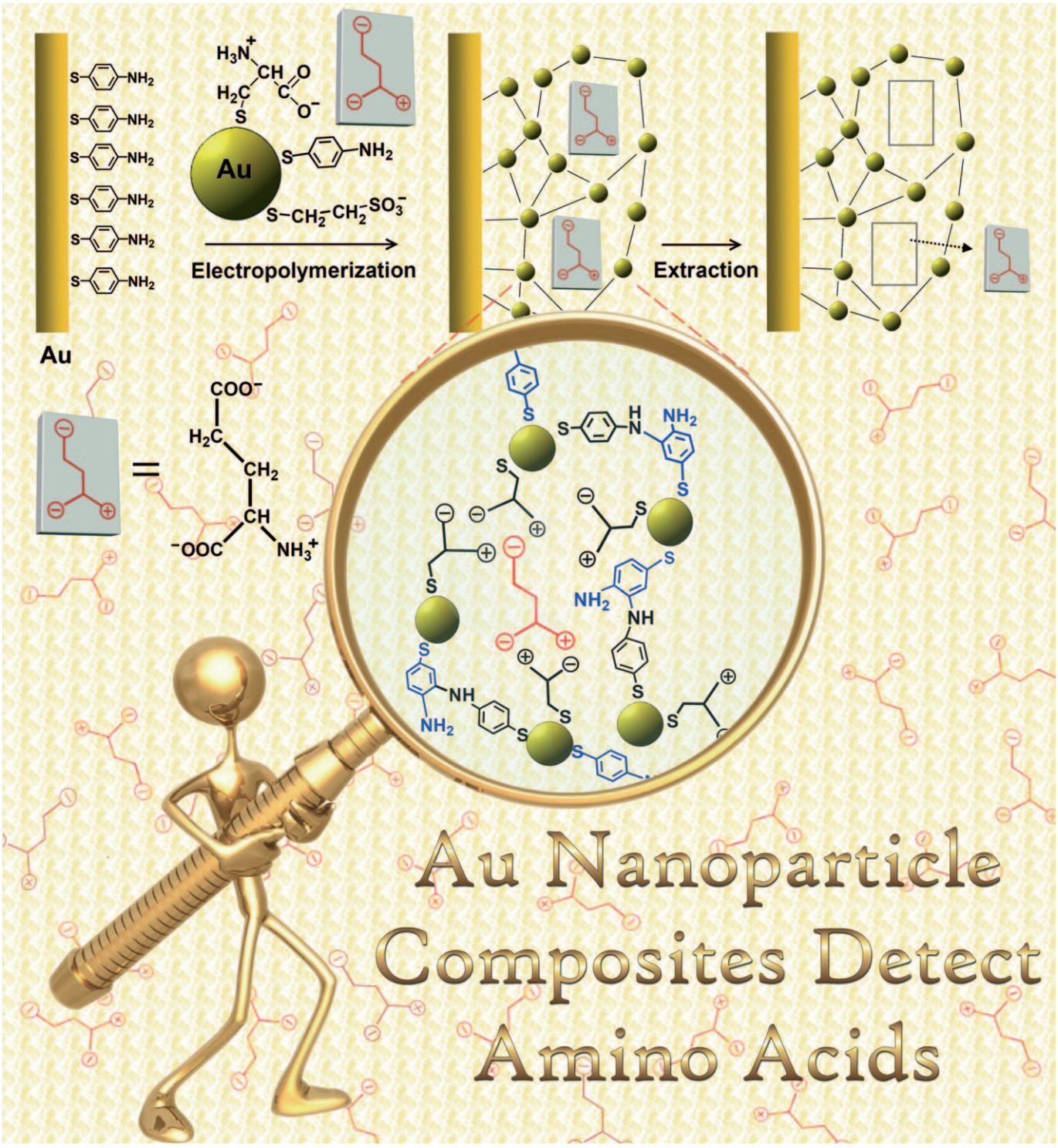

Abstract: Au nanoparticles (NPs) functionalized with thioaniline and cysteine are used to assemble bis-aniline-bridged Au-NP composites on Au surfaces using an electropolymerization process. During the polymerization of the functionalized Au NPs in the presence of different amino acids, for example, L-glutamic acid, L-aspartic acid, L-histidine, and L-phenylalanine, zwitterionic interactions between the amino acids and the cysteine units linked to the particles lead to the formation of molecularly imprinted sites in the electropolymerized Au-NP composites. Following the elimination of the template amino acid molecules, the electropolymerized matrices reveal selective recognition and binding capabilities toward the imprinted amino acid. Furthermore, by imprinting of L-glutamic or D-glutamic acids, chiroselective imprinted sites are generated in the Au-NP composites. The binding of amino acids to the imprinted recognition sites was followed by surface plasmon resonance spectroscopy. The refractive index changes occurring upon the binding of the amino acids to the imprinted sites are amplified by the coupling between the localized plasmon associated with the Au NPs and the surface plasmon wave.

Keywords: amino acids · chiroselectivity · gold · molecular imprinting · nanoparticles · surface plasmon resonance

Introduction

Amino acids play an important role in numerous metabolic and signal transduction processes. For example, among the different amino acids, glutamic acid acts as a neurotransmitter,[1] and the increase of extracellular L-glutamic acid concentrations as a result of neuronal or mental disorders was reported to cause brain damages.[2] Despite the significance of developing selective sensor systems for glutamic acid, only a few studies described the assembly of biosensors and sensors for glutamate. Several amperometric biosensors that use enzymes to transform glutamic acid into redox-active products have been reported.[3] Also, molecularly imprinted polymers have been used as matrices for the enantioselective detection of L- or D-glutamic acids; for example, imprinted polypyrrole films deposited on piezoelectric crystals have been used for the enantioselective microgravimetric detection of the optical isomers using the quartz crystal microbalance technique.[4] Similarly, the electropolymerization of *o*-phenylenediamine and dopamine, in the presence of glutamic acid as an imprint molecule, yielded an imprinted copolymer that allowed the enantioselective capacitive sensing of L- and D-glutamic acids.[5] Also, the selective functionalization of mesoporous SiO_2 enabled the specific entrapment of glutamic acid into the nanoscale pores of the silica support, and the fluorescence detection of the neurotransmitter.[6] The different sensor systems suffer, however, from insufficient sensitivity or selectivity (e.g., discrimination of the structurally related aspartic acid).

Surface plasmon resonance (SPR) spectroscopy is a versatile method to probe refractive index changes that occur on the surface of metals, such as gold, as a result of chemical events.[7] The technique was widely applied to develop SPR sensors and biosensors,[8] but its usefulness is limited by the degree of refractive index changes that occur upon the binding of the analyte and control the observable SPR changes. While the association of high-molecular-weight substrates, such as proteins, to the sensing surface is sufficient to induce refractive index changes that permit observable SPR changes at low coverage, the analysis of low-molecular-weight substrates usually lacks the necessary sensitivity. Amplified SPR sensing has been achieved by the conjugation of latex particles,[9] liposomes,[10] or proteins[11] to the recognition complex. Specifically, metallic nanoparticles (NPs), such as Au or Ag NPs, have been used as labels for amplified SPR analyses of sensing events. The coupling of the localized plasmon of the NPs with the surface plasmon wave, associated with the thin film, was found to shift the SPR energy, and, thus, to enhance the SPR changes.[12] Indeed, numerous studies have used Au NPs as amplifying labels for the SPR analysis of immuno-complexes,[13] DNA hybridization,[14] and to follow biocatalytic transformations.[15] The effects of the sizes of the Au NPs and their coverage on the resulting SPR shifts has been discussed.[16] Also, the amplified SPR detection of low-molecular-weight substrates (e.g., dopamine) was reported by the incorporation of Au NPs into a molecularly imprinted polymer matrix.[17]

Recently, we reported on the ultrasensitive SPR detection of trinitrotoluene (TNT) using a bis-aniline-crosslinked Au-NP matrix assembled on a Au surface.[18] We demonstrated that the electropolymerization of thioaniline-modified Au NPs onto a thioaniline-functionalized Au surface, in the presence of picric acid as a TNT imprint analog, yielded a molecularly imprinted Au-NP matrix for analyzing TNT. The imprinted, crosslinked, Au-NP composite revealed high affinity and selectivity for the association of TNT through π-donor–acceptor interactions and sterically constrained binding sites. This led to the ultrasensitive detection of the TNT explosive (detection limit: 1×10^{-14} M). In the present study, we extend this sensing platform to develop amplified SPR sensors for the selective analysis of amino acids, particularly, the selective and chiroselective analysis of glutamic

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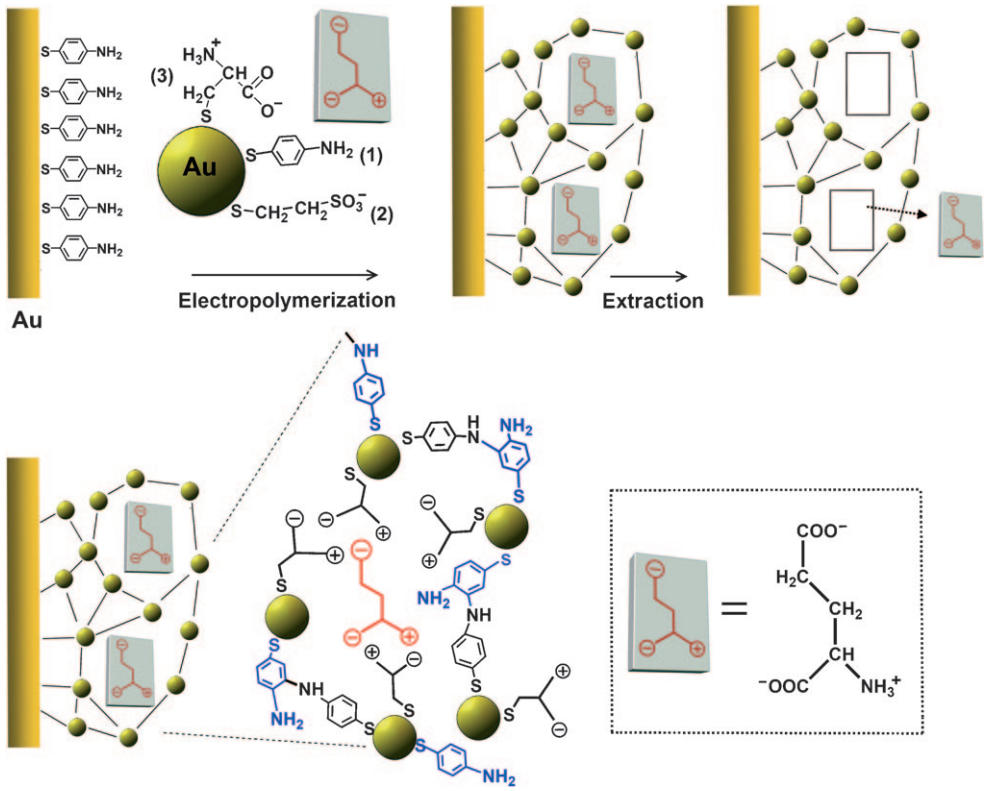
acid. We demonstrate that by the co-functionalization of the thioanline-modified Au NPs with cysteine units, functional electropolymerizable Au NPs that bind amino acids through complementary zwitterionic electrical interactions are yielded, allowing the synthesis of imprinted matrices for the different amino acids.

Results and Discussion

Au nanoparticles (NPs), ≈4.0 nm, were functionalized with a capping monolayer consisting of 4-mercaptoaniline (**1**), mercaptoethane sulfonic acid (**2**), and cysteine (**3**). While the thioaniline units act as electropolymerizable components, the mercatoethane sulfonate units enhance the stability and water solubility of the NPs, and the cysteine units provide the ligands for the recognition of amino acids by means of zwitterionic and hydrogen-bond intermolecular interactions, Scheme 1. The modified Au NPs were electropolymerized on a Au-coated glass surface in the absence or in the presence of the amino acid imprint to yield the bis-aniline-crosslinked Au-NP composite. The imprinted amino acid was washed off of the electropolymerized Au-NP composite, which resulted in the formation of specific molecular contours in the Au-NP composite, Scheme 1. The sensing matrix was prepared by the application of ten electropolymerization cycles between −0.35 and 0.8 V in the Experimental Section between −0.35 and 0.8 V versus a Ag wire, following the application of a constant potential, $E=0.8$ V versus Ag wire (QRE) for 60 min. Coulometric analysis of the redox wave associated with the bis-aniline bridging units indicated that the content of Au NPs in the electropolymerized matrices generated in the absence or presence of the imprint molecules was identical. Parallel microgravimetric quartz-crystal-microbalance experiments revealed that the content of Au NPs associated with the Au surface was approximately 2×10^{13} NPs cm^{-2}, a value that translates to about four random, densely packed, Au-NP layers. The binding of the guest amino acid to the imprinted or non-imprinted Au-NP matrices was monitored by following the reflectance changes of the surface plasmon waves due to the changes of the dielectric properties of the composite resulting from binding of the amino acid to the matrix. The changes in the dielectric properties adjacent to the NPs perturb the coupling of the localized plasmon of the NPs with the surface plasmon wave, thus leading to a shift in the SPR spectrum.

Figure 1A depicts the SPR spectrum of the L-glutamic acid-imprinted bis-aniline-crosslinked Au-NP matrix before (curve a) and after the addition of glutamic acid (20 μM, curve b). The shift in the SPR spectrum upon the addition of the amino acid is attributed to the association of the glutamic acid to its corresponding imprinted sites in the Au-NP composite (for a detailed discussion of the binding process and related control experiments, vide infra). Figure 1B depicts the sensogram corresponding to the reflectance



Scheme 1. Imprinting of glutamic acid molecular recognition sites during the electropolymerization of a bis-aniline-crosslinked Au-NP composite on a Au surface for the sensing of glutamic acid.

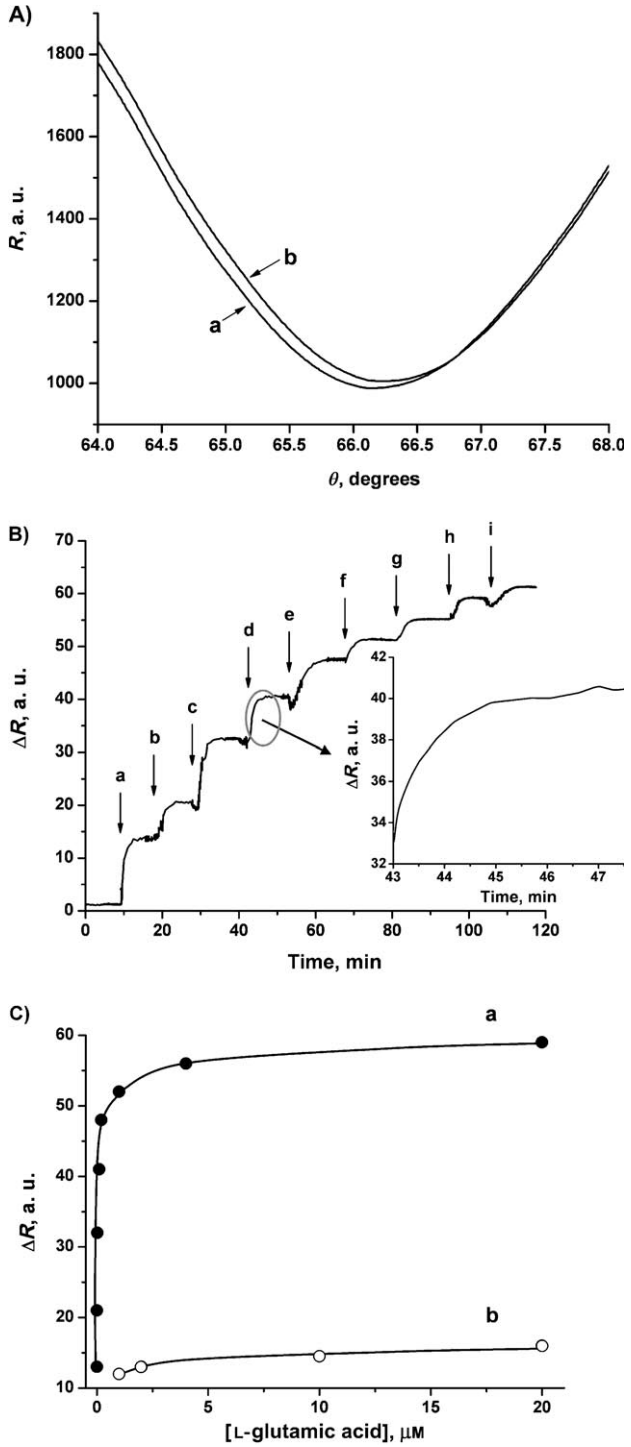


Figure 1. A) SPR curves corresponding to the L-glutamic acid-imprinted bis-aniline-crosslinked Au-NP composite: a) before the addition of L-glutamic acid and b) after the addition of L-glutamic acid, 20 μM. B) Sensogram corresponding to the changes in the reflectance intensities, at $\theta = 64.5°$, upon addition of variable concentrations of L-glutamic acid: a) 2 nM, b) 4 nM, c) 20 nM, d) 100 nM, e) 200 nM, f) 1 μM, g) 4 μM, h) 20 μM, i) 100 μM. Arrows indicate the time of addition of the analyte. Inset: enlarged time-dependent reflectance changes. C) Calibration curves relating the reflectance changes to the concentration of added L-glutamic acid on the: a) L-glutamic acid-imprinted bis-aniline-crosslinked Au-NP composite and b) non-imprinted bis-aniline-crosslinked Au-NP composite. All measurements were performed in a 0.1 M HEPES buffer solution (pH 7.2).

changes of the L-glutamic acid-imprinted Au-NP composite upon addition of variable concentrations of L-glutamic acid. The inset in Figure 1B shows the time-dependent reflectance changes upon reacting the sensing matrix with L-glutamic acid (20 μM) indicating that the response time of the surface is about 5 min. Figure 1C shows the calibration curves corresponding to the reflectance changes observed upon the analysis of L-glutamic acid by the imprinted Au NPs composite (curve a), and the non-imprinted Au-NP composite (curve b). The results clearly show that L-glutamic acid is effectively sensed by the imprinted Au-NP composite. The reflectance changes increase as the concentration of L-glutamic acid is elevated, and they level off at a glutamic acid concentration of 4 μM. The glutamic acid is sensed with a detection limit corresponding to 2 nM. In this concentration range, the non-imprinted Au-NP matrix almost does not respond to added glutamic acid. The successful detection of L-glutamic acid by the imprinted Au-NP composite is attributed to the formation of specific molecular contours (recognition sites) for the amino acid during the electropolymerization process. The imprinting process is driven by ionic interactions and hydrogen bonds between the glutamic acid imprint and the cysteine units associated with the Au NPs. Indeed, an attempt to imprint L-glutamic acid by the electropolymerization of thioaniline-functionalized Au NPs that lacked the cysteine component failed to give an active sensing surface. The association of L-glutamic acid to the imprinted sites follows a Langmuir-type binding [Eq. (1)], from which the association constant (K_{ass}) of L-glutamic acid to the imprinted sites was estimated to be $5.0\times10^{8}\,M^{-1}$ [Eq. (2); see Supporting Information]. In Equation (2) θ is the number of sites occupied by the L-glutamic acid molecules and α is the total number of binding sites.

$$\text{L-glutamic acid} + \text{imprinted sites} \rightleftharpoons \underset{(\alpha-\theta)}{\text{L-glutamic acid bound sites}} \quad \underset{(\theta)}{} \tag{1}$$

$$K_{ass} = \frac{\theta}{(\alpha-\theta)[\text{L-glutamic acid}]} \tag{2}$$

The selectivity of the resulting imprinted Au-NP composite is a major aspect to consider. Figure 2A shows the responses of the L-glutamic acid-imprinted surface upon interaction with L-glutamic acid (curve a), and upon interacting the surface with variable concentrations of L-histidine (curve b) or L-aspartic acid (curve c). Sensible reflectance changes are observed only at higher concentrations of the non-imprinted amino acids, and these level off to substantially lower saturation values. The reflectance changes in the presence of L-histidine or L-aspartic acid are attributed to a low density of imperfect imprinted sites that reveal low af-

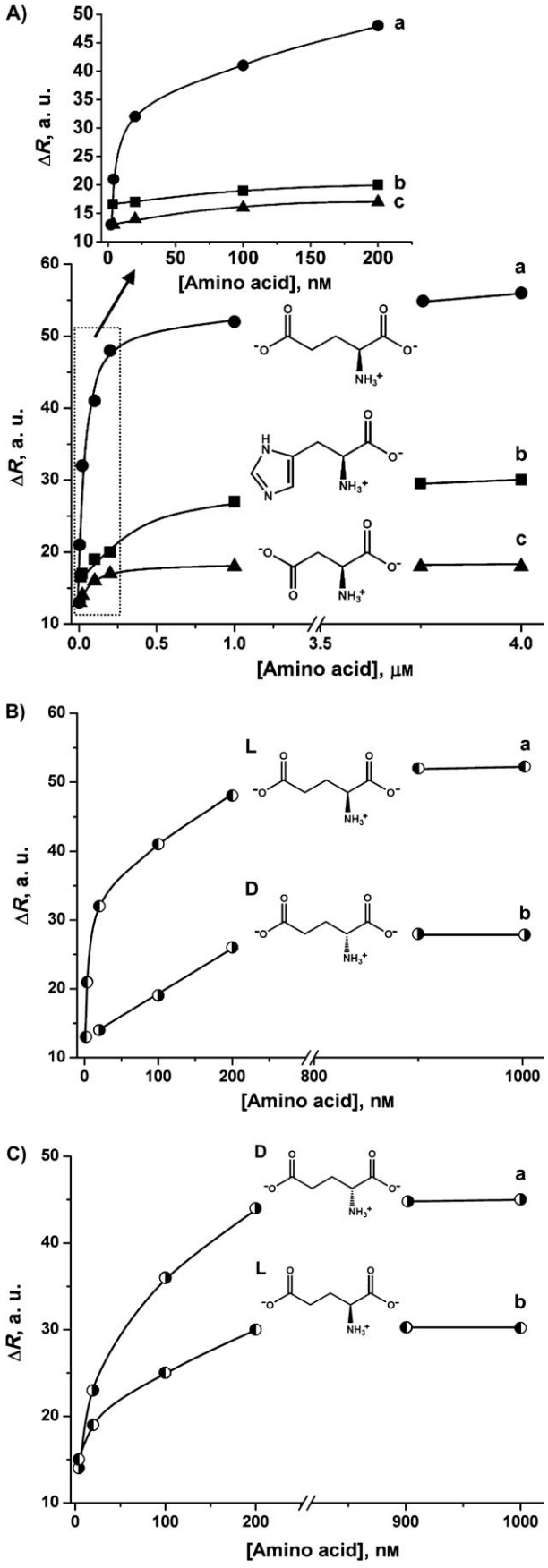


Figure 2. A) Calibration curves corresponding to the analysis of various concentrations of a) L-glutamic acid, b) L-histidine, and c) L-aspartic acid on an L-glutamic acid-imprinted bis-aniline-crosslinked Au-NP composite. The inset shows the lower concentration region of the calibration curves. B) Calibration curves corresponding to the chiroselective analysis of various concentrations of a) L-glutamic acid and b) D-glutamic acid on an L-glutamic acid-imprinted bis-aniline-crosslinked Au-NP composite. C) Calibration curves corresponding to the chiroselective analysis of various concentrations of a) D-glutamic acid and b) L-glutamic acid on a D-glutamic acid-imprinted bis-aniline-crosslinked Au-NP composite.

finity to the foreign amino acids. At high bulk concentrations of the foreign amino acids, these imperfect sites are occupied, but due to their low coverage and low association constants, the saturated reflectance changes values remain low. The results clearly demonstrate that the L-glutamic acid-imprinted Au-NP composite reveals impressive selectivity toward the sensing of the imprinted substrate. Particularly, the results indicate that the structurally related aspartic acid lacks affinity to the imprinted sites, albeit it is slightly smaller in size as compared to L-glutamic acid. However, L-aspartic acid, which is structurally similar to L-glutamic acid, reveals reflectance changes that are lower in magnitude as compared to the values observed for L-histidine (compare curves c vs. b in Figure 2A). The slightly higher reflectance changes for L-histidine are attributed to π–π interactions between the imidazole moiety and the bis-aniline bridging units. These lead to a slightly higher affinity of L-histidine to the sensing matrix.

Besides the steric selectivity for sensing the imprinted L-glutamic acid, we find that the imprinting procedure can also lead to chiroselective recognition of the imprinted amino acids. Toward this goal the L-cysteine-functionalized, thioaniline-modified, Au NPs were used to imprint either L- or D-glutamic acid. The diastereoisomeric interactions of L-cysteine/L-glutamic acid or L-cysteine/D-glutamic acid complexes are anticipated to yield molecularly imprinted sites that discriminate between the L- and D-glutamic acid enantiomers. Figure 2B (curves a and b) shows the reflectance changes of the L-glutamic acid-imprinted composite upon interaction with L- and D-glutamic acids, respectively. The reflectance changes upon interaction with the imprinted enantiomer are about two-fold higher than the reflectance changes in the presence of the D-glutamic acid. Also, the imprinted Au-NP matrix responds to lower concentrations of L-glutamic acid as compared to the D-glutamic acid. These results imply that the L-glutamic acid-imprinted composite reveals chiroselectivity, and that the binding affinity of L-glutamic acid to the imprinted sites is enhanced with respect to the binding affinity of D-glutamic acid to the imprinted sites. A Langmuir-type analysis of the respective calibration curves indicates that the association constant of L-glutamic acid to the L-glutamic acid-imprinted sites is $K_{ass}^{L/L} = 5.0 \times 10^8\,M^{-1}$, whereas the association constant of D-glutamic acid to the L-glutamic acid-imprinted sites is $K_{ass}^{D/L} = 2.5 \times 10^7\,M^{-1}$. Upon the imprinting of D-glutamic acid into the Au-NP composite, the chiroselective binding of the two enantiomers is reversed. Figure 2C (curves a and b) shows the reflectance

changes of the D-glutamic acid-imprinted matrix upon interaction with D-glutamic acid and L-glutamic acid, respectively. The sensing interface reveals enhanced reflectance changes in the presence of D-glutamic acid, implying that the D-glutamic acid-imprinted Au-NP matrix reveals higher affinity for association of this substance. Thus, we conclude that by the imprinting of the enantiomers, the chiroselective association of the imprinted amino acids is achieved.

It should be noted that the non-imprinted Au-NP composite does not reveal any structural selectivity or chiroselectivity toward the different amino acids. All three amino acids (L-glutamic acid, L-histidine, or L-aspartic acid) exhibited similar minute reflectance changes, $\Delta R = 5 \pm 2$, at concentrations lower than 500 μM. Also, the non-imprinted Au-NP composite did not show any distinguishable reflectance changes upon interaction with L- or D-glutamic acids, despite the diastereoisomeric interactions of the two enantiomers with the L-cysteine-functionalized composite. We, thus, conclude that the selective and chiroselective detection of L- or D-glutamic acids originates from the imprinting procedure and the formation of specific recognition sites for the association of the imprinted amino acids.

Indeed, the selective detection of amino acids by imprinting molecular recognition sites into the Au-NP matrix is a general method. To exemplify its versatility, we synthesized selective Au-NP sensing matrices for L-histidine and L-phenylalanine. The bis-aniline-crosslinked Au-NP composite was electropolymerized on a Au surface in the presence of L-histidine to yield the L-histidine-imprinted matrix. Figure 3A shows the reflectance changes of the composite (after washing off the imprint L-histidine) upon interaction with variable concentrations of L-histidine (curve a). For comparison, the reflectance changes of the imprinted matrix upon interaction with L-phenylalanine (curve b) and L-glutamic acid (curve c) in the same concentration range are presented. Clearly, the L-histidine-imprinted matrix reveals affinity for the binding of the imprinted substrate, while lacking binding affinities for the foreign amino acids (L-glutamic acid and L-phenylalanine). The association constant of L-histidine to the L-histidine-imprinted sites was estimated to be $3.3 \times 10^8\,\text{M}^{-1}$. Similarly, L-phenylalanine was imprinted in the Au-NP composite (Figure 3B). The resulting L-phenylalanine-imprinted matrix revealed the effective sensing of the imprinted substrate, detection limit, 1.2×10^{-8} M, whereas L-glutamic acid or L-histidine were not recognized by the L-phenylalanine-imprinted interface.

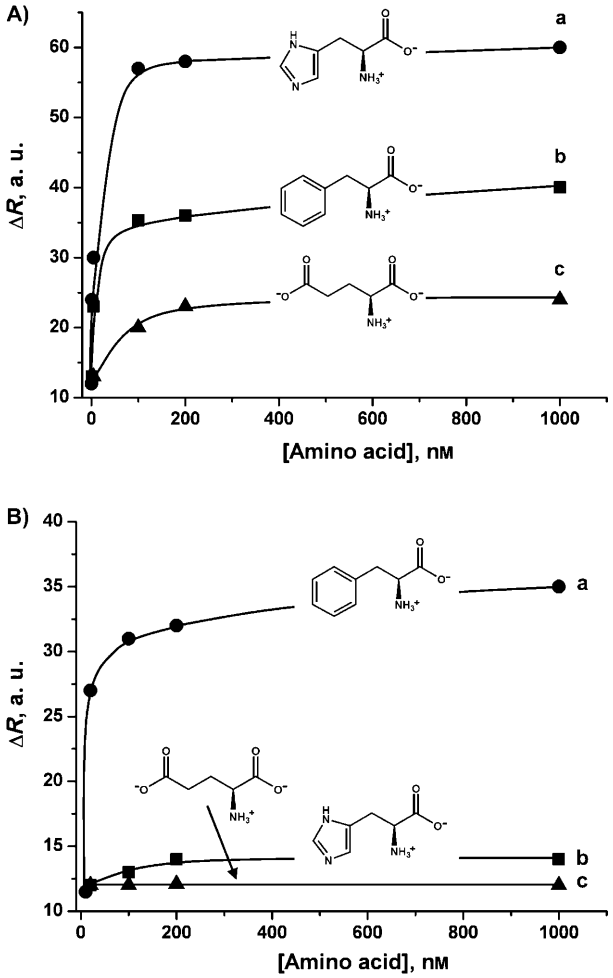


Figure 3. A) Calibration curves corresponding to the analysis of various concentrations of a) L-histidine, b) L-phenylalanine, and c) L-glutamic acid on an L-histidine-imprinted bis-aniline-crosslinked Au-NP composite. B) Calibration curves corresponding to the analysis of various concentrations of a) L-phenylalanine, b) L-histidine, and c) L-glutamic acid on a phenylalanine-imprinted bis-aniline-crosslinked Au-NP composite.

Conclusion

The present study has demonstrated the use of cysteine-functionalized, electropolymerizable, thioaniline-capped, Au NPs as active materials for the preparation of stereoselective and chiroselective matrices for the sensing of amino acids. The electrostatic and hydrogen-bond interactions between the respective amino acids and the cysteine units associated with the NPs drive the formation of the imprinted molecular recognition sites during the electropolymerization process. We also demonstrate that the changes in the dielectric properties of the Au-NP composite, upon the binding of the amino acids to the imprinted sites, are sufficient to affect the coupling between the localized plasmon of the NPs and the surface plasmonic wave, thus inducing a measurable change in the surface plasmon resonance (SPR) spectra. It should be noted that the sensing matrices used in our study were not optimized. Systematic alteration of the L-cysteine loading on the Au NPs, which may increase the concentration of the imprinted guest molecules, and the control of the number of electropolymerization cycles used to synthesize the sensing matrices, are anticipated to affect and control the sensing functions of the composites. Furthermore, the use of other amino acids as constituents of the capping layer of the NPs could also control the imprinting efficiencies. The study highlights, however, the development of a sensitive, label-free, sensing platform in which ligand-

functionalized, electropolymerizable Au NPs are used to prepare molecularly imprinted stereoselective and chiroselective sensing matrices.

Experimental Section

Synthesis of the functionalized Au nanoparticles: Au nanoparticles (Au NPs) functionalized with cysteine, 2-mercaptoethane sulfonic acid, and *p*-aminothiophenol were prepared by mixing a solution of $HAuCl_4$ (197 mg) in ethanol (10 mL) and a solution of mercaptoethane sulfonate (12 mg), L-cysteine (8 mg) and *p*-aminothiophenol (8 mg) in methanol (5 mL). Glacial acetic acid (2.5 mL) was added to this mixture, and the resulting solution was stirred in an ice bath for 1 h. Subsequently, an aqueous solution of $NaBH_4$ (1 M, 7.5 mL) was added dropwise, resulting in a dark colored solution associated with the presence of the Au NPs. The solution was stirred for 1 h in an ice bath, and then for 14 h at room temperature. The particles were successively washed and centrifuged (twice in each solvent) with methanol, ethanol, and diethyl ether. A mean particle size of about 4.0 nm was estimated using TEM measurements.

Chemical modification of the electrodes: *p*-Aminothiophenol-functionalized electrodes were prepared by immersing the Au slides for 24 h in a *p*-aminothiophenol ethanolic solution (50 mM). In order to prepare the bis-aniline-crosslinked Au-NP composite on the electrode, the surface-tethered *p*-aminothiophenol groups were electropolymerized in a 0.1 M HEPES buffer solution (pH 7.2) that contained *p*-aminothiophenol-functionalized Au NPs (2 mg mL^{-1}). The polymerization was performed by the application of ten potential cycles between −0.35 and 0.8 V versus Ag wire quasi-reference electrode, at a potential scan rate of 100 mV s^{-1}, followed by applying a fixed potential of 0.8 V for 60 min. The resulting films were then washed with the background buffer solution to exclude any residual monomer from the electrode. Similarly, amino acid-imprinted bis-aniline-crosslinked films were prepared by adding 50 mM of the selected amino acid to the Au NPs mixture prior to the electropolymerization process. The extraction of the amino acid from the film was carried out by immersing the electrodes in a 0.1 M HEPES solution, pH 7.2 for 2 h at room temperature. The removal of the amino acid from the electropolymerized film was verified by monitoring the SPR curve that reached a constant value.

Instrumentation and measurements: A surface plasmon resonance (SPR) Kretschmann-type spectrometer NanoSPR 321 (NanoSPR devices, USA), with a LED light source ($\lambda = 650$ nm) and a prism refraction index $n = 1.61$, was used. The SPR measurements were performed using a home-built fluid cell. Electropolymerization was performed using a Pt wire (diameter 0.5 mm) counter electrode and a Ag wire quasi-reference electrode ($d = 0.5$ mm), which were installed in the cell (volume 0.5 mL, working electrode area 0.2 cm^2). A PC-controlled (Autolab GPES software) electrochemical analyzer potentiostat/galvanostat (μAutolab, type III) was employed.

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