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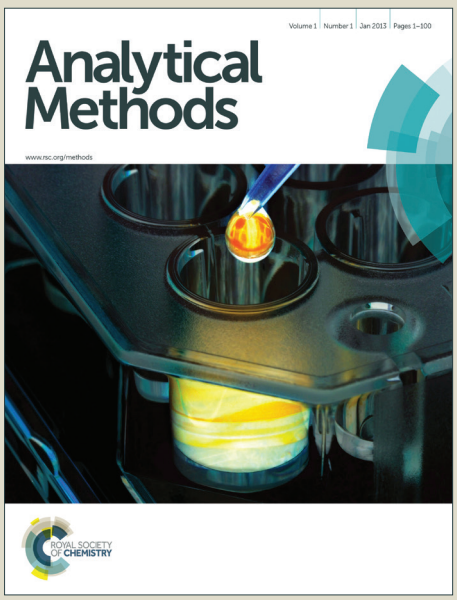

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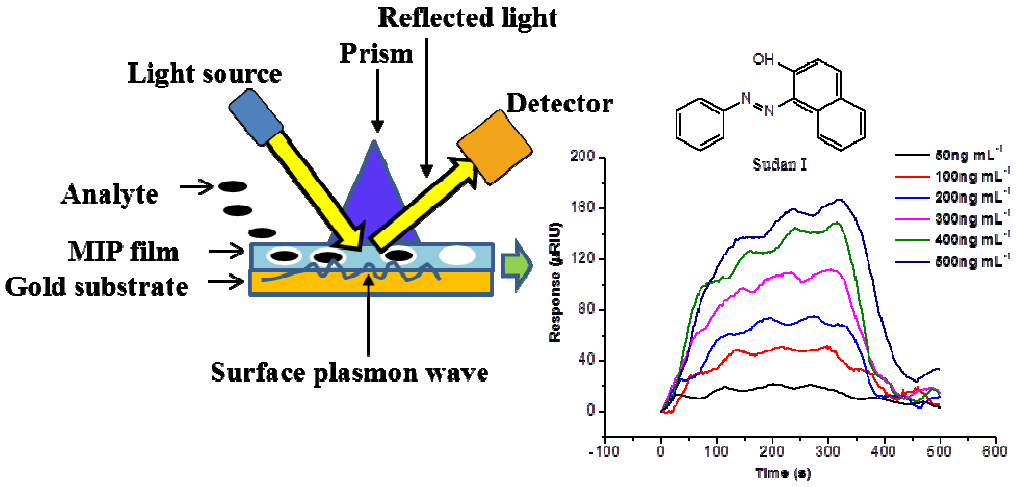
Graphical abstract



Highly sensitive and selective surface plasmon resonance sensor for detection of Sudan dyes based on molecularly imprinted nanofilm

Molecularly imprinted polymer based surface plasmon resonance sensors for Sudan dyes detection

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Abstract

The demanding task of monitoring illegal dyes in foodstuffs requires sensitive detection methods. Here, a surface plasmon resonance (SPR) sensor, combined with a 75±5-nm-thick molecularly imprinted polymer (MIP) nanofilm as recognition element, is used for the selective detection of Sudan dyes. The MIP-SPR sensor chip was fabricated by anchoring the MIP nanofilm of Sudan I on a gold chip *via* surface-initiated *in situ* polymerization. The surface morphology and thickness of the MIP films were characterized by Atomic force microscopy (AFM) and scanning electron microscopy (SEM) .The adsorption properties of the sensor chip were studied through application as an SPR sensor. The MIP-SPR sensor exhibited excellent adsorption capacity, and high sensitivity and selectivity for Sudan dyes. The SPR response was linearly proportional to the concentration of Sudan I over the range 50–400 $ng \cdot mL^{-1}$. Based on a signal-to-noise ratio of three, a detection limit of 30 $ng \cdot mL^{-1}$ was established with a 400-s response time. Non-imprinted SPR sensors were compared in terms of selectivity with MIP-SPR sensors and it was found that all the recognition coefficients (α) for Sudan I–IV were greater than 1. The MIP-SPR sensor also had reproducible response over three equilibration-adsorption-regeneration cycles.

Keywords: Molecular imprinting, Surface plasmon resonance, Sudan dyes, Surface initiated polymerization

Introduction

Sudan dyes (Sudan I, II, III and IV) are industrial azo dyes that are commonly used in waxes, inks, plastics, oils and polishes.[1] They have been classified as Category 3 carcinogens by the International Agency for Research on Cancer (IARC) because they can induce liver and bladder cancer in animal models.[2] Therefore, they are prohibited worldwide at any concentration. Unfortunately, because of their colorfastness and low price, these dyes (particularly Sudan I) have been found recently in foodstuffs such as chili powder and sauce in China and Europe.[3,4] This situation has led to an increasing demand for rapid, simple and selective methods for identification and quantification of such compounds in foodstuffs. Currently, a number of analytical methods have been proposed,[4-7] such as liquid chromatography combined with photometry[8-10] and mass spectrometry.[11-13] These methods detect low levels, but they are time consuming, they need large solvents volumes, and they generate large amounts of waste. Immunological methods, such as enzyme-linked immunosorbent assays (ELISA), have also been used for rapid screening,[14] but they need biological materials that are expensive and unstable. As a label-free, rapid and highly selective and sensitive assay technique, surface plasmon resonance (SPR) has considerable potential as an effective alternative for the detection of dyes.

SPR is an optical technique that measures changes in the refractive index of a medium near a metal surface.[15] SPR-based biosensors have attracted attention since the early 1980s,[16,17] and have been applied in a variety of areas, such as food safety, environmental contaminants, and clinical diagnosis.[18-21] Even though SPR methods perform rapid measurements in real time, they require recognition-sensing materials that are usually costly and involve unstable, natural antibodies and enzymes. Thus, numerous attempts have been made to develop artificial receptors as recognition elements in SPR analysis. Molecular imprinting, a technique for creating synthetic tailor-made

receptors capable of selectively recognizing and binding target molecules with high affinity, has become a powerful and preferred approach. Molecular-imprinted polymers (MIPs) are extensively cross-linked and synthesized in the presence of template molecules and functional monomers. Initially, the template and functional monomers form a complex. After co-polymerization, the functional groups are "frozen" in a specific position by the cross-linked polymeric network. Subsequent removal of the template molecules leads to empty cavities in the polymer structure that are complementary to the template in terms of size, shape and positioning of chemical groups.[22] MIPs have gained wide acceptance as molecular recognition materials in sensors because of their robustness, low fabrication costs and reusability, in contrast to their biological counterparts.[23] The efficacy of MIPs as artificial receptors for SPR sensors has recently been demonstrated.[24-26]

Two methods, namely, thermal radical polymerization and photo-initiated polymerization have been used to assemble an MIP film on an SPR gold chip.[27-31] Zhao *et al.*[27] reported that the surface of a gold chip was first modified with alkyl sulfhydrate, soaked in a polymerization mixture to which was added the initiator azobisisobutyronitrile (AIBN), and then irradiated with ultraviolet light (UV) at 4 °C for 2 h. In this way, an MIP-SPR sensor for the detection of nicosulfuron herbicide was developed. Hao *et al.*[28] prepared a morphine-MIP mixture that was polymerized between gold-coated (50-nm thickness) collated glass under nitrogen at 60 °C for 4 h. The MIP-film-coated SPR sensor exhibited high sensitivity and specificity to morphine. Here, we immobilized the initiator onto an alkanethiolate self-assembled monolayer on a gold substrate, which was a deviation from the above references. By immobilizing a radical initiator prior to the introduction of the prepolymerization mixture, the polymerization reaction was confined to the vicinity of the gold surface, resulting in a covalently attached MIP coating. Thus, better homogeneity of the distributed imprinted cavities could be obtained on the surface. Two methods (thermal polymerization *vs.* photo-polymerization) were compared for the synthesis of the MIP nanofilms. The best technique was chosen on the basis

of nanofilm characterization by atomic force microscopy (AFM), scanning electron microscopy (SEM), and the SPR sensor. Linearity, sensitivity, selectivity, and reproducibility of the MIP nanofilm were also evaluated with the SPR sensor.

Experimental

Materials and reagents

Ethylene glycol dimethacrylate (EGDMA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methacrylic acid (MAA) was purchased from Tianjin Damao Reagent Plant (Tianjin, China) and vacuum distilled to remove inhibitors prior to use. 2, 2-Azobisisobutyronitrile (AIBN) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Diphenylketone 1,2-diphenyldiazene, 2-naphthol and Sudan I-IV (the purities: >99%) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). The HPLC-grade acetonitrile were obtained from Merck (Darmstadt, Germany). All other reagents used were of analytical grade and obtained from Tianjin Damao Reagent Plant (Tianjin, China). Milli-Q ultrapure water (resistivity = 18.2 M Ω cm) was used in the whole experiments. All solutions were filtered through corning cellulose acetate membranes with 0.22 μm pores prior to use. The gold-coated glass SPR sensor chips (Au thickness, approximate 50 nm) were obtained from Reichert Inc. (NY, USA).

Instruments

The SPR measurements were carried out on a SR7000DC system (Reichert Inc., NY, USA). AFM imaging was performed using the MFP-3D SA instrument (Asylum Research, Santa Barbara, California, USA). SEM imaging was performed using the XL30 ESEM-TMP instrument (Philips-FEI, Amsterdam, Netherlands). Water used for making buffers and aqueous solutions was taken from a Millipore system (Millipore, Boston, Massachusetts, USA).

In situ preparation of MIP nanofilms

To clean the gold surface, the chip was first immersed in 10 mL of fresh piranha solution (3:1, 70% H_2SO_4: 30% H_2O_2) for 5 min followed by washing with copious amounts of deionized water and ethanol. Subsequently, the chip was placed in 1 mM of 1-dodecanethiol in ethanol and kept at room temperature for 24 h to form an alkanethiol self-assembled monolayer (SAM). Finally, the chip was rinsed with water and ethanol, and the gold surface was dried under nitrogen. A prepolymerization solution was prepared by dissolving Sudan I (template, 0.04 mmol) and MAA (monomer, 0.24 mmol) in 3 mL of dimethylformamide (porogen) in a test tube and agitating the solution. The test tube was then sealed at room temperature for 30 min. Ethylene glycol dimethylacrylate (cross-linker, 0.48 mmol) was added, and the mixture was degassed in an ultrasonic bath for 5 min, and then purged with nitrogen for 10 min.

For photo-initiated polymerization, the SAM gold chip was placed in 2 mL of 0.2 M benzophenone in ethanol, and kept in the dark at room temperature for 1 h. The initiator-covered chip was dried under nitrogen and immediately transferred into the prepolymerization solution, followed by irradiation with a 365-nm UV lamp for 2 h. In the case of thermal radical polymerization, the SAM gold chip was paced in 2 mL of 0.2 M AIBN in ethanol at room temperature for 1 min. The initiator-covered chip was dried under nitrogen and immediately transferred into the prepolymerization solution, and baked in a vacuum oven at 60 °C for 6 h.

After both polymerization steps, the film-coated SPR chip was washed with 5% acetic acid (v/v) in acetonitrile for 6 h to remove the templates. As a control, corresponding non-imprinted polymer (NIP) films were prepared in the same manner, without template molecules in the prepolymerization solution.

SPR experiments

A complete SR7000DC SPR system consists of a dual-channel SPR refractometer, a syringe pump (SR7500), an autosampler (SR7100), and fluidics consisting of tubing with fittings. The SR7000DC SPR sensor is a Kretschmann configuration based on angle modulation that uses a 780-nm light-emitting diode source. The SPR signal was detected with a 3700 pixel linear-array charge coupled device and the measurements were obtained using Scrubber TM software. Analyte adsorption on the sensing surface was quantified by measuring the change in SPR angle, which was converted to refractive index units (where 1 RIU = 0.073°). The SPR angle ranges from 60–80°, which corresponds to a refractive index range of 1.32–1.60 for the sensor substrate. The SPR angle resolution was less than 10^{-4} μRIU, and the sensitivity of the instrument was 0.35 μRIU. The analyte or wash solution was syringe pumped from a reservoir into the flow cell. The autosampler has a minimum injection volume of 2.5 μL.

The film-coated SPR chip was installed in the instrument so that the gold side was in contact with the flow cell. The entire SPR system was initially flushed for at least 5 min with ultrapure H_2O at a flow rate of 300 μL min^{-1}. Then, the system was injected with 0.5% sodium dodecyl sulfate (v/v), 0.1 M H_3PO_4, 0.1 M NaOH and 50 mM glycine (pH 9.5) in sequence, at a flow rate of 15 μL/min to clean tubing contamination from previous runs. Before the experiments were started, acetonitrile was used to stabilize the instrument until a stable baseline was achieved. All the experiments were carried out at 25±0.5 °C.

Results and discussion

Preparation and characterization of MIP nanofilms

The preparation of MIP films played a key role in high sensor performance. As noted above, we integrated an SAM with an MIP layer on the gold surface. The preparation involved four steps (see

Fig. 1): (i) modification with 1-dodecanethiol to form a thiol SAM on the gold surface, (ii) covalent linkage of the initiator to the SAM; (iii) formation of the Sudan I imprinted nanofilm either by thermal radical polymerization or photo-initiated polymerization; (iv) removal of the imprinted Sudan I molecules from the nanofilm.

Because SPR signals depend on the surface properties of the film, the MIP film formed should have a thickness and roughness that are suitable for the detection of Sudan I using SPR.[32] AFM was used to probe surface morphologies of the film-coated chip in a noncontact mode. Fig. 2 illustrates the topographies of a 2×2-μm area of a bare gold chip (a), an MIP-film-coated chip *via* thermal polymerization (b), and an MIP-film-coated chip *via* photo-polymerization (c). The AFM images revealed large differences in the surface roughness among the three chips. The surface of the bare gold chip was compact and smooth, while the MIP film-coated chips were covered with many protuberances, which are likely caused by imprinted cavities in the MIP films. Furthermore, the MIP film *via* thermal polymerization was much more homogeneous and was not easily removed from the gold surface, compared with the MIP film *via* photo-polymerization. When 100 ng·mL^{-1} Sudan I solution was injected onto the film-coated SPR chips, the MIP-SPR sensor *via* thermal polymerization displayed an increase in sensor response (48 μRIU), whereas a much lower signal was observed for the MIP-SPR sensor *via* photo-polymerization (28 μRIU), as seen in Fig. 3. Given these results, we used thermal polymerization to synthesize MIP films for the following work.

The thickness of the MIP film *via* thermal polymerization was characterized by SEM. An SEM image of an MIP film cross-section is displayed in Fig. 4, where the Au and MIP layer thicknesses were 52 nm and 75±5 nm, respectively. The homogeneous MIP film was suitable as a recognition element for an SPR sensor.

Kinetic studies with MIP-SPR sensor

The MIP-SPR sensor was used for real-time detection of Sudan I in acetonitrile. It was exposed to a series of Sudan I standard solutions with concentrations ranging from 50–500 ng·mL^{-1} (Fig. 5a). After each measurement, Sudan I molecules were removed from the coating by washing with pure acetonitrile for 2 min. The sensor had a fast response time of 400 s, enabling multiple measurements over short periods of time. An increase in concentration caused an increase in sensor response, which also demonstrated the presence of the molecular-imprinted layer on the gold surface. Thus many imprinted sites specific to Sudan I were created on the gold surface that non-covalently interact with Sudan I. Adsorption of the dye at the imprinted sites resulted in a change of refractive index that was detected *via* the SPR sensor. The sensor response reached a plateau at 500 ng·mL^{-1} because the accessible binding sites were saturated. A calibration plot of sensor response (μRIU) *vs.* concentration for the range 50–400 ng·mL^{-1} exhibits good linearity (R^2=0.9905, Fig. 5b). Based on a signal/noise ratio of three, the detection limit was estimated to be 30 ng·mL^{-1}.[33] Therefore, we can conclude that the MIP nanofilm is an excellent Sudan I recognition element for an SPR sensor.

Selectivity

Sudan I-IV dyes and the reference compounds 1,2-diphenyldiazene and 2-naphthol were used to characterize the selectivity of the MIP-SPR sensor; the NIP-SPR sensor was used as a comparison. As shown in Fig. 6a, the Sudan II–IV dyes were similar in shape to Sudan I and contained two of the same functional groups (-N=N-, -OH), while 1,2-diphenyldiazene and 2-naphthol were only partially similar. Fig. 6b illustrates the MIP-SPR and NIP-SPR response to each analyte at the same concentration (100 ng mL^{-1}). The recognition coefficient α, defined in Eq. 1 where R_{MIP} and R_{NIP} are the responses due to the MIP and NIP films, was used to evaluate the recognition ability.[34]

$$\alpha = \frac{R_{MIP}}{R_{NIP}} \quad (1)$$

When α>1, the MIP has selectivity for the analyte.[35] For Sudan I–IV, α=4.36, 3.33, 3.18, and 3.08, respectively. For 1,2-diphenyldiazene and 2-naphthol, α was around 1. Thus MIP-SPR could clearly recognize not only the template Sudan I but also structurally related Sudan II–IV. In contrast, the NIP-SPR gave almost the same response for all the compounds. These low signals resulted from weak and nonspecific adsorption caused by the random arrangement of the functional groups in the NIP films. The distinct differences between the MIP and NIP sensors indicate that molecular imprinting was the source of selectivity. Moreover, the importance of similar molecular shapes and functional groups was supported in the selective recognition by the MIP film. The imprinting of Sudan dyes is facilitated by the formation of dihydrogen bonds between the functional groups (-N=N-, -OH) of Sudan dyes and the carboxyl group in MAA, as shown in our previous report.[36]

Reproducibility

The adsorption of template molecules by the MIP-SPR sensor was reversible. To demonstrate the reproducibility of the MIP-SPR sensor response, three equilibration-adsorption-regeneration cycles were repeated with a 300-ng·mL^{-1} Sudan I solution. Initially, as shown in Fig. 7, acetonitrile was injected until a stable baseline was reached. Sudan I was then absorbed onto the MIP film-coated chip. Finally, the baseline was restored with acetonitrile and the MIP film-coated chip was regenerated. The SPR response exhibited a slight decrease from the recycling, which may be caused by incomplete removal of the templates.[28] By comparison, the NIP-SPR had a lower response without significant change. Thus the imprinting process established recognition memory and specificity for the MIP-SPR sensor, which can be reused reproducibly at least 20 times without loss of recovery (data not shown).

Conclusions

We have fabricated and characterized an MIP-SPR sensor for enhanced, specific detection of Sudan dyes (I, II, III, and IV). The MIP nanofilm was prepared by *in situ* surface-initiated thermal polymerization on a bare Au SPR chip in the presence of a template Sudan I molecule. The so formed MIP layer with a thickness of 75±5 nm featured a homogeneous structure, high selectivity, and fast adsorption/desorption kinetics. The MIP-SPR sensor exhibited a linear response for the detection of Sudan I in the range of 50–400 ng·mL^{-1} (R^2=0.9905) with a detection limit of 30 ng mL^{-1}. Selectivity studies indicated that the imprinted cavities formed in the nanofilm had high binding affinity for four kinds of Sudan dyes simultaneously. These results suggest that the combination of SPR sensing with MIP films has potential for practical monitoring of Sudan dyes in foodstuffs and this new strategy may be expanded to other small organic molecules.

Acknowledgements

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Figure captions

Fig. 1. Schematic representation of preparation for MIP nanofilm by surface initiated polymerization.

Fig. 2. AFM images of the chips. (a) the bare gold chip; (b) the MIP film-coated chip *via* thermal polymerization; (c) the MIP film-coated chip *via* photo-polymerization**.**

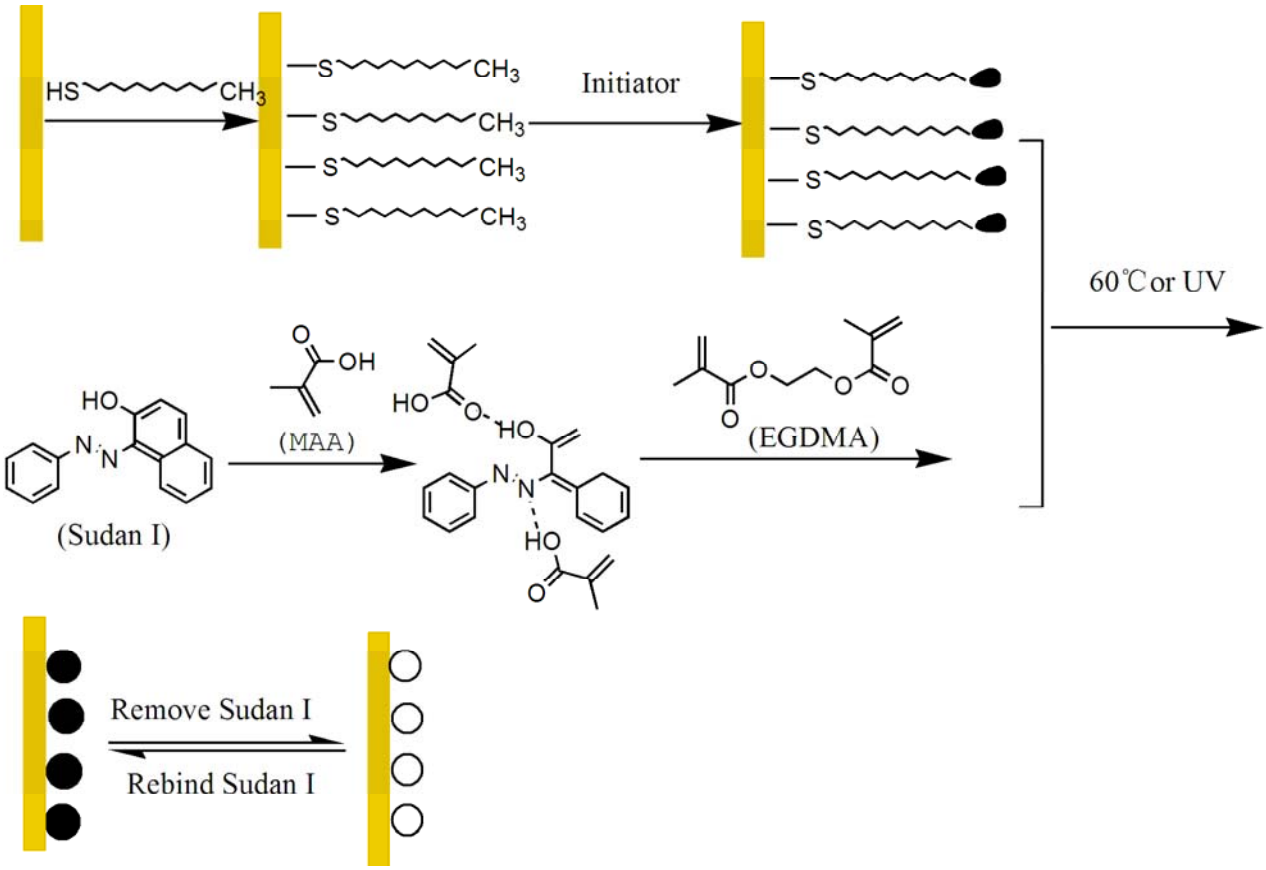
Fig. 3. The responses of MIP-SPR sensors *via* two methods. (a) thermal polymerization; (b) photo-polymerization.

Fig. 4. SEM image of the MIP nanofilm *via* thermal polymerization.

Fig. 5. Real-time detection of Sudan I in acetonitrile with MIP-SPR sensor. (a) the responses of MIP-SPR sensor to varying concentrations of Sudan I solution; (b) calibration plot. The Y axis displayed the response of MIP-SPR sensor. The X axis displayed the concentration of Sudan I solution.

Fig. 6. (a) Chemical structures of Sudan I–IV dyes, 1,2-diphenyldiazene and 2-naphthol; (b) selectivity of the MIP-SPR and NIP-SPR sensor. Analyte concentration was 100 ng mL^{-1}.

Fig. 7. Reproducibility of MIP- SPR and NIP-SPR sensor. All measurements were performed with 300 ng mL^{-1} of Sudan I.



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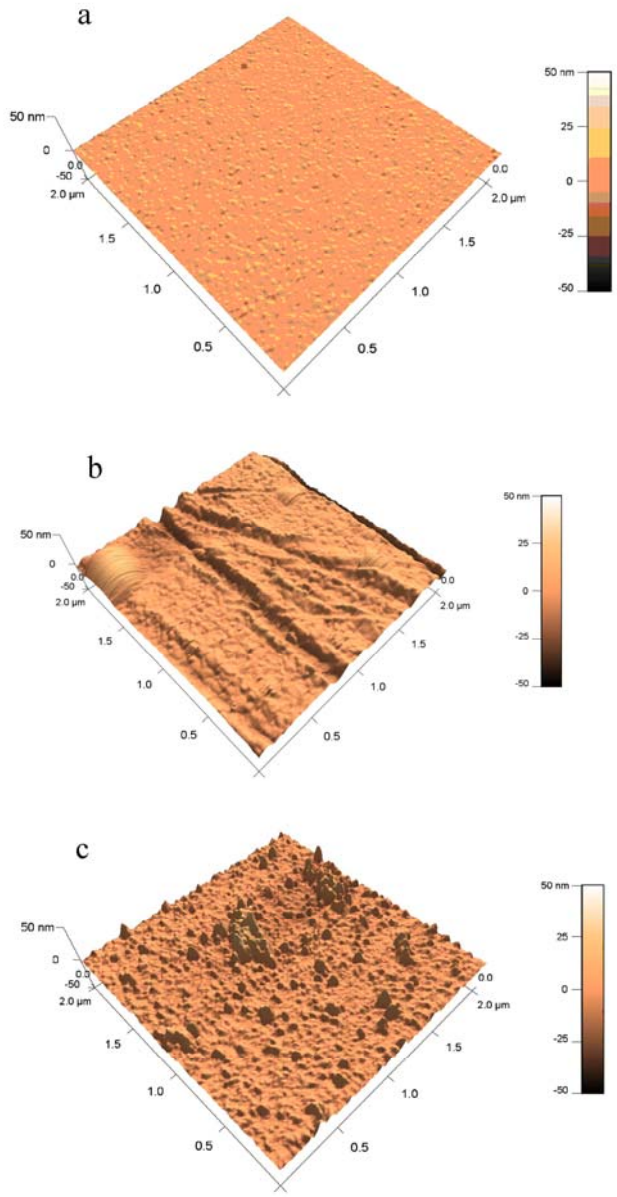

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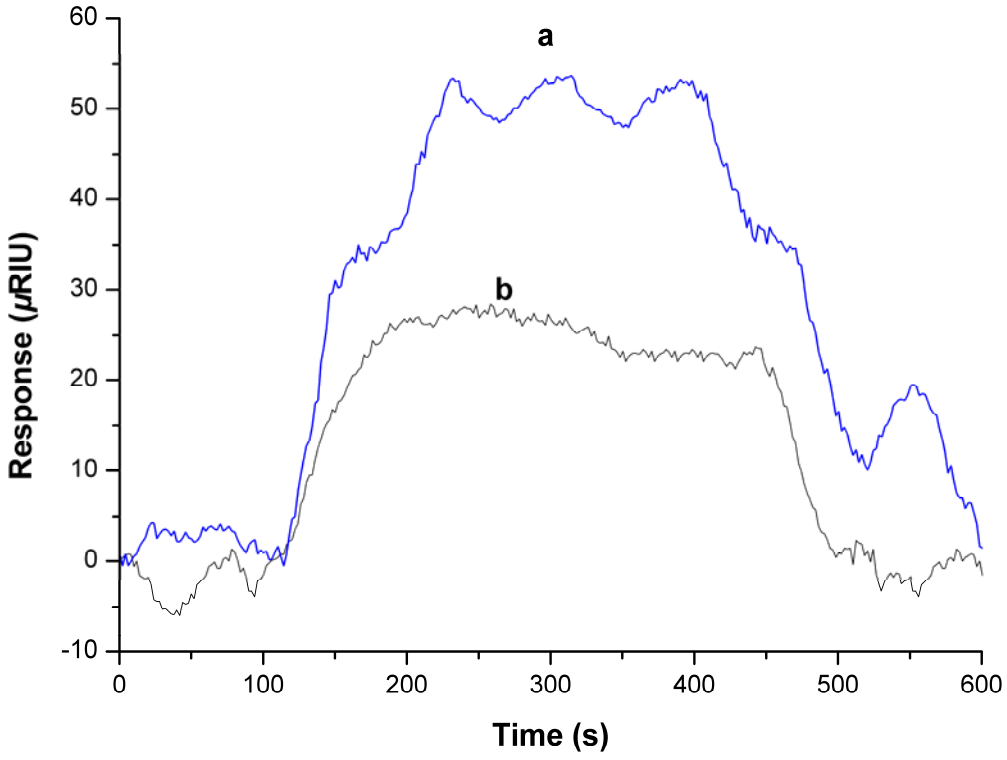

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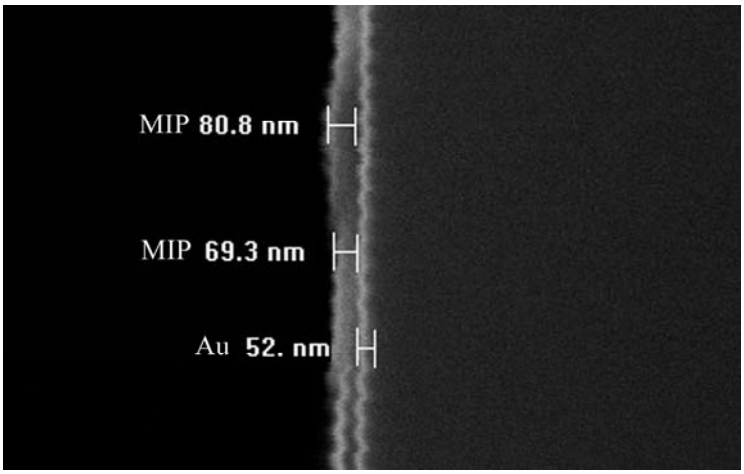

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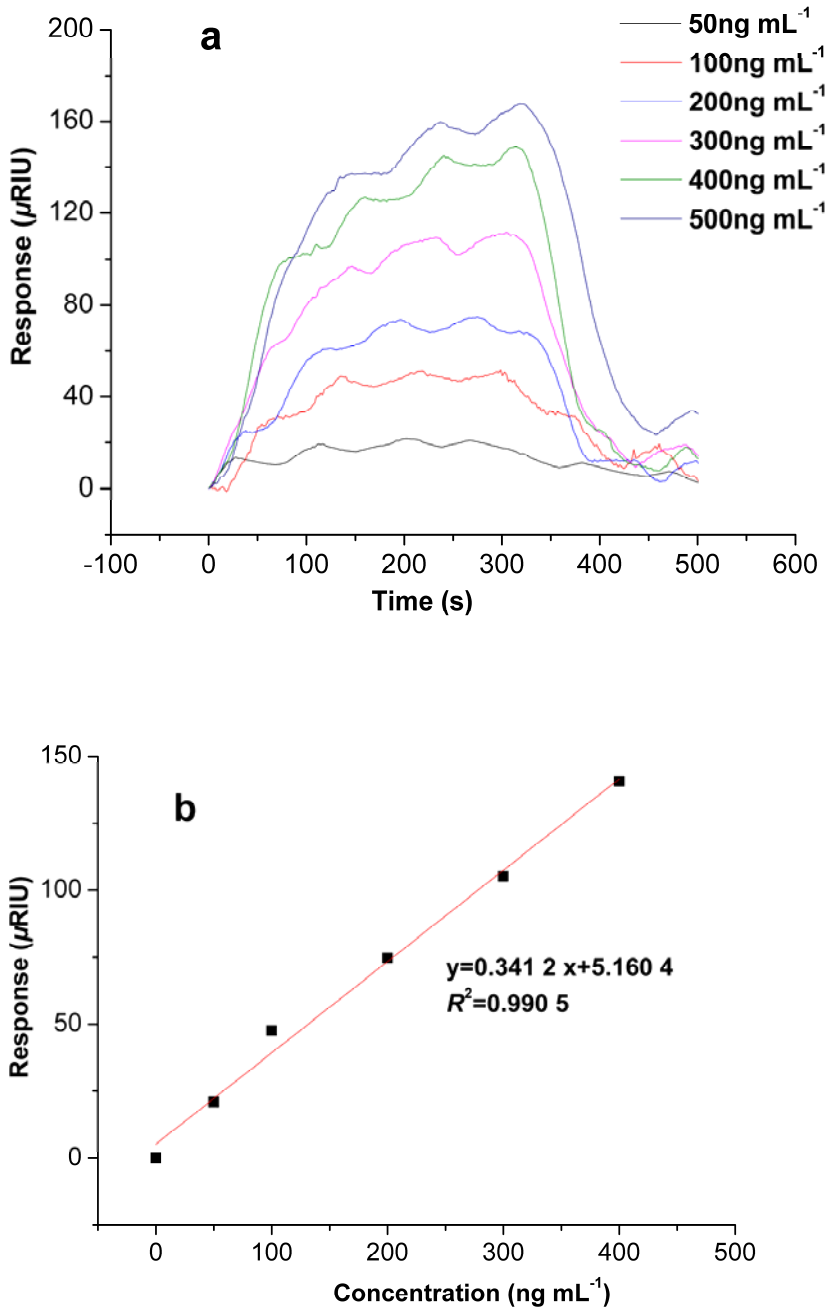

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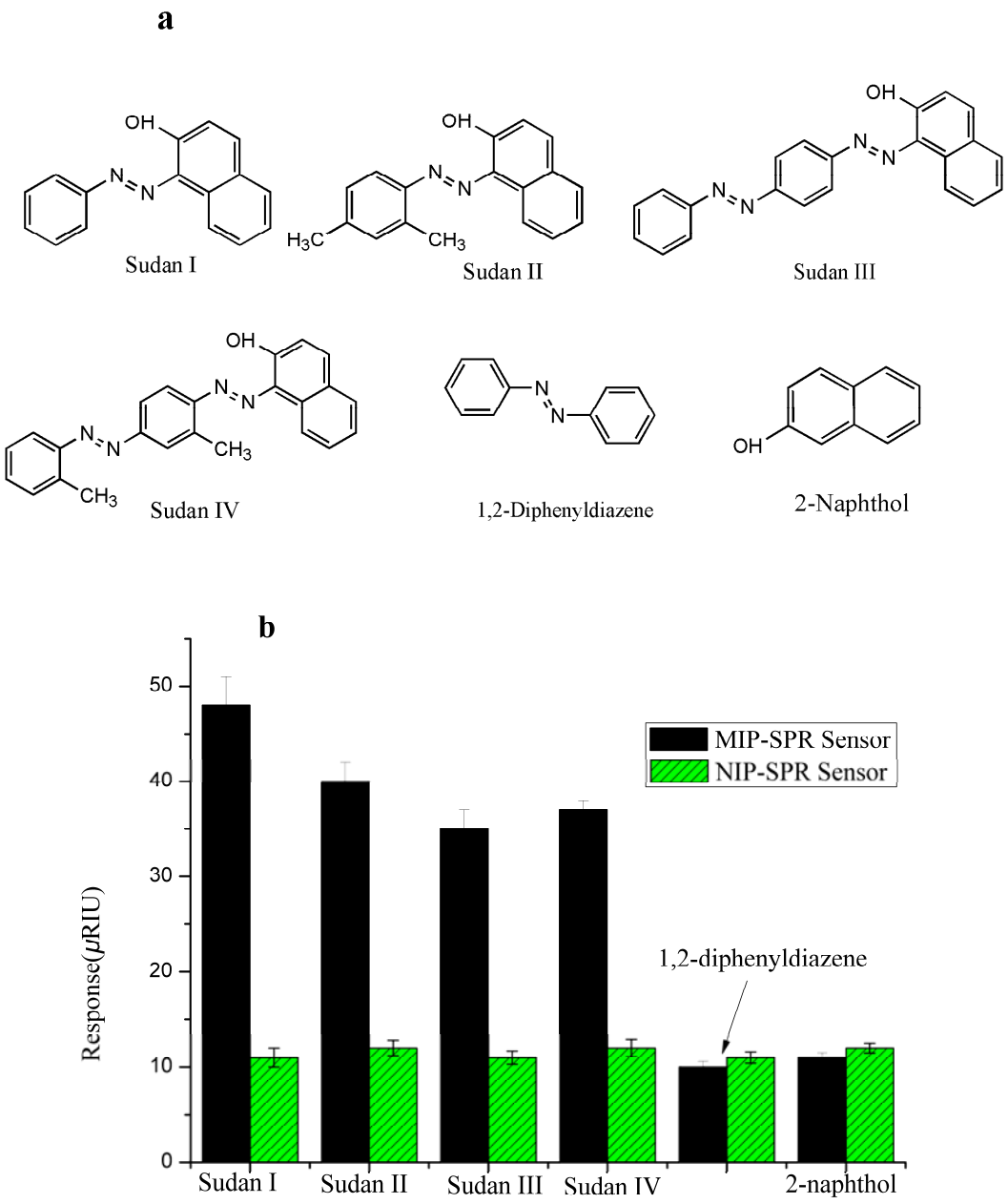

Fig. 6. (a) Chemical structures of Sudan I–IV dyes, 1,2-diphenyldiazene and 2-naphthol; (b) selectivity of the MIP-SPR and NIP-SPR sensor. Analyte concentration was 100 ng mL^{-1}.

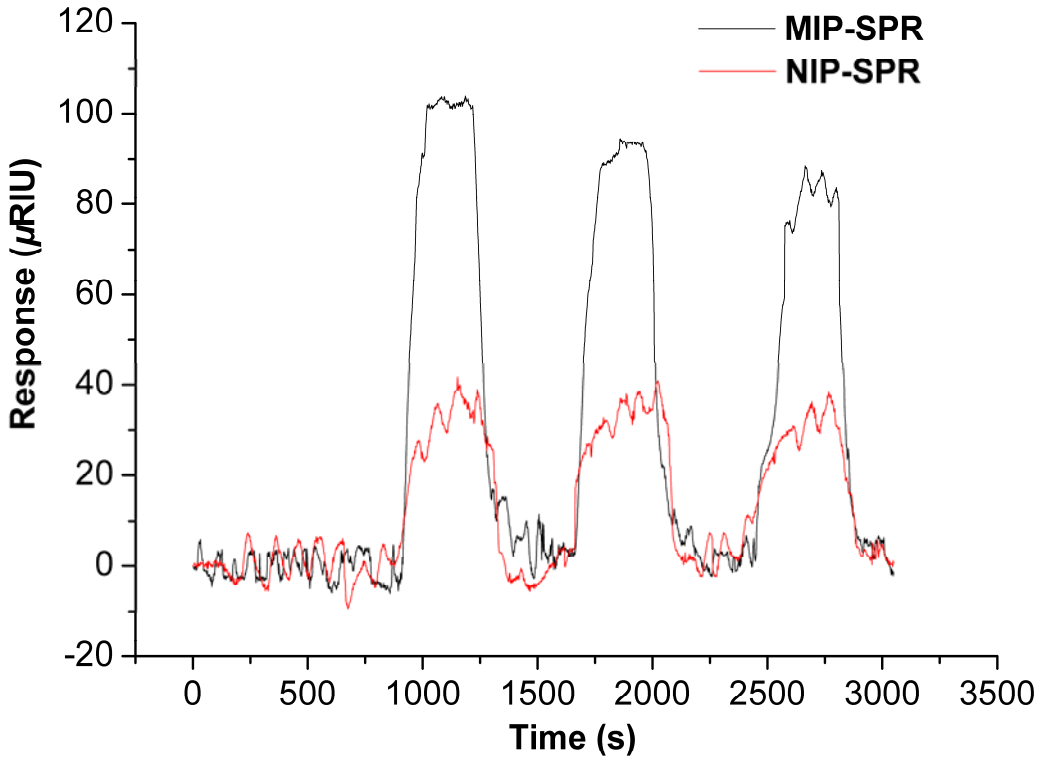

Fig. 7. Reproducibility of MIP- SPR and NIP-SPR sensor. All measurements were performed with 300 ng mL^{-1} of Sudan I.