

Photoconjugation of Molecularly Imprinted Polymer Nanoparticles for Surface-Enhanced Raman Detection of Propranolol

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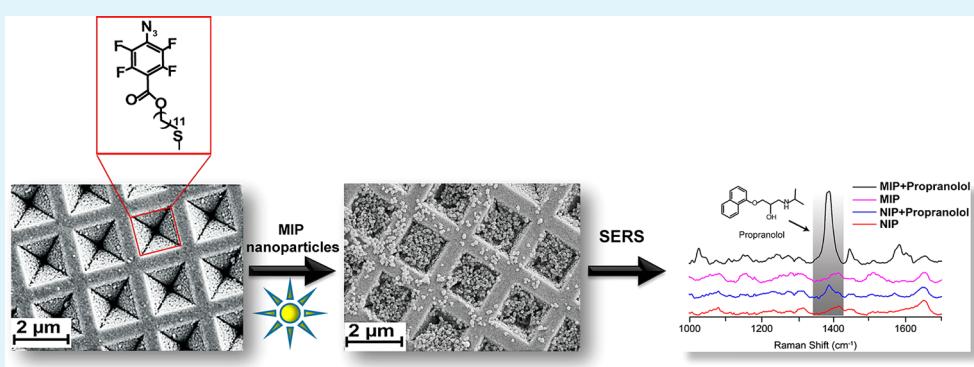
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Supporting Information



ABSTRACT: We report a simple and versatile method to covalently immobilize molecularly imprinted polymer (MIP) nanoparticles on a Raman active substrate (Klarite) using a disulfide-derivatized perfluorophenylazide (PFPA-disulfide). Gold-coated Klarite was functionalized with PFPA-disulfide via a gold–sulfur bond. Upon light radiation, the available azido groups were converted to highly reactive singlet perfluorophenyl nitrene that undergoes a CH insertion reaction and form covalent bonds with the MIP nanoparticles. The resulting surfaces were characterized using scanning electron microscopy and surface enhanced Raman spectroscopy to study the morphology and template affinity of the surfaces, respectively. The Raman measurements clearly show a dose-responsive signal when propranolol binds to the MIP surface. Because the MIP particles were covalently attached to the Raman active substrate, the sensing surface was stable and could be reused after regeneration in acetic acid solution. The MIP-based Raman sensor was used successfully to detect propranolol in urine samples (7.7×10^{-4} M). Our results show that the high selectivity of MIPs and the fingerprint Raman identification can be integrated into a compact sensing unit using high-efficiency photoconjugation. Thus, the method proposed is reliable, efficient and fast for fabricating label-free chemical sensors.

KEYWORDS: molecularly imprinted polymers, propranolol, perfluorophenylazide disulfide, photocoupling, Klarite, surface-enhanced Raman spectroscopy, urine

INTRODUCTION

Detection of drug molecules in biological, industrial, and environmental samples is gaining importance due to different health concerns.¹ Various analytical methods have been reported to detect propranolol in pharmaceutical materials, a commonly used beta blocker to treat anxiety, hypertension, and panic. The analytical methods based on conductometry,² spectrophotometry,³ and high-performance liquid chromatography (HPLC)^{4,5} are highly accurate. However, these analytical methods often require time-consuming sample preparation steps, skilled laboratory personnel, and complex instrumentation. For routine analysis, it is more attractive to use chemical

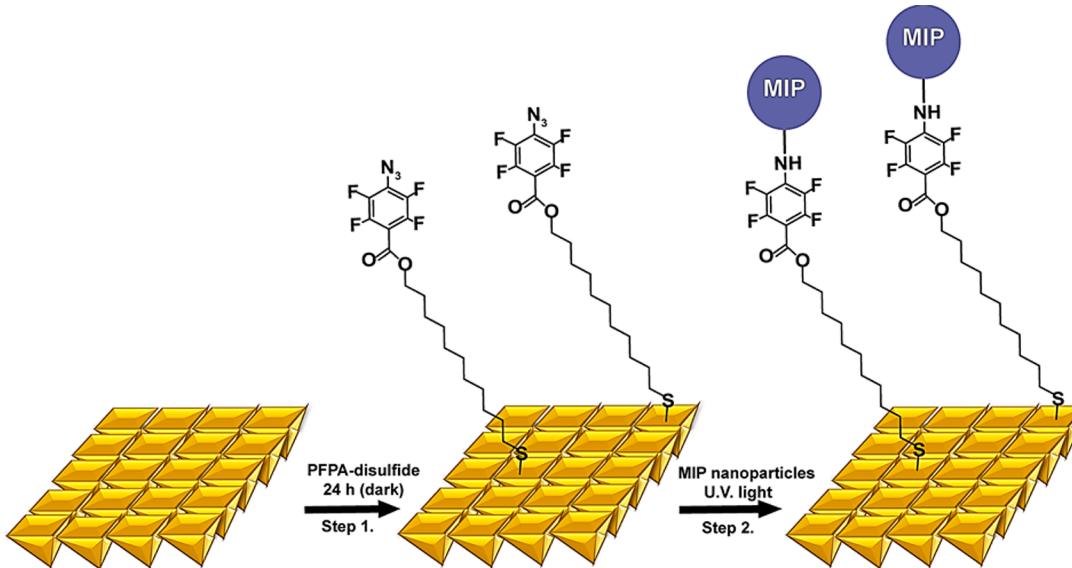
sensors with a high molecular selectivity, because these analytical devices can be more easily operated for analysis of complex samples. Recently, molecularly imprinted polymers (MIPs) have emerged as substitutes for biological recognition materials (e.g., enzymes, antibodies) to enable selective detection of analytical targets due to that MIPs have outstanding stability, durability and cost effectiveness.⁶

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Scheme 1. Immobilization of MIP Nanoparticles on a PFPA-Functionalized Klarite Surface Using Photoconjugation Chemistry



Step 1: functionalization of Klarite with PFPA-disulfide. Step 2: photoconjugation of MIP nanoparticles on PFPA-functionalized Klarite.

MIPs are cross-linked polymers synthesized in such a way that they retain binding cavities for the template molecules used during polymerization.^{7–9} MIPs can be interfaced with a sensitive transducer, making these polymers an efficient tool for sensitive and easy quantification of propranolol. Different MIP-based chemical sensors have been reported using potentiometric,^{10,11} electrochemical,¹² and quartz-crystal microbalance (QCM)^{13–15} as detection systems. Compared to these, surface enhanced Raman spectroscopy (SERS) measures analyte-specific vibrational spectrum and is less-affected by cross-reactivity from interfering molecules.^{16,17} Different methods have been reported for introduction of MIPs to SERS surfaces for chemical sensing. Kosterwa et al.¹⁸ prepared MIP layers on gold- and silver-coated microscopic slides, and used SERS to study the uptake and release of (2S,3S)-(+)-di-O-benzoyltartaric acid and N-benzyloxycarbonyl-(L)-aspartic acid. Kantrovich et al.^{19,20} used a nano fountain pen to print MIP precursors on a SERS substrate (Klarite), and prepared MIP dots on the substrate by *in situ* polymerization. The MIP dots on the Klarite were then used to monitor propranolol using SERS. In the reported methods, the MIPs were not firmly bonded to the Raman substrate, which is required if the sensing surface is designed for repeated use. To construct robust and reusable Raman sensors, we followed an interesting approach using the photochemistry based on perfluorophenylazide (PFPA).²¹ PFPAs are heterobifunctional coupling reagents with functional groups that can be used to attach to transducer surfaces and a terminal azido group that undergoes a CH insertion or C=C addition reaction with organic materials upon UV activation.^{22,23} PFPAs have been used to immobilize graphene,²⁴ polymers²⁵ and small molecules²⁶ on different substrates. Pei et al.²⁷ and Norberg et al.²⁶ used polymer-grafted PFPAs to immobilize carbohydrates on a QCM crystal and studied the real-time lectin-carbohydrate interactions. Stein et al.²⁸ also showed covalent and oriented protein attachment on PFPA-functionalized SPR chip to detect antibody binding.

Previously, Chaudhary et al.²⁹ reported the photoconjugation of MIP nanoparticles on PFPA-modified silicon and glass surfaces. The MIP nanoparticles immobilized on the surfaces

retain their propranolol binding selectivity. In this work we report, for the first time, the direct immobilization of MIP nanoparticles on a highly Raman-active substrate (Klarite) using PFPA-based photoconjugation for the successful detection of propranolol using this new surface enhanced Raman scattering sensor. The PFPA-disulfide has an alkyl chain of 11 carbons (PFPA-C₁₁-S₂). The disulfide group forms stable bonds with the gold coated on the Klarite substrate. The exposed azide group was used to form covalent bond with the MIP nanoparticles upon UV activation (Scheme 1).³⁰ Using this approach the MIP particles were packed in the patterned cavities on the Klarite substrate and could withstand repeated washing steps. We show that the MIP-based Raman sensor can detect propranolol at a low concentration (3×10^{-6} M) and in complex biological samples. Although detection of β -blockers in urine has been reported earlier using MIPs as solid phase extraction (SPE) sorbents^{31–33} and MIPs as room temperature phosphorescent sensors,³⁴ the analytical process can be greatly simplified by integrating MIPs with Raman transducer into a compact chemical sensor. The simple and compact SERS system studied in this work is used as a model to demonstrate the general applicability of our construction method.

EXPERIMENTAL SECTION

Materials. Acetone, acetic acid (glacial, 100%), acetonitrile (99.7%), and azobis(isobutyronitrile) (AIBN, 98%) were purchased from Merck (Darmstadt, Germany). Methacrylic acid (MAA, 98.5%) was purchased from ACROS (Geel, Belgium). Propranolol hydrochloride (99%) supplied by Fluka (Dorset, U.K.) was converted into free base form before use. Trimethylolpropane trimethacrylate (TRIM, technical grade) and toluene were bought from Sigma-Aldrich. Before use, AIBN was recrystallized from methanol. All other chemicals were used as received. PFPA-disulfide was synthesized according to the literature.³⁵

Klarite substrates were purchased from Renishaw Diagnostics (D3 Technologies, Ltd.). These SERS active substrates are comprised of Au coated inverse pyramidal wells with an opening area of $1.5 \times 1.5 \mu\text{m}$ and a depth of $1 \mu\text{m}$.

Methods. Synthesis of MIP Nanoparticles. The propranolol imprinted MIP nanoparticles were synthesized using precipitation polymerization as reported by Yoshimatsu et al.³⁶ Briefly, the template

molecule (*R,S*)-propranolol (0.53 mmol) was dissolved in 40 mL of acetonitrile in a 150 × 25 mm borosilicate glass tube equipped with a screw cap. The functional monomer (MAA, 1.31 mmol), the cross-linking monomer (TRIM, 2.02 mmol) and the initiator (AIBN, 28 mg) were then added. The solution was purged with a gentle flow of N₂ for 5 min and sealed under N₂. The borosilicate glass tube was fixed horizontally in a Stovall HO-10 hybridization oven (Greensboro, NC) and rotated at a speed of 20 rpm. The temperature was ramped from 20 to 60 °C within 20 min and, thereafter, was kept at this temperature for 24 h. After polymerization, the particles were collected by centrifugation. The template was removed by batch mode-solvent extraction with methanol containing 10% acetic acid (v/v), until no template could be detected from the washing solvent by spectrometric measurement (UV absorption at 290 nm). The polymer particles were finally washed with acetone and dried in a vacuum chamber. For comparison, nonimprinted polymer (NIP) nanoparticles were synthesized in the same way except for the omission of the template during the polymerization.

Photoconjugation of MIP Nanoparticles on Klarite. Klarite is a chemically etched silicon wafer patterned with inverse pyramid wells coated with a thin layer of gold.^{37,38} In this structure, analyte molecules that are closely located to the pyramid walls can generate detectable Raman signal due to the creation of Raman “hot spots”. The gold coated pits of the Klarite wells confines the localized plasmons thus amplifying the vibrational signals due to the attached analyte molecules.³⁹ Clean Klarite substrates were immersed in PFPA-disulfide solution (10 mM) in chloroform for 24 h [cf. Scheme 1 (Step 1)] followed by rinsing in the solvent.⁴⁰ A drop (50 μL) of MIP nanoparticle suspension (2 mg/mL) in acetonitrile was deposited on the PFPA-functionalized Klarite and allowed to stand for 15–20 min to evaporate the solvent. The surfaces were then illuminated with a UV lamp for 30 min through a 280 nm filter [cf. Scheme 1 (Step 2)]. The nanoparticle-conjugated surfaces were rinsed thoroughly in acetonitrile to remove the physisorbed nanoparticles and dried under a nitrogen flow. The same procedure was used to immobilize the NIP nanoparticles on the Klarite substrates.

Regeneration of Sensing Surfaces. Propranolol was eluted from the immobilized MIP nanoparticles by incubating the sensing surfaces in 20% acetic acid in methanol for 1 h. The surfaces were dried and then incubated in acetonitrile for 1 h to wash away the acid, followed by drying with a N₂ flow.

SERS Measurements on Different Substrates. Propranolol standard solution was prepared in acetonitrile. Klarite substrates, with and without the immobilized polymer particles, were dipped into the standard solution for 3 h, rinsed in acetonitrile, and dried before the Raman measurement.

Detection of Propranolol in Urine Samples. Urine was collected from a healthy 27-year-old female and stored at −20 °C before use. The urine (1 mL, pH = 6–7) was mixed with 1 mL of acetonitrile. The solution was then centrifuged at 12000 rpm for 10 min before the supernatant was collected. A 7.7×10^{-4} M propranolol (free base) was added to the above supernatant. The pH of the supernatant remained at 6–7 after the addition of propranolol. The MIP-coated sensing surface was dipped in the supernatant for 3–5 h, then rinsed in acetonitrile and dried before the Raman measurement.

Apparatus. Raman measurements were carried out using an iRaman system from B&W Tek, Inc., equipped with an excitation laser at 785 nm, spot size of 80 μm and a source power of 325 mW. The fiber optics probe was maintained at 6 mm from the sample surface during the measurement. Raman spectra were collected in the range of 200–2000 cm^{−1} with a spectral resolution of 3 cm^{−1}. For each spectrum, 10 scans were averaged, and each scan took 1 s. The spectra were recorded using the BWSpec software and analyzed with Origin Pro 9.0 software. The spectra were background subtracted, smoothed using the Savitzky–Golay method and normalized with respect to the polymer signal.

Photoconjugation was done using a medium-pressure Hg UV lamp (450 W) from Hanovia, Ace Glass Inc. (Vineland, NJ) and a N-WG 280 nm optical filter from Schott, Germany.

Scanning electron microscopy (SEM) characterization for the SERS substrates was carried out using a SEM LEO 1560 electron microscope (Zeiss, Oberkochen, Germany) operated at a voltage of 10 kV. The SEM analysis was carried out directly on the samples without prior metal coating.

RESULTS AND DISCUSSION

Preparation and Characterization of MIP-Coated SERS Substrates. To prepare a robust Raman substrate that has a predefined molecular binding selectivity, we used PFPA-based photoconjugation to immobilize MIP nanoparticles on a Klarite surface. The MIP nanoparticles used in this study have high molecular binding selectivity, which was confirmed in a previous radioligand binding analysis.³⁶ The process of nanoparticle immobilization was completed in two steps under very mild reaction conditions (Scheme 1). After the sample preparation, the obtained sensing surfaces are ready for measuring analytical samples using Raman scattering.

Figure 1 shows the SEM morphology of a Klarite substrate before and after being coated with the MIP nanoparticles. In

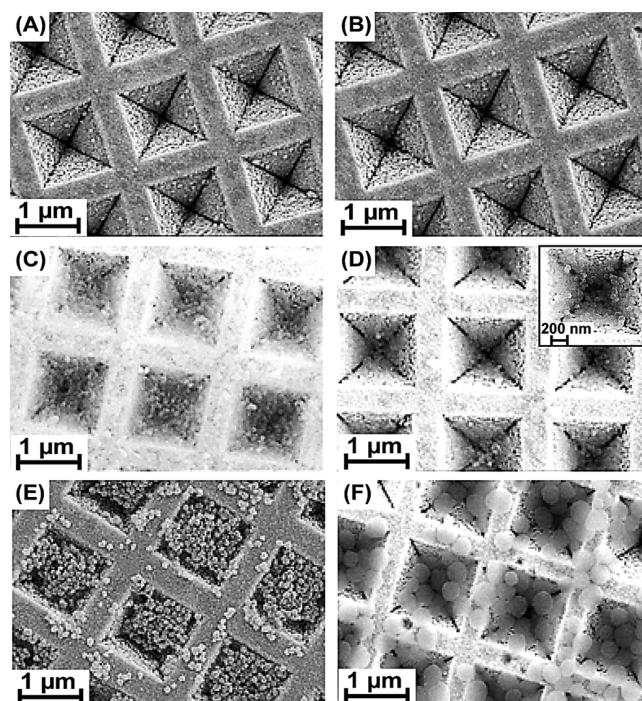


Figure 1. SEM images of (A) clean Klarite; (B) Klarite loaded with MIP nanoparticles, after solvent washing; (C) MIP nanoparticles photoconjugated on PFPA-functionalized Klarite; (D) MIP nanoparticles photoconjugated on PFPA-functionalized Klarite, regenerated by washing with acetic acid; (E) MIP nanoparticles photoconjugated on PFPA-functionalized Klarite, regenerated by acid-washing and after sputtering 10 nm Au; and (F) NIP nanoparticles photoconjugated on PFPA-functionalized Klarite, without acid regeneration.

Figure 1A, the surface in the inverted pyramid wells of the Klarite substrate is seen, with the sputtered gold colloids clearly visible on the wall. Although the size of the inverted pyramid wells (1.5 × 1.5 × 1 μm) is large enough to accommodate the MIP nanoparticles (approximately size of 150 nm), almost no MIP nanoparticles can be observed after the particle-loaded substrate was washed (Figure 1B). On the contrary, using the PFPA-mediated photoconjugation, the MIP nanoparticles were firmly attached to the wells, as shown in Figure 1C. This

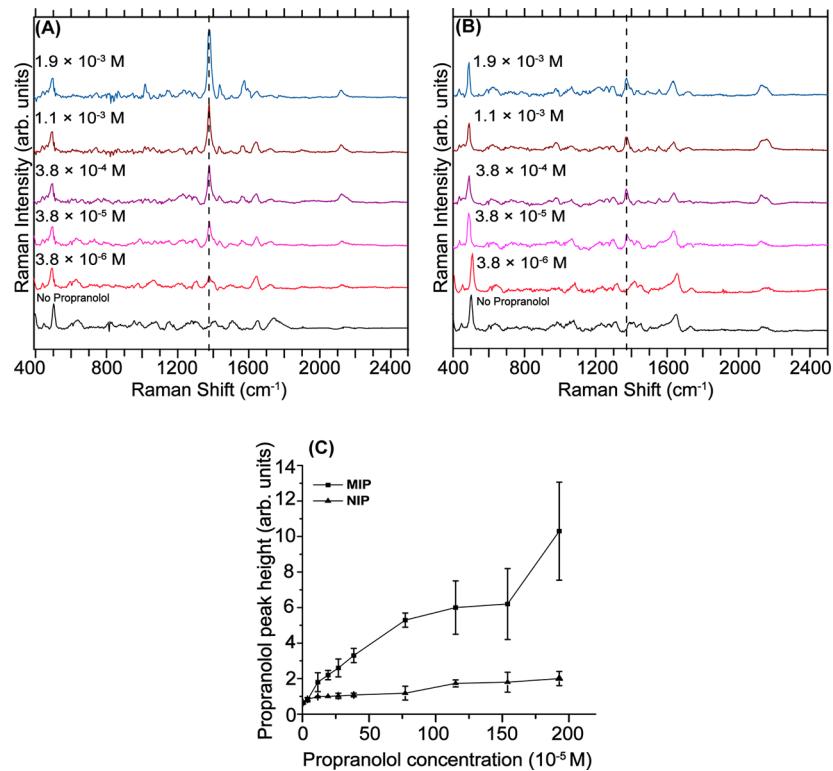


Figure 2. (A) Raman spectra obtained from the MIP-coated surface before and after binding propranolol at different concentrations. (B) Raman spectra obtained from the NIP-coated surface before and after binding propranolol at different concentrations. (C) Intensity of propranolol signal measured using the MIP- and the NIP-coated substrates. The propranolol peak at 1385 cm^{-1} was normalized against the polymer peak at 1650 cm^{-1} .

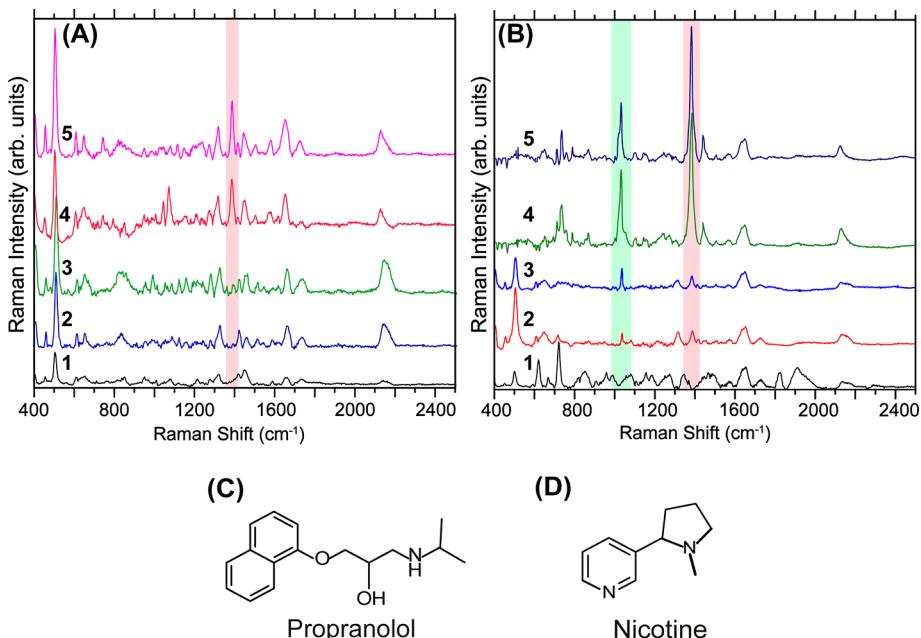


Figure 3. (A) Raman spectra recorded on (1) a MIP-coated surface, (2) a MIP-coated surface after the first regeneration, (3) a MIP-coated surface after the second regeneration, (4) a MIP-coated surface exposed to 3.85×10^{-4} M propranolol, (5) a regenerated MIP-coated surface exposed to 3.85×10^{-4} M propranolol. (B) Raman spectra recorded on (1) a MIP-coated surface, (2) a MIP-coated surface exposed to a solution containing propranolol (3.85×10^{-5} M) and nicotine (3.85×10^{-5} M), (3) a MIP-coated surface exposed to a propranolol solution (3.85×10^{-5} M) and nicotine (3.85×10^{-4} M), (4) a MIP-coated surface exposed to a propranolol solution (1.54×10^{-3} M) and nicotine (1.54×10^{-3} M), (5) a MIP-coated surface exposed to a propranolol solution (3.85×10^{-3} M) and nicotine (3.85×10^{-3} M). (C) Chemical structure of propranolol. (D) Chemical structure of nicotine.

covalent attachment allows the loaded nanoparticles to remain stable inside the wells and unremoved during acid washing

(Figure 1D), a condition commonly used to regenerate MIPs for repeated use. Note that the poor resolution in Figure 1C,D

is due to the direct imaging of the MIP-loaded substrates without sputtering conductive metal (to allow the same substrate to be used in later Raman experiment). To show more clearly the presence of stable nanoparticles after the acid washing, we sputtered 10 nm Au on the regenerated surface and then collected additional SEM image (Figure 1E). The SEM image after Au sputtering (Figure 1E) shows clearly the dense MIP particles inside the Klarite wells. Compared to the MIP nanoparticles, the NIP nanoparticles have a larger particle size (approximately size of 300 nm) and can be distinguished more easily inside the pyramid wells (Figure 1F).

Detection of Propranolol Using MIP-Based SERS. The MIP-coated Klarite substrate was tested as a Raman active surface to detect propranolol at different concentrations. The experiments were carried out by first incubating the substrate in propranolol solution (3.8×10^{-6} to 1.92×10^{-3} M), then measuring the characteristic signal caused by the propranolol that bound to the MIP-coated substrate. As seen from Figure 2A, the MIP surface produces a strong Raman band at 1385 cm^{-1} (marked with the vertical dashed line) due to the naphthalene moiety of the propranolol molecule,⁴¹ as seen from the chemical structure of the propranolol molecule in Figure 3C. The Raman band starts to appear when the propranolol concentration reaches 3.8×10^{-6} M. At higher concentration, additional vibrational bands from propranolol at 1440 and 1577 cm^{-1} also become visible. We should mention that without the immobilized MIP particles, the Klarite substrate itself gave a much weaker propranolol signal (Supporting Information, Figure S1). Here, the MIP nanoparticles immobilized on the Klarite acted as affinity adsorbents to bind the propranolol and to bring the analyte close to the underlying Raman active substrate. As a result, the MIP-coated Klarite was able to produce the strong characteristic analyte signal even from a dilute propranolol solution. To verify that the effective signal generation is caused by the specific propranolol binding to the MIP, we also tested NIP-coated Klarite to study its response to the different propranolol solutions. As seen from Figure 2B, the NIP-coated Klarite produced much weaker propranolol signal due to the lack of high affinity sites for propranolol. By normalizing the propranolol signal at 1385 cm^{-1} against the intrinsic polymer band at 1650 cm^{-1} , we obtained a dose-response curve for propranolol for both the MIP- and the NIP-coated Klarite (Figure 2C). Obviously, the MIP-coated Klarite produced significantly higher analytical signal than the NIP-coated Klarite. The nonlinear increase of propranolol signal in the high concentration range (7.71×10^{-4} to 1.54×10^{-3} M) may be due to the saturation of the propranolol binding sites in the MIP nanoparticles. At the highest concentration (1.92×10^{-3} M), propranolol may adsorb on the surface through nonspecific binding and leads to increased Raman signal. To test the reusability of the MIP-coated substrate, we also tried to regenerate the sensing surface by washing the MIP-coated substrate in acetic acid solution. From Figure 3A it can be seen that after the bound propranolol was eluted from the MIP surface, the Raman band at 1385 cm^{-1} disappeared. This band appears again after the regenerated MIP surface was exposed to the same propranolol solution (Figure 3A). After two times of regeneration, the MIP surface still gave the same propranolol response, suggesting that the MIP nanoparticles are firmly attached on the surface and retain their specific binding. The SEM image in Figure 1D,E also confirmed that the MIP

nanoparticles remained in the Klarite wells after the acid washing.

To demonstrate that the MIP-based SERS sensor can detect propranolol in the presence of possible interfering compounds, we exposed the MIP-coated surface to a propranolol solution containing nicotine, at different concentrations (0.038 – 3.85×10^{-3} M) and recorded the Raman spectra. To ensure fair competition between the two compounds at all the different concentration levels, we used equivalent amounts of nicotine and propranolol in the competition experiments. In this way, we could evaluate the average binding sites in the MIP and minimize the influence of inhomogeneity of the binding sites, which is well-known for molecularly imprinted polymers. As seen from Figure 3B, being an organic amine (cf. Figure 3D), nicotine can get physisorbed to the MIP-coated surface (containing carboxyl groups) and generated a characteristic Raman band at 1034 cm^{-1} .^{42,43} In spite of this interference, the propranolol signal at 1385 cm^{-1} was still easily detected. As the analyte concentration increases, the intensity ratio between propranolol and nicotine becomes larger. This phenomenon may be explained by the saturation of the binding sites that are close to the particle surface by propranolol, which displaces nicotine from the MIP surface and leads to a reduced nicotine signal in the Raman spectra. From this result, it is clear that the fingerprint identification provided by Raman spectroscopy can help to solve the cross-reactivity problem associated with molecularly imprinted polymers. In other words, combining Raman spectroscopy with MIPs has a synergistic effect and leads to more reliable analytical results.

Detection of Propranolol in Urine Sample. To prove that the MIP-coated Klarite can be used to measure propranolol in more complex samples, we tested the sensing surface using urine samples spiked with propranolol. Figure 4

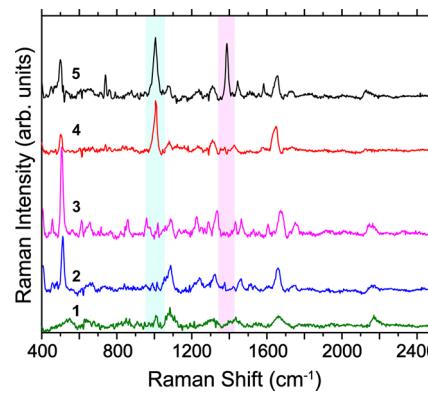


Figure 4. Detection of propranolol in urine samples. Raman spectra of (1) urine spiked with 7.7×10^{-4} M propranolol measured on a Klarite substrate, (2) MIP-coated Klarite, (3) NIP-coated Klarite, (4) urine spiked with 7.7×10^{-4} M propranolol measured on NIP-coated Klarite substrate, and (5) urine spiked with 7.7×10^{-4} M propranolol measured on MIP-coated Klarite substrate.

shows the results of the SERS measurements using the different Klarite surfaces. As observed from the spectra, the MIP-coated Klarite allowed propranolol (at 7.7×10^{-4} M) to be detected unambiguously (curve 5). The sharp peak at 1003 cm^{-1} was caused by the C–N stretching in urea that was abundant in urine.⁴⁴ The occurrence of the urea signal in Figure 4 can be explained as a result of nonselective physisorption. The concentration of urea in a healthy person's urine is about 9.3

g L^{-1} (0.16 M),⁴⁵ which is more than 200 times higher than the added propranolol. The fact that the MIP-coated Klarite still gives clear propranolol signal, in spite of the large excess of urea, provides strong evidence that the specific propranolol binding remains on the MIP-coated Klarite. Because the signal intensities for urea measured on the MIP- and NIP-coated substrates are similar, we can conclude that urea adsorption is nonselective, and the physisorbed urea does not affect specific propranolol binding to the imprinted sites in the MIP.

In contrast to the MIP-coated surface, neither the Klarite alone (curve 1) nor the NIP-coated Klarite (curve 4) gave any detectable propranolol signal under the same condition. When measured on the NIP-coated Klarite, propranolol standard solution (3.8×10^{-4} – $1.1 \times 10^{-3} \text{ M}$) gave a weak Raman band at 1385 cm^{-1} (Figure 2B). This signal did not occur when propranolol in urine ($7.7 \times 10^{-4} \text{ M}$) was measured on the NIP-coated Klarite. The reason for this variation is that many substances contained in urine can affect propranolol physisorption, particularly at the nonselective sites such as the NIP surface. For the specific propranolol binding to the MIP, the substances in urine have much less effect. Therefore, it can be concluded that only the MIP-coated Klarite can act as a useful sensing surface to measure propranolol in the complex urine sample.

CONCLUSIONS

We have developed a new method to covalently attach MIP nanoparticles on Raman active substrate and demonstrated that the obtained surface can be used to detect a model drug in complex biological samples. In this work, we used propranolol-imprinted nanoparticles as the molecular recognition component and SERS as the spectroscopic transducer. The combination of molecular binding selectivity with the fingerprint identification led to unambiguous detection of propranolol in urine. The covalent attachment of the nanoparticles on the Raman-active surface was achieved using straightforward photoconjugation. SEM inspections showed the successful nanoparticle immobilization on Klarite with a high stability, allowing the sensing surface to be regenerated by acid washing. The photoconjugation method to integrate MIP with Raman substrate is simple, robust, and effective. Although the model substrate (Klarite) used in this work is relatively expensive, it can be replaced by other Raman-active surfaces, for example, porous alumina membranes with a gold coating.⁴⁶ The method developed for sensor construction has a general applicability. Given that a large number of organic MIP nanoparticles can now be synthesized with high binding selectivity for different analytical targets, and these MIP nanoparticles can act as modular building blocks to be conjugated to different transducer surfaces using the photocoupling method reported here, we can expect a large number of robust, MIP-based chemical sensors to be developed for different analytical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsami.5b09500](https://doi.org/10.1021/acsami.5b09500).

Raman spectra of propranolol measured on Klarite and MIP-coated Klarite. (PDF)

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Notes

The authors declare no competing financial interest.

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