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Surface plasmon resonance sensor for femtomolar detection of testosterone with water-compatible macroporous molecularly imprinted film

Qingwen Zhang [a], Lijing Jing [a], Jinling Zhang [b], Yamin Ren [a], Yang Wang [a], Yi Wang [b,*], Tianxin Wei [a,*], Bo Liedberg [b]

[a] Key Laboratory of Cluster Science of Ministry of Education, Beijing Institute of Technology, Beijing 100081, China
[b] Centre for Biomimetic Sensor Science, Nanyang Technological University, Singapore 637553, Singapore

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ABSTRACT

A novel water-compatible macroporous molecularly imprinted film (MIF) has been developed for rapid, sensitive, and label-free detection of small molecule testosterone in urine. The MIF was synthesized by photo copolymerization of monomers (methacrylic acid [MAA] and 2-hydroxyethyl methacrylate [HEMA]), cross-linker (ethylene glycol dimethacrylate, EGDMA), and polystyrene nanoparticles (PS NPs) in combination with template testosterone molecules. The PS NPs and template molecules were subsequently removed to form an MIF with macroporous structures and the specific recognition sites of testosterone. Incubation of artificial urine and human urine on the MIF and the non-imprinted film (NIF), respectively, indicated undetectable nonspecific adsorption. Accordingly, the MIF was applied on a surface plasmon resonance (SPR) sensor for the detection of testosterone in phosphate-buffered saline (PBS) and artificial urine with a limit of detection (LOD) down to 10^{-15} g/ml. To the best of our knowledge, the LOD is considered as one of the lowest among the SPR sensors for the detection of small molecules. The control experiments performed with analogue molecules such as progesterone and estradiol demonstrated the good selectivity of this MIF for sensing testosterone. Furthermore, this MIF-based SPR sensor shows high stability and reproducibility over 8 months of storage at room temperature, which is more robust than protein-based biosensors.

Molecular imprinting is a well-established technique for the preparation of polymers with specific recognition sites of molecules [1,2] and cells [3] on various applications [4,5], including artificial antibodies [6,7], chromatographic separations [8], sensors [9,10], and mimic enzyme catalysis [11,12]. In general, molecularly imprinted polymers (MIPs)[1] are prepared by copolymerization of functional monomers and cross-linkers at the presence of template molecules in a porogen solvent. After the subsequent removal of the templates from polymer matrix, complementary cavities in memory of size, shape, and orientation of their binding sites to the target molecules are formed. The previously developed MIPs have demonstrated robustness and high selectivity for the recognition of target molecules predominantly in organic solvent media [13–15]. However, the MIPs employed for selective binding targets in aqueous conditions is an issue that should be addressed because many target molecules of interest are present only in aqueous solution such as body fluids, drinks, and waste water. Therefore, it is necessary to develop water-compatible MIPs for binding the targets in a specific and selective manner [16–19]. The major challenge on the preparation of MIPs with high selectivity in aqueous media is to overcome the nonspecific hydrophobic binding of targets that is often induced by water. To achieve this, various approaches have been used, including tuning the polarities of porogens and incorporating polar or nonpolar comonomers or cross-linkers into the MIP matrix [20]. In addition, MIPs with macroporous structures such as inverse opal structure have been fabricated for label-free detection of small molecules in water medium [21,22]. These macrostructures provides high accessibility for molecules binding into the polymer

* Corresponding authors. Fax: +65 67912274 (Y. Wang), +86 10 68945482 (T. Wei).
E-mail addresses: yiwang@ntu.edu.sg (Y. Wang), txwei@bit.edu.cn (T. Wei).

[1] *Abbreviations used:* MIP, molecularly imprinted polymer; PS NP, polystyrene nanoparticle; SPR, surface plasmon resonance; MIF, molecularly imprinted film; UV, ultraviolet; NIF, non-imprinted film; PBS, phosphate-buffered saline; LOD, limit of detection; HEMA, 2-hydroxyethyl methacrylate; EGDMA, ethylene glycol dimethacrylate; MAA, methacrylic acid; AU, artificial urine; SAM, self-assembled monolayer; SP, surface plasmon; SEM, scanning electron microscope; ELISA, enzyme-linked immunosorbent assay.

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matrices. However, this sensor relying on the diffraction or color changes of the thick MIPs has limited sensitivity. Here we have developed a procedure for the preparation of water-compatible MIPs by introducing monomer with high polarity and a macroporous structure by removing polystyrene nanoparticles (PS NPs) from the polymers. The MIPs with thickness of approximately 180 nm, which is comparable to the penetration depth of surface plasmon resonance (SPR), were well-controlled and synthesized in situ on an SPR sensor chip. This functionalized SPR sensor chip was further employed for the specific detection of testosterone in aqueous solution.

SPR is an optical phenomenon that enables the detection of mass changes on the surface of metallic substrates. It has been widely developed as a rapid, label-free, and real-time assay technique for highly sensitive detection of chemical and biological analytes [23], including large molecules such as antibodies [24], protein [25,26], pathogen [27], and nucleic acids [28]. SPR has also been reported for the detection of small molecules with high sensitivity. For instance, SPR sensors enable the detection of hormone at the picogram per milliliter (pg/ml) range [29] and trinitrotoluene (TNT) down to tens of femtomoles (fM) based on the molecularly imprinted gold nanoparticles [30]. Testosterone is a steroid hormone that acts as an indicator of a great many pathological conditions such as a prostate cancer biomarker and a stimulant that is often illegally used to increase muscle mass and strength in athletic sports. In general, the daily production rates of testosterone are 4 to 12 mg in young men and 0.5 to 2.9 mg in young women [31]. Conventional testosterone analysis is performed by immunoassays [32,33] and chromatography [34,35]. The immunoassays typically provide high sensitivity but require long incubation/washing steps and rely on the antibodies that have limited thermal stability, whereas the chromatography suffers from the weak specificity and insufficient sensitivity. SPR sensors in combination with MIPs can address this issue for the detection of small molecules at high sensitivity and selectivity [36,37]. In addition, an SPR sensor chip with a three-dimensional binding matrix such as hydrogel and MIPs containing a porous structure could improve the sensitivity because it provides higher binding capacity [38] and allows the target molecules to diffuse rapidly to the recognition sites.

In this study, a water-compatible macroporous molecularly imprinted film (MIF) was prepared on an SPR sensor chip through ultraviolet (UV) photo-polymerization. The macroporous structure of the MIF was developed through removal of PS NPs from the polymerized MIPs. The MIF with a high porosity was expected to provide a high capacity and high accessibility for the target molecules reaching to the recognition binding sites, thereby resulting in high sensitivity for rapid detection of the corresponding targets. The selectivity of the MIF was investigated on two other analogues, namely estradiol and progesterone. Furthermore, the nonspecific adsorption of serum, artificial urine, and human urine was evaluated on macroporous MIF and non-imprinted films (NIFs), respectively. This macroporous MIF was finally used for SPR sensing of testosterone in phosphate-buffered saline (PBS) buffer and artificial urine with a limit of detection (LOD) down to 10^{-15} g/ml. The sensor performance of the MIF in female and male human urine was also investigated. In addition, the stability and reproducibility of this SPR sensor chip were investigated after 8 months of storage at room temperature.

Materials and methods

Materials

Testosterone (MW = 288.42, cat. no. A0277793) and benzophenone (BP, wt% = 99%, cat. no. A0253552) were purchased from Acros Organics (China). 17β-Estradiol (cat. no. 11657, wt% = 98%), progesterone (USP, cat. no. 15925), 2-hydroxyethyl methacrylate (HEMA, wt% = 96%), and ethylene glycol dimethacrylate (EGDMA, analytical grade, wt% = 98%) were purchased from Aladdin Reagent (China). PS NPs (100 nm, 25 mg/ml, cat. no. 619820) were purchased from Polysciences (Germany). Methacrylic acid (MAA, analytical grade, wt% = 99%) and 1-dodecanethiol (wt% = 98%, cat. no. LBB0J03) were purchased from J & K Scientific (China). PBS was prepared from 140 mM NaCl, 10 mM phosphate, and 3 mM KCl, and pH 7.4 was adjusted by HCl and NaOH. Ethanol, acetic acid, and acetone were all analytical grade and purchased from Beijing Tongguang Fine Chemicals (China). MAA, HEMA, and EGDMA were distilled under reduced pressure before use. All of the other reagents were used as received. Rabbit serum was purchased from Sigma–Aldrich (cat. no. R9133, Singapore). Artificial urine (AU) solution at pH 6.0 was prepared by dissolving 2.0 mM citric acid, 25 mM $NaHCO_3$, 170 mM urea, 2.5 mM $CaCl_2$, 90 mM NaCl, 2.0 mM $MgSO_4$, 10 mM Na_2SO_4, 7 mM KH_2PO_4, 7 mM K_2HPO_4, and 25 mM NH_4Cl in water [39]. The pH of the solution was adjusted to 6.0 by the addition of 1.0 M HCl. Human urine samples were obtained from female and male volunteers.

Sensor surface functionalized with macroporous MIF

First, the gold chip was immersed in 1 mM 1-dodecanethiol ethanol solution for 24 h to form a self-assembled monolayer (SAM), which will react with the benzophenone to form free radicals for anchoring polymers. The testosterone-imprinted film was synthesized based on UV photo polymerization. Next, 0.036 g of testosterone was mixed with 42 μl of MAA and 61 μl of HEMA in 4.518 ml of ethanol. The mixture was sonicated for 20 min and pre-polymerized for 4 h at room temperature. Subsequently, the mixture was added to 355 μl of cross-linker EGDMA, 25 μl of PS NPs, and 0.02 g of benzophenone as initiator. After being treated with nitrogen gas for 10 min, the reaction solution was injected into a homemade flow cell in contact with the SAM modified gold chip for in situ polymerization. The thin MIF embedded with PS NPs (PS–MIF) was formed on the gold chip by UV irradiation (λ = 365 nm, irradiation power of 2 W/cm^2). The formation of the MIF on gold chip was real-time monitored by SPR reflectivity changes and the angular spectra. Afterward, the PS NPs were removed from PS–MIF by rinsing with acetone overnight to form the macroporous film. Finally, the macroporous MIF was rinsed with a mixture of ethanol and acetic acid (v/v = 1:1) to remove the template molecule testosterone from the MIF and form the recognition cavities. For a blank control, a macroporous NIF was prepared through removal of PS NPs from the same polymers without imprinting of template molecules. In addition, a conventional MIF was synthesized through the polymerization of the same monomers and template molecules but without PS NPs. The thickness of the MIF was determined with Alpha Step IQ Surface Profiler (KLA-Tencor, Milpitas, CA, USA) by carefully scratching the film with a needle to form a scratch down to the glass with approximately 100 μm in width. The underneath gold film with a thickness of 50 nm was taken into account.

SPR implementation

In the experiment, an optical setup based on SPR spectroscopy was employed [40] (Fig. 1). A light beam emitted from a stabilized HeNe laser (2 mW, λ = 632.8 nm) passed through a polarizer to become a transverse magnetically polarized beam and was coupled to a LASFN 9 glass prism (90°, n_p = 1.845) with an optically matched sensor chip to its base by using immersion oil. A flow cell with a volume of approximately 0.5 ml was pressed against the sensor surface to flow liquid sample over the substrate surface