Surface plasmon resonance sensor for biomimetic detection of progesterone with macroporous molecularly imprinted polymers prepared by visible light

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Abstract. Reversible addition-fragmentation chain transfer (RAFT) polymerization mechanism was used for the preparation of molecularly imprinted polymers (MIPs) film using 4-cyano-4-[dodecylsulfanylthiocarbonylsulfonyl]pentanoic acid (CDTPA) as chain transfer reagent and visible light initiator. In addition, the effect of CDTPA concentration on the polymerization rate was monitored, establishing that too high CDTPA concentration would inhibit the polymerization rate. The MIPs film was characterized by contact angle measurement, Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM) which showed the successful grafting of MIPs films onto surface plasmon resonance (SPR) sensor chip. The response of progesterone sensing ranged from 10^{-16} to 10^{-6}mol/L with low detection limit of 1×10^{-16}mol/L in PBS buffer (pH 7.4) and the MIPs film exhibited good selectivity, reproducibility and stability. Moreover, the sensors had been successfully applied to detect progesterone in tap water, lake water and human urine turning out with wide detection range and low detection limit.

1. Introduction
Molecularly imprinted polymers (MIPs) are the artificial receptors of targeted molecule and this combination is an analogue of natural antibody-antigen system. MIPs has advantages of lower cost, easier synthesis, better stability and repeatability compared to other artificial biological sensor and take great use of biology, chemistry, medicine, etc. There are different ways to initiate the reaction of MIPs synthesis, commonly including thermoinitiation, electrical initiation and photoinitiation. Since the photoinitiation has so many advantages, it is used widely in the field of controlled radical polymerization. Reversible addition-fragmentation chain transfer (RAFT) is one of the controlled radical polymerization and general technology for preparation of polymers with low polydispersity and functional terminal groups. The main feature of RAFT is to introduce a suitable chain transfer agent into the conventional free radical polymerization system which can effectively control the reaction by balancing free radical intermediates to mediate the growth of chain free radicals.

With the development of RAFT polymerization, thiocarbonyl compounds take part in the UV light controlled radical polymerization, no matter photoinitiator is used or not. However, during RAFT polymerization, the irreversible decomposition of chain transfer agent under UV irradiation in the duration of RAFT polymerization unavoidably leads to the permanent premature termination. Thus undermining the “living” character at relatively high conversions or long irradiation time. Furthermore, phototoxicity of UV light limits their application in the biological and biomedical fields.
To overcome the restriction, Cai et al. [10-13] confirmed that RAFT polymerization can be initiated by visible light and it was utilized afterwards [14-17]. Visible light can avoid the adversity caused by high-energy UV light during the polymerization process and overcome the disadvantage of template instability while preparing MIPs in UV light. But until now, visible light initiated polymerization of molecularly imprinted polymers is rare.

Surface plasmon resonance (SPR) sensors provide with high sensitivity, no marking, fast response and real-time monitoring, etc. They have been widely used in various fields of detection in combination with MIPs. Regarding, environmental detection, Ayankojo and Erdem et al. [18, 19] realize the detection of antibiotics amoxicillin and enterococcus faecalis in water by combining MIPs and SPR technique. In terms of food security, Zhang and Altintas et al. [20, 21] established MIP-SPR sensors for detection of different antibiotics in foods. In the field of biology, Kidakova et al. [22] utilized BSA-MIPs film combined with SPR technology based on surface-initiated active-radical photopolymerization and micro-contact imprinting to successfully detect bovine serum albumin which was further developed for A sensory system for clinically relevant proteins.

Progesterone is the main progestational hormone secreted by ovaries with biological activities which are crucial for maintenance of human health. Unstable secretion of progesterone content will negatively affect the normal physiological activities in human body. Now, there are many ways to detect progesterone such as high performance liquid chromatography, immunoassay, gas chromatography and mass spectrum, etc [23,24]. But these methods need complicated sample processing with high cost and poor stability and require organic phase rather than aqueous phase for successful detection. However, water content in human body is as high as 70 percent. So the development of simple, efficient and highly sensitive detection method of progesterone in water phase is particularly significant. Recently, Nezhadali et al. [25] used bulk MIPs prepared by chemical oxidation of pyrrole with FeCl₃ to selectively extract progesterone hormones from environmental and biological samples, and achieved good results. But this method needs a long time (24h) to prepare the MIPs and complicated post processing. Present study using Methacrylic acid (MAA) and acrylamide (AM) as functional monomer, progesterone as template molecule, ethylene glycol dimethacrylate (EGDMA) as cross-linker, 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDTPA) as initiator and chain-transfer agent, with no existence of other photoinitiator or catalyst, beginning with grafting of the MIPs film on the surface of SPR sensor chip in visible light which is simple and time-saving. The prepared sensor chips were successfully used for practical samples such as tap water, lake water and human urine with high sensitivity and stability.

2. Experimental

2.1. Materials and apparatus

Methacrylic acid (MAA) and acrylamide (AM) were obtained from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China). Progesterone, Estradiol, testosterone and estrone were purchased from J&K Scientific Ltd (Beijing, China). Ethylene glycol dimethacrylate (EGDMA), 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDTPA) and cysteamine were purchased from Aladdin Regent Company (Shanghai, China). N, N’-dicyclohexylcarbodiimide (DCC) was obtained from Macklin Biochemical Co., Ltd (Shanghai, China). NaH₂PO₄·2H₂O, Na₂HPO₄·12H₂O and H₂SO₄ were purchased from Beijing chemical plant (Beijing, China). NaCl, acetonitrile, ethanol and acetic acid were bought from Tong Guang Fine Chemicals Company (Beijing, China). MAA and EGDMA were distilled under reduced pressure before use. Phosphate buffered saline (PBS) was prepared from 150mmol/L NaCl and 20mmol/L phosphate. The pH value was adjusted using HCl and NaOH. The lake water used in the actual sample test was taken from the artificial lake in the campus (there are many fish in the lake, filtered twice at atmospheric pressure), and the urine samples used were from the same woman.

Contact angle instrument (Powereach, JC2000C, Shanghai Zhongchen Digital Technical Apparatus Co., China), UV-Vis spectrometer (U-3310, Hitachi, Japan), scanning electron microscopy (Zeiss
SUPRATM 55 SAPPHIRE, Germany), Nicolet 6700 FTIR spectrometer (Thermo Scientific, U.S.A.), light emitting diode (LED) point source irradiator with visible light ($\lambda = 450$nm, irradiation power of 3 W/cm$^2$) and ultraviolet light ($\lambda = 365$nm, irradiation power of 3 W/cm$^2$), SPR setup (home-built) based on Kretschmann configuration with 632.8nm laser source. The SPR substrate consisted of a glass slide (LaSFN9, refractive index = 1.845) with a metal layer (Au 50nm in thickness).

2.2. Surface modification of SPR chips
LaSFN9 substrates coated with gold film (50nm) were placed in an ethanol solution of cysteamine (5mmol) for 24h. After 24h, the substrates were washed with pure ethanol three times and dried with nitrogen. 16.5mg DCC was weighed in 40mL acetonitrile homogenized by ultrasonication for 10min and then placed in refrigerator for 20min to activate DCC. Then 16.0mg CDTPA (1.0mmol/L) was dissolved in the above solution. Finally, the cysteamine modified chips were placed in it for 24h. Afterwards, the chips were washed several times with acetonitrile and dried under a stream of nitrogen.

Progesterone (15.7mg), MAA (16.2μl) and AM (1.42mg) were dissolved in 2mL acetonitrile at room temperature for 3h in order to obtain the pre-complex of template and functional monomer. Addition of AM to MIPs, the polar monomer copolymerization changes the polarity of the MIPs matrix resultantly improving its specific binding, limited by a certain specific concentration to avoid higher non-specific adsorption [26]. Monomer ratio n(MAA): n(AM) = 95: 5 was chosen to prepare MIPs film. The cross-linker (EGDMA, 85.5mL) and RAFT agent (CDTPA, 4.0mg) were then added to the pre-polymerization solution, the final polymerization reaction solution was obtained after thorough mixing. The modified chips were installed on the SPR and the reaction solution was introduced to reaction cell. The reaction cell was illuminated by the LED visible light ($\lambda$=450nm) point source (maintaining the distance between the light source and the reaction cell to be about 1cm). Real time monitoring of polymerization kinetics was done in SPR. After polymerization, washing solution (acetic acid : PBS = 1:9, pH = 7.4) was used to remove the template molecules from the MIPs film. Non-imprinted polymers (NIPs) were prepared by the same method without adding progesterone molecule. The specific preparation process is shown in figure 1.

![Figure 1. Formation of MIPs film](image-url)
2.3. Characterization of MIPs film

MIPs films were synthesized and characterized by FTIR spectrometer, contact angle instrument and scanning electron microscope.

2.4. Detection by MIPs in SPR

The detection by MIPs and NIPs film in SPR was carried out at room temperature. A series of sample solutions with progesterone concentration of $10^{-16}$mol/L, $10^{-14}$ mol/L, $10^{-12}$mol/L, $10^{-10}$mol/L, $10^{-8}$ mol/L and $10^{-6}$mol/L prepared in PBS (pH = 7.4) were sequentially monitored for 16min in SPR sensor chip at a fixed flow rate of 1.0mL/min. The final reflectivity changes (ΔR) of each sample measured is the average of three parallel measurements.

3. Results and discussion

3.1. Optimization of irradiating light source

In order to select the appropriate wavelength of visible light to initiate the reaction, the UV-Vis absorption spectra of CDTPA solution with different concentrations prepared in different solvents are shown in figure 2. From the figure, it can be seen clearly that solvents have minor effect on the position and intensity of absorption peaks. With the concentration of 0.1mM, CDTPA has strong absorption in the ultraviolet region of 280-320nm, which is attributed to the π-π* transition of C=S group. Changing concentration to 5.0mM, CDTPA showed significant absorption in the visible region of 420-480nm due to the n-π* forbidden transition of the C=S group. The maximum absorption wavelength was around 450nm. Therefore, a visible light source with a wavelength of 450nm was selected for the following research.

In order to explore the photolysis effect of visible light ($\lambda= 450$ nm) and UV light ($\lambda= 365$ nm) on CDTPA reagent, a series of acetonitrile solutions with concentration of 0.1mmol/L CDTPA were exposed to aforementioned light sources for 0-150min, respectively. The UV-Vis spectra are shown in figure 3 (A) & (B). With the increase of illumination time, the absorbance of the solution decreases continuously with unaltered position of the absorption peak indicating the conversion of weak C-S bond in CDTPA to form alkyl active radicals and trithiocarbonate-based inactive radicals. The considerable change in the trend of absorbance verses illumination time in Visible and UV light source can be observed figure 3 (C). From figure, it is observed that the photolysis of CDTPA is fast under UV light which increases the concentration of free radicals produced by CDTPA in a short time leading to increased grafting rate, rendering it difficult to control the accurate termination of polymerization. Under visible light irradiation, the photolysis of CDTPA is slow, which provides considerable time for chain transfer, thus making the polymerization more controllable, resulting in uniformity of MIPs film. Therefore, the visible spot light source with a wavelength of 450nm is selected as the initiating light source for grafting MIPs film onto the surface of SPR chips.
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Figure 2. UV-Vis absorption of 0.1 mmol/L CDTPA in different solvents, inset graph is the visible light absorption of 5.0 mmol/L CDTPA in different solvents

Figure 3. U.V-Vis spectra of 0.1 mmol/L CDTPA acetonitrile solution irradiated by different wavelength light sources for 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min, 120 min and 150 min (A) $\lambda =450$ nm; (B) $\lambda =365$ nm; (C) Variation in illumination time verses absorbance values of two light sources

3.2. Effect of CDTPA concentration on polymerization rate

The polymerization time required to prepare MIPs films of the same thickness at different CDTPA concentrations, maintaining same conditions is shown in table 1. In the CDTPA concentration range of 0 to 5.0 mmol/L, the time required for polymerization is shortened with the increase of concentration which indicates the accelerated rate of polymerization. This can be attributed to the enhanced concentration of active radicals produced by photolysis of CDTPA. Increase in concentration after 5.0 mmol/L the results are inversely leading to decelerated polymerization rate. Dependence of photolysis rate of CDTPA on photoirradiation in the beginning causes increased rate at initial stage (see figure 3A) but at higher concentration, the delayed effect of the undecomposed CDTPA on the
polymerization cannot be neglected as it significantly reduces the concentration of the growing chain radicals by the RAFT equilibrium to suppress the polymerization rate. The above experimental results also prove the dual roles of CDTPA as initiator and chain transfer agent in the polymerization process. In order to reduce cost and save time, MIPs film was prepared by 5.0mmol/L CDTPA.

3.3. Contact angle measurement
The contact angle values of Au, cysteamine-Au, CDTPA-cysteamine-Au and the MIPs film modified Au were obtained as 89.43°, 18.64°, 93.77° and 75.41°, respectively, as shown in figure 4. The cysteamine molecule has a -SH group at one end and a hydrophilic -NH₂ group at the other. When cysteamine contacts the gold film, the -SH groups self-assemble on the surface making interior side of gold film through Au-SH bond while the -NH₂ groups are at the terminal, away from the gold film. So the surface of the gold film modified with cysteamine has strong hydrophilicity giving 18.64° as the contact angle. Subsequently, CDTPA molecule was self-assembled on the surface, one end of which was -COOH groups and the other end was hydrophobic alkyl chain. DCC condensating agent can combine -COOH in CDTPA with -NH₂ at the end of cysteamine through dehydration at room temperature. At this time, hydrophobic alkyl groups at the terminal of chain increase the contact angle to 93.77°. When the MIPs film was grafted onto the surface of the gold, the contact angle (75.41°) was greatly reduced indicating that the MIPs film had good hydrophilicity.

Table 1. Effect of different CDTPA concentrations on the polymerization rate

| Concentration of CDTPA(c) | Time of polymerization(t) | SPR resonance angle change value before and after MIPs polymerization (Δθ=θ_after-θ_before), n=3 times |
|---------------------------|---------------------------|------------------------------------------------------------------------------------------------------------------|
| 0                         | 5 h                       | 0                                                                                                               |
| 1.0 mmol/L                | 3 h                       | 0.5°                                                               | 10±0.04°                                                          |
| 2.5 mmol/L                | 70 min                    | 10±0.01°                                                          |
| 5.0 mmol/L                | 43 min                    | 10±0.07°                                                          |
| 7.5 mmol/L                | 56 min                    | 10±0.01°                                                          |
| 10.0 mmol/L               | 62 min                    | 10±0.01°                                                          |

Figure 4. Contact angle measurements: (A) Au; (B) cysteamine-Au; (C) CDTPA-cysteamine-Au; (D) MIPs film coated Au

3.4. SEM analysis
The surface morphology and cross section of MIPs and NIPs films were characterized by SEM, the results are shown in figure 5. It can be seen from the figure that the surface of the MIPs film is rough and there are many nano-size cavities uniformly distributed. These holes provide a specific binding site for template molecules to be attached on imprinted sites provided by MIPs. However, the surface of NIPs thin films is compact and there are no cavities pointing to absence of template molecules.
consequently lacking the imprinting sites with recognition function. Figure 5C shows that the thickness of MIPs films is about 100nm. The SEM images show that MIPs and NIPs films were successfully grafted onto the surface of SPR chip.

Figure 5. SEM images: (A) MIPs film; (B) NIPs film; (C) cross section of MIPs film

3.5. FTIR analysis
In figure 6, the number and position of infrared peaks in the spectrogram indicate the presence of different functional groups constituting MIPs and NIPs. The peak at around 1735cm⁻¹ is the characteristic peak of the stretching vibration of the C=O group [27,28]. The peaks near 1264cm⁻¹ and 1113-1165cm⁻¹ represent the antisymmetric and symmetric vibrations of C-O-C groups [29]. The peaks observed at 2964cm⁻¹ and 1450cm⁻¹ represented the antisymmetric stretching and bending vibration of methyl and methylene, respectively. The above results further indicate that MIPs and NIPs films have been successfully synthesized on the surface of SPR chips.

Figure 6. FTIR spectra of MIPs film and NIPs film

3.6. SPR analysis

3.6.1. Specific affinity. In order to evaluate the efficiency of MIPs films, a series of progesterone sample solutions with concentration of 10⁻¹⁶mol/L, 10⁻¹⁴mol/L, 10⁻¹²mol/L, 10⁻¹⁰mol/L, 10⁻⁸mol/L and 10⁻⁶mol/L were prepared in PBS (pH = 7.4) buffer solution and monitored through SPR in ascending order of concentration. The absorption of each sample was monitored for 16min and then filled with pure PBS buffer for 6min. From figure 7, it can be seen that the reflective light intensity increases gradually with the increasing concentration of samples. This is due to the combination of template molecules and specific recognition sites in the MIPs films, which leads to the change of refractive index, and increase the corresponding SPR resonance angle (figure 7A). In the concentration range of 10⁻¹⁶-10⁻⁶mol/L, the reflectivity changes (ΔR) is linear with the concentration of progesterone sample (figure 7C), the linear regression equation is $y=0.2037x+3.7918$, and $R^2=0.99956$.

3.6.2. Selectivity. Three progesterone analogues, estrone, testosterone and estradiol, were selected to ensure the selectivity of MIPs. Four sample solutions with concentration of 10⁻⁸mol/L were
investigated in MIPs film respectively, each sample was measured three times. The results are shown in figure 8. Compared with the three analogues, the MIPs film has a higher absorption capacity for the progesterone molecule in the same time (16 min), and the reflectivity change ΔR (%) in progesterone is 0.46%, estradiol 0.14%, estrone 0.13% and testosterone 0.12%. The reason is that the imprinted cavity formed by the MIPs film is identical in structure to the progesterone molecule, making it specific and selective in detection. Although the other three analogues are similar in structure to progesterone, the slight difference causes their weak interaction with the binding site, resulting in poor absorption. The same procedure was repeated with NIPs film. The response (ΔR) towards progesterone, estradiol, estrone and testosterone are 0.10%, 0.18%, 0.12% and 0.09%, respectively which ascertains previous results in need for compulsory presence of effective recognition sites and imprinted cavities for efficient detection, lacking which rendered NIPs film unable to absorb.

Figure 7. (A) Angular reflectivity spectra of MIPs:(1) after washing, (2)-(7) absorption samples of $10^{-16}$ to $10^{-6}$mol/L progesterone, inset graph is the enlarged part of resonance angles; (B) Absorption kinetics of progesterone from $10^{-16}$ to $10^{4}$mol/L; (C) Calibration curves for detection of progesterone in PBS (pH=7.4, n = 3, RSD = 3.6-8.9%)
3.6.3. **Reproducibility and stability.** Five absorption-desorption cycles were carried out for progesterone sample solution with concentration of $10^{-5}\text{mol/L}$ providing 15min absorption time. Then pure PBS (pH 7.4) was introduced for elution until the reflected light intensity was restored to the initial value and the next absorption was carried out. After five consecutive cycles, the change value of reflectivity ($\Delta R$) is obtained, as shown in figure 9. Compared with the first absorption, the $\Delta R$ value of each absorption cycle is about 100%, 97.78%, 92.92%, 80.76% and 71.89% which depicts better reproducibility of SPR sensor chips. After storing sensor chip under room temperature for 35 days, the absorption of $10^{-10}\text{mol/L}$ progesterone sample solution was performed with favorable results with 66% of original value of $\Delta R$ (figure 10) which showed the SPR sensor chips based on MIPs has a long lifetime and excellent stability.

3.6.4. **Practical implementation of sensor in real life samples.** In order to evaluate the performance of MIPs in real life samples; tap water, lake water and human urine were taken for absorption. A high concentration progesterone sample solution of $10^{-2}\text{mol/L}$ was prepared with acetonitrile and then a series of sample solutions with different concentrations were prepared separately by dilution with tap water, lake water and human urine. SPR was used to monitor the absorption properties of MIPs films in variety of sample solutions. The results are shown in table 2. It shows that the biosensor based on MIPs films can be successfully applied for the detection of progesterone in real environment and human urine.
Figure 10. (A) The absorption kinetics of MIPs film toward $10^{-10}$ mol/L progesterone after 0 and 30 days; (B) the reflectivity changes for rebinding of the MIPs film after 0 and 30 days.

| Validation parameters | Tap water | Lake water | human urine |
|-----------------------|-----------|------------|-------------|
| Linear range (mol/L)  | $10^{-14}$-$10^{-6}$ | $10^{-13}$-$10^{-7}$ | $10^{-11}$-$10^{-7}$ |
| Linearity (R²)        | 0.99943   | 0.9977     | 0.9949      |
| Slope                 | 0.0656    | 0.08007    | 0.15143     |
| Intercept             | 1.1060    | 1.21339    | 1.94659     |
| LOD (mol/L)           | $1.66\times10^{-15}$ | $7.55\times10^{-15}$ | $3.94\times10^{-13}$ |

4. Conclusion
In this paper, based on the RAFT rule, progesterone MIPs film on the surface of SPR sensor chip by visible light polymerization was successfully prepared for the first time. By optimizing the MIPs film in terms of CDTPA concentration, the MIPs film with the best efficiency was selected for further characterization: contact Angle measurement, SEM, FTIR. The MIPs film successfully grafted on the surface of the SPR chip can effectively be used to detect progesterone molecules, in real life samples with aqueous phase environment, promising high sensitivity, selectivity and stability owing to the existence of specific recognition and binding sites. It is inferred that Preparation of MIPs films using CDTPA as chain transfer agent and initiator in visible light is more suitable as it provides with moderate rate of polymerization compared with UV light. Moreover, optimized visible light polymerization makes it easier to control the thickness of MIPs films, providing with better uniformity and reproducibility of sensor chips which can realize large-scale production. This study predicts the possibility of low energy polymerization with cost saving methodology.

Acknowledgments
This research was supported by the National Natural Science Foundation of China (No. 20771015) and the National “111” Project of China's Higher Education (B07012).

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