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ORIGINAL PAPER

Novel Fluorescent Membrane for Metronidazole Sensing Prepared by Covalent Immobilization of a Pyrenebutyric Acid Derivative

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Abstract In the present paper, we report the fabrication of a new sensing membrane for fluorescence detection of metronidazole (MNZ). Briefly, a pyrenebutyric acid derivative, 2-(methacryloyloxy) ethyl-4-(1-pyrenyl) butanoate (MPB) with a double bond, was synthesized and copolymerized with 2-hydroxyethylmethacrylate (HEMA) on the activated glass surface by thermal initiation in the presence of cross-linker. The sensor responds linearly to metronidazole in the concentration range of 1.23~35.48 mg.L^{-1} in aqueous solution with a detection limit of 0.36 mg.L^{-1}. The lifetime is enhanced by covalently immobilizing the pyrenebutyric acid derivative on glass slide, which hinders leaching of the dye from the membrane. The sensor could be regenerated after use by washing in methanol (RSD=2.42 %), and it shows sufficient stability, and selectivity. Interference of other pharmaceuticals on membrane performance is discussed. The developed membrane has been successfully applied for the direct determination of metronidazole in human serum sample without pretreatment.

Keywords Metronidazole · Fluorescence quenching · Sensing membrane · Pyrenebutyric acid derivative · Serum

Introduction

Metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, has been demonstrated to have a great potential in antiprotozoal and antibacterial activities [1]. Basically, the metronidazole may penetrate the plasma membranes of bacteria and inhibit the replication of their DNA. Hence, the metronidazole has been widely used for the treatments of trichomoniasis, giardiasis, amoebiasis, acute ulcerative gingivitis, and dental infection in spite of being a bacterial mutagen and rodent carcinogen [2–4]. So far, several analytical methods, such as gas chromatography [5, 6], high-performance liquid chromatography (HPLC)[7–12], voltammetric [13–15] chemiluminescence [16, 17] capillary electrophoresis [18, 19] have been established for metronidazole measurement. However, these methods have some drawbacks such as time consuming, narrow range of determination, requirement of extraction, the use of nonaqueous systems, etc. Hence, it is important to develop an alternative method for metronidazole determination with a high degree of selectivity and sensitivity.

Fluorescence method has been widely used as a highly sensitive tool for determining and imaging biological-relevant events [20]. With an advance of fluorescence technology, many conventional organic fluorophores have been developed for versatile applications [21]. Pyrene is a particularly attractive dye because of its typical excimer/monomer emission property and extremely long decay time (400 ns, versus less than 10 ns for most organic fluorophores) [22, 23]. Zhu and co-workers have reported that fluorescence of pyrenebutyric acid can be quantitatively quenched by metronidazole and fabricated polymer membrane containing the dye by solvent casting method [24]. However, in the system pyrenebutyric acid dye molecules were immobilized by weak physical embedment into the membrane, so they were found to have significant leakage from the membrane accompanying with a decrease of emission intensity. Covalent immobilization [25–27] effectively prevents the leakage of the fluorescent dye from the membrane. In this article, we developed a new sensing membrane through immobilization of a pyrenebutyric acid derivative by covalent bond on glass slide. In brief, a polymerisable pyrenebutyric acid derivative MPB was synthesized and copolymerized with HEMA on the activated glass surface by thermal initiation in the

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presence of a cross-linker. The sensing membrane formed on glass slide was observed to have an excellent response to metronidazole in buffer solution and also display excellent reversibility, reproducibility, and selectivity during measurements. It is proved that the sensing membrane can also be used in human serum.

Experimental

Reagents and Chemicals

1-Pyrene butyric acid (PBA), 2-hydroxyethyl methacrylate (HEMA), 3-(trimethoxysilyl) -propyl methacrylate (TMSPMA), and ethylene glycol dimethacrylate (EGDMA) were purchased from J&K Chemicals, China. Metronidazole (MNZ) was obtained from Hangzhou Dayangchem Co. Ltd., China. Dicyclohexylcarbodiimide (DCC), 4-dimethylamiopryidine (DMAP), organic solvents, hydrogen peroxide, and 0.22 μm filters were commercially obtained from Sinopharm Chemical Reagent Co., Ltd, China. 2,2-azobisisobutyronitrile (AIBN) was supplied by Shanghai Reagent Factory, China. Before use, AIBN was re-crystallized in methanol. KH_2PO_4-HCl buffer solution at pH4.0 was used in the measurements. Double distilled water was made by a quartz apparatus from de-ionized water and used throughout all experiments.

Apparatus and Spectroscopic Measurement

All fluorescence spectral measurements were conducted on a Fluorescence Spectrophotometer (CRT 970 Shanghai, China) with a tunable excitation light source. Steady-state extinction and emission spectra of the immobilized dyes on the glass slide were collected placing the glass slide in the cuvette at a right angle of 90° to the excitation beam at the back of the glass slide, as shown in Fig. 1. Metronidazole

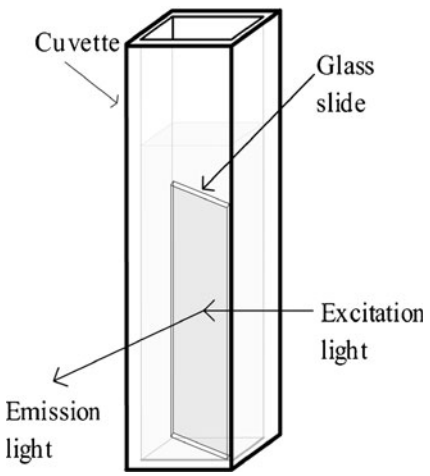

Fig. 1 Schematic diagram of the fluorescence measurement of MPB immobilized on glass slides inside cuvette

sensing was also tested in human serum. The serum was collected by filtering human blood through a 0.22 μm filter followed by diluting with a KH_2PO_4-HCl buffer solution at pH=4.0 to 50 % serum. The metronidazole was added to the diluted serum to obtain the solutions of 8, 16, and 24 mg L^{-1}, respectively. The recovery and relative standard deviation were calculated on the basis of emission intensity of the MPB-immobilized slide.

The pH measurements were carried out on a HANNA 211 pH meter (Italy). A DZF - 6020 Vacuum oven (Shanghai Heng Technology Co. Ltd) was used for the polymerization. ^{1}HNMR spectra were recorded on a ZAB-HS-400 (Varian) NMR spectrometer.

Synthesis of 2-(methacryloyloxy)ethyl-4-(1-pyrenyl) butanoate (MPB)

To covalently bind the dye on the glass, 2-(methacryloyloxy) ethyl-4-(1-pyrenyl)butanoate (MPB), a pyrene butyric acid derivative, was first synthesized through a direct esterification reaction between 1-pyrene butyric acid (PBA) and 2-hydroxyethylmethacrylate (HEMA) with dicyclohexylcarbodiimide (DCC) as condensation agent and 4-dimethylamiopryidine (DMAP) as catalyst, respectively (shown in Fig. 2.) [28–30].

Briefly, a solution of DCC (0.60 g, 3.1 mmol) in THF (7.0 ml) was added dropwise over the course of 20 min to 10.0 ml THF solution containing HEMA (0.33 g, 2.5 mmol), PBA (0.5 g, 1.7 mmol), and DMAP (0.05 g, 0.4 mmol) at 0°C. The reaction mixture was allowed to warm up to ambient temperature and continuously stirred for 36 h under nitrogen. The product was collected by purifying on silica gel column chromatography. A mixture of petroleum ether and ethyl acetate (7:3) was used for eluting the product (0.21 g, 42%).

^{1}HNMR (400 MHz, $CDCl_3$): δ=1.916 (s, 3H), 2.18–2.26 (m, 2H), 2.49–2.51 (m, 2H), 3.38–3.42 (m, 2H), 4.36(s, 4H), 5.54 (s, 1H), 6.1(s, 1H), 7.27–8.33 (m, 9H).

IR (KBr), ν (cm^{-1}): 1746.03, 1708.59, 1245.50 (due to C=O group)

These spectral data clearly demonstrated that the product is an ester. There is no IR signal around 3,500 cm^{-1} representing the absence of –OH group of HEMA and PBA. Thus, the product is regarded to be pure and free from HEMA and PBA.

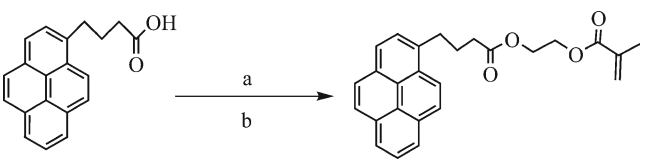

Fig. 2 Synthesis of 2-(methacryloyloxy) ethyl-4-(1-pyrenyl) butanoate. (**a**) 2-hydroxyethylmethacrylate (**b**) Dicyclohexylcarbodiimide, 4-dimethylamiopryidine, tetrahydrofuran, stir, 36 h, 43 %

Silanization of the Glass Slides

In this study, MPB was covalently bound on the glass slide through copolymerization with methacrylate monomer. In the experiment, the glass slides were first silanized using 3-(trimethoxysilyl)-propyl methacrylate (TMSPMA). Typically, the glass slides (1.1 cm, 2.2 cm) were cleaned in a piranha solution (7:3 v/v 98% H_2SO_4/ 30% H_2O_2) at 90°C for 1 h [27, 31] followed by rinsing with water and blow-drying with a stream of filtered nitrogen. Thereafter, the slides were immersed in a fresh 20 mmol solution of TMSPMA in toluene for 12 h, and then washed with toluene and chloroform, respectively. After gently dried under N_2 stream, the slides were cured in a vacuum oven at 100°C for 2 h to fully condense TMSPMA molecules onto the glass surfaces.

Immobilization of the MPB on the Modified Glass Surface

Pre-polymer solution was prepared with 0.03 g (0.231 mmoL) of HEMA, 0.003 g (0.0075 mmoL) of MPB, 0.0075 g (0.038 mmoL) of EGDMA, 0.001 g (0.006 mmoL) of AIBN dissolved in 2.0 ml ethanol, sonicated for 5 min. The mixture was transferred into a glass tube and the silylated glass slides were inserted. The solution was first deoxyenized with nitrogen for 5 min and then placed in an oven at 60°C for 6 h. Subsequently, the glass slides were transferred into another glass tube and filled with N_2 at 60°C for 24 h. Finally, the glass slides were Soxhlet extracted with methanol for 8 h to remove free monomers including MPB molecules.

Result and Discussion

Spectral Characteristics

In this study, MPB molecules were covalently bound in the polymer membrane on the glass slide via copolymerization with HEMA as monomer and EGDMA as crosslinking agent. Prior to the reaction, the glass slides were silanized using 3-(trimethoxysilyl)-propyl methacrylate (TMSPMA). A small amount of EGDMA was included as crosslinking agent in the reaction solution. Consequently, a stable polymer membrane was formed on the glass slide, which could be demonstrated by the change of water contact angle on the slide surface. Bare glass has a contact angle of 22° which changed to 77° due to the polymer immobilization.

Immobilized MPB on the glass slide could be evaluated by ensemble fluorescence spectra upon excitation at 347.0 nm in methanol, shown in Fig. 3. Two emission bands at 378.5 nm and 395.0 nm are almost same with the emission maxima of the free dye in solution. We also noticed that the emission bands of MPB in the membrane became broader than that of the free dye, supporting that the dye molecules were indeed immobilized in the polymer membrane leading to the significant restriction on their movements. On the other hand, the shape of emission band was not significantly altered implying that the most dye molecules were presented as monomer in the membrane.

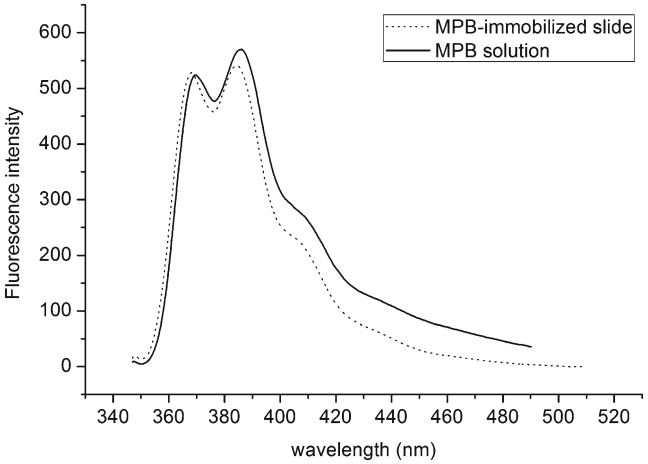

Fig. 3 Fluorescence emission spectra of the dye in solution and glass surface. The spectra were measured in methanol with λex=347 nm

Effect of pH

The emission intensity of immobilized MPB in the membrane was found to be efficiently quenched by metronidazole in buffer solution, implying that they could respond to metronidazole. The response was also found to be pH sensitive. The pH effect on the response performance was investigated by exposing the MPB-immobilized slide to a KH_2PO_4-HCl buffer solution with a pH value from 2.5 to 10, containing 2 mg.L^{-1} of metronidazole. As shown in Fig. 4, the maximum

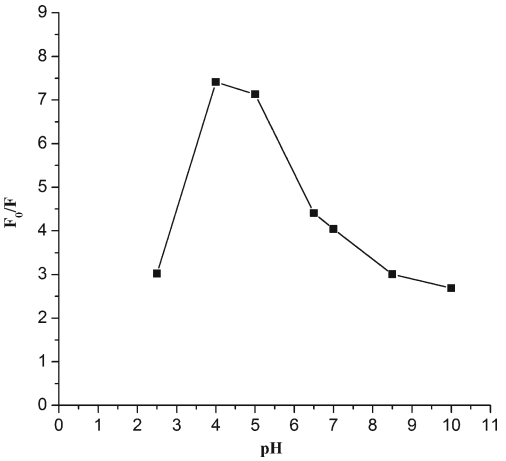

Fig. 4 Effect of pH on the F_0/F of MPB-immobilized slide in the presence of 2.0 mg.L^{-1} metronidazole, λex=347 nm

quenching efficiency appeared at pH=4.0. Hence, the buffer solution at pH=4.0 was used in the subsequent experiments.

Response of the Membrane to Metronidazole

Metronidazole measurements were performed by immersing an MPB-immobilized slide in metronidazole buffer solutions having different concentrations. The emission intensity from the MPB-immobilized slide was observed to decrease gradually with increase of metronidazole concentration in buffer solution, leading to a quantitative correlation between them (Fig. 5). The change on the emission maximum at 378.5 nm was selected in the analysis. A quantitative correlation was created as described in Equation (1),

$$\mathrm{Ln}(F_0/F) = 0.0555[\mathrm{MNZ}] - 0.0165 \quad (1)$$

In which, F_0 and F denote the emission intensities in the absence and presence of metronidazole in solution, respectively, and [MNZ] represents the concentration of metronidazole in buffer solution. It is noticed that the Ln (F_0/F) value can be linearly plotted against the metronidazole concentration in solution within a range of 1.23–35.48 mg.L^{-1} (inset of Fig. 5), representing that the MPB-immobilized slide can be used to quantitatively determine the metronidazole in a wide range of concentration in solution. It can be confirmed that the slide can be used as metronidazole optical sensor based on the fluorescence quenching [32].

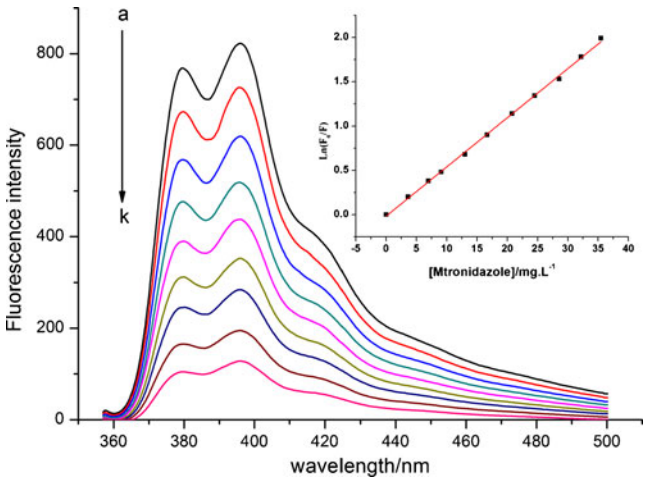

Fig. 5 The quenching curve of MPB-immobilized slide with increasing concentration of metronidazole in 0.01 M KH_2PO_4-HCl buffer (*pH*=4.0). [MNZ]: (**a**) 0, (**b**) 3.61, (**c**) 6.98, (**d**) 9.09, (**e**) 13.04, (**f**) 16.67, (**g**) 20.79, (**h**) 24.53, (**i**) 28.57, (**j**) 32.20, (**k**) 35.48 mg.L^{-1}. λex=347 nm. Inset: The calibration cure of fluorescence intensity natural logarithm [Ln (F_0/F)] to the concentration MNZ , fitting results: Ln (F_0/F) = 0.0555 [MNZ]−0.0165 Ln (F_0 / F)=0.0555 [MNZ] −0.0165, R^2=0.9989, LOD=0.36 mg.L^{-1}

The Stability of the Membrane

The dye molecules were covalently bound on glass slide, and thus, they could not be released into solution leading to a good stability. The stability of MPB-immobilized slide was evaluated in the research by monitoring the emission intensity from the slide with an interval of 100 min and the total time over 600 min (Fig. 6). It showed that the emission intensity remains almost unchanged supporting the MPB-immobilized slide was durable in the solvent and UV light, and owned good stability in the subsequent sensing measurements.

Reproducibility of the Membrane

It was noticed that MPB-immobilized slides had fast respond to the change of metronidazole concentration in solution and displayed an excellent reversibility in the measurements. Moreover, the emission intensity of the used slide was almost completely recovered by washing it with methanol (Fig. 7). The relative standard deviation of the emission intensity from the repetitive measurements was calculated to be 2.42 % (n=6), which was evaluated with 20.0 mg.L^{-1} of metronidazole buffer solution, representing its excellent reproducibility.

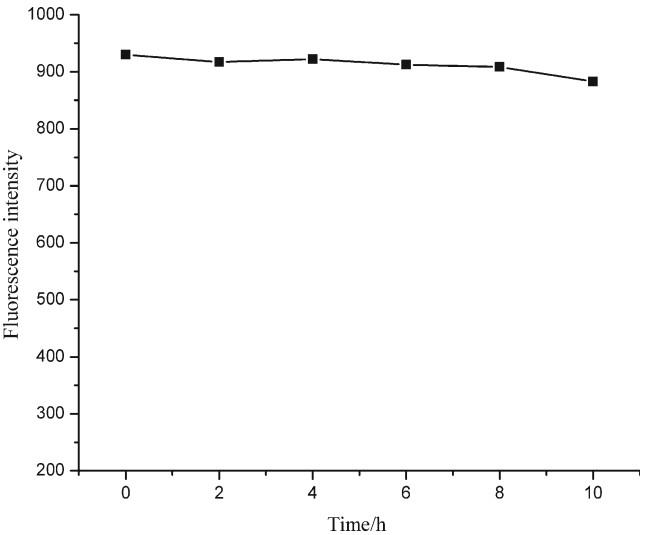

Fig. 6 Stability of fluorescence intensity of the membrane fabricated with MPB in continuously immersed in buffer medium *pH*=4.0, λex=347 nm

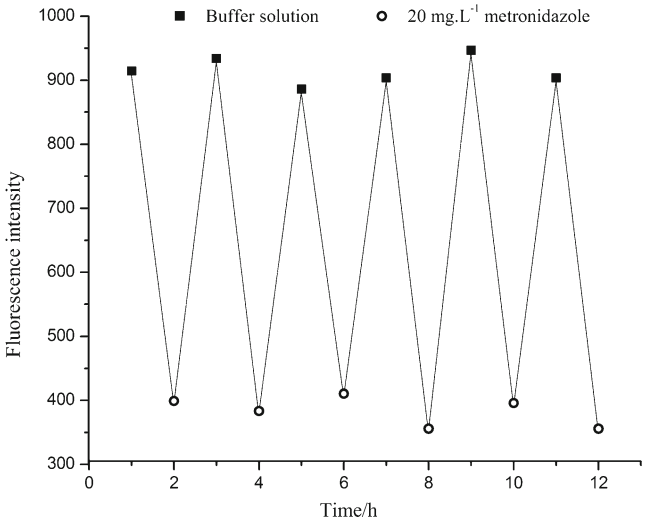

Fig. 7 The membrane exposed to 20 mg.L^{-1} metronidazole ○ and buffer solution *pH*=4.0 ■ in-between to return to the base- line, λex=347 nm

Interference Study

In application of the sensor for biological samples, it is important to know the interference from other pharmaceuticals. In this study, the interference measurements were conducted in a solution containing 5.0 mg.L^{-1} of metronidazole and 50.0 mg.L^{-1} of other pharmaceuticals. The data was collected and listed in Table 1, showing that lidocaine, lamivudine, or zidovudine could not cause obvious interference, whereas, amiodarone had a significant interference. Therefore, there is a need to further improve the selectivity of MPB-immobilized slide for metronidazole sensing. So far, the construction of sensing membrane using molecular imprinting technique is in process in our laboratory. More results will be reported with development of the research.

Table 1 Effect of interferants on fluorescence intensity of the MPB-immobilized slide in the presence of 5.0 mg.L^{-1} MNZ

Interferant	Concentration/ mg.L^{-1}	Relative error $[(F_2-F_1)/F_1] \times 100$
Lidocaine	50	−2.47
Lamivudine	50	−3.30
Amiodarone	50	34.75
Zidovudine	50	−2.84

Table 2 Recovery test of metronidazole added to the serum sample by using the MPB-immobilized slide (n=3)

Added/mg.L^{-1}	Found/mg.L^{-1}	Recovery (%)	RSD (%)
8.00	8.37	104.62	0.99
16.00	13.54	84.63	1.83
24.00	24.36	101.50	1.22

Analytical Application

To test the applicability of MPB-immobilized slide, metronidazole measurement was performed in human serum. In the experiment, the metronidazole was dissolved in the human serum to a certain concentration. An MPB-immobilized slide was immersed in the serum solution to measure the emission signal. It was shown that the emission intensity changed with the change of metronidazole concentration. The regression curve in the serum was also calibrated using the same approach for buffer solution. Using calibrated regression curve, the concentration of metronidazole in the serum solution could be estimated according to the emission intensity of the slide. The result showed, in Table 2, that estimated concentrations of metronidazole in the serum solutions are approximately consistent to the actual concentrations, representing that the current method is reliable for the sensing measurement. There was no additional sample pretreatment for the measurement, implying that the method is simple and fast.

Conclusion

In summary, we fabricated a new fluorescent membrane for the determination of metronidazole in aqueous solution. The pyrenebutyric acid derivative was covalently immobilized on glass slide by polymerization. The sensing membrane exhibited fast and quantitative response toward metronidazole in both buffer and human serum solution within a broad range of concentration. And the covalent immobilization offered excellent stability for the sensing membranes in the measurements. The MPB-immobilized slide was also found to possess excellent reversibility, reproducibility, and selectivity. All these features demonstrate that the MPB-immobilized slide has a great potential for clinical applications.

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