Fluorescent, Molecularly Imprinted Thin-Layer Films Based on a Common Polymer

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ABSTRACT: Fluorescent, molecularly imprinted polymer thin films, with cyclic guanosine 3′,5′-monophosphate (cGMP) as a template and 1,2-diphenyl-6-vinyl-1*H*-pyrazole-[3,4-*b*]-quinoline as a fluorescent receptor, were prepared according to a method based on commercially available poly (methyl methacrylate). This method of preparation predicts photoinduced crosslinking in the mixture of polymer chains and involved components. The advantages of this method are the relative simplicity of its preparation and the fact that a common polymer can be used. The spin-coated thin-layer films of imprinted and nonimprinted polymers were studied with the use of fluorescence microscopy with a scanning range of 80 × 80 μm. A strong fluorescence quenching effect was observed when a cGMP-imprinted film was incubated in aqueous solutions of cGMP, but a comparatively small effect was observed for a nonimprinted polymer and when an imprinted film was incubated with cyclic adenosine 3′,5′-monophosphate (cAMP). The separation factor by the imprinted polymer was determined to be 2.55 for cGMP against cAMP. The obtained polymeric sensor appeared to be stable during subsequent measurements after rewashing and readsorption. The homogeneity of the surface of the polymer film, dependent on the method of film preparation, was also studied. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 229–235, 2007

Key words: fluorescence; molecular imprinting; molecular recognition; sensors

INTRODUCTION

The molecular imprinting of polymers has become a widely used method that provides synthetic polymers with specific binding sites. Molecularly imprinted polymers (MIPs) are attracting increasing interest for a variety of applications. Sensing systems based on the molecular imprinting of polymers have been used as filling for chromatographic columns,[1,2] elements for drug screening and delivery,[3] and sensing plates in sensory systems.[4,5] In general, the synthesis of MIPs involves the association of appropriate functional monomers around a template molecule followed by polymerization in the presence of a crosslinker and photoinitiator. After the extraction of the polymer and removal of the template, the obtained molecular cavities are ready for the subsequent adsorption and recognition of the target molecule—the template. The recognition sites are specific for the template molecule in both shape and size and in chemical functionality. Additionally, the MIPs are stable chemically and mechanically. Although the most effective method of MIP preparation is the copolymerization of functional monomers in the presence of the template, new methods of preparation have appeared recently.[6,7] These methods have been developed with the aim of obtaining easy-to-prepare and chipper functional polymers to be used for separation,[8] drug delivery,[9] or sensory systems.[10] The need for an easy and fast methodology for MIP preparation prompted us to develop an MIP sensory system based on a commercially available polymer, poly(methyl methacrylate) (PMMA). We expect that the preparation method will give a material efficient in the selective adsorption of the adsorption of template, with separation factor comparative to data of some commonly used MIPs.[11] However, it also seems important that less complicated processing based on a commercially used polymer will increase the availability.

Some examples of the molecular imprinting of polymers based on a phase-inversion method involving commercially available polymers such as poly (vinyl chloride), polystyrene, and polyacrylonitrile have been successfully used for the preparation of recognition materials for dibenzofuran.[12,13] Recently, poly (ethylene-*co*-vinyl alcohol) and a biological polymer,

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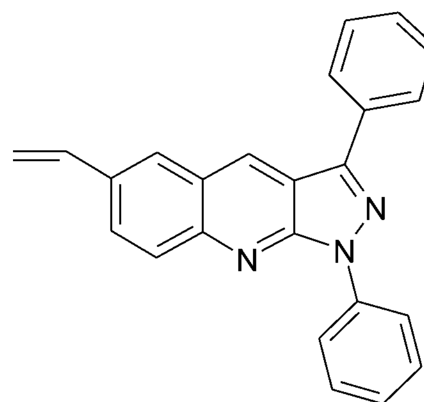
dextran, were molecularly imprinted with α-amylase[14] and successfully tested as recognition materials.

The molecular imprinting of polymers involving a particular target molecule—a template, imposes restrictions on the choice of the components, such as the monomer and/or polymer, receptor, transducer, and solvent, as well as suitable processing. The template determines the polymer structure as well as the analytical methods, and the whole strategy due to MIP development has to be correlated. Our strategy for the development of an optical sensor based on molecular imprinting technology is to use a fluorosensor as a functional monomer,[15–18] so we have two functions, a receptor and a transducer, in one. This approach has the advantage that it provides less signal divergences and additionally limits the number of components within the system.

In the reported case, the target molecules are nucleotides in particular cyclic nucleotides, and the general idea of these studies is to incorporate a fluorescent molecule as a transducer into the sensing system to obtain an optical sensor. Cyclic nucleotides have very important functions in many biochemical processes, acting as second messengers in cell metabolism. Despite the great importance of cyclic nucleotides to living organisms, there have been only a few examples of their use as templates in molecular imprinting.[15–18] However, studies on other compounds connected to cyclic nucleotide functions have also been reported.[19] The purpose of this report is an optical sensor for cyclic guanosine 3′,5′-monophosphate (cGMP), a major intracellular mediator of extracellular signals such as nitric oxide and natriuretic peptides. In general, major cyclic nucleotides are similar in structure and differ from one another by functional group arrangements; that is why the use of a particular nucleotide as a template in the processing of MIPs can provide valuable information about the importance of these functional groups for the recognition process.

As mentioned earlier, this approach considers an application of a fluorescent, specific molecule both as a receptor and as a signal transducer. 1,2-Diphenyl-6-vinyl-1*H*-pyrazole-[3,4-*b*]-quinoline (PAQ), whose structure is shown in Scheme 1, has been used here as the functional monomer. It belongs to the family of pyrazolequinoline derivatives. The functional vinyl group derived in pyrazolequinoline provides an easy incorporation of the receptor into the polymer matrix. The pyrazolequinoline derivatives are typical charge-transfer compounds that are very sensitive to environmental interactions. The influence of the solution polarity on the photophysical properties of PAQ as a free molecule in solution have been widely studied.[20,21] We have also studied the photophysical properties of this particular derivative of pyrazolequinoline as a photoactive element anchored at the polymer surface.[4] It has been concluded there that the incorporation of PAQ to the polymer surface does not affect the photophysical properties in comparison with the properties in solution. It is worth mentioning that the PAQ and other compounds from the pyrazolequinoline family have been already used as dopants in polymer films.[22,23] However, no pyrazolequinoline derivative has been used as a receptor in MIPs.



Scheme 1

This report describes the processing of an MIP based on a PMMA (commercial sample) and involving a fluorescent receptor, PAQ, to obtain an optical sensor for cyclic nucleotides. We report here the monitoring of the sensing system with the use of fluorescence spectroscopy and microscopy. Additionally, the aqueous environment used for analytical purposes increases the attractiveness of the preparation methodology. This method of sensor preparation is simple and cheap, and it can be used for other purposes as well.

EXPERIMENTAL

Materials

The preparation method of fluorescent, molecularly imprinted thin polymer films was based on commercially available PMMA (100,000 g/mol) obtained from Solvent Wistol S. A. (Oswiecim, Poland). PAQ was prepared as described elsewhere.[24] Benzoin ethyl ether and sodium salts of cyclic nucleotides—cyclic adenosine 3′,5′-monophosphate (cAMP) and cGMP—were purchased from Sigma–Aldrich Co. (St. Louis, MO; Poznan, Poland) and used as received. Aqueous solutions of nucleotides were made with the use of double-distilled, deionized water. Tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich. Both solvents were spectroscopic-grade and were checked for impurities by absorption and fluorescence measurements.

Sample preparation

The method of MIP preparation, as illustrated in Figure 1(a), involves three general steps: (1) complex-

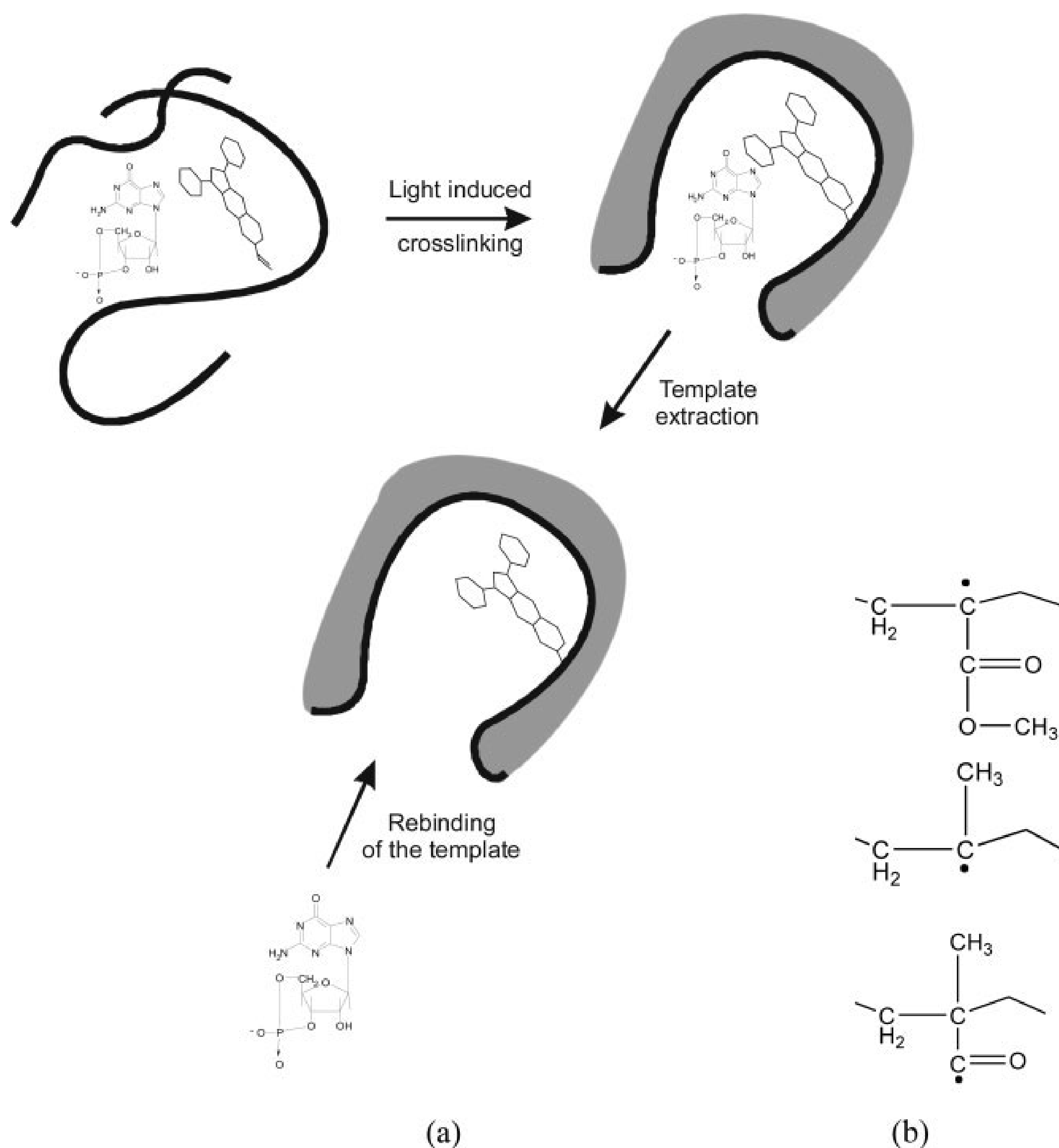


Figure 1 (a) Molecular cavity formation and (b) possible products of the UV irradiation of PMMA.

ation between a fluorescent functional monomer and a template either in one of the solvents within the two-component mixture or at the interface, (2) light-induced crosslinking of the polymer and the complex in the presence of a photoinitiator, and (3) drying and soaking of the final product. The complexation takes place in a mixture containing a $10^{-4}M$ solution of PAQ in THF and a $10^{-4}M$ solution of cGMP in DMSO in a 1 : 1 (v/v) ratio. Although we studied, using fluorescence spectroscopy, the interaction between the precursors PAQ and cGMP in solution,[21] the stoichiometry of the complexation in the THF and/or DMSO environment is unknown; the mode of PAQ in organic solvents protonated with acid addition shows the possibility of 1 : 1 and 1 : 2 stoichiometry, which depends on the derivatives of pyrazolequinoline.[25] The mixture of the precursors was purged with argon for 0.5 h to eliminate oxygen. Afterwards, the mixture was stored in darkness overnight at room temperature to stabilize it and to allow the complexation. Then, the solution was mixed with 5% (w/w) PMMA dissolved in THF and left overnight to equilibrate and to form a suitable structure in the mixture. Although PMMA dissolved slightly better in THF than in DMSO, we did not observe a noticeable inhomogeneity in the distribution of the fluorescence of the film and the same sensing sites. Because PMMA is characterized by a small absorption of UV light and is rather resistant to crosslinking when irradiated by UV,[26] a strong α-cleavage polymerization photoinitiator, benzoin ethyl ether, was added (5% of the PMMA volume) to increase the number of active centers able to crosslink, to increase the probability of anchoring the PAQ fluorosensor and/or PAQ/cGMP complexes to

the PMMA chain during the preparation, and to increase the probability of the crosslinkage of the statistically distributed chains. Benzoin ethyl ether has been already used as an initiator for MIP polymerization[27,28] and appears to be an effective photoinitiator of crosslinking. Within this approach, we predicted the statistical anchoring of the PAQ/cGMP complex to PMMA side chains involving PAQ vinyl group activation due to either benzoin ethyl ether breakup or the activation of the PMMA backbone or side groups. PMMA irradiated by UV light undergoes degradation through chain breaking but also through side-group abstraction and/or destruction with three possible radicals, as shown in Figure 1(b), which are able to bound the complex. All lead to radical formation.[26] In this approach, we assume that it is most important to fix the complex, but the position in which the PAQ/cGMP complex is anchored at the polymer chain is not important at all. Additionally, the initiator incorporation leads to a reduction of the time necessary to complete the preparation. The reduction of the exposure time is also important in view of the overlap of the cGMP absorption and the light source wavelength emission range. The limitation of the exposition time decreases the probability of cGMP mutations, damage, and degradation, which might occur during exposure to UV irradiation.[29]

The final mixture was cast onto quartz plates with either the drop-casting or spin-coating method. The polymer films were exposed to UV irradiation with a maximum at 350 nm in an atmosphere of argon. Subsequent storage in an oven for 24 h at a pressure of 0.06 cmHg at 60°C was applied to eliminate solvent leftovers, particularly THF, and to finish the processing through thermal stabilization. After the main preparation and storage, the films were extracted in 10 cm^3 of deionized water for 24 h until no change in the fluorescence was observed, and no available cGMP site stood filled; DMSO leftovers were expected to be removed after the washing of the polymer in water. Subsequent incubation of the film in 10 cm^3 of an aqueous solution of $10^{-4}M$ cGMP for 24 h provided readsorption of the template. The influence of cGMP adsorption on the MIP fluorescence was studied by steady-state and time-correlated fluorescence microscopy. The corresponding nonimprinted polymer was prepared in the same way, except that no cGMP template was added. The nonimprinted polymer was treated with the same procedure. Although this approach differs from that previously reported, the procedure was typical.[15,18]

Experimental setup

The fluorescence signal of cGMP-imprinted and nonimprinted polymer films was collected with a Microtime 200 confocal lifetime microscope from Picoquant GmbH (Berlin, Germany). The setup included an inverted optical microscope (IX-71, Olympus, Tokyo, Japan). The sample holder was positioned on an XY piezo-scanning stage with a scanning range of 80 × 80 μm and a resolution of 1 nm. The images were obtained by the scanning of the sample over a 100× objective (Olympus). The emitted photons were collected in an epifluorescence mode, after passing the appropriate filters, by a single photon avalanche diode.

The sample was excited by a diode laser (5 mW of power), with an average emission in the range of 370–380 nm and a maximum intensity at 375 nm, purchased from Picoquant. The Microtime 200 software was used for system control, data acquisition, and data processing.

Steady-state fluorescence spectra were acquired with a Spex Fluorolog 3 spectrometer with the spectral bandwidth of a 2-nm band pass for both excitation and emission shutters and were collected with a Peltier-cooled R636-10 photomultiplier detector (Hamamatsu, Hamamatsu City, Japan).

RESULTS AND DISCUSSION

The preparation of the film and the formation of the molecular imprint with the application of a commercially available polymer are shown in Figure 1(a,b) and described in detail in the Experimental section. In the first step, the various components prearrange to form a supramolecular structure around the previously formed complex between a functional monomer fluorosensor and the template. This approach considers polymer chains surrounding the complex to be a result of weak noncovalent interactions, such as van der Waals forces. The second step involves the light-induced crosslinking of such an organized polymeric

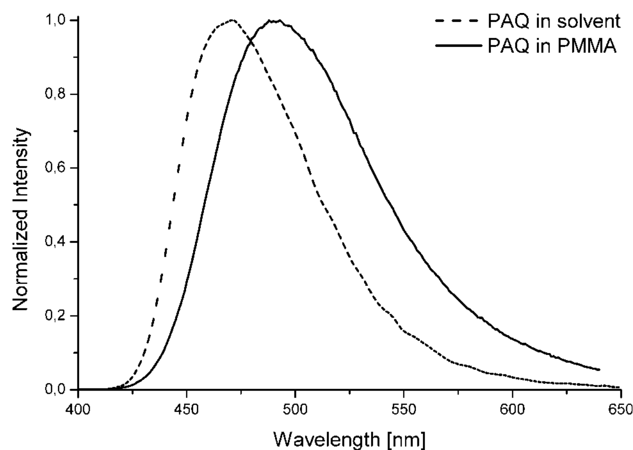


Figure 2 Normalized florescence spectra of PAQ diluted in $10^{-5}M$ THF and in a film of PMMA obtained by the spin coating of PMMA in a 5% (v/v) THF solution and $10^{-5}M$ PAQ.

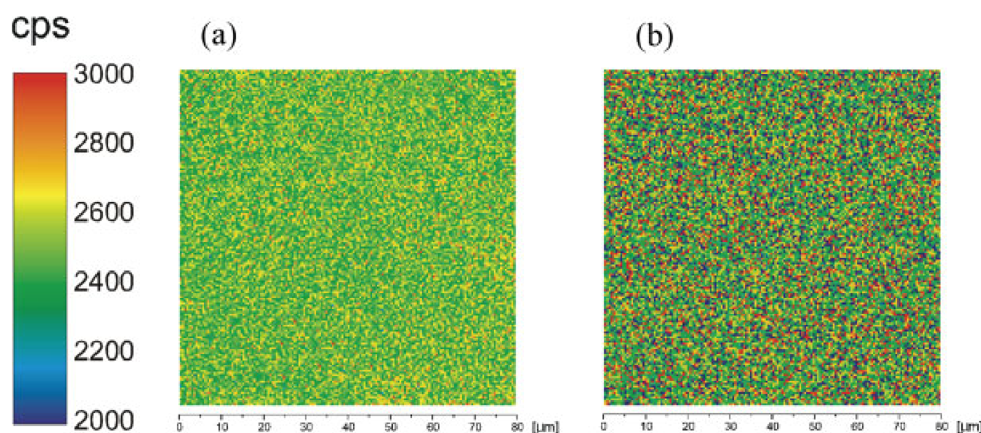


Figure 3 Fluorescence intensity images of (a) spin-coated (~500 nm) and (b) drop-cast (~5 μm) molecularly imprinted polymer thin films recorded with the use of fluorescence microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mixture in an inert atmosphere in which the oxygen concentration at the polymer surface is sufficiently low that it does not affect the process. The subsequent extraction and the template removal lead to the formation of a molecular cavity able to adsorb the template. Not only does the molecular cavity recognize the template by the shape and size, but the electrostatic interactions play a noticeable role in the recognition mechanism as well. Figure 2 shows normalized fluorescence spectra of a PAQ fluorosensor in solution and as a molecule incorporated into a polymer film. The incorporation of the molecule into the polymer leads to a redshift and broadening of the emission spectrum. The latter observation can be attributed to an increasing variety of interactions within the stiff film and is usually observed for fluorophore-labeled polymers.[30] The shift of the maximum of the emission toward a lower energy at the wavelength scale might be due to a loss of excitation energy for some interactions and nonradiative processes within the polymeric cavity. Generally, no change in the character of an MIP fluorescence emission spectrum after washing and reincubation has been observed.

Figure 3 shows two fluorescence microscopy images of MIP thin films with a PAQ molecule as a receptor and cGMP as the template. The films were obtained with the use of spin-coating (left image) and drop-casting methods (right image). Those images show the distribution of the fluorescence intensity over an 80 × 80 μm^2 area of the polymer film surface. There are noticeable differences in the distribution of the fluorescence intensity. They suggested a better homogeneity of the film produced with the use of the spin-coating technique. The spin-coated surface is supposed to be more stable in terms of the fluorescence signal distribution, whereas the drop-cast sample has stronger disorder of the fluorescence signal. Although only small areas of the sample are presented, similar differences have been observed for the whole sample surface. From the point of view of reproducibility, the spin-coating method thus offers better, more homogeneous samples than the drop-casting method.

The fluorescence spectra from the cGMP imprinted film before and after incubation in an aqueous solution of cGMP are compared in Figure 4. One can observe a significant decrease in the fluorescence intensity and no change in the character of the fluorescence spectrum. This decrease in the emission is due to the quenching of fluorescence after incubation of the cGMP-imprinted polymer film in an aqueous solution of cGMP and after readsorption of the nucleotide into the film. The empty binding sites, which appeared after extraction, were capable of subsequent adsorption of cGMP from the solution, and that is now detected as a change in the fluorescence intensity.

The effect of the fluorescence change for cGMP-imprinted polymeric films incubated in the presence

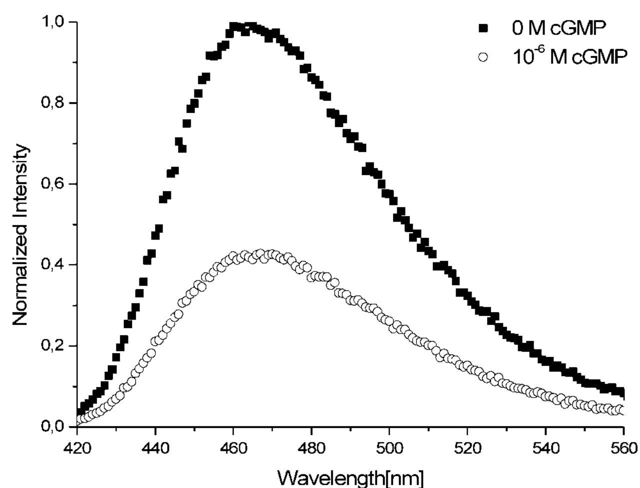


Figure 4 Effect of fluorescence quenching on the MIP spectrum before and after incubation in $10^{-6}M$ aqueous cGMP.

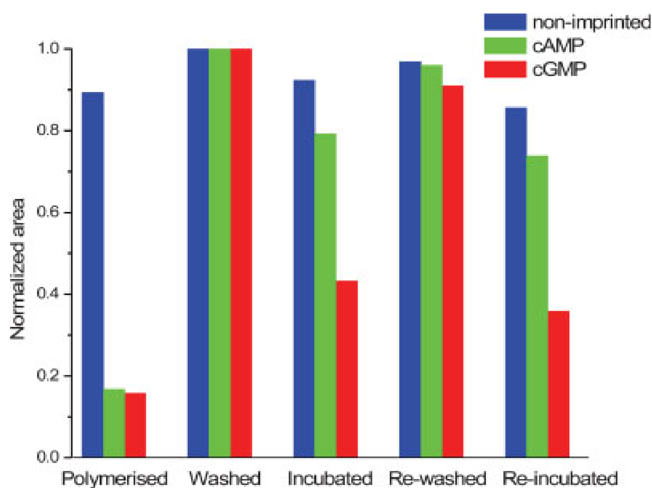


Figure 5 Selectivity effect on the fluorescence from molecularly imprinted and nonimprinted films incubated in an aqueous solution of either $10^{-6}M$ cGMP or $10^{-6}M$ cAMP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of cGMP and cAMP and, for comparison, the results of the same measurements for a nonimprinted polymer that underwent the same procedure of extraction and readsorption are shown in Figure 5. cAMP has to be chosen as similar in size and shape to determine the selectivity of adsorption of the cGMP-imprinted polymer. The integrated emission was measured to assess the fluorescence emission.

One can see in Figure 5, columns 1–4, that after the film preparation, the fluorescence of the nonimprinted polymers and that of the imprinted polymers are significantly different. The fluorescence from the nonimprinted polymer film is relatively high, whereas for the imprinted film, the emission is reduced to around 20% of that of the nonimprinted film. During the process of molecular imprinting, the PAQ/cGMP complexes are fixed in the crosslinked, porous polymer, and the fluorescence of the fluorophore is reduced, being quenched by cGMP. After the extraction of the nucleotide, the fluorescence of the imprinted film significantly increases, as seen in Figure 5, column 5, in comparison with that of the nonimprinted one. This is the reason that the cGMP molecules, which are involved in the complex formation with PAQ and entrapped in the film, have been removed during extraction. The cGMP release causes a significant (ca. 80%) increase in the film fluorescence, as shown in Figure 5, columns 2 and 4. After the same process, almost no change in the emission from the nonimprinted polymer has been observed. The subsequent incubation in an aqueous solution of cGMP causes a decrease in the fluorescence of about 60%. As one could expect, for the cGMP-imprinted film incubated in an aqueous solution of cAMP, the quenching of fluorescence is significantly less (ca. 20%). In our case, the extraction and rebinding have been done twice for every polymer film with no noticeable differences in the fluorescence measurements, as shown in Figure 5. This material has been used twice but seems to be robust enough to be further used repeatedly.

CONCLUSIONS

The foregoing discussion has shown that the application of a simplified method to produce molecularly imprinted films can give a material with properties relatively similar to those of films prepared according to common MIP protocols. The use of a commercially available polymer for the processing of the sensory system increases the attractiveness of the material. A spin-coated film has shown satisfactory and better homogeneity than a drop-cast film in terms of the fluorescent molecule distribution in the whole film. The selectivity of the film against the template is moderate, and the separation factor is 2.5. Studies to improve these characteristics are therefore worthwhile.

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