Thin-Layer Molecularly Imprinted Sensors Studied by Fluorescence Microscopy

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Fluorescence microscopy was used to study a molecularly imprinted thin-layer polymeric sensor for nucleotides (MIP), in which 1,2-diphenyl-6-vinyl-1H-pyrazole-[3,4-b]-quinoline (PAQ) was used as a fluorescent functional monomer and the nucleotide, cyclic 3',5'-guanosine monophosphate (cGMP) as a template. The effects of extraction of the template and its subsequent re-adsorption on the fluorescence of the films were monitored using steady-state and time-resolved fluorescence microscopy. Six-fold fluorescence quenching of the MIP upon the analyte adsorption was observed, whereas the non-imprinted polymer (NIP) fluorescence stayed essentially unaffected. The results of steady state and time-resolved fluorescence microscopy show that the intensity and lifetime distributions are strongly affected by the molecular interactions during the extraction and re-adsorption processes. Widening of the distribution is observed when cGMP is extracted from the sensor, and narrowing of the distribution when cGMP is absorbed from its aqueous solution to the MIP. [DOI: 10.1380/ejssnt.2010.293]

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I. INTRODUCTION

Molecular imprinting of polymers is a method that produces synthetic polymers having pre-determined recognition properties [1, 2]. The wide diversity of interactions that can lead to specific and selective adsorption of the target molecules into the molecularly imprinted polymers (MIPs) has attracted significant interest of such systems during the last two decades. MIPs have been used in filling of chromatography columns [3, 4], catalysis [5], immunoassays [6], drug screening [7] and sensors [8, 9]. The synthesis of molecularly imprinted polymers involves the process of co-polymerisation of the functional monomers and cross-linker in the presence of a template. Removal of the template after the polymerisation provides the empty sites complementary to the template not only in shape and size, but also in the distribution of functional groups in the sites. Then, these sites are able to recognize and selectively adsorb the target molecule. Importantly, the selectivity towards recognition of the template depends on the quality of the molecular cavity [10].

Although molecular imprinting is conceptually understandable and seems to be relatively easy to apply for any target molecule, there are still uncertainties that are difficult to recognize and to explain [11, 12]. It is well established that different spatial configurations of the supramolecular structure formed by the template and functional monomers association strongly depend on the conditions of polymerisation [13]. Consequently, the arrangement of the polymer network formed around the template during polymerization, as well as the quality of extraction, affect the properties of the sites and, indirectly, the molecular recognition. The interaction of fluorescent functional monomers with the template can give information not only about the binding phenomenon but about the quality of the site as well [10, 14]. Given this fact, the template and the receptor would seem to be the most significant elements in the molecularly imprinted polymeric sensor preparation. In the case of optical sensing systems, the study of the template-receptor interaction is vital to the improved design of MIPs. Therefore, study on thin-polymer MIPs by fluorescence microscopy as a tool having potential to support the analysis is promising [9, 15].

We report here a comprehensive, quantitative study on the ability to recognize cGMP by thin-layer MIPs using steady-state and time-resolved fluorescence microscopy, which is in contrast to a previously presented study that employed conventional fluorescence spectroscopy [9]. Steady-state microscopy confirmed those results obtained with the use of conventional spectroscopy. We also discuss the usefulness, and applicability, of time-resolved fluorescence microscopy to investigate the distribution homogeneity or heterogeneity of binding sites within the thin-layer MIP.

II. EXPERIMENTAL

The template molecule, cyclic guanosine 3’5’-monophosphate (cGMP) was dissolved in DMSO yielding 10^{-5} mol·L^{-1} solution. Then, 2 ml of the fluorescent functional monomer 1,2-diphenyl-6-vinyl-1H-pyrazole-[3,4-b]-quinoline (PAQ) [16] in THF (10^{-5} molcotL^{-1}) was added to 2 ml of cGMP solution and the whole was left overnight to assure formation of the supramolecular assembly between PAQ and cGMP. Next 20 ml of poly(methyl methacrylate) (PMMA) dissolved in THF

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and incorporated in the MIP layer. The fluorescence was excited by a diode laser at 375 nm and collected by a Peltier cooled photomultiplier at 490 nm [9]. The fluorescence spectra (FM) of the MIP being quenched in the presence of cGMP are shown in Fig. 2(a). The intensity of fluorescence in the FM images decreases with the application of higher concentrations of cGMP. The influence of cGMP on fluorescence of the NIP (Fig. 2(b)) is negligible. Therefore, the existence of imprinting sites with the MIP can be confirmed. Importantly, no significant changes occur in the shape of the fluorescence spectra during the whole cycle of the experiment.

A. Fluorescence intensity imaging microscopy

The recognition of cGMP by MIP can be assessed by comparison of the distributions of the fluorescence intensity obtained from the FM images for the MIP and NIP, respectively. The distributions of the intensity around the most probable value of the fluorescence intensity during the processing are shown in Fig. 3. Noticeably, a significant increase of the full width at the half maximum of the distribution after extraction of the MIP is observed when the template is removed, and the interior of the molecular cavity loses its equilibrium under the interactions with cGMP. By this a greater variability of environmental interactions with the fluorophore is detected. The full width at the half maximum of the distribution of fluorescence intensity from the imprinted polymer increases more than twice, whilst the changes in the values of the distributions for the non-imprinted polymers are negligible. A significant increase (about six times) in fluorescence intensity of the MIP, after extraction of cGMP from the film, is observed while the fluorescence intensity of the NIP increases much less (∼10%). The change observed for the NIP is assumed to be due to interactions between cGMP and PAQ at the surface of the film. Similar results were obtained using conventional spectrophotometer-based steady-state fluorescence spectroscopy, as reported previously [9]. The spectrophotometer-based acquisition gives, however, only a general picture of phenomena occurring, whereas microscopy gives us opportunity to study the system more in detail.

The distributions of fluorescence intensity demonstrate how the adsorption of cGMP affects the photophysical processes, and particularly the dissipation of excitation energy of the PAQ fluorophore. It is worth mentioning that while intensity measurements are relatively simple and accurate, they are often inadequate. In fluorescence microscopy is often impossible to precisely control the amount of probe at the each point of the image [17]. Photobleaching, phototransformation or diffusive processes are the main reasons affecting the intensity measurements done by fluorescence microscopy. It is of particular importance for thin layer MIPs, for which a little change in thickness may cause significant difference in fluorescence signals. In order to further explore these phenomena in detail time-resolved fluorescence microscopy measurements were done.
Counts

Relative intensity [a.u.]

1000

1200

1400

1600

Counts

Relative intensity [a.u.]

1000

1200

1400

1600

200

400

600

800

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

FIG. 2: Normalised fluorescence emission spectra of (a) the MIP and (b) NIP before and after overnight incubation in the $1 \times 10^{-2}$ M solution of cGMP.

![Fluorescence intensity distributions](image)

FIG. 3: Fluorescence intensity distributions of the imprinted (a) and non-imprinted (b) polymer: 1–before extraction, 2–after extraction, and 3–after rebinding.

B. Fluorescence lifetime imaging microscopy of MIPs

Fluorescence intensity decay, as measured by time-domain methods, is represented in terms of the multi-exponential model

$$I(t) = \sum_i A_i \exp(-t/\tau_i),$$

where the $A_i$ are the pre-exponential factors and the $\tau_i$ are the decay times. There is a wide variety of molecular interactions within the local environment that can influence the fluorescence lifetimes. The measured fluorescence lifetime is usually shorter than the radiative one due to other decay rates that depend on these interactions. The lifetime distributions taking into account the existence of two or more different forms of the probe represent their fractions depending on the analyte concentration. Nevertheless, in polymers one of the fractions can be independent on the analyte concentration. It can be due to the interaction of the fluorophore with the polymer itself. In this case all forms can be excited at the same wavelength but their decays will be different. Under these conditions the sample displays fluorescence lifetimes that are characteristic of analyte-free and analyte bound forms of the fluorescent functional monomer. The change in lifetime is due to binding of the analyte, and it may result from changes in radiative and non/radiative decay rates. By establishing the relative contributions of the lifetimes, depending on concentration, one can establish the fraction of active cavities. Numerous molecular interactions can be responsible for creation of a complex or non-exponential decay. If a single fluorophore is present in two different environments, bound and free, then the pre-exponential factors often represent the fraction of the molecules in each environment.

Since the fluorosensor incorporated into the MIP is covalently bound to polymer it is involved into two types of interactions, namely covalent and noncovalent. The first is a high energy bond, and is fixed to the polymer at the room temperature in an aqueous environment; the second depends on the environment, and on the presence of the template inside of the molecular cavity as it was originally established through polymerization.

In our case the pyrazolequinoline receptor was evenly distributed in the polymer film and, as we assume, is situated inside the molecular cavities which are distributed inside the polymeric film. The parameters obtained for the MIP sensor from time-resolved measurements are shown in Table I. The data show a significant increase of the fluorescence lifetime after extraction of the template cGMP from the film, from 15 ns to 25 ns. After re-incubation with cGMP, the fluorescence lifetime decreases again to almost the same value (16 ns) while there is virtually no change of the lifetime during the same processing of the non-imprinted polymer. The observed variability of the fluorescence lifetime of the non-imprinted polymer is related to the fluctuation of the lifetime during the measurements. A fluorescence lifetime increase of 10 ns as a result of the extraction process is due to the removal of...
### TABLE I: Fluorescence lifetime measurements for cGMP imprinted and non-imprinted polymer films.

|                | Imprinted Lifetime [ns] | Width of the distribution [ns] | Non-imprinted Lifetime [ns] | Width of the distribution [ns] |
|----------------|-------------------------|-------------------------------|-----------------------------|-------------------------------|
| Polymerisation | 15.3                    | 7.6                           | 15.2                        | 7.7                           |
| Extraction     | 25.1                    | 11.7                          | 16.2                        | 9.1                           |
| Re-adsorption  | 16.1                    | 8.5                           | 14.9                        | 8.1                           |

![Histograms of lifetimes of fluorescence of the cGMP imprinted film (a) and of the blank polymer film (b). (1) Before extraction, (2) after extraction, and (3) after rebinding.](image)

### TABLE II: The PAQ fluorescence quenching in the presence of various quenchers in solution and MIP as represented by Stern-Volmer constants.

| Molecule       | Solution $K_{SV}$ [L·mol$^{-1}$] | MIP $K_{SV}$ [L·mol$^{-1}$] |
|----------------|----------------------------------|------------------------------|
| Guanosine      | 5.5±0.2                          | 0.25±0.003                   |
| GMP-Na$_2$     | 3.8±0.2                          | 0.01±0.002                   |
| cGMP           | 3.1±0.2                          | 0.045±0.005                  |
| cAMP           | 1.2±0.1                          | 0.005±0.001                  |

The existence of the imprinting sites, as demonstrated by the comparison of imprinted and non-imprinted polymers, does not indicate anything about the selectivity of the MIP. In order to demonstrate the selectivity, the MIP was exposed to three different structural analogues of cGMP; namely guanosine, GMP, and cAMP. An equivalent experiment has been performed using free PAQ molecule in solution [18]. In that study, the most pronounced quenching was caused by guanine. It has been observed that, in solution, the PAQ fluorescence is affected by guanosine $>$ GMP $>$ cGMP. After the introduction of the functional monomer to create the fluorescent cGMP imprinted polymer, the strongest interaction was observed by cGMP, then guanosine and then GMP. This
indicates that recognition was also induced by the spatial hindrance of the template within the polymer, which can be directly translated to selectivity of the MIP. The selectivity of adsorption of the molecularly imprinted polymers is observed because of the size and shape of the molecule which was imprinted. Also, the electronic structures formed by arrangement of the functional groups fixed by the polymerization represents the cavities that selectively adsorb analytes from the surrounding. The results of study, both in solution, and with the use of the MIP, are represented by Stern-Volmer constants, and are collected in Table II.

IV. CONCLUSIONS

We have performed fluorescence microscopy measurements of a biomimetic polymeric sensor obtained using molecular imprinting. We showed that the distributional approach, combined with fluorescence microscopy, is advantageous over the classical one due to improved precision and a shorter time of acquisition. Advantageously, to synthesize the MIP, we used a simple method based on a commercially available polymer, PMMA. Additionally, the pyrazolequinoline receptor incorporated into the polymer makes a noninvasive measurement by the MIP possible. The results of steady-state and time-resolved fluorescence microscopy indicate that quenching of the MIP fluorescence occurs when the nucleotide/template was adsorbed into the imprinted cavity. Because of this, the imprinted films are able to recognize cGMP, the imprinted structure, and can be easily compared with the non-imprinted polymer. When structural analogues of cGMP were compared to cGMP itself, it was found that cGMP selectivity was much higher in solution. Our current efforts are focused on establishing a new method to analyze MIPs via lifetime distributions on the basics of the ratio between fluorescence lifetimes of bound and unbound fluorescent receptors, which can correspond to the ratio occupied and unoccupied molecular cavities.

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