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Fluorescent molecularly imprinted polymer studied by time-resolved fluorescence spectroscopy

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Abstract

Molecularly imprinted polymers (MIPs) with template-selective recognition sites and incorporated fluorosensor were prepared against adenosine 3′,5′-cyclic monophosphate (cAMP). The time-resolved fluorescence decay analysis was used to investigate the specificity and affinity of the binding of template molecules to the MIP. The fluorescence decays were modelled in terms of lifetime distributions and two fluorescence lifetimes were observed for the MIPs. The lifetime distributions are interpreted in terms of the heterogeneity of the functionalised imprinted cavities. Quenching of fluorescence of the imprinted polymer with increasing concentration of aqueous cAMP was observed from the fluorescence lifetime parameters data. The mechanisms of interactions between the cAMP and fluorosensor molecules inside the imprinted cavity in comparison with the interactions in solution are discussed.

Keywords: Imprinted polymers; Fluorescent polymers; Time-resolved fluorescence

1. Introduction

Synthetic biosensors that rely on naturally occurring recognition elements such as enzymes and antibodies, particularly those based on molecular imprinting technology are actually of great interest [1,2]. The molecular imprinting technology is a co-polymerisation method for the preparation of synthetic material that exhibits the molecular recognition phenomenon [1,2]. In this technology, a template molecule is associated with a functional monomer to form a complex by means of covalent linkages or non-covalent interactions. The cross-linking monomer is then added to establish a network around the complex. Subsequent removal of the template molecules leave behind functionalised cavities that are able to recognise the template molecule. The spatial features and bonding preferences of the template molecule are left inside the imprinted polymer network. Molecular imprinting has been used extensively during the last decade as a versatile method for creating macromolecular matrices that display selective molecular recognition behaviour, which has found application in a number of areas [3–9]. In our previous studies [9], it was established that the fluorescent sensor, which was incorporated into the polymer, responds effectively in interactions with the template molecule by changes in the polymer fluorescence. This fluorosensor was introduced as a functional monomer forming complexes with the template molecules in the polymerising mixture, as shown in Scheme 1. This fluorosensor, whose fluorescence was sensitive to interactions with nucleotides [10], caused the polymer itself to moderate the transducer signal by its own functionality. Molecular imprints that contain a fluorescent sensor, polymerisable chromophore *trans*-4-[*p*-(*N*,*N*-dimethylamino)-styryl]-*N*-vinylbenzylpyridinium chloride (vb-DMASP) (**1** in Scheme 1), were prepared against adenosine 3′,5′-cyclic monophosphate (cAMP) (**3** in Scheme 1). Fluorescence signals from the chromophores covalently bound to a solid matrix can reveal information about their local environment through changes in their photophysical properties studied by steady state emission and/or time-dependent fluorescence measurements. The steady state fluorescence measurements of the MIPs [9] showed a quenching of the output fluorescence signal upon binding of cAMP ions on the imprinted sites. Previous studies showed a high selectivity of fluorescent MIPs in the rebinding of the template molecules; the association constant for the binding of cAMP ions to the MIP matrix was $10^5\ M^{-1}$ [9].

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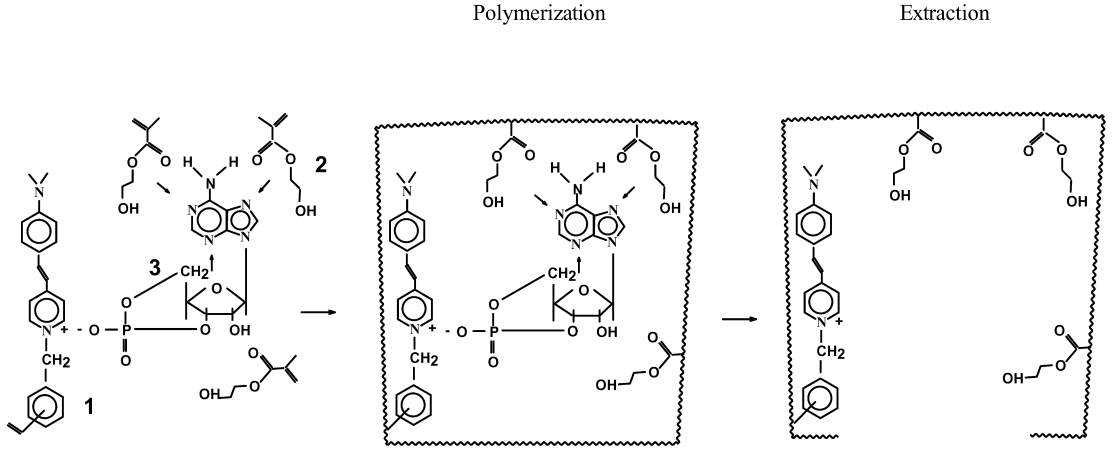

Scheme 1. Imprint formation.

The steady state fluorescence of the vb-DMASP dye in liquid solvents was studied [10–12]. In particular, we investigated the effects of viscosity [11] and polarity [10,12] on the fluorescence emission quantum yield of the dye. We found that the dye exhibited a strong solvatochromic behaviour resulting from a large increase of the dipole moment (~18 D) upon excitation. This indicates that its excited state has a considerable charge-transfer character, and thus the fluorescence is strongly sensitive to environmental interactions. We also reported a marked enhancement in the fluorescence quantum yield of the dye in the presence of nucleotides in unbuffered and buffered aqueous solutions. Adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) sodium salts had a particularly strong affect on the fluorescence of the dye. The vb-DMASP molecule relates to donor–acceptor substituted stilbenes [13–16] such as dimethylamino-cyano-stilbene [13,14] and styrylpyridiniums [11]. We believe that after polymerisation, the vb-DMASP chromophore is located in the cavity as is shown in Scheme 1. In this state, photoisomerisation dynamics as well as the excited state structure of the chromophore are strongly affected by the cavity's internal environment and architecture. Following polymerisation, the template molecule was extracted, leaving behind a molecular cavity that has a three-dimensional structure, which is complementary to cAMP. The three-dimensional network of the polymer allows for very effective recognition of the template molecule [9]. The quenching of the fluorescence from the MIP with an incorporated vb-DMASP chromophore, if the polymer was incubated with aqueous cAMP, suggests a strong interaction between the chromophore ion and the cAMP ion due to stable complex formation. These observations do not correlate with the enhancement of fluorescence from the chromophore during interaction with nucleotides in an aqueous solution [10], as this would suggest different mechanisms of molecular interactions when the chromophore is free or immobilised in the polymer matrix.

In imprinting technologies, it is of critical importance to be able to define the specific properties of the polymer and particularly of the imprinted cavity, in detail. These specific properties include the size and shape of the cavity as well as the number and type of functional groups involved in the binding of the target ions. However, these very important characteristics of the imprinted cavities are specific properties of polymer relating to internal micro-organisation of the MIPs. All the above-mentioned properties of imprinted polymer can affect its fluorescence decay and photophysical parameters describing the decay kinetic.

We have conducted these studies of the system with the aim of finding more effective detection method of the molecularly imprinted polymers specificity, as well as in hopes of obtaining more information on the binding mechanism and effectivity.

2. Experimental details

2.1. Materials

trans-4-[*p*-(*N*,*N*-Dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride (vb-DMASP) was prepared as described in the earlier paper [10]. The MIP was synthesised using molecular imprinting technology, which is schematically described in Scheme 1 and was described previously in detail [9]. The functional monomers (the fluorescent monomer vb-DMASP (**1**), 2-hydroxyethyl methacrylate (HEMA) (**2**) and trimethylolpropane trimethacrylate (TRIM) (HEMA and TRIM were purchased from Aldrich Chemical) were polymerised in the presence of a template/print molecule,

adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP) (**3**). In agreement with the first step of the imprinting process in Scheme 1, the functional monomer vb-DMASP, which has a positive charge, promotes an association with the negatively charged nucleotide ion to form a complex. After the polymerisation step, the spatial features and bonding preferences of the template, cAMP, were left inside the cross-linked, polymerised material. The solid polymers were grounded to particles of 45–106 μm. The third step was to remove the template molecules from the polymer. The template molecules (cAMP) along with the rest of monomers were removed by a long term extraction process (24 h) using water/methanol (7:3, v/v) followed by pure methanol for 24 h. The polymer was dried overnight, at 35 °C in a vacuum, then stored in the dark. A non-imprinted/blank polymer was prepared under the same conditions but without cAMP salt in the polymerising mixture, thus there were no cavities which would be complementary to cAMP.

The cyclic nucleotides, adenosine 3′:5′-cyclic monophosphate sodium salt (cAMP) and guanosine 3″:5′-cyclic monophosphate sodium salt (cGMP) were purchased from Sigma Chemical. Aqueous solutions of the nucleotides were prepared and all measurements were done using double-distilled deionised water.

2.2. Measurements

The imprinted and non-imprinted powder polymers, 150 mg in weight, were wetted overnight and then suspended in double-distilled deionised water. The fluorescence decays were measured using excitation wavelength 469 nm and fluorescence emission was collected at the maximum of the steady state fluorescence emission spectra, 600 nm. These wavelength data result from previously reported steady state fluorescence studies of the MIPs [9]. The data for polymers in the presence of guest molecules (cAMP, cGMP) were collected after the incubation of the polymer in the presence of a proper guest molecule aqueous solution at 25.0 ∓ 0.5 °C, for 90 min from its addition; this was to be in the region of a steady response of the fluorescent polymer as was reported in the previous paper [9]. Fluorescence emission decays were measured with a time-correlated single photon counting apparatus from Edinburgh Instruments, equipped with a hydrogen-filled coaxial flash lamp excitation source. The excitation and emission wavelength was chosen using a monochromatours, based on diffraction grating, with a 2 nm bandpass. The measurements were carried out with the emission monitored at a 90° angle to the excitation. A standard cubic cuvette was applied for the liquid samples, and a triangular one was applied for the polymers. Front face measurement geometry was used at a 45° angle to the excitation and emission beams. The holder position was that the scattered light was eliminated from the emission beam reaching the detector. To eliminate orientational effects in the fluorescence decays, a polariser was placed in the emission beam at the magic angle of 54.7°. The instrumental profile was obtained by replacing the sample with ludox as a scatterer. The data were collected in 1023 channels to 10,000 counts in the peak, and the time-calibration was 0.053 ns per channel. The decay instrumental pulse width at half-maximum was about 1.5 ns and was stable within the experiment. Each component of the instrument was optimised as was recommended by Edinburgh Instruments. The data were analysed by a least squares reconvolution procedure [17] using the software package provided by Edinburgh Instruments. How good of a fit it was judged in terms of a χ^2 value and residuals distribution, and values for χ^2 of 1.3 or lower were interpreted as indicating the appropriateness of the kinetic model.

3. Results and discussion

3.1. Inhomogeneous fluorescence decay kinetics

Generally, it was observed that the fluorescence decays of polymeric systems are almost always non-homogeneous [17,18]. A traditional method of dealing with these data is to fit the decay curves with two or three exponential terms of fitting functions. The results are usually believed to be due to different pathways of energy dissipation and/or to different emitting species. Frequently the obtained exponential terms do not possess physical meaning in a model of mechanism of photophysical processes, which we can describe for our specific system. To avoid this problem in the analysis of heterogeneous fluorescence decay kinetics for probe molecules in micellar system [19], adsorbed on silica surfaces [20–23] and on zeolites [24], and for studies of dipole–dipole energy transfer between the silica and excited aromatic probe [25], lifetime distribution analysis was used, without arbitrarily assuming the existence of two or three exponential terms. The decay rates obtained from applications of the lifetime distribution analysis shown for studies of fluorescence decay kinetics from aromatic molecules adsorbed on silica surfaces [20–24] were mostly bimodal. The bimodal distribution was explained in terms of the original heterogeneity of silica surface, which are known to be both geometrically and energetically heterogeneous; there exist various silanol groups, which have various activities.

We used the lifetime distribution model to analyse the time-resolved fluorescence spectroscopy studies of imprinted fluorescent polymer. Although fluorescence techniques have been reported to probe the imprinted polymers effectivity, never before have time-resolved fluorescence measurements been used to characterise imprinted polymer with incorporated fluorophore. We used the least squares reconvolution procedure in analysis of the integrated fluorescence kinetic data and the ‘Distribution Analysis’ program from the software package provided by

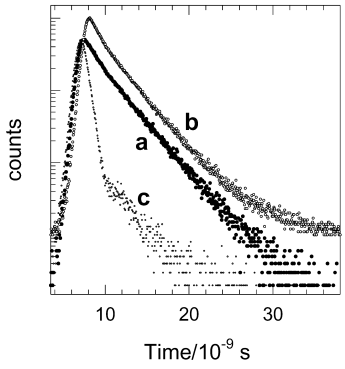

Fig. 1. Fluorescence decay kinetics of imprinted polymer (a), non-imprinted polymer (b) and flash lamp pulse (c). Powder polymer was suspended in deionised water and decays were measured using excitation at 469 nm and fluorescence was collected at 600 nm.

Edinburgh Instruments. The distribution kinetic model fits a decay by weighting a series of individual exponential lifetimes (up to 100). The relative weighting of each peak corresponds to the relative weighting of the standard exponential fitting. One can find a wider description of the fitting methods in literature [17]. In the case of our measurements of the MIPs, all the fluorescence decay kinetics were inhomogeneous in contrast with the fluorescence decays of vb-DMASP in solutions. The homogeneous decay kinetics were observed for vb-DMASP in a methanol solution and in a mixture of glycerol and water [26]. Homogeneous decays were even observed for vb-DMASP in very viscous solutions of 80% glycerol in water. We do not expect any natural heterogeneity from the polymer surface and only a fact of existence of two types of fluorescent cavities. One of the types is the mechanically opened type during grinding and then extracted and accessible to the analyte. Second is the inaccessible to the solvent and analyte, closed inside the bulk polymer. However, we have to consider the possibility of existence of *cis*- and *trans*- isomeric forms of the substituted styrylpyridinium molecules. The main deactivation process competing with the fluorescence of the chromophore seems to be *cis–trans* photoisomerisation accomplished by twisting about the central double bond, characteristic for substituted stilbenes [13–16]. Two lifetime parameters were obtained by Duveneck et al. [15] when they studied binding of stilbenes to cyclodextrins. They suggested that the double-exponential fluorescence decays of *trans*-stilbene in the presence of β-cyclodextrin result from dynamic equilibrium of loose and tightly bound conformation of the complex. In the present studies of MIPs with incorporated vb-DMASP chromophore, we are considering a dynamic equilibrium of *cis*- and *trans*- conformations of the fluorosensor molecules forming different complexes with template in the polymerising mixture. After the polymer network formation, the chromophore vb-DMASP stays in the earlier established conformation frozen in the rigidified matrix.

Fig. 1 presents two examples of fluorescence decay kinetics obtained from the imprinted and non-imprinted polymers. A program of 'Distribution Analysis' from the package provided by Edinburgh Instruments was used to analyse the data of fluorescence decay kinetics of the MIPs. Table 1 shows the results of the analysis of decay kinetics of fluorescence from polymer aqueous suspension for three independent samples of both imprinted and non-imprinted polymers. Fig. 2(A) presents the lifetime distributions for the imprinted polymer samples and Fig. 2(B) for the non-imprinted polymer. The distributions suggest large variability for both polymers. Although we could not know what the most important factor affecting the lifetime distributions and the fluorescence decay kinetics obtained from the polymer would be, we suspect that during the grinding of the polymer some internal electronic micro-organisation of the cavities around the fluorosensor molecule would be destroyed.

Recently, Dai et al. [21] reported on time-resolved fluorescence studies of uranyl adsorbed on uranyl-imprinted sol–gel silica polymers. They showed that the width parameter of the distribution obtained from the decay analysis using the Kohlraush–Williams–Watts model presented a broader distribution of decay rates for control/blank silica than for the imprinted polymer sample. In their case, however, uranyl was the template molecule. In our case, the fluorescent sensor is covalently bound and, as can be seen in Scheme 1, is located in the polymer cavity. Most of the cavities in the imprinted polymer are thought to be created by the template molecules forming a uniform micro-organisation. Although, the mean lifetime parameters

Table 1
Parameters of lifetime distribution analysis of fluorescence decays for imprinted and non-imprinted polymers. The τ_1, τ_2 are the lifetime parameters, and B_1, B_2 referring to the relative contribution (%) of quantum yields of the respective species. Excitation wavelength was 469 nm and emission wavelength 600 nm

Polymer	Lifetimes (ns)			Relative contribution (%)			χ^2
	τ_1	τ_2	τ_3	B_1	B_2	B_3	
Imprinted	0.158 ± 0.111	2.79 ± 0.49	–	17.3	82.7		0.94
	0.155 ± 0.053	2.76 ± 0.49	–	24.3	75.7		1.21
	0.092 ± 0.022	1.13 ± 0.54	2.86 ± 0.40	21.2	0.8	77.9	1.17
Non-imprinted	0.126 ± 0.011	2.76 ± 0.56		2.7	97.3		0.86
	0.108 ± 0.027	1.54 ± 0.16	3.01 ± 0.35	19.3	16.4	64.3	0.97
	0.103 ± 0.038	2.70 ± 0.6		14.2	85.8		0.96

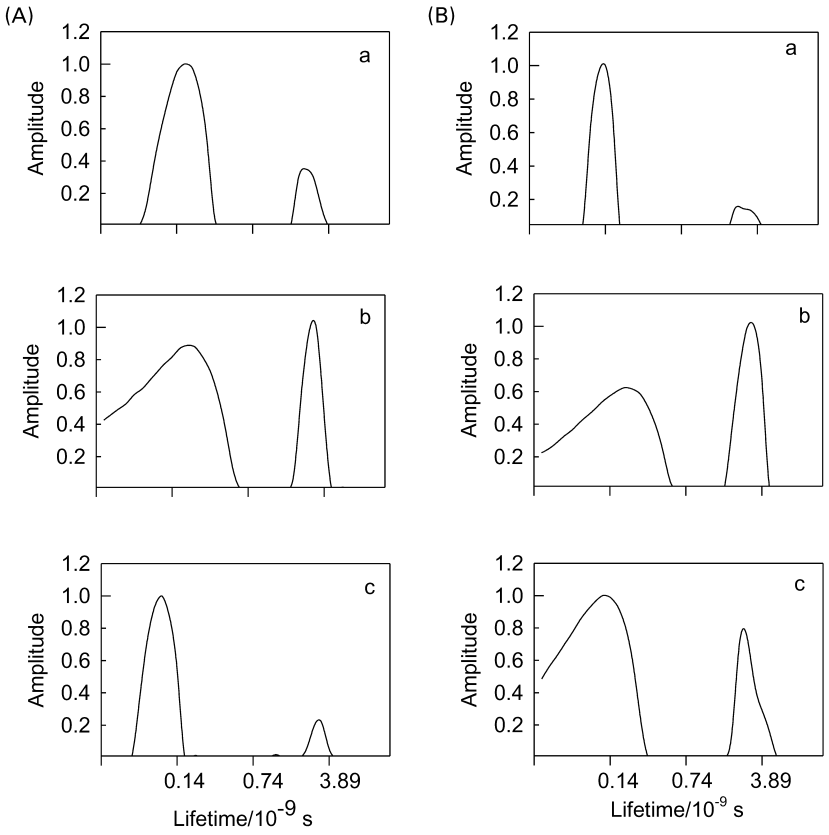

Fig. 2. Fluorescence lifetime distributions for three samples of imprinted polymer (A) and for three samples of non-imprinted polymer (B). The fluorescence decays were measured exciting at 469 nm and emission was collected at 600 nm.

are similar within the experimental error each of the samples presents individual lifetime distribution. However, there is no significant difference between the lifetime parameters of imprinted and non-imprinted polymer samples in Table 1, especially since shorter lifetime values were used, which are nearly at the limit of the equipment. The time-resolution limit in measurements derived from a single photon counting instrument was discussed by O'Connor and Phillips [27]. They used the Zimmerman and Cutler [28] method to evaluate the time-resolution for instruments with a flash lamp source of excitation. Two instrumental pulse profiles were measured. The deconvolution of one pulse profile with the other yielded the time-resolution of the instrument. Although their measured value for a system similar to ours was 50 ps, they suggested raising it to 100 ps to be sure. Although we optimised each component of the instrument and the instrumental pulse was stable during the experiments, for our instrument, we assumed the time-resolution 100 ps to be accurate.

3.2. Fluorescence lifetime studies of cAMP binding

The measurements of fluorescence decay kinetics were taken before and after incubation of the cAMP imprinted polymer for 90 min in an aqueous solution containing cAMP salt. All the fluorescence decay kinetics were inhomogeneous. The results of the lifetime distribution analysis of the kinetics of MIPs after incubation in aqueous cAMP and cGMP are presented in Table 2. Although, some of the analysis show very low fraction of third component, two components in the lifetime distribution analysis of the decays were accepted. The mean lifetimes are gathered in Table 3. The data of the mean lifetimes show a decrease in both lifetime parameters with an increasing concentration of cAMP, but as well some change of fraction of the components in the decays are noticeable. The quenching of the MIPs fluorescence in the presence of cAMP, observed from the results, correlate well with the steady state measurement data [9]. However, these studies suggest that the mechanism of fluorescence quenching is somewhat more complicated then we expected after the steady state fluorescence measurements [9].

We found previously [10,12] that the fluorescent monomer vb-DMASP, when its free in aqueous solution, displays an enhancement in fluorescence in the presence of both nucleotides: cAMP and cGMP. The fluorescence of the fluorophore, which demonstrate charge transfer properties under excitation, can be enhanced by the dipole–dipole interaction when the distance between the fluorophore and the nucleotide is high enough, like in solution used to be. The dipole–dipole interactions rigidify the structure of the fluorophore and that causes reduction of the rate constant of

Table 2
Parameters of lifetime distribution analysis of the fluorescence decays for imprinted polymers after incubation with aqueous nucleotides. Excitation wavelength was 469 nm and emission wavelength 600 nm

	Lifetime (ns)			B_1 (%)	B_2 (%)	B_3 (%)	χ^2
	τ_1	τ_2	τ_3				
cAMP (M)							
10^{-5}	0.133 ± 0.097	2.72 ± 0.59	–	20.5	79.5	–	0.86
	0.125 ± 0.055	2.70 ± 0.26	–	16.0	84.0	–	0.98
	0.146 ± 0.035	2.72 ± 0.42	–	25.0	75.0	–	1.24
10^{-4}	0.107 ± 0.050	2.66 ± 0.68	–	8.3	91.7	–	0.89
	0.163 ± 0.042	2.68 ± 0.72	–	10.6	89.6	–	1.11
	0.093 ± 0.068	2.65 ± 0.32	–	8.8	91.2	–	1.19
10^{-3}	0.073 ± 0.023	1.10 ± 0.08	2.98 ± 0.30	17.5	11.9	70.6	1.15
	0.103 ± 0.051	2.68 ± 0.09	–	15.9	84.3	–	1.07
	0.076 ± 0.023	2.83 ± 0.45	–	10.5	89.5	–	0.99
	0.112 ± 0.034	2.65 ± 0.38	–	17.5	82.5	–	0.95
	0.141 ± 0.049	2.67 ± 0.08	–	14.5	85.5	–	1.23
CGMP (M)							
10^{-3}	0.109 ± 0.055	2.76 ± 0.42	5.01 ± 0.24	21.7	75.5	2.9	0.88
	0.212 ± 0.028	2.87 ± 0.35	–	25.3	74.7	–	0.81
	0.165 ± 0.082	2.81 ± 0.08	–	20.6	79.4	–	1.34

non-radiative processes of deactivation of the excitation [10].

In the case of interactions inside of solid polymer cavity, the adsorbed ion of cAMP has the ability of tightly bound stable complex/exciplex formation with the fluorosensor, which is located inside. The interactions of the ions inside the cavity induce charge transfer that result in fluorescence reduction. This is a static quenching leading rather to changes in the fractions of the short and/or long living components. Decrease of the percentage of the first (short living) component in the fluorescence decays with rising concentration of cAMP in Table 3 is accompanied by increasing value of the percentage amount of the second (long living) component. Simultaneously the cAMP molecule may interact with the fluorosensor non-specifically. Possibly, some of the fluorosensors can be located inside of the cavities, but some of them can at the surface of the polymer particles. These type of interactions can result in the fluorescence lifetime reduction, especially observed for the short living component, as can be seen in Fig. 3, curve 1. The reduction of fraction amount of the short living component in the fluorescence decay kinetics is accompanied by increase of amount of the longer living component of the decays. All the results suggest that two different mechanisms of interactions of the ions of fluorosensor and analyte can affect the fluorescence of the fluorosensor involved there. First are the interactions inside the tightly bound

Table 3
The mean lifetimes and adequate pre-exponentials for fluorescence kinetic measurements of three independent samples of imprinted polymer after incubation with aqueous nucleotides. Excitation wavelength was 469 nm and emission wavelength 600 nm

	Lifetime (ns)		B_1 (%)	B_2 (%)
	τ_1	τ_2		
cAMP (M)				
0.0[a]	0.14 ± 0.07	2.79 ± 0.08	22 ± 2	78 ± 2
10^{-5}	0.13 ± 0.03	2.71 ± 0.05	21 ± 9	80 ± 9
10^{-4}	0.12 ± 0.08	2.66 ± 0.04	9 ± 3	91 ± 3
10^{-3}[a]	0.10 ± 0.07	2.70 ± 0.03	15 ± 8	85 ± 8
cGMP (M)				
10^{-3}	0.16 ± 0.10	2.84 ± 0.06	22 ± 5	78 ± 5

[a] The data are means for five independent samples measurements.

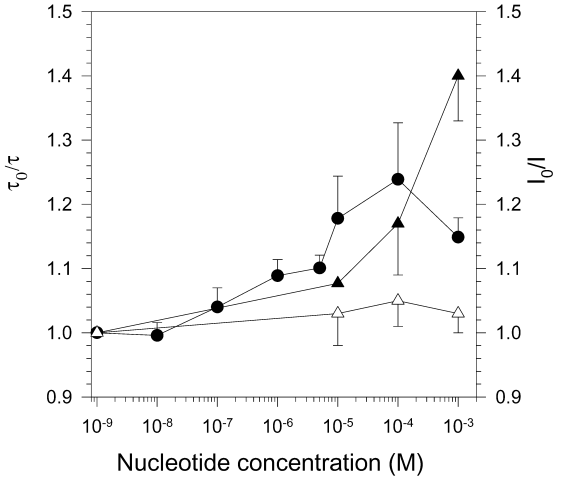

Fig. 3. Lifetimes and intensity of fluorescence of the imprinted polymer in the presence of various concentrations of cAMP. τ_0 and τ are the lifetimes of fluorescence in the absence and presence of nucleotide, respectively. (1) filled triangles, refers to the short living component of the fluorescence kinetics, (2) empty triangles, refers to the long living component of the kinetics, and (3) circles, refers to fractional intensity of the polymer fluorescence before I_0 and I after incubation of the polymer in aqueous cAMP [9].

complex/exciplex formatted inside the cavity, and second are the long distance dipole–dipole interactions.

Thus our fluorescent imprinted polymer displays a combined static and dynamic interactions resulting in fluorescence changes in the presence of aqueous cAMP when measured using two techniques: steady state and time-dependent fluorescence measurements. Fig. 3 shows effect of concentration of cAMP on the fluorescence lifetime, τ_0/τ (curve 1 and 2, from data in Table 3) and on the fluorescence intensity, I_0/I (curve 3, from data gathered in previous paper [9]) from the imprinted polymer. In addition some representative of the lifetime distribution spectra of MIPs shown in Fig. 4 together with that of the polymer after incubation with aqueous cAMP show new, analytically valuable, information about the binding process. A shift and a widening of the peaks in the lifetime distribution spectra towards the shorter lifetime side for the imprinted polymer in presence of an increasing concentration of aqueous cAMP can be observed. However, the shorter lifetime parameter seems to be more sensitive for interactions with the nucleotide.

An estimate of the association constant (K_a = [NP]/[N][P]) for association of cAMP (N) with the imprinted polymer (P) to form a complex (NP) was obtained from the following equation [9]:

$$\frac{I_0}{I} = \frac{1 + K_a[N]}{1 + (k_{np}/k_p)K_a[N]} \quad (1)$$

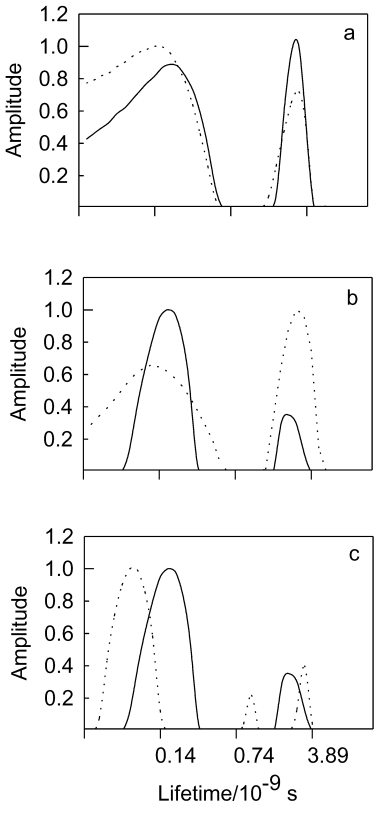

Fig. 4. Lifetime distributions of cAMP imprinted polymer before (continuous line) and after incubation with aqueous cAMP (dotted line) of different concentration: (a) 10^{-5} M, (b) 10^{-4} M and (c) 10^{-3} M of cAMP.

where I_0/I is the fractional fluorescence from steady state measurements, and k_{np} and k_p are constants relating to the proportionality of the complex cAMP-polymer and polymer concentrations. The data in Fig. 3, curve 3, from steady state fluorescence measurements were fit to Eq. (1), as we reported in the previous paper [9], giving the K_a value of $(4 \pm 2)10^5$ M^{-1}. Unfortunately, the combined dynamic and static quenching mechanism, shown in literature for systems, which are forming stable complexes and being suspended in solution are able to maintain dynamic contact with the quencher [29], gave rather non-satisfying results.

At this moment we cannot describe the mechanism of the photophysical processes for the fluorophore located inside the imprinted cavity in bulk polymer. The mechanism is more complicated than we expected. There may be different interaction patterns between the template-cAMP and the fluorophore incorporated in the polymer. As we discussed in Section 3.1, the samples demonstrate large in-homogeneity, resulting not only from the polymerisation process but as well as from the sample preparation i.e. grinding of the polymer bulk to particles of 45–106 μm. The imprinted cavities, which were opened by the grinding, represent big variability of the depth. Some of them are dip but some are very shallow. In the last case, the fluorophore molecule behaves as attached to the polymer surface. This introduces possibility of a long distance interactions with the nucleotide diluted in water. These interactions could lead to enhancement of the fluorescence, as it was for the fluorophore in solution [10,12]. In addition some suggestions about the presence of two possible isomeric forms of vb-DMASP incorporated in polymer matrix, obtained from Raman spectroscopy studies (we are working on this problem), made it more complicated. We can also conclude that the affinity of the cAMP-imprinted polymer for the template molecule corresponds to that of other MIPs reported from different laboratories; some of them are presented in Table 1 of the previous paper [9].

3.3. Selectivity of MIPs for binding of cAMP

To assess the selectivity of the interaction of the cAMP-imprinted polymer, the effects of a structurally related molecule cGMP on polymer fluorescence decays were examined. Fig. 5 shows the lifetime distribution spectra of fluorescence kinetics for cAMP-imprinted polymer before and after incubation with a 10^{-3} M aqueous cGMP solution. In contrast to cAMP, the presence of cGMP in aqueous solution induced increase in the mean lifetimes of fluorescence, as the data gathered in Table 2. However, in contrast to the behaviour of the lifetime distribution spectra of MIPs incubated with aqueous cAMP in Fig. 4, a shift of the lifetime distribution spectra toward the longer fluorescence lifetime was observed for the MIP incubated with aqueous cGMP. A similar effect was observed when we used a non-imprinted/blank polymer—a polymer obtained following the same protocol but without the presence of a template

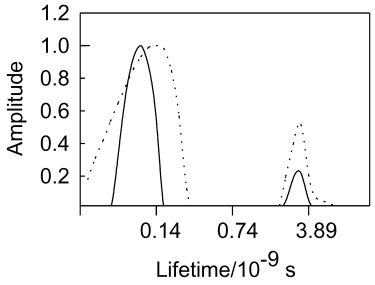

Fig. 5. Lifetime distributions of cAMP imprinted polymer before (continuous line) and after incubation with aqueous cGMP (dotted line) of concentration 10^{-3} M.

molecule in the polymerisation mixture. Fig. 6 shows the lifetime distribution spectra for non-imprinted polymer in water and that after incubation for 90 min in a 10^{-4} M aqueous cAMP. It has been previously shown that the ability of the imprinted polymer to discriminate between cAMP and cGMP is not due to pH or ionic strength [9], but rather because the template-cAMP molecule casts empty space and forms a complex with the fluorosensor present there. Since memory exists in the polymer defined by the cavity, this complex corresponds to 1% before polymerisation. Short distance ion interactions and tight/stable complex formation are the most important effects when the imprinted polymer is incubated with the template molecule. In the case of the non-imprinted polymer, there are no cavities and only long distance dipole–dipole interactions can take place between cAMP and the fluorophore. The steady state fluorescence of the vb-DMASP ion in an aqueous solution in the presence of cAMP and other purine nucleotides enhanced quantum yield [10]. The same effect was observed for the non-imprinted polymer. Only long distance interactions of the fluorophore ion with the nucleotide ion are possible, because the nucleotide can never enter the polymer to form a tight complex. The interactions of fluorophore incorporated in the cAMP-imprinted polymer with aqueous cGMP may be explained in a similar way. Since the cGMP molecule cannot enter the polymer, only long distance interactions between the dipoles are possible. These studies of time-resolved fluorescence of MIPs with an analysis of lifetime-distribution, shown in Figs. 4–6, provide strong evidence for the binding selectivity of cAMP-imprinted polymer.

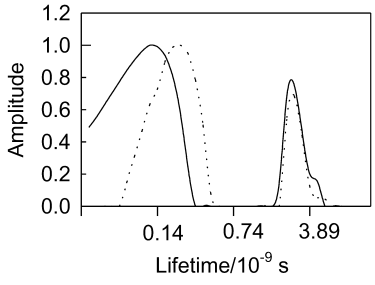

Fig. 6. Lifetime distributions of non-imprinted polymer before (continuous line) and after incubation with aqueous cAMP (dotted line) of concentration 10^{-4} M.

4. Conclusions

The quenching of fluorescence from cAMP-imprinted polymer when it binds cAMP was observed using two different techniques: steady state fluorescence intensity measurements and time-resolved fluorescence decays analysis. The results obtained with use both techniques provide strong evidence for the selective binding of the template molecule to cAMP-imprinted polymer. The nature of the complex of cAMP and the fluorosensor in the molecularly imprinted cavity can be viewed as a tightly bound form (inside the cavity) leading to a relatively shorter lifetime (quenching of fluorescence) and decrease of its percentage amount. Oppositely when the cAMP molecule does not enter the cavity, no tightly bound complex with the fluorosensor is formed, only larger distance interactions such as in solution are possible (enhancement of fluorescence). Such a model corroborates the fact that imprinted material possesses enhanced selectivity toward a template molecule ion.

We observed the quenching of fluorescence of the MIPs in about 16% of the fluorescence lifetime change, as shown in Table 3. Approximately the same percentage of fluorescence change was obtained from steady state fluorescence measurements [9]. However, only a part of the imprinted sites remained available after the extraction of the template molecules from the imprinted cavities, and only this part, about 18% of the polymer volume, can actively bind the cAMP molecules. However, the accessible recognition sites situated on the surface of particles obtained after the step of grinding of the polymer seems to be noticeable damaged. The observed variability of the fluorescence lifetime distributions from sample to sample in Fig. 2(a) and (b) confirms it. The time-resolved fluorescence provides some new possibilities for detection and monitoring of the specific imprinted polymers.

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