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SELECTIVE RECOGNITION OF CYCLIC GMP USING A FLUORESCENCE-BASED MOLECULARLY IMPRINTED POLYMER

Nguyen Thi Kim Thanh [a], Daniel L. Rathbone [b], David C. Billington [c] & Nicholas A. Hartell [d]

[a] Department of Chemistry, University of New Orleans, New Orleans, LA, 70148, U.S.A.

[b] Pharmaceutical Sciences Research Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

[c] L'Oreal Recherche, 1 Avenue Eugene Schueller, BP 22, Aulnay sous Bois, CEDEX, 93601, France

[d] The School of Pharmacy, Department of Pharmacology, University of London, WC1N 1AX, UK

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SELECTIVE RECOGNITION OF CYCLIC GMP USING A FLUORESCENCE-BASED MOLECULARLY IMPRINTED POLYMER

Nguyen Thi Kim Thanh,[1,2,*] Daniel L. Rathbone,[1] David C. Billington,[1,3] and Nicholas A. Hartell[1,4]

[1]Pharmaceutical Sciences Research Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK
[2]University of New Orleans, Department of Chemistry, New Orleans, LA 70148, USA
[3]L'Oreal Recherche, 1 Avenue Eugene Schueller, BP 22, 93601, Aulnay sous Bois, CEDEX, France
[4]The School of Pharmacy, Department of Pharmacology, University of London, WC1N 1AX, UK

ABSTRACT

Guanosine 3′,5′-cyclic monophosphate (cGMP) plays a role as a second messenger in many different biological systems. Given the ubiquitous nature of cGMP, a simple method of detecting cGMP is of interest. To that end a fluorescent polymer with recognition sites for cGMP has been prepared.

*Corresponding author. Fax: 001-504-280-325; E-mail: nguyen.tk.thanh@uno.edu

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Its selectivity and sensitivity were investigated and a dose-dependant decrease in fluorescence of the polymer in the presence of cGMP was observed. In contrast, virtually no effect was detected upon application of the structurally similar molecules, guanosine 5′-monophosphate (GMP) and adenosine 3′,5′- cyclic monophosphate (cAMP), thus demonstrating the high selectivity of this polymer. The association constant for the binding of cGMP to the imprinted polymer was determined in order of $3 \times 10^5\,M^{-1}$. A fluorescent, molecularly imprinted polymer that selectively recognises cGMP may have a useful application as a fluorescent chemosensor for cGMP detection in biological samples.

Key Words: Molecularly imprinted polymer (MIP); Fluorescent monomer; Molecular recognition; cGMP; cAMP

INTRODUCTION

Guanosine 3′,5′-cyclic monophosphate (cGMP) plays an important role as a second messenger in many different biological systems including those involved in the regulation of blood flow, retinal light transduction[1] and the acquisition of memory.[2] This cyclic nucleotide exerts its effect directly by modulating cGMP-gated ion channels or indirectly as a potent substrate for cGMP-stimulated protein kinases or cGMP-dependent phosphodiesterases. Given the ubiquitous nature of cGMP a simple method of detecting cGMP and related second messengers is of interest. Currently, signaling molecules such as cGMP are assessed using radioimmunoassay techniques[3] however, this approach is both costly and technically demanding. Molecularly imprinted polymers (MIPs) that selectively recognize compounds of interest provide an alternative approach.

The imprinted polymer consists of sites of specific molecular arrangement. This is achieved by formation of a pre-polymerisation complex between complementary monomers and the template molecule. Subsequent polymerisation in the presence of a crosslinker, in a porogenic environment, results in the production of a macroporous polymer capable of specific molecular recognition after removal of template molecules.[4] Apart from the obvious specific recognition properties of the imprinted polymer, its other characteristics such as physical and chemical stability and durability (resistance to elevated temperatures and pressures; and

inertness towards chemicals) are highly appealing. Imprinted polymers have many biological and pharmaceutical applications. They are used as a stationary phase for analytical chromatographic and electrophoretic separations (HPLC, thin layer chromatography, capillary electrophoresis, electrochromatography) and especially for chiral separations.[4–7] They are used for controlled release, drug monitoring devices and as biological receptor mimetics, biomimetic assays,[8–10] and as sensors for low weight organic molecules.[11] Imprinted polymers can also be used in combinatory chemistry for in automated assays to screen libraries of potential drug candidates[12] and used as the adsorbent for solid phase extraction.[4] They have also found their way to other applications such as catalysis, molecularly imprinted membranes and even cell surface imprinting.[4]

In spite of the recent upsurge in publications concerning imprinted polymers there is a relative dearth of reports regarding fluorescent imprinted polymers.[13–17] There is, however, work on imprinted polymers for detection of fluorescent analytes[10,18] or for non-fluorescent analytes by competitive displacement by the analyte of a fluorescent compound.[19] In the cases where the polymers, rather than the analytes, contain covalently-bound fluorophores, these are fabricated by means of fluorescent functional monomers. Once the cross-linked imprinted polymer has been obtained the fluorophore is covalently located in the polymer cavities. The imprinting template associates non-covalently with the fluorophore, usually by hydrogen bonding, and the presence or absence of the template in the cavity may be detected via changes in the fluorescence properties of the polymer. Fluorescence detection is highly advantageous due to its high sensitivity and non-destructive nature. Recently, a fluorescent polymer that recognizes the related cyclic nucleotide second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP) has been reported.[16]

EXPERIMENTAL

Materials

Guanosine-3,5′-cyclic monophosphoric acid trihydrate (cGMP-H) and adenosine-3′,5′-cyclic monophosphoric acid monohydrate (cAMP-H) were purchased from ICN Biomedicals Inc. (Germany). TMPTA and AIBN were obtained from Acros Ltd, UK, and Aldrich Ltd, UK, respectively. The methanol used was reagent grade. Aqueous solutions of the nucleotides were prepared in distilled water.

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Preparation of Fluorescent Monomer

The fluorescent monomer, 2-acrylamidopyridine was prepared from 2-aminopyridine as previously described.[20] Briefly, it was synthesised from 2-aminopyridine and acryloyl chloride in dichloromethane in the presence of triethylamine.

Preparation of Polymers

The fluorescent imprinted polymer was synthesized by radical polymerisation of 2-acrylamidopyridine, the template cGMP and the cross-linking agent. A stirred solution of cGMP-H (5 mg, 12.5 μmol), 2-acrylamidopyridine (2.0 mg, 13.5 μmol), AIBN (0.156 g, 0.95 mmol) and TMPTA (10 mL, 37.2 mmol) in methanol (30 mL) was deoxygenated by alternate application of vacuum and argon (5×). The reaction mixture was stirred under argon overnight at 38°C and for two days at 60°C. A white solid polymer was collected by filtration and washed with methanol (5 × 50 mL) and dried under high vacuum. A non-imprinted control polymer containing no cGMP was prepared under the same conditions. The template molecule cGMP was extracted from the polymer particles with methanol–water 3 : 7 (v/v) in a soxhlet apparatus for four days. This polar solvent mixture was chosen, as it created an environment unfavourable for template-imprinted polymer hydrogen bonding and ion pairing.[4] The free cGMP imprinted polymer was then dried under high vacuum. The polymer was then ground to a powder using a mortar and pestle. The product was sieved to obtain particles smaller than 38 μm.

Characterising the Fluorescent Properties of the Control and Imprinted Polymers

The fluorescence characteristics of a stirred suspension of the polymers (0.75 mg/mL) were measured at excitation wavelengths 355, 450, 485, and 490 nm with an LS 50 Perkin Elmer Luminescence Spectrometer.

Measurement of the Effect of Nucleotides on the Fluorescence of cGMP Imprinted Polymer

GMP, cAMP, or cGMP (0.25 μmol) were added to methanol (10 mL) containing dried imprinted polymer (100 mg). The mixtures were sonicated

for 15 min and left to stand overnight, then sonicated for 2 h and finally stirred for 45 min. The resulting polymers in the presence of nucleotide were filtered and washed quickly with methanol (3×50 mL), then dried under high vacuum. Ten milligram of each of the GMP-bound, the cAMP-bound, the cGMP-bound and the cGMP-free polymers were placed in triplicate in a 96 well fluorescence plate. Methanol (0.25 mL) was added to each well to avoid scattering of the finely powdered polymer during fluorescence measurements. Controls comprised methanol only and blank wells. The plate was read using a Wallac Victor 1420 multilabel counter using a range of excitation/emission filter combinations. The data presented are averages of the results and are quoted as fluorescent counts per mg of polymer.

Examination of the Specificity and Sensitivity of cGMP Imprinted Polymer

A 96-well fluorescence plate was prepared with wells in quintuplet containing (a) imprinted polymer suspension in water (0.2 mL, 4.5 mg/mL) and (b) control, non-imprinted polymer suspension in water (0.2 mL, 4.5 mg/mL) containing cGMP at concentrations of 0.1, 1, 5, 10, 50, 100, and 500 μM. The mixtures were incubated at ambient temperature for 0.5 h and then the fluorescence response was measured in triplicate using a Wallac Victor 1420 spectrofluorimeter. I_0 and I represent the absolute fluorescent intensities of the polymers in the absence and presence of the nucleotide, respectively. The results presented are means and standard error of the mean. The same procedure was repeated with cAMP in the presence of the cGMP imprinted polymer.

RESULTS AND DISCUSSION

Preparation of Molecularly Imprinted Polymers

2-Acrylamidopyridine, synthesised from 2-aminopyridine and acryloyl chloride,[14] was selected as a suitable fluorescent monomer because it is compact, highly fluorescent and has suitable hydrogen bond donor and acceptor sites available[20] for interaction with the cyclic nucleotide template. TMPTA, a tri-functional cross linker, formed the bulk of the polymer under free radical conditions initiated thermally in the presence of AIBN. The free-acid form of cGMP (cGMP-H) was used in order to facilitate interaction with the fluorescent monomer. Owing to the UV absorption profiles of both the template and fluorescent monomer, polymerisation

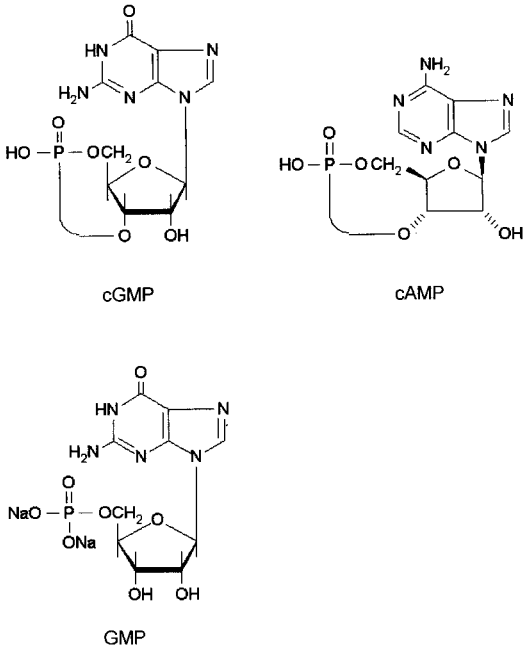

Figure 1. The structures of cGMP, cAMP, and GMP.

initiation was by thermal decomposition of AIBN rather than by UV irradiation at low temperature. Due to the low solubility profile of cGMP in most organic solvents, it was necessary to use methanol as the porogen. Although this is not ideal because of the hydrogen bonding properties of methanol, an imprinted polymer was obtained which was able to discriminate between cGMP and the similar molecules GMP and cAMP (Fig. 1). The template molecule was removed from the imprinted site by washing the polymer with a sufficiently polar solvent system. A control polymer was prepared in the same way but without the imprinting molecule.

The Effect of cGMP on the Fluorescence of cGMP Imprinted Polymer

Figure 2 shows the emission spectra of the control (solid line) and imprinted polymer (dotted line) at different excitation wavelengths (355, 450, 485, and 490 nm). As can be seen from Fig. 2 the fluorescence

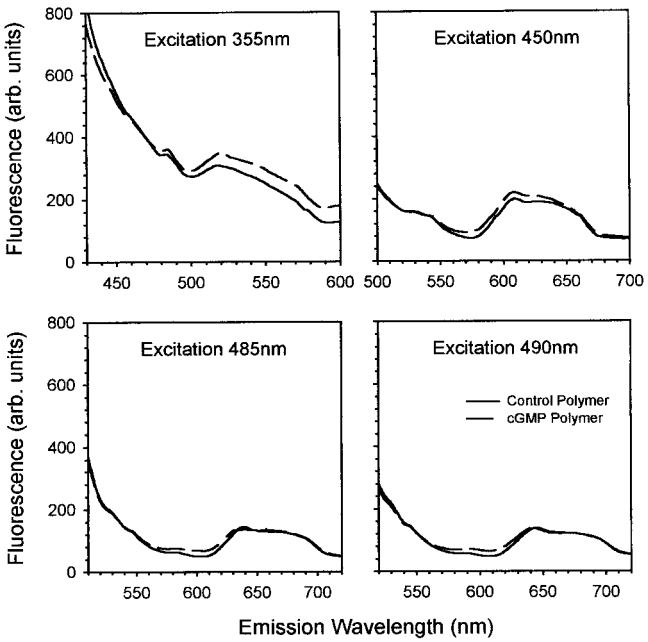

Figure 2. Emission fluorescence profile of control (solid line) and cGMP (dashed line) imprinted polymers at excitation wavelengths 355, 450, 485, and 490 nm.

responses of the control and cGMP-free imprinted polymers were very similar both in shape and intensity. This is because in both polymers, cGMP was absent, either during the polymerisation or being extracted out after polymerisation. Exposing the imprinted polymer to its template molecule resulted in quenching of fluorescence by a factor of approximately 2.5 across the range of excitation and emission pairs investigated (Fig. 3). The greatest absolute change in fluorescence occurred at E_{ex} 355/E_{em} 460 nm. Exposure of the polymer to GMP, cAMP the most similar structure molecules under identical conditions did not result in any significant quenching of fluorescence.

The Specificity and Sensitivity of cGMP Imprinted Polymer

The imprinted polymer was exposed to a range of concentrations of aqueous cGMP and cAMP (Fig. 4). cAMP did not induce in any significant change in the fluorescence response of the imprinted polymer over the range

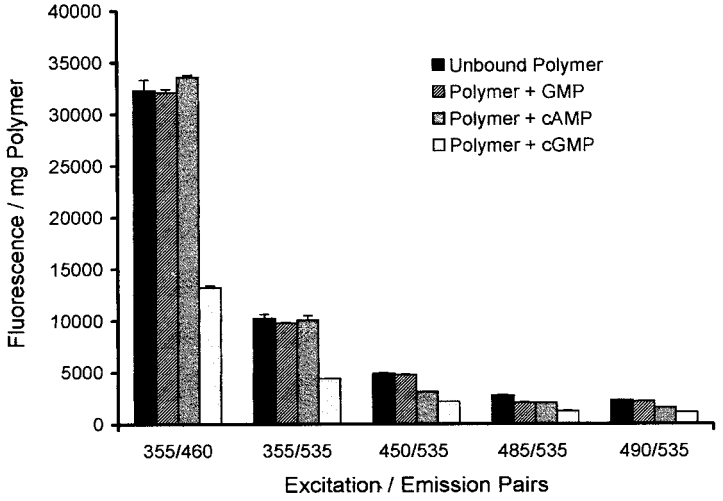

Figure 3. Fluorescence response of the imprinted polymer in the absence of cGMP, and in the presence of GMP, cAMP, or cGMP. The pairs of numbers on the horizontal axis denote the combinations of excitation and emission wavelengths used (nm).

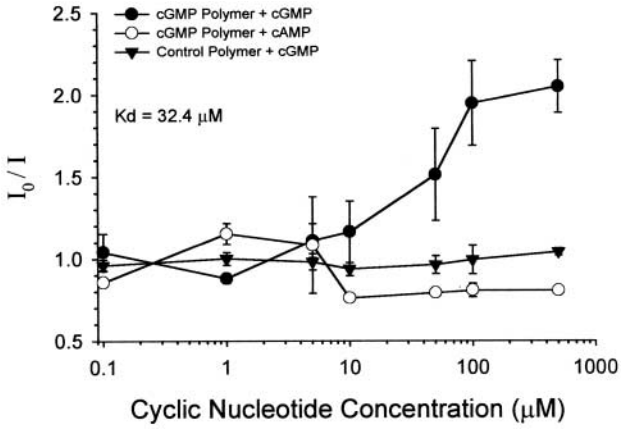

Figure 4. Fluorescence of control and cGMP imprinted polymers in the presence of increasing concentrations of cGMP and cAMP. Data were measured 30 min after incubation with the nucleotide. I_0 and I are the fluorescence intensities of the polymers measured in the absence and presence of nucleotide, respectively. Graphs show the fluorescence intensities measured at an emission wavelength of 460 nm following excitation at 355 nm. The data are the means and standard errors of at least three replicates at each nucleotide concentration.

0.1–1000 μM. In contrast cGMP induced a concentration-dependent quenching of fluorescence reaching a maximal level at around 100 μM. Saturation at higher concentrations presumably reflects saturation of the accessible sites on the polymer. Exposure of the control polymer to cGMP had no effect. The association and dissociation constants for cGMP were calculated to be $3 \times 10^5\,M^{-1}$ and 32.4 μM, respectively. These results are similar to those reported by Turkewitsch et al who used the more complex fluorescent monomer *trans*-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride in the preparation of their fluorescent chemosensor for cAMP.[16]

CONCLUSIONS

Imprinted polymer cavities selective for cGMP, prepared using the easily-obtained fluorescent functional monomer, 2-acrylamidopyridine, were found to display high affinity and selectivity for aqueous cGMP over GMP and cAMP. This fluorescent molecularly imprinted polymer containing both recognition element and the measuring element for the fluorescent detection of cGMP may be useful in the detection of this molecule in biological systems, and the same principle could potentially be used for practical fluorescent sensors for many other compounds.

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