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Fluorescent Molecularly Imprinted Polymers in Sensing of cAMP and cGMP

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The cyclic nucleotides are biomolecules taking part in many intracellular processes [1]. Among cyclic nucleotides, 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) are particularly important due to their numerous functions. Those cyclic nucleotides have been recognized as crucial second messengers involved in the regulation of physiological processes, such as platelet aggregation, long term potentiating, or blood pressure regulation [2]. Their intracellular activity processes through phosphodiesterases, nucleotide-gated channels and protein kinases [3]. Both cAMP and cGMP plays a relevant role in diseases such as cancer, diabetes and, particularly, in cardiovascular diseases [4]. Since their discovery various approaches have been taken to quantifying cGMP and cAMP in biological samples. Molecular imprinting of polymers is one of the methods used to detect or distinguish between cGMP and cAMP.

The history of molecular imprinting is usually traced back to the experiments of Dickey [5] performed in the 40s and 50s of the last century. Those experiments were designed to create affinity for dye molecules in silica gel and were inspired by a theory of Linus Pauling who postulated how antibodies are formed and their interactions with an antigen might proceed [6]. The silica's investigated by Dickey are usually considered to be the first imprinted materials. Imprinting in organic polymers first appeared in early 1970s, when covalent imprinting in vinyl polymers was reported [7]. Non-covalent imprinting, in the form we know today, was introduced about a decade later [8].

In order to prepare a MIP, three main components are necessary: functional monomer(s), crosslinker and a target analyte called template. The components are initially pre-mixed and subsequently left to arrange in a configuration complementary to the template either by the formation of covalent bonds, or by self-association via non-covalent interactions. In many cases, a porogenic solvent or non-reactive polymer is added to improve overall MIP porosity that facilitates template diffusion through a MIP. After initial pre-arrangement, the pre-mix is polymerised either thermally, using UV light or in the electropolymerisation process. Then the template is removed by washing with a "good" template solvent or by washing combined with chemical treatment (e.g. using acids). After the washing process, a porous material is obtained that contains nascent imprint sites complementary to the template in shape and functionality. Those vacant imprinted sites are then available for rebinding of the template or its structural analogues. Typically with a MIP, a non-imprinted polymer (NIP) is prepared to provide with a reference response/signal. This is the most commonly used way to confirm the successful imprinting. The NIP approach, however, has one major drawback; its porosity is significantly different when compared to the porosity of a MIP. In NIP the porosity is usually reduced due to the absence of the analyte. The reduced NIP porosity prevents from free analyte diffusion though the polymer. Therefore, the applicability of NIP for MIP referencing is still the topic of public scientific debate. In the case of fluorescent molecularly imprinted polymer (F-MIP), a fluorophore, which interacts with the template and simultaneously is a signal transducer, is one of the functional monomers. This fluorophore is commonly called fluorescent functional monomer (FFM). The change in FFM properties, either emission and/or decay time, gives information upon binding of the template. In this view, the present article focuses on the application of F-MIPs for cGMP and cAMP detection.

In one of the first attempts, a F-MIP was prepared for determination of cAMP [9]. A *trans*-4-[1,4-(*N*,*N*-dimethylamino) styryl]-*N*-vinylbenzylpyridinium chloride (vb-DMASP) dye was used as an FFM. In this F-MIP, the positively charged vb-DMASP associated in complex with the negatively charged cAMP. The F-MIP was prepared in bulk and then ground and sieved to obtain smaller particles that facilitated washing cAMP out of the F-MIP. The washed F-MIP was then exposed to various cAMP concentrations. Upon interaction with cAMP, a noticeable decrease in F-MIP fluorescence at 595 nm was measured in the cAMP concentration range from 10 to 100 μM. This F-MIP had high affinity and selectivity for aqueous cAMP, as confirmed by a cross-reactivity test with the structurally similar cGMP. The complex stability constant K_{MIP} was equal to $(3.5 \pm 1.7)\times10^5$ M^{-1}. In similar example, time-resolved fluorescence spectroscopy was applied for vb-DMASP-based cAMP imprinted F-MIP [10,11]. Those measurements also showed a decrease in fluorescence with an increase in cAMP concentration. In the fluorescence decays analysis, two major components were identified and assigned to two different types of cavities present in the F-MIP. The first decay component was associated with open imprinted sites, accessible for binding, whereas the second component was assigned to inaccessible sites hindered inside the bulk polymer. These studies revealed that the imprinted sites are mainly situated inside the F-MIP and are inaccessible for fast and effective mass transfer. In order to address this issue, a thin-layer cAMP-imprinted F-MIP was synthesized [12]. Similarly to the bulk F-MIP, thin-layer F-MIP fluorescence was quenched when exposed to cAMP. Noticeably, the quenching was much stronger yielding the complex stability constant K_{MIP} up to 1×10^6 M^{-1}. The higher K_{MIP} value was reasoned by improved cAMP accessibility to the imprinted sites available for re-binding.

In another approach, a thin-layer cGMP-imprinted F-MIP was prepared using 1,3-diphenyl-6-vinyl-1H-pyrazolo[3,4-b]quinoline (PAQ) as an FFM [12-15]. In this piece of research, both steady-state

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and time-resolved fluorescence microscopy was used to investigate F-MIP in the presence of cGMP. Similarly to the vb-DMASP-based F-MIP, for the PAQ-based F-MIP fluorescence quenching was observed upon interaction with cGMP yielding the complex stability constant K_{MIP} was up to 1×10^{6} M^{-1}. Noticeably, the use of time-resolved fluorescence microscopy was reported for the first time and constituted a new approach for studying thin-layer F-MIPs. In fluorescence microscopy experiments, the fluorescence decays were collected at various locations on the thin-layer F-MIP. These decays were then analysed using a fluorescence lifetime distribution (FLD) method [14]. Both a full width at half maximum (FWHM) and distribution position was taken as a measure to analyze cGMP binding. Initially after polymerisation, the MIP FLD was narrow (~7 ns) and positioned at around 15 ns. When cGMP was extracted from the F-MIP, the FLD moved up to 25 ns and became broader up to 12 ns. Simultaneously, the FLD for the NIP was changed insignificantly. After cGMP re-binding, the FLD became narrower (8.5 ns) and returned to its initial position before cGMP washing (~16 ns). The control NIP showed no response to the cGMP rebinding. Since for the MIP, the FWHM and FLD position both for the fluorescence lifetime and intensity did not return exactly to their initial values. The washing was repeated, but no further influence was observed. The alternation of FLD broadening and narrowing accompanied by its shift, can be explained by interaction with cGMP. When cGMP is present in the imprinted site, it interacts with PAQ and consequently, PAQ depopulates its excited state non-radiatively. Therefore, the fluorescence decay time is shorter. After extraction, no cGMP interacts with PAQ and its fluorescence decay time increases. When F-MIP is again exposed to cGMP, the non-radiative processes are again dominating over the radiative processes and PAQ fluorescence is quenched again. The cross-reactivity test was performed towards structurally similar biomolecules and yielded the cross-selectivity factors equal to 1.5, 2.5, and 5.1 for guanosine, cAMP, and cCMP, respectively.

In similar investigations 2-acrylamidopyridine was used as the FFM in bulk F-MIP to detect cGMP [16]. The F-MIP sites showed high affinity to cGMP and significant selectivity over structurally similar GMP and cAMP. The concentration-dependent F-MIP fluorescence quenching reached a saturation at 100 μM that gave the complex stability constant K_{MIP} equal to 3×10^{5} M^{-1}. These results stayed in good agreement with findings of other authors [9,13].

Currently, commercial colorimetric assays are conventionally used to distinguish between cAMP and cGMP. Even though biochemical detection assays are routinely used, the cyclic nucleotides sensing still remains a challenge. Therefore new, robust and cost effective solutions, such as F-MIPs, constitute a promising alternative for efficient nucleotide sensing.

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