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Short communication

Cyclic GMP recognition using ratiometric QD-fluorophore conjugate nanosensors

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ABSTRACT

Novel luminescent ratiometric nanosensors (QD-NAPTHs) were prepared based on cadmium telluride (CdTe655) quantum dots as luminescent nanoscaffolds with naphthyridine dyes as fluorescent receptors. This biosensing bifluorophoric nanosystem has been designed to achieve detection of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) in buffered media. Cyclic GMP is a secondary messenger that is an important factor for detecting cancer, diabetes and, cardiovascular diseases. Due to low concentration levels, even in pathological conditions, sensitive cGMP detection remains a challenge for modern biomedical diagnostics. Here, QD-NAPTH nanosensors were tested in the presence of a target nucleotide and with various structural cGMP analogues. Steady-state fluorescence spectroscopy was used to monitor a change in the nucleotide concentration. A 5-fold increase in naphthyridine fluorescence with a simultaneous decrease in QD luminescence was observed after adding 50 μM of cGMP. Using this novel nanosystem with ratiometric detection, it was possible to recognize cGMP with limit of detection (3σ) equal to 70 ng/ml. Moreover, the enhancement in fluorescence upon interaction with the target nucleotide constitutes a favourable approach towards the detection of cGMP in buffered media. These bifluorophoric nanosensors have a potential for application in fluorescence microscopy imaging and *in-vitro* assays.

1. Introduction

Cyclic GMP is intracellularly active through phosphodiesterases, nucleotide-gated channels, and protein kinases (Vaandrager and Jonge, 1996). It regulates ion channel conductance, glycogenolysis, and cellular apoptosis (Beavo, 1995). cGMP has been found to be physiologically relevant in the development of diseases such as cancer, diabetes and, particularly, cardiovascular diseases (Browning et al., 2009; Giannini et al., 2008). Because of cGMP's role in numerous physiological processes, new sensing devices to detect and monitor cGMP are highly desirable and would provide valuable tools both for clinicians and researchers.

In the last decade, various optical methods have been widely used for the detection of various nucleotides and nucleosides, mostly cGMP or cyclic adenosine monophosphate (cAMP) (Zhou et al., 2011). Conventionally, in biochemistry nucleotide-specific antibodies or DNA strands are used to detect nucleotides *in vitro* or *in vivo* (Gierschik et al., 1986). Noticeably, biological receptors such as antibodies or aptamers possess better affinity to the target with sensitivity around 10 pg/ml (Tsugawa et al., 1991), but they are fragile and require complicated preparation methodology when compared to chemical sensors. With regard to antibodies, QD-based chemical sensors are advantageous since they are much more robust and reliable. Fluorescent sensors to detect nucleotides and nucleosides have also been developed as small molecular probes (Moro et al., 2010; Turkewitsch et al., 1996), nanosensors (Cywinski et al., 2011), molecularly imprinted polymers (Cywinski et al., 2007; Wandelt et al., 2004) and antibodies or aptamers (Li and Ho, 2008). The nucleotide-targeted probes and sensors work on the basis of either a formation of multiple hydrogen bonds between a base and a complementary moiety of a fluorophore (Amemiya et al., 1997), or a cation–anion pairing between positively-charged fluorophore and negatively-charged phosphate groups (Turkewitsch et al., 1998) or through the combination of both mechanisms (Cywinski et al., 2010).

Quantum dots (QDs) are versatile inorganic probes with unique photophysical properties, including broad absorption spectra and a narrow and size-dependent luminescence. These extraordinary properties, together with the common features of colloidal systems, such as their mobility in liquid phases and high surface-to-volume ratio (enabling high sensitivity and usability in

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high-throughput bioanalysis), make them suitable candidates for biosensing. Appropriately surface functionalized QDs linked to biomolecules, such as DNA, proteins or biocompatible polymers are potentially applicable to cellular imaging, drug delivery, and as nanosensors for clinically significant analytes *in vivo* and *in vitro* (Gerion et al., 2001). QDs have a major role in medical and biological research due to their excellent spatial and temporal resolution, as well as their high sensitivity. Unlike other fluorescent labels, QDs do not display unfavourable properties such as luminescence self-quenching and photobleaching; both of which are considerable drawbacks in bioanalysis (Gerion et al., 2001; Medintz et al., 2005).

In recent years, several approaches have been developed to detect nucleotides using QDs. Cadmium sulphide (CdS) QDs have been recently reported to mediate the formation of nanorods build up in the presence of 5′-GMP (Kumar and Kumar, 2009). In another example adenosine triphosphate (ATP) detection was achieved using cadmium selenide QDs functionalized with trimethylammonium (Callan et al., 2008). In this case, the QD luminescence increased 5 times when ATP was present in solution. Moreover, the QDs were selective against other adenosine nucleotides. Another interesting example is represented by QDs with imprinted nanoshells for guanosine recognition (Diltemiz et al., 2008; Neogi et al., 2004). In all aforementioned examples, however, nucleotide detection was realised via QDs functionalized with non-fluorescent and non-specific receptors. A fluorescent and more specific receptor increases the possibility to achieve more selective and sensitive cGMP detection.

Due to their ability to form triple or quadruple hydrogen bonds, naphthyridines have attracted considerable interest over the past two decades. They have been successfully used for the molecular recognition of guanine, tartaric acid, carboxylic acid and amino acids (Goswami et al., 2001; Lu et al., 2006; Yoshimoto et al., 2007). It has been shown that acetoamino naphthyridines bind via triple hydrogen bonds with guanine preferentially over the other nitrogenous bases of adenine, cytosine, thymine and uracil. This property has been demonstrated using supramolecular chemistry, HPLC separation, and in molecular sensing and mapping of DNA sequences (Corbin et al., 2001; Feibush et al., 1987; Nakatani et al., 2000). Organic nanoparticles based on benzofuran-naphthyridine linked molecules have also been developed, and their fluorescence was observed in aqueous media (Sun et al., 2006).

Here we report on the specific base pairing between bi-luminescent CdTe655 quantum dot/naphthyridine (QD-NAPTH) nanosensors and a mononucleotide, guanosine 3′,5′-cyclic monophosphate (cGMP), which, to the best of our knowledge, has not yet been presented. Our approach makes it possible to measure simultaneously both the rising naphthyridine fluorescence signal and the quenched QD luminescence. This ratiometric format allow for more sensitive cGMP detection than in the case of free dye in solution and to reduce the influence of non-specific interactions.

2. Materials and methods

2.1. Materials

All the chemicals used for synthesis and spectroscopy were purchased from Sigma-Aldrich (Taufkirchen, Germany) and used without any further purification. The amine-terminated Polyetylene glycol (12 ethylene glycol units) PEG-NH2 was purchased from Thermo Scientific. All solvents both for chromatography and spectroscopy, as well as labware, were from Roth (Karlsruhe, Germany).

2.2. Synthesis of the CdTe-naphthyridine nanosensors

The naphthyridine receptor was synthesised as described previously (Cywinski et al., 2011). To prepare CdTe-naphthyridine nanosensors 100 μg of carboxylated CdTe quantum dots (PlasmaChem GmbH, Berlin, Germany) were dispersed in 1 ml 50 mM MES buffer at pH 7.2. Then, 0.2 mg of *N*-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, and 0.5 mg of N-hydroxysulfosuccinimide was added and left to react for 10 min at room temperature. 2 mg of the receptor molecule and 100 μg of PEG-NH2 in ethanol were then added and left to react for 2 h. The nanosensors were subsequently washed in MES buffer containing 100 mM ethanolamine. Finally, the nanosensors were multiply washed (up to 8 times) with 10 ml of MES/ethanol (4:1) and twice with pure MES buffer and concentrated to 1 ml to be used for further analysis.

2.3. Absorption and fluorescence measurements

The electronic absorption spectra were collected using a UV/vis/NIR spectrometer Lambda 750 from Perkin Elmer. Steady-state fluorescence spectroscopy experiments were carried out using a FluoroMax 4 spectrofluorometer (Horiba Jobin Yvon GmbH, Unterhaching, Germany). Both for emission and excitation the slits were set to 2 nm bandpass. Acquisition time was set 1 s for each nm. The samples were excited at 450 nm and fluorescence emission was collected in a spectral window from 470 nm to 800 nm. The emission spectra were corrected with respect to the detector response and scattered light. Polystyrene particles functionalised using the naphthyridine dye were prepared according to a protocol described previously (Cywinski et al., 2011).

For fluorescence spectroscopy experiments, each time an aliquot (10 μL) of the final dispersion was diluted in 3 ml of 0.1 M HEPES buffer at pH 7.4 and this solution was transferred to a 1×1 cm^2 quartz cuvette (Hellma GmbH, Jena, Germany). Small aliquots (≥ 3 μl) of the analyte 1 mM solution (up to a total volume of 15 μL) were then added to the cuvette to study the influence of the nanosensor's lminescence. The pH value remained constant throughout all the measurements, as measured using a pH metre (Hanna Instruments, Woonsocket, MI, USA). All measurements were done at room temperature.

3. Results and discussion

3.1. Recognition principle

The cGMP recognition is based on mimicking the interactions that, in nature, occur in DNA between nucleotide bases. In DNA, guanine primarily binds to cytosine via triple hydrogen bonding (Scheme 1a) while, in the case of the QD-NAPTH nanosensors, this bonding is mimicked by the naphthyridine present on a QD surface (Scheme 1b).

Unlike non-specific sterical nanoparticle stabilisation by long aliphatic chains, the naphthyridine functionalised QD can be specifically stabilised in the presence of the negatively charged nucleotide. This specific stabilisation together with cooperativity of the recognition sites can be considered as a measure for the nanosensor selectivity. A similar feature has been already observed for polymeric nanoparticles (Cywinski et al., 2011). The cGMP recognition using QD-NAPTH proceeds as follows: initially the QD-NAPTH nanosensors show high QD luminescence and low dye luminescence. When cGMP is present in solution, it binds to the dye increasing its fluorescence. Simultaneously, the nucleotides interact with the QD surface. Those interactions can be observed as an increase in dye characteristic fluorescence with simultaneous decrease in QD fluorescence. Additionally, the nucleotides play the