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Design, synthesis and evaluation of a fluorescent peptidyl sensor for the selective recognition of arsenite

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Abstract—A series of fluorescently labelled phytochelatin-like peptide sequences has been designed and synthesised as molecules with the ability both to bind to arsenite and to report this binding event. The peptides have been evaluated against a panel of biologically significant ions. The results of these initial studies are reported herein, and a structure which displays selectivity for As(III) over As(V) and other anions has been identified.

Arsenic is a toxic element, exposure to which leads to a wide variety of medical conditions, including cancers, anaemia, dermatological diseases, and damage to most major organs and the nervous system.[1,2] The molecular basis by which arsenic effects disease is not well understood, in part because there are no sensitive, non-destructive, real-time methods by which to study the element in biological systems. New methods for monitoring arsenic levels, both in the environment and in biomedical samples, and also for studying the biochemistry of this element, need to be developed. To this end, preliminary studies aimed at utilising fluorescent peptide motifs as selective sensors for arsenic have been undertaken.

Arsenic speciation in aqueous solution is dependent on the redox environment and pH of the solution. The main arsenic species present in natural waters are inorganic arsenate (As^{V}) and arsenite (As^{III}), the latter being the most toxic species. At physiological pH, arsenite exists as a neutral species (H_3AsO_3) and arsenate is negatively charged ($HAsO_4^{2-}$).[1] In vivo, inorganic arsenic is converted to a variety of other species, including small molecule organo-arsenic species. Biomethylation of arsenic has long been considered a detoxification pathway, but it is now known that trivalent methylated arsenic metabolites, such as methyl and dimethyl arsinous acids (MMA and DMA), are more toxic than arsenite itself.[2]

Small peptidic motifs, which bind specifically to arsenic (both arsenate and arsenite) are currently unknown. However, it is known that arsenite reacts with the thiol groups of cysteine residues.[3] The active sites of arsenite oxidase[4] and arsenate reductase[5] both contain cysteine residues and residues which are positively charged at physiological pH, such as arginine. It is also known that phytochelatins (PCs) are produced by plants in response to exposure to heavy metals such as lead, mercury and cadmium. The PCs are a family of peptides with the consensus sequence $(\gamma\text{Glu-Cys})_n\text{-Gly}$, $n = 2$–11, and are named such that a phytochelatin where $n = 2$ is termed PC_2.[6] Some studies have shown that exposure to arsenic also increases the production of these peptides in some plants.[7,8]

In initial studies, we synthesised a series of naphthyl-labelled phytochelatin-like sequences, in which the γGlu residue of the PC was replaced with Lys, generating an overall positive charge on the peptides at neutral pH. This change was introduced in an attempt to lower the affinity of the peptide for positively charged ions. Arsenic binding to the peptide sequence was to be reported via quenching of the fluorescence of an attached naphthyl (Napth) group, as shown in Figure 1. These species were not expected to bind arsenate ions but might be expected to bind arsenite. Moreover, they can also form the starting point for the subsequent design of sequences that might detect arsenate in concert with relevant redox activity.

Keywords: Arsenic; Arsenite; Peptide sensor; Fluorescence.
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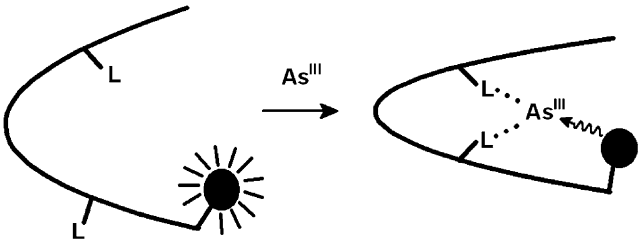

Figure 1. Schematic representation of a fluorescent peptide before arsenite binding, and the fluorescence-quenched peptide after arsenite binding.

Two peptides **1** and **2** were synthesised,[9] which would be expected to possess no distinct secondary structure. Peptide **2** is directly analogous to PC_2, in that it contains dithiol functionality, and this gives a more sensitive fluorescence response upon exposure to excess arsenite than the tripeptide **1**. The most likely explanation for this is that there are a greater number of thiol residues/peptide available in **2** to bind arsenite. These may include 1:1 and 1:2 complexes of peptide:arsenite. No increased response to added arsenate was observed for peptide **2** (see Fig. 2).

In an attempt to improve selectivity, a secondary structure was then introduced in the design and synthesis of peptide **3**.[9] This peptide, via the inclusion of a proline residue had the propensity to form a hairpin turn,[10] in which the two cysteine–lysine regions would be positioned on either side of the turn and opposite each other, for potential arsenite binding. Unfortunately this sequence demonstrated no net enhancement in either the sensitivity, or selectivity of **2** for As^{III}.

Peptide **4** was designed and synthesised[9] to possess a tighter hairpin turn than **3**, because a greater pre-organisation of the peptide binding-site should result in improved binding of arsenite.[10] At high concentrations (100 equiv) of arsenite, this peptide displayed a 50% quenching of the original (unbound) naphthyl fluorescence.

In all of the dithiol-containing systems, arsenite binding was shown to occur via the cysteine thiols. This was accomplished by oxidation of the peptide with DMSO[11] prior to incubation with arsenite, forming the corresponding intramolecular disulfide-bridged compounds. In each case, the treated peptide no longer exhibited any significant change in fluorescence response upon exposure to arsenite.

Since peptides **2** and **4** were found to be much more sensitive to arsenite than arsenate, at high concentrations, it was of interest to test the fluorescence quenching response of these peptides with other biologically significant ions such as sulfate, phosphate, lead and copper (see Fig. 3). The fluorescence responses for phosphate and sulfate were very similar to arsenate, most likely due to the structural similarity of these anions. The fluorescence emissions of both **2** and **4** were quenched significantly, in the presence of cations such as lead (Pb^{2+}) and copper (Cu^{2+}), which are known to bind thiol donors with high affinity.[12] Although the peptides were designed to have a net positive charge, further elaboration of the sequence may be necessary to place the positive charge much closer in space to the thiol-rich binding site in order to influence the binding of positively charged ions.

In conclusion, peptide **4** possesses significant sensitivity to arsenite. As such, **4** appears a most promising motif for exploitation in a second generation of arsenite sensors. Work to this end is currently on-going in our laboratories and aims to both enhance arsenite sensitivity and selectivity, as well as to alter the fluorescence reporting mechanism of peptide **4** such that the molecule acts as a 'switch on' sensor.

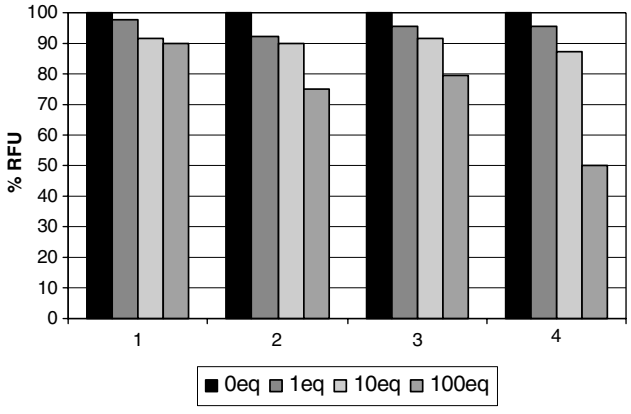

Figure 2. Naphthyl fluorescence quenching response of the four peptides when exposed to increasing equivalents (equiv) of As^{III}. Compound **1** Napth-Cys-Lys-Gly, **2** Napth-Cys-Lys-Cys-Lys-Gly, **3** Ac-Lys-Cys-Thr-Pro-(D)Ala-Val-Cys-Lys(Napth)-Gly, **4** Napth-Cys-Pro-Gly-Cys-Lys-Lys. Fluorescence quenching of peptides (5 μM) observed upon sequential addition of $NaAsO_2$. Samples prepared in 50 mM HEPES (pH 7.0) containing 150 mM NaCl. The experiment was carried out in duplicate, ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 320$–350 nm for all peptides) with spectra acquired at 25 °C and baseline corrected using a sample of buffer solution.

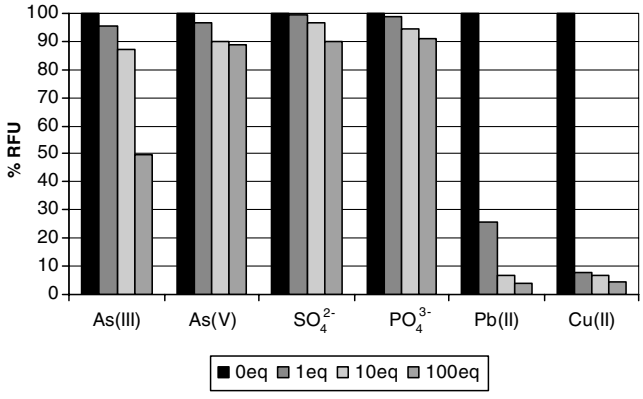

Figure 3. Naphthyl fluorescence quenching response of peptide **4**, Napth-Cys-Pro-Gly-Cys-Lys-Lys, when exposed to increasing equivalents (equiv) of panel of ions. Fluorescence quenching of peptide (5 μM) observed upon sequential addition of $NaAsO_2$, Na_2HAsO_4, Na_2SO_4, Na_2HPO_4, $Pb(NO_3)_2$, $CuSO_4$. Experimental conditions as for Figure 2.

Acknowledgements

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Supplementary data

Response of oxidised versus reduced peptide **4** upon exposure to As^{III}. Response of peptides to As(V). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.08.058.

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9. Peptide sequences were assembled on a Rink amide MBHA resin support using standard Fmoc chemistry with an amino acid/HOBt/HBTU/DIPEA (4:4:4:6 equiv with respect to resin) protocol, for all residues except cysteine. Cysteine couplings were carried out using Fmoc-Cys(Trt)-OPfp and according to the HBTU/collidine method of Angell, Y. M.; Alsina, J.; Abericio, F.; Barany, G. *J. Pept. Res.* **2002**, *60*, 292–299, to prevent racemisation. All peptides were derivatised using 1-naphthylacetic acid, under the standard amino acid coupling conditions; peptides **1**, **2** and **4** were derivatised at the *N*-terminal and peptide **3**, was derivatised at the Lys 8 side chain, which had been previously been Mtt protected. All peptides were cleaved from the resin using trifluoroacetic acid/water/trisopropylsilane/ethanedithiol (93:2.5:2.5:2) and purified by RP-HPLC, under the conditions stated below. The presence of free thiol was confirmed in all cases by performing an Ellmans test (Novabiochem catalog, 2004/5, 3.23).
The identity of each of the peptides was confirmed by high resolution mass spectrometry (HRMS) using electrospray (ES) as the ionisation method. Purity was verified by RP-HPLC analysis on a C18, 5 μm column, using a 2–70% water/acetonitrile gradient, $\lambda = 254$ nm. The RP-HPLC and ESI-MS data, together with extinction coefficients of the naphthyl group ($\lambda_{ex} = 280$ nm) for each peptide are given below.
Peptide **1**: HRMS (ES) calculated for $C_{23}H_{31}N_5O_4SNa$ $[M+H]^+$ 496.1994, found 496.2001. HPLC 21.83 min. ε_{nap} (MeOH) = 6195 M^{-1} cm^{-1}.
Peptide **2**: HRMS (ES) calculated for $C_{32}H_{48}N_8O_6S_2Na$ $[M+H]^+$ 727.3036, found 727.3056. HPLC 22.06 min. ε_{nap} (MeOH) = 6231 M^{-1} cm^{-1}.
Peptide **3**: HRMS (ES) calculated for $C_{51}H_{78}N_{12}O_{12}S_2Na$ $[M+H]^+$ 1137.5201, found 1137.5232. HPLC 22.86 min. ε_{nap} (MeOH) = 6289 M^{-1} cm^{-1}.
Peptide **4**: HRMS (ES) calculated for $C_{37}H_{55}N_9O_7S_2Na$ $[M+H]^+$ 824.3564, found 824.3572. HPLC 23.23 min. ε_{nap} (MeOH) = 6319 M^{-1} cm^{-1}.
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