Fluorescent nitric oxide detection using cobalt substituted myoglobin†

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We report two advances in optical biosensing of nitric oxide (NO). Firstly, we developed an improved biomolecular gated system for fluorescent transduction of heme–NO binding. Secondly, through a cobalt substitution, the detection limit for NO was decreased an order of magnitude lower than that of native myoglobin.

Nitric oxide (NO) sensors are useful for a variety of applications ranging from pollution monitoring to clinical diagnostics and biomedical research.1–3 Elevated NO levels in breath have been used as a marker for the diagnosis of airway inflammation in asthma and chronic obstructive pulmonary disease, allowing early diagnosis and intervention.4 Bioimaging of in situ NO production has allowed the elucidation of its many biological roles.5 While there are a number of NO sensors commercially available,6–8 there is a need for cheap and compact NO breath sensors to enable early detection of asthma attacks and new methods for detecting NO in vivo. Biosensors could provide a useful option for developing cheaper and more selective NO sensors.6,7

Heme proteins are ideal recognition elements for gases such as oxygen and NO. Soluble guanylate cyclase (sGC), a heme protein, can bind nitric oxide in preference over other gases such as oxygen.9 While a number of heme proteins have been tested for their suitability as NO optical sensors, they do not have the sensitivity provide by techniques such as chemiluminescence or electrochemistry. Furthermore, immobilisation of heme proteins can adversely affect both their response and reversal times.10–14

In order for heme proteins to be effectively used as optical biosensors, their sensitivity needs to be increased and limits of detection (LOD) needs to be reduced. In this study, we sought to improve the sensitivity of heme protein based optical transducers using two approaches. The first involves developing an efficient fluorescent method to detect NO binding to heme proteins. The second involves using a metal substituted myoglobin, cobalt myoglobin (CoMb) to decrease the LOD for NO.

We employed a relatively new fluorescent method known as biomolecular gating.15 In this approach, the fluorescence intensity of a fluorophore is modulated by the differing amount of light absorbed by a heme protein in the NO-bound or NO-free state. The heme protein effectively acts as a wavelength selective filter, limiting the exciting light reaching the fluorophore (Scheme 1).15

In the first reported biomolecular gating system, Strianese et al.15 employed two heme proteins; cytochrome c peroxidase and myoglobin to measure NO and O2 binding respectively. Fluorescein immobilised on a nitrocellulose membrane, excited at 450 nm, was used as the fluorophore. Although the authors

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Scheme 1 Cartoon representation of the principle of biomolecular gating. Myoglobin (Mb) acts as a filter modulating the emission of cyan fluorescent protein (CFP). (A) Represents the scenario without nitric oxide present. (B) Represents the scenario with NO bound to Mb, leading to a shift in the Soret peak from 430 nm to 420 nm. This spectral shift leads to a quenching of emission from CFP.
were able to convert absorption changes into either an increase or decrease in fluorescence, quantitative analysis of gas binding was not reported.

The key to an efficient molecular gating system is to optimise the overlap between the excitation band of the fluorophore and one or more of the absorption bands of the gas binding protein. In order to improve the efficiency of biomolecular gating we chose a fluorophore with a large spectral overlap with heme proteins in the 400–430 nm region. Cyan fluorescent protein (CFP) with absorption maxima at 435 nm and 455 nm has large spectral overlap with reduced, oxidised and nitric oxide bound myoglobin (Fig. 1). Fluorescent proteins in biosensors are also advantageous to small molecular dyes such as fluorescein as they have greater photostability.

A further modification to the procedure reported by Strianese et al. was to have both the fluorophore and NO binding protein in solution rather than immobilising the fluorophore on a membrane.

With CFP as the fluorophore, we used excitation wavelengths of 400–430 nm. The changes in the position of the Soret peak with increasing dissolved NO concentration (generated by diethylamine NONOate, see ESI†) were converted into quantitative fluorescence changes (Fig. 2). When exciting at 420 nm, the increase in absorption at 420 nm of myoglobin with NO (Fig. 2B) led to a decrease in CFP emission at 480 nm (Fig. 2C). Conversely, the decrease in myoglobin absorption at 430 nm (Fig. 2B) led to an increase in CFP fluorescence when exciting at 430 nm (Fig. 2C). No fluorescence changes were seen with the addition of NO to CFP.

The biomolecular gating system with myoglobin and CFP was optimised for the “gating” effect of myoglobin. Optimal concentrations of 15 μM and 0.65 μM for myoglobin and CFP respectively were determined. Using the optimised conditions, there was ~85% decrease in the initial fluorescence signal with excitation at 420 nm, substantially greater than the 20% decrease previously reported with fluorescein.\textsuperscript{15}

Initially, both 420 and 430 nm were used as excitation wavelengths. However, excitation at 420 nm was found to give improved reproducibility and larger changes in emission compared to excitation at 430 nm. Using an excitation wavelength of 420 nm, with myoglobin, a linear region between 0–30 μM NO was obtained and it was possible to determine the dissociation constant ($K_d$) of NO binding (Fig. 3).

We hypothesised that an iron to cobalt substitution could improve the limit of detection for NO. CoMb (where iron protoporphyrin IX has been substituted with cobalt protoporphyrin IX) has been used previously to study the function of oxygen binding heme proteins such as hemoglobin and myoglobin. CoMb has a number of properties which could make it ideal for use as a NO sensor. Firstly, Co\textsuperscript{3+}Mb has a lower oxygen affinity (50–100 times larger partial pressure of oxygen, $p_{O_2}$) than native myoglobin,\textsuperscript{17,18} while showing an intrinsically
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We used cobalt substituted myoglobin for this study as cobalt substitutions in the protein have been well characterised. There are a number candidate heme proteins which could be used in a NO sensor, such as Tt H-NOX (Table 1).\textsuperscript{9,14,23} The findings presented here suggest that it could be worthwhile investigating cobalt substitutions in a range of heme proteins to determine if lower detection limits can be obtained.

**Conclusions**

Using CFP and myoglobin we have developed an improved molecular gating system which allowed quantitative analysis of NO binding. The LOD of a myoglobin nitric NO detection system was improved from 10 μM to 1 μM through an iron to cobalt substitution. These are promising advances in the development of optical biosensors for nitric oxide.

**Notes and references**