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Fluorescence Biosensing in Nanopores

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Abstract—**Hydrated nanopores offer a unique environment for studying biological molecules under controlled conditions and fabricating sensors using fluorescence. Silica nanopores for example are non-toxic, biologically and optically compatible with protein, and can be easily synthesized to entrap protein and exclude potentially interfering macromolecules, while transmitting analytes of interest. A well known problem when polymerizing orthosilicates to fabricate silica sol-gel nanopores is the release of alcohol, which denatures proteins. We will describe how using the fluorescence of PRODAN (6-propionyl-2-(N,N-dimethylamino) naphthalene) to monitor methanol generated during polymerization has helped define a protocol with enhanced biocompatibility. The improved biocompatibility of sol-gel nanopores synthesized using tetramethyl orthosilicate (TMOS) has been demonstrated by preserving the unstable native trimer form of allophycocyanin (APC) for up to 500 Hrs without the need to covalently binding the subunits together. This has enabled the observation of native APC trimer by means of its fluorescence in a pore down to the single molecule level. In this paper we demonstrate how PRODAN and another polarity sensitive dye, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, Nile red (NR) report on pore polarity and successfully extend protein encapsulation to nanochannels of alumina (Al_2O_3). Improved biocompatibility of nanopores has potential impact in nanomedicine where the ability to study single biomolecules is a primary goal as it underpins our understanding of disease pathology and therapeutics at the most fundamental level. In sensing also the advantages of nanopore isolation of metabolite-specific protein for detecting non-fluorescent metabolites has been demonstrated. Similar approaches can in principle be developed for both single-molecules and lab-on-a-chip sensors.**

I. Introduction

Molecular fluorescence has a number of key attributes for use in biosensing. For example it is sensitive, specific through spectral tuning and ubiquitous through both naturally occurring and synthetic fluorophores. The advantages of fluorescence decay time measurements in particular over intensity measurements have opened up new avenues in sensing, and are being widely explored for metabolites, particularly glucose [1]. The combination of fluorescence sensing with nanotechnologies, to use what are essentially nanocuvettes, is particularly appealing [2].

Light quanta emitted from single fluorophores can be collected according to two different experimental approaches, either from molecules undergoing free translational diffusion in solution or from molecules immobilized in a specific volume element, *e.g.* the excitation volume in a confocal microscope. Experimentally the easiest case is to record photon bursts emitted from single molecules as they diffuse in and out through a detection volume. Due the small excitation volume in confocal experiments, often only a few femtoliter large, the residence time is very short, but since data can be collected over a long period of time, although on different molecules, a high S/N ratio can often be achieved. Burst analysis is then employed to extract equilibrium and kinetic parameters.

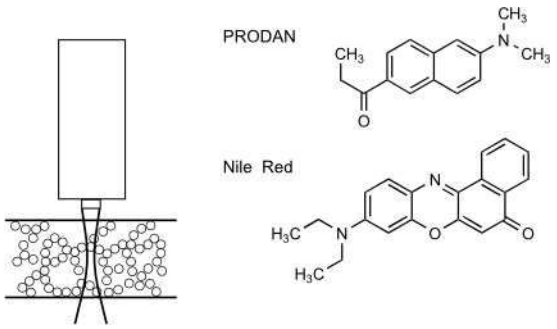

Fig. 1. Left: Illustration of a microscope objective with focus set inside a silica sol-gel. Right: Structures of dyes used in this work.

In order to observe events that occur on a time-scale much slower than the translational diffusion constant, often encountered in protein folding processes, molecules must be immobilized in the detection volume for an extended period of time, and further, immobilization methods must be used that introduce the least disturbance to the molecule of interest. Immobilization techniques commonly used can be divided into two classes, tethering to a surface and entrapment techniques. The tethering to a surface can either be covalent or no-covalent but in both cases suffers from the introduction of specific surface-molecule interactions that may be difficult to predict. Entrapment can be achieved in solvated organic gels like agarose or acrylamide gels. However there is always the issue of specific interactions between the organic molecules. Also, large pore sizes may allow the protein to diffuse. The entrapment in inorganic silica matrices offers some unique advantages as has been demonstrated with green fluorescent protein [3] using orthosilicates as a precursor molecule. However, orthosilicates releases alcohol during the polymerization process that may denaturate some proteins as for example been observed in entrapment experiments on the light harvesting protein APC. To investigate APC in its trimeric form Loos *et. al.* [4] did not use the native form but covalently cross-linked APC and immobilization was achieved by using a polyvinyl alcohol film. The

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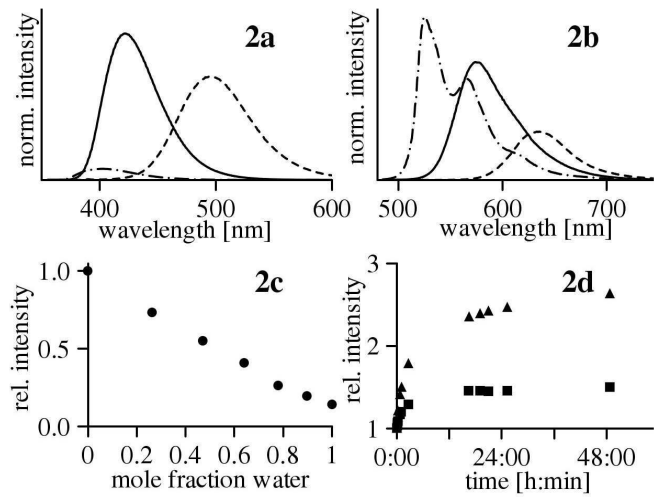

Fig. 2. **a** and **b** shows the emission spectra of PRODAN and Nile red, respectively, in solvents of different polarity: cyclohexane (dash dot), dioxane (solid) and methanol (dash). **c** Dependence of Nile Red fluorescence intensity on the mole fraction water in methanol. **d** Time dependence of PRODAN (▲) and Nile Red (■) fluorescence intensity in a TMOS sol-gel.

encapsulation of APC directly into a silica sol-gel prepared from orthosilicates requires that alcohol is removed. A protocol for this approach without cross-linking has recently been reported and successfully used at the single-molecule level [5].

II. Materials and Methods

All chemicals were purchased from sigma-Aldrich and used as received. The preparation of silica nanopores can be summarized as:

$Si(OCH_3)_4 + 4\ H_2O \rightarrow Si(OH)_4 + 4\ CH_3OH$ (Hydrolysis reaction catalyzed by acid)

$nSi(OH)_4 \rightarrow$ porous network of $(SiO_2)_n + 2n\ H_2O$ (Sequence of condensation reactions leading to formation of discrete nanometer sized SiO_2 particles [6, 7] and eventually a gel).

The protocol we have developed can be summarized by:

1. Sonicate 4.4 ml TMOS, 5 ml H_2O, 0.1ml HCL (nH_2O/nTMOS =9.4)
2. Hydrolysis for 24 Hrs till MeOH production ends (at 4 C to prevent gelling)
3. Vacuum distil for 3-5 mins at 300 mbar, 50 C to remove MeOH
4. Add 15 ml PBS (pH 7.4) and 15 ml borate buffer (pH 9.2)
5. Add protein in PBS pH 7.4

To successfully use inorganic silica sol-gel for encapsulation the pore environment must be tailored, both in terms of pore size and solvent composition. Silica entrapment has the additional advantage that the matrix is optically transparent down to ultraviolet region, and opens up studies in a wider optical window as compared to organic entrapment techniques base on agarose or acrylamide gels. In this report we discuss how two fluorophores can be used to monitor the polarity of the porous silica environment, PRODAN and NR. Structures of dyes are shown in Fig. 1 with a schematic illustration of a silica sol-gel with an encapsulated biomolecule. We also show how protein encapsulation can be successfully achieved using hydrated alumina (Al_2O_3) channels of 200 nm diameter, which was used as received.

Steady-state fluorescence spectra and fluorescence polarization were recorded using a FluoroMax fluorimeter (Horiba Jobin Yvon, Edison USA) and fluorescence decay time measurements using a FluoroCube, (Horiba Jobin Yvon IBH, Glasgow UK). Fluorescence spectra of APC in alumina nanopores were obtained using an α-SNOM confocal microscope (WITec, Ulm, Germany).

III. Results and Discussion

The fluorophores PRODAN and NR, Fig.1, are liphophilic and highly solvatochromic probes. They are often used to investigate polarity and viscosity in nanoscale systems [8]. The sensitivity towards the environment is illustrated in Fig. 2a and 2b where the emission spectra are presented for each fluorophore in three different solvents, cyclohexane, dioxane and methanol. Here the area under respective spectrum reflects the relative quantum yield. In the preparation of silica sol-gels starting from teramethyl orthosilicates water is used in the initial hydrolysis process and methanol is released. Since methanol is the critical component that needs to be removed for successful bioencapsulation it is interesting to compare the relative quantum yield of the fluorophores when dissolved in methanol/water mixtures. In Fig. 2c we show the relative quantum yield for NR at various water concentrations measured as mole fraction in the solution. The sensitivity is significant; an 80 % drop in intensity can be observed when going from pure MeOH to neat water. The sensitivity is similar to that observed for PRODAN, however, PRODAN has a lower solubility in water and shows a time-dependent aggregation where an additional blue-shifted peak gradually appears as water is added and the relation to MeOH concentration is therefore more complex.

The relative fluorescence intensity, $I_{rel}(t)$ for both dyes as function of polymerization time for a typical TMOS sol-gel is shown in Fig. 2d. Recorded data can be fitted to the expression

$$I(t) = 1 - e^{-\frac{t}{\tau_p}} \qquad [1]$$

where t is the polymerization time and τ_p a time constant reflecting the concentration of released MeOH and thus indirectly reporting on the underlying polymerization kinetics. Fitting the data to equation 1 shows that for NR and PRODAN τ_p equals 170 min and 137 min, respectively. It can also be noticed there is gradual hypsochromic shift in the emission spectra, as is shown for PRODAN in Fig. 3a. As is expected, the spectrum recorded 8 minutes after mixing of the precursors is closer to the spectral profile recorded for PRODAN in water as compared to the spectral profile recorded in MeOH. However, after 48 hrs PRODAN emission spectra is shifted towards the spectra in MeOH.

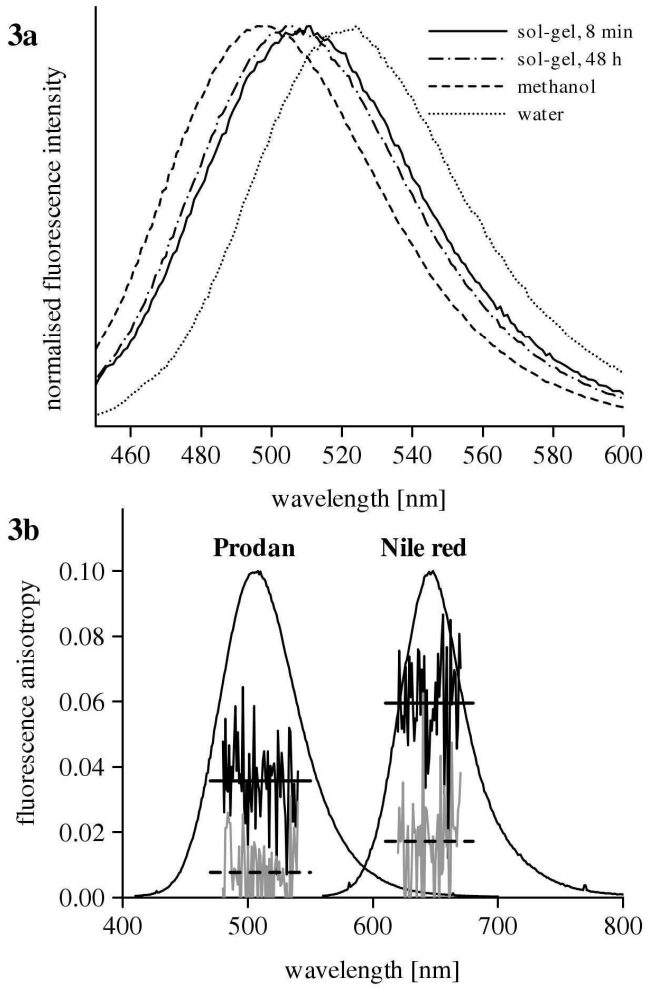

Fig. 3. **a** Emission spectra of PRODAN in a TMOS sol-gel 8 min after mixing (solid line) and 48 hrs after mixing (dashed dot). Emission spectra recorded on fluorophores dissolved in neat water (dotted) and MeOH (dashed) are also shown. **b** Steady-state anisotropy for PRODAN and Nile Red in TMOS sol-gels (black) and in MeOH (grey).

It is well known that the surface of silica particles are negatively charged at neutral and alkaline pH. It is thus often observed that positively charged molecules and ions non-covalently attach to the particles and thus report on an environment at the particle surface. As NR and PRODAN both are electrically neutral, minimal electrostatic interaction with the surface of the silica particles is expected. To determine the localization of probes we have carried out steady-state fluorescence anisotropy experiments. The steady-state anisotropy r can be calculated from the expression

$$r(\lambda) = \frac{I_{vv}(\lambda) - I_{vh}(\lambda)}{I_{vv}(\lambda) + 2I_{vh}(\lambda)}, \qquad [2]$$

where $I_{vv}(\lambda)$ and $I_{vh}(\lambda)$ indicates polarized emission spectra and the subscript refers to the orientation of the excitation and emission polarizer, respectively. In Fig. 3b the steady state anisotropy is compared for PRODAN and NR when dissolved in methanol and when in silica sol-gels. In both cases the steady state anisotropy is slightly higher as compared to fluorophores in neat MeOH. However, the anisotropy is much lower than what would be expected for a fluorophore attached to a silica particle. For example, the hydrodynamic radius of NR is ~0.5 nm which according to Stokes-Einstein equation should give a correlation time of about ~70 ps in neat methanol. If NR is attached to a silica nanoparticle of radius 5 nm the steady-state anisotropy can be estimated to be ~0.39. However, for NR the measured mean anisotropy is $r = 0.06 \pm 0.02$ which indicates that the fluorophore is located in the solvent phase. These results on PRODAN and NR have a wider bearing as the molecule BADAN, which is similar to PRODAN, has recently been shown to be very promising for glucose sensing as a label for a glucose binding protein derived from *E. coli* [9].

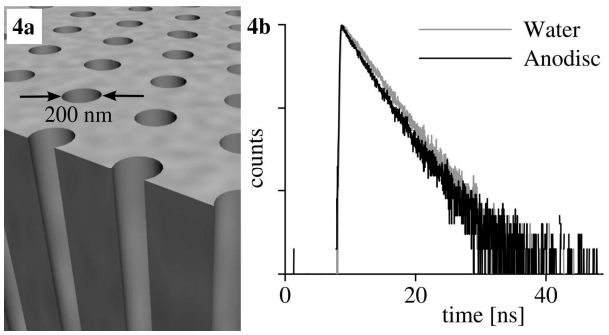

Fig. 4. **a** shows a schematic of a porous anodisc. **b** shows the fluorescence decays recorded on Rhodamine 6G in neat water and in water filled porous alumina.

Porous anodized alumina (Fig. 4a) has been used as a template for nanorods [10]. Here we assess and characterize it as a possible alternative to silica in biosensing. Fig. 4b shows the fluorescence decay of a typical dye used in sensing, rhodamine 6G. The fluorescence decay time in hydrated alumina of 3.62 ns is similar to the 3.92 ns found in bulk water, the difference probably reflecting the dye being attached to the walls and the associated difference in refractive index.

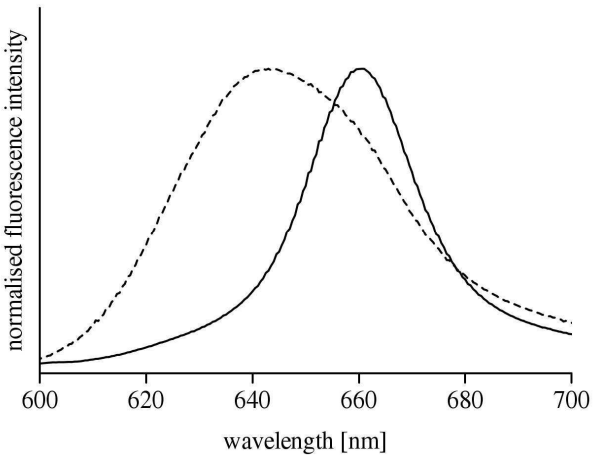

Fig. 5. Emission spectra of APC in a porous anodisc (solid). The emission spectra of APC in monomeric form (dotted) is shown for comparison.

Although the fluorescence is rapidly depolarized this is probably due to light scattering and not due to the dye residing in the pore water unattached to the walls. Fig. 5 shows the trimer of APC can be preserved in alumina nanopores as evidenced by the fluorescence spectrum being similar to that in silica nanopores [5] and is distinct from the monomer in bulk solution (also shown). However, one drawback in using alumina without blocking the pores is leaching of the dye or protein.

IV. CONCLUSIONS

Fluorescence can be used to optimize nanopores for encapsulation in biosensing applications using properties of polarity sensitive dyes such as NR and PRODAN. Even highly unstable proteins used in biosensors can be successfully encapsulated in their native form given careful engineering.

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