A FRET-based method to study the activity of electron or oxygen transfer proteins and redox enzymes

Zauner, G.

Chapter IV

Type-3 copper proteins as biocompatible and reusable oxygen sensors[#]

[#] published as G.Zauner, M.Strianese, L.Bubacco, T.J.Aartsma, A.W.J.W.Tepper, G.W.Canters, *Inorganica Chimica Acta* 2008, 361, 1116-1121.

Abstract

Fluorescently labeled type-3 copper proteins have been proposed previously as solution oxygen sensors by using a FRET mechanism. Herein, we describe how this principle can be adapted to sense O_2 by means of proteins immobilized in optically transparent silica matrices. Specifically, the protein, hemocyanin from *Octopus vulgaris* N-terminally labeled with Cy5, is immobilized in two different kinds of optically transparent silica matrices, which appear to be a promising platform for enzyme encapsulation. The presented results provide proof of principle that fluorescently labeled proteins immobilized in a silica matrix can be implemented in a reusable, biocompatible and stable oxygen measuring device that might lead to new developments in the field of optical biosensing.

Introduction

Oxygen is one of the most important substances for sustaining aerobic life. Thus, there is a continuous need in various fields, such as chemical or clinical analysis or environmental monitoring, to improve available oxygen sensing techniques. Different methods for oxygen detection based on titration, amperometric measurement, chemiluminescence or thermoluminescence have been reported. The Clark type electrode provided a revolutionary device in its time to measure oxygen; it is considered the method of choice for many applications (1). The Clark cell is reliable, but is bulky, consumes the analyte and can be compromised by electrical interference.

Recently new examples of a protein-based method to sense O_2 were reported (2;3). They make use of so-called type-3 copper proteins, the active site of which consists of a pair of Cu ions. Examples are provided by the hemocyanins (Hcs). When the Cu ions in Hc are in the reduced (Cu(I)) state the metal site can reversibly bind O_2. Hcs function as oxygen transporters in arthropods and molluscs. Another example is tyrosinase (Ty) in which the reduced Cu_2 site also binds O_2 reversibly but which, in addition, can oxygenate or oxidise phenolic substrates. In this case the bound oxygen serves as an electron acceptor and a source of oxygen.

The binding of oxygen by these proteins can be followed optically. While the $Cu(I)_2$ form of the protein does not exhibit any features in the visible region of the optical spectrum the binding of oxygen gives rise to a strong (ε= 16-20 $mM^{-1}cm^{-1}$) charge transfer (CT) band at 340nm and a much weaker (ε around 1 $mM^{-1}cm^{-1}$) CT band in the 500- 600nm wavelength range (4). Thus, the absorbances at 340 and 570 nm are a direct measure for the concentration of the oxygen bound species and, therefore, of the oxygen concentration.

Erker *et al* and Decker *c.s.* showed that a considerable gain in sensitivity can be obtained by employing fluorescence instead of absorption detection (2;5). By attaching to the protein a fluorescent label whose emission spectrum overlaps with the 570 nm CT band of the protein, the label fluorescence becomes (partially) quenched due to fluorescence resonance energy transfer (FRET) to the CT band. Since the latter is only present when O_2 is bound, the amount of quenching directly reports on the concentration of the O_2-bound form and thus on the O_2 concentration.

Recently we showed that even higher sensitivity can be reached by employing a different scheme based on sensitised fluorescence (3). The method makes use of the intrinsic fluorescence of the protein - which originates from the tryptophans in the protein - when excited in the near UV. The Trp fluorescence band overlaps with the CT band of the O_2 bound protein at 340 nm and, again, the amount of quenching is a measure for the O_2 concentration in solution. The sensitised fluorescence technique now employs a fluorescent label for which the Trp emission serves as the excitation light source. When the Trp fluorescence is (partially) quenched the label fluorescence diminishes accordingly. Moreover, when the label emission happens to overlap with the 570 nm CT band of the protein, the label fluorescence is additionally quenched, thereby amplifying the contrast between the oxygen-free and oxygen-bound protein. Thus, in this set-up, the excitation source (Trp emission) as well as the quenching of the label fluorescence become sensitive to the binding of oxygen. An added and important advantage of this scheme is that the excitation light and the background fluorescence can be efficiently separated from the label fluorescence since they occur in distinct regions of the spectrum.

In order to demonstrate the potential of this protein- based method for a solid-state O_2 sensing device, we combined this approach with an immobilization technique. This has the potential to provide a more stable and, above all, reusable sensor. Two requirements for an optically based oxygen sensor are: a) transparency and b) inertness of the matrix chosen for the immobilization. These requirements are met by silica

based matrices, which, especially in the last decade, have become an established tool for enzyme encapsulation giving rise to biocatalysts that can be easily recycled (6). By tuning the polymerization reaction conditions (e.g. pH) these so-called sol-gel materials can be designed for a given specific application meaning that the gels can be tailored to a range of porous textures, network structures, surface functionalities and processing conditions. Furthermore, the manufacture of the sol-gel does not require harsh reaction conditions, which is an advantage when working with the often delicate proteins that have to be incorporated in the matrix. It allows proteins to retain their native structure, spectroscopic properties and (catalytic) activity upon encapsulation into the matrix.

The compounds by which the silica network is built around the enzyme are termed precursors. The two most commonly used compounds are:

1) Alkoxide TetraMethOxySilane (TMOS), which is by far the best studied and the most used among a variety of precursors now available (6). The resulting sol-gels are excellent for applications in biosensors (7).
2) Sodium metasilicate (Na_2SiO_3) commonly termed waterglass, which has as its main advantage that no alcohols - which could be harmful for the biomolecule - are generated as byproducts during the encapsulation step (6;8).

In this study, we report on the immobilization of hemocyanin from *Octopus vulgaris*, an endemic species from the Mediterranean sea, labeled with Cy5 at the N-terminus into a silica matrix to obtain a biocompatible and reusable oxygen sensor. The protein has been encapsulated in the above mentioned sol-gel matrices, and the fluorescence of the materials in response to the oxygen concentration in the surroundings has been studied.

Experimental

General

Hemocyanin (Hc) from arthropod *Carcinus aestuarii* and from molluscs *Octopus vulgaris* and *Rapana thomasiana* were prepared following the procedures described elsewhere (9). The purity of the Hcs was checked spectrophotometrically by measuring the ratio of the absorbances at 340 nm and 278 nm and comparing them with literature values (A_{340}/A_{278} = 0.21 (arthropod Hcs) or A_{340}/A_{278} = 0.25 (molluscan Hcs)) (10). Alexa568 and Alexa350 NHS-ester were from Molecular Probes (Leiden, The Netherlands), Cy5 and Cy3 NHS-ester were purchased from Amersham Biosciences (Freiburg Germany) and Atto655 and Atto390 NHS-ester were purchased from ATTO-TEC Biolabeling and Ultraanalytics (Siegen, Germany). 50mM stock solutions of the dyes were prepared by dissolving the powders in water-free DMSO. All purification steps during protein labeling were performed using PD-10 gel-filtration columns (Amersham Pharmacia). Sodium silicate, Dowex 50WX8-100 ion-exchange resin and TMOS (tetramethyl orthosilicate) were obtained from Sigma Aldrich.

Protein labeling

Hc was labeled at the N-terminus in potassium phosphate 100 mM, pH 6.8, using procedures reported in literature (3). Labeling ratios were in the range of 0.2 -1 (dye molecule/protein), as determined from the absorption spectra of the labeled proteins using the extinction coefficients of the 280 nm protein absorption (10) and of the labels as stated by the manufacturers respectively.

Protein encapsulation in TMOS sol-gel

The preparation of silica gels and the encapsulation of proteins were undertaken with pure TMOS, according to a previously reported procedure (7). TMOS (15.22 g) was mixed with milliQ water (3.38 g) in a 1:2 molar ratio followed by the addition of 20 µl of 10 mM HCl. The reaction mixture was sonicated for 20 minutes. Upon addition of buffer (potassium phosphate 100mM, pH 6.8) in a 1:1 (volume) ratio and roughly l ml of labeled protein solution (end conc. of protein: 5-10 µM) the mixture was degassed to remove possible airbubbles. Before gelation 150 µL of the sol solution was quickly poured onto a home made device (quartz slide Heraeus 3 quality with a 1 mm thickness, a 30 mm height and a 8 mm length) yielding a roughly 0.6 mm thick sol-gel layer on top of the quartz slide. Activation of quartz slides with "Piranha solution" (30% H_2O_2 and concentrated H_2SO_4 in a 1:3 volume ratio) was performed before pouring the sol solution on top. Samples were kept sealed with parafilm and aged at 4 ºC overnight before the measurements. Methanol, a byproduct of the TMOS-based sol-gel process, was removed by washing the samples with potassium phosphate buffer (100 mM, pH 6.8) several times.

Protein encapsulation in sodium silicate sol-gel

Sol solution was prepared as described previously (8). Sodium silicate solution (0.83 ml) was mixed with milliQ water in a 1:4 (volume) ratio and the resulting solution was vortexed. Upon addition of a strongly acidic cation-exchange resin (Dowex 50WX8-100), the pH of the solution was lowered to a value of 7.0. The resin was filtered off by vacuum filtration and the labeled enzyme (end conc.: 5-10 µM) was added, the mixture was degassed to remove the possible airbubbles. Pouring, quartz activation and aging was performed as described above.

Absorbance and fluorescence measurements

Absorption spectra were recorded on a Cary-50 Spectrophotometer. Fluorescence spectra and time traces were measured on a Cary Eclipse Spectrophotometer by fixing the glass or quartz slides into a home made device in a 10x10 mm airtight quartz fluorescence cuvette (Hellma Benelux bv, Rijswijk, Netherlands) and applying front-face illumination (11). Measurements were performed both in the gas phase and in potassium phosphate buffer (100 mM, pH 6.8) at room temperature. High quality argon (< 1 ppm O_2) was blown over the sample or into the surrounding buffer solution to deoxygenate the sample while for oxygenation a flow of pure O_2 was used. In the case of gas phase measurements the gas was humidified by passing it through potassium phosphate buffer (100 mM, pH 6.8) before it was passed into the sample.

Förster radius calculations

R_0 values for FRET from Cy5 to the 570 nm absorption of Hc were determined as previously described (11) from the equation $R_0=0.211(J\kappa^2 n^{-4}\Phi_D)^{1/6}$ (Å). Here κ^2 is an orientation factor, n – refractive index, Φ_D – fluorescence quantum yield of the donor and J – spectral overlap integral, defined as $J= \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda)d\lambda$, where $F_D(\lambda)$ is the fluorescence intensity of the donor, $\varepsilon_A(\lambda)$ - the extinction coefficient in [M^{-1} cm^{-1}] of the acceptor at wavelength λ with λ expressed in nanometers. The refractive index was assumed to be 1.4 and the orientation factor κ^2 was taken to be 2/3. Φ_D for Cy5 was taken to be 0.27 (12).

The distance (R) from the Cy5 to the active site was estimated as R= (d +1)nm ± 0.5nm (adding 1nm to the calculated distance d accounts for the approximate length of the linker chain), where d is the distance from the attachment point of the dye (N-terminus). The distance d was estimated to be 2.6 nm from the Hc crystal structure

for *Octopus vulgaris* [PDB 1JS8 , (13)]. The distance (6.2 nm) to the type-3 site of the other oxygen binding sites was too large to contribute to any additional quenching effect. In total 7 tryptophan residues per protomer are surrounding the active site. A quantitative treatment of the quenching of the sensitised fluorescence of the label was not attempted at this stage. (For further details on the estimation of the distances between donor and acceptor as well the influence of κ, please also see chapter 2.)

Results and Discussion

We first deal with the experiments performed in bulk solution (section bulk experiments). They provide a reference for judging the sol-gel samples. The sol-gel experiments are dealt with in the next section (section sol-gel matrices). The last section deals with a number of controls.

Bulk experiments

Initially three different hemocyanins, (Hcs), (from the arthropod *Carcinus aestuarii* and from the molluscs *Rapana thomasiana* and *Octopus vulgaris*) were selected and labeled with various dyes at their N-termini, as described elsewhere (3). The dye switching ratio (*SR*) is defined by $SR = (F_{deoxy}-F_{oxy})/F_{deoxy}$, in which F_{oxy} and F_{deoxy} are the emission intensities of the fully oxygenated and the fully deoxygenated protein upon direct excitation of the fluorophore. They have been determined for all three Hcs in solution (Table 4.1). The *SR* was measured also for the tryptophan residues of each Hc (unlabeled) (Table 4.1). In two similar studies the *SRs* for Trp (92%) and the attached dye TAMRA-SE (50%) have been measured for Hc of the tarantula *Eurypelma californicum (2;5).*

Dye	λ_{abs} (nm)	λ_{em} (nm)	*SR* Hc *Carcinus*	*SR* Hc *Octopus*	*SR* Hc *Rapana*
Alexa350	346	440	19%	44%	54%
Cy3	550	570	22%	40%	53%
Cy5	645	665	33%	55%	23%
Atto655	663	684	35%	42%	13%
Alexa568	580	602	33%	53%	57%
Trp	-	334	80%	68%	78%

Table 4.1. Switching ratios (SR, expressed as percentage) obtained in bulk for three different hemocyanins upon direct excitation of the dye at the wavelength of maximum absorption (λabs).

For further experiments we focused on hemocyanin from *Octopus vulgaris* (*O.v.*) labeled with Cy5. The typical architecture of an *Octopus* hemocyanin, which consists of a string of seven globular functional units (14;15) (designated a to h, about 50kDa each), was verified on a 5% SDS- page gel (data not shown).

In order to check the quality of our samples, we recorded the spectroscopic features of this protein (for details see the experimental section). Figure 4.1A shows the absorption spectrum of (*O.v.*) Hc in the oxygenated and the deoxygenated state and the CT band around 570 nm (inset). The overlap of this band with the emission spectrum of Cy5 can be judged from Figure 4.1B. In the Hc doxygenated state the 570 nm CT band is missing. The difference in spectral overlap modulates the quenching of the label fluorescence.

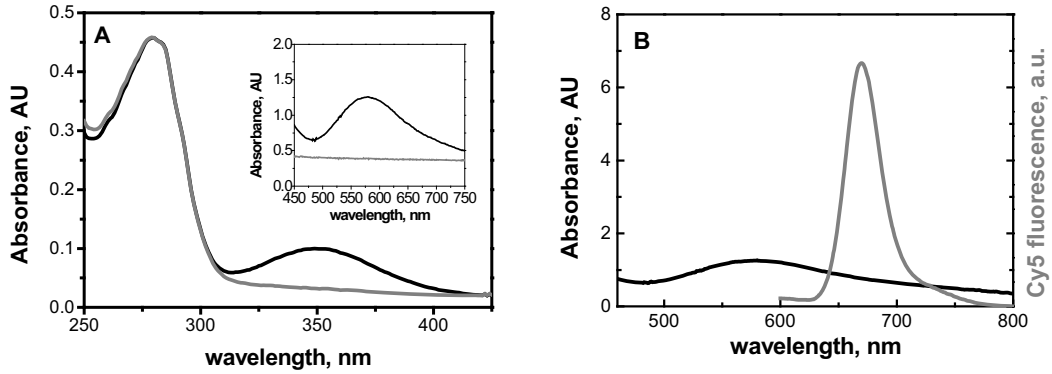

Figure 4.1. A. Absorption spectrum of oxygenated (black) and oxygen- free (grey) *Octopus vulgaris* Hc and the CT band around 570nm (inset; oxy: black; deoxy: grey). **B.** Spectral overlap of the 570 nm band of *O.v.* Hc (black) with the emission spectrum (λ_{em}= 665 nm) of Cy5 (grey). Protein concentration: 2.1 mM.

The previously reported 'sensitised fluorescence approach', where the dye, attached to the protein, is excited by the emission of endogenous Trp residues, can also be observed with this construct. An oxygenation/deoxygenation time-trace was recorded by exciting at two wavelengths alternatingly (with ~5 second intervals) at the dye absorption maximum (see Table 4.1) and at the absorption maximum of the tryptophan (295 nm). The dye emission was followed at 665 nm. Figure 4.2 shows typical traces of the dye response. A switching ratio of approximately 50 ± 5% for the direct excitation of Cy5 and of 70 ± 5% for the excitation through the tryptophans was observed. The *SR* observed by exciting at 295 nm is in accordance with our previous findings (3).

Taking into account the theoretically estimated R_0 value of 2.7 nm in the oxygen bound state a FRET efficiency of roughly 0.56 for direct excitation of Cy5 is predicted, which is in good agreement with the experimental value (for further details see experimental section).

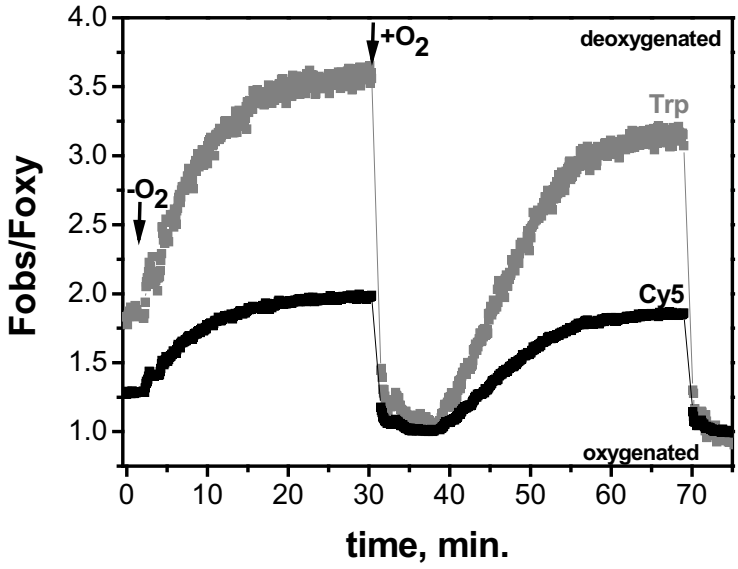

Figure 4.2. Reversible oxygenation and deoxygenation of a solution containing Hc labeled with Cy5 (2.4 μM). Grey trace: excitation of the dye through the Trp emission (λ_{ex} 295 nm); black trace: direct excitation of Cy5 at 645 nm.

The complete removal of [O_2] from the sample, by passing Argon through the solution, takes around 30 minutes. The fraction of O_2-free protein changes accordingly. Oxygenation occurs in a much shorter time period, which is related to the relatively low K_d for oxygen (22 μM) (16). From figure 4.2 it is clear that the minimum and maximum levels of the fluorescence decrease with increasing number of cycles. We ascribe this to damage inflicted to the Hc by the bubbling of the Ar or oxygen through the solution. For the sol-gel encapsulation experiments dealt with in the next section we focused again on the Cy5 *O.v.* hemocyanin construct.

Sol-gel matrices

Undoped sol-gels

Absorption spectra of the undoped sol-gels (TMOS- and waterglass- based as described in the experimental section) were measured on a quartz support and confirmed the transparency of the samples in the 250-800 nm range (Figure 4.3A). Fluorescence from the sol-gel, upon excitation at 295 nm and at 645 nm, was virtually absent for the TMOS case; for the waterglass-based sample a weak band around 380 nm was obtained when excited at 295 nm (Figure 4.3B and 4.3C). In all cases the emission intensity observed is negligible in comparison with that of the doped samples (see below).

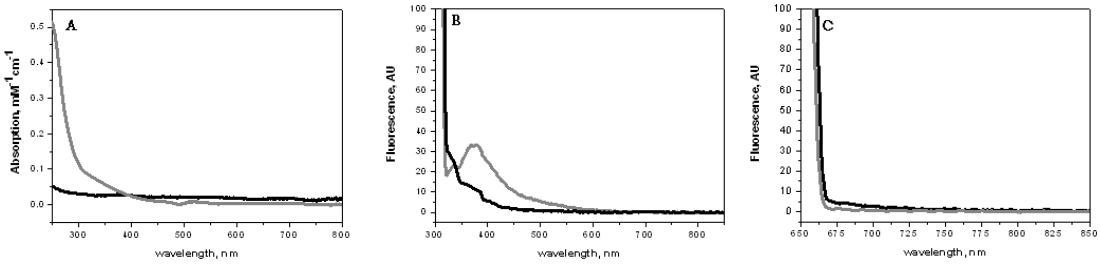

Figure 4.3. A. Absorption spectra of two undoped sol-gels on a quartz support; grey: waterglass based; black: TMOS based. **B.** Fluorescence spectra of two undoped sol-gels on a quartz support upon excitation at 295 nm; grey: waterglass based; black: TMOS based. **C.** Fluorescence spectra of two undoped sol-gels on a quartz support upon excitation at 645 nm; grey: waterglass based; black: TMOS based. Thickness of the all the sol-gels prepared was around 0.6 mm.

Hc doped sol-gels

The fluorescence intensity of the encapsulated Cy5-labeled Hc was monitored as a function of time during the change from an oxygen saturated to an oxygen free environment and vice versa for a) the sample immersed in a gaseous atmosphere and b) the sample immersed in solution. Two parameters were determined: a) the *SR* and b) the time response as described in the previous section. The first two time-traces shown in Figure 4.4 were measured on two different samples in a gaseous surrounding, one waterglass- and the other TMOS-based.

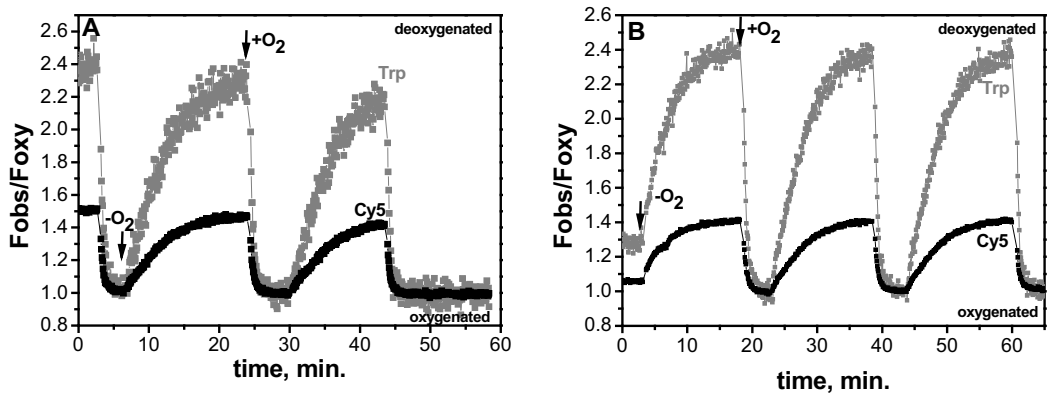

Figure 4.4. Fluorescence intensity as a function of time for hemocyanin Cy5 upon deoxygenation and oxygenation; excitation at 295 nm (grey) and 645 nm (black); protein concentration 5-10 μM. The sample was immersed in a gaseous atmosphere. A: waterglass based sol-gel; B: TMOS based sol-gel.

In the gas phase the deoxygenation response time (about 16 mins) for both sol-gels (waterglass and TMOS) appears to be twice as short as in solution (about 32 minutes). This probably reflects the faster diffusion of the oxygen in a gaseous atmosphere than in solution.

Concerning the *SRs*, values of roughly 30% for the direct and circa 60% for the indirect excitation of the dye were observed both for the waterglass- and the TMOS-based sol-gels. These values are smaller than the ones obtained in the bulk. Possibly a

fraction of encapsulated enzyme is not accessible to the substrate (6) or has been damaged and lost its capability to bind O_2. This would increase the background fluorescence and cause a decrease of the *SR*.

For the measurements of TMOS- and waterglass-based samples in surrounding buffer, *SRs* of 25% in case of direct excitation of the dye and 50% for excitation through the Trps were obtained (data not shown). The response time was 32 minutes for both sol-gels, comparable to the bulk experiments (30 minutes). The 'shelf life' of our samples was found to be at least 15 days upon storage at 4 degrees as judged by the lack of decrease in either the *SR* or the response time of the samples.

Controls

Several experiments were performed as controls: 1) The fluorescence intensity of neither unreacted Cy5 nor bovine serum albumin labeled with Cy5, both individually encapsulated in a sol-gel matrix, depended on the [O_2]. 2) The buffer solution surrounding the quartz slides was checked for leaking of the labeled protein out of the matrix. After prolonged incubation of the solgel in a buffer solution (one to two days), the buffer showed no trace of dye fluorescence or absorption. As already reported, the mechanical strength of silica gels is often preventing the leaking of biomolecules (17). 3) Tryptophan fluorescence has been used to study stability and flexibility of proteins upon entrapment (18). For example, the emission maximum of correctly folded Hc occurs at 331 nm, as reported by (19), while a shift to higher wavelength indicates that initially buried Trp residues become exposed to the solvent, possibly signalling denaturation of the protein. Hence, we measured Trp emission spectra of encapsulated Hc over several weeks. After storing our sol-gels for 7 weeks at four degrees, no significant alterations in the bandshape or intensity of the Trp emission could be detected, suggesting that the enzyme is stable in the matrix.

Conclusions

The present data provide proof of principle that the previously published results on 'sensitized' fluorescence for oxygen sensing remain valid when labeled hemocyanin is encapsulated in a polymeric sol-gel matrix. It has been reported that TMOS is less favourable than waterglass for the immobilization of biomolecules due to generation of methanol as a side product which might lead to denaturation of the encapsulated protein (17;20). In our experiments this appears not to be the case as we observe the same activity in both sol-gel matrices. The relative robustness of the hemocyanin we are using might contribute to this feat (21). The present implementation of labeled Hc into an oxygen sensor features a fast response, biocompatibility, reusability and outstanding stability. Furthermore, the large variety of Hc types, characterized by different oxygen affinities, provides a broad range of oxygen sensing capabilities. Our findings might be significant for possible applications in the fast growing field of oxygen and/or biosensing.

Acknowledgements

We dedicate this manuscript to Ed Solomon on the occasion of his 60th birthday in recognition of his ground breaking contributions to our understanding of the biological chemistry of copper. This work is supported by the Stichting voor Fundamenteel Onderzoek der Materie (FOM) with financial aid from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) and and by the European Community through the EdRox network under Contract No. MRTN-CT-2006-035649.

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