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Carbon nanotube-based biosensors

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Abstract

An easy and rapid detection of hazardous compounds is crucial for making on-the-spot irreversible decisions at airport security gates, luggage storage rooms, and other crowded public places, such as stadia, concert halls, etc.

In the present study we carried out a preliminary investigation into the possibility of utilizing as advanced nano-biosensors a mutant form of the bovine odorant-binding protein (bOBP) immobilized onto carbon nanotubes. In particular, after immobilization of the protein on the carbon nanotubes we developed a competitive resonance energy transfer (RET) assay between the protein tryptophan residues located at the positions 17 and 133 (W17 and W133) and the 1-amino-anthracene (AMA), a molecule that fits in the binding site of bOBP. The bOBP–AMA complex emitted light in the visible region upon excitation of the Trp donors. However, the addition of an odorant molecule to the bOBP–AMA complex displaced AMA from the binding site making the carbon nanotubes colorless.

The results presented in this work are very promising for the realization of a color on/color off b-OBP-based biosensor for the initial indication of hazardous compounds in the environment.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Odorant-binding proteins (OBPs) are small extracellular proteins belonging to the lipocalin superfamily. They have been supposed to play a role in peri-receptorial events of odor detection by carrying, deactivating, and/or selecting odorant molecules [1–3]. The OBPs share a conserved folding pattern, an eight-stranded β-barrel flanked by an α-helix at the C-terminal end of the polypeptide chain. The β-barrel creates a central apolar cavity whose role is to bind and transport hydrophobic odorant molecules. These proteins reversibly bind odorant molecules with dissociation constants in the nano-/micromolar range. Although their functions are still not fully understood, OBPs are also believed to participate in the deactivation of odorant molecules [4–6]. OBPs have been identified in a variety of species, including pig, rabbit, mouse, and rat. Bovine OBP (bOBP) shows a peculiar three dimensional (3D) structure, characterized by domain 'swapping'. In solution at neutral pH it appears as a dimer in which each monomer is composed of the classical lipocalin fold, with a large buried cavity internal to the beta-barrel forming the binding site for odorant molecules. The absence of a Gly after position 121 in the sequence and the lack of the disulfide bridge between the C-terminal and the beta-barrel, which are both strictly conserved in other sequences of mammalian OBPs, were identified as the determinants for the domain swapping. This hypothesis is supported by the finding that a mutant bOBP in which a glycine residue was inserted after position 121, showed a monomeric structure. Recently a 'deswapped' triple mutant bOBP (Gly 122+, W64C, H156C) has been obtained, in which a Gly residue is inserted after position 121 and the two residues in positions 64 and 156

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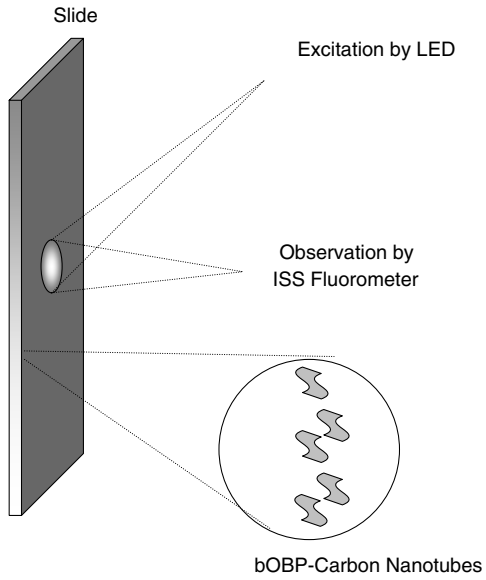

Figure 1. Experimental sample geometry for fluorescence front-face measurements.

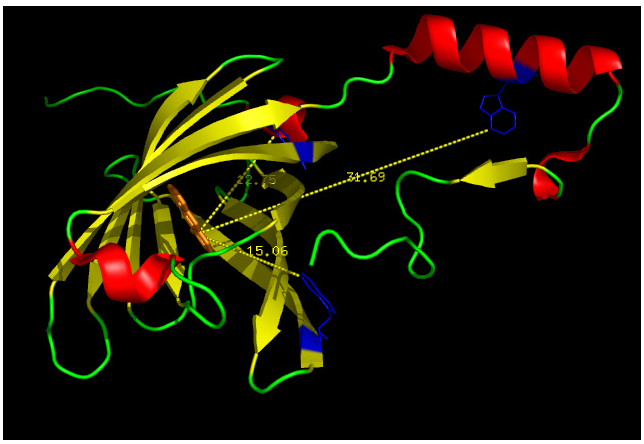

Figure 2. Structure of bOBP.

are replaced by Cys residues, in order to restore the disulfide bridge common to the lipocalin family [7–9]. This protein has been over-expressed in *E. coli* and it has been demonstrated that it binds compounds that are usually utilized for the preparation of explosives and/or compounds produced upon an explosion [10, 11].

In this work we utilized this mutant form of bOBP to develop a new sensing concept: a light on/off carbon nanotube-based biosensor for easy and rapid detection of harmful analytes present in the environment.

2. Materials and methods

2.1. Materials

Carbon nanotubes were from Sigma. All the chemicals from different commercial sources, were of the purest grade available. A brief description of bovine OBP purification and the functionality test with 1-amino-anthracene is given below [9, 12].

3. Protein production

A 6xHis affinity tag was placed at the N-terminal of bOBP formed by polymerase chain reaction (PCR) using specific primers. The fused cDNAs were sub-cloned in the expression vector pT7-7 and the expression of the proteins, in BL21-DE 3 *E. coli*, was realized as reported above for the recombinant form of the mutant bOBP [9]. The purification of the proteins was obtained by affinity chromatography with an Ni-NTA agarose (Quiagen, Germany) according to the manufacturer's instructions, followed by a second chromatographic step on the anion exchange column Recourse Q (Amersham Biosciences, Italy), in FPLC. The purity of bOBP preparation was determined by SDS-PAGE and protein concentrations were calculated based on the absorbance values at 280 nm ($48\,000\ M^{-1}\ cm^{-1}$ for bOBP). Functionality of the bOBP form was determined by direct titrations using the fluorescent ligand 1-amino-anthracene as previously reported [9, 12]. Briefly, 1 ml samples of 1 μM OBP, in 20 mM Tris-HCl buffer pH 7.8, were incubated overnight at 4 °C in the presence of increasing concentrations of AMA (0.156–10 μM). Fluorescence emission spectra between 450 and 550 nm were recorded with a Perkin-Elmer LS 50 luminescence spectrometer (excitation and emission slits of 5 nm) at a fixed excitation wavelength of 380 nm and the formation of the AMA–OBP complex was followed as an increase of the fluorescence emission intensity at 480 nm. The dissociation constants of the AMA–OBP complexes were determined from the hyperbolic titration curves using the nonlinear fitting program of Sigma Plot 5.0 (Cambridge Soft. Corp., Cambridge, MA, USA). The concentrations of the AMA–OBP complexes were determined on the basis of emission spectra obtained by incubating AMA (0.1–10 μM) with saturating amounts of both OBP forms.

4. Fluorescence microscopy

A 2P laser (Mira, Coherent, Santa Clara, CA) pumped by 6.5 W of 532 nm light (*green*) from a Verdi solid state laser (Coherent) generates femtosecond 820 nm pulses at 80 MHz (*magenta*). The infrared (IR) laser beam, directly coupled to a microscope (Zeiss Axiovert 135, Zeiss, Jena, Germany), is expanded by a beam expander (BEX), attenuated by neutral density filters and passed to the X–Y scanner (SCN), which projects the scanned beam onto the objective (OBJ) (Zeiss Apo C 40×, numerical aperture (NA) = 1.2 water immersion) and muscle fiber (MUS). The IR power impinging on the muscle is 65 mW. Fluorescent light (*yellow*) is collected by the objective, passed by the same scanner, and reflected by the dichroic mirror M3 into photomultipliers 1 and 2, which detect orthogonally polarized light passed by crossed analyzers AN1 and AN2. Since the fluorescent light is scanned again on the way to the detectors, it is termed descanned detection. Alternatively, mirror M5 can be substituted by a dichroic filter to pass the fluorescent light to another set of photomultipliers 3 and 4. Since the fluorescent light does not

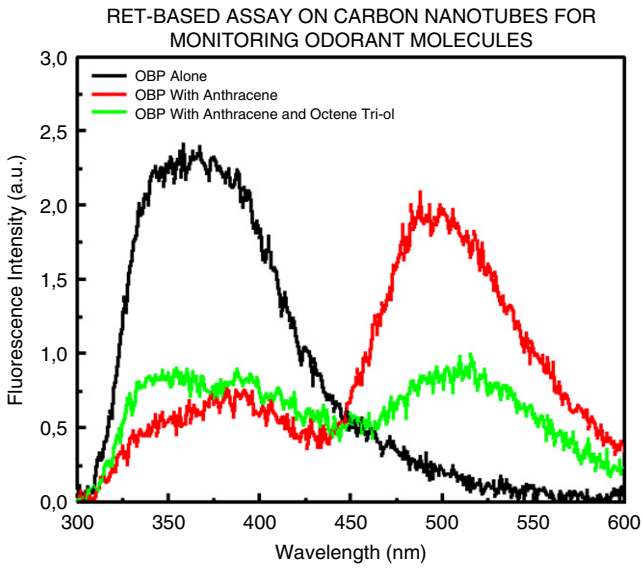

Figure 3. Fluorescence emission spectra of bOBP alone, in the presence of AMA and AMA ligand.

pass through the scanner, it is termed non-descanned detection. The significant advantage of this mode of detection is that the distance between the detectors and the sample is shortened and that the fluorescent light does not enter the microscope at all, and hence does not pass through the scanner or is not attenuated by the internal optics. Unless otherwise stated, all the experiments were performed in non-descanned mode. The 351 + 364 nm light from the ultraviolet (UV) laser (*blue*) (Enterprise, Coherent) is made collinear with the IR beam by the dichroic filter FT395. A fast-shutter SHT (Vincent Associates, Rochester, NY, model T132) is opened for 10 ms to admit UV light to the sample.

5. Fluorescence spectroscopy

Emission spectra were obtained with an ISS K2 spectrofluorometer. The excitation was set at 295 nm in order to exclude the tyrosine contribution to the overall fluorescence emission. Front-face measurements were performed on bOBP immobilized onto CN in the absence and in the presence of AMA and the ligand according to figure 1.

6. Results and discussion

In figure 2 is shown the structure of bOBP. In particular it is possible to notice that the molecule 1-amino-anthracene (AMA) fits very well into the bOBP binding site. A static analysis of the 3D structure of bOBP shows that the protein tryptophan residues are located in proximity to AMA, that is AMA and the protein indole residues are at a distance (Foster distance) allowing a resonance energy transfer (RET) phenomenon. We questioned whether it would be possible to design a carbon nanotubes (CTs) competitive RET assay based on the Trp donor–AMA acceptor for sensing the presence of odorant molecules in the environment.

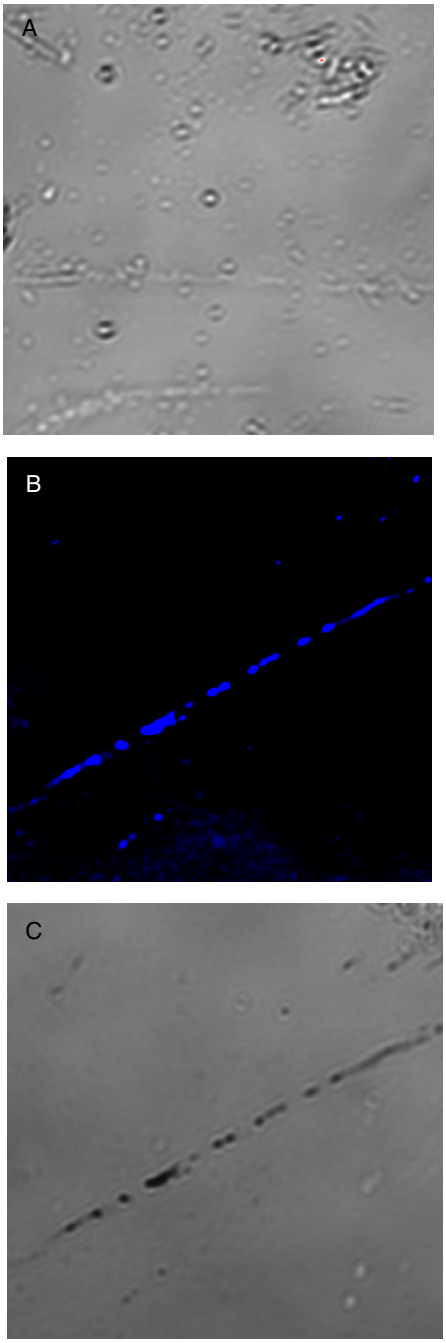

Figure 4. Optical microscope image of CTs (A) with bOBP–AMA (B), and CTs with bOBP–AMA-ligand (C).

We verified that bOBP could be immobilized onto CTs by simple incubation of the CTs-bOBP at room temperature for 10 min in aqueous buffers (phosphate buffer, pH 6.5). In fact, after several washings of the bOBP-treated CTs, the absorbance and fluorescence measurements confirmed that a large amount of bOBP was still present on the CTs surface.

Figure 3 shows the emission spectra of CTs-bOBP alone (black line), upon addition of AMA (red line) and upon addition of an excess of 1-octen-3-ol, the natural ligand of bovine OBP [12].

The resonance energy transfer process observed upon addition of AMA shows that the close interaction between the

Trps of bOBP and AMA results in a high efficiency process of RET between the donor and acceptor. Since 1-octen-3-ol is present in large excess, the addition of the ligand to the bOBP–AMA complex results in a displacement of AMA from the bOBP binding site, and in turn to a decreased efficiency of the RET.

As the emission of AMA was in the visible region of light, we wondered whether it would be possible to design a light on/off nano CT-based biosensor.

Figure 4 shows the confocal microscopy images of bOBP-treated CTs alone (figure 4(A)), in the presence of AMA (figure 4(B)), and after addition of 1-octen-3-ol (figure 4(C)). It is possible to observe a spectacular emission of blue light due to RET upon excitation of Trp at 290 nm. In fact, the CTs become completely blue in color. The addition of the competitive odorant molecule, 1-octen-3-ol, displacing AMA from the OBP ligand binding site, makes the CTs colorless.

The results shown above demonstrate that bOBP can serve as a probe for the development of an optical biosensor for odorant molecules present in the environment. Additional studies are needed to obtain a bOBP-based sensor that displays larger spectral changes, as we feel that it is important to covalently immobilize bOBP onto CTs. In addition, the use of different fluorophores for RET assays could allow larger spectral variations since RET is a through-space interaction which occurs whenever the donor and the acceptor are within the Forster distance (R_o) and does not require change in the probe microenvironment. For these reasons, we are confident that bOBP can be used with long wavelength donors and acceptors to devise a sensor for odorant and, in general, volatile molecules to use for safety and homeland security. Since the measurements can be easily performed by using an LED as an excitation source, one may envisage a polarization-based device with an external calibrated standard. The main advantage of using this method is the obtainment of ratiometric polarization measurements that are not influenced by light instability and sample perturbation. Finally, one can imagine a variety of OBP mutants covering a wide range of ligand binding constants, each labeled with a different fluorophore.

In conclusion, the recombinant bOBP appears to be a valuable source for the development of innovative and stable CT-based biosensors for safety and homeland security.

Acknowledgments

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