Research Article

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A photosensitizing fusion protein with targeting capabilities

Abstract: The photodynamic treatment for antimicrobial applications or anticancer therapy relies on reactive oxygen species generated by photosensitizing molecules after absorption of visible or near-infrared light. If the photosensitizing molecule is in close vicinity of the microorganism or the malignant cell, a photocytotoxic action is exerted. Therefore, the effectiveness of photosensitizing compounds strongly depends on their capability to target microbial or cancer-specific proteins. In this study, we report on the preparation and preliminary characterization of human recombinant myoglobin fused to the vasoactive intestinal peptide to target vasoactive intestinal peptide receptor (VPAC) receptors. Fe-protoporphyrin IX was replaced by the photosensitizing compound Zn-protoporphyrin IX. Taking advantage of the fluorescence emission by Zn-protoporphyrin IX, we show that the construct can bind prostate cancer cells where the VPAC receptors are expressed.

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Introduction

Photodynamic therapy (PDT) of cancer is an adjuvant therapeutic procedure that combines an otherwise harmless compound (the photosensitizer, PS) with visible light and molecular oxygen to achieve the photoinduced killing of malignant cells [1–5]. Cytotoxicity is obtained through the action of reactive oxygen species (ROS), mostly singlet oxygen, produced by the interaction between the excited states of the PS and molecular oxygen [6]. The short lifetime of ROS (the lifetime of singlet oxygen in water is about $3\,\mu s$ [7] and that of $O_2^{-\cdot}$ radical is ca. $1\,\mu s$ [8]) means that the oxidant action of these species is exerted only against molecules located within some 200 nm from their generation site [9,10], which requires the photoactive compound to be brought in close vicinity of sensitive cellular components.

Precise and effective delivery of PS molecules to their biological targets is still an open issue in PDT. Their low water solubility, which reduces bioavailability, is normally addressed using suitable carrier systems [11–15]. Among possible carriers, proteins offer the advantage of being inherently biocompatible. Particularly, PSs can be covalently conjugated with peptides or proteins that target specific cells [12]. Antibodies are an obvious choice [12–18], although their recombinant production in mammalian cells is costly and time-consuming. Therefore, single-domain antibodies called nanobodies were proposed [16]. The use of short peptides to introduce targeting properties in supramolecular complexes between a PS and a carrier for PDT applications was also suggested. In one strategy, short peptides are grafted on nanoparticles [19,20] or supramolecular assemblies [21] functionalizing with PS molecules. Peptides were also conjugated directly to PS molecules [12,22,23].

As an alternative to covalent conjugation to amino acid residues, which might result in heterogeneous products, the delivery of hydrophobic PSs can take advantage of their spontaneous binding to internal hydrophobic protein pockets of proper size. Within the binding pocket, PSs preserve their monomeric, photoactive excited states, leading to fluorescence emission and generation of ROS [24–30].

Proteins capable of spontaneously binding PSs can be engineered to encompass a signaling peptide endowed with the desired target specificity [31,32]. Recombinant carriers that include both the PS-binding moiety and the targeting peptide do not require additional chemical modifications – which would need further purification steps – unlike chemically conjugated peptides [12]. Moreover, several peptides targeting drug receptors overexpressed in cancer cells have been characterized for both diagnostic purposes [33] and therapeutic applications [34] and their incorporation in the sequence of a recombinant protein is straightforward. The combination of PSs with these functionalized nanostructures consisting of PS-binding proteins fused with targeting peptides has the potential for intelligent drug delivery with theranostic capabilities [35].

Among protein-based photosensitizing compounds, zinc-substituted myoglobin (ZnMb) is of special interest. The photosensitizing properties of ZnMb were reported by Lepeshkevich et al. [36]. In their study, the iron ion at the center of the heme moiety of Mb was replaced with a Zn(II) ion. Just like Fe-protoporphyrin IX, Zn-protoporphyrin IX is bound to the protein matrix using a coordinate bond to the proximal HisF9. This introduces a photoactive cofactor in the heme pocket that can photosensitize the production of $^1O_2$ with yield $\Phi_\Delta = (0.9 \pm 0.1)$ [36]. We have recently broken these properties to obtain a photosensitizing material that is effective against planktonic Staphylococcus aureus, taking advantage of weak interactions between the protein and the bacterial wall [37].

The superior photosensitizing properties of ZnMb could be made more effective if targeting properties are introduced into the protein carrier by fusing the myoglobin gene with that coding for a short peptide sequence that binds the molecular species of interest.

In this study, we explored this concept and reported on the preparation and preliminary characterization of human recombinant myoglobin genetically fused to the vasoactive intestinal peptide (VIP). VIP is a 28-amino acid peptide that belongs to the glucagon/secretin superfamily and is an agonist of the VPAC1 and VPAC2 G-protein-coupled receptors [38,39]. VIP basic peptide contains three lysines (no. 15, 20, and 21) and two arginines (no. 12 and 14) [40,41]. VPAC1 is overexpressed in several frequently occurring human tumors, including breast, prostate, pancreas, lung, colon, stomach, liver, and bladder carcinomas, as well as lymphomas and meningiomas [42,43]. In particular, VPAC1 was identified in prostate cancer cells, where overexpression was reported [42,44]. VPAC receptors were suggested to play a major role in the progression and angiogenesis of several malignancies [45]. Reverse transcriptase polymerase chain reaction studies, as well as functional studies using a specific agonist and antagonist for each receptor subtype, peptide binding, and adenylate cyclase stimulation, show that VPAC1 and VPAC2 receptors are present in prostate cancer cell line (PC3) cells [46,47], although more recent data suggest that the expression of VPAC1 might be rather low [48].

The overexpression of VPAC1 on several common neoplastic tissues is receiving attention both for tumor imaging and targeted treatment by coupling cytotoxic agents to VIP analogs [49], as functional studies demonstrated a VIP-binding affinity for VPAC1 of 0.6 nM [50]. VIP analogs have been exploited to develop contrast agents mostly based on radionuclide-labeled probes for molecular imaging [45]. Nanoparticles designed for VIP drug delivery have also been developed [51].

In this study, we showed that the recombinantly expressed fusion protein myoglobin-VIP (Mb-VIP) is endowed with targeting capability towards cells overexpressing VPAC1, and can be turned into a photosensitizing compound by replacing Fe-heme with Zn-heme (ZnMb-VIP). The photosensitizing properties and the fluorescence emission by ZnMb-VIP make the compound a potentially theranostic agent.

Materials and methods

Gene

The synthetic gene encoding human myoglobin (NCBI Reference Sequence: NP_001349775.1) was codon-optimized for expression in *Escherichia coli*. The gene was fused at the N-terminal with a hexahistidine tag followed by a tobacco etch virus (TEV) protease recognition site. At the C-terminus, the gene was in-frame with a sequence encoding a thrombin recognition site followed by encoding the VIP peptide (HSDAVFTDNYTRLRKQMAVKKYLNS-ILN) [52]. The thrombin site was added to remove the VIP peptide from the final product if needed. The synthetic gene (Geneart, Life Technologies) was subcloned in a pET28a plasmid (Novagen, Merck group), which harbored a kanamycin resistance gene between the NcoI and SalI restriction sites. The final construct – named pMbVIP – was verified

through sequencing. The plasmid was finally transformed into BL21 cells, BL21 star cells, Rosetta cells, and BL21 cells harboring the Takara plasmids (Takara Bio Inc., Shiga, Japan) for an initial expression screening. BL21 cells harboring the Takara 4 plasmid, which encodes for chaperones groES, groEL, and tig, were subsequently used for expression.

Expression of MbVIP

Single colonies of BL21 cells, BL21 star cells, Rosetta cells, and BL21 cells harboring the Takara plasmids (Takara Bio Inc., Shiga, Japan) were transformed with pMbVIP and isolated from Luria-Bertani (L.B.) agar plates containing the appropriate antibiotics. They were then inoculated in 50 mL of L.B. culture medium containing the appropriate antibiotics. The following day, 10 mL of the cultures were inoculated in 1 L of L.B. or DM1 medium and grown at 37°C until an absorbance of 0.4 OD was achieved. At that point, 1 mM isopropyl ß-D-1-thiogalactopyranoside was added, and the culture was allowed to grow at different temperatures ranging from 20 to 42°C, in the presence and absence of iron chloride ($FeCl_2$) or hemin. Cells were recovered by centrifugation, resuspended in phosphate-buffered saline (PBS) buffer, and lysed by sonication. The proteins were then purified with immobilized metal affinity chromatography using a TALON® Superflow™ resin (Cytiva) and finally brought in a solution containing 100 mm of NaH_2PO_4 at pH 7. The proteins were then concentrated to around 1 mg/mL. The integrity of the proteins obtained from the various cultures was tested by matrix-assisted laser desorption/ionization (MALDI) spectrometry (MALDI TOF/TOF 4800 Plus, AB SCIEX), quantified by sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and characterized spectroscopically (Cary4000, Agilent). Since expression in BL21 cells transformed with the Takara 4 plasmid in the presence of hemin and at 20°C offered the best results, the subsequent growth was carried out under these conditions, in the presence of 30 μg/mL kanamycin and 20 μg/mL chloramphenicol in all media. Upon induction, 1 μL tetracycline was also added to the cultures.

Heme exchange of MbVIP

Fe-heme was exchanged using a published protocol [37]. Briefly, the protein at 1 mg/mL concentration was first partially denatured by lowering the pH of its solution to around 2.8. Butanone was then added to extract Fe-heme. Butanone was then removed by extensive dialysis against a buffer containing 100 mm of NaH_2PO_4 at pH 7. Aliquots of a Zn-protoporphyrin IX solution at 10 mm concentration were then slowly added to the protein solution until a small molar excess was reached. Excess Zn-heme was removed by diafiltration, and the protein solution was brought to 0.3 mg/mL concentration.

Preparation of ZnMb

As a non-targeted control, horse Mb was prepared as Zn-protoporphyrin IX complex (ZnMb) as described [37].

Cell cultures

PC3 human prostate adenocarcinoma cells were grown in Ham F12 nutrient mixture, supplemented with 5% FBS and 1% penicillin–streptomycin solution. PC3 cells were seeded on Nunc plates (ThermoFisher) at 60–80% confluence and were used after incubation at 37°C and 5% CO_2 for 24–48 h.

Spinning disk microscopy

Fluorescence imaging was performed with a spinning disk confocal microscope comprising an inverted microscope body (TiE, Nikon Instruments, Yokohama, Japan), an incubation system (OKOLAB, Naples, Italy), four laser lines (405, 488, 561, 640 nm) and two spinning disks containing about 20,000 pinholes coupled to the same number of microlenses (CSU-X1, Yokogawa, Tokyo, Japan), necessary to focus the excitation laser light. The fluorescence light was collected by an oil immersion 100× 1.4NA objective (Nikon Instruments, Yokohama, Japan) and acquired by an electron multiplying charge coupled device (EMCCD) camera (Ixon3 897 Andor, Oxford Instruments, Oxford, UK), characterized by high sensitivity and temporal resolution.

Time-lapse analysis

The cells placed in the Nunc have been positioned inside a suitable housing installed on the spinning disk that keeps cells at the temperature of 37°C, RH 95%, and a
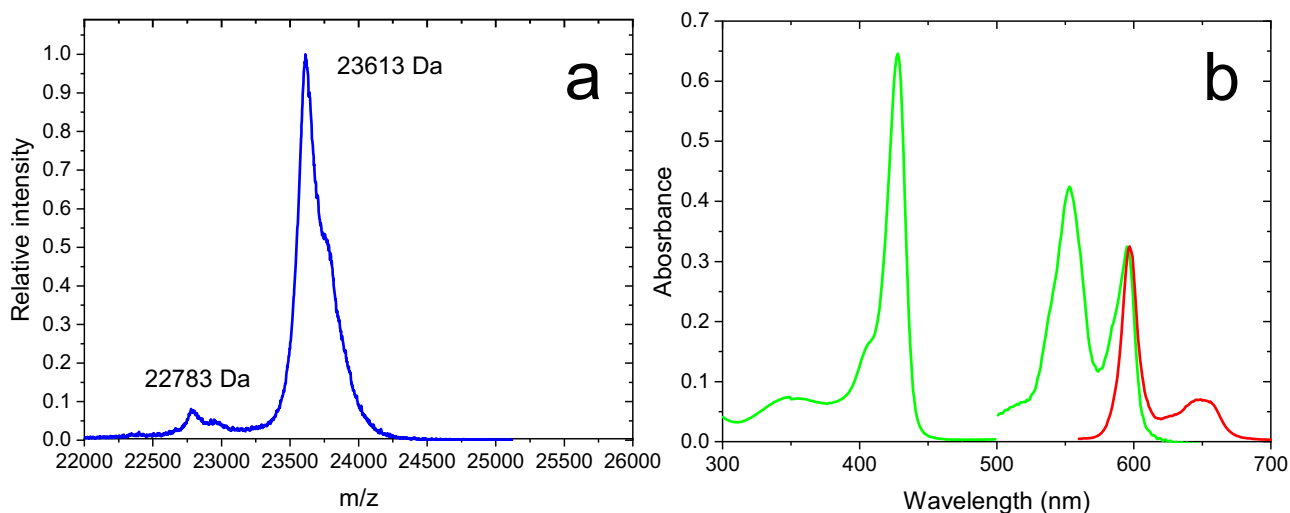
Figure 1: Characterization of MbVIP. (a) MALDI mass spectrum of Mb-VIP purified from BL21 cells cotransformed with the Takara 4 plasmid and grown overnight at 20°C. (b) Absorption (green) and fluorescence emission (red) spectra for ZnMb-VIP (4 µM) in PBS buffer. T = 20°C.

flow of 0.6 L/min. Different areas of the Nuncs were selected to follow the behavior of the cells in different parts of the sample at the same time. The time-lapse was activated by acquiring one frame each 2 min for the first 14 min and 1 frame each 5 min up to 69 min. Excitation 561 nm, emission 592/22 nm, exposure time 100 ms. For all the images, excitation was 561 nm, emission 592/22 nm, exposure time 100 ms, the pixels' area 512 × 512, and the pixel size was 0.23 mm. The first frame was acquired without PS, after which, once the acquisition
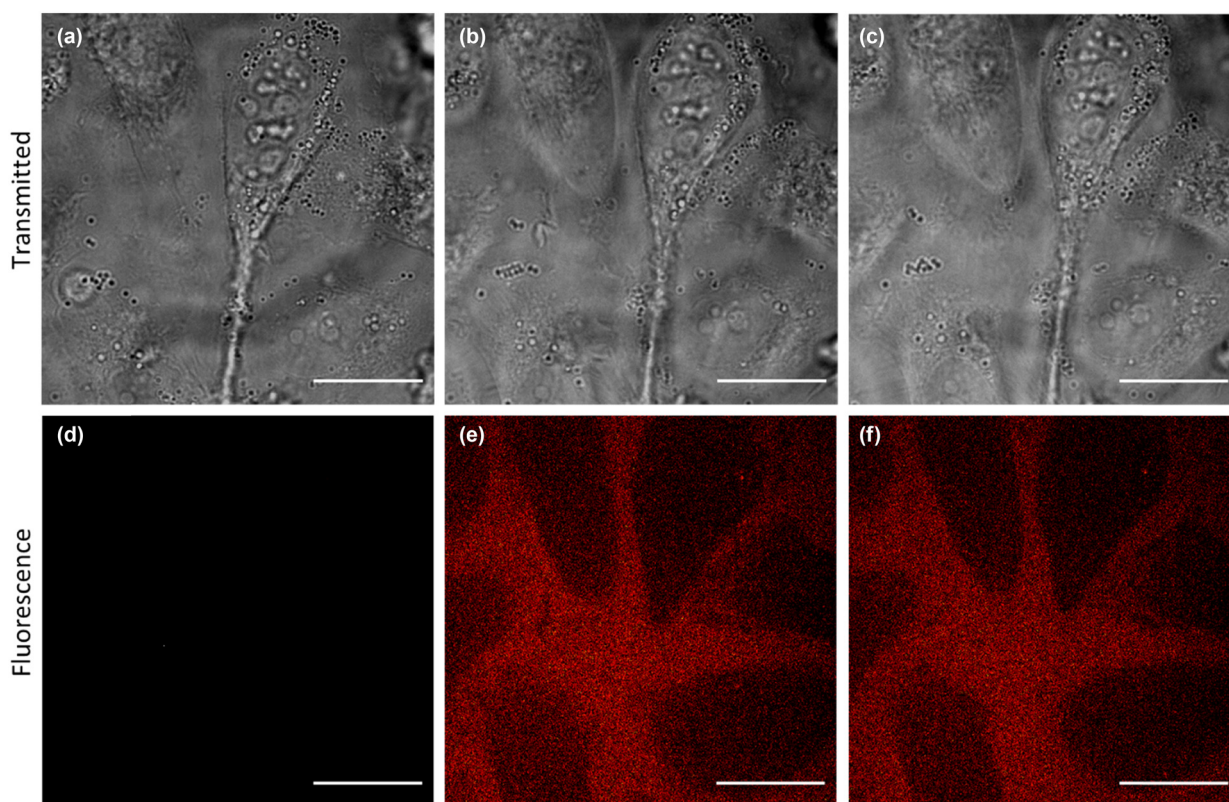

Figure 2: Fast confocal spinning disk fluorescence imaging of PC3 cells treated with ZnMb (1 µM) at 0 min (a and d), 19 min (b and e), and 59 min (c and f) of accumulation time. Time-lapse one frame each 2 min for the first 14 min and 1 frame each 5–59 min. Excitation 561 nm, emission 592/22 nm, exposure time 100 ms. For all the images, the pixel's area is 512 × 512, and the pixel size is 0.23 µm. 0-time was collected before the PS is added to the solution. Scale bar 5 µm.

of the frame was completed, PS was added and the accumulation over time and the consequent photodamage were followed.

Results and discussion

Protein expression and characterization

Preliminary attempts at expressing Mb-VIP were carried out in BL21 cells, BL21 star cells, Rosetta cells, and BL21 cells harboring the Takara plasmids, grown in either L.B. broth or DM1 medium. The growth temperature after induction varied between 20 and 42°C. The addition of either $FeCl_2$ or hemin at different concentrations was also tested. All expression attempts but those in cells containing the Takara 4 plasmid resulted in poor yields and partial hydrolysis of the C-terminus, as assessed by MALDI mass spectrometry. Indeed, the protein – with an expected molecular weight (M.W.) of 23,807 – partially exhibited an M.W. around 800 Da (data not shown), similar to that obtained upon digestion with thrombin. Digestion of the fraction with low M.W. with thrombin did not decrease further the observed m/z, indicating that the observed proteolysis occurred at the C-terminus. The expression in cells harboring the Takara 4 plasmid in the presence of hemin and at 20°C led to the highest yield and minimal hydrolysis at the C-terminus (Figure 1a). Upon heme substitution with Zn-heme, the absorption spectrum of the protein shows an intense Soret band peaked at 428 nm, and two Q-bands centered at 554 and 595 nm (Figure 1b) [36]. The fluorescence emission spectrum is characterized by an intense narrow band centered at 597 nm and a lower intensity band around 650 nm [36,37].

Interaction of PS compounds with PC3 cells

To assess the possible nonspecific interactions between ZnMb and VPAC1 expressing PC3 cells, we first incubated
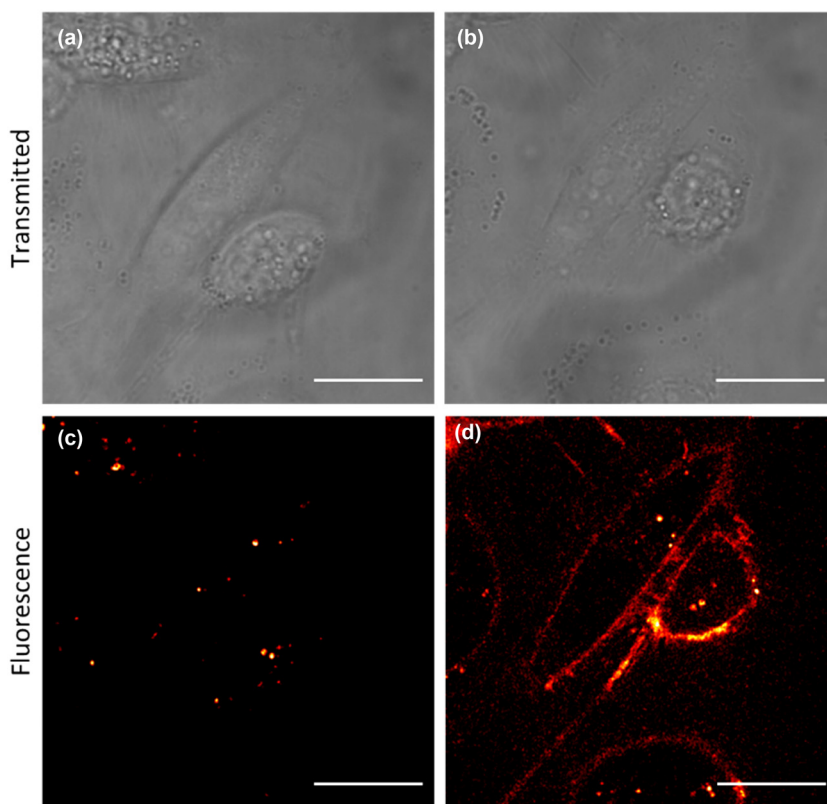

Figure 3: Fast confocal spinning disk fluorescence imaging of PC3 cells before (a and c) and 4 min (b and d) after treatment with ZnMb-VIP (1 μM). Transmitted light (a and b), confocal fluorescence (c and d). Excitation 561 nm, emission 592/22 nm, exposure time 100 ms. For all the images, the pixel's area is 512 × 512, and the pixel size is 0.23 μm. 0-time is collected before the PS is added to the solution. Scale bar 20 μm.

PC3 cells with ZnMb and collected images with a fast confocal spinning disk microscope. Over time, fluorescence was observed from the bulk solvent but not from cell components, which remained dark even after 1 h incubation (Figure 2). This is consistent with the lack of high-affinity binding of the water soluble ZnMb with the plasma membrane, as recently demonstrated for the complex between hypericin and apomyoglobin [27]. In that case, through confocal fluorescence microscopy, we showed that when apomyoglobin (myoglobin without heme) is used to transport the PS hypericin to tumor cells, the PS payload is rapidly and efficiently transferred to the plasma membrane, for which hypericin has a higher affinity. On the other hand, the protein carrier showed no interaction with the plasma membrane and remained solubilized in the surrounding medium [27].

To assess the capability of ZnMb-VIP to target VPAC1 receptors, PC3 cells were incubated with ZnMb-VIP and time-lapse images were collected from time 0 (before the ZnMb-VIP was added) on, for about 1 h (Figure 3). When ZnMb-VIP was added to PC3 cultures, fluorescence appeared on the plasma membrane after only ca. 2–4 min (Figure 3d), indicating high-affinity binding. Given the high affinity of VIP for VPAC1, this receptor is the most likely candidate for binding. It is predicted that unspecific binding of ZnMb-VIP through VIP direct insertion in the plasma membrane is unlikely to occur, considering its amino acid composition, which, according to the hydropathy scale of Kyte and Doolittle [53], does not show a relevant propensity to bind membranes.

For a long time, extensive damage to the cell structure was observed (data not shown), which will be the subject of future functional investigations of the present compound.

Conclusion

Human recombinant myoglobin fused with VIP allowed to obtain a supramolecular complex where the globin domain preserved the well-known capability to bind and transport the heme, and the VIP domain can bind to its VPAC1 receptor. The system has the advantage of binding the modified Zn-heme, a well-characterized PS endowed with the red fluorescence emission. In comparison to other formulations, where PS molecules are covalently bound post-translationally to the recombinant proteins at reactive side chains of amino acids (typically Lys, Cys, or Glu) to a different extent from each preparation, the fusion construct binds stoichiometrically in the heme

pocket. As such, the construct is a well-defined, single chemical species. An additional advantage of this small size, water-soluble protein is that it is stable fold (the protein is stable also under acidic conditions, with a pK_a of about 3.8) [54], which allows its use under harsh environmental conditions too. While the system is interesting in itself for targeting several tumors characterized by VPAC1 over-expression, we emphasized that the construct can be easily adapted to target other receptors by introducing a different targeting peptide in place of VIP. Future studies will assess the potential of the compound for PDT.

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