Zn phthalocyanine conjugation to H2-ul aptamer for HER2-targeted breast cancer photodynamic therapy: Design, optimization and properties

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ABSTRACT: Tetrasulfonated Zn phthalocyanine was conjugated to a ssDNA aptamer moiety to produce a HER2 targeted photosensitizer. The tetraconjugate obtained was subjected to purification and the monoconjugate was successfully isolated, purified and characterized. This monoconjugate retained the ability to bind to HER2 as well as the potency to generate singlet oxygen.

KEYWORDS: cancer targeting, photodynamic therapy, HER2, aptamer, phthalocyanine.

INTRODUCTION

Breast cancer is known to be associated with a number of biomarkers that include Epidermal Growth Factor receptor 2 (EGFr2), also known as HER2, ErbB2, Neu, CD340 or P185. HER2 is a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptor (EGFR or ErbB) family. It is overexpressed in approximately 15% to 25% of breast carcinoma specimens [1], and is hence a target for breast cancer therapies. However, lack of easily synthesizable high-affinity ligands is a major limiting factor. Therefore, researchers are attempting to develop new types of ligands more suitable for high-throughput screening and chemical analysis that includes synthetic aptamers.

Aptamers are small, single-stranded DNA (ssDNA) or RNA molecules [2] and have been previously selected against a variety of targets ranging from small molecules to cell surface protein, with high affinity and specificities comparable to those of monoclonal antibodies [3]. These protein-binding nucleic acids (ssDNA or RNA molecules) are selected from random pools based on their ability to bind other molecules [4]. Aptamers can be designed to be highly specific and resistant to nuclease digestion and can be chemically synthesized in bulk as opposed to antibodies that often require sacrifice of living animals. Aptamers can be selected using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [5]. As a result, aptamer-drug conjugates are increasingly used to optimize drug delivery system [6].

On another hand, photodynamic therapy (PDT) is a treatment modality based on the generation of singlet oxygen or other reactive oxygen species upon irradiation of a photosensitizer (PS) at appropriate wavelengths. This modality found application mainly in anti-cancer issues [7] but also in antibacterial treatments [8] and in the cure of age-related macular degeneration. Amongst the different photosensitizing units, phthalocyanines have a precious advantage due to their maximum absorption at NIR wavelengths in the phototherapeutic window [9]. In addition, phthalocyanines' properties can be modulated and optimized by playing with their metalation and/or substitution patterns [10]. Therefore, each new modification of phthalocyanines needs a procedure optimization and verification for retaining their photoproperties.

◊ SPP full member in good standing

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Since the development of modern anti-cancer PDT, three generations of PSs have been developed, the latest one focusing mainly on surrounding the photodynamic effect in tumorous tissues. This can be achieved by tumor-site specific activation of photosensitizers – the PS being activated in tumor-specific conditions [11], either acidity [12], gluthatione superior concentration [13] or the presence of specific enzymes or receptors [14] – or effective targeting *via* conjugation or combination to antibodies [15] or smaller peptidic units [16].

So far, only a few molecular or nanophotosensitizers have been conjugated or combined to aptamers [17]. Nevertheless, aptamer conjugation onto phthalocyanines has not yet been performed, despite their ability to target specific cancer cells and the advantages of phthalocyanines over other photosensitizing cores.

RESULTS AND DISCUSSION

Design and synthetic strategy

Commercially available tetrasulfonic acid phthalocyanine and their salts have been widely used in PDT [18]. It was also chosen because the sulfonic acid function is further modifiable. The selected aptamer is a single-stranded DNA of 54 nucleotides known for its affinity for HER2 receptor [19]. This aptamer had the following sequence: 5′-NH2-(CH2)6-GGGCCGTCGAACACGA GCATGGTGCGTGGACCTAGGATGACCTGAGTA CTG-TCC-3′ and it is designated hereafter as **H2-ul**. Its NH$_2$ extremity is available to perform coupling reactions. Formation of sulfonamide bonds by EDC/NHS coupling in water appeared suitable.
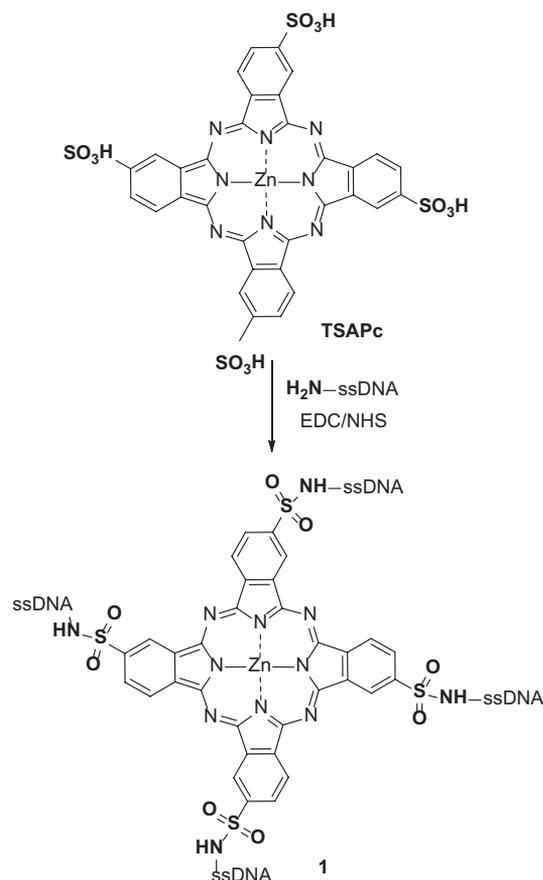
Tetra-aptamer conjugate 1

Our first attempts aimed at preparing the tetra-aptamer phthalocyanine conjugate (Scheme 1). 4.1 equivalents of aptamers reacted with one equivalent of phthalocyanine. The reaction was followed by UV-visible spectroscopy (Fig. 1).

Final addition of cold ethanol to the reaction mixture induced precipitation of the conjugate and of the remaining unreacted **H2-ul**. The classical electrophoresis method was used for final purification, and the gel confirmed conjugation (Fig. 2). Unfortunately, the acidic buffered conditions used to recover the conjugate from the gel led to the conjugate degradation.

Mono-aptamer conjugate 2

Another conjugate purifiable without electrophoresis was designed (Scheme 2). The idea is based on the fact that an excess of TSAPc would be eliminated during the final precipitation of the reaction mixture in cold ethanol, hence allowing purification by washing/precipitation/
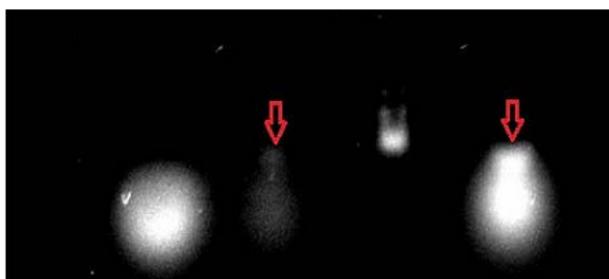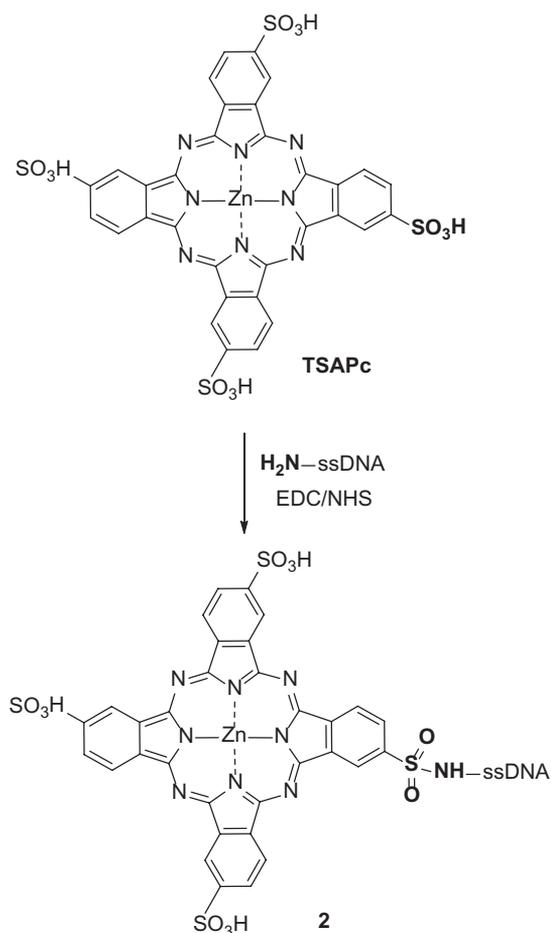

Scheme 1. Preparation of tetra-aptamer phthalocyanine conjugate **1**

Fig. 1. UV-vis spectra of **H2-ul** (red), **TSAPc** (green) and the crude conjugate **2** (blue) in water

centrifugation without the need of electrophoresis. Several experimental conditions have been explored to determine the optimal ones. The use of a 20-fold excess of phthalocyanine allowed to ensure that only a mono-conjugate is obtained. Performing the reaction in DMSO in which **TSAPc** is much less aggregated than in water

Fig. 2. Electrophoresis of (from left to right): **TSAPc**, conjugate **1**, DNA ladder reference and **H2-ul**

Fig. 3. UV-vis spectra of **TSAPc** (green), **H2-ul** (red) and of the conjugate **2** (blue) in water

was a crucial improvement. The addition of triethyamine to the reaction mixture was also beneficial.

UV/vis spectra of the conjugate showed distinguishable peaks associated with ssDNA as well as TSAPc indicating the successful conjugation of ssDNA with TSAPc (Fig. 3).

Reverse-phase HPLC experiments confirmed the purity of the conjugate (Fig. 4). The shorter retention time of the conjugate, compared to **TSAPc**, indicates that the presence of the aptamer moiety increases the hydrophilicity of the molecule.

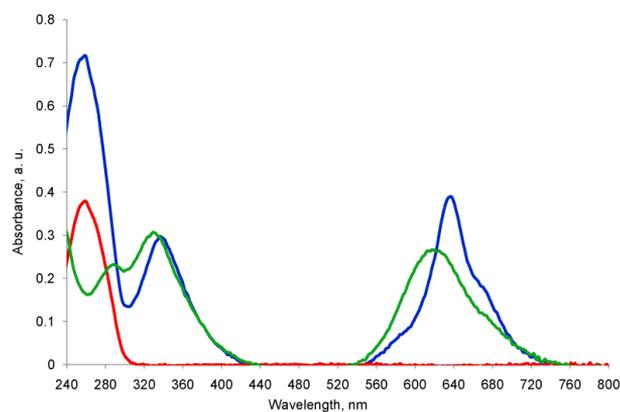**Scheme 2.** Preparation of the mono-aptamer phthalocyanine conjugate **2**

Singlet oxygen generation

The ability of conjugate **2** to generate singlet oxygen was compared to that of TSAPc (Fig. 5). Although the presence of the aptamer moiety slightly lowers the SOG ability of the phthalocyanine core, it remains satisfying for photodynamic purposes.

Binding assays

The binding of conjugate **2** to HER2 receptor was investigated using HER2 protein-coated magnetic beads. The conjugate **2** was first denatured by heating at 60 °C for 10 min and quickly cooled on ice for 5 min and incubated at 25 °C for 10 min before the binding assays were carried out as a pre-requisite condition before the binding assays. This allowed the aptamer to attain its native confirmation that is required for its binding with the target protein (HER2). Human serum albumin (HSA) — coated magnetic beads were used as a negative control. The extent of varying concentrations of conjugate **2** binding with fixed concentration of HER2 protein was measured spectrophotometrically at 260 and 340 nm for the ssDNA and Pc portions of the conjugate **2**, respectively (Fig. 6). The bound complex (conjugate **2**+HER2-magnetic beads) was magnetically separated and the residual unbound/free conjugate **2** was measured to calculate the % conjugate **2** bound to the target protein (Fig. 7). Here, HSA-coated magnetic beads were utilized as negative control as the conjugate **2** was specifically designed to bind HER2.

EXPERIMENTAL

Materials and methods

Tetrasulfonic acid phthalocyanine was purchased from Santa Cruz biotechnology (sc-264509A, Lot #G2811). Reverse-phase HPLC were performed on an Agilent 1100 HPLC system, using a C18 reverse-phase
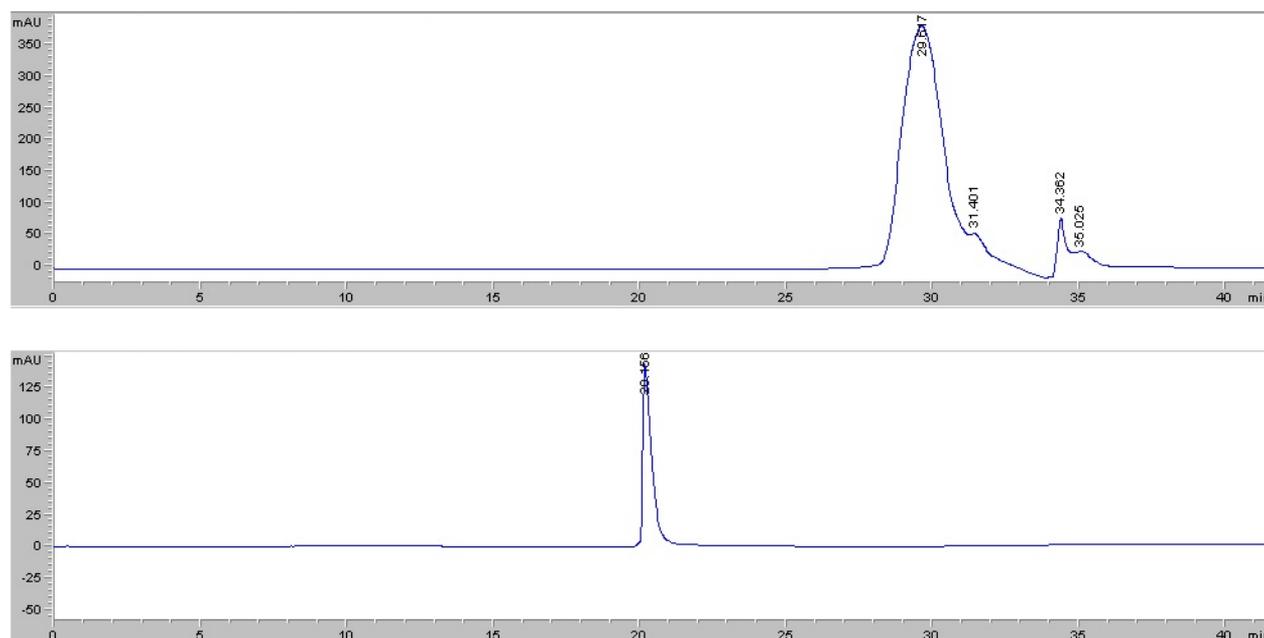
Fig. 4. Reverse-phase HPLC spectra of **TSAPc** (top) and of conjugate **2** (bottom). Solvent: water 96% / isopropyl alcohol 4%. Flow rate: 0.1 mL/min
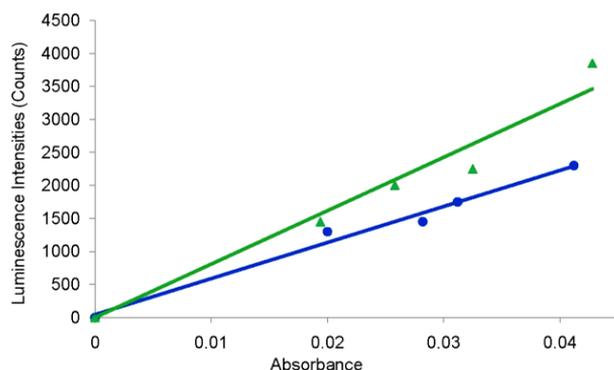
Fig. 5. Phosphorescence emission intensities of $^1O_2$ at 1270 nm *vs.* absorbance of **TSAPc** (green) and conjugate **2** (blue) in ethanol with a few drops of DMSO at 298 K (λ_{ex} 597 nm)

column. A mixture of 96% water/4% isopropyl alcohol was used as a solvent system with a flow rate of 0.1 mL/min. UV-vis was recorded using a Thermo Scientific Nanodrop 2000. The gel electrophoresis image was taken in Bio-Rad Gel Doc EZ System. Singlet oxygen generation was measured in ethanol with a few drops of DMSO by the optical method which is based on comparison between singlet molecular oxygen phosphorescence in the near infrared region at 1270 nm. The phosphorescence signal of $^1O_2$ was recorded on a Horiba-JobinYvon Fluorolog-3R spectrofluorimeter using 450 W Xenon arc lamp as a light source equipped with high sensitive Hamamatsu PMTcooled housing detector (300–1700 nm). In this system, a high cutfilter (1250 nm) was used in order to prevent the interference light below 1250 nm.

Preparation of conjugate 2

A solution of **TSAPc** in DMSO (20 μL, 1148 μM) was mixed with triethylamine (2 μL) for 5 min. DMSO solutions of EDC (5 μL, 0.5 mg/mL) and NHS (5 μL, 0.5 mg/mL) were added to the mixture and mixed in room temperature for 30 min. **H2-ul** in DMSO (10 μL, 114.8 μM) was added and the mixture was mixed at room temperature for 1.5 h. An aqueous solution of sodium acetate (10 μL, 3M, pH = 5.6) was added and the mixture was mixed vigorously. Ice-cold isopropanol (-20 °C, 100 μL) was then added, mixed and incubated overnight in 4 °C. It was centrifuged in 4 °C at 14000 rpm for 10 min. After discarding the supernatant, the precipitate was washed with 100% ethanol, centrifuged in 4 °C at 14000 rpm for 10 min, and the supernatant was discarded. The resulting precipitate was washed three times with 70% ethanol/30% water, centrifuged in 4 °C at 14000 rpm for 10 min, and the supernatant was discarded.

Preparation of HER2 protein coated magnetic beads

Pure and carrier free recombinant HER2 (ErbB2) protein (R&D Systems®) and human serum albumin (HSA, Sigma-Aldrich) were first conjugated with surface activated magnetic Dynabeads® M-270 Carboxylic Acid (Invitrogen, USA) using the carbodiimide coupling method as described by the manufacturer. Briefly, about 100 μL corresponding to 2×10^8 magnetic beads were washed with 25 mM MES buffer, pH 5. The washed beads were activated by suspending in a mixture of 50 μL of 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) (50 mg/mL) and 50 μL of *N*-hydroxysuccinimide (NHS)
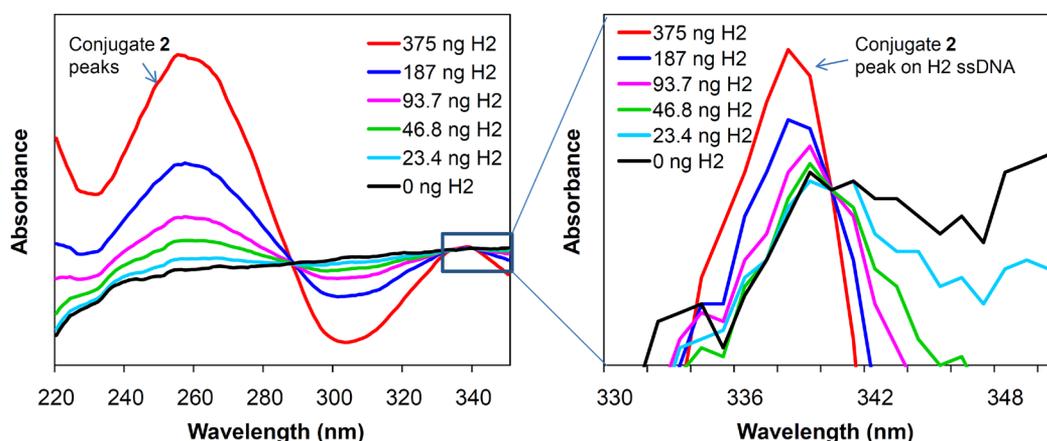
Fig. 6. Left: Spectra showing peaks at 260 nm derived from ssDNA region of conjugate **2** at various concentrations used for binding with target HER2 protein. Right: A magnified portion of spectra showing peak at 340 nm derived from PC in conjugate **2**. These samples were subjected to binding assays and the %binding of conjugate **2** with constant protein concentration on magnetic beads

solution (50 mg/mL) and incubated for 30 min at room temperature with gentle mixing. Following incubation, the EDC/NHS activated magnetic beads were washed twice with 100 µL of 25 mM MES buffer, pH 5 and incubated with 100 µL of 27 µg of HER2 or HSA protein. The HER2/HSA protein coated beads were magnetically separated and washed three times with 100 µL of PBS, pH 8 containing 0.05% tween 80 and finally resuspended in 100 µL of PBS, pH 7.4 containing 0.1% BSA and stored at 4 °C. Resulting HER2/HSA protein coated beads were utilized for *in vitro* binding assays with H2 ssDNA aptamers conjugated with conjugate 2.

Binding assays

To a series of reaction tubes, a fixed amount (~10^4) of HER2 (test) or HSA (control) protein coated magnetic beads were added and washed three times with 1X binding buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02% Tween 20). The magnetic beads were separated on a magnet and the supernatant was eliminated. Next, incubation of protein-coated beads for 1 h at 25 °C with varying concentrations of conjugate **2** (0~375 ng) in replicate experiments ($n = 3$). The beads were magnetically separated and the supernatant containing unbound/ residual conjugate **2** was measured using a Nanodrop spectrophotometer (Thermo). The concentration of bound conjugate **2** was calculated based on the measured concentration of unbound ssDNA from conjugate **2** with respect to initially added concentrations. Average concentrations measured were plotted and the standard deviations are shown as errors (see Figs 6 and 7).
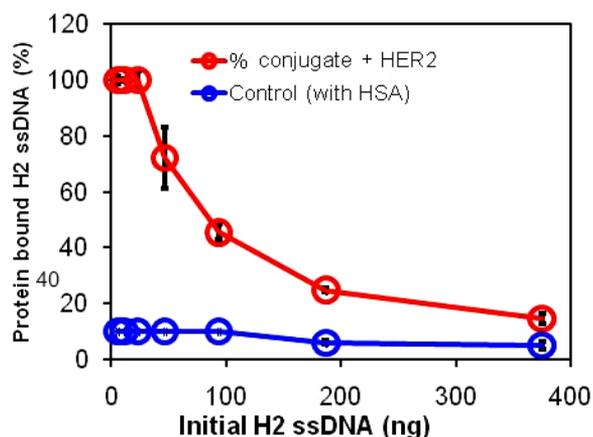
Acknowledgments

Fig. 7. Percentage of conjugate **2** bound to target proteins (HER2/HSA) after 1 h incubation with HER2/HSA protein coated magnetic beads followed by magnetic separation of protein-bound conjugate **2**. The residual unbound conjugate **2** was measured spectrophotometrically at 260 nm (ssDNA) and 340 nm (PCs)

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