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Peptide-functionalized ZCIS QDs as fluorescent nanoprobe for targeted HER2-positive breast cancer cells imaging

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ABSTRACT

In this paper, the synthesis of alloyed $CuInZn_xS_{2+x}$ quantum dots (ZCIS QDs), their transfer into aqueous solution via a polymer coating technique, and the use of these nanocrystals to selectively target HER2-positive cells, are reported. By optimizing first the ZnS shell deposition process onto the $CuInS_2$ core, and next the encapsulation of the dots with the amphiphilic poly(maleic anhydride-alt-1-octadecene) (PMAO) polymer, water-dispersible ZCIS QDs were successfully prepared. The nanocrystals with a photoluminescence quantum yield of 35% were purified via centrifugation and ultracentrifugation and high quality nanoparticles with narrow size distributions and surface charges were obtained. After verifying the biocompatibility of PMO-coated ZCIS QDs, we coupled these nanocrystals with the LTVSPWY peptide and demonstrated via MTT assay that both bare and the peptide-linked QDs exhibit low cytotoxicity. The HER2-mediated delivery of the peptide-linked QDs was confirmed by confocal microscopy. This study indicates that as engineered QDs can efficiently be used as fluorescent nanoprobes for selective labelling of HER2-positive SKBR3 cancer cells.

1. Introduction

Semiconductor nanocrystals, also called quantum dots (QDs), have stimulated a great deal of research in recent years owing to their excellent optical properties originating from quantum confinement effects and large surface-to-volume ratio. QDs possess symmetric and narrow photoluminescence (PL) emission, tunable emission by crystal size, shape and composition, high photostability and brightness, longer fluorescence lifetime than organic dyes, and finally broad absorption spectra which enables a simultaneous excitation of multicolored nanocrystals with a single excitation source [1,2].

During the last two decades, binary II–VI and IV–VI semiconductors have been intensively studied in order to obtain high quality QDs in a reliable way, and therefore suitable for various applications like biomedical labelling, photovoltaics, and light-emitting diodes [3,4]. However, their toxicity mainly attributed to the presence of heavy metals (like Cd, Hg or Pb) shed a doubt on future applicability of such QDs, especially in the biomedical and industrial areas [5–9]. Therefore, the engineering of heavy metal-free QDs has become an emerging field. In this direction, ternary I–III–VI QDs like $CuInS_2$ (CIS) are extremely promising candidates as they do not contain toxic elements and due to their stable PL and high quantum efficiency [10–13]. In order to enhance the PL quantum yield (QY) of CIS QDs, a ZnS shell is introduced at the periphery of the core. The shell introduction results in the substitution of In^{3+} and Cu^{+} atoms by Zn^{2+} atoms and generates quaternary $CuInZn_xS_{2+x}$ QDs composed of Cu, In, Zn and S (commonly called ZCIS) with a higher bandgap than the starting CIS QDs [11,14,15].

The ZCIS nanocrystals are mostly produced in organic solvents using dodecanethiol (DDT) both as ligand and as sulfide source [6,16]. Thus, their surface modification aiming at QDs transfer into water is essential to use them in biological applications. For this purpose, two basic approaches have been developed. The first relies on the replacement of hydrophobic surface ligands on QDs surface by hydrophilic ones, generally thiol derivatives [17]. In

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the second strategy, an amphiphilic polymer is introduced at the periphery of the dots and stabilizes QDs aqueous dispersions through hydrophobic van der Waals interactions between QDs capping ligands and the hydrophobic parts of the polymer. Although the hydrodynamic size of such nanohybrids increases, their colloidal stability is generally higher under harsh biological conditions compared to the water-dispersible QDs obtained via the ligand exchange procedure [18,19]. The cheap and commercially available amphiphilic poly(maleic anhydride-alt-1-octadecene) (PMAO) polymer has been chosen in this study since it enables long-term colloidal stability of various nanoparticles in aqueous media [20]. Moreover, surface carboxylate groups obtained after hydrolysis of anhydride units allow further modifications like the anchorage of targeting ligands. A PMAO-mediated transfer into aqueous solution has been already used for Fe_3O_4 [21], γ-Fe_2O_3, FePt/Fe_xO_y, Au [22], lanthanide-doped upconverting $NaYF_4$ [23], manganese ferrite nanoparticles and for CdSe/CdS, Zn-doped $AgInS_2$ QDs [24]. To date, only two reports of Cheng et al. [25] and Guo et al. [26] described the PMAO-mediated water-dispersion of ZCIS QDs. Recently, Speranskaya et al. reported the overcoating of ZCIS QDs using PMAO followed by cross-linking with Jeffamine to minimize nonspecific interactions in biological media [27]. These reports clearly demonstrate that hydrophobic particles can easily be transferred into water using PMAO.

Statistically about 30% of all diagnosed breast cancers exhibit higher human epidermal growth factor receptor 2 gene *ERBB2* (hereafter *HER2*) amplification and/or overexpression of HER2 corresponding protein [28,29]. It has been proved that HER2 overexpression correlates with more aggressive tumor growth, enhanced rates of metastases and the poor prognosis for patients with breast cancers [30]. HER2 at low levels may be found in some normal tissues but much higher expression is associated with some tumors growth what makes it useful as a target for therapy and/or imaging contrast agents. Besides breast cancer, HER2 overexpression has also been found in colorectal, non-small-cell lung, ovarian, stomach, uterine, prostate, head and neck cancers [31].

The currently used protocols to determine *HER2* status (negative or positive and level of expression) of patients include immunohistochemistry and fluorescence in situ hybridization (FISH). The *HER2* status assessed by immunohistochemistry has been found to show problems with reproducibility and poor concordance between laboratories [32]. On the other hand, the FISH method, which determines the *HER2* gene status in the nucleus, is an expensive technique that requires special equipment but it is very sensitive. Moreover, due to the decrease in fluorescence signals in standard FISH assays, the results are often lost after a few weeks [31].

Besides the receptor status assessment, HER2-targeted imaging might also be useful in an image-guided cancer surgery or in the detection of micrometastasis. The organic dyes, although routinely used in fluorescence imaging or in the FISH method, possess some limitations like photo-bleaching, that make them often not adapted for bioimaging purposes [33,34].

One of the most explored anti-HER2 targeting ligand is the Herceptin antibody (also called Trastuzumab) [35]. This antibody can also be used as a therapeutic agent due to its blocking receptor activity, which inhibits cancer cells proliferation and angiogenesis process. Mandal et al. conjugated ZCIS and core/shell CdSe/ZnS QDs with Herceptin to label HER2-positive breast cancer cells (SKBR3) and demonstrated that the detection sensitivity of HER2 on SKBR3 cells with ZCIS QDs is as efficient as for Cd-containing QDs [36]. However, the use of Herceptin has some limitations like a risk of cardiotoxicity, for instance [37,38]. Moreover, the high molecular weight and poor tissue penetration of antibodies make them less attractive in terms of targeting ligands than a single domain anti-HER2 antibody or small peptides. Recently, Rakovich et al. [39] developed various conjugates based on a single domain anti-HER2 antibody and CdS/ZnS QDs or Alexa dye, in order to improve the immunohistochemical methods for screening and early detection of HER2 in lung and breast cancers. The results revealed that the QDs-based nanoprobes are superior in staining of lung cancer cells with different expression of HER2 comparing to Alexa-based conjugates.

Peptides are hydrophilic and exhibit excellent permeability abilities, small side effects and high affinity to the receptor. They are readily produced and might be easily chemically modified. These properties make them good candidates as targeting molecules [40]. The LTVSPWY peptide is a well-known HER2 binding peptide and exhibits weak immunogenic properties. In this study, we designed a novel heavy metal-free QDs-based nanoprobe, functionalized with a peptide for HER2-targeted imaging. We first developed a new method to coat ZCIS QDs with PMAO and demonstrated that the obtained nanocrystals exhibit high colloidal stability (>four months in PBS buffer), high PL QY (35%), and low cytotoxicity. The LTVSPWY HER2-binding peptide was further covalently coupled to the nanocrystals. The peptide-linked QDs were demonstrated to be suitable fluorescent nanoprobes with high specificity to HER2-positive cancer cells.

2. Materials and methods

2.1. Materials

Indium acetate ($In(OAc)_3$, 99.99%), zinc acetate ($Zn(OAc)_2$, 99.99%), copper iodide (CuI, 99.999%), dodecanethiol (DDT, >98%), oleylamine (OA, 70%), 1-octadecene (ODE, 90%), polymaleic anhydride-alt-1-octadecene, average Mn 30,000–50,000 (PMAO), 2,2′-(ethylenedioxy)bis(ethylamine) (diaminoPEG, 98%), *N*-hydroxysuccinimide (NHS, 98%), N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), fetal bovine serum (FBS), concanavalin A from canavalia ensiformis (Jack bean), FITC conjugate, Type IV (ConA-FITC), Fluoroshield™ with DAPI (DAPI), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT, 98%), Dulbecco's Modified Eagle's Medium (DMEM), HEPES (⩾99.5%), paraformaldehyde (PFA) and phosphate buffer (PBS) were purchased from Sigma–Aldrich. LTVSPWY peptide (95%) was purchased from Lipopharm.pl (Poland). All the chemicals were used without further purification. SKBR3 and NIH/3T3 cell lines were provided by American Type Culture Collection (ATCC, Menassas, VA, USA). MSU1.1 fibroblasts were obtained from Prof. C. Kieda (CBM, CNRS, Orléans, France).

2.2. Synthesis of $CuInS_2$/ZnS QDs (ZCIS QDs)

ZCIS QDs were synthesized using the $Zn(OAc)_2$–OA complex for the introduction of the ZnS shell at the periphery of CIS core QDs according to the synthetic procedure we recently developed [41], with slight modifications. Briefly, CuI (0.14 mmol), $In(OAc)_3$ (0.2 mmol) and ODE (50 mmol) were loaded in a 100 mL three-neck flask under argon flow. The reaction mixture was further degassed by heating at ~75 °C under vacuum for 20 min and then backfilled with argon. DDT (8.3 mmol) was subsequently injected, and the temperature of the reaction solution was raised to 210 °C. The reaction time was fixed to 20 min. In a separate flask, anhydrous zinc acetate (1.8 mmol) was dissolved in an OA/ODE mixture (4.1 mmol/10 mmol, respectively) under nitrogen by raising of temperature with a heat gun up to 200 °C. The solution was maintained at 80 °C after dissolution of the reagents.

After 20 min of core growth, a first portion of Zn^{2+}–OA/ODE mixture (0.5 mL) was injected into the crude core solution main-