Colloids and Surfaces B: Biointerfaces 146 (2016) 9–18

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

# iRGD peptide as effective transporter of $CuInZn_xS_{2+x}$ quantum dots into human cancer cells

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ARTICLE INFO

*Article history:*
Received 22 February 2016
Received in revised form 15 April 2016
Accepted 16 May 2016
Available online 17 May 2016

*Keywords:*
$CuInZn_xS_{2+x}$ QDs
iRGD peptide
Cell labelling
Tumor spheroids
Tumor-targeted delivery

ABSTRACT

In this paper, iRGD peptide-mediated quantum dots (QDs) delivery was studied. In the first step, dodecanethiol-capped $CuInZn_xS_{2+x}$ (ZCIS) QDs were prepared and subsequently transferred into water using a standard and facile ligand exchange approach involving 3-mercaptopropionic acid (MPA). ZCIS@MPA nanocrystals possess a photoluminescence quantum yield (PL QY) of 25%, a PL emission centered at ca. 640 nm and low distributions in size and shape. Next, the iRGD peptide was electrostatically associated to ZCIS@MPA QDs. After cytotoxicity evaluation, the tumor-targeting and penetrating activities of the iRGD/QD assembly were investigated by confocal microscopy. The experiments performed on various cancer cell lines revealed a high penetration ability of the assembly, while the bare QDs were not internalized. Additionally, imaging experiments were conducted on three-dimensional multicellular tumor spheroids in order to mimic the tumor microenvironment *in vivo*. iRGD/QD assemblies were found to be evenly distributed throughout the whole HeLa spheroid contrary to normal cells where they were not present. Therefore, iRGD/QD assemblies have a great potential to be used as targeted imaging agents and/or nanocarriers specific to cancer cells.



## 1. Introduction

To date, semiconductor nanocrystals, also called quantum dots (QDs), have gained much attention as fluorescent probes due to their unique optical properties including size, shape and composition-tunable photoluminescence (PL) emission, high photostability, long fluorescence lifetime, and broad absorption spectrum [1,2]. So far, cadmium-containing QDs like CdSe and CdTe have been extensively investigated as biological labels in fluorescence imaging for biomedical research or diagnostic purposes. Nevertheless, the safety of these dots has become a big concern in terms of *in vivo* applications due to their intrinsic toxicity [3,4]. To overcome this obstacle, cadmium-free alternatives such as I-III-$VI_2$ type QDs, like $CuInS_2$ (denoted CIS hereafter), have been proposed as promising candidates for *in vitro* and *in vivo* visible or near infrared (NIR) fluorescence imaging [5,6]. To increase both the stability and the PL QY of CIS QDs, a ZnS shell is generally introduced at their periphery via a high temperature decomposition process leading to the production of alloyed $CuInZn_xS_{2+x}$ QDs (denoted ZCIS). The highly emissive ZCIS QDs obtained are generally capped with dodecanethiol (DDT). The surface modification of these nanocrystals to make them hydrophilic is pivotal for biological applications [7]. Various modifications have been developed to achieve the ZCIS QDs transfer in aqueous solution such as ligand exchange with dihydrolipoic acid coupled to poly(ethylene glycol) 1000 [8], coating with the amphiphilic polymer poly(maleic anhydride-alt-1-octadecene) (PMAO) [6], or with a silica shell [9], for instance. Ligand exchange using thioacids is one of the most common methods to disperse dots in water and affords small-sized and negatively-charged particles. The dots obtained are less cytotoxic than positively-charged ones as demonstrated by Nagy et al. [10].

Although QDs-based fluorescence labelling is attractive, the low QDs penetration through cell membranes and their non-specific delivery into the cells are major problems. Therefore, numerous studies were devoted to develop strategies for QDs-targeted internalization into the living cells including conjugation with folic acid

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http://dx.doi.org/10.1016/j.colsurfb.2016.05.041
0927-7765/

or with specific antibodies (Abs) to cancer cells [11–13]. The use of so-called homing-peptides is another approach developed to overcome cell membrane barrier and deliver QDs into cells. Each healthy or diseased tissue contains organ- or disease-specific molecular tags on its vasculature that constitute a vascular 'zip code' system [14]. The number of "homing peptides" that recognize specific types of cells is rapidly increasing. The advantage of these peptides is their capacity to recognize specific phenotypes and thus, home onto a desired location. Furthermore, some of these peptides not only recognize the targeted cell but also have the capacity to translocate across its cellular membrane and are therefore named "cell-penetrating-homing peptides". The key feature of these peptides is that their sequences hold the target molecular address. Consequently, they offer a great potential as vectors for drug delivery purposes, as they show the desired selectivity and the capacity to introduce cargoes into a specific cell target [15]. One of such tumor-homing and tumor-penetrating peptide is the cyclic peptide iRGD. This peptide consists of a RGD motif with a protease site and a cryptic Cend Rule (CendR) motif (R/KXXR/K). The iRGD peptide is first recruited through its RGD motif to αv integrins, which are overexpressed on tumor endothelial cells, then CendR motif interacts with neuropilin-1 (NRP-1), and thereby increases tumor vascular permeability that triggers the activation of a transport pathway [16–18]. It has been reported that this peptide works either as conjugated to the payload or when it is co-administered with small drug compounds, nanoparticle-based therapeutics or monoclonal antibodies [19]. Recently, iRGD peptide-conjugated to type II CdTe/CdS QDs have been successfully applied for recognition of cancer cells in mice xenografted with pancreatic tumor cells [20]. Previous studies concerning RGD peptide have also shown that a probe prepared by linking this peptide to near-infrared emitting QDs (NIR QDs-RGD) can target integrin αvβ3 expressed in the endothelial cells of tumor angiogenic vessels *in vivo* into glioblastoma tumor cells [21–23] as well as human oral squamous cell carcinoma [24].

Moreover, co-administration of iRGD could also improve the delivery of therapeutic agents into tumor parenchyma and, in some cases, was even more effective in delivering than conjugation [25]. One example of such *in vivo* treatment is the co-injection of the tumor penetrating peptide iRGD with multistage tumor-microenvironment-responsive DOX-Au nanoparticles that enhance tumor penetration and tumor treatment efficiency [26].

While the covalent linkage of a biomolecule to a carrier requires the expertize in conjugation chemistry as well as purification and subsequent evaluation of biomolecule functionality, the non-covalent binding simplifies the path to clinical application, thereby providing a versatile way to enhance the delivery of imaging agents into tumors.

Nanoparticles delivery to cells has been mostly studied using monolayer cell culture models, which do not obviously reflect the *in vivo* environment [27]. As nanoparticles need to cross membrane barriers and effectively enter into three-dimensional tissues, more recent investigations have progressed to the use of cell-seeded scaffolds or cells spheroids to recreate 3D models of cells in a tissue environment [28]. Multi-cell-layer studies are a step toward mimicking the 3D architecture of the *in vivo* tissue. The movement of molecules through these more complex, fibrous networks is far less predictable than the movement in liquids like culture medium. The 3D model better reflects the tumor microenvironment and therefore introduces more insights in the case of studies of new diagnosis and therapy methods [29].

In this paper, we first synthesized ZCIS QDs and transferred these dots into water using a standard and facile ligand exchange approach involving 3-mercaptopropionic acid (MPA). We further studied the non-covalent association between these QDs and the iRGD peptide. Finally, we present a detailed investigation of ZCIS@MPA QDs delivery effectiveness achieved by the iRGD peptide. The iRGD/QD assemblies are efficiently internalized by studied human cancer cell lines as well as 3D model of cellular spheroids, and show therefore a great potential to be used as targeted imaging agents and/or nanocarriers to 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%), 3-mercaptopropionic acid (MPA, ≥99%), Dulbecco's modified Eagle's medium (DMEM), Hanks Balanced Salt solution, fetal bovine serum (FBS), Trypsine-EDTA (0.25%), penicillin-streptomycin, Hoechst 33258 and agarose were purchased from Sigma-Aldrich and used as received. WST-1 Cell Proliferation Assay Kit was purchased from Clontech. 4,6-Diamidino-2-phenylindole (DAPI) and Oregon Green 488 Phalloidin staining dyes were obtained from Molecular Probes (Life Technologies). The iRGD peptide (CRGDKGPDC, molecular weight 948,180) was synthesized by Lipopharm (Poland) with purity in the range of 93–98%. All other materials/reagents were obtained as noted in the text.

### 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 based on our previous report [30]. In a typical experiment, 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 parallel, anhydrous $Zn(OAc)_2$ (1.8 mmol) was dissolved in an OA/ODE mixture (4.1 mmol/10 mmol, respectively) under nitrogen by raising of temperature up to 200 °C. Once the reagents were dissolved, the solution was maintained at 80 °C. After 20 min of core growth, a first portion of the $Zn^{2+}$-OA/ODE mixture (0.5 ml) was injected into the crude core solution maintained at 210 °C under argon flow. This step was repeated every 15 min (total injected volume: 4.5 ml). Finally, the reaction was cooled down to room temperature and 5 ml of toluene were added. ZCIS QDs were precipitated with ethanol and the mixture centrifuged. The supernatant was decanted and the isolated solid was redispersed in toluene, and reprecipitated by adding ethanol. The centrifugation and precipitation procedure was repeated five times for the purification of ZCIS QDs.

### 2.3. Synthesis of MPA-capped ZCIS QDs (ZCIS@MPA)

500 mg of purified ZCIS QDs were dispersed in toluene (5 ml) and MPA (4.2 ml) was added. The reaction mixture was vigorously stirred and heated to reflux for 72 h. During this time, the transparent solution became turbid. After cooling to room temperature, the resulting nanocrystals were centrifuged and washed three times with chloroform. The pellet was subsequently redispersed in 5 ml of MPA water solution (1 M, pH 10) and refluxed for another 24 h. The nanocrystals were purified by washing with isopropanol three times.