Acta Biomaterialia 14 (2015) 197–207

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Multifunctional calcium phosphate nanoparticles for combining near-infrared fluorescence imaging and photodynamic therapy

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ARTICLE INFO

Article history:
Received 29 August 2014
Received in revised form 20 November 2014
Accepted 14 December 2014
Available online 19 December 2014

Keywords:
Calcium phosphate nanoparticles
Photodynamic therapy
NIRF optical imaging
Apoptosis
Vascularization

ABSTRACT

Photodynamic therapy (PDT) of tumors causes skin photosensitivity as a result of unspecific accumulation behavior of the photosensitizers. PDT of tumors was improved by calcium phosphate nanoparticles conjugated with (i) Temoporfin as a photosensitizer, (ii) the RGDfK peptide for favored tumor targeting and (iii) the fluorescent dye molecule DY682-NHS for enabling near-infrared fluorescence (NIRF) optical imaging in vivo. The nanoparticles were characterized with regard to size, spectroscopic properties and uptake into CAL-27 cells. The nanoparticles had a hydrodynamic diameter of approximately 200 nm and a zeta potential of around +22 mV. Their biodistribution at 24 h after injection was investigated via NIRF optical imaging. After treating tumor-bearing CAL-27 mice with nanoparticle-PDT, the therapeutic efficacy was assessed by a fluorescent DY-734-annexin V probe at 2 days and 2 weeks after treatment to detect apoptosis. Additionally, the contrast agent IRDye® 800CW RGD was used to assess tumor vascularization (up to 4 weeks after PDT). After nanoparticle-PDT in mice, apoptosis in the tumor was detected after 2 days. Decreases in tumor vascularization and tumor volume were detected in the next few days. Calcium phosphate nanoparticles can be used as multifunctional tools for NIRF optical imaging, PDT and tumor targeting as they exhibited a high therapeutic efficacy, being capable of inducing apoptosis and destroying tumor vascularization.

1. Introduction

Photodynamic therapy (PDT) is a promising tumor treatment modality, which allows the local generation of reactive oxygen species by selective activation of a photosensitizing agent via visible laser light [1]. Thus, the specific destruction of tumor tissue with simultaneous preservation of the healthy surrounding tissue is guaranteed. Successful treatments have already been realized with the photosensitizer Temoporfin (mTHPC), which is used as the liposomal formulation Foscan® [2,3] in clinics. Despite these observations, these and most other photosensitizers do not show any target-specific binding in tumors. Therefore, after systemic administration of the agents, the whole body and especially the skin exhibit a high photosensitivity, such that patients have to avoid direct contact with light [4,5]. Hence, it is of great interest to develop photosensitizers that show precise enrichment in tumors but not in other regions of the body [6,7].

One rising field in this regard is the use of multifunctional nanoparticulate carrier systems, which enable the loading of a photosensitizer together with other components that enhance the specificity and imaging qualities of the carriers [8–10]. These features make a combination of diagnostic imaging, drug delivery and therapy [11–14] feasible. Concerning cell-specific targeting, the conjugation of functionalized nanoparticles with receptor-binding molecules can selectively increase their uptake by target cells [15]. Various targeting moieties, such as antibodies, aptamers or small molecules, have already been used [16]. Among them, RGD peptides are particularly suitable for enhancing intratumoral probe enrichment. RGD peptides specifically bind to $\alpha_v\beta_3$ integrins on the activated endothelium of angiogenic blood vessels, they can easily be conjugated to nanoparticles and they are cheaper than antibodies [17–20].

To track the nanoparticles in vivo, near-infrared fluorescence (NIRF) optical imaging in the range of 650–900 nm [21] is an appropriate technology due to its low autofluorescence and the high penetration depth of the NIR light. Interestingly, the use of

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http://dx.doi.org/10.1016/j.actbio.2014.12.009
1742-7061/

nanoparticles as carrier systems for NIRF-emitting dyes was shown to improve their admission, prolong their circulation time in the body, intensify their fluorescence and improve their photostability [22–24].

Among the different nanoparticle varieties currently available, calcium phosphate nanoparticles are very attractive [25–31] as they exhibit high biocompatibility, small size, low toxicity, high biodegradability, easy preparation and suitability for functionalization [32]. In particular, the attachment of an antibody or dye to calcium phosphate nanoparticles has shown the potential for cell-specific targeting or diagnostic fluorescence imaging [33–35]. Also, the utilization of these nanoparticles for PDT has already been demonstrated [36–38]. However, calcium phosphate nanoparticles have never hitherto been synthesized that can unite all of these aspects – NIRF optical imaging, favored tumor targeting and PDT – at once.

In this study, we designed multifunctional calcium phosphate nanoparticles made up of a calcium phosphate core as the carrier, an NIRF dye as an imaging tool, enabling localization in the body, the photosensitizer mTHPC as the drug for the PDT of tumors, and finally the RGDfK peptide for specifying delivery to endothelial cells in tumors, thus causing less skin photosensitivity compared to free mTHPC. Furthermore, the suggested nanoparticles should prospectively allow a reduction in the hydrophobicity of mTHPC. Our multifunctional nanoparticles will be shown to be suitable for PDT. In this context, the nanoparticles will be first characterized regarding size, spectroscopic properties and internalization into tumor cells in vitro. As a precondition for PDT in vivo, intratumoral enrichment will be assessed with simultaneous verification of their suitability for in vivo NIRF optical imaging. Finally, the potential of the nanoparticles for inducing apoptosis and destroying tumor vascularization after PDT will be analyzed in mice xenografts. Here we use a self-designed annexin V probe that addresses phosphatidylserine on apoptotic tumor cells, together with an integrin-targeting RGD-probe for detecting tumor vascularization.

2. Materials and methods

2.1. Cell line and animals

For in vitro experiments and as xenografts, CAL-27 cells (tongue–squamous epithelium carcinoma cells, DSMZ, Braunschweig, Germany) were used. They were cultured in Dulbecco's modified Eagle's medium (GlutaMAX™, Gibco®, Darmstadt, Germany) supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 humidified atmosphere.

For in vivo experiments, female athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu} (nu/nu), Harlan Laboratories GmbH, Venray, The Netherlands) of approximately 20–25 g body weight were housed with food and water ad libitum under standard conditions. For generating xenografts, 2×10^6 CAL-27 cells in Matrigel™ (BD, Heidelberg, Germany) were implanted subcutaneously into the posterior back of mice aged 8–12 weeks and grown until they reached a diameter of approximately 5 mm. All experiments were carried out with isofluorane-anesthetized (Actavis, Munich, Germany) animals and with the approval of the regional animal committee, as well as in accordance with international guidelines on the ethical use of animals.

2.2. Preparation of multifunctional calcium phosphate nanoparticles

Silica-modified calcium phosphate nanoparticles with an inner shell of the stabilizing polymer polyethylenimine (PEI) were synthesized as described previously [34]. The preparation pathway is shown schematically in Fig. 1. After modification with a silica shell, the nanoparticles were dye-labeled to enable NIRF optical imaging by adding 200 µl of DY682-N-hydroxysuccinimidyl ester in dimethylsulfoxide (DY682-NHS, 1 mg ml^{-1}, absorption: 690 nm, emission: 709 nm, Dyomics GmbH) to 10 ml of the CaP/PEI/SiO_2 dispersion under stirring, followed by stirring for 30 min at room temperature. For the functionalization with thiol groups, the DY682-labeled nanoparticle dispersion was added to a mixture of 40 ml of ethanol and 50 µl of (3-thiolpropyl)trimethoxysilane (MPS, Sigma-Aldrich, 95%) and stirred for 8–10 h at room temperature. Then the CaP/PEI/SiO_2/DY682/SH particles were purified by ultracentrifugation and redispersed in 8 ml of water by ultrasonication. DY682-labeled thiol-terminated calcium phosphate nanoparticles were loaded with meta-tetra-hydroxyphenyl-chlorin (mTHPC; Biolitec Research GmbH) as follows: 200 µl of mTHPC in 2-propanol (1 mg ml^{-1}) was added to 8 ml of the CaP/PEI/SiO_2/DY682/SH dispersion under stirring, followed by stirring for 1 h at room temperature. The CaP/PEI/SiO_2/DY682/SH/mTHPC particles were collected by ultracentrifugation and redispersed in 8 ml of water by ultrasonication.

For covalent conjugation, 500 µl of RGDfK peptide in PBS (cyclo(-Arg-Gly-Asp-D-Phe-Lys) trifluoroacetate salt, 1 mg ml^{-1}, Bachem AG) were activated with 200 µl of the crosslinker 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulpho-*N*-hydroxysuccinimide ester sodium salt (4 mM, p.a., Sigma-Aldrich) for 3–4 h at room temperature. Next, 5 ml of the CaP/PEI/SiO_2/DY682/SH/mTHPC nanoparticles were added to the activated crosslinker and peptide mixture and reacted for 24 h at 4 °C.

After the conjugation reaction, the nanoparticles were purified by centrifugation for 15 min at 14,500 *g* and redispersed in 5 ml of water (UP50H, Hielscher, Ultrasound Technology, sonotrode 2, cycle 0.8, amplitude 70%, 8 s).

For long-term storage, the dispersions were freeze-dried. For this, 15 mg of D-(+)-trehalose dihydrate (Aldrich) was added to 1 ml of the dispersion (CaP/PEI/SiO_2/DY682/SH, CaP/PEI/SiO_2/DY682/SH/mTHPC, CaP/PEI/SiO_2/DY682/SH/mTHPC-RGDfK), which was then shock-frozen with liquid nitrogen. This system was lyophilized for 48 h at 0.31 mbar and −7 °C [39].

2.3. Characterization of multifunctional calcium phosphate nanoparticles

Scanning electron microscopy (SEM) was performed with an ESEM Quanta 400 instrument with gold/palladium-sputtered samples. Dynamic light scattering (DLS) and zeta potential determinations were performed with a Zetasizer nanoseries instrument (Malvern Nano-ZS, laser: $\lambda = 532$ nm) using the Smoluchowski approximation and taking the data from the Malvern software without further correction. The particle size data refer to scattering intensity distributions (*z*-average). Ultracentrifugation was performed at 25 °C with an Optima XL-I instrument (Beckman-Coulter). Absorption spectra were recorded with a Varian Cary 300 spectrophotometer. Emission spectra were recorded with an Agilent Cary Eclipse spectrophotometer. Freeze-drying (lyophilization) was performed with a Christ, Alpha 2-4 LSC instrument.

2.4. Cellular uptake of multifunctional calcium phosphate nanoparticles

For determining the cellular uptake of the nanoparticles, CAL-27 cells were grown for 2 days in a chamber slide. They were then incubated for 24 h with 1 nmol (with respect to the dye concentration) of the nanoparticle formulation conjugated with DY682 and mTHPC (NP-DY682-mTHPC). Cell nuclei were counterstained with 2 µg ml^{-1} Hoechst-33258 (Applichem, Darmstadt, Germany). Cells were analyzed with an EVOS fluorescence microscope (AMG, Bothell, WA, USA) using a filter for Hoechst-stained cell nuclei