Biomaterials 34 (2013) 7143–7157

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Multifunctional calcium phosphate nano-contrast agent for combined nuclear, magnetic and near-infrared *in vivo* imaging

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ARTICLE INFO

Article history:
Received 20 May 2013
Accepted 30 May 2013
Available online 21 June 2013

Keywords:
Calcium phosphate nanoparticles
NIR contrast
Magnetic contrast
Nuclear contrast
Tri-modal *in vivo* imaging

ABSTRACT

Combination of three imaging techniques such as nuclear, magnetic and near-infrared fluorescence can aid in improved diagnosis of disease by synergizing specific advantages of each of these techniques such as deep tissue penetration of radiation signals, anatomical and functional details provided by magnetic contrast and better spatial resolution of optical signals. In the present work, we report the development of a multimodal contrast agent based on calcium phosphate nanoparticles (nCP), doped with both indocyanine green (ICG) and Gadolinium (Gd^{3+}), and labeled with 99m-Technetium-methylene diphosphonate (^{99m}Tc-MDP) for combined optical, magnetic and nuclear imaging. In order to obtain the desired tri-modal contrast properties, the concentrations of ICG, Gd^{3+} and ^{99m}Tc were optimized at ~0.15 wt%, 3.38 at% and ~0.002 ng/mg of nCP, respectively. The leaching-out of ICG was protected by an additional coating of polyethyleneimine (PEI). Toxicological evaluation of the final construct carried out on healthy human mononuclear cells, red-blood cells and platelets, showed excellent hemocompatibility. *In vivo* multimodal imaging using mice models revealed the ability to provide near-infrared, magnetic and nuclear contrast simultaneously. The nanoparticles also showed the potential for improved MR based angio-imaging of liver. Retention of intravenously administrated nanoparticles in the liver was reduced with PEGylation and the clearance was observed within 48 h without causing any major histological changes in vital organs. Thus, we developed a non-toxic tri-modal nano-contrast agent using calcium phosphate nanoparticles and demonstrated its potential for combined nuclear, magnetic and near-infrared imaging *in vivo*.

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1. Introduction

Development of nanoparticle-based multimodal contrast agents is gaining great interest in the field of diagnosis and therapy [1–4]. Multimodal imaging is a technique where two or more imaging modalities are combined to obtain specific advantages of various methods simultaneously. For example, combining physiological characteristics provided by techniques such as positron emission tomography (PET) or single photon emission tomography (SPECT) with anatomical and molecular details provided by computed tomography (CT) and magnetic resonance imaging (MRI) has great potential in disease diagnosis, treatment planning and prognosis analysis [5,6]. So far, there has been significant progress in the area of developing combined PET–CT, PET–MRI and SPECT–MRI systems [7,8]. Compared to CT, MRI has specific advantageous due to the non-ionizing means of imaging, much better soft tissue contrast, physiological and functional imaging capabilities, diffusion weighted imaging or dynamic contrast enhanced imaging modes that can help in detecting metastasis to lymph nodes and specific amino acid profiles [9]. Further, if we can combine PET–MRI or SPECT–MRI with near-infrared (NIR) fluorescence imaging, it will be possible to conduct image guided surgical procedures by correlating the PET/SPECT and MRI data with intra-operational NIR images [10].

In the present clinical practice, each of these imaging modalities needs separate contrast agents such as Fluorine-18 fluorodeoxyglucose (^{18}F-FDG) for PET, 99m-Technetium (^{99m}Tc) for SPECT, 2-gadolinium diethylenetriamine-pentaacetic acid (Gd–DTPA) for

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0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.05.077

MRI and Indocyanine green (ICG) for optical imaging. The pharmacokinetic profiles of each of these contrast agents differ significantly and getting them simultaneously to a particular diseased site for combinatorial imaging is a major challenge. In addition, simultaneous use of multiple contrast agents may increase the risk of cumulative toxicity. Ideally, this issue can be overcome by developing a single contrast agent that can provide multiple contrast properties together with biocompatibility. With the emergence of nanotechnology, there have been serious efforts in developing multimodal contrast agents using polymers, lipids, liposomes, inorganic particles [11–15]. Most of the reports are based on developing nanoparticles for combined magnetic and optical imaging, where a magnetic core (mostly iron oxide) is either surrounded by a fluorescent quantum dot shell (CdSe, CdTe) or embedded in a nanoparticle co-loaded with fluorescent dyes [16,14]. Recently, Jokerst et al. reported on the application of Gd^{3+} and FITC loaded silica nanoparticles for ultrasound guided stem cell implantation and MRI based long term cell tracking [15]. The toxicity of CdSe, CdTe quantum dots [17] as well as silica like materials [18] and the aggregation of organic dyes within nanoparticles leading to fluorescent quenching [19] are the major limitations of such systems. Nanoparticles combining optical and nuclear imaging modalities are also being developed [20–22]. Generally, optical and nuclear contrast moieties were conjugated to reactive carboxyls or amines in chelates such as tetraazacyclododecane–tetra acetic acid (DOTA) or peptides [20,23]. In addition to bimodal contrast agents, recently, tri-modal agents were also developed for combined optical, nuclear and magnetic contrast imaging [24]. Xie et al. reported an FeO based tri-modal agent [24] labeled with Cy5.5 and ^{64}Cu-DOTA where the radio-labeling efficiency varied from 5 to 88% [25]. In another report, an aptamer conjugated cobalt ferrite nanoparticle surrounded by rhodamine within a silica shell labeled with ^{67}Ga citrate was reported [26]. Both the above works used iron oxide nanoparticle which is a T_2 contrast agent giving dark contrast which may be confused with bleeding, calcification and susceptibility artifacts compared to T_1 agents based on Gd^{3+} [27]. In another interesting work, ^{18}F-labeled lanthanide doped NaYF4 nano-phosphors were reported for combined nuclear, magnetic and up-conversion luminescent properties [28]. In addition to low up-conversion quantum yield of NaYF4, the biocompatibility of rare-earth fluorides is not well understood.

Recently, the application of calcium phosphate nanoparticles for the development of diagnostic [29,30], drug [31], gene [32] or siRNA [33] delivery agents is being widely researched upon. Calcium phosphate, being the mineral component of human bone and teeth, is an appropriate choice for the development of non-toxic contrast agents. It is used clinically for bone tissue regeneration and as adjuvant for vaccines [34,35]. Barth et al. reported development of ICG doped calcium phosphosilicate nanoparticles for targeted *in vivo* NIR imaging of breast and pancreatic tumor [36] as well as for photodynamic therapy of leukemia in mice models [37]. Calcium phosphosilicate nanoparticles had an improved ICG loading efficiency and quantum yield compared to polymeric nanoparticles [29]. In our earlier study, we reported on the development of Eu^{3+} and Gd^{3+} doped hydroxyapatite nanoparticles (nHAp) for combined optical, magnetic and X-ray contrast imaging [38]. Chen et al., also reported the development of Eu^{3+} and Gd^{3+} doped calcium phosphate nanospheres and hydroxyapatite nanorods for multimodal imaging together with drug delivery [39,40]. But the emission of Eu^{3+} was not in the NIR range that was suitable for *in vivo* imaging. Considering this limitation we have doped NIR emitting dye, ICG, into nHAp [41].

In the present work, we report a tri-modal contrast agent based on calcium phosphate nanoparticles for combined optical, magnetic and nuclear imaging and demonstrated its *in vivo* application using mouse models. Calcium phosphate nanoparticles (nCP) were doped with ICG and Gadolinium (Gd^{3+}) and surface labeled with ^{99m}Tc-MDP such that all the three contrast properties could be derived from a single nanoparticle. To the best of our knowledge, this is the first report on tri-modal contrast agent based on calcium phosphate nanoparticles for combined near-infrared, magnetic and nuclear imaging. In further discussions we refer this tri-modal contrast agent as multifunctional nCP (MF-nCP). We have discussed the optimization of various synthesis parameters to obtain the best tri-modal contrast properties *in vivo*. The effect of polyethylene glycol (PEG) capping on biodistribution is also discussed.

2. Materials and methods

2.1. Synthesis of MF-nCP

All the precursors were prepared in endotoxin free water and the synthesis was carried out under highly sterile conditions. In a typical reaction procedure, 20 mL of 0.05 M calcium chloride ($CaCl_2$, Sigma, USA) was mixed with 20 mL 0.01 M trisodium citrate, 1 mM ICG (Sigma, USA) and 0.1 M Gadolinium nitrate ($Gd(NO_3)_3$) for 30 min at room temperature (~25 °C). Volume of ICG taken was varied from 15 μL to 72 μL that corresponded to weight % of ICG to calcium phosphate ranging from 0.05 to 0.24% (total yield of undoped calcium phosphate was 30 mg). Volume of $Gd(NO_3)_3$ was varied from 150 μL to 2.5 mL that corresponded to Gd atomic % varying from 1.5 to 25% (wrt Ca). Later, the effective Gd^{3+} doping was characterized by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). Then, 5 mL of 0.03 M diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, S.D Fine Chemicals, India) mixed with 2 mL of 3 N ammonium hydroxide (NH_4OH) was added drop wise to $CaCl_2$–ICG–Gd^{3+} mixture under constant stirring to obtain ICG and Gd^{3+} doped calcium phosphate nanoparticles (ICG-Gd-nCP). The precipitate was washed and dispersed in 30 mL endotoxin free water. 40 mL of 0.00025 weight% polyethyleneimine (PEI) (average molecular weight 25,000, Sigma, USA) was added to the ICG-Gd-nCP and stirred for 30 min to obtain PEI capped ICG-Gd-nCP. The pH during the entire process was maintained at ~9 by NH_4OH. The precipitate was then centrifuged at 6000 rpm for 3 min and washed with endotoxin free water at least two times. Next, conjugation of ^{99m}Tc-MDP to the prepared ICG-Gd-nCP was to be carried out. ^{99m}Tc-MDP conjugate was prepared by adding ^{99m}Tc (sodium pertechnetate solution) to the MDP cold-kit (Board of Radiation and Isotope Technology, India. Kit contents: Methylene diphosphonate – 10 mg, tin(II) chloride dehydrate – 1 mg, ascorbic acid – 1.8 mg) and incubating at room temperature for 10 min. A labeling efficiency of >95% was ensured by chromatographic analysis. Different volumes of ^{99m}Tc-MDP (radioactivity ranging from 60 to 300 μCi) was treated with 1 mL of ICG-Gd-nCP (20 mg/mL) and allowed to stand at room temperature for 30 min. After incubation, the sample was washed twice to remove unconjugated radionuclide to obtain MF-nCP. Radioactivity of the conjugates and the supernatants obtained on washing steps were measured in a radionuclide dose calibrator (Capintec, USA). Radiation protection techniques were practiced while handling the radioactive material.

Polyethylene glycol capping (PEGylation) of MF-nCP was carried out using N hydroxysuccinimide (NHS) activated branched PEG molecules (molecular weight 40,000, Jenkem technology, USA). 1 mg/mL of MF-nCP dispersed in phosphate buffered saline (PBS – pH 7.4) was treated with 13 μL of 15 mM NHS–PEG at 4 °C for 2 h. The conjugated sample was then washed in PBS twice in order to obtain the final sample.

2.2. Characterization of MF-nCP

Crystallinity of MF-nCP was studied by X-ray powder diffraction using PANalytical X Pert-pro system fitted with Cu-Kα source. Composition of the material was characterized by Fourier Transform Infrared (FTIR) spectra of KBr supported samples using PerkinElmer Spectrum RX1. The particle size analysis was carried out using Scanning electron microscope (SEM) [JEOL JSM-6490 LA] and dynamic light scattering (Nano ZS, Zetasizer Nanoseries, Malvern). Zeta potential of the sample was measured using Nano ZS, Zetasizer Nanoseries, Malvern. Fluorescence excitation and emission spectra of 1 mg/mL of sample were recorded using HORIBA-JOBINYVON Fluoromax 4 Spectrofluorometer. NIR fluorescence imaging of samples were carried out in a multispectral imaging station (Kodak Multispectral *in vivo* imaging system FX pro, USA) using a band pass excitation filter of 760 ± 15 nm and an emission filter of 830 ± 15 nm. 100 μL of 5 mg/mL of each sample was taken in a 96 well plate and imaged for an exposure time of 30 s. A fixed region of interest (ROI) was chosen for each sample and the average fluorescence intensity over the ROI was analyzed for fluorescence intensity comparison between different samples. To study the effect of different capping agents, 100 μL of the supernatants collected after each washing step of capped samples was analyzed and their fluorescence intensity was compared.

Quantitative evaluation of the efficiency of Gd^{3+} doping was carried out using ICP-AES (Thermo Electron IRIS INTREPID II XSP DUO), for which doped nCP was

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