European Journal of Pharmaceutics and Biopharmaceutics 87 (2014) 132–141

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Biodegradable human serum albumin nanoparticles as contrast agents for the detection of hepatocellular carcinoma by magnetic resonance imaging

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ARTICLE INFO

Article history:
Received 11 December 2013
Accepted in revised form 12 December 2013
Available online 21 December 2013

Keywords:
Human serum albumin
Nanoparticles
Human hepatocellular carcinoma (HCC)
Magnetic resonance imaging (MRI)
Gd-DTPA
Rhodamine 123

ABSTRACT

Tumor visualization by magnetic resonance imaging (MRI) and nanoparticle-based contrast agents may improve the imaging of solid tumors such as hepatocellular carcinoma (HCC). In particular, human serum albumin (HSA) nanoparticles appear to be a suitable carrier due to their safety and feasibility of functionalization. In the present study HSA nanoparticles were conjugated with gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) using carbodiimide chemistry. The nanoparticles had a uniform spherical shape and a diameter of 235 ± 19 nm. For better optical visualization in vitro and in vivo, the HSA-Gd nanoparticles were additionally labeled with rhodamine 123. As shown by confocal microscopy and flow cytometry analysis, the fluorescent nanoparticles were readily taken up by Huh-7 hepatocellular carcinoma cells. After 24 h incubation in blood serum, less than 5% of the Gd(III) was released from the particles, which suggests that this nanoparticulate system may be stable *in vivo* and, therefore, may serve as potentially safe T1 MRI contrast agent for MRI of hepatocellular carcinoma.

1. Introduction

Early diagnosis of premalignant and malignant lesions is essential for improving the current poor prognosis of patients with hepatocellular carcinoma (HCC). Magnetic resonance imaging (MRI), which produces high resolution three-dimensional images delineating morphological features of the specimen, is an effective clinical strategy to diagnose solid malignancies such as HCC [1]. To enhance contrasting between different tissues, contrast agents are used that shorten the relaxation parameters (T1 and T2) of water. Among different kinds of contrast agents, the Gd-based contrast agents are employed in the majority of the cases. The Gd-chelates, such as Gd-diethylenetriaminepentaacetic acid (Gd-DTPA), shorten the longitudinal relaxation time and are thus positive contrast agents and are widely used for both vascular imaging and tumor imaging.

However, the Gd-chelates are rapidly eliminated by renal filtration, resulting in short imaging time and lack of specificity to target organs. To overcome these drawbacks, contrast agents prepared by nanotechnology have been studied for many years and showed favorable potential in tumor diagnosis. Thus, polymeric Gd complexes based on dextrans, polylysine derivatives, or dendrimers as well as Gd chelate grafted latex nanoparticles, liposomes, and micelles have been successfully employed [2–4]. Nanoparticle-based contrast agents can be functionalized to enable specific targeting of tumors, including HCC. Thus, Liu et al. reported the use of anti-vascular endothelial growth factor receptor antibody and Gd-DTPA-conjugated NPs for the visualization of HCC in MRI of H22 hepatoma xenografted nude mice [5].

Among the various macromolecular carriers, albumin appears to be particularly advantageous, as it is stable, biodegradable, non-toxic, non-immunogenic, and can be readily modified with ligands due to the presence of numerous and variable functional

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http://dx.doi.org/10.1016/j.ejpb.2013.12.010

groups. Previously, it has been reported that human serum albumin (HSA) nanoparticles with attached apolipoprotein E can be used to deliver drugs across the blood–brain barrier (BBB) [6–8]. In addition, HSA NPs conjugated with Gd-DTPA and coupled with transferrin or folic acid as targeting moieties appear to be potential contrast agents for the brain, as well as other organs (i.e. heart, liver, and skeletal muscles) [9,10].

Gd-DTPA is one of the most extensively used contrast agents for MRI due to its strong paramagnetic properties. However, its short half-life in blood and the lack of specificity of low-molecular-weight contrast agents like Gd-DTPA for target organs limit its usability [11,12]. For this reason, Gd-DTPA-conjugated nanoparticles were developed as a potential MRI contrast agent for molecular diagnosis and *in vivo* imaging. It is well known that Gd-DTPA linked to macromolecules, for example, Gd-labeled albumin (Galbumin®, BioPal Inc., USA) and polylysine dendrimer with Gd-DOTA complexes (Gadomer-17) enabled higher contrast levels over a longer time period [13,14]. In the present study, Gd-DTPA conjugated HSA nanoparticles (HSA-Gd-Rho) were evaluated regarding their performance to deliver this contrast agent to HCC cells. Additionally, the covalent attachment of DTPA to HSA in solution and subsequent nanoparticle formation was compared to nanoparticles where the DTPA was attached after their formation regarding their ability to chelate Gd^{3+}. Furthermore, a fluorescent label was added to enable tracking of the nanoparticles in tissues.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA, fraction V, purity 96–99%, batch 028K7550, 65,000 Da) was obtained from Sigma. Diethylenetriaminepentaacetic acid dianhydride (DTPAa) and $GdCl_3$, glutaraldehyde, rhodamine 123, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (Steinheim, Germany). Other chemicals were of analytical grade, commercially available, and used as received. Human blood plasma and serum were obtained from the local German blood bank (DRK Blutspendedienst, Frankfurt am Main, Germany).

2.2. Cell culture

The Huh-7 hepatocellular carcinoma cell line originating from human liver epithelial cells was used in this study [15]. The Huh-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin at 100 mg/ml and penicillin at 100 U/ml. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were trypsinized, resuspended, and precultured before use.

2.3. Preparation of nanoparticles

2.3.1. Preparation of HSA nanoparticles

The HSA nanoparticles were prepared by a desolvation process [16,17]. Briefly, 100 mg HSA (0.0015 mmol) was dissolved in 1.0 ml of 10 mM sodium chloride solution, the pH of the solution was adjusted to 8.4, and then this solution was filtered through a 0.22 μm filtration unit. The nanoparticles were formed by desolvation using continuous addition of ethanol (4.0 ml at the rate of 1 ml/min) under constant stirring at room temperature. After desolvation, 59.74 mg of glutaraldehyde (0.5968 mmol, 58.8 μl of 8% aqueous glutaraldehyde solution) was added to achieve cross-linking of the particles. This amount of glutaraldehyde corresponds to 100% of the theoretic micromolar amount required for the quantitative cross-linking of the 59 primary amino groups in micromolar amounts of HSA [18,19]. To investigate the influence of the amount of glutaraldehyde, the cross-linking was performed also using the 2-fold molar excess (200%) of glutaraldehyde (119.38 mg or 1.926 mmol, 117.5 μl of 8% aqueous glutaraldehyde solution). The resulting particles were purified by three cycles of differential centrifugation (16,100 *g*, 10 min) and redispersed to the original volume in water.

2.3.2. HSA nanoparticles modified with covalently attached Gd-DTPA [HSA-(Gd-DTPA)]

The HSA-(Gd-DTPA) nanoparticles were prepared according to the method described previously [20]. Briefly, 5 mg DTPAa (0.0140 mmol) was added to 100 mg of HSA (1 ml of 100 mg/ml solution), the pH was adjusted to 9, and the mixture was incubated at room temperature for 3 h under constant shaking at 600 rpm. The DTPA-conjugated HSA was dialyzed for 24 h, and 0.5 mg of $GdCl_3$ (0.0019 mmol) was then added and immediately chelated to form a stable compound. Then HSA-Gd-DTPA was desolvated with ethanol to form the HSA-Gd-DTPA nanoparticles that were subsequently cross-linked with 100% and 200% amounts of glutaraldehyde, as described above.

Alternatively, the HSA-Gd-DTPA NPs were prepared by covalent binding of DTPA to the previously formed HSA nanoparticles [5]. 5 mg of DTPAa (0.0140 mmol) was added to 1 ml of the HSA NP suspension (100 mg HSA in 1 ml of phosphate buffer, pH 8), the pH was adjusted to 9, and then the suspension was incubated at room temperature for 3 h under constant shaking. To separate the unbound DTPAa, the DTPA-conjugated HSA NP was purified by four cycles of differential centrifugation (16,100 *g*, 10 min) and redispersion in 1.0 ml of water. Then 0.5 mg of $GdCl_3$ (0.0019 mmol) was added to the HSA-DTPA suspension. The mixture was incubated at 21 °C for 30 min under constant shaking. Then the HSA-(Gd-DTPA) nanoparticles were subjected to dialysis against deionized water to remove excess of Gd^{3+}. The arsenazo test was used to confirm the absence of free Gd(III) in the nanoparticle suspension [21].

2.3.3. Fluorescently labeled HSA-(Gd-DTPA) nanoparticles

The freshly prepared solution of 1 mg EDC (0.0052 mmol) in 0.1 ml phosphate buffer (pH 8) was added into the HSA-Gd-DTPA nanoparticle suspension, and the mixture was incubated for 15 min under constant shaking. Then 50 μg of rhodamine 123 (0.1313 μmol) was added to the activated nanoparticles, and the suspension was incubated at room temperature for 3 h under constant shaking and purified by three cycles of differential centrifugation (16,100 *g*, 10 min) and redispersion in 1.0 ml of water to obtain the rhodamine 123-labeled HSA-Gd(DTPA) nanoparticles (HSA-Gd-Rho).

2.4. Characterization of nanoparticles

The resulting nanoparticles were characterized using a Zetasizer 3000 (Malvern Instruments Ltd., Malvern, UK): mean diameter and polydispersity were measured by photon correlation spectroscopy (PCS); the zeta-potential was assessed using Laser Doppler micro-electrophoresis in a palladium electrode dip cell. For size measurements the samples were diluted 1:50 with purified water; the scattering angle was 173°; and the temperature was set to 25 °C. All measurements were repeated 3 times.

The encapsulation efficiency and loading of rhodamine 123 was evaluated indirectly by measuring the amount of unbound rhodamine in the supernatant after separation of the particles by centrifugation. The concentration of rhodamine 123 was measured spectrophotometrically at 503 nm. The relative encapsulation efficiency (EE) and loading (L) of rhodamine were calculated as follows: