Colloids and Surfaces B: Biointerfaces 129 (2015) 39–46

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Preparation and *in vitro* characterization of chitosan nanobubbles as theranostic agents

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ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form 21 January 2015
Accepted 8 March 2015
Available online 14 March 2015

Keywords:
Gd complex
Nanobubbles
MRI
Theranosis
Ultrasound
Controlled release

ABSTRACT

Theranostic delivery systems are nanostructures that combine the modality of therapy and diagnostic imaging. Polymeric micro- and nanobubbles, spherical vesicles containing a gas core, have been proposed as new theranostic carriers for MRI-guided therapy. In this study, chitosan nanobubbles were purposely tuned for the co-delivery of prednisolone phosphate and a Gd(III) complex, as therapeutic and MRI diagnostic agent, respectively. Perfluoropentane was used for filling up the internal core of the formulation. These theranostic nanobubbles showed diameters of about 500 nm and a positive surface charge that allows the interaction with the negatively charged Gd-DOTP complex. Pluronic F68 was added to the nanobubble aqueous suspension as stabilizer agent. The encapsulation efficiency was good for both the active compounds, and a prolonged drug release profile was observed *in vitro*. The effect of ultrasound stimulation on prednisolone phosphate release was evaluated at 37 °C. A marked increase on drug release kinetics with no burst effect was obtained after the exposure of the system to ultrasound. Furthermore, the relaxivity of the MRI probe changed upon incorporation in the nanobubble shell, thereby offering interesting opportunity in dual MRI-US experiments. The ultrasound characterization showed a good *in vitro* echogenicity of the theranostic nanobubbles.

In summary, chitosan drug-loaded nanobubbles with Gd(III) complex bound to their shell might be considered a new platform for imaging and drug delivery with the potential of improving anti-cancer treatments.

1. Introduction

A typical theranostic agent is a system that provides imaging support to a therapeutic treatment [1–4].

The theranostic approach appears to be particularly relevant for improving the cure of cancer or other important diseases at an early stage, as it has the potential to image the pathological tissues and, at the same time, to monitor the delivery kinetics and biodistribution of a drug, thereby obtaining important benefits in terms of tuning therapy and doses, and reducing adverse side effects.

Nano-sized systems are promising theranostics because their sizes favor a prolonged circulation time after intravenous administration, and good loading capacity. Various nanotechnological systems have been considered for carrying imaging probes and drugs, *e.g.* polymeric nanoparticles, micelles, vesicles, and liposomes [5,6]. Ideally, for theranostic purposes, the nanocarrier should be stable (*i.e.* no drug release in body districts different from the biological target), easily tunable, with a high payload capacity, and with multifunctional properties. Microbubbles and nanobubbles are spherical particles with a core–shell structure filled up with a gas, which gives them acoustically active properties [7–9]. Microbubbles mean diameters are generally between 1 and 8 micrometers. The shell can be mainly composed of proteins, lipids or polymers, whereas the core can be filled up with various gases. Currently, microbubbles are on the market as contrast agents for Ultrasound (US) imaging. Under US acoustic pressure, they are able to produce volumetric oscillations detectable by clinical US scanners. Besides the well established diagnostic applications [10–12], microbubbles have been recently investigated as delivery systems for drugs and genes.

Nanobubbles (NBs) are bubbles of sub-micron size, which are primarily designed to increase the stability and improve the biodistribution of the transported drug to the pathological region. Indeed, microbubbles are not able to extravasate from the bloodstream

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http://dx.doi.org/10.1016/j.colsurfb.2015.03.023
0927-7765/

due to their larger size. Nanobubbles do and have been already investigated as gene delivery systems [13–15].

Lipid nanobubbles for US imaging detection have been proposed for the *in vivo* contrast-enhanced imaging of tumor tissues for applications in the field of drug delivery [16].

The use of MRI detectable microbubbles for theranostic purposes has been already proposed. Gd-DTPA loaded microbubbles of PLGA were designed as multimodal contrast agents for both US and MRI [17]. Albumin-based microbubbles filled with decafluorobutane gas and containing Gd-DTPA covalently linked to Human Serum Albumin (HSA) were used to enhance the detection of inflamed sites within the vascular wall [18].

Bubbles for MRI detection has been also proposed by Feshitan et al., who prepared lipid-based theranostic microbubbles loaded with a paramagnetic Gd(III) complex to guide focused ultrasound surgery [19].

Bubbles made of chitosan have been already prepared as oxygen delivery system for the potential treatment of hypoxic tissues [20,21] and, recently, chitosan-based nanobubbles have been reported for US-triggered DNA delivery [22]. We deemed of interest to design chitosan nanobubbles as dual MRI/US theranostic tool to deliver both a MRI probe and a drug.

For this purpose, in this work a chitosan-based formulation containing prednisolone phosphate (PLP) as model drug and the paramagnetic complex Gd-DOTP as T_1-MRI agent (positive contrast) has been prepared and tested *in vitro*.

Perfluorocarbons have been used for contrast agent preparation because of their stability, biological inertness and low water solubility [23]. *In vivo*, they are excreted from lung capillaries, where they can escape from the bubble core and enter the alveolus to be exhaled. Perfluorocarbons can act as bubble stabilizer being able to increase the lifetime of bubbles in the bloodstream [24,25].

Perfluoropentane vapor, due to the extremely low solubility in water (4×10^{-3} mol/m^3), can remain inside the bubbles and it is able to dissolve the water-soluble gases present in the blood.

For these reasons, the latter molecule was selected for the herein presented system. Furthermore, perfluoropentane can tolerate intrabubble gas pressure larger than 1 atm, thus stabilizing the system. Besides these favorable features, we selected perfluoropentane because it is liquid at room temperature (b.p. = 29 °C), thus allowing an easy preparation set up. It could convert to a gas at body temperature (37 °C), though the presence of the Laplace pressure can increase the gas boiling temperature within the nanobubble structure.

The Laplace pressure is the pressure difference between the inside and the outside of a bubble (or a droplet), given as:

$$\Delta P = P_{\text{inside}} - P_{\text{outside}} = \frac{2\sigma}{r}$$

where P_{inside} and P_{outside} are the pressures inside and outside a bubble respectively, σ is the interfacial tension, and r is the bubble radius.

However, it has been demonstrated that the liquid to gas phase transition of perfluoropentane inside the nanobubbles can be activated by US through the Acoustic Droplet Vaporization (ADV) mechanism [26].

PLP is a glucocorticoid drug used for the treatment of inflammatory diseases and, in chemotherapy, to reduce adverse side effects of anticancer drugs. It was also proposed as anticancer drugs itself due to the ability to inhibit angiogenesis [27]. Recently, PLP encapsulated in liposomes showed an increased anti-tumor efficacy, which is attributed to an enhanced tumor accumulation of the drug loaded in the liposomal formulation caused by the EPR effect [28]. More recently, an amphiphilic Gadolinium complex was added to liposomes containing PLP to obtain paramagnetic liposomes suitable for their MRI-guided visualization in mice [29]. The choice of PLP was primarily driven by its amphiphilic nature (that allows both the interaction with the bubble interface) and the presence of a negative charge (that favor the interaction with the cationic chitosan). The anionic paramagnetic complex Gd-DOTP was chosen as MRI agent, in virtue of the well-documented ability of lanthanide-complexes of DOTP ligand to interact with cationic systems [30,31].

The aim of this work is the development of new stable theranostic chitosan-based nanobubbles containing the MR imaging (Gd-DOTP) and the drug (PLP) companions. The theranostic system is characterized *in vitro* to assess the MRI and echogenic potential.

2. Materials

Ethanol 96° was purchased from Carlo Erba (Milan, I). Epikuron 200® (dipalmitoyl phosphatidylcholine 95%) was a kind gift from Degussa (Hamburg, D). Palmitic acid, perfluoropentane, Pluronic F68, chitosan (low molecular weight, 50–70 kDa, DD = 75–85%), prednisolone phosphate (PLP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gd-DOTP was kindly provided by Bracco Imaging SpA (Colleretto Giacosa (TO), Italy). Ultra-pure water was obtained using a system 1-800 Milli-Q (Millipore, FL, USA). Tetradecylphosphoric acid (C14) was synthesized as previously reported [22]. All the other reagents were of analytical grade.

2.1. Preparation of the theranostic chitosan nanobubbles

A multistep method was purposely developed to prepare the theranostic nanobubbles. Firstly, 300 microliters of an ethanolic solution of Epikuron® 200 (1%, w/w) containing a co-surfactant were added under stirring to 500 microliters of perfluoropentane at room temperature. Then, 4.8 ml of ultrapure water were slowly added to the mixture (under mild stirring) until the formation of an emulsion. Subsequently, the system was homogenized for three minutes at 12,000 rpm using a high-shear homogenizer (Ultraturrax, IKA, Germany) in an ice-bath. The third step consisted of the drop-wise addition of an aqueous solution of chitosan (pH = 5.0, 2.7, w/w) that formed the nanobubble polymeric shell. For tuning the formulation sizes, two different co-surfactants were tested: palmitic acid (C16) and tetradecylphosphoric acid (C14), thus obtaining two types of nanobubble formulations: C14-NB and C16-NB.

Finally, 200 μl of an aqueous solution of Gd-DOTP (Fig. 1A) at the concentration of 4.4 mM were drop-wise added under stirring to the two types of pre-formed nanobubbles in aqueous suspension. The so-obtained formulations (Gd-C14-NB and Gd-C16-NB) were incubated for 30 min under stirring to facilitate the binding of the negatively charged Gd complex with the cationic nanobubble chitosan shell. Then, free Gd-DOTP (as well as other soluble components unbound to NBs) was removed by dia-ultrafiltration using a TCF2 system (Amicon) with a dialysis membrane cut off of 100 kDa.

After the purification, an aqueous solution of the stabilizer agent Pluronic F68 (0.01%, w/w) was added under stirring to the aqueous nanobubble suspensions.

The drug prednisolone phosphate (Fig. 1A) was loaded to the NB formulation upon addition of the drug (2 mg/ml) to the ethanolic solution of Epikuron®. Then, the preparation procedure was carried out as reported above. Blank nanobubbles (C14-NB and C16-NB) were prepared as control. All the nanobubble samples were stored at 4 °C.

2.2. Determination of perfluoropentane surface tension

The surface tension of perfluropentane as such and perfluoropentane plus Epikuron 200® and PLP ethanol mixture at the same