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First *in vivo* MRI study on theranostic dendrimersomes

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ABSTRACT

Amphiphilic Janus-dendrimers are able to self-assemble into nanosized vesicles named dendrimersomes. We recently synthesized the 3,5-C_{12}-EG-$(OH)_4$ dendrimer that generates dendrimersomes with very promising safety and stability profiles, that can be loaded with different contrast agents for *in vivo* imaging. In this contribution, nanovesicles were loaded with both the Magnetic Resonance Imaging (MRI) reporter GdDOTAGA$(C_{18})_2$ and the glucocorticoid drug Prednisolone Phosphate (PLP), in order to test their effective potential as theranostic nanocarriers on murine melanoma tumour models. The incorporation of GdDOTAGA$(C_{18})_2$ into the membrane resulted in dendrimersomes with a high longitudinal relaxivity ($r_1 = 39.1\ mM^{-1}\ s^{-1}$, at 310 K and 40 MHz) so that, after intravenous administration, T_1-weighted MRI showed a consistent contrast enhancement in the tumour area. Furthermore, the nanovesicles encapsulated PLP with good efficiency and displayed anti-tumour activity both *in vitro* and *in vivo*, thus enabling their practical use for biomedical theranostic applications.

1. Introduction

Despite the rapid advances in diagnostic and therapeutic protocols, cancer still remains one of the most challenging domains of investigation in modern medicine. The limitations or the failure of current treatments encouraged the research for innovative approaches to cancer disease, in the effort to improve the early-stage detection and specifically target the therapy [1].

By combining both therapeutic and diagnostic properties in one single platform, theranostic systems are emerging medical tools that hold great promises in enhancing the therapeutic outcome of cancer therapy [2–5]. Unlike conventional approaches, the theranostics integrates specific molecular targeting, therapeutic activity, and imaging in one multifunctional medical system, very often based on the use of nanosized carriers. Some of the most attractive features of the nanoparticle-based theranostics include the opportunity to specifically deliver different pharmaceutically active molecules, to monitor their biodistribution *in vivo*, and, in some cases, also to control/trigger the release of the drug at the target site [5,6].

So far, a very wide and continuously extending array of nanoplatforms has been considered for biomedical applications, but few of them have received so much interest in the theranostic field as liposomes. These vesicles, composed of a phospholipidic bilayer, already proved their ability to load both imaging-responsive molecules and drugs, and to act as theranostic agents in several pathologic scenarios, especially in oncology [5,7].

Following the work on liposomes, other nanosized bilayered vesicular structures have been proposed. Among them, dendrimersomes (DSs) are vesicles self-assembled from amphiphilic Janus-dendrimers, described for the first time by Percec et al. in 2010 [8]. Based on these initial studies, we argued whether DSs could be employed as reliable alternative to liposomes in the advanced biomedical protocols, especially considering the advantages in terms of cost and easiness of synthesis of the dendrimeric constituents of the membrane. Therefore, we initially evaluated the potential of these nanocarriers to act as MRI agents by loading paramagnetic Gd-complexes either in the aqueous core or in the membrane of the particles [9–11]. Furthermore, we recently synthesized a new low generation Janus-dendrimer (3,5-C_{12}-EG-$(OH)_4$, Chart 1, left) that has been demonstrated to form Gd-loaded dendrimersomes of high stability and biocompatibility [10].

In this contribution, we report on the first *in vivo* testing of the theranostic potential of DSs composed of the 3,5-C_{12}-EG-$(OH)_4$ dendrimer, co-loaded with a drug (Prednisolone Phosphate, PLP, Chart 1) and the lipophilic MRI agent GdDOTAGA$(C_{18})_2$ (Chart 1), whose excellent MRI performance has been very recently demonstrated [11]. Since liposomal formulations of PLP already proved to be effective in the treatment of melanoma [12–18], the herein investigated theranostic

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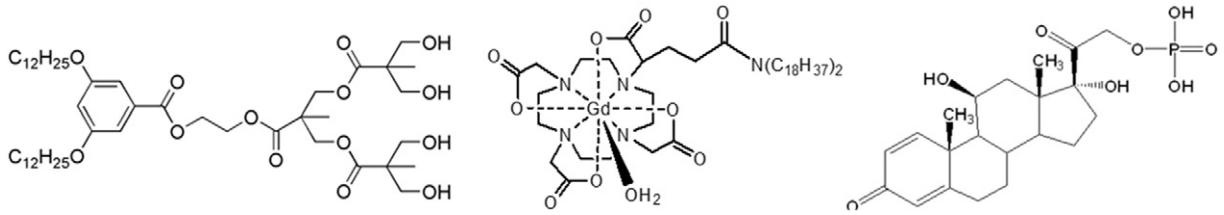

Chart 1. Structures of the dendrimersomes components. Left: 3,5-C_{12}-EG-$(OH)_4$ Janus dendrimer (main constituent of the dendrimersomes); middle: GdDOTAGA$(C_{18})_2$ complex (MRI agent incorporated in the dendrimersome bilayer); right: Prednisolone Phosphate (PLP, antitumour drug encapsulated in the inner core of the dendrimersomes).

platform was tested *in vivo* on a syngeneic murine melanoma model and its therapeutic efficacy was compared to that provided by a corresponding liposome-based formulation.

2. Materials and methods

2.1. Chemicals

The phospholipids 1,2-distearoyl-sn-glycero-3 phosphoethanolamine-*N*-[carboxy(polyethyleneglycol)-2000] ammonium salt (DSPE-PEG2000-COOH) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The 3,5-C_{12}-EG-$(OH)_4$ Janus dendrimer was synthesized as described elsewhere [10]. Briefly, the amphiphile was obtained by linking the hydrophobic block 3,5 bis-dodecyl substituted benzoyl ether to a generation 1 of 2,2-bis(hydroxymethyl)propanoic acid, using ethylene glycol as spacer between the two moieties. The first synthesis intermediate, a 2-hydroxyethyl 3,5-(didodecyloxy)benzoate was obtained by reacting 3,5-(didodecyloxy)benzoic acid with an excess of ethylene glycol in the presence of 4-(dimethylamino)pyridinium *p*-toluenesulfonate (DPTS) and dicyclohexylcarbodiimide (DCC). Following the same coupling procedure, 2,2,5-trimethyl-1,3-dioxane-5-carboxylic acid was reacted with the free hydroxyl group of the first synthesis intermediate. Deprotection of the acetonide was carried out in a 1:1 mixture of 6 M HCl and THF to get a generation zero intermediate. The final compound was obtained by repeating once again the coupling and the deprotection steps.

GdDOTAGA$(C_{18})_2$ was synthesized in a four steps-process starting from the DOTAGA$(t\text{Bu})_4$, as it was already reported in literature [11]. Briefly, the free carboxylic acid of DOTAGA$(t\text{Bu})_4$ was activated by formation of the *N*-hydroxysuccinimidyl ester, which was then reacted with dioctadecylamine in pyridine at 70 °C. The free ligand was obtained after *t*Bu esters deprotection using a 1:1 mixture of TFA and dichloromethane and the Gd(III) complex was prepared by reacting the ligand with $GdCl_3$ in methanol at 50 °C overnight. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, USA), and used as received unless otherwise noted.

2.2. Nanovesicles preparation

DSs and liposomes were prepared by the thin film hydration method [19]. Briefly, to prepare the stock samples with a concentration of 80 mg/ml in the final vesicle suspension useful to perform further experiments, the appropriate amounts of amphiphilic materials GdDOTAGA$(C_{18})_2$, DSPE-PEG2000-COOH, and 3,5-C_{12}-EG-$(OH)_4$ dendrimer (or the phospholipid DPPC) were weighted according to the molar ratio 20:5:75, and then dissolved into chloroform (8 ml). After dissolution, the organic solvent was slowly evaporated, so that the amphiphilic components distributed in a thin homogeneous film at the bottom of a round flask. After 2 h under vacuum, the film was hydrated at 50 °C with an isotonic buffered solution containing PLP at 50 mg/ml, in addition to NaCl and 4-(2-hydroxyethyl)-1- piperazineetha nesulfonic acid (HEPES) with a final whole osmolarity of *ca.* 300 mOsm and pH of 7.4. The vesicular suspension was then extruded several times through polycarbonate filters (Lipex extruder, Northern Lipids Inc.) with pore diameters decreasing from 1 μm to 200 nm. Finally, to completely remove the non-encapsulated drug, an exhaustive dialysis was carried out at 4 °C against an isotonic buffer consisting of a pH 7.4 solution 0.15 M of NaCl and 0.004 M of HEPES, in a total volume of 2 l and for a minimal duration of 16 h, with one buffer renewal after the first 4 h.

2.3. Nanovesicles characterization

The mean hydrodynamic diameter and the polydispersity of the vesicles were determined by Dynamic Light Scattering (DLS, Zetasizer Nano 90 ZS, Malvern, UK).

The relaxometric characterization of GdDOTAGA$(C_{18})_2$-bearing nanoformulations was carried out to define the longitudinal relaxivity r_1 (at 25 °C and 37 °C) as a function of the applied magnetic field. Specific details about such measurements are available on the Electronic Supplementary Information (ESI). The total concentration of Gd in the samples was determined by a relaxometric measurement (at 20 MHz) after mineralization. Briefly, the samples were diluted 1:2 with concentrated HCl (37%) and stored overnight at high temperature (120 °C) in a sealed glass ampoule to ensure the complete release of the metal ions from the complexes and to obtain free Gd(III) aqua-ions. The R_1^{obs} measurement (at 25 °C) allowed for the accurate estimation of the Gd concentration through a calibration line obtained by using standard solutions of $GdCl_3$.

The total amount of PLP loaded into the vesicles was measured by analytical high-performance liquid chromatography (HPLC), carried out on a Waters Alliance System (separations module: 2695 model) equipped with a photodiode array detector (model 2998) and a Waters Atlantis C_{18} reverse phase column (4.6 mm × 150 mm, 3.5 μm). The chromatographic separation took 37 min in gradient mode, with a re-equilibration time of 18 min to restore the initial conditions. Gradient composition started from 95% of A (H_2O containing 0.1% Trifluoro Acetic Acid, TFA)-5% of B (CH_3CN added with 0.1% TFA) and it was maintained for 2.5 min. Then, B was raised to 30% at 15 min, 60% at 30 min, and finally 100% at 35 min remaining until the end of the analysis. The total amount of PLP in the vesicular suspension (5 mg of amphiphilic material per mL of hydration solution) was quantified by monitoring the eluate through UV detection (detection wavelengths set between 200 and 400 nm). The drug quantification was performed at $\lambda = 278$ nm [20]. In order to determine the drug-loading efficiency of the nanocarriers, the PLP content of samples was then compared to the initial amount of PLP present in the hydration solution (0.103 M).

2.4. Cells

All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and resulted negative for mycoplasma test (MycoAlert™ Mycoplasma Detection Kit, Lonza Sales AG, Verviers, Belgium). Murine fibroblasts (NIH/3T3), melanoma (B16.F10) and human umbilical vein endothelial (HUVEC) cells were cultured as monolayers at 37 °C in a 5% CO_2-containing humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% (*v*/v) of heat-inactivated foetal bovine serum, 2 mM of Glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. At 80%