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Self-assembly triggered by self-assembly: Optically active, paramagnetic micelles encapsulated in protein cage nanoparticles ☆

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ABSTRACT

In this contribution, optically active and paramagnetic micelles of the ligand 1,4,7,10-tetraaza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10) have been incorporated inside capsids of the cowpea chlorotic mottle virus (CCMV) protein through a hierarchical process of self-assembly triggered by self-assembly. The DOTAC10 ligand was used to complex Gd^{III}, in order to form paramagnetic micelles, as well as to encapsulate an amphiphilic Zn^{II} phthalocyanine (ZnPc) dye that optically confirmed the encapsulation of the micelles. The incorporation of ZnPc molecules in the paramagnetic micelles led to high capsid loading of both Gd^{III} and ZnPc, as the micelles were stabilized by the amphiphilic dye encapsulation. The resulting protein cage nanoparticles (PCNs) show an improved r_1 relaxivity, suggesting the possible use of these nanostructures as contrast agents (CAs) for magnetic resonance imaging (MRI). Since the encapsulated ZnPc dye also has a potential therapeutic value, the present results represent a first step towards the consecution of fully self-assembled PCNs for multimodal imaging and therapy.

1. Introduction

Well-defined nanostructured materials are responsible for the recent success of a wide range of biomedical technologies in molecular imaging, diagnostics and therapy. A major advantage of the use of nanomaterials in these fields is the possibility to integrate, within them, various molecular functional components. In this way, nanostructures become suitable 'carriers' for both therapy and diagnosis, as well as for multimodal imaging agents able to aid the contrast of different imaging modalities simultaneously [1–6]. In this respect, many types of imaging techniques are available, ranging from those that enable whole-organism anatomical imaging (e.g., magnetic resonance imaging, MRI) [7,8] to others that provide images with subcellular resolution (e.g., optical imaging) [9–12].

The self-assembly of biomolecules such as the coat proteins (CPs) of virus capsids offer great opportunities for this goal [13–20], leading to monodisperse platforms where different chemical species can be self-organized through covalent or non-covalent bonding. In particular, protein cages have been utilized as nanometre-sized carriers of covalently attached Gd^{III}-based contrast agents (CAs) [21] for MRI [22–31], or of porphyrins [32–35] and phthalocyanines (Pcs) [36] as near-infrared light-absorbing molecules with potential applications in biomedicine. Chelates of the Gd^{III} ion represent the predominantly used MRI CAs, while Pc derivatives are well-known chromophores with different biomedical applications. The synergy of MRI CAs and photoactive organic molecules into biocompatible nanoparticles is indeed highly desirable, yet it is not an easy task from the synthetic point of view. While virus capsids are excellent alternative nanosized scaffolds for achieving high loading of CAs [37], their covalent modification often requires extensive synthetic procedures. Regarding the encapsulation of photoactive dyes, for the same reason, we previously employed a supramolecular strategy to encapsulate tetrasulfonated Zn^{II} phthalocyanine (ZnPc-S4) in the inner cavity of empty cowpea chlorotic mottle virus (CCMV) capsids (i.e., without RNA inside) [36]. Although this work represented a promising approach for photodynamic therapy (PDT), ZnPc-S4 strongly aggregates inside these protein cage nanoparticles (PCNs), forming stacks by π–π and hydrophobic interactions. This type of aggregation is known to quench the photophysical and photochemical properties of Pcs, and is therefore a problem that needs to be solved.

Herein, we describe a straightforward method for the incorporation of amphiphilic Gd^{III} complexes and Zn^{II} phthalocyanine (ZnPc) molecules into CCMV PCNs. Stable and dual-mode functional nanoparticles (i.e., suitable for MRI and optical purposes) can be easily obtained in this way through a hierarchical process of self-assembly triggered by self-assembly. For this goal, we worked under the hypothesis that the

☆ Dedicated to the memory of our friend and colleague Dr G. Christian Claessens, who regretfully passed away last June 2012.

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undesired aggregation of ZnPc molecules can be avoided using an amphiphilic derivative susceptible to be encapsulated inside micelles. In turn, the undesired disassembly of micelles upon dilution can be overcome by confinement within CCMV capsids. As at neutral pH CCMV CPs are known to self-assemble in the presence of negatively charged templates [38–40], negative micelles were expected to trigger the CP self-assembly by acting as 'nucleation points' (Fig. 1) [41].

The main building block of our paramagnetic micelles is the Gd^{III} complex of the amphiphilic ligand 1,4,7,10-tetraaza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (Gd-DOTAC10, **1**), known to show enhanced r_1 relaxivity in its aggregated form [42]. Moreover, the DOTAC10 ligand (**2**) was used to optically confirm the encapsulation of micelles within CCMV capsids by entrapment of the ZnPc dye (**3**), whose amphiphilic nature favors its inclusion into the micelles. Interestingly, the incorporation of this ZnPc derivative in the paramagnetic micelles formed by **1** stabilized them and led to a noticeably higher Gd^{III} loading of the capsids. As a consequence, these purely self-assembled PCNs, which contain both Gd^{III} and dye molecules (see below), show an improved r_1 relaxivity.

2. Experimental section

Commercial grade reagents were purchased from Sigma Aldrich and TCI Europe, and were used without further purification unless otherwise stated. Solvents were dried over molecular sieves. Deuterated solvents were purchased from Cambridge Isotope Laboratories. All chemicals used for the preparation of buffers were of analytical quality and were dissolved in ultrapure (Milli-Q) water.

The synthesis of compounds **1**–**3** and their precursors, as well as their characterization by nuclear magnetic resonance (NMR), mass spectrometry (MS) and UV–vis spectroscopy, is described in the Supplementary Material (sections S1 and S2). The encapsulation of the studied micellar systems into CCMV PCNs is described in the first two paragraphs of the Results and discussion section.

The purification of CCMV, removal of RNA and isolation of the CP were carried out according to literature procedures [13]. The purity of the viral CP was characterized by fast protein liquid chromatography (FPLC; elution volume of CP = 2.3 mL) and UV–vis spectroscopy, using the following buffers: 50 mM sodium acetate, 1 M sodium chloride and 1 mM sodium azide (pH 5). The FPLC was equipped with a Superose 6 size exclusion column 10/100 GL (GE Healthcare) with a bed volume of 2.4 mL. For each run, the injection volume was 100 μL. The capsid concentration in each sample used for further elemental analysis was determined by measuring its UV absorbance at 280 nm, using a $\varepsilon_{CP} = 24080\ M^{-1}\ cm^{-1}$.

2.1. Total reflection X-ray fluorescence (TXRF)

Total reflection X-ray fluorescence (TXRF) measurements were performed by a Bruker S2 Picofox TXRF (Bruker AXS GmbH, Karlsruhe, Germany) portable system. The primary X-rays were generated by an air-cooled low power X-ray tube using a molybdenum anode. The excitation settings were 50 kV and 750 mA. Measurements were performed by signal integration over 1500 s. Quartz glass disks were used as sample carriers, which were previously cleaned in 1 N nitric acid followed by flushing in water for 1 h at 80 °C. After that, they were dried, coated with silicon solution for better wettability and dried again. Samples for TXRF analysis were prepared by adding equal amounts of the gallium internal standard (10 mg/L) and the sample to be analyzed (volume). After vortexing, a drop of the solution was placed on the quartz disk and dried under vacuum for 15 min. For control purposes, elemental analysis of the empty capsid (in buffer) was conducted and the obtained values for each sample were corrected. The quantification is performed by the Bruker S2 Picofox TXRF software (Spectra, Version 6.1.5.0), based on the known quantity of the gallium internal standard added.

2.2. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) images were obtained using analytical TEM FEI instruments. In order to prepare the samples, 5 μL of the desired sample was applied onto Formvar-carbon coated grids (from Electron Microscopy Sciences). After leaving the sample for 1 min, the excess of liquid was removed using a piece of filter paper. Uranyl acetate (5 μL, 1% w/v) was then applied and the drying procedure was repeated. The particle size distribution of the newly formed assemblies (after purification by FPLC) was determined by analyzing over 200 particles observed in TEM images.

2.3. MRI experiments

MRI experiments were carried out on a 14.1 T (600 MHz) Avance II NMR Spectrometer from Bruker (Karlsruhe, Germany), equipped with a vertical narrow bore magnet (14.1 T), a great B_0 compensation unit (BGU-II) and 3 great 1/60 amplifier units (X, Y and Z) also from Bruker.

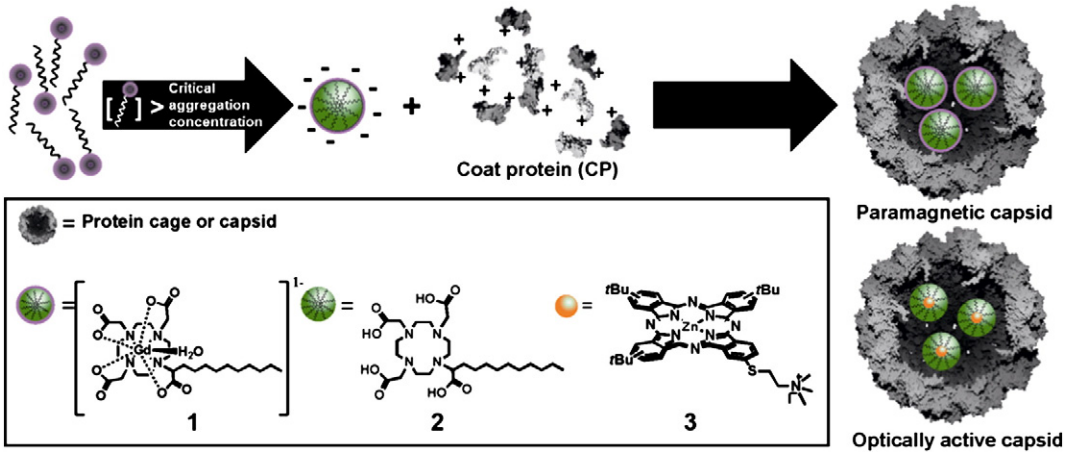

Fig. 1. Schematic representation of the 'self-assembly triggered by self-assembly' process (top row): micelles are formed when the monomer concentration is above the critical aggregation concentration (cac) value. The resulting negatively charged micelles act as 'nucleation points' for the self-assembly of CCMV coat proteins (CPs), yielding stable paramagnetic protein cage nanoparticles (PCNs). Legend: structure of Gd-DOTAC10 (**1**), DOTAC10 (**2**) and the amphiphilic ZnPc dye (**3**). For the synthesis of optically active PCNs (bottom row, right), the same process is followed but using DOTAC10/ZnPc micelles as templates for the CP self-assembly. The ZnPc localization within micelles (depicted by yellow spheres) is only schematic, and multiple site-isolated dye molecules can actually be encapsulated in each micelle.