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Protein nanocages for self-triggered nuclear delivery of DNA-targeted chemotherapeutics in Cancer Cells

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ABSTRACT

A genetically engineered apoferritin variant consisting of 24 heavy-chain subunits (HFn) was produced to achieve a cumulative delivery of an antitumor drug, which exerts its cytotoxic action by targeting the DNA at the nucleus of human cancer cells with subcellular precision. The rationale of our approach is based on exploiting the natural arsenal of defense of cancer cells to stimulate them to recruit large amounts of HFn nanoparticles loaded with doxorubicin inside their nucleus in response to a DNA damage, which leads to a programmed cell death. After demonstrating the selectivity of HFn for representative cancer cells compared to healthy fibroblasts, doxorubicin-loaded HFn was used to treat the cancer cells. The results from confocal microscopy and DNA damage assays proved that loading of doxorubicin in HFn nanoparticles increased the nuclear delivery of the drug, thus enhancing doxorubicin efficacy. Doxorubicin-loaded HFn acts as a "Trojan Horse": HFn was internalized in cancer cells faster and more efficiently compared to free doxorubicin, then promptly translocated into the nucleus following the DNA damage caused by the partial release in the cytoplasm of encapsulated doxorubicin. This self-triggered translocation mechanism allowed the drug to be directly released in the nuclear compartment, where it exerted its toxic action. This approach was reliable and straightforward providing an antiproliferative effect with high reproducibility. The particular self-assembling nature of HFn nanocage makes it a versatile and tunable nanovector for a broad range of molecules suitable both for detection and treatment of cancer cells.

1. Introduction

Cancer is the third cause of mortality in the world and the global burden of cancer continues to increase [1]. The primary option for the treatment of most solid tumors is surgery, followed by adjuvant chemotherapy to prevent the onset of metastasis. However, in recurrent cancer, the first clinical approach is chemotherapy [2–4]. The main advantage of chemotherapy resides in the systemic action towards both primary and metastatic tumors. However, non-selective activity causes severe side effects that strongly affect the therapeutic outcomes.

Doxorubicin (DOX) is one of the most widely used chemotherapeutics in the treatment of solid tumors, although the development of resistance and the occurrence of severe side effects, including cardiotoxicity and myelosuppression, caused by high dosages, limits its efficacy in the clinical practice [5]. DOX presents chemical suboptimal characteristics, including poor solubility and easy metabolism to doxorubicinol [6], while the entry of DOX into cancer cells basically occurs by diffusion. However, even at low concentrations, the process reaches saturation, which drastically limits the uptake of the compound [7]. In addition, DOX is subjected to the effect of multi-drug resistance mechanisms (MDR) that remove the drug from the cytoplasm, preventing it to exert its cytotoxic action [7]. Therefore, the increase of DOX therapeutic index is of utmost importance in cancer research. Recent effort has led to novel DOX delivery strategies, including the use of liposomes or inorganic nanoparticles with the aim to reduce the drug-related toxicity and to escape from MDR mechanisms [8–10].

Apoferritin nanoshells have been proposed to be a promising and versatile solution [11]. Ferritins are a family of iron storage proteins composed of a regular assembly of 24 subunits to form a spherical cage architecture with an external size of ~12 nm [12,13]. Mammalian ferritins consist of a mixture of two different types of self-assembling subunits known as H (heavy) and L (light) chain [14]. H chain includes a catalytic ferroxidase site, which catalyzes the oxidation of Fe(II) to Fe(III), while L chain plays a role in the iron nucleation process. Ferritin inner cavity has a diameter of 8 nm to enclose a core of hydrated iron oxide, which can contain up to 4000 iron atoms [12]. This cavity has been exploited

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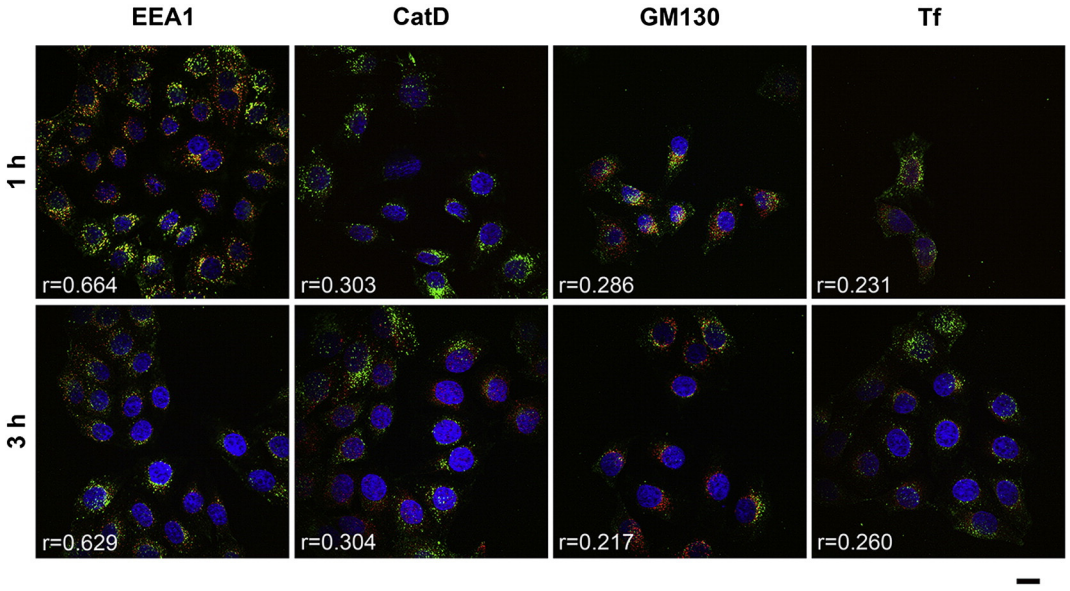

Fig. 1. Intracellular localization of HFn nanoparticles. Confocal microscopy merges images of HeLa cells, incubated for 1 h or 3 h at 37 °C with 0.1 mg mL^{-1} of HFn (green). Early endosomes, lysosomes, Golgi and recycling endosomes were recognized with early endosome marker EEA1, lysosomal protein CatD, Golgi marker GM130 and recycling endosome marker Tf antibodies, respectively, and labeled with an anti-mouse secondary antibody conjugated with Alexa Fluor 546 (red). Nuclei were stained with DAPI (blue). Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in nanomaterial chemistry as a reaction chamber for the template synthesis of nanoparticles with a well-defined size and shape and a narrow size distribution [14–19]. Moreover, since the subunits can be disassembled at acidic pH and reassembled by bringing back the pH to neutrality in a shape-memory fashion, apoferritin can be exploited for the encapsulation of various organic molecules thus representing an interesting scaffold for the development of a biocompatible drug delivery system [20]. Ferritin is specifically cross-recognized in humans by the receptor of transferrin 1 (TfR1), which is found overexpressed in many types of tumor cells but not in normal cells and healthy tissues [21]. Recently, an RGD-modified apoferritin cage was demonstrated to improve the delivery of DOX in the cytoplasm of a glioblastoma cell line [11].

Ferritin plays a key role in the oxidative metabolism by converting Fe^{2+}, which is a source of toxic reactive oxygen species (ROS), into inoffensive Fe^{3+} [12]. This protective mechanism is particularly important in the nucleus, where it is needed to shield DNA from iron-induced oxidative damages [22]. In eukaryotic cells, the nuclear pore complex is responsible for translocation of molecules into the nucleus, partly by passive diffusion provided that the molecular size is small enough (≤40 kDa) [23]. However, larger molecules, including proteins, can be efficiently transported through the involvement of signal- and energy-dependent pathways, usually exploiting "nuclear localization signals" (NLS) [24]. These may include a peptide sequence that can bind to importin β, which in turn binds to the nuclear pore complex [25], or short consensus sequences not involving the interaction with importins [26]. Recent evidence has been provided that ferritin is translocated into the nucleus by an active transport mechanism [22,27]. Available data suggest that the H subunit is involved in nuclear translocation mechanisms, which, however, occurs without any NLS involvement [28]. It has been observed that if as low as 15% of the monomeric H-ferritin is deleted or replaced, nuclear translocation is inhibited and ferritin is confined in the cytoplasm [29]. Although the size of monomeric H-ferritin (21 kDa) allows passive diffusion into the nucleus [30], the efficiency of translocation suggests the involvement of an active mechanism, in which the import of an integral ferritin cage cannot be ruled out despite its molecular weight (~450 kDa). Indeed, macromolecules with a diameter of up to 39 nm are capable to penetrate the nucleus *via* an active signal-mediated transport [31].

Based on the above considerations, we reasoned that a 24-H subunit variant of apoferritin (HFn) would facilitate the cumulative delivery of encapsulated DOX directly inside the nucleus, potentially reducing DOX dosages and mitigating MDR effects. Therefore, we produced the monomeric H subunit by recombinant engineering, which proved valuable in self-assembling in apoferritin-like nanocages. We envisaged that HFn could be a good candidate nanocarrier specific for nuclear delivery of chemotherapeutics, as: 1) HFn could be easily loaded with a broad range of drugs, including DOX; 2) HFn was expected to sensitively and selectively recognize tumor cells exploiting the binding with TfR1; 3) H subunits were found in monomeric form in the nucleus [29], suggesting a disassembly mechanism inside or in close proximity of the nucleus, which would allow the drug to be intranuclearly released; 4) as cancer cells exhibit greater ROS stress than normal cells do [32–34], nuclear translocation of HFn should be also favored in cancer cells in response to oxidative stimuli; and 5) DOX could be passively released out of HFn shell inducing a DNA damage, which, in turn, could further trigger HFn nuclear translocation.

The intent of this work was to investigate the interaction of HFn with a model TfR1-positive cancer cell line, to assess the increased cytotoxic efficacy of DOX incorporated in HFn, to study DOX release in cancer cells and to demonstrate enhanced and self-triggered nuclear delivery.

2. Materials and methods

2.1. HFn nanocage design

The cDNA encoding for the heavy chain of human ferritin, modified by inserting the restriction sites for *NdeI* and *NotI* (respectively in 5′ and 3′), was synthesized by Eurofins MWG Operon and subcloned into the vector pET30b(+) from Eurofins MWG Operon to express the HFn under the control of a T7 promoter, as reported in Figure S1b (Supporting Information). The resulting plasmid pET30b/HFn was used to transform *Escherichia coli* expression strain BL21(DE3) by heat-shock method. The recombinant expression vector was confirmed by restriction endonuclease digestion and DNA sequencing.

2.2. HFn expression in E. coli and purification

BL21(DE3)/pET30b/HFn cells were grown at 37 °C in Luria Bertani kanamicin medium until OD_{600nm} = 0.6 and induced with 0.5 mM isopropyl β-D-1-tiogalactopiranoside (IPTG) for 2 h and 30 min. Then,