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High hydrostatic pressure encapsulation of doxorubicin in ferritin nanocages with enhanced efficiency

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ABSTRACT

Human ferritin (HFn) nanocaging is becoming an appealing platform for anticancer drugs delivery. However, protein aggregation always occurs during the encapsulation process, resulting in low production efficiency. A new approach using high hydrostatic pressure (HHP) was explored in this study to overcome the problem of loading doxorubicin (DOX) in HFn. At the pressure of 500 MPa and pH 5.5, DOX molecules were found to be encapsulated into HFn. Meanwhile, combining it with an additive of 20 mM arginine completely inhibited precipitation and aggregation, resulting in highly monodispersed nanoparticles with almost 100% protein recovery. Furthermore, stepwise decompression and incubation of the complex in atmospheric pressure at pH 7.4 for another period could further increase the DOX encapsulation ratio. The HFn-DOX nanoparticles (NPs) showed similar morphology and structural features to the hollow cage and no notable drug leakage occurred for HFn-DOX NPs when stored at 4 °C and pH 7.4 for two weeks. HFn-DOX NPs prepared through HHP also showed significant cytotoxicity *in vitro* and higher antitumor bioactivity *in vivo* than naked DOX. Moreover, This HHP encapsulation strategy could economize on DOX that was greatly wasted during the conventional preparation process simply through a desalting column. These results indicated that HHP could offer a feasible approach with high efficiency for the production of HFn-DOX NPs.

1. Introduction

Protein cages have attracted growing interest for targeted delivery and controlled release of therapeutics due to the nanoscale size and capacity of drug encapsulation (Ghisaidoobe and Chung, 2015; Putri et al., 2015). Ferritin is a highly symmetrical multimeric protein cage consisting of 24 subunits, with external and internal diameters of 12 and 8 nm, respectively (Fan et al., 2013; Jutz et al., 2015). Additionally, ferritin shows great solubility and stability (Heger et al., 2014). Different from other protein cages, ferritin can actively target tumor tissues where the ferritin receptor is overexpressed (Li et al., 2010). Doxorubicin (DOX), cis-platinum, 5-fluorouracil, daunorubicin, and atropine have been reported to be loaded in recombinant human ferritin and successfully delivered to neoplastic tissue and organs (Heger et al., 2014; Lei et al., 2016). These HFn-drug NPs are demonstrated as having antineoplastic properties in human sarcoma, pancreatic cancer, glioblastoma, melanoma, and colon cancer. Moreover, they are compared favourably with the naked species (Falvo et al., 2016; Lei et al., 2016; Liang et al., 2014).

Encapsulating drugs into HFn is the first step to developing this smart nanoparticle as a clinically used anticancer agent. An efficient encapsulation method should allow both high drug loading ratio and high recovery for ferritin, which would decrease the amount of HFn in a single dose when administered in clinic settings and benefit the practical production. Until now, three encapsulation methods have been developed. The pH-based method was first utilized for encapsulation of anticancer drugs in HFn (Simsek and Kilic, 2005). The HFn cage is disassembled at extreme acidity of pH 2.5 and then reassembled at elevated pH in the presence of drugs, allowing them to be encapsulated during reassembling process. This pH-based method loads approximately 20–30 DOX molecules in one HFn cage generally with a low protein recovery (Bellini et al., 2014; Blazkova et al., 2013). Gradually

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changing the pH value from acid to neutral could improve the recovery to about 50% (Kilic et al., 2012), but the severe acidic condition used to disrupt the cage structure would irreversibly damage the protein's partial conformation, resulting in adverse effects for stability (Kim et al., 2011).

There are pores that exist on the shell of HFn, allowing the entry or release of metal ions to regulate them at normal levels in the body (Ebrahimi et al., 2015; Fan et al., 2011; Theil et al., 2008). Therefore, using metal ions to carry DOX into the cage interior through these pores is another loading method for preparation of HFn-DOX NPs (Zhen et al., 2013). Metal ions could chelate DOX by binding to the carbonyl and phenolate oxygen at neutral pH conditions (Beraldo et al., 1985; Feng et al., 2000). The complexes of metal ions and DOX are then transferred to the HFn cavity by interaction with metal binding sites (Lawson et al., 1991; Watt et al., 1988). However, even at low HFn concentrations, HFn molecules are prone to precipitate during the metal ion-based loading process. Recently, a urea-based method has been developed for encapsulation of DOX into HFn by mixing the two components in the presence of 8 M urea firstly. The mixture is then dialyzed against gradient concentrations of urea buffer containing DOX (Lei et al., 2016; Liang et al., 2014). For this method, although as high as 33 DOX molecules could be loaded in one HFn cage, protein precipitation still cannot be avoided; moreover, there is a great waste of DOX during the dialysis process.

Nowadays, a modified HFn was reported by genetically fused with PAS (proline, alanine, and serine) polymers to cover on the surface of the native cage with the aim to improve its circulation time in body. After such modification, the PAS-containing constructs encapsulated over twice as many doxorubicin molecules as HFn and achieved more than 90% recovery of protein (Falvo et al., 2016; Fracasso et al., 2016). However, the extra surface modification might destroy the tumor-specific binding of natural ferritin and disturb the biocompatibility and *in vivo* performance because of the altered surface physicochemical properties of ferritin (Li et al., 2012; Liang et al., 2014; Lin et al., 2011). From this perspective, the natural HFn should still not be neglected when developing the protein-nanocages based anticancer therapeutics.

High hydrostatic pressure (HHP) is a newly developed technique that could reversibly change protein conformation in the scope of 200–300 MPa. It could impair the inter-molecular and intra-molecular hydrophobic interactions and increase protein solubility by the hydration of those buried hydrophobic residues (Crisman and Randolph, 2008; Nucci et al., 2014; Roche et al., 2012). Recently, HHP has been used to refold proteins from inclusion bodies or aggregates, which could achieve a relative high yield compared with other traditional methods (Rodrigues et al., 2014; Seefeldt et al., 2009). HHP could also be used to inactivate viruses by reversibly disrupting interactions between assembling units. Picornavirus is inactivated at 200–300 MPa by loosening the subunits and releasing the VP4 outside the capsid (Goncalves et al., 2007; Oliveira et al., 1999). Many viruses could be fully inactivated by HHP without drastic structural changes of the capsid, as demonstrated by spectroscopic measurements and electron microscopy analysis (Dumard et al., 2013; Ishimaru et al., 2004; Lou et al., 2015).

In this study, HHP was exploited for encapsulation of DOX in HFn instead of using pH or urea to disassemble the protein cage. It was found that DOX could be loaded in HFn cages at certain pH values during the HHP process. Concentrations of DOX and the additives NaCl, urea, and arginine, as well as the operation protocols after pressurization all had great effects on the encapsulation efficiency. At the optimized conditions, a high DOX loading ratio was achieved and most importantly, almost 100% protein could be recovered without any soluble aggregates. The HFn-DOX prepared through HHP showed comparable stability and anticancer bioactivity with counterparts prepared through other methods. In the end, the mechanism for the encapsulation through HHP is also proposed.

2. Materials and methods

2.1. Materials and equipment

Recombinant human ferritin was provided by the Institute of Biophysics, Chinese Academy of Sciences. Doxorubicin was supplied by Meilun (Dalian, China). All the other reagents were analytical grade and homemade.

HPP.L3 600/0.6 high-pressure equipment (HuaTaiSenMiao, Tianjin, China) was utilized for all the encapsulation experiments, which could provide pressure up to 600 MPa with an effective volume of 600 mL.

2.2. Preparation of HFn-DOX NPs through HHP

The stock solution of doxorubicin (20 mg/mL) was prepared using double distilled water. Encapsulation was carried out according to the following procedures. Briefly, HFn of 1.5 mg/mL was mixed well with DOX (0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 mg/mL) in 1 mL buffer with different pH levels and additives. Subsequently, the solution was placed in disposable syringes and pressurized (150–500 MPa) for 16 h at room temperature using HPP.L3 600/0.6 high-pressure equipment. After decompression, the solution was centrifuged at 12,000 rpm for 15 min and the supernatants were loaded on a Sephadex G25 desalt column (16 mm × 25 mm, GE Healthcare, USA) pre-equilibrated with 200 mM PB (pH 7.4) and connected to a AKTA Purifier 100 (GE Healthcare, USA). HFn-DOX fractions were collected and subjected to the concentration analysis of protein and DOX to determine the protein recovery and drug loading ratio.

Four HHP strategies with different post-treatment protocols were operated as follows: (1) 500 MPa for 16 h and direct decompression; (2) 500 MPa for 16 h followed by stepwise decompression with a grade of 50 MPa every 10 min; (3) 500 MPa for 16 h, direct decompression to atmospheric pressure, and incubation with DOX at pH 5.5 for another 16 h; and (4) 500 MPa for 16 h and direct decompression, removing the excess DOX, exchanging the pH to 7.4 through desalting, dialyzing HFn-DOX with 20 mM PB buffer at pH 7.4 with 5 μg/mL of DOX at 4 °C for 16 h (molecular weight cut-off 3000 Da; Thermo Scientific, USA) followed by dialyzing against water overnight to remove the free DOX. Eventually, the HFn-DOX NPs were pooled for further analysis.

2.3. Determination of the DOX loading ratio

The DOX loading ratio was calculated according to the absorbance of HFn-DOX at 280 and 480 nm by using an Ultrospec 2100 pro UV/Visible spectrophotometer (GE Healthcare, USA). The DOX concentration (C_{DOX}) and the concentration of HFn (C_{HFn}) were determined by measuring absorbance at 480 nm and 280 nm separately (Eqs. (1) and (2)). Standard linear curves of DOX and HFn were determined by serial concentrations of DOX (1–40 μg/mL) and HFn (0.1-1.2 mg/mL). The loading ratio (N) was calculated according to Eq. (3). The molecular weights of HFn and DOX are 450 kDa and 543.5 Da, respectively.

$$A_{280}(total) = A_{280}(HFn) + A_{280}(DOX) \quad (1)$$

$$A_{480}(total) = A_{480}(DOX) \quad (2)$$

$$N = \frac{C_{DOX} \times M_{HFn}}{C_{HFn} \times M_{DOX}} \quad (3)$$

2.4. Drug leakage study of HFn-DOX NPs

The leakage of DOX from HFn-DOX NPs was evaluated in buffers of pH 7.4, 5.5, and 3.5 at 4 °C. A Sephadex G25 desalt column (16 mm × 25 mm, GE Healthcare, USA) was utilized to remove the leaked DOX from the 500 μL sample in 20 mM PB at pH 7.4, and the remaining DOX was calculated by the method mentioned above to