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Engineered drug-protein nanoparticle complexes for folate receptor targeting

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ABSTRACT

Nanomaterials that are used in therapeutic applications need a high degree of uniformity and functionality which can be difficult to attain. One strategy for fabrication is to utilize the biological precision afforded by recombinant synthesis. Through protein engineering, we have produced ~27-nm dodecahedral protein nanoparticles using the thermostable E2 subunit of pyruvate dehydrogenase as a scaffold and added optical imaging, drug delivery, and tumor targeting capabilities. Cysteines in the internal cavity of the engineered caged protein scaffold (E2 variant D381C) were conjugated with maleimide-bearing Alexa Fluor 532 (AF532) and doxorubicin (DOX). The external surface was functionalized with polyethylene glycol (PEG) alone or with the tumor-targeting ligand folic acid (FA) through a PEG linker. The resulting bi-functional nanoparticles remained intact and correctly assembled. The uptake of FA-displaying nanoparticles (D381C-AF532-PEG-FA) by cells overexpressing the folate receptor was approximately six times greater than of non-targeting nanoparticles (D381C-AF532-PEG) and was confirmed to be FA-specific. Nanoparticles containing DOX were all cytotoxic in the low micromolar range. To our knowledge, this work is the first time that acid-labile drug release and folate receptor targeting have been simultaneously integrated onto recombinant protein nanoparticles, and it demonstrates the potential of using biofabrication strategies to generate functional nanomaterials.

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1. Introduction

Functional nanoparticles have promising potential for broad applicability, but many challenges exist in chemically synthesizing materials at such small sizes. In contrast, natural biological systems have been highly successful in this endeavor, and therefore nature-inspired macromolecular architectures can be used as the basis upon which new types of materials are made [1,2]. In this work, we demonstrate that the detailed control which genetic engineering provides in defining the polymeric architecture of proteins can be coupled with synthetic strategies to introduce new multiple functionalities into a nanoparticle scaffold. This approach enables the creation of defined and uniform nanoparticles that are potentially applicable for drug delivery.

Our biomimetic scaffold is a virus-like protein nanoparticle modeled from the E2 subunit of a pyruvate dehydrogenase multienzyme complex. It is composed of 60 identical subunits, contains 12 openings which lead to a hollow internal cavity, has an outer hydrodynamic diameter of ~27 nm, and is stable up to ~80 °C [3,4]. Unlike other protein-based nanoparticles, it is non-viral in origin. Our research has demonstrated that the internal, external, and subunit–subunit interfaces of the E2 protein scaffold can be individually engineered for various capabilities such as drug encapsulation, modulation of immune response, and pH-dependent disassembly, respectively [5–8].

The size of this protein assembly has a decisive relevance for intracellular drug delivery applications. Others have reported that the optimal size range for cellular particle uptake is approximately 25–75 nm [9,10], and particles of this size also accumulate in tumor tissues due to the enhanced permeability and retention effect [11,12]. Tumor targeting can also be actively implemented by attaching ligands to nanoparticles that target tumor-associated receptors. This approach utilizes the over-expression of receptors on the surface of tumor cells relative to normal cells [13,14] and have included folate, transferrin, and integrin binding receptors [13–15].

Of these, the folate receptor (FR) has been often investigated and used. FR is a membrane glycoprotein with a molecular weight of 38–40 kDa, and it is overexpressed in various tumors such as ovarian, brain, lung, and breast cancers [16–19]. The corresponding targeting ligand, folic acid (FA, vitamin B9), is involved in one-carbon metabolic transfer reactions, is critical in nucleotide synthesis and the viability of proliferating cells, and exhibits a high affinity for FR ($K_d \sim 10^{-10}$ M) [17,18]. FA can be coupled to

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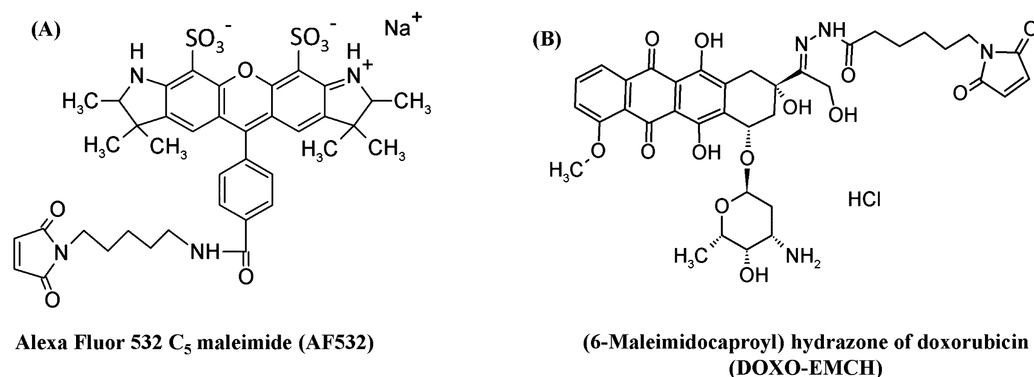

Fig. 1. Chemical structures of (A) Alexa Fluor 532 C_5 maleimide and (B) DOXO-EMCH.

anti-tumor drugs directly [18,20] or to various macromolecular delivery systems, including liposomes, micelles, polymers, or inorganic particles [16,17,21,22].

To our knowledge, genetically engineered protein-based nanoparticles combining the dual functionalities of acid-responsive drug release and FA targeting have not yet been reported. In this work, we fabricate an E2 protein scaffold in a recombinant *Escherichia coli* system and further extend the utility of these nanoparticles by simultaneously incorporating imaging/targeting and drug/targeting abilities through chemical conjugation. The fluorescent molecule Alexa Fluor 532 (AF532) and a derivative of the anti-cancer drug doxorubicin (DOX), the (6-maleimidocaproyl)hydrazone derivative of doxorubicin (DOXO-EMCH), which both contain a thiol-reactive maleimide group (see Fig. 1), were coupled to the internal cavity of the E2 variant D381C [5]. Attachment of FA to the outer nanoparticle surface was achieved through a polyethylene glycol (PEG) linker. PEGylation is a typical strategy to modulate the immune response and increase the nanoparticle circulation time *in vivo* [23], and our prior work showed that PEGylation of the E2 surface significantly decreased the nonspecific cellular uptake [8]. We examine cellular uptake of our functionalized protein-based nanoparticles in cell lines expressing high and low amounts of FR, and we measured the cytotoxicity of these particles in cancer cells. Our results demonstrate the broad potential of utilizing biomimetic structures and biofabrication strategies as viable approaches to extend nanotechnology applications.

2. Materials and methods

2.1. Materials

Sodium chloride (NaCl), sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), sodium phosphate dibasic, sodium phosphate monobasic, *N*,*N*-dimethylformamide (DMF), and magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) were supplied by EMD. Alexa Fluor 532 C_5 maleimide (AF532) and Hoechst 33342 were obtained from Invitrogen. Dimethyl sulfoxide (DMSO) and cysteine·HCl were purchased from Thermo Scientific. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was from Pierce. Tris base, bovine serum albumin, potassium phosphate monobasic, and potassium phosphate dibasic were from Fisher Scientific. A heterobifunctional polyethylene glycol (PEG) linker (2000 Da) with folic acid (FA) and N-hydroxysuccinimide (NHS) at each respective end (FA-PEG-NHS) and a PEG linker (2000 Da) functionalized with maleimide and NHS at each respective end (Mal-PEG-NHS) were obtained from Nanocs. The (6-maleinimidocaproyl) hydrazone derivative of doxorubicin (DOXO-EMCH) was synthesized and characterized as previously described [24] and doxorubicin hydrochloride (DOX) was from Yic-Vic. DOX and DOXO-EMCH were dissolved in 10 mM sodium phosphate (pH 5.8) and used within 30 min. Dulbecco's Modified Eagle's Medium (DMEM), DMEM without folic acid, and CelLytic M cell lysis reagent were purchased from Sigma–Aldrich. PBS was from MP Biomedicals. Fetal bovine serum (FBS), L-glutamine, and penicillin–streptomycin were obtained from Mediatech. Folic acid was from Fisher BioReagents. Non-fat dry milk was from LabScientific. KB, A549, and HeLa cells were obtained from ATCC.

2.2. Protein expression and purification

The E2 protein scaffold consists of 60 identical subunits, with no surface-accessible cysteines on the native wild-type scaffold. To conjugate guest molecules into the hollow cavity of the E2 protein scaffold, the aspartic acid at position 381 of the E2 scaffold was mutated to cysteine, resulting in 60 thiols (one per subunit) in the internal surface of the nanocapsule (mutant designated as D381C) [3]. Guest molecules, such as the fluorescent marker AF532 and anticancer drug doxorubicin, can be conjugated to D381C through these thiols in a fast Michael addition [5].

Detailed experimental procedures for the mutagenesis, expression, and purification of D381C have been reported in previous studies [3,5]. In summary, we performed site-directed mutagenesis, cloned the gene into the expression plasmid, and transformed the plasmid into *E. coli* BL21(DE3). Cells induced to express protein nanoparticles were harvested and lysed. Protein nanoparticles were purified with a fast protein liquid chromatography (FPLC) system (ÄKTA, Amersham Biosciences) using Q Sepharose and Superose 6 PG columns.

2.3. Chemical functionalization of recombinant protein nanoparticles

The nanoparticles that were synthesized for this study and their abbreviations are summarized in Fig. 2. Conjugation of AF532 to D381C (D381C-AF532) was performed following protocols similar to those previously described [5]. Purified protein scaffolds D381C were mixed with AF532 at a ratio of 1 subunit: 2.5 molecules at room temperature for 2 h, followed by 4 °C overnight incubation. Unbound AF532 were removed by desalting columns (Zeba, 40 kDa MWCO, Pierce) following the vendor protocol. Samples were loaded onto columns equilibriated with phosphate buffer and centrifuged. The unbound AF532 was retained in the resin while D381C-AF532 was recovered in the flow-through.

We conjugated polyethylene glycol (PEG) onto the external surface lysines of D381C-AF532. Briefly, FA-PEG-NHS and Mal-PEG-NHS were dissolved in DMSO under argon. D381C-AF532 was then mixed with FA-PEG-NHS or Mal-PEG-NHS at a ratio of 1 subunit: 5 PEG linkers at room temperature for 1 h. Since the internal thiols are

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