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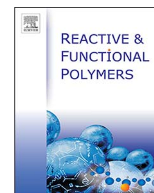

Size-tunable protein–polymer hybrid carrier for cell internalization

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ABSTRACT

We describe a polymer-templated protein nanoball (PTPNB) system as an *in-situ* platform for the preparation of polymer-protein core-shell capsules containing hydrophobic cargos. These structures boast high protein activity through soft immobilization and maintain a consistent protein orientation through a specific interaction between the protein and polymer. We demonstrate the potential of this PTPNB system through bio-conjugation, encapsulation, and size-control of nanoballs in a one-pot process, and furthermore characterize the effects of nanoball size on endocytosis.

1. Introduction

Polymer-protein core-shell hybrid structures are in the limelight as the new generation of nanocarriers due to the biocompatibility of proteins and the versatile functionalities of proteins. Ligand–receptor interactions are particularly important for surface recognition on particular cells [1–5].

Several approaches have been employed to prepare polymer–protein core–shell structures. One approach is the self-assembly of protein-polymer hybrid amphiphiles after conjugation of protein and polymer [6–10]. A second approach conjugates protein directly onto the polymer particles [11–23]. Our group has developed protein-based, carrier-loaded metal nanoparticles by conjugating histidine (His)-tagged green fluorescent protein (GFP) onto nickel-complexed, nitrilotriacetic acid-functionalized polystyrene (Ni^{2+}-NTA-PS) particles [24]. In these approaches, the overall process requires a minimum of two steps: bioconjugation and structure formation. A third approach, discussed herein, is the *in-situ* formation of protein-coated polymeric particles. In this method, bioconjugation and structure formation occur simultaneously in a single step [25–27].

To realize the commercialization of advanced materials based on protein–polymer hybrid structures, we need to overcome the complex production procedures and inherently low functional efficiency of hybrid materials. The key parameters related to functional efficiency are 1) the degree of protein activity preservation, 2) the controllability of protein orientation, 3) the encapsulation ability, and 4) the size-tunability of the hybrid structures. The key to process simplification is to implement a one-step protocol such that all events take place simultaneously. These events include 1) protein–polymer conjugation, 2) core-shell structure formation, 3) cargo encapsulation, 4) size control and 5) protein orientation control.

With the aforementioned parameters in mind, we report a polymer-templated protein nanoball (PTPNB) system as a sophisticated platform to form protein-based functional nanocarriers. This study is the first to describe an *in situ* method for the preparation of cargo-loaded, size-tunable protein–polymer carriers that exploits specific NTA-Ni^{2+}-His interactions between the polymer and protein to control protein orientation. Since NTA-Ni^{2+}-His conjugation is fast, selective, and non-covalent [28], this PTPNB system based on NTA chelation does relatively little damage to proteins and is amenable to a one-pot protocol with protein orientation control through selective binding.

This research builds on previous reports detailing the synthesis of polymer–protein core-shell particles. In the precedent study, a one-pot system was shown to be generally applicable to the various proteins and allowed for the control of particle size [29]. Since this method is relatively unique, it is important to confirm that cargo encapsulation does not negatively impact the functional strength of the nanocarrier system. In most biomedical applications, intracellular uptake depends on the size of the nanocarrier. Therefore, size controllability is important for tuning nanocarrier efficacy [30].

In this study, PTPNBs loaded with hydrophobic cargo were prepared with controlled sizes through a simple, one-step process. Furthermore,

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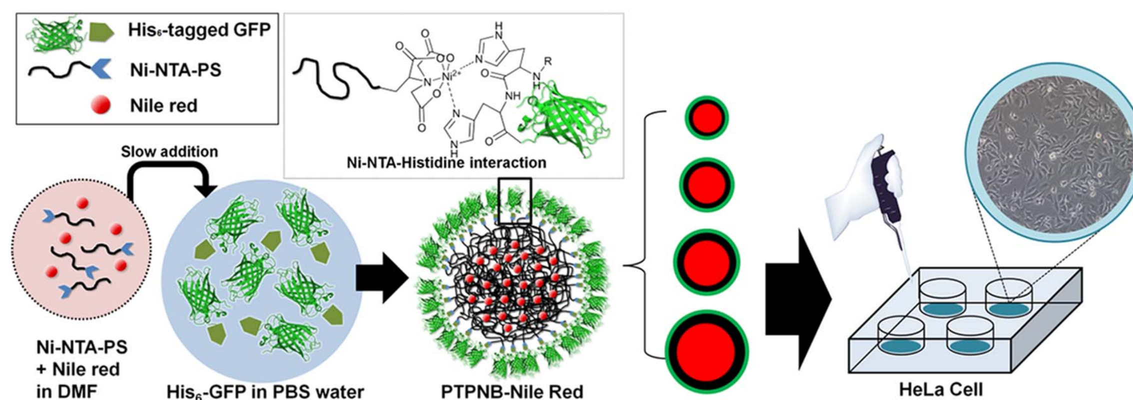

Scheme 1. Preparation of polymer-templated protein nanoballs (PTPNBs) of various size, loaded with Nile Red, and their cellular uptake.

the effects of PTPNB size on internalization was demonstrated with human cervical cancer (HeLa) cells. The results show that this PTPNB system, shown in Scheme 1, is a viable means of preparing functional nanocarriers for cell theranostics. Ni^{2+}-NTA-PS was introduced as a representative hydrophobic polymer, while GFP tagged with 6 × His (His$_6$-GFP) was used to show the existence of a protein coat. Nile Red was chosen as a hydrophobic cargo since the blue shift of its emission spectrum can be monitered in the hydrophobic microenvironment in the particle core [31,32].

2. Experimental section

2.1. Measurements

Transmission electron micrographs (TEM) were obtained on a Hitachi H-7600 instrument (Tokyo, Japan) at 80 kV. TEM samples were prepared by dipping a carbon-coated TEM grid into respective solutions. Dynamic light scattering (DLS) was performed with a 90Plus Particle Size Analyzer from Brookhaven Instruments Corporation (Holtsville, NY, USA). Photoluminescence (PL) spectra were recorded using an Ocean Optics HR4000CG composite-grating spectrophotometer (Dunedin, FL, USA) with excitation and emission wavelengths of 450 and 550 nm, respectively. Fluorescence optical micrographs were obtained on a Nikon TE2000-U microscope (Tokyo, Japan). Nanoparticles were imaged on a Leica TCS SP8 inverted microscope (Wetzlar, Germany). All images were analyzed with Leica software (LAS X) and visualized with a HCX PL APO 100 × objective lens (numerical aperture, 1.40) using a 458-nm argon laser and HyD detector (462–520 nm) for GFP excitation and emission, and a 550-nm argon laser HyD detector (560–700 nm) for Nile Red excitation and emission. The cellular uptake of PTPNB-Nile Red was investigated by confocal microscopy (LSM 710; Carl Zeiss, Oberkochen, Germany).

2.2. Methods

2.2.1. Preparation of PTPNB-Nile Red

Ni^{2+}-NTA-PS (0.1 mg, 1.75×10^{-5} mmol) and Nile Red (0.02 mg, 6.28×10^{-5} mmol) were dissolved in 0.2 mL of dimethylformamide (DMF). The resulting mixture was added at a rate of 0.02 mL/h, using a syringe pump, to 5 mL of phosphate buffer solution (PBS, 10 mM, pH 7.5) containing His$_6$-GFP (0.41 mg, 1.48×10^{-5} mmol) under rapid stirring at 25 °C in a glass vial. After this addition, the mixture was left stirring for 1 day and any unincorporated Nile Red was removed by filtration through a syringe filter (0.45 µm). The resulting particle structure and structural stability were characterized by DLS and TEM measurements. Particle optical properties was investigated by acquiring and evaluating PL spectra and fluorescence optical micrographs.

2.2.2. Size control of PTPNB-Nile Reds

Four stock solutions were prepared by dissolving Ni^{2+}-NTA-PS and Nile Red (0.02 mg, 6.28×10^{-5} mmol) in 0.2 mL of DMF containing different amounts of polymer: 0.025 mg, 0.05 mg, 0.1 mg and 0.2 mg. The remaining synthetic and characterization methods were the same as those described above for PTPNB-Nile Red.

2.2.3. Calculation of encapsulation efficiency

To determine the amount of incorporated Nile Red, a standard solution was prepared by dissolving 0.02 mg Nile Red and 0.1 mg Ni^{2+}-NTA-PS in 5.2 mL DMF. This represents the 100% standard solution and contains the same weight concentration of PTPNB-Nile Red as used in the particle preparation protocol described above. Additional standard solutions of 2–5% were made by diluting the 100% standard. PTPNB-Nile Reds were dissociated by dissolving in 100% DMF after lyophilization. The EE (*i.e.*, amount of loaded dye/amount of injected dye) of each PTPNB-Nile Red sample was determined by comparing the sample emission intensity against a standard curve.

2.2.4. Cellular uptake of PTPNB-Nile Red

To evaluate the cellular uptake and deposition of PTPNB-Nile Red into living cells, HeLa cells (gift from Nano Entek Inc., Seoul, Republic of Korea) were pre-cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (*v/v*) fetal bovine serum (FBS) and 1% (v/v) HyClone™ penicillin-streptomycin (Pen-strep) at 37 °C in a humidified incubator with 5% CO_2. When the confluency of the cells reached 85%, they were sub-cultured to obtain equal cellular activity and seeded into a 96-well opaque plate (1.0×10^4 cells/well) containing 100 µL of cell medium. After culturing for 24 h and removing the supernatant, PTPNB-Nile Red in 100 µL of medium was loaded into the wells of the same 96-well opaque plate and incubated for 1 h to allow for cellular uptake. After uptake, the cells were washed three times with PBS to remove any unabsorbed PTPNB-Nile Red, and fixed with 4% paraformaldehyde for 5 min. Each well was then washed three times with PBS to remove any unused paraformaldehyde. The degree of cellular uptake of PTPNB-Nile Red was investigated by confocal microscopy. The endocytosis of PTPNB was viewed and analyzed using a z-stack procedure, which captured images of the cells at every 2 µm of depth.

3. Results and discussion

3.1. In situ formation of PTPNB loaded with Nile Red (PTPNB-Nile Red)

Ni^{2+}-NTA-PS was synthesized by atom transfer radical polymerization (ATRP) using an NTA-end-functionalized amidic initiator in accordance with a previous report (Scheme S1) [33]. The molecular weight (M_n) and molecular weight distribution (M_w/M_n) of the polymer were 5620 g/mol and 1.28, respectively (Fig. S2). The NTA functionality of the resulting polymer was confirmed by proton nuclear

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