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# An albumin nanocomplex-based endosomal pH-activatable on/off probe system

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ABSTRACT

Albumin has gained considerable interest as a material for fabricating nanoparticulate systems due to its biomedical advantages, such as biocompatibility and chemical functionality. Here, we report a new pH-sensitive albumin nanocomplex prototype with a zinc-imidazole coordination bond. Albumin was conjugated with 1-(3-aminopropyl)imidazole and $mPEG_{10\,kDa}$-NHS, and the resulting albumin conjugate (PBI) was then modified with either Cy5.5 or BHQ-3. The newly formed albumin nanocomplex (C/BQ-PBI Zn NCs: ~116 nm) system was facilely self-assembled around pH 7.4 in the presence of $Zn^{2+}$, but it quickly disassembled in an acidic environment (~pH 5.0). Based on this pH-sensitivity, C/BQ-PBI Zn NCs emitted strong near-infrared fluorescence and released $Zn^{2+}$, turning "off" at pH ~7.4 (e.g., plasma) and "on" at pH ~5.0 (e.g., endo/lysosomes in tumor cells) on account of fluorescence resonance energy transfer. C/BQ-PBI Zn NCs displayed significant cytotoxicity due to an increase in cellular $Zn^{2+}$ in response to endosomal pH (~5.0) in breast cancer MCF-7 cells and lung adenocarcinoma A549 cells. Particularly, confocal laser scanning microscopic images showed a strong fluorescence signal caused by the disassembly of C/BQ-PBI Zn NCs in the endosomal region of MCF-7 cells. Based on these results, we believe that this albumin nanocomplex is an attractive biocompatible tumor targeting probe carrier for the theranostic purpose.


## 1. Introduction

Near-infrared fluorescence (NIRF) imaging is an effective way of recognizing tumor tissues, and a variety of unique NIRF probes have been developed to improve diagnostic effectiveness [1]. Especially, activatable on/off probe systems that respond to micro-environmental triggers significantly augment the intensity of fluorescence emission in tumors than in other tissues [2]. Fluorescence resonance energy transfer (FRET) is the best known mechanism of transferring energy from donor NIR dye (e.g., Cy5.5) to acceptor NIR dark quencher (e.g., black hole quencher: BHQ-3), which enables strong fluorescence emission at the desired site or conditions [3]. Energy transfer efficiency is highly dependent on the distance (generally <10 nm) between the donor and acceptor [4]. Therefore, a delivery system that increases the distance between the two by disassembly, thereby quelling and quenching in response to physiological environment of tumors, is useful for tumor-specific visualization.

Nanoparticulate delivery systems have been viewed as an efficient tool for diagnosis and treatment of malignant tumors because nanoparticles accumulate in the leaky vasculature of tumor tissues via passive tumor targeting due to the enhanced permeability and retention (EPR) effect [5]. Furthermore, nanoparticles with specific ligands can be more effectively internalized into tumor cells by endocytosis. Unlike the normal physiological pH of 7.4, the microenvironmental pH of tumors can be as low as ~6.8, and that of endo/lysosomes can be ~5.0 [2]. Hence, pH-sensitive nanoparticles that are stable at normal pH but unstable at endo/lysosomal pH of tumors can be used as a specific visualization tool via FRET imaging.

Albumin has many advantages as a protein carrier because it is physicochemically stable, biodegradable, biocompatible, and

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non-immunogenic (and so safe) in humans. It shows good accumulation in malignant or inflamed tissues [6,7]. Furthermore, its surface functional groups, such as amines and carboxylates, can be modified with many targeting ligands for active tumor targeting. A paclitaxel-loaded albumin-based nanoparticle formulation (Abraxane®) was approved by the Food and Drug Administration (FDA) in 2005 for treatment of metastatic breast cancers [8]. Therefore, albumin is considered as one of the most attractive materials for preparing nanoparticles for the purpose of clinical application.

Imidazole ($pK_a$: ~6.8) is sensitive to the pH of tumor microenvironment because the unsaturated nitrogen is protonated under acidic conditions [2,9]. This property has enabled the use of pH-dependent ionization in the tumor-targeting micelle system. Imidazole can also form a zinc ion coordination bond. Thus, imidazole molecules can bind together in the presence of $Zn^{2+}$ by chelation. The bond strength is sufficient to allow formation of histidine-based polyethylene glycol (PEG) hydrogels [10].

Zinc exerts significant antitumor activity via apoptosis and necrosis [11–13]. Many studies have shown that zinc ion selectively kills tumor cells with little or no toxicity to normal cells. Especially, the cytotoxicity of zinc involves the generation of reactive oxygen species. Previous studies have clearly shown that intracellular hypocalcemia can induce overproduction of ROS (reactive oxygen species) because $Ca^{2+}$ activates various enzymes (e.g., proteases, dehydrogenases, and hydrolytic enzymes) that generate ROS. Zinc increases intracellular $Ca^{2+}$, and thus, it induces overproduction of ROS. A study has also demonstrated that the accumulation of ROS disrupts redox homeostasis. Overproduction of ROS can induce ER stress and DNA damage that results in activation of p53, which is part of the apoptosis machinery of tumor cells [14]. Using this strategy, zinc oxide (ZnO)-based nanoparticles have exhibited meaningful cytotoxicity in many cancer cell lines.

Herein, we report a zinc-coordinated pH-sensitive nanocomplex (hereafter termed as NCs) that consists of two types of PEG-bovine serum albumin (BSA)-imidazole (PBI) modified with either Cy5.5 or BHQ-3, which is capable of turning "off" at pH 7.4 (e.g., blood circulation) due to self-assembly, and "on" at pH 5.0 (e.g., endo/lysosomes in tumor cells) due to disassembly, emitting a strong NIRF (Fig. 1).

## 2. Materials and methods

### 2.1. Materials

BHQ-3 succinimidyl ester (BHQ-3-NHS) and methoxy-polyethylene glycol *N*-hydroxysuccinimide (mPEG-NHS, Mw 2, 5, and 10 kDa) were purchased from Biosearch Technologies (Petaluma, CA, USA) and NOF Corporation (Tokyo, Japan), respectively. Bovine serum albumin (BSA) and Cy5.5 NHS ester dye (Cy5.5-NHS) were purchased from Sigma Aldrich (St. Louis, MO, USA) and GE Healthcare (Piscataway, NJ, USA), respectively. LysoTracker® Green DND-26 was purchased from Molecular Probes, Inc. (Eugene, OR, USA). DMEM and fetal bovine serum (FBS) were purchased from Capricorn (Ebsdorfergrund, Hesse, Germany). Trypsin and penicillin were purchased from Corning (Corning, NY, USA). All other reagents were obtained from Sigma-Aldrich unless otherwise specified.

### 2.2. Synthesis of imidazole-modified BSA (BI)

A portion (100 ml) of BSA (1 g) dissolved in 100 mM MES buffer (pH 4.75) containing 1-(3-aminopropyl)imidazole (1 g) was added to *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (3.067 g). Especially, an excessive amount of this imidazole derivative was added to the BSA solution in order to prevent self-aggregation of BSA molecules. The reaction was allowed to continue while stirring for 6 h at room temperature and it was stopped by adding 4 ml of 4 M acetate buffer (pH 4.75). The resulting solution was concentrated by using an ultrafiltration concentrator with a molecular weight cut-off of 30 kDa (Millipore, Beverly, MA, USA) and washed five times with deionized water (DW). The resulting solution was lyophilized and stored at −20 °C until required.

### 2.3. Preparation of PEGylated BI (PBI) and PBI Zn nanocomplex (PBI Zn NCs)

BI (10 mg) was dissolved in 5 ml of 10 mM PBS buffer (pH 8.0) and mixed with different equivalent amounts (1, 2, and 4) of mPEG-NHS (Mw 2, 5, and 10 kDa) versus BI for 6 h at room temperature. Unreacted PEG was removed by ultrafiltration with the same buffer. $ZnSO_4$ (10 mg) was added to each 5 ml of PBI solution (2 mg/ml), and the final pH and $ZnSO_4$ concentration of the solutions were adjusted to 8.0 and 0.1% (w/v), respectively, at a 1 mg/ml concentration of PBI Zn NCs. The desired Mw and optimal ratio of mPEG-NHS were determined according to the size and turbidity of the newly prepared PBI Zn NCs.

### 2.4. Matrix assisted laser desorption/Ionization-time of flight (MALDI-TOF) mass spectrometry

To evaluate molecular masses, the mass spectra for BSA, BI, and PBI (4 eq. vs. BI, Mw 2, 5, and 10 kDa) were obtained using an UltrafleXtreme MALDI-TOF spectrometer (Bruker, Boston, MA, USA) with a Perpetual™ ion. Spectra were recorded in a linear mode with an acceleration voltage of 25 kV. Protein samples were dissolved in 10 mM PBS (pH 7.4) at a concentration of 1 mg/ml. As a matrix, a saturated solution of sinapinic acid in 0.1% trifluoroacetic acid in water–acetonitrile (1:1) was used. Protein and matrix solutions were mixed at a 1:1 volume ratio, 1 μl of the sample mixture was added to the target plate and the spots were dried at room temperature to allow sample crystallization prior to insertion into the instrument.

### 2.5. Determination of the optimal concentration of $ZnSO_4$

PBI Zn NCs was prepared according to the protocol mentioned above (Section 2.3). To determine the optimal concentration of $ZnSO_4$, various $ZnSO_4$ concentrations ($ZnSO_4$; 0.025, 0.05, 0.1 and 0.2% w/v) of PBI Zn NCs were prepared. The optimal concentration of $ZnSO_4$ was determined according to the size and turbidity of the newly prepared PBI Zn NCs.

### 2.6. Preparation of Cy5.5 and BHQ-3 conjugated PBI Zn NCs (C/BQ-PBI Zn NCs)

PBI (1 mg; 4 eq. Mw 10 kDa) was covalently modified with Cy5.5-NHS (4 μg, 0.5 ml) and BHQ-3-NHS (9 μg, 1.5 ml) in 10 mM PBS buffer (pH 8.0), respectively. Cy5.5-modified PBI and BHQ-3-modified PBI were mixed at a 0.1% $ZnSO_4$ concentration and at an adjusted pH of 8.0.

### 2.7. Characterization of PBI Zn NCs

Mean particle size and zeta potential of PBI Zn NCs were measured by dynamic light scattering (Nano ZS90, Malvern, Worcestershire, UK) with a 90° scattering angle for optimum detection at a concentration of 2 mg/ml. Mean particle size of PBI Zn NCs was measured at different pHs (5.0, 5.5, 6.0, 6.5, 7.0, and 7.4) in 10 mM PBS buffer. Zeta potentials were measured at pHs of 5.0, 6.5, and 7.4 in DW. Also, the turbidity of PBI Zn NCs (1 mg/ml) was measured by using a Genequant 100 spectrophotometer (GE