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A water-soluble and biocompatible polymeric nanolabel based on naphthalimide grafted poly(acrylic acid) for the two-photon fluorescence imaging of living cells and *C. elegans*

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ABSTRACT

Polymeric nanoparticles (NPs) with two-photon (TP) activity were prepared by grafting a naphthalimide fluorophore onto poly(acrylic acid) to yield an amphiphilic polymer, which self-assembled in water. The NPs were characterized using various analytical techniques such as transmission electron microscopy, dynamic light scattering and spectroscopic measurements. The *in vitro* and *in vivo* biocompatibilities of the NPs were assessed by a cytotoxicity assay using HeLa cells and a feeding assay using *Caenorhabditis elegans* (*C. elegans*) as a small animal model, respectively. Finally, TP fluorescence imaging (FI) of living cells and *C. elegans* labelled with the NPs were observed by TP confocal microscopy. The experimental outcomes demonstrated that the NPs had sufficient water-dispersity and biocompatibility, had TP fluorescence activity, were resistant to pH variation and illumination, and were physically stable. TP FI revealed that the NPs could enter living cells and were primarily located in the cytoplasm. In addition, the NPs were ingested by *C. elegans* during the feeding process and were recognized and taken up by the active transport system of the intestinal cells. These findings indicated the feasibility of using the developed NPs as a nanolabel for TP FI. Moreover, with numerous modifiable carboxyl groups on its surface, the NPs could act as a platform to build multifunctional probes for potential applications in biosensing and assay labeling.

1. Introduction

Fluorescence imaging (FI) is a method often used in biomedical science because it allows for noninvasive observation of living cells and organisms with high resolution and sensitivity. Fluorescence labels are at the core of FI. Small molecule organic dyes are mostly used as fluorescence labels [1–3], but their applications have often been hindered by their drawbacks, such as poor biocompatibility, chemical instability, rapid photobleaching, sensitivity to pH and so on. Embedding organic dyes into nanoparticles (NPs) has proven to be an efficient method to overcome the limitations of organic dyes, and it provides a broad platform for the design of multifunctional structures [4–6]. Thus, dye-loaded NPs have attracted significant attention for use in FI during the past several years. Silica NPs have been extensively adopted for the loading of fluorescence dyes [7,8]. Investigations have indicated that additional surface coatings are usually needed for these carriers to increase the label's water-solubility and photostability. In comparison to silica NPs, organic polymeric NPs are more versatile. Using various polymer materials and formulation processes allows for the tunability of the physicochemical and physiological properties of NPs. Currently, polymeric NPs play important roles in drug delivery systems [9–13], and they also display great potential for the encapsulation of dyes, which would result in nanolabels with promising and viable applications [14–18].

At present, dye-loaded polymeric fluorescence NPs (PFNPs) are commonly fabricated via the noncovalent encapsulation of dyes into a polymeric nanomatrix. However, their stabilities are challenged by dye leakage, which is unfavorable for their application as nanolabels. In addition, the reported PFNPs were mainly applied for conventional one-photon FI, and only a few of them can be cited as examples of nanolabels for two-photon (TP) FI to date [22–24]. Compared to one-photon FI, TP FI is more attractive because of the longer wavelength excitation (690–1000 nm), which results

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in minimal phototoxicity, weak autofluorescence and high resolution and penetration depth [19–21]. Therefore, it is important to develop polymeric nanolabels with water-solubility, covalently immobilized dyes and TP activity for TP FI in various biological systems.

Poly(acrylic acid) (PAA) is a biocompatible hydrophilic polymer. The abundant carboxyl groups along its backbone make it easy to modify. Thus, it has been used as a scaffold to product polymeric NPs for biomedical applications [25–27]. In addition, it has also been utilized for the surface modification of inorganic NPs such as silica NPs [28,29], quantum dots [30] and Ag nanoclusters [31] to endow them with biofunctional surfaces or to increase their suitability in biological fields. 1,8-Naphthalimide derivatives are widely used as fluorescent dyes for biological sensing and imaging. Owing to their good TP activity, they have been exploited as scaffolds in TP fluorescence probes for cysteine, F^-, hydrogen sulfide, etc., [32–34] in recent years. In this work, we grafted PAA with *N*-butyl-4[(2-aminoethyl)amino]-1,8-naphthalimide (BN), a naphthalimide dye that acts as both a TP fluorophore and hydrophobic chain, to prepare an amphiphilic polymer. Then, the polymer self-assembled to form fluorescent NPs (defined as BNPA NPs). The structure and luminescence properties of the BNPA NPs were investigated by various analytical techniques such as transmission electron microscopy (TEM), dynamic light scattering (DLS) and spectroscopic measurements. Furthermore, its *in vitro* and *in vivo* biocompatibilities were assessed by a cytotoxicity assay using HeLa cells and a feeding assay using *Caenorhabditis elegans* (*C. elegans*) as a small animal model, respectively. Finally, the TP FI of living cells and *C. elegans* labelled with BNPA NPs were observed by TP confocal microscopy. The results indicated that the BNPA NPs were suitable as nanolabels for the TP FI of living cells and *C. elegans*.

2. Materials and methods

2.1. Materials and chemicals

BN was synthesized according to the method previously described [35]. PAA (Mw 2.6 kD) was from Kaite Chemical Co. (Zhengzhou, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) from Sigma was dissolved in PBS at a concentration of 5.0 mg mL^{-1}. Other materials were obtained from commercial sources and used without further purification. Millipore-Q water was used for all experiments.

2.2. Preparation and characterization of BNPA NPs

$SOCl_2$ (2.0 mL) was added dropwise to a solution of 0.5 g PAA in DMF at 35–40 °C with stirring. The acid chloride reaction was allowed to continue for 1 h, after which the extra $SOCl_2$ was removed by rota-evaporation to give a poly(acryloyl chloride) solution. A half gram of BN in 10 mL of DMF was slowly added to the above solution, and the reaction proceeded for 8 h. After, the mixture was subjected to dialysis at room temperature with stirring at 400 rmp. The dialysate was replaced every 2 h for the first 12 h of the dialysis and then 6 times each day. The dialysis procedure took approximately 4 days, during which time the fluorescence intensity of the dialysate was monitored at 540 nm using an excitation wavelength of 444 nm. When a fluorescence emission was no longer observed, the liquid in the dialysis tube was lyophilized, and the BNPA NPs were obtained as a yellow powder.

DLS and zeta potentials were measured in a Malvern Zetasizer Nano ZEN3600 (UK). TEM images of the NPs were taken with a JEM-2100 TEM. TEM samples were prepared by dropping 0.15 mg mL^{-1} of NPs onto to a TEM copper grid and drying the grid in air overnight. To enhance the image contrast, the samples on the TEM grids were stained with tungstophosphoric acid. FTIR spectra were acquired on a Nicolet 7199 spectrometer using the KBr pellet technique. ^1H NMR spectra were collected using a Bruker AV-400 NMR spectrometer at 400 MHz with a mixed solvent of [D_6]DMSO and D_2O. UV–vis absorption spectra were recorded on a Shimadzu UV-1700 spectrophotometer. One-photon fluorescence was measured on a Hitachi F-4600 fluorescence spectrophotometer. TP fluorescence were measured using a mode-locked Ti:Sapphire femtosecond pulsed laser (Coherent Mira 900) excited at 800 nm.

2.3. MTT assay

The HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified environment containing 5% CO_2. Before the experiments, the cells were pre-cultured until confluence was reached.

For the MTT assay, the cells were seeded into a 96-well plate and cultured to allow for cell attachment. Then, the culture media was removed and replenished with 180 μL/well of fresh media and 20 μL/well of BNPA NP solutions at different concentrations. When the cells were exposed to the BNPA NPs for 24 h, 20 μL of MTT solution were added to each well. After 4 h of incubation, the MTT-medium solution was carefully removed and replaced with 200 μL of DMSO. The plate was placed on a shaking table and shaken for 5 min. Finally, the absorbances of the wells were measured at 570 nm to calculate cell viabilities.

2.4. Flow cytometry analysis

The HeLa cells were incubated in a culture medium containing 10 μg mL^{-1} of the BNPA NPs at 37 °C in a 24-well plate for different time periods. The cells were then harvested after washing with PBS and trypsinized and centrifuged at 1000 rpm for 5 min. Finally, the cells were re-suspended in 500 μL of PBS and analyzed by FACSCalibur flow cytometry (BD Biosciences, San Jose, USA).

2.5. TP FI of living cells

The HeLa cells were plated on glass coverslips in a 24-well plate. Labeling was carried out by incubating the cells with 10 μg mL^{-1} of the BNPA NPs in the culture medium for 30 min at 37 °C. The cells were then washed three times with PBS and fixed with 4% paraformaldehyde. The labeled cells were observed by a Zeiss Confocal Microscope LSM710 with TP excitation at 800 nm.

2.6. Nematode strains and culture conditions

The *C. elegans* wild-type strain (N2) was maintained at 20 °C on NGM (Nematode Growth Medium) agar plates supplemented with *Escherichia coli* (*E. coli*) OP50. To synchronize the growth stages of the nematodes, gravid adult worms were treated with hypochlorite, and then the fertilized eggs were collected. The collected eggs were cultured overnight in an S-medium at 15 °C in the absence of food until hatching. *E. coli* strains were routinely grown in Luria broth at 37 °C overnight, pelleted by centrifugation, frozen at −70 °C, and then re-suspended in the nematode S-medium.

2.7. Feeding assay

Assays were performed in 96-well plates based on the method described in the literature [37]. Approximately 50–60 synchronized N2 nematodes in 10 μL of S-media were added to 90 μL of the *E. coli* suspension containing various concentrations of the BNPA NPs in