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Aggregation-enhanced fluorescence in PEGylated phospholipid nanomicelles for *in vivo* imaging

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ABSTRACT

We report polymeric nanomicelles doped with organic fluorophores (StCN, (Z)-2,3-bis[4-(N-4-(diphenylamino)styryl)phenyl]-acrylonitrile), which have the property of aggregation-enhanced fluorescence. The fluorescent nanomicelles have two unique features: (1) They give much brighter fluorescence emission than mono-fluorophores. (2) The nanomicelles with amphiphilic copolymers [e.g., phospholipids-PEG (polyethylene glycol)] make the encapsulated fluorophores more stable in various bio-environments and easy for further conjugation with bio-molecules. After chemical and optical characterization, these fluorescent nanomicelles are utilized as efficient optical probes for *in vivo* sentinel lymph node (SLN) mapping of mice. The StCN-encapsulated nanomicelles, as well as their bioconjugates with arginine-glycine-aspartic acid (RGD) peptides, are used to target subcutaneously xenografted tumors in mice, and *in vivo* fluorescence images demonstrate the potential to use PEGylated phospholipid nanomicelles with aggregation-enhanced fluorescence as bright nanoprobes for *in vivo* diagnosis of tumors.

1. Introduction

In recent years, photoluminescence based bioimaging forms a major thrust of bio-photonics [1]. Much focus has been given to the development of efficient, inexpensive, stable, and tunable exogenous optical agents in biological systems, such as quantum dots/rods [2–6], silica nanoparticles [7–9], metal nanoparticles [10,11], carbon nanomaterials [12,13] and up-converting nanophosphores [14–17]. Although organic fluorophores exhibit remarkably high photoluminescence quantum efficiencies, their applications in the area of biological imaging are still limited due to their intrinsic hydrophobic property and instability in bio-environments. To overcome these problems, some approaches (e.g., phospholipid nanomicelles encapsulation) have been adopted.

Phospholipid-PEG nanomicelles can be utilized to encapsulate various fluorophores and drugs, and have many advantages in bio-applications: (1) They possess no cytotoxicity; (2) The preparation process of phospholipid-PEG nanomicelles is much simpler than that of other nanocarriers, such as silica nanoparticles [7–9] and gold nanoparticles [10,11]; (3) A large hydrophobic core in the nanomicelle, which arises due to the presence of long acyl chains of phospholipids, can facilitate the loading of high concentrations of hydrophobic molecules per micelle; (4) The long PEG chains in nanomicelles can improve the long-time circulation of nanoparticles in an animal body and help to avoid capture/degradation by reticuloendothelial systems (RES), and this is very important for *in vivo* animal experiments [18–20]. Self-assembled constructions of phospholipid-PEG nanomicelles have been widely utilized in the applications of drug delivery [21,22] and *in vivo* animal imaging [23] in the past several years.

However, there is still an obstacle for fluorophore doped nanomicelles. Most commonly used organic fluorophores suffer from an aggregation-induced fluorescence quenching phenomenon and their fluorescence emission decreases when they are highly loaded in phospholipid-PEG nanomicelles. Thus, it would be very useful if

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certain fluorescent materials, which have aggregation-induced enhanced emission properties, could be encapsulated in phospholipid-PEG nanomicelles and applied in fluorescence bioimaging. Recently, some groups have successfully synthesized these "special" fluorescent materials and demonstrated that they could exhibit enhanced emission when aggregated in certain solvents, or fabricated into solid films [24,25]. Furthermore, some other groups also began to use these fluorescent materials for various *in vitro* cell imaging experiments [7,26–28]. However, to the best of our knowledge, aggregation-enhanced emission dyes have still not been applied in *in vivo* animal imaging.

In this paper, (Z)-2,3-bis[4-(N-4-(diphenylamino)styryl)phenyl] acrylonitrile (StCN), a D–A–D type stilbene derivative with cyano group (as fluorescence acceptor group) in the center and vinyl and triphenylamine (as fluorescence donor group) in both ends, was synthesized as previously reported [29]. The intermolecular orientation is interrupted by the bulky cyano group during the formation of aggregation, which prevents the parallel overlap of fluorophores from becoming face-to-face oriented H-aggregations, but facilitates the formation of head-to-tail oriented J-aggregations [30,31]. Subsequently, StCN-encapsulated phospholipid-PEG (StCN@PEG) nanomicelles with a diameter of ~20 nm were prepared, and excellent chemical stability of the nanomicelles was demonstrated. The absorption and emission properties of StCN@PEG nanomicelles with various loading densities were characterized with an absorbance spectrophotometer and photoluminescence spectroscopy, and no noticeable aggregation-induced fluorescence quenching or emission peak-wavelength shift was observed. We then used the StCN@PEG nanomicelles for *in vitro* stain of HeLa cells, and their biological uptake by tumor cells was confirmed with fluorescence microscopy. To demonstrate the potential of StCN@PEG nanomicelles as bright fluorescent probes for *in vivo* animal imaging, we first used them for sentinel lymph node (SLN) mapping, which is a key process in SLN biopsy (SLNB) for cancer staging and surgery [32]. Subsequently, we developed a fluorescence imaging protocol based on StCN@PEG nanomicelles as contrast probes for *in vivo* diagnosis of tumors of mice. As is well known, nanoparticles have a property to be preferentially taken up by malignant tissues (e.g., tumors) due to the "enhanced permeability and retention" (EPR) effect [33,34]. We demonstrated the passive targeting of tumors using StCN@PEG nanomicelles as fluorescent agents in nude mice, which bear subcutaneous lung tumor xenografts. Furthermore, StCN@PEG nanomicelles bioconjugated with arginine-glycine-aspartic acid (RGD) peptides were used for *in vivo* tumor targeting. RGD peptides, which have high binding affinity to the $\alpha_v\beta_3$ integrin receptor [16,35–39], are promising new tools for imaging of tumors. The integrin $\alpha_v\beta_3$ is a type of cell-surface receptors that are overexpressed at the endothelium of growing blood vessels (vasculature) associated with tumor growth (angiogenesis) [40,41]. It plays an important role in angiogenesis and tumor cell metastasis, and is currently being evaluated as a target for new diagnosis and therapeutic treatment of tumors *in vivo* [35,36]. Our experimental results revealed that RGD peptide-conjugated StCN@PEG nanomicelles provided higher targeting efficiency to the subcutaneous lung tumor xenografts of mice than StCN@PEG nanomicelles without RGD peptides.

2. Experimental section

2.1. Materials and instruments

(Z)-2,3-bis[4-(N-4-(diphenylamino)styryl)phenyl]-acrylonitrile (StCN) was synthesized by the Department of Advanced Materials, Hannam University, Daejeon, Korea. The detailed information, including preparation and spectroscopic characterizations, were described in Ref. [29]. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-DSPE-5000) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide[poly(ethylene glycol)] (DSPE-PEG-Maleimide-3400) were purchased from Creative PEGWorks, Inc. Chloroform, 3,3′-diethylthiadicarbocyanine iodide (DTDC), Nile Red, hydrochloric acid and sodium hydroxide were obtained from the Chemical Reagent Department of Zhejiang University. RGD peptides (PCI-3686-PI) were purchased from Peptides International, Inc. Cell-culture products, unless otherwise mentioned, were purchased from Gibco. All the reagents, which were not specially pointed out, were analytical grade, and deionized (DI) water was used in all the experimental procedures.

Transmission electron microscopy (TEM) images were taken by a JEOL JEM-1230 transmission electron microscope operating at 160 kV in bright-field mode. A Shimadzu 2550 UV–vis scanning spectrophotometer and a HITACHI F-2500 fluorescence spectrophotometer were used to measure the absorption and photoluminescence (PL) spectra of samples.

2.2. Preparation of StCN@PEG nanomicelles

Typically, 1.5 mL of StCN solutions in chloroform (1 mg/mL) were injected into 100 μL mPEG-DSPE solutions in chloroform (10 mg/mL). The mixture solution was then evaporated and dried under vacuum in a rotary evaporator at 70 °C. Next, 2.5 mL of DI water was added into the solid lipidic mass obtained, and the solution was sonicated for 2 min. After that, an optically clear suspension containing StCN@PEG was prepared. As excess mPEG-DSPE molecules were used to encapsulate StCN molecules, the small loss of StCN in the experimental process could be negligible. The loading density (StCN/[StCN + mPEG-DSPE] in wt%) of StCN in StCN@PEG nanomicelles was calculated by the weight ratio of the materials put into the reaction. By varying the added quantities of StCN and mPEG-DSPE solution, nanomicelles with different StCN loading densities (20 wt%, 40 wt%, 60 wt%) were prepared by the same method (Table S1).

2.3. Preparation of RGD-conjugated StCN@PEG nanomicelles

First, maleimide-functionalized StCN@PEG nanomicelles were prepared by mixing 1.5 mL StCN solutions (1 mg/mL in chloroform), 80 μL mPEG-DSPE solutions (10 mg/mL in chloroform) and 10 μL DSPE-PEG-maleimide solutions (10 mg/mL in chloroform) together. After evaporated and dried under vacuum, 2.5 mL PBS (pH = 7.4, 10 mM) solutions were added into the obtained lipidic film, and the solution was sonicated for 2 min to obtain StCN@PEG-maleimide nanomicelles. The StCN@PEG-maleimide nanomicelles were then conjugated with thiolated RGD through specific thiol–maleimide reactions. Briefly, 1 mL PBS solution of StCN@PEG-maleimide nanomicelles was mixed with 0.3 mL thiolated RGD peptide solution (24 mg/mL) and incubated for 2 h in room temperature. The resulting dispersion was further centrifugated at 12,000 rpm in a 0.2 μm membrane filter for 15 min to remove the excess unreacted RGD molecules and the pellets blocked on the membrane (mainly containing bioconjugates) were redispersed in 1 mL of PBS (pH = 7.4, 10 mM) and kept at 4 °C for further use.

2.4. Release kinetics and chemical stability analyses

For release kinetics studies, 1 mg StCN@PEG (60 wt% of StCN loading) nanomicelles were incubated with 1 mL Tween-20 solutions (1% in DI water) at 40 °C. After a certain time, the sample was spin-filtered using microfuge membrane-filter (NANOSEP 100K OMEGA, Pall Corporation, USA) at 12,000 rpm for 15 min (spinfiltration). The filtrated solution, which passed through the