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Protease Sensitive Fluorescent Nanofibers

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Abstract

We report the design and synthesis of enzyme responsive nanofibers. The fibers are composed of self-assembled hydrophobic β -sheet peptides incorporating protease sensitive domains, fluorescent reporters and hydrophilic polyethyleneglycol (PEG) units. Using urokinase plasminogen activator (uPA) as a model system, nanofibers were developed to release fluorescent fragments upon uPA incubation. These protease sensitive nanofibers may have considerable biomedical applications as diagnostic sensors or for protease assisted drug deliveries.

Keywords

Nanofiber; self-assembly; fluorescence; sensor; protease

INTRODUCTION

A variety of nanomaterials have shown promise in biomedical applications (1–3). Over the past few years, several synthetic materials have emerged from biological systems and have been developed as sensors or for drug delivery. Self-assembly peptides can provide a high degree of flexibility and smartness when constructed into bio-responsive materials (4). For instance, self-complementary nanofibers have been proposed as scaffolds for tissue engineering, drug delivery, and imaging agent development (5,6). These fibers are typically assembled from simple building blocks driven by non-covalent interactions. Peptide nanofibers have been designed, by using the biotin-sandwich approach, to provide a specific and prolonged local myocardial delivery of survival factor, ICF-1 in cell-based therapies (7). Another study designed heparin-tagged nanofibers as sustained release system for growth factors such as FGF-2 or VEGF to promote angiogenesis (8). Importantly, these assemblies are often quite stable and homogenous in biological conditions.

One of the approaches to fine-tune bio-responsiveness is to incorporate environmentally sensitive chemical moieties into nanomaterials. For example, we have previously demonstrated that protease-responsive matrices composed of multiple peptide substrates (kldkl-SGRSANA-dklkl-NH₂, small letters indicated D-amino acid, and the capital letters indicated L-amino acid) could be synthesized (9). Here, the self-assembly nature of β -sheets was adapted to develop highly stable nanofibers. The core sequences were designed to incorporate (Fig. 1): 1) a hydrophobic β -sheet segment, [kldl]₃ (10), which was consisted of D-amino acids to avoid non-specific proteolytic digestion at the C-terminus, 2) an α -methyl substituted polyethylene glycol (mPEG) hydrophilic polymer attached at the N-terminus to prevent potential

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aggregation, 3) a specific peptide substrate (SGRSANA) positioned in the middle of the hydrophobic and hydrophilic segments and has been employed previously for in vivo protease imaging (11,12). The substrate is highly degradable by uPA (13), an enzyme known to have distinct roles in tumor growth, invasion, migration and angiogenesis by facilitating the breakdown of extracellular matrix (ECM) through proteolytic cascades (14–16), and finally 4) a conjugated fluorescein derivative, 5(6)-fluorescein isothiocyanate (FITC), as an optical reporter.

EXPERIMENTAL PROCEDURES

Chemicals

All solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All D and L amino acids were supplied by Novabiochem (San Diego, CA). α -methyl substituted polyethyleneglycol N-hydroxysuccinimide ester (mPEG₂₀₀₀-NHS ester) was purchased from Nektar Therapeutics (San Carlos, CA). Amiloride, anisole, ethandithiol, 5(6)-fluorescein isothiocyanate (FITC), thioanisole, urokinase (EC: 3.4.21.73) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Peptide Synthesis

Peptide synthesis was performed on an automated solid-phase peptide synthesizer (ABI-433A, Applied Biosystems, Foster City, CA) employing the traditional N α -Fmoc methodology on Rink Amide resin (280 mg, 0.1 mmol). Side-chain protections were Arg(Pbf), Asn(Trt), Lys(Boc), and Ser(tBu). Lys(ivDde) was used for selective deprotection on solid phase prior to FITC conjugation. All amino acids (10 eq.) were attached to the resin by step-wise elongation using 2(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt)/N,N-diisopropylethylamine (DIEPA) as the coupling reagent in N-methylpyrrolidinone (NMP). mPEG-NHS (avg. MW = 2470, 500 mg, 0.2 mmol) in DMSO (4 mL) was coupled to the peptide resin (140 mg, 0.05 mmol), initiated with the addition of DIEPA (1 mL) and was subjected to further gentle shaking for 3 hr at room temperature. The resulting resin was washed three times with 2% (v/v) hydrazine monohydrate in DMF (10 mL) for 5 min to selectively remove ivDde (4,4-dimethyl-2,6-dioxo-cyclohexylidene)3-methylbutyl side-chain amino protecting group. FITC (151 mg, 0.4 mmol) and DIEPA (1 mL) were dissolved in DMSO (4 mL). The solution was then added to the resin and allowed further shaking for 3 hr at room temperature. After drying with methanol, the peptide was cleaved from the resin with addition of a cleavage cocktail containing TFA/thioanisole/ethandithiol/anisole: 90/5/3/2 (5 mL) for 3 hr at room temperature. The filtrate was collected and precipitated into methyl-tert-butyl ether at 4°C. Crude mPEG-peptide conjugates were purified by high performance liquid chromatography (HPLC) (Ranin, Woburn, MA). MALDI-TOF mass spectrometry (Tufts Medical School, Core Facility, Boston, MA) confirmed the average mass ions for all synthetic conjugates. The concentrations of all peptides were determined by UV absorbance for their attached FITC moieties ($\epsilon = 65 \times 10^{-3} \text{ M}^{-1}\text{cm}^{-1}$ at 490 nm) in methanol.

The synthesis of nanofibers 1, 2, 3

The peptide (mPEG₂₀₀₀-BK(FITC)SGRSANA-[kdl]₃, 20 mg) or the control sequence (mPEG₂₀₀₀-BK(FITC)SGASNRA-[kdl]₃, 20 mg) was dissolved in 50 % (v/v) acetonitrile in water (5 mL). The solution mixtures were stirred vigorously for overnight inside a fume hood to evaporate the solvent. The resulting fibers **1** were then purified by size-exclusion chromatography (P-10 Bio-gel, Bio-Rad, Hercules, CA). Concentrations were determined by UV absorbance for their attached FITC moieties in methanol. To control the lengths of the preparations, the nanofibers solutions were passed through a mini-extruder (Avanti polar lipids Ltd., Alabaster, AL) using polycarbonate membranes (Whatman, Florham Park, NJ) with 0.4 μm and 0.2 μm pore sizes for the synthesis of 200 nm **2** and 100 nm **3** fibers, respectively.

After 10 passages, the solutions were collected and further purified by size-exclusion chromatography to remove the smaller fragments. The amounts were determined by UV absorbance for their attached FITC concentrations in methanol.

Transmission electron microscopy

All images were recorded by transmission electron microscopy (Tecnai G² Spirit BioTWIN, Hillsboro, OR) equipped with an AMT 2k CCD camera at Harvard Medical School (Department of Cell Biology, Boston, MA). All samples were negative stained with uranyl formate (17). Briefly, the samples were diluted with water and absorbed onto the formvar/carbon coated grids. Excess samples were blotted off on a filter paper. The grids were then floated on small drops (5 μ L) of uranyl formate solutions 2 % (v/v) for a few minutes and then blotted off. The samples were dried and examined in the TEM.

Enzyme digestion study by fluorescence imaging

Fluorescence imaging was performed by a reflectance fluorescence imaging system that described previously. Band-pass filters (Omega Optical, Brattleboro, VT) were employed to adjust the corresponding excitation wave bands for FITC. Stock nanofibers **3** (400 μ M) in PBS buffer (10 mM, pH 7.4, 50 μ L) or control fibers (400 μ M) containing a scrambled uPA substrate sequence (SGASNRA) in PBS buffer (10 mM, pH 7.4, 50 μ L) were loaded onto individual wells of a clear-bottom 96-well plate (Corning, Corning, NY). PBS (10 mM, pH 7.4, 50 μ L) or amiloride (4 mM) dissolved in PBS (10 mM, pH 7.4, 50 μ L) was added to the corresponding wells. After 24 hr incubations at room temperature, the plates were imaged with the exposure time of 10 min per image for four images. The entire wells were chosen as the region of interest (R.O.I.) and the relative fluorescence intensities were further analyzed by using computer software (Kodak Digital Science 1D software, Rochester, NY). To investigate the fluorescence activation of **3** were uPA-dependent, stock nanofibers **3** (400 μ M) in PBS buffer (10 mM, pH 7.4, 50 μ L) were loaded onto the wells of a clear-bottom 96-well plate. Different concentrations of urokinase (from 0–250 nM, 0–9.45 units, 150 μ L) were then added to the corresponding wells. After 24 hr incubations at room temperature, the plates were imaged as described above. For kinetic experiments, the enzyme treated and untreated samples were imaged every four hours in a period of 24 hr.

RESULTS AND DISCUSSION

The complete peptide-polymer conjugate (mPEG₂₀₀₀-BK(FITC)SGRSANA-[kldl]₃) was synthesized in solid phase employing the standard Fmoc chemistry on Rink amide resins. Following peptide synthesis, mPEG-NHS with average molecular weight of 2470 Da was attached at the N-terminus. To introduce the fluorescein reporter group, Lys(ivDde) was employed as the amino acid building block. The protecting group, ivDde, was selectively removed by hydrazine prior to fluorophore conjugation. The cleaved peptides were purified by HPLC and the average molecular mass ($[M + H]^+ = 4755$) was confirmed by MALDI-TOF.

The morphology of any designed assemblies can be influenced by modifying hydrophobic/hydrophilic ratios(18). The employed hydrophobic beta-sheet segment ([kldl]₃)(10) and several similar ionic self-complementary peptides are known to form amyloid-typed fibers (19,20). Nanofibers assembled by dissolving pegylated peptides in a mixture of acetonitrile and water, followed by solvent evaporation. Nanofibers assembled spontaneously into unique structures of homogenous diameter and length (Fig. 2A and B). The diameters of fiber **1** observed by TEM were extremely narrow (approx. 4 nm). In contradiction to previous reports (19,21), we did not observe cylindrical tubes (approx. 50 nm in diameter). Intermediate helical ribbons with the longer fibers (> 1 μ m), indicating an early sign of lamination, were found

infrequently (Fig. 2C and D). The nanofibers did not fuse into nanotubes (20), presumably because the conjugated mPEG₂₀₀₀ in **1** sterically prevents aggregation.

To prepare homogenous nanofibers, the assembled samples were physically disrupted into shorter fragments by passing them through a mini-extruder. This procedure is similar to routinely used methods for producing homogenous liposomes. Depending on the pore sizes of the employed polycarbonate membranes, nanofibers with approximately 200 nm **2** and 100 nm **3** in lengths were obtained (Fig. 3). The fragmented fibers (2 μ M) had similar physical properties as the original fibers **1**, and there was no aggregation, nor crosslinking between fibers. Further experiments are undertaken to study the long-term stability of these fibers.

The conjugated FITC fluorophores of **3** positioned in close proximities resulted in near complete quenching (96 %) of fluorescence in intact nanofibers (Fig. 4A). When compared with the same concentrations of free FITC, the normalized (at 494 nm) absorption spectrum in PBS buffer was broadened and had an increased peak maximum (at 460 nm) (Fig. 4B). In contrast, when **3** was completely dissociated in methanol, the absorption overlapped with the free FITC (Fig. 4C), thus providing evidence that fluorescence quenching of **3** is caused by structural orientations of the nanofiber assemblies.

The core nanofiber peptide was designed to contain an uPA cleavage site (SGRSANA) for proteolytic digestion. We hypothesized that the assembled nanofibers could thus respond to uPA by releasing FITC conjugated fragments (mPEG₂₀₀₀-BK(FITC)SGR) when cleaved (Fig. 1). To study enzymatic activation, the nanofibers **3** (100 μ M) were incubated with uPA (250 nM) for 24 hr. We observed an up to 4-fold increase in fluorescence intensity (Fig. 5A), which was uPA-concentration dependent (Fig. 5B). In the presence of amiloride (1 mM), an inhibitor of uPA, fluorescence remained at the baseline level. Enzyme selectivity was further confirmed by using control nanofibers. Here, the substrate cleavage site was substituted with a scrambled peptide sequence (SGASRNA). As expected, in the presence of uPA, there was no activation of the control nanofibers. To study digestion kinetic, nanofibers **3** were analyzed serially over time. In the absence of uPA, the fluorescence baseline remained constant with time suggesting that the formulations were stable. The fluorescence increases reached the plateau after 8 hr using 250 nM of uPA. Further experiments are undertaking to analyze the morphological changes of the nanofibers after enzymatic digestions.

In summary, using hydrophobic β -sheet peptides as core materials, we have developed a new type of protease sensitive nanofiber. The materials are easy to synthesize and self-assemble into fibers with homogeneous nanometer diameters. The lengths of these fibers can be controlled by membrane filtration. Proof-of-principle experiments demonstrated that these nanofibers could be made protease sensitive and biodegradable. These novel materials could have important applications in vivo protease imaging or as enzyme responsive biopolymers for systemic drug delivery.

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Abbreviations

B

beta-alanine

DIEPA

N,N-diisopropylethylamine

ECM	extracellular matrix
FITC	5(6)-fluorescein isothiocyanate
HBTU	2(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	N-hydroxybenzotriazole
ivDde	4,4-dimethyl-2,6-dioxo-cyclohexylidene)3-methylbutyl
mPEG	α -methyl substituted polyethylene glycol
NMP	N-methylpyrrolidinone
PEG	polyethyleneglycol
uPA	urokinase plasminogen activator

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Figure 1.

Design of uPA sensitive nanofibers. The fibers are composed of multiple PEG₂₀₀₀-peptide conjugates (mPEG₂₀₀₀-BK(FITC)SGRSANA-[kldl]₃-NH₂, where B = *beta*-alanine). Each conjugate consists of an uPA peptide substrate (SGRSANA, L-amino acid) inserted in between the hydrophilic (mPEG₂₀₀₀) and hydrophobic ([kldl]₃, D-amino acid) fractions. In addition, a fluorescein isothiocyanate (FITC) is introduced onto individual peptides as the optical reporter. In 10mM of PBS buffer (pH 7.4), the mPEG₂₀₀₀-peptide conjugates self-assemble spontaneously to form filaments with organized structures, so that the fluorophores are arranged in close proximity and result in fluorescence quenching. Upon addition of uPA, the substrate site is cleaved, leading to release of the fluorescent hydrophilic fragments (mPEG₂₀₀₀-BK(FITC)SGR) and resulting in fluorescence recovery.

Figure 2. TEM images of protease sensitive nanofibers. Images of fibers are shown at different magnifications (A–B). All samples were negatively stained with uranyl formate to provide contrast. Some fibers self-assemble to form super coil networks (black arrow, C–D).

Figure 3.

Size control of nanofibers. TEM images of 200 nm nanofiber **2** (A–B) and 100 nm nanofiber **3** (C–D). All samples were negatively stained with uranyl formate to provide contrast. (E) Schematic presentation indicates the synthesis of nanofiber **3**.

Figure 4.

Optical properties of 100 nm nanofiber **3**. (A) Relative fluorescence intensities of FITC and fibers in PBS buffer (10 μ M). The nanofiber was 30 $\times$ less fluorescent than free FITC. The absorption spectra of FITC and nanofiber **3** in (B) PBS buffer and (C) methanol. The spectra were normalized at 494 nm.

Figure 5.

Enzyme responsiveness. (A) Activation of 100 nm nanofiber **3** (100 μ M) in different conditions at 24 hr after incubation with uPA (250 nM) in 10 mM of PBS buffer (pH 7.4) at room temperature. No enzyme was added in the negative control. Nanofibers containing a scrambled uPA substrate sequence (SGASNRA) were synthesized as control fibers. Amiloride (1 mM) was used as the inhibitor to co-incubate with enzyme. The concentrations of nanofibers or the controls were 100 μ M. All samples were imaged in a 96-well plate by a reflectance imaging system. Individual wells were chosen as the region of interest (R.O.I.). (B) Fluorescence intensities of nanofibers (100 μ M) at 24 hr after incubation with various uPA concentrations (0–250 nM). (C) Fluorescence intensities plotted against time (hr) following activation of nanofiber **3** (100 μ M) with (■) or without uPA (250 nM) (◇).