Colloids and Surfaces B: Biointerfaces 132 (2015) 132–137

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Synthesis of poly(sulfobetaine methacrylate)-grafted chitosan under γ-ray irradiation for alamethicin assembly

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ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form 8 May 2015
Accepted 11 May 2015
Available online 19 May 2015

Keywords:
Alamethicin
Biocompatibility
Chitosan
Sulfobetaine
Self-assembly

ABSTRACT

Interaction between peptide and lipid membrane plays a major role in biological activity of membrane-active peptide. We describe here a new biocompatible polymeric assembly to support membrane peptide. Specifically, chitosan-*graft*-poly(sulfobetaine methacrylate) (CS-*g*-PSBMA) was synthesized for alamethicin assembly by controlled polymerization under γ-ray irradiation. The graft copolymer could self-assemble into micelles in distilled water for supporting alamethicin. The assembly of alamethicin with CS-*g*-PSBMA micelles in aqueous solutions was related with the ratio of alamethicin/CS-*g*-PSBMA: the more alamethicin, the smaller sizes of the hybrid complex. Moreover, alamethicin penetrated into the hydrophobic cores of CS-*g*-PSBMA micelles while displayed secondary helical conformation in the complex. The results indicate that CS-*g*-PSBMA assemblies can be used to support membrane peptide.

1. Introduction

The biological activities of membrane-active peptides are determined to a great extent by their assembly with phospholipid bilayer [1,2]. Membrane environment significantly affects the structural properties and functionality of membrane-active peptides [3,4]. It has been realized that the reconstitution of membrane peptides in vitro is important for practical application [5–8], and many trials were performed to accommodate functions of membrane peptides with lipid bilayers [9–17]. However, biological lipid bilayers are often fragile and subject to disintegration when exposed to air [18,19], which limits the utilization of membrane peptides in practical devices.

Meanwhile, polymeric nanoscopic entities may be an alternative to lipid membranes due to excellent stability and chemical functionality, which has shown great potential in drug delivery [20]. Until now, there are a few reports about polymeric assembly as structural materials to reconstitute membrane peptides. For example, Meier et al. [21] used giant block copolymer vesicles for supporting alamethicin in ion-carrier controlled precipitation of calcium phosphate; Taubert et al. [22] reported reactive amphiphilic block copolymers as mimetics of biological membranes for combination of protein; and Liang et al. [23] reported that proteorhodopsin could be spontaneously reconstituted into glassy state amphiphilic block copolymer membranes via a charge-interaction-directed reconstitution mechanism. The improved stability can be obtained in membrane protein–polymer complex [23–28].

Among membrane peptides, alamethicin is a 20-residue membrane-active peptide, and has an amphiphilic structure that can interact with lipid membrane [29–31]. Several materials have been used for studying the interactions between peptides and polymeric membranes. For example, Chen et al. [10] reported membrane thinning effect induced by the alamethicin in diphytanoylphosphatidylcholine bilayers; and Jelinek et al. [9] found that the phospholipid/polydiacetylene vesicle solutions had visible color changes upon binding of alamethicin. However, there are some concerns (such as biocompatibility and functionalization) need to be addressed about polymeric assembly, and it is still significant to develop a new polymeric assembly for supporting membrane peptides.

In this study, we report a biocompatible polymeric assembly to support membrane peptides (Scheme 1). Specifically, poly(sulfobetaine methacrylate) (PSBMA) was first grafted onto chitosan via controlled polymerization under γ-ray irradiation [32]. Chitosan (CS) is a natural polysaccharide that is regarded as a

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http://dx.doi.org/10.1016/j.colsurfb.2015.05.019
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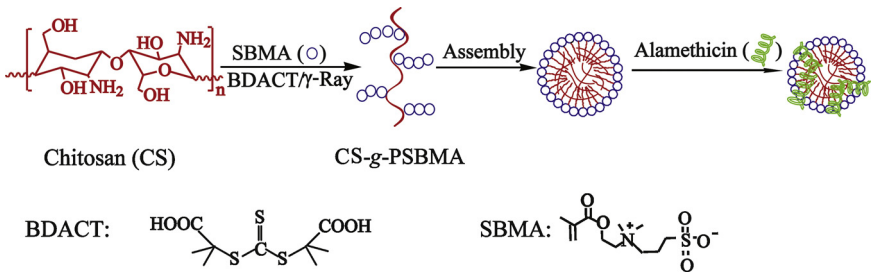

Scheme 1. The schematic for synthesis of CS-*g*-PSBMA particles for the assembly with alamethicin.

nonimmunogenic, nontoxic, and biocompatible polymer [33–35]. Furthermore, zwitterionic PSBMA is also noncytotoxic and biocompatible [36], and is similar to surface phosphorylcholine structure of cell membrane [37,38]. Therefore, the polymeric assembly of CS-*g*-PSBMA copolymer may be a good biocompatible system for supporting membrane peptides. It is expected that alamethicin may self-assemble well with CS-*g*-PSBMA micelles.

2. Experimental

2.1. Materials and reagents

Chitosan (deacetylation degree = 95.2%, $\bar{M}_n$ = 50, 000 g/mol) was obtained from Golden-Shell Biochemical Co. Ltd. (Zhejiang, China). Rhodamine B (95%) and alamethicin (95%) were purchased from J&K Chemical (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT). Sulfobetaine methacrylate (SBMA, 97%), fluorescein isothiocyanate (FITC, 90%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zoliumbromide (MTT, 98%), 5-doxylstearic acid (5-DS) and 16-doxylstearic acid (16-DS) were purchased from Sigma–Aldrich (Shanghai, China). Human dermal fibroblasts (HDF) were purchased from the Hygia Biotech Co. (Suzhou, China). S,S′-Bis (R,R′-dimethyl-R″-acetic acid) trithiocarbonate (BDACT) was synthesized according to a literature method [39]. All other chemical agents were used as received.

2.2. Characterization methods

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were taken by a Varian INVOA-400 instrument working at 400 MHz. Fourier transform infrared (FT-IR) spectrum was recorded on a Varian-1000 spectrometer: the samples were ground with KBr crystals, and the mixture was then pressed into a pellet for IR measurement. Field-emitting scanning electron microscopy (SEM) images were obtained on a Hitachi S-4700 microscope with an accelerating voltage of 15 kV. The molecular weights and polydispersities of the free homopolymers were determined with a Waters 1515 gel permeation chromatograph (GPC) equipped with a detector (RI 2410) and Ultrahydrogel columns (PL aquagel-OH MIXED-M) at 30 °C, with PEO standard samples and 0.2 mol/L $NaNO_3$, 0.1 mol/L NaH_2PO_4, 30% (v/v) CH_3OH aqueous solution as the eluent at the flow rate of 1.0 mL/min. Malvern Zetasizer with irradiation (He–Ne laser, 632.8 nm) was used to determine Z-average size distribution of the nanoparticles. Absorbance in MTT was measured at 570 nm with a Synergy 2 microplate reader (BioTek, USA). An UltraView VoX Confocal Microscope was used for confocal microscopy studies, while alamethicin and nanoparticles were fluorescently labeled with FITC and activated Rhodamine B-NHS, respectively [16]. The measurements were performed with Circular Dichroism (CD) spectra acquired on an AVIV Model 420 circular dichroism spectrometer (Aviv Inc., USA) with a 0.1 cm optical path length, and scans were recorded between 190 and 260 nm with 2 nm acquisition steps. Electron spin resonance (ESR) data was taken by JES-FA200 electron paramagnetic resonance spectrometer (JEOL, Japan).

2.3. Synthesis of CS-g-PSBMA copolymer

A typical recipe for the graft polymerization is described as follows [32,40]: BDACT (0.0212 g, 0.08 mmol) was dissolved in acetone (2.4 mL), and then was added into 1% HCl aqueous solution (5.6 mL) of chitosan (0.12 g) and SBMA (0.12 g, 0.43 mmol) in a 10 mL ampule. The contents were purged with nitrogen for 20 min to remove oxygen, and then the ampule was flame-sealed and placed in an insulated room with a ^{60}Co source (10 Gy/min) for 16.7 h. After polymerization, the mixture was dialyzed against distilled water with a membrane (MWCO = 25,000 Da) for 72 h to remove PSBMA homopolymers, unreacted SBMA, acetone and HCl. Finally, the dialyzate was lyophilized to give chitosan graft copolymer (CS-*g*-PSBMA). The graft content was determined by gravimetry. Three samples of graft copolymers with different graft contents of PSBMA chains were synthesized by changing the feed of SBMA (0.12 g, 0.2 g and 0.3 g).

In order to ensure the free PSBMA has been removed completely by dialysis (MWCO = 25,000 Da), we did the model experiments with homopolymers for the purification of the graft copolymer. We synthesized homopolymer PSBMA without chitosan under the same conditions of graft polymerization, and the homopolymer may be an indicator for the free PSBMA in the grafting polymerization mixture. The homopolymers were characterized by GPC measurements, and the results were depicted in Fig. S1 (ESI). For the free PSBMA with CS-*g*-PSBMA, the homopolymer was obtained, and could be removed completely with the membrane (MWCO = 25,000 Da) within 72 h. The result suggested that the free PSBMA could be removed completely from the polymerization mixture by dialysis. According to the related references [41,42], the homopolymer can be used as an indicator for the molecular weight and polydispersity index of grafted polymers.

2.4. Assembly of alamethicin with graft copolymer

CS-*g*-PSBMA (0.5 mg) was dissolved in 1% HCl aqueous solution (1.0 mL), and the solution was dialyzed against distilled water with a membrane (MWCO = 3500 Da) for 72 h to remove HCl. Then it was mixed with a solution of alamethicin in ethanol (0.0625 mg/mL, 1.0 mL). The mixture was dialyzed against distilled water with a membrane (MWCO = 1000 Da) for 24 h. The assembly was investigated by SEM, confocal microscopy and CD spectra.

Three samples of alamethicin/CS-*g*-PSBMA were synthesized by changing the concentrations of alamethicin in ethanol (0.0625, 0.125 and 0.25 mg/mL; 1.0 mL).

2.5. The cytotoxicity of CS-g-PSBMA by MTT assay

The cytotoxicity of CS-*g*-PSBMA was investigated using HDF cells by MTT assay. Under a humidified (5% CO_2) atmosphere,