Chemical Engineering Journal 347 (2018) 923–931

Contents lists available at ScienceDirect

Chemical Engineering Journal

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

Chitosan-graft-PAMAM loading nitric oxide for efficient antibacterial application

Guowei Li[a,1], Siming Yu[a,1], Wei Xue[a,b], Dong Ma[a,*], Wu Zhang[b,c,*]

[a] Key Laboratory of Biomaterials of Guangdong Higher Education Institutes, Department of Biomedical Engineering, Jinan University, Guangzhou 510632, China
[b] The First Affiliated Hospital of Jinan University, Jinan University, Guangzhou 510632, China
[c] School of Stomatology of Jinan University, Jinan University, Guangzhou 510632, China

HIGHLIGHTS

- Polyamidoamine dendrimer-grafted chitosan (CS-PAMAM) was synthesized as a NO donor by a fast and simple click reaction.
- The NO donor CS-PAMAM showed good biocompatibility and high NO loading efficiency.
- The as-prepared CS-PAMAM/NONOate showed significant antibacterial activity.

ARTICLE INFO

Keywords:
Antibacterial activity
Chitosan
Nitric oxide
PAMAM

ABSTRACT

Fabrication of nitric oxide (NO) donors with good biocompatibility and high NO loading efficiency for antibacterial applications remains a big challenge. In the present work, using low molecular weight chitosan (CS) with high water solubility as the backbone, we designed and synthesized a poly(amidoamine) (PAMAM) dendron-grafted CS using a fast and simple copper-catalyzed azide-alkyne cyclization reaction of azide-modified CS ($CS\text{-}N_3$) and propargyl focal point PAMAM dendrons with the third generation. NO was loaded on CS-PAMAM via a reaction with its secondary amine groups, which resulted in the formation of CS-PAMAM/NONOate with a maximal NO loading amount of 1.7 μmoL/mg. The CS-PAMAM/NONOate showed significant antibacterial activity against both Gram-negative *E. coli* and Gram-positive *S. aureus*, where 2.5 mg of CS-PAMAM/NONOate showed an inhibition zone with a diameter of about 20 mm. Moreover, the CS-PAMAM/NONOate presented negligible toxicity on normal mouse embryonic fibroblast cells, suggesting excellent biocompatibility. These findings indicate that the prepared CS-PAMAM/NONOate is promising for use in wound dressing industry as wound healing and treatment of anti-bacterial infections.

1. Introduction

Bacterial infection is recognized as one of the most prominent public health concerns [1]. In clinics, the most common way to treat bacterial infections is the use of antibiotics, although their therapeutic efficiency is being threatened by the increased antibiotic resistance of bacteria [2]. As a consequence, the necessary therapeutic goals can only be met by greatly increasing antibiotic doses [3], which are harmful to human health and could also accelerate the evolution of bacterial resistance and produce superbugs [4]. Therefore, the development of novel antibacterial agents is urgently needed.

In recent years, nitric oxide (NO), which plays an important role in physiological and pathophysiological processes, has attracted much attention due to its potential antibacterial properties [5,6]. It has been reported that NO can serve as a potent antibacterial agent against a broad spectrum of bacteria by means of a reaction with free radical superoxide (O_2^{*-}), which results in reactive byproducts like peroxynitrite (-OONO) and dinitrogen trioxide (N_2O_3) [7,8]. These by products can cause severe nitrosative and oxidative stresses on bacteria, leading to bacterial membrane disruption and cell dysfunction [9,10]. Due to its multi-mechanistic antibacterial behavior, NO is an ideal antibacterial reagent for combating bacterial resistance [11]. However, the gaseous state and short half-life of NO limit its clinical antibacterial application. Therefore, several NO carriers (NO donors) have been developed [12].

Dendrimers have been widely used in biomedicine due to their 'tree-like' architecture and high density of functional groups [13]. Among them, poly(amidoamine) (PAMAM) is commonly used as a NO donor

* Corresponding authors at: Jinan University, Guangzhou 510632, China (D. Ma and W. Zhang).
E-mail addresses: tmadong@jnu.edu.cn (D. Ma), tzhangwu@jnu.edu.cn (W. Zhang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cej.2018.04.159
Received 19 January 2018; Received in revised form 21 April 2018; Accepted 23 April 2018
Available online 24 April 2018
1385-8947/

because of its abundant secondary amine groups, which make it a suitable scaffold for high NO payloads [14]. The advantages of NO-based therapy dendritic effects have been highlighted by PAMAM's use as the NO donor [15]. Schally et al. developed the propargyl focal point PAMAM dendrons with different generations. Their secondary amines were subsequently modified with NO donors to establish NO payloads of 1.0 μmoL/mg. This NO-loaded PAMAM was then used in biomedical applications and showed a promising higher efficacy in the field of polymer therapeutics [16]. Worley et al. synthesized the NO-loaded quaternary ammonium-PAMAM with the generation from 1 (G1) to 4 (G4) [17]. Our recent work also demonstrated the high NO loading efficiency of PAMAM (G_3) [18]. It was found that with the increase of PAMAM generations, the total NO payload increased. However, the increasing cytotoxicity of the high generation PAMAM limits its future clinical applications. In our previous research, it was confirmed that a highly efficient low-toxicity dendrimer-containing polymer could be obtained by conjugating a number of low generation dendrons to a multifunctional polymer. The obtained new polymer demonstrated high efficiency of the high generation dendrimer as well as low toxicity of the low generation dendrimer [19].

In this work, we aimed to design and construct a NO donor with high payload and low toxicity by conjugating PAMAM-G_3 with low toxicity to a multifunctional polymer. The co-polymer was expected to exhibit a high payload property of high generation PAMAM and low toxicity of PAMAM-G_3. Chitosan (CS) is a natural polysaccharide and has been widely used as a biomedical material due to its excellent biocompatibility and biodegradability [20–24]. Moreover, CS's plentiful functional groups can be modified by a wide range of organic molecules to improve its performance [25]. Previous work has indicated that PAMAM-conjugating CS (CS-PAMAM) can be prepared by using a copper-catalyzed azide-alkyne cyclization reaction, where the resulting CS-PAMAM demonstrated high water solubility, biocompatibility, and high gene delivery efficiency [26,27]. However, there have been no reports on the use of CS-PAMAM for NO loading or for antibacterial applications.

Herein, using low molecular weight CS with high water solubility as the backbone, CS-PAMAM was synthesized via a fast and simple copper-catalyzed azide-alkyne cyclization reaction of azide-modified CS (CS-N_3) and propargyl focal point PAMAM dendrons (alkynyl-PAMAM-G_3). The obtained CS-PAMAM conjugate integrated the merits of CS and PAMAM, showing good biocompatibility from CS and low-generation PAMAM-G_3, as well as high NO payload of the high-generation PAMAM. After the NO loading, the physicochemical properties of CS-PAMAM/NONOate were characterized in detail and its antibacterial activity was systematically evaluated against Gram-negative *E. coli* and Gram-positive *S. aureus* using a series of assays.

2. Materials and methods

2.1. Materials

CS (MW < 2000 Da) was purchased from Jinan Haidebei Marine Bioengineering Co. Ltd. (Shandong, China). N-N-dimethylformamide (DMF), sodium azide, methyl bromoacetate, dimethyl sulfoxide, ethanediamine, methyl acrylate, propargylamine, and sodium ascorbate were obtained from Aladdin (Shanghai, China). Methanol and copper sulfate pentahydrate were bought from Chemical Reagent (Guangzhou, China). Lysogeny broth, tryptic soy broth, and lysogeny agar were obtained from Huankai Microbial Sci. & Tech. Co. Ltd. (Guangdong, China). Total Nitric Oxide Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). NO gas was purchased from Gndgas (Zhaoqing, China). The LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) was purchased from Thermo Fisher Scientific (Waltham, USA). *E. coli* (ATCC25922) and *S. aureus* (ATCC29213) were obtained from Guangzhou Southern Medical University (Guangzhou, China). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Biyuntian (Shanghai, China).

2.2. Synthesis of CS-PAMAM

2.2.1. Synthesis of CS-N_3

Azidoacetic acid (N_3-CH_2-COOH) (1.01 g) was dissolved in 25 mL of DMF, then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (2.85 g) and N-hydroxysuccinimide (1.72 g) were added to the solution. After 1 h activation, CS (1.63 g) in 20 mL of water was mixed with the above solution and the reaction continued for another 24 h under continuous stirring. After the reaction, the product was dialyzed (MW 2000 D) against water for 3 days and then lyophilized to obtain the oily azidoacetic acid with a yield of 78%. ^{1}H NMR (300 MHz, D_2O), δ (ppm): 3.5–4.0 (m, H-2, H-3, H-4, H-5, H-6), 4.87 (d, H-1), 1.98 (t, $NHCOCH_2$). The characteristic signal at 1.98 (t, $NHCOCH_2$) indicated a successful coupling of azidoacetic acid (N_3-CH_2-COOH) with CS backbone (Fig. S1).

2.2.2. Synthesis of alkynyl-PAMAM-G_3

Propargylamine (1.5 g) was dissolved in 5 mL of methanol under nitrogen atmosphere in an ice-water bath, followed by an addition of 10 mL of a methyl acrylate methanol solution (0.94 g/mL) over a period of 2 h. The mixture was first stirred at 0 °C for 1 h and then at room temperature for another 24 h. The unreacted methyl acrylate and methanol were removed by rotary evaporation and the final product was vacuum-dried and labeled PAMAM-$G_{0.5}$ ($G_{0.5}$ refers to generation 0.5).

PAMAM-$G_{0.5}$ (5.36 g) was dissolved in 20 mL of methanol under nitrogen atmosphere in an ice-water bath, followed by an addition of 30 mL of ethanediamine (21.2 g) methanol solution (0.76 g/mL) over a period of 2 h. The mixture was stirred at 0 °C for 1 h and then at room temperature for another 24 h. Subsequently, the unreacted ethanediamine and methanol were removed by rotary evaporation and the final product was vacuum-dried and labeled PAMAM-G_1. The reactions were performed twice more to obtain alkynyl-PAMAM-G_3. ^{1}H NMR (300 MHz, D_2O), δ (ppm): 2.20 (t, 1H, -CHC-), 2.459 (m, 28H, -CH_2CONH-), 3.29 (m, 28H, -CONHCH_2-), 2.6–3.0 (protons next to amines), 3.42 (d, -CHC-CH_2-). ^{1}H NMR data clearly indicated the synthesis of third generation alkynyl-PAMAM (Fig. S2).

2.2.3. Synthesis of CS-PAMAM

In this work, CS-PAMAM was synthesized via a fast and simple copper-catalyzed azide-alkyne cyclization reaction of CS-N_3 with alkynyl-PAMAM-G_3. Briefly, CS-N_3 (0.48 g) was added slowly to 50 mL of aqueous alkynyl-PAMAM-G_3 (0.079 g/mL) under nitrogen atmosphere with continuous stirring. Copper sulfate pentahydrate (0.98 g) and sodium ascorbate (3.12 g) were then added and reacted at 40 °C for 48 h. The mixture was dialyzed (MW 1500 D) against water for 3 days and lyophilized to obtain CS-PAMAM with a yield of 58%.

2.2.4. Synthesis of CS-PAMAM/NONOate

For NO loading, CS-PAMAM (0.5 g) and sodium methoxide (62.5 mg) were dissolved in 25 mL of absolute methanol with stirring for 0.5 h. The mixture was then transferred to a high-pressure reactor. The reactor was purged with high-purity nitrogen at 20 psi for 15 min and immediately filled with NO gas. The final pressure of the reactor was maintained at 80 psi for 3 days at room temperature. After that, NO was removed by filling with high-purity nitrogen at 20 psi. The products were washed 3 times with acetone and then precipitated by excess of cold diethyl ether. CS-PAMAM/NONOates was obtained after drying and storing the reaction products in a desiccator at −20 °C.

2.3. Characterization

The chemical structures of CS-N_3, alkynyl-PAMAM-G_3 and CS-PAMAM were analyzed by ^{1}H NMR spectroscopy (300 MHz) using deuterium oxide (D_2O) as the solvent. For the Fourier-transform