Biomaterials 34 (2013) 8450–8458

Contents lists available at ScienceDirect

Biomaterials

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

Polysaccharide-based biomaterials with on-demand nitric oxide releasing property regulated by enzyme catalysis

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ARTICLE INFO

Article history:
Received 25 June 2013
Accepted 12 July 2013
Available online 30 July 2013

Keywords:
Nitric oxide
Controlled release
β-galactosidase
Chitosan
Cardiovascular biomaterials

ABSTRACT

The regulatory role of nitric oxide (NO) in cell signaling has been well recognized. Clinically, NO deficiency is known to be associated with severe vascular disorders, especially in patients with long-term diabetes. Exogenous compensation of NO is a promising therapeutic strategy, although the lack of stable NO compounds often lead to unsatisfactory clinical outcomes. In the present study, we report a stable comb-shaped polymer (CS–NO) using glycosylated NO compound as pendent chains and chitosan (CS) as backbone for controlled NO release. The on-demand release of NO is achieved by controlling the decomposition process of the CS–NO polymer, which is blocked by galactose and only occurs in the presence of glycosidase, making the NO releasing kinetic closely correlate with the glycosidase concentration. In addition, due to its high stability, the CS–NO polymers can also be processed into supportive membrane or injectable hydrogel, further demonstrating its clinical potential. Indeed, we report that the NO-releasing membrane inhibited platelet adhesion, prolonged activated partial thromboplastin time (APTT) as shown in the platelet-rich-plasma (PRP) assay. We also observe enhanced human umbilical vein endothelial cell growth yet suppressed vascular smooth muscle cell proliferation on the NO-contained membrane *in vitro*. Furthermore, *in vivo* administration of CS–NO solution significantly enhanced angiogenesis in diabetic mice with hind-limb ischemia. Protective effect of CS–NO was also observed against limb necrosis. Given the physiological importance of NO, the CS–NO polymer may be considered a promising option in therapeutic development against vascular disorders and diabetic feet.

1. Introduction

Cardiovascular disease (CVD) is a major cause of mortality accountable for 30% of global deaths per annum [1]. In recent years, the development of biomaterials has provided a variety of therapeutic options to improve interventional therapies, with coronary stent, cardiac patch and artificial vascular graft all showing clinical success [2]. The effectiveness of biomaterial-based treatments is often determined by parameters such as the physiochemical performance of the matrix material and its biofunctionality. As a result, developing biomaterials that are able to deliver biological mediators on demand has attracted increasing attention due to their additional bioactivities [3].

Nitric oxide (NO) is an essential signaling molecule initially reported in the cardiovascular system [4–9]. It is endogenously produced via the activity of nitric oxide synthase (NOS), an enzyme that have been identified in different cell types. Three main isoforms of NOS have been classified to date including the endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [5]. NO deficiency is often associated with advance in age as a result of decreased eNOS expression. Recently NO deficiency has also been implicated in chronic disorders including cardiovascular diseases (CVDs) and diabetes. Indeed, NO has been shown to induce artery dilatation and inhibit platelet adhesion, both of which are

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http://dx.doi.org/10.1016/j.biomaterials.2013.07.045

beneficial in the management of CVDs. Compounds that can decompose to release NO have therefore been developed as exogenous NO donors for clinical use.

Diazeniumdiolates (NONOates) is by far the most extensively investigated NO donor, which has a predictable NO releasing half-life ranging from a few seconds to several hours [10]. However, clinical application of NONOate is limited by its unstable chemical composition, making it difficult to store or deliver NO in a controlled manner. In addition, difficulties in conjugation between the donor molecules and matrix materials also remain to be solved since the combination has to be stable yet sensitive enough to achieve the desirable spatio-temporal accuracy of NO release [11]. The easiest and most commonly practiced method is to blend the NO compound into polymers to obtain an NO-doping polymer system [12,13]. However, this method often results in uneven distribution of the NO compound, which may lead to NO accumulation at high concentration, causing local cytotoxicity and initial burst release.

Given the problems mentioned above, we have previously designed and synthesized a different NO donor compound, of which the terminal oxygen (O^2) is protected by a galactose molecule. Decomposition of this NO compound can only take place in the presence of glycosidase, which breaks the glucosidic bond and enables NO release [14]. In this study, we have further modified the molecular structure of Gly-NONOate by introducing an azide group at the end of its molecular chain. Under conditions of the [3 + 2] cycloaddition click reaction, this newly modified compound can be covalently linked to chitosan, which has alkynyl as side groups, to form a comb-shaped polymer, CS–NO (Scheme 1). Here we report that the as-synthesized CS–NO is chemically stable as injectable solution, supportive membrane, and fibrous scaffold. The physiological relevance of CS–NO was evaluated in aspects of hemocompatibility, cell proliferation using endothelial cells and smooth muscle cells. *In vivo* application of CS–NO was also investigated in diabetic mice with hind-limb ischemia.

2. Experimental section

2.1. Materials

Chitosan (M_n = 50,000) with deacetylation degree of 90% was purchased from Haidebei bioengineering company (China). 4-pentynoic acid (95%) was purchased from Sigma. N-(3-(dimethylamino)propyl)-N-ethylcarbodiimide hydrochloride (EDC·HCl, 99%) and L-ascorbic acid sodium salt (99%) were obtained from Alfa Aesar. Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 98%) and other reagents were purchased from Tianjin sixth Reagent Company. Galactosidase (8.9 units/mg) was also bought from Sigma. All other reagents unless otherwise noted were used as received without further purification. The Gal-NONOate was synthesized by a modified method (Supporting information, SI Scheme 1) [14].

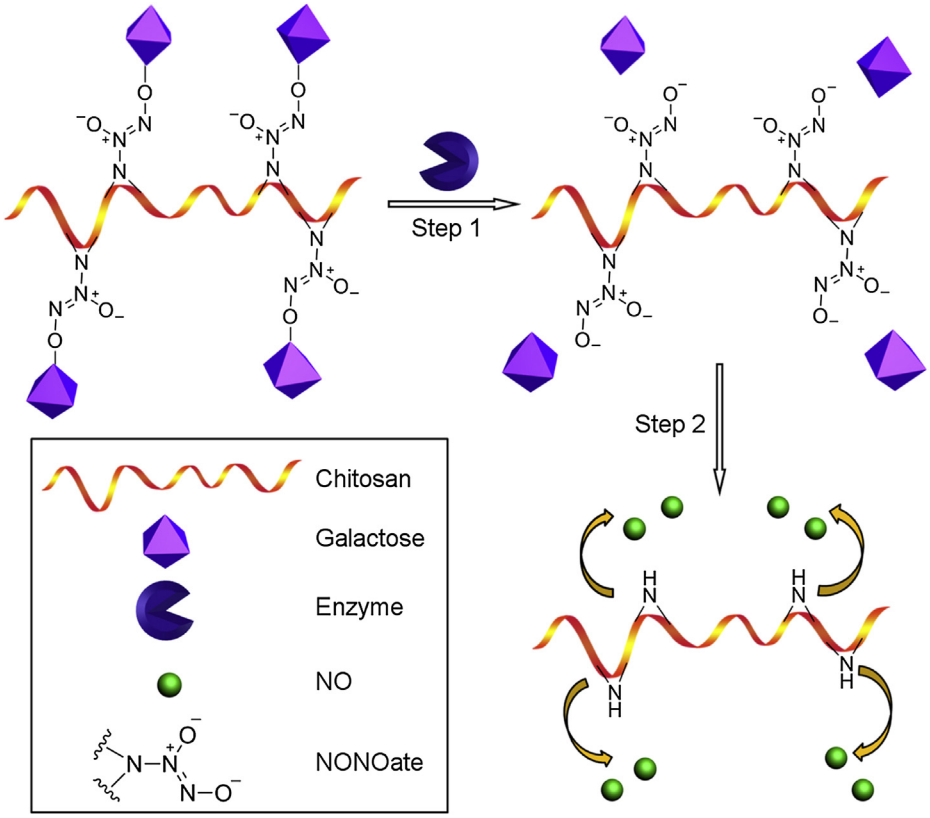
2.2. Synthesis of alkyne-substituted chitosan (alkynyl-CS)

Chitosan of 0.5 g was dispersed in 50 mL deionized water. Certain amount of pentynoic acid was added and stirred in ice bath. Subsequently, hydrochloric acid (HCl) solution of 1 M was added dropwise until the chitosan was completely dissolved. The pH was adjusted to about 5 by adding 1 M aqueous NaOH. EDC was added into chitosan solution by three times at time interval of 30 min, and the final mole ratio of EDC to pentynoic acid ([EDC]/[Pentynoic acid]) was 2:1 [15]. The reaction lasted for 24 h at room temperature. The products were purified by consecutive dialysis against 5 mM HCl containing 1 wt% NaCl for 2 days, 3 mM HCl for 1 day, 1 mM HCl for 2 days and deionized water for 3 days at 4 °C [16]. The dialyzate was replaced every 24 h. The products were finally obtained by lyophilization.

The chemical structure of alkynyl-CS was analyzed by ^{1}H NMR (400 MHz, D_2O).

2.3. Synthesis of CS–NO by click chemistry

100 mg alkynyl-CS was dissolved in 15 mL deionized water under the protection of nitrogen atmosphere. To this solution, Gal-NONOate, $CuSO_4$, and sodium ascorbat was added at the molar ratio 1:0.2:0.4. The reaction proceeded at 37 °C for 24 h under N_2 atmosphere (Fig. 1a). After stopping the reaction, the products were dialyzed against deionized water for 3 days, and finally lyophilized. The entire reaction



Scheme 1. Illustration for the decomposition of CS–NO under the catalysis of enzyme.