Nitric oxide releasing PLGA microspheres for biomedical applications

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ABSTRACT

We encapsulated an *S*-nitroso species (*S*-nitroso-*N*-acetylpenicillamine (SNAP)) in biodegradable PLGA microspheres that release nitric oxide (NO) for weeks in a continuous and controlled manner. The rate and duration of NO release are tunable by varying the formulation (e.g., SNAP loading, acid terminated or ester-capped PLGA and lactic-glycolic acid ratio). The NO release rate can also be tuned to desired levels by use of copper ions or light. The encapsulation of the SNAP crystals in the polymer leads to highly stable microspheres when stored at room temperature in the dark. The PLGA-SNAP microspheres can be used for several biomedical applications where extended and controlled NO release is desired (e.g. in wound healing), due to its substantial antimicrobial effects and ability to promote angiogenesis. To prepare new types of NO-releasing wound dressings, we incorporated the PLGA-SNAP microspheres into ointments and alginate films (PLGA-SNAP/alginate) that could potentially be used for treatment of chronic wounds (e.g., diabetic ulcers) and skin burns.

Keywords: nitric oxide, PLGA microspheres, controlled release, wound healing

1 INTRODUCTION

Nitric oxide (NO) is a well known endogenous gaseous molecule with potent antimicrobial [1][2][3], vasodilating, smooth muscle relaxant [4], and growth factor stimulating effects [5]. The efficiency of gaseous NO as a therapeutic agent (e.g., inhaled for pulmonary treatments) is already well established [6]; however, its wider biomedical utility is hindered by its cumbersome administration, very short lifetime (seconds) and short diffusional distance (some hundred μm) [7]. To overcome the drawbacks of using gaseous NO, many NO donor molecules have been developed and studied. The most frequently used NO donors are low-molecular-weight molecules, such, *S*-nitroso, *N*-nitroso, *C*-nitroso, metal ion-NO complexes, heterocycles and diazeniumdiolates [8][9]. The mechanism of the NO release from these donors can be based on spontaneous decomposition, redox or catalytic processes. Upon exposure to aqueous media, NO donors typically yield an initial NO burst release behavior, and many of the NO donors and/or their decomposition products are toxic or carcinogenic. Very high NO release is undesirable, as it can react with other reactive oxygen species to produce secondary reactive nitrogen species such as peroxynitrite. A major disadvantage of prevalent diazeniumdiolate (NONOate) donor molecules is that they are precursors of carcinogenic nitrosamines [10]. The most critical challenge in the development of new NO delivery systems is the formation of a local, long-term NO release at an optimal concentration to achieve a given therapeutic effect while minimizing toxic effects. Thus, NO donors are usually attached to, or encapsulated within, polymers in order to mimic endogenous NO release for biomedical applications [11][12][13].

Endogenously produced NO plays a crucial role in wound helaing, mediates vasodilation, inhibits platelet aggregation and protects against pathogens. In the proliferative phase, NO stimulates fibroblasts, keratinocytes, endothelial cells and vascular endothelial growth factor (VEGF) production. NO can also accelerate angiogenesis, augmented interfollicular stem cell recruitment, enhance hair follicular reconstruction and increase collagen deposition in burn wounds [14].

The topical supplementation of NO during wound healing can be enormous, especially in burn wound healing and in diabetic chronic wound healing, where the immune system or the endogenous NO production is impaired. Toward this goal, we now encapsulate a *S*-nitrosothiol (RSNO) NO donor in biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres in order to provide extended NO release to support wound healing and prevent infections. PLGA is the major excipient in numerous controlled release products [15] approved by the Food and Drug Administration (FDA) and the applied NO donor (*S*-nitroso-*N*-acetylpenicillamine - SNAP) and its decomposition products are expected to exhibit excellent biocompatibility as the precursor of SNAP, penicillamine, is an FDA approved chelating agent [16].

Herein, we investigate different ways to apply PLGA encapsulated SNAP (PLGA-SNAP) microspheres, i.e. incorporate them in ointments and hydrogels, for potential wound healing applications.

2 MATERIALS AND METHODS

2.1 Synthesis of SNAP

SNAP was synthetized as previously described. [17] Briefly 2 g of *N*-acetylpenicillamine (NAP) (Fluka) was dissolved in a mixture of 50 mL methanol, 16.7 mL H_2O, 8.3 mL HCl and 2.5 mL H_2SO_4. Twenty mL of 1 M $NaNO_2$ was then slowly added to the mixture. The color of the solution turned to dark red. The solution was placed onto ice, and by blowing N_2 over the solution, the methanol was evaporated until crystals appeared (~3 h). The green crystals were collected by solution filtration, washed with ice cold water and vacuum dried. The whole process was performed with protection from light to avoid the decomposition of SNAP. Before encapsulation in PLGA, SNAP was micronized by a CryoMill (Retsch, Düsseldorf, Germany). Five hundred mg SNAP was cryomilled in two 5 mL stainless steel jars, with 16 stainless steel 3 mm balls in each. After 7:50 min long precooling with liquid nitrogen at 5 Hz, SNAP was ground for 40 min at 20 Hz, then freeze-dried (-41 °C, 0.160 mbar) for 1 day. Prior to freeze-drying the cryomilled SNAP was flash frozen in liquid nitrogen.

2.2 SNAP Loaded PLGA Microsphere Preparation

SNAP loaded microspheres were prepared by a solid-in-oil-in-water emulsion water evaporation technique. 300-400 mg PLGA 50/50 (free carboxylic acid and ester endcapped, M_w = 24,000-54,000) polymer (Evonik Röhm GmbH, Darmstadt, Germany) was dissolved in 1 mL of methylene-chloride. Then, 60-100 mg of cryomilled SNAP was added and homogenized at 10 000 rpm for 1 min with a Tempest IQ^2 homogenizer (The VirTis Co., Gardiner, NY, USA) equipped with a 10 mm shaft. Four mL of 5% (w/V) PVA solution was immediately pipetted into the solution and vortexed (Genie 2, Fisher-Scientific Industries, Inc., Bohemia, NY, USA) at the highest speed for 1 min, then poured into 100 mL of 0.5% (w/V) PVA solution under rapid stirring with a magnetic stirbar and hardened for 3 h. The final microspheres were then sieved and washed with 500 mL DI water. The fraction with diameter between 20 μm and 125 μm was collected and freeze-dried (-41 °C, 0.160 mbar) for 2 days. Prior to freeze-drying, the microspheres were flash frozen in liquid nitrogen.

2.3 Measurement of SNAP Loading of PLGA Microspheres

To determine the SNAP loading in the microspheres, 5 mg of microspheres were dissolved in 1 mL acetonitrile and the concentration of SNAP was measured by absorbance at 340 nm using a Synergy Neo Microplate Reader (BioTek U.S., Winooski, VT, USA).

2.4 PLGA-SNAP/alginate Hydrogel Preparation

Sodium alginate (Sigma) was dissolved in DI water with continuous stirring overnight. PLGA-SNAP microspheres were then suspended in the sodium alginate solution, poured into a Petri dish and degassed with vacuum. Alginate gels were crosslinked with $CaCl_2$ or $CuCl_2$ solution. PLGA-SNAP/alginate gels were characterized right after cross-linking or stored dried, then rehydrated with DI water before use.

2.5 Scanning electron microscopy

For scanning electron microscopy (SEM) imaging, PLGA-SNAP samples were coated with 40 nm gold using a sputter coater (Desk II, Denton Vacuum Inc., Hill, NJ, USA) for 120 s. Scanning electron micrographs were taken by a Hitachi S-3200N Variable Pressure SEM (Hitachi High-Technologies Corp., Tokyo, Japan). The applied accelerating voltage was 15 keV. For cross-sectional images, samples were cut using a razor-blade on glass slide prior to coating.

2.6 Nitric oxide release measurement by Nitric Oxide Analyzer

For NO release experiments, samples were incubated in 5-10 mL release media in amber 50 mL centrifuge vials with gently shaking at 37°C. Nitric oxide release experiments were performed by using an ozone-chemiluminescence technology-based Sievers Nitric Oxide Analyzer (NOA, Model 280i, GE Analytical Instruments, Boulder, CO, USA). During NO measurement, NO was purged out from the release media via a N_2 purge gas.

Light modulated NO release measurements were performed in a transparent vial exposed to a tungsten halogen lamp equipped with a Fostec DCR II EKE cold light source (Schott-Fostec, LLC, New York, USA) set at its highest intensity.

3 PLGA-SNAP MICROSPHERES FOR NO DELIVERY

SNAP was encapsulated in biodegradable PLGA microspheres with diameter of 20-125 μm using a solid in oil in water emulsion and solvent evaporation method (Figure 1). The encapsulation efficiency of SNAP was > 40% in all cases. The loadings were between 6-13%, increasing with the amount of added SNAP.

The NO release of PLGA-SNAP microspheres was tested in PBS buffer, pH 7.4, containing 100 μM EDTA (PBSE), 1 mM ascorbic acid, and variable amounts of Cu^{2+} (PBSACu). The testing was conducted at 37°C, in the dark (Figure 2 and Figure 3). In PBSE buffer, the EDTA can coordinate Cu^{2+} ions, inhibiting the decomposition of SNAP. However, the release of NO is controlled without an NO burst even in the presence of decomposing agents like Cu^{2+} and a

high concentration of ascorbic acid. The NO release was continuous for 2 weeks in the carboxylic acid terminated PLGA was used and for over 4 weeks in the case of the ester terminated PLGA. The amount of NO was also tunable by varying the SNAP loading of PLGA microspheres (data not shown).

A B

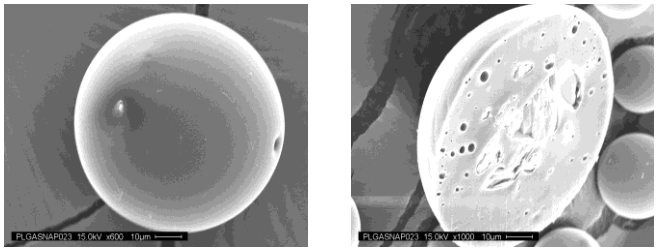

Figure 1. Scanning electron micrograph of PLGA-SNAP microsphere (A) and its cross-section (B) with the SNAP crystals within.

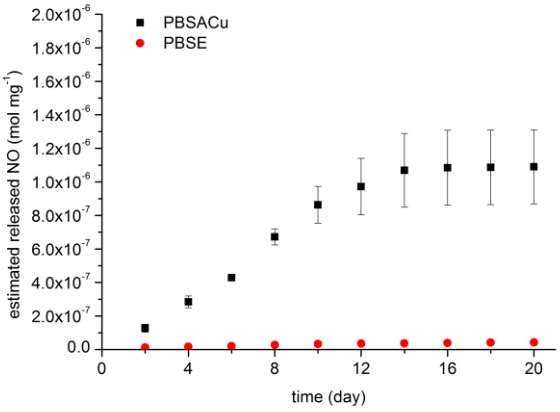

Figure 2. NO release of free carboxylic acid terminated PLGA-SNAP microspheres in PBSE and PBSACu release media changed at every timepoint

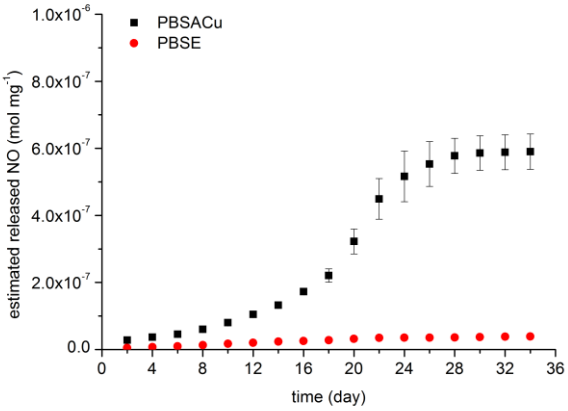

Figure 3. NO release from ester terminated PLGA-SNAP microspheres in PBSE and PBSACu release media changed at every timepoint

The NO release also can be induced and kept at desired levels by shining light on the microspheres. Dry particles released 75% of their loading in the form of NO in 20 h (Figure 4), and the level of released NO was tunable by the intensity of the light (data not shown).

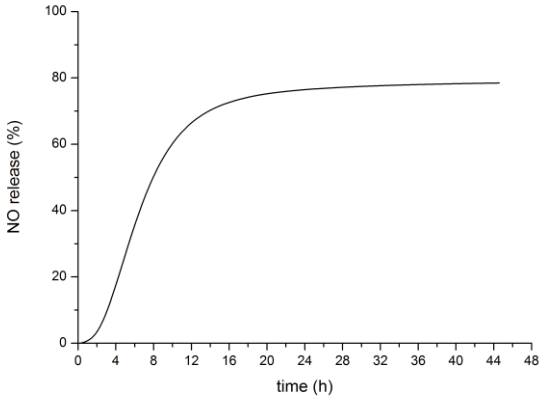

Figure 4. Light modulated NO release from ester terminated PLGA-SNAP microspheres at room temperature

The encapsulation of the SNAP crystals in the polymer led to highly stable microparticles that retained > 90% of their NO release properties over a month period when stored at room or elevated (37°C) temperature in the dark.

4 PLGA OINTMENT AND PLGA-SNAP/ALGINATE HYDROGEL FOR WOUND HEALING

PLGA-SNAP microspheres can be used for several biomedical applications where extended and controlled NO release is desired (e.g. in wound healing) since NO has substantial antimicrobial effects and promotes growth of new blood vessels.

We incorporated the PLGA-SNAP particles in hydrophobic ointment, and we were able to induce the NO release by using a simple light source. The PLGA-SNAP particles are highly stable at room temperature in the absence of humidity; thus, we expect appropriate stability of the microspheres in the ointment, which could be administered directly onto the wound and activated by a light source.

To prepare a new type of NO releasing wound dressing, we also incorporated our PLGA-SNAP microspheres into alginate hydrogels (PLGA-SNAP/alginate) (Figure 5).

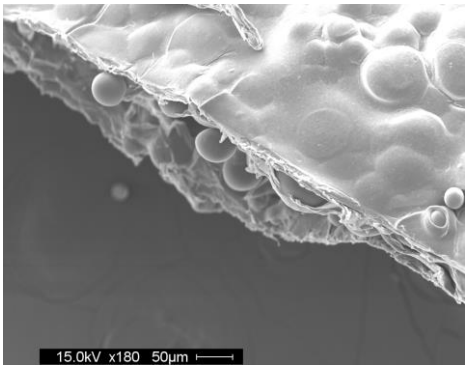

Figure 5. Cross-sectional scanning electron micrograph of PLGA-SNAP/alginate film

Alginate hydrogel dressings have great potential in wound healing applications due to their smoothness, flexibility and elasticity. They also can be removed without much trauma. Since such dressings can absorb high amount of water, it can protect the wound from drying out or can be used as an occlusive dressing. Since trace amounts of copper ions are desired for efficient NO release of the PLGA-SNAP microspheres in the absence of light, the alginate hydrogel can be used as the source of copper ions by crosslinking the hydrogel with Cu^{2+}. Indeed, Cu^{2+} cross-linked alginate hydrogels were recently reported as effective wound dressing [18]. The PLGA-SNAP incorporated Cu^{2+}-alginate film continuously released NO at endothelial NO release levels for over a one week period (Figure 6). Hence, such materials can potentially be employed in chronic and burn wound management.

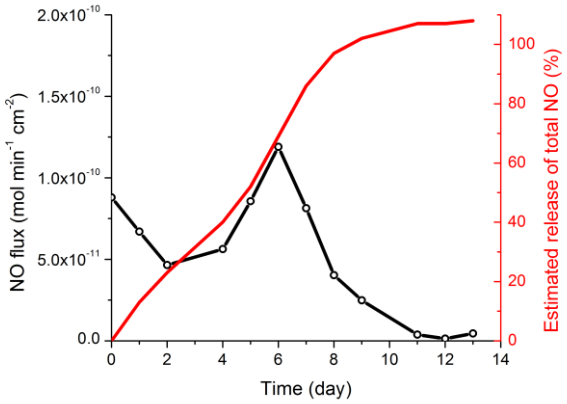

Figure 6. Estimated NO release of PLGA-SNAP/alginate film

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