Lysozyme-Triggered Epidermal Growth Factor Release from Bacterial Cellulose Membranes Controlled by Smart Nanostructured Films

GUILHERME FADEL PICHETH,[1,2] MARIA RITA SIERAKOWSKI,[1] MARCO AURELIO WOEHL,[1] LUCY ONO,[3] AXEL RULF COFRÉ,[4] LUANA PASETTI VANIN,[1] ROBERTO PONTAROLO,[2] RILTON ALVES DE FREITAS[1]

[1]Biopol, Chemistry Department, Federal University of Paraná, Curitiba 81531-980, Paraná, Brazil
[2]CEB, Pharmacy Department, Federal University of Paraná, Curitiba 80210-170, Paraná, Brazil
[3]Yasuyoshi Hayashi Microbiology Laboratory, Basic Pathology Department, Federal University of Paraná, Curitiba 81531-990, Paraná, Brazil
[4]ICC, Fiocruz, Curitiba 81350-010, Paraná, Brazil

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ABSTRACT: A novel wound-dressing biodevice, sensitive to lysozyme, an enzyme commonly found at infected skin wounds, was assembled by the layer-by-layer deposition of nanopolymeric chitosan and alginate films onto oxidized bacterial cellulose membranes incorporated with epidermal growth factor (EGF). Distinct EGF release profiles were obtained according to specific stimuli caused by infection. In *in vitro* conditions simulating noninfected wounds, the EGF rate and burst release effect were reduced by three deposited layers (M_t/M_∞ of 0.25 at 3 h) in a process dependent on the porosity of the compact chitosan–alginate complex. The importance of the organized structure was revealed when an infected wound was simulated by adding lysozyme to the release medium, thus inducing the formation of a loosely polyelectrolyte architecture that caused rapid EGF diffusion (M_t/M_∞ of 0.75 at 30 min). The results indicate that the nanopolymeric layers were capable of slowly releasing EGF as required for normal wound repair and rapidly undergoing architectural transitions that allow the diffusion of massive amounts of drug to enhance the process of re-epithelialization. In summary, the proposed system comprises the roles of both wound dressing and local delivery mechanism to recognize infections and respond with a burst of EGF release. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3958–3965, 2014

Keywords: bacterial cellulose; layer-by-layer; chitosan; alginate; nanotechnology; wound dressing; mathematical model; sustained release; *in vitro* models; protein delivery

INTRODUCTION

Local delivery of healing agents, such as growth factors (GFs), strives to induce faster and more effective skin repair. The reconstruction of wounded skin often requires occlusion of the damaged area to accelerate the process of re-epithelialization, avoid infections, and reduce exudates and local pain.[1] Although occlusive dressings are widely utilized in the treatment of burns and chronic wounds,[2] few systems are capable of simultaneously protecting the skin against infections and releasing bioactive drugs in a sustainable fashion. Therefore, the development of sensitive materials for sustained drug release as well as controlled cell proliferation, migration, and differentiation[3] represents a fundamental advance in current regenerative medicine.

Among the most successful wound dressings, bacterial cellulose (BC) membranes accelerate the process of tissue repair in chronic wounds, skin burns, and bones.[4] BC produced by *Gluconobacter xylinus* was first introduced as an ideal wound dressing material because of its high *in vivo* biocompatibility and unique microfibrillar structure that provides flexibility, high water retention capability, and gas exchange.[5] Additionally, BC membranes maintain a physical barrier that reduces pain and bacterial infection and allows drug transfer into the wounded region.[6] For all of these characteristics, BC is utilized in several biomedical applications, such as microblood vessels, bone and cartilage tridimensional grafts, and burn treatment.[2]

Novel strategies for damaged skin treatment include the utilization of molecules with the capability to induce faster re-epithelialization.[7] This family of molecules is represented by GFs, which are signaling peptides that show great potential to accelerate the wound healing process with their ability to induce cells to proliferate and establish a new matrix in the wounded gap.[8] Among the key regulators of keratinocyte proliferation at the injury edge, epidermal GF (EGF) acts as a motogen and a mitogen in the epidermis to ensure wound closure.[9]

Although EGF is widely utilized in epithelial wound repair, its rapid hydrolysis by proteolytic enzymes requires a controlled release according to the lesion type, phase, and/or the coadministration of anti-proteolytic agents. Additionally, GF concentrations fluctuate according to lesion characteristics: chronic and infected wounds present increased amounts of metalloproteinases that diminish GF bioavailability at the injury bed[10] and demand higher amounts of EGF for optimal treatment. Nevertheless, in venous and diabetic ulcers, GFs are trapped within fibrin cuffs and become unavailable for tissue repair[11];

Correspondence to: Rilton Alves de Freitas (Telephone: +55-41-3361-3260; Fax: +55-41-3361-3186; E-mail: rilton@ufpr.br, rilton@quimica.ufpr.br)

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therefore, exogenous EGF is needed to ensure wound closure. In summary, delivery of EGF with distinct release rates is required to enhance skin repair in wounds that are susceptible to and incur infection. Although many release systems succeed in delivering EGF, few are capable of altering the amounts of drug diffused instantaneously according to tissue requirements.

In this study, we report the development and characterization of a novel nanoengineered biodevice for "smart" sustained release of an EGF-mimicking peptide at damaged skin areas that are either noninfected or infected. This novel sensitive delivery system is based on thin and flexible oxidized BC (OBC) membranes coated with nanopolymeric layers through a spray-assisted layer-by-layer (sLbL) technique[12] and composed of oppositely charged polyelectrolytes: chitosan, a linear polysaccharide composed of *N*-acetyl-D-glucosamine and D-glucosamine bonded via β-(1→4) linkages that presents naturally antimicrobial and hemostatic activities[13]; and alginate, a copolymer of (1→4) linked β-D-mannuronic acid and α-L-guluronic acid.[14]

MATERIALS AND METHODS

Chemicals

Chitosan was obtained from Purifarma (São Paulo, Brazil). Acetic acid, alginate, sodium hydroxide, 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO), hydrochloric acid, sodium bromide, sodium hypochlorite, rhodamine B isothiocyanate (RITC), fluorescein isothiocyanate, Dulbecco's modified Eagle medium, amphotericin B, Bacto agar, enrofloxacin, penicillin G potassium, and lysozyme from chicken egg white (96,381 U/mg) were obtained from Sigma Chemical Company (St. Louis, Missouri). The EGF-mimicking peptide was purchased from Caregen, Company (Kunpo, Republic of Korea). Ultrapure water was obtained from a MilliQ system using 0.45-μm cellulose ester filtration membranes from Millipore Company (Billerica, Massachusetts). BC membranes were kindly donated by Bionext® (São José dos Pinhais, Brazil).

Chitosan was previously purified[15] and solubilized in 0.5 mol/L acetic acid at fixed concentrations of 1 mg/mL. The degree of deacetylation of the chitosan was 82.3%, the weight average molar mass (M_w) was 87.8 × 10^3 g/mol, and the ζ-potential was +14 ± 5.2 mV at pH 4.5. Alginate was solubilized in ultrapure water for 24 h and used without further purification at 1 mg/mL. The M_w of alginate was 92.0 x 10^3 g/mol and the ζ-potential was −20 ± 6.1 mV at pH 8.0 (Supporting Information 1.1).

Oxidation of BC Membranes

The BC membranes were oxidized according to a protocol described in Sierakowski et al.[16] (Supporting Information 1.2).

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy

Infrared spectra were recorded with a VERTEX 70 FT-IR spectrometer (Bruker Company, Billerica, Massachusetts) equipped with an attenuated total reflection (ATR) accessory. All spectra were obtained from a total of 16 scans with a resolution of 4 cm^{-1} in the range of 400–4000 cm^{-1}.

X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) analyses were performed using a commercial system (VG Microtech ESCA 3000, Sussex, UK) equipped with Al Kα radiation sources with a base pressure in the experimental chamber lower than 10^{-9} mbar. The spectra were obtained at a takeoff angle of 45° using a hemispherical energy analyzer with an overall energy resolution of approximately 0.8 eV. The binding energy scales were referenced by setting the C–C component of the C1s signal at 285.0 eV.[17] The XPS analyses were performed by assuming core level spectra with Gaussian line shapes after standard Shirley background subtraction.

sLbL Deposition

Polymer deposition on TEMPO-modified membranes was performed using spray methodology.[18] The samples were immobilized in a glass stand and nebulized with an air-propellant device (AIR-BOY; Carl Roth, Lauterbourg, Bas-Rhin, France) containing solutions of chitosan (1 mg/mL pH 4.5) and alginate (1 mg/mL pH 8.0) in an alternating fashion for 2 s interspersed with a washing step with ultrapure water for 3 s. The membranes were coated with up to four polymeric layers and dried in an oven at 37°C for 24 h.

Atomic Force Microscopy

Atomic force microscopy (AFM) analyses were performed in air using an Agilent microscope (Agilent Technologies, Santa Clara, California) and Pico Image software (Agilent Technologies). Images were obtained in tapping mode with Vistaprobes® (Nanoscience Instruments, Inc., Phoenix, Arizona) silicon tips (nominal spring constant of 48 N/m and resonance frequency of ~180 kHz) by scanning three distinct areas of 4 × 4 μm^2 on each sample. All membrane images were collected after 24 h of drying at 37°C. The data were analyzed with Gwyddion Software (Czech Metrology Institute, Brno, Morávia, Czech Republic).

In Vitro Cytotoxicity Assay

The cytotoxicity of the membranes was evaluated using the agar overlay assay[19] (Supporting Information 1.3).

Confocal Fluorescence Microscopy

Fluorescein isothiocyanate-labeled EGF and RITC-labeled chitosan were prepared following protocols described by Clayton et al.[20] and Ma et al.,[21] respectively (Supporting Information 1.4).

In Vitro Drug Release

The dry OBC membranes were cut into round shapes with a diameter of 1.5 cm (1.5 ± 0.4 mg) and left to swell in 2000 μg/mL EGF-mimicking peptide solution at 22°C for 48 h. Afterwards, the OBC membranes were dried in an oven at 37°C for 24 h and coated by sLbL as described in section *sLbL Deposition*.

The *in vitro* drug release experiments were performed in 20-mL flasks containing 10 mL of 0.1 mol/L phosphate-buffered saline (PBS) buffer at 37°C (pH 5.5/sodium azide 200 ppm as a preservative)[22] with or without lysozyme (10,000 U/mL),[23] under constant stirring. At each sampling time point, 2 mL were withdrawn and immediately replaced with fresh medium. The release experiments were repeated in triplicate for each sample. The released amounts of EGF were analyzed by high-performance liquid chromatography (HPLC) in a Shimadzu Prominence LC-20AT/CBM-20A (Kyoto, Kansai, Japan) with a C18 column (Symmetry®) and UV detector at 210 nm. The mobile phase was composed of 60% (v/v) acetonitrile and 40%