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Self-Assembly Stereo-Specific Synthesis of Silver Phosphate Microparticles on Bacterial Cellulose Membrane Surface For Antimicrobial Applications

Bernardo Bayón[a], Maximiliano L. Cacicedo[a], Vera A. Álvarez[b], Guillermo R. Castro[a,*]

[a] Laboratorio de Nanobiomateriales, CINDEFI, Facultad de Ciencias Exactas, Universidad Nacional de La Plata - CONICET (CCT La Plata), Calle 47 y 115, B1900AJL La Plata, Argentina

[b] Grupo de Materiales Compuestos (CoMP), INTEMA (National Research Institute of Material Science and Technology), Facultad de Ingeniería, Universidad Nacional de Mar del Plata, Solís 7575, B7608FDQ Mar del Plata, Argentina

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ABSTRACT

In situ self-assembly stereo-specific synthesis of silver phosphate (AgP) microparticles (MPs) in one side bacterial cellulose (BC) membrane surface and loading of ciprofloxacin provides BC scaffolds with a broad-spectrum antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The BC membranes containing AgP-MPs can be loaded > 5 times with ciprofloxacin compared to naked BC. The reuse of ciprofloxacin-loaded AgP-BC scaffolds in fresh cultures of both tested microbes showed high inhibition haloes without any signal of microbial resistance or scaffold activity decay after 30 days of assays. Additionally, microbial biofilms of *E. coli* and *S. aureus* were mostly killed by AgP-BC scaffolds loaded with ciprofloxacin in 1 h. The biophysical characterization of the hybrid AgP-BC scaffolds was performed by scanning electron microscopy (SEM), vibrational spectroscopy (FTIR), calorimetric techniques and X-ray diffraction (XRD). SEM images display AgP-MP spheroid structures with a high homogenous distribution on BC scaffold surfaces. The properties of the novel AgP-BC matrices are very promissory for biomedical applications, like a therapeutic dressing patches for the prevention and treatment of microbial infections in skin wounds and burns.

1. Introduction

The world population suffering chronic ulcers, scalds, severe burns and wounds is rising because of an adverse social impact in many low- and middle-income countries that is challenging medical and research areas. Particularly, skin wound therapies are one of the riskiest treatments in episodic and chronic pathologies such as diabetes, severe burns and others, mostly in medium/long-term hospitalized patients because of the high probability to suffer bacterial infections. For example, approximately 265,000 diseases per year were caused only by skin burns in low- and middle-income countries according to the World Health Organization (WHO) 2017 report. Furthermore, in 2014 the WHO reported 422 million people worldwide with diabetes consuming between 15% and 25% of the health care resources in their countries. Also, at least 25% of diabetic patients develop chronic ulcers during their lifetime, and the ultimate consequence without proper care is that they must undergo lower limb amputations. Since open wounds are the preferred access of pathogens to be spread in the body, the first and main therapeutic approach is the systemic and local administration of drugs. Besides, systemic drug administration sometimes does not provide an optimal therapy because of the high concentration of pathogens localized in the wounds and the presence of multidrug resistant (MDR) microorganisms. Additionally, due to the prevalence of MDR microorganisms in many pathologies around the world, the WHO declared a global antibiotic emergency.

Local treatment using transdermal patches combining therapeutic molecules and containing different biocide principles can be excellent alternatives to treat MDR microorganisms with high efficiency, low costs of production, ease of manipulation and replacement without the requirement need for specific medical facilities and trained personnel. Transdermal patches for different applications with contraceptive and anticholinergic activities and containing nicotine to treat tobacco addiction are available on the market.

Bacterial cellulose (BC) is a natural polymer biosynthesized by many microorganisms, but the most popular is *Komagataeibacter hansenii* (formerly *Gluconacetobacter hansenii*) [1, 2]. BC is composed of pure cellulose nanofiber mesh consisting of β-(1 → 4) glucose chains that is synthesized at the air/liquid interface as a three-dimensional asymmetric network of nanofibrils during bacterial growth [2, 3]. The BC matrix structure has a high-water content (about 99%) and displays

* Corresponding author.
E-mail address: grcastro@quimica.unlp.edu.ar (G.R. Castro).

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distinctive properties such as high purity, high degree of polymerization (up to 8000 glucose units), high crystallinity (70%–80%) and mechanical stability. The BC high water content and purity make the matrix biocompatible for multiple medical applications, particularly for tissue engineering and skin wound healing [4, 5]. However, native BC has no antimicrobial activity. In the last decade, BC composites showing biological activity were explored particularly for skin repair and tissue engineering. The most common polymers to tailor BC scaffolds are alginates, hyaluronic acids and gelatins. The addition of polymers to BC confers novel and interesting properties to the scaffolds including mucoadhesivity and the ability of being a molecular carrier [5, 6].

The interest in silver as an antibacterial agent, used since ancient times, was recently rediscovered by research community and extensively studied in the last ten years [7, 8]. The main advantages of silver devices are based on their low toxicity to humans, broad antimicrobial spectrum and low probability to produce bacterial resistance compared to traditional antimicrobials. In fact, silver alone or combined with other molecules is currently used for controlling bacterial growth in a variety of applications, including dental implants, catheters, skin creams and clothes [9]. The strong silver biocide activity was attributed to several antimicrobial mechanisms such as damage of cell scaffold proteins, blocking of RNA transcription, disruption of DNA binding and replication [10].

Metallic silver nanoparticles with a diameter between 20 nm and about 110 nm showed different cytotoxic values, but in all cases cellular and/or inflammatory response and/or genotoxicity in mammalian cells were reported [11, 12] The toxicity of metallic silver nanoparticles was associated with the cellular uptake of Ag^{+1} acting inside the cells [13]. Also, the toxicity of the metallic silver nanoparticles was inversely proportional to de diameter of the nanoparticles. Metallic silver nanoparticles of low dimensions (i.e., 10 nm diameter) were much more toxic than particles of large diameters [11, 13].

Silver phosphate microparticles (AgP-MPs) developed in our laboratory are in the size range of 1.3 μm to 1.5 μm (with a distribution higher than 90% of the microparticle population) and at least 14 times bigger than metallic silver nanoparticles but with similar bactericidal effect [14]. Another advantage of AgP-MPs is that they are a mesoporous material with the ability to absorb and contain molecules with different molecular weights and physicochemical properties keeping their biological activities. The tuning of mesoporous structures determines the interactions within the loads and consequently, the controlled release kinetics [15].

Hybrid devices based on silver salts and polymers could improve the antimicrobial activity against pathogens, but only few reports can be found in the literature and the mechanisms are not fully understood [16, 17]. Among biopolymers, alginate, cellulose, pectin and others were lately reported as potential “green carriers” for drug delivery [18]. Natural polymers provide excellent platforms for molecular loading and release because of their gelling properties, GRAS status by FDA, and some of them can be considered smart molecules since they are responsive to environmental conditions (e.g., pH, temperature, ionic strength).

Fluoroquinolones comprise a large family of antibiotics widely used for the treatment of microbial infections in mammals because they inhibit DNA gyrase and topoisomerase IV present only in bacteria and cause microbial cell death. The second-generation fluoroquinolones such as ciprofloxacin (Cipro) were considered the worldwide battle horse for the treatment of bacterial infections. Besides, the oral administration of Cipro requires high antibiotic doses because of low antibiotic solubility and the trend to π-stacking causing low drug bioavailability and gastrointestinal associated problems. The encapsulation of fluoroquinolones seems to be an alternative to reduce the undesirable secondary effects and extend the drug release. Particularly, gel structures have been reported to be efficient for the encapsulation of fluoroquinolones increasing antibiotic efficiency, reducing the antibiotic dose, and extending the antimicrobial activity of the antibiotic [19, 20]. In previous work in our laboratory, mesoporous AgP-MPs were developed for the encapsulation of levofloxacin showing high drug encapsulation efficiency, high surface area, small pore size, and high bactericidal activity [14].

The aim of the present work was to develop bacterial cellulose scaffolds containing a dual antimicrobial activity conferred by self-assembly of silver phosphate microparticles and ciprofloxacin for potential application in skin wound/burn dressing. The BC systems were characterized by infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), X-ray diffraction (XRD) and scanning electron microscopy (SEM). Ciprofloxacin loading on BC scaffolds and kinetic release from the matrices were analyzed. Antimicrobial assays were carried out with *Escherichia coli* (Gram(−) bacteria) and *Staphylococcus aureus* (Gram(+) bacteria), the main causative agents of skin infections.

2. Materials and Methods

2.1. Chemicals and Media

All reagents used were of analytical grade purchased from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or local suppliers. Deionized water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic) and further purified by using MilliQ Direct QUV apparatus equipped with a UV lamp.

2.2. Bacterial Cellulose

The synthesis of bacterial cellulose (BC) by *Komagataeibacter hansenii* (ATCC 23769) was performed in a medium containing ($g\,l^{-1}$): 25.0 mannitol, 5.0 yeast extract, 3.0 peptone, and adjusted to pH = 6.5 with 0.1 M NaOH solution before sterilization. The culture was maintained statically in 96-well plates at 30 °C for 6 days. The BC membranes were collected from the plates and washed with distilled water. BC purification was performed by incubating the scaffolds in 100 mM NaOH at 50 °C for 24 h followed by successive washes with distilled water and adjustment of the pH to 7.0. Later, the BC membranes were sterilized by autoclaving (121 °C for 20 min).

2.3. Synthesis of Silver Phosphate Microparticles in BC Scaffolds

The BC system containing nanostructured micro-hybrid particles (AgP-Ms) was developed by colloidal crystallization in the presence of sodium tripolyphosphate (Na-TPP) as follows: BC membranes were immersed in a vial containing 60 mM sodium tripolyphosphate ($Na_5P_3O_{10}$) at 25 °C for 20 min under low speed stirring. Later, a solution containing 100 mM $AgNO_3$ was poured into the vial, and stirred at 250 rpm for other 20 min. Each BC film was washed twice with distilled water.

2.4. Infrared Spectroscopy (FTIR)

BC membranes were examined in an infrared spectrometer (Thermo Scientific Nicolet 6700) equipped with attenuated total reflection (ATR). The samples were analyzed at room temperature in the 400–4000 cm^{-1} spectral range with 4 cm^{-1} resolution and 32 scans.

2.5. X-Ray Diffraction (XRD) Analysis

The crystal diffraction patterns of dried scaffold samples were collected using Analytical Expert Instrument (Philips 3020, The Netherlands) using CuKα radiation (1.54 Å) and scans in the 2Θ range 0° to 60° with 0.04 step size and 1.00 seg/s/step at room temperature. The generator voltage and the current were set at 40 kV and 35 mA respectively at room temperature.