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Ephemeral biogels to control anti-biofilm agent delivery: From conception to the construction of an active dressing

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ABSTRACT

Chronic wound colonization by bacterial biofilms is common and can cause various complications. An anti-biofilm strategy was developed around the co-entrapment of a commercially available antiseptic, PHMB (polyhexamethylene biguanide 4 mg mL^{-1}), with EDTA (Ethylen diamine tetra acetic acid, 20 mM) in a gelatin gel. The two active compounds act synergistically against bacterial biofilms, but their efficiency is strongly reduced (16-fold) when entrapped inside the 5% gelatin gel, and they weaken the mechanical properties (50-fold) of the gel. Increasing the gelatin concentration to 7% allows for good mechanical properties but large diffusional constraints. An active ephemeral gel, a chemical gel with controlled hydrolysis, was conceived and developed. When the ephemeral gel was solubilized after 48 h, PHMB delivery increased, leading to good anti-biofilm activity. The various gels were examined over 24 and 48 h of contact with *P. aeruginosa* and *S. aureus* biofilms, two types of bacterial biofilms frequently encountered in chronic wounds. The ephemeral gel eradicated the dense biofilms ($> 6.10^7$ CFU·cm^{-2}) produced by either single or mixed strains; a similar efficiency was measured for biofilms from strains of both laboratory and clinical origin. The formulation was then adapted to develop a dressing prototype that is active against biofilms and fulfils the requirements of an efficient wound care system.

1. Introduction

An ageing population and the increase in diabetes has led to an increase in the number of people bearing chronic wounds, creating a serious public health problem [1,2]. A chronic wound induces pain, nauseous odour, risk for amputation and patient isolation. Unfortunately, wound management is complex, particularly for biofilm-contaminated wounds, which reveal a remarkable resistance to antibiotics [3–5]. To eradicate biofilms, four strategies have been developed: prevention of bacteria aggregation, inhibition of their defence system, disruption of the biofilm, and elimination of biofilm bacteria [6]. Some antiseptics are efficient against biofilm bacteria, and their activity may be improved by various molecules that disturb biofilm organisation. Previously, we developed an anti-biofilm strategy that combines the destruction of bacteria and the disruption of the biofilm matrix using a mixture of Prontosan®, Ethylen diamine tetra acetic acid (EDTA) and Esperase® in solution, which led to a 99.999% and 99.76% decrease in dense *P. aeruginosa* and *S. aureus* biofilm populations ($> 6.10^7$ CFU·cm^{-2}), respectively, after 24 h of contact.

To care for a chronic wound, dressings are required. Thus, there is a need to develop a scaffold that can entrap and release active compounds and easily be adapted to a dressing. Until now, few biomaterials have been described that efficiently deliver anti-biofilm agents [7].

Hydrogel dressings play an important role in wound management, as they control moisture balance and promote the healing process. In addition, hydrogels are also frequently used for molecular delivery, as they can entrap a large variety of molecules. Thus, we prepared a gel containing an antiseptic, EDTA and an enzyme to eradicate biofilms.

Gelatin gels, with the use of enzymes, were chosen because various networks can be obtained, resulting in different gel properties [8], and because they have already been adapted for controlled molecular delivery [9]. Moreover, the entrapment of various molecules is easily achieved by adding the molecules to the solution mixture prior to gelatin crosslinking.

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In addition, gelatin is a good precursor for the generation of ephemeral gels, which are remarkable tools for accomplishing the delivery of both small and large molecules [10]. These particular gels have network properties that evolve with time so that diffusion can be controlled; moreover, they can be made into dressings [11].

The aim of the present study is to create a biogel system that entraps and releases various agents in order to eradicate bacteria organized in biofilms.

Prontosan®, a commercial preparation, has been shown to be efficient against biofilms of both Gram negative and Gram positive bacteria. In the present study, we have chosen to use polyhexamethylene biguanide (PHMB), one of the two components of Prontosan®, as the active reagent. It interacts with bacterial membranes and modifies the membrane permeability, leading to cellular death [12]. PHMB is biocompatible and shows a low cytotoxicity on skin; dermal exposure to a dose of 5 g kg^{-1} showed no sign of acute toxicity in rats [13].

The chelating agent, EDTA, targets metalloprotease inhibition and contributes to disrupting the cellular membrane or potentiating antibacterial actions [14–16].

A non-specific serine protease without antibacterial activity, Esperase®, induces a strong disruption of the biofilm matrix with a significant release of matter [17]. This enzyme can also participate in wound debridement [18]. Finally, a serine protease is also suited to turn a gelatin gel into an ephemeral gel.

In the present study, an anti-biofilm strategy was developed around the co-entrapment of a commercially available antiseptic, PHMB, with EDTA in a gelatin gel. The mechanical properties of such a gel were optimized and its efficiency for eradicating biofilms was evaluated. Then, to increase their efficiency, PHMB and EDTA were co-entrapped in ephemeral gels, chemical gels with controlled hydrolysis by a protease, and their diffusion from the gel was measured and improved. The ephemeral gels were tested on monospecies biofilms from *Pseudomonas aeruginosa* and *Staphylococcus aureus*, then on mixed biofilms of both strains. As the developed treatment appeared able to eradicate all biofilms from strains of both laboratory and clinical origin, the ephemeral gel was turned into an active dressing taking into account the requirements of an efficient wound care system.

2. Materials and methods

2.1. Bacteria and growth conditions

Both of the used strains, *P. aeruginosa* CIP 103 467 and *S. aureus* CIP 4.83, were purchased from Institut Pasteur, Paris. They were stored at − 20 °C in a culture medium containing 30% glycerol. *P. aeruginosa* was grown in LB broth Lennox (Difco) at 37 °C, and *S. aureus* was grown in TS Bacto™ soy broth, Lennox (Difco) at 37 °C.

Two strains of *P. aeruginosa* and *S. aureus* were also extracted from a mixed biofilm evidenced in a chronic wound (collected by Dr. I. Fromantin, Institut Curie, France, in the common study ANR-10-TECS-019). These strains are referred to as “clinical strains”.

Bacteria precultures were grown in TS broth (Difco) for *S. aureus* and in LB broth (Difco) for *P. aeruginosa* at 37 °C without shaking and were then calibrated in the appropriate medium.

2.2. Chemicals

All solutions were prepared in 50 mM Tris buffer at pH 7.4.

PHMB was obtained from Pareva, France. Esperase® was obtained from Sigma (P5860). Type B gelatin, extracted from ox bone using an alkaline process, was provided by Rousselot (103-10-51) and presented a bloom of 240, which means a rather high strength for a standard (6.67%) gelatin gel.

2.3. Preparation of chemical and ephemeral gelatin gels

Gelatin solutions containing PHMB and EDTA at the concentrations mentioned in the text were prepared at 40 °C. Transglutaminase was added to the solution, and the gelation was maintained at 37 °C for 2 h. The final concentrations were either 5 or 7% (w/V) gelatin and 1 U mL^{-1} transglutaminase. The obtained gels are referred to as “chemical gels”.

To obtain the ephemeral gels, Esperase®, at the desired concentration, was added to the mixture prior to transglutaminase.

Gelatin gels were cut into 14 mm diameter and 3 mm thick pastilles.

2.4. Biofilm assay

Biofilms were formed on 14 mm glass coverslips. Bacterial cells from the overnight cultures at 37 °C were re-suspended in the culture medium to reach an optical density ($OD_{595\ nm}$) of 0.001. Then, 24-well plates (Polystyrene Clear Plates, Corning) containing glass slides were inoculated with the bacterial suspension (1 mL per well) and incubated at 37 °C for 24 h. The wells were rinsed twice with a sterile saline solution ([NaCl] = 9 g L^{-1}). The gels containing the antimicrobial agents were then brought into contact with the biofilm for 24 h or 48 h at 37 °C.

2.5. Plate counting procedure after treatment

After rinsing twice with saline solution, the biofilms were detached from the glass coverslips by 2 cycles of gentle sonication for 10 min in two 500 µL portions of saline solution. The supernatants were collected and sampled. The samples underwent a series of seven dilutions from 1 to 10^{-7}. Each diluted suspension was spread on agar and incubated for 24 h at 37 °C. Agar gels were counted, and the bacteria concentration is expressed as CFU·cm^{-2}. A non-specific agar gel was used for total bacteria counting (PCA), and a cetrimide agar gel and a Baird Parker agar gel were used to evaluate the exact number of *P. aeruginosa* and *S. aureus* colonies, respectively.

2.6. Rheology

Rheology measurements were performed using an Anton Paar MCR301 rheometer, operating in the oscillatory mode with a strain of deformation of 1%, a frequency of 1 Hz and a 50 mm plane/plate geometry at constant temperature (37 °C). The storage modulus G’ and loss modulus G” were recorded as a function of time. The gelling time was estimated when $G' = G''$.

For ephemeral gels, the resolubilization time was determined to be the time when $G' < 1$.

2.7. Antimicrobial diffusion on agar

Bacterial cells from the overnight cultures at 37 °C were re-suspended in culture medium to reach an optical density ($OD_{595\ nm}$) of 0.001. Then, the suspension was spread on PCA agar. A pastille of gel containing the antimicrobial agents was placed in the centre of the agar gel. After 24 h at 37 °C, the diameter of the inhibition ring was measured.

2.8. PHMB release

Gelatin gels (200 µL) containing PHMB at various concentrations were directly formed in 2.5 mL cuvettes. After 2 h storage at 37 °C to allow gel formation, the gels were covered with 2 mL of buffer and continuously agitated at 37 °C. PHMB diffused in a unidirectional manner from the bottom of the gel to the upper liquid phase. At regular intervals, PHMB diffusion was measured as follows: a 460 µL sample was added to 40 µL of sodium acetate (0.1 g L^{-1}), 100 µL of eosin