CONCEPTION AND ELABORATION OF BIOGELS TO DELIVER ANTI-BIOFILM AGENTS

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Over the past 15 years, the impact of biofilms on persistent infections and their potential role on chronic wounds have been extensively documented. However, up to now, no efficient system to deliver anti-biofilm agents has been described. The aim of this study is to conceive a "smart" dressing against biofilms in chronic wounds. We developed an innovative biogel system containing various anti-biofilm agents to improve eradication of biofilm pathogenic bacteria. The anti-biofilm strategy consisted in preventing bacterial colonization, disrupting the biofilm and eradicating pathogen bacteria (< 10^3 CFU/mL). Combination of PHMB (54 mg/mL), an antiseptic agent and EDTA (10mM) a cation chelating agent, eradicated *P. aeruginosa* and *S. aureus* biofilms. These anti-biofilm agents were entrapped in gelatin gels which have the capacity to deliver molecules with controlled release. This combination in the biogel affected *in vitro* biofilms depending on the gelatin and antiseptic concentrations. The use of ephemeral gels, where the gelatin network hydrolysis by enzymes was programmed and timed-controlled, permitted to stimulate the agent release and enhanced our results on various biofilms. Chronic wounds are a common and expensive problem in public health. Gelatin biogels have a great potential to entrap and deliver antibiofilm agents over a longer period and at smaller concentrations than the current wound care treatments.

Keywords: biogel, anti-biofilms, drug delivery.

INTRODUCTION

Many pathologies and age may deregulate healing, leading to chronic wounds which do not heal within 6 weeks. The curing duration is depending on the weakness of the patient (Menke, 2007). Moreover, chronic wounds are very sensitive to bacterial colonization which can evolve to infection.

In recent years, the presence of biofilms on persistent infections and their potential negative role on wound healing have been extensively studied (Steven, 2004). Biofilms are structured communities of microorganisms surrounded by a polymeric matrix; they are found on a wide range of biotic and abiotic surfaces. The biofilm extracellular polymeric matrix contains different types of exopolymers such as polysaccharides, proteins, DNA and lipids (Flemming, 2010). Biofilms seem to be associated to a lengthened inflammatory phase (Singh and Barbul, 2008). The main clinical problem of biofilm-associated infections is the treatment failure due to the high resistance level to antibiotics and other antimicrobial drugs. Nowadays, the current health care strategies against biofilm infected wounds are still poorly developed (Ammons, 2010).

As presented here, we conceived and elaborated a biogel system containing various anti-biofilm agents (fig.1) to improve eradication of biofilm pathogenic bacteria and prevent wounds against a pathogenic bacterial colonization.

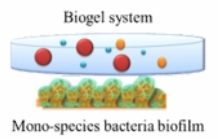

Figure 1. Anti-biofilm strategy

The originality of our system consisted in the combination of two different types of molecules: an antiseptic usually applied in chronic wound care and a chelating agent active against MMPs which are over-expressed in chronic wounds. Several compounds were tested. We selected respectively PHMB (PolyHexaMéthylène Biguanide) which is one of the active compounds of Prontosan®, an antiseptic solution with anti-biofilm properties commercialized by B. Braun and EDTA, a divalent cation chelator known to have activity against biofilms for a large range of bacteria (Banin, 2006).

EXPERIMENTAL METHODS

Materials

Gelatin was provided by Rousselot (103-10-51). It is extracted from ox bone through an alkaline process (pI 4.7). The enzymes used in this study are a serine protease, Esperase (P5860 – Sigma) and a Tranglutaminase from microbial origin, produced by Ajinomoto. PHMB is a solution provided by Pareva.

Both strains, *Pseudomonas aeruginosa* CIP 103 467 and Staphylococcus aureus CIP 4.83 were provided by Institut Pasteur, Paris. *Pseudomonas aeruginosa* was grown in LB broth (Lennox) and *Staphylococcus aureus* in TS bacto™ soy broth (Lennox) both at 37°C.

Preparation of Gelatin Gel

Gelatin powder was solubilized in Tris –HCl buffer 50mM pH 7.4 at 40°C for 30min. The solution was composed of 5 or 7% of gelatin (W/V) and 1.5 U/mL of transglutaminase (final concentration). Various molecules can be added into the solution so as to be finally entrapped in the gel. Gelation was performed at 40°C. The obtained gel was irreversible with temperature.

Rheology

Rheology measurements were performed using a Rheostress Anton Paar MCR301, operating in the oscillatory mode with a deformation strain of 1% and a frequency of 1 Hz. A 50 mm plate/plate geometry was used. The storage modulus G' and loss modulus G'' were recorded as a function of time. Temperature (37°C) was controlled using a Peltier device.

Biofilm Formation and Bacteria Assay

Bacteria were suspended overnight at 37°C and re-suspended in culture medium at 0.001 DO_{595nm}. The 24-well plates containing 14 mm-diameter glass slides were inoculated with the bacterial suspension (1mL/well) and incubated at 37°. The culture medium was changed every 12h. After 24 hours of biofilm growth, the wells were rinsed twice with saline solution ([NaCl] = 9 g. L-1). The solutions or gel to be tested were then brought into contact with the biofilm for 24h at 37°C. The viable biofilm bacteria were quantified on agar after glass slide sonication to detach the biofilm. The control was a medium solution diluted in saline solution with the same ratio as the one used for the treatment.

RESULTS AND DISCUSSION

Gelatin Gel Properties

Gel Formation

A gel is a soft matter composed of a liquid phase entrapped in a polymer network. Gelatin has the capacity to form a hydrogel which aqueous phase represents 95% of the gel mass. A 5% (W/V) gelatin solution may spontaneously turn to a gel, but only below 30°C. At the same protein concentration, a chemical gel may be formed at 37°C by the action of transglutaminase, an enzyme, which forms covalent bonds between lateral chains of the protein.

Rheological measurements (fig.2, white symbols) evidenced a viscoelastic behavior; the gel reached an elastic modulus, G', of 800 Pa after 5h. The gelatin solution turned to a gel only 9 min after the transglutaminase addition (G' ≥G'').

Viscoelastic Properties

The gelatin gel has the capacity to entrap molecules in its aqueous phase. The aim of this study was to conceive a drug delivery system from this gel. However, encapsulation of antiseptic agents decreased the mechanical properties of the gel (fig. 2). So, the formulation was adapted by increasing the gelatin concentration to 7% (W/V) to obtain a handeable gel (G'/G''>10).

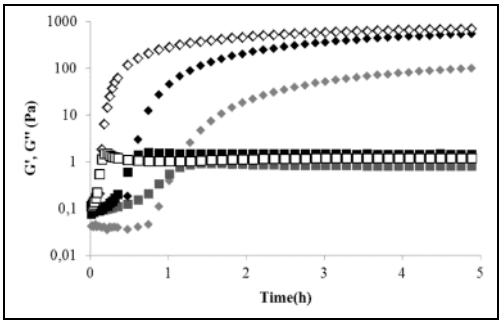

Figure 2. Rheological properties of a chemical gelatin gel over time. The gel was tested with (grey and black symbols) and without (white symbols) 4 mg/mL of PHMB for a gelatin concentration of 5% (white and grey symbols) or 7% (black symbols). G' is represented by diamonds and G'' by squares.

The results highlighted the influence of PHMB on the mechanical properties of the gel. After 5h (fig.2, compare white and grey symbols), the 5% gelatin gel entrapping PHMB was 7 times less elastic than the control gel. Furthermore, gelation was delayed as the gel time (time where G'=G'') was increased 7 times. Using a more concentrated gelatin solution allowed to reduce the gel time and obtain satisfactory elastic properties. The addition of EDTA at the used concentration (20 mM) did not disturb the gel formation and properties (data not shown).

Diffusion of Molecules from the Gel

Influence of Molecules Properties

Molecules entrapped into a gelatin gel diffuse to the external environment according to their and gel properties. To visualize and predict diffusion, some molecules were used with different charges and weights. The anti-biofilm strategy consisted in delivering a treatment on a wound potentially contaminated by a biofilm. This biofilm can be assimilated to a soft gel. So, our experimental diffusion model was a gelatin gel entrapping molecules layed on a soft alginate gel. The diffusion from one gel to the other was studied over time.

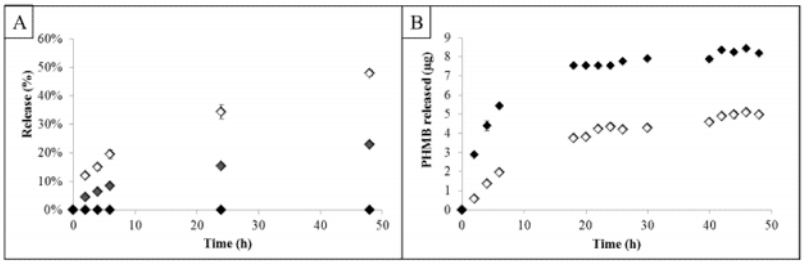

Figure 3. Diffusion kinetics of molecules from a 7% gelatin gel over time: [A] Diffusion kinetics from a gelatin gel to a 1% alginate gel of molecules of various molecular weights: 300Da methylene blue (white symbols), 70kDa dextran (grey symbols) and 2,000kDa blue dextran (black symbols). [B] Diffusion kinetics of PHMB at 4 mg/mL (white symbols) or at 8 mg/mL (black symbols) (98 and 196µg respectively per gel) from a gelatin gel to a solution.

The diffusion kinetics of molecules is theoretically dictated by diffusion laws: the release depends on the molecule size and charge as well as on gel network structure (data not shown). This was verified with our two gels; the smaller the molecule was, the faster it diffused from one gel to another (fig.3[A]). But, when the molecule size was larger than the mesh size of the gel network, the molecule was blocked into the gel, (see blue dextran behavior of fig.3[A]).

The antiseptic chosen in the project is PHMB, a small size molecule. Its diffusion from the gel should be fast. Nonetheless, only 4% of the initial PHMB concentration included in the gel diffused in the surrounding solution within 24h (fig.3[B]). The drug seemed to be retained in the gel. This can be explained by the positive charge of PHMB at physiological pH, which is opposite to the gelatin network charge. Thus it diffusion to a gel or a biofilm could be limiting.

Effect of Anti-Biofilm Agents

PHMB and EDTA Effects on Biofilm

Some studies suggest that combining EDTA with an antibiotic can enhance its activities against bacteria [Lambert (2004)]. The effect of combined PHMB and EDTA was thus tested on biofilms of *P. aeruginosa* and *S. aureus*, two pathogenic bacteria frequently encountered in chronic wounds. In a first step the activity of antiseptic

molecules in solution was measured. The number of viable bacteria was calculated after 24h of treatment on the biofilm.

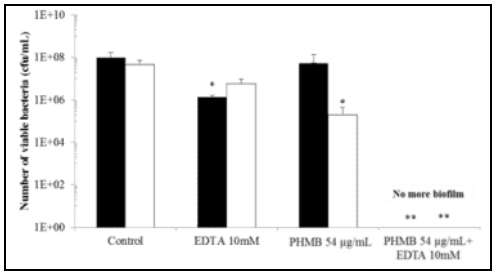

Figure 4. Anti-biofilm effect of EDTA and PHMB on biofilm of *P. aeruginosa* (black bars) and *S. aureus* (white bars)

From these results (fig.4), it was obvious that the addition of EDTA potentialized the effect of PHMB. EDTA was more efficient on *P. aeruginosa* biofilm than on *S. aureus* with a decrease of the biomass higher than 2 logarithms. PHMB alone showed no effect on *P. aeruginosa* biofilm and a reduced effect on *S. aureus* biofilm while, at the same concentration, it was totally bactericidal on planctonik bacteria of both strains (data not shown). However, a very high anti-biofilm effect (> 99.999%) was obtained with PHMB combined to EDTA, showing a synergistic effect of the two compounds. Then, the active compounds were entrapped into a gel.

Anti-Biofilm Effect of the Gel

Entrapment of PHMB with EDTA in a cross-linked 7% gelatin gel was possible (data not shown). Even if the elastic properties are weak when a high antiseptic concentration is used (G' = 100 and 50 Pa, after 5h of gelation, for respectively 4 and 8 mg/mL of PHMB), the gels were still handleable. To test the anti-biofilm effect, these gels were brought into contact with the biofilm during 24h.

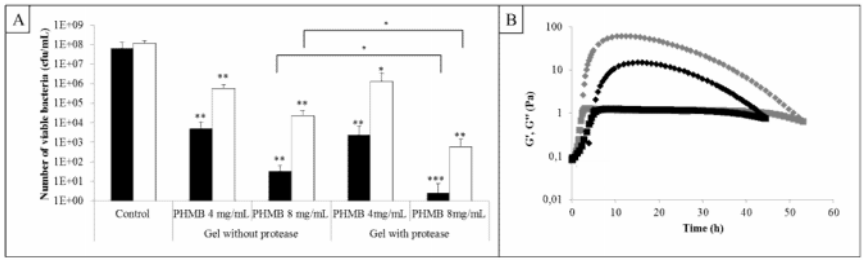

Figure 5. Anti-biofilm effect of the 7% gelatin gel containing 10mM EDTA and PHMB with or without protease: [A] Biomass evaluation after 24h of treatment entrapped in a gelatin gel containing or not a protease. *P. aeruginosa* is represented by black bars and *S. aureus* by white bars; [B] Rheological properties of a gelatin gel containing 4 mg/mL (grey symbols) or 8 mg/ml (black symbols) of PHMB, EDTA and a protease. G' is represented by diamonds and G'' by squares.

The treatment entrapment in a gel decreased molecule efficiency on the biofilm (compare fig.4 with fig.5 [A]). Indeed, the PHBM concentration had to be increased from 54µg/mL to 4 and 8 mg/mL to induce a significant biomass reduction highlighting a high sensitivity of *P. aeruginosa* biofilm to active molecules. This sensitivity was 60 and 400 times higher than that of *S. aureus* for respectively 4 and 8 mg/mL (fig.5[A]). However, the biofilms were not totally eradicated in these conditions.

The aim of the project was to reach a biomass reduction of 5 logarithms; limit not reached with the treatment on the *S. aureus* biofilm. As shown on figure 3[B], the gelatin network disturbed active molecule diffusion. To improve the results, the gel formulation had to be optimized. An innovative drug delivery system, developed in ERRMECe laboratory (Klak, 2012), allows a gelatin gel formation followed by a controlled-time resolubilization, leading to enhanced molecular release; this ephemeral gel is named Enzgel. This gel is based on the action of two antagonistic enzymes, one generating and the other cleaving covalent bonds, respectively a transglutaminase and a protease. Thus, a protease, Esperase, was added to the gelatin solution containing the treatment; a gel was first formed which later re-solubilized (fig 5[B]). The protease decreased the elastic properties and increased gelation time; but the gel was still handleable (G'/G'' >10). The network hydrolysis was complete after 51h and 41h for respectively 4 and 8 mg/mL of entrapped PHMB. This system promoted molecule diffusion from the gel to the surrounding medium allowing their anti-biofilm activity to express (fig.5[A]). For 8 mg/mL of PHMB, the biomass was reduced by 5 logarithms for *S. aureus* biofilm and by 7 logarithms for *P. aeruginosa* biofilm, an improvement of 40 and 11 times compared to the gel without protease. The efficiency rates fixed in the project were thus fulfilled.

CONCLUSION

In the United States, 8 million people are affected by chronic wounds which lead to a cost of 20 billion dollars per year. The presence of biofilm may extend the healing delay. Our strategy to limit or eradicate biofilms was based on two major points: destabilize the biofilm and its matrix; eliminate pathogenic bacteria by the use of an antiseptic.

The use of EDTA enhanced the PHMB effect on biofilm. The great potential of gelatin gel to entrap and deliver agents over a longer period was used. The use of an ephemeral gel, where gelatin network hydrolysis was programmed and time-controlled, allowed stimulating the molecule release and enhance treatment activity against biofilms. Finally an efficient system, able to control the bacteria level in various biofilms was conceived and elaborated.

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