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Bacterial protease triggered release of biocides from microspheres with an oily core

Marina Craig[a,b,c,*], Mona Amiri[a,1], Krister Holmberg[a,c]

[a] Chalmers University of Technology, Chemical and Biological Engineering, Applied Surface Chemistry, Gothenburg, Sweden
[b] Mölnlycke Health Care, Gothenburg, Sweden
[c] SuMo Biomaterials, VINN Excellence Centre, Chalmers University of Technology, Gothenburg, Sweden

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ABSTRACT

This study deals with controlled release of drugs to a *Staphylococcus aureus* infected site from microspheres with an oily core and a polymeric shell. The intended use of the microspheres is for chronic wounds and the microspheres may be administered in the form of a wash liquid or incorporated in a gel. Chronic wounds often carry infection, and the use of microspheres with drug release triggered by the bacterial infection is therefore of interest. A lipophilic drug or a model of the drug was dissolved in an oil and the oil phase was dispersed into an o/w emulsion. A nanofilm shell was then assembled around the oil droplets with the layer-by-layer technique using the two biodegradable polypeptides anionic poly-L-glutamic acid (PLGA) and cationic poly-L-lysine (PLL). Since *S. aureus* exudes proteases such as glutamyl endopeptidase (V8) during colonization and infection, its substrate specificity was key when assembling the nanofilm. Since V8 is known to be substrate specific to the Glu-X bond, PLGA was chosen as the terminating layer of the nanofilm. Crosslinking the nanofilm after assembly lead to increased stability of the microspheres. It was shown that in a non-infectious environment, *i.e.* when a human wound enzyme, HNE (human neutrophile elastase), was present, the microspheres remained intact. The staphylococcal protease V8, on the other hand, readily catalyzed degradation of the microspheres, thus releasing the drug when triggered by the infectious environment.

1. Introduction

Due to underlying causes such as diabetes, obesity, venous insufficiency, immobility, *etc.*, wound healing can be severely delayed, which means that a so-called chronic wound may develop. A balance between human proteases and protease inhibitors is needed for a wound to heal; however, in a chronic wound, the balance is impaired [1]. Due to an increased amount of proteases the pH of the chronic wound increases, which makes it a favorable environment for bacterial colonization [2–4]. Thus, bacterial infections often develop in chronic wounds, which in turn increase the pH further [4,5]. Treatment of such wound infections is not trivial because excessive use of bactericides can be harmful for the wound healing process. There is therefore a need for an 'intelligent' administration of the active substance, *i.e.* some kind of infection triggered delivery. The inflammation phase is part of the healing cascade, a stage which also can be caught in a loop causing chronic inflammation in the wound. There is consequently an opportunity for combination therapy. Discussions regarding inflammation in chronic wounds mainly caused by the bacterial biofilm are ongoing [6]. Wound debridement to remove bacterial biofilm combined with topical treatment of antimicrobial drugs has been suggested as an alternative to combining antibacterial and anti-inflammatory drugs as a way to combat both infection and inflammation [7].

Microspheres with an oily core and a polymeric shell can be used for distributing lipophilic substances in an aqueous medium [8–10]. Such microspheres can have a wide variety of applications and they are of particular interest for controlled release of actives, *e.g.* biocides in paints [11], additives in food, pharmaceuticals, *etc.* [12]. Tunable features such as environment responsive shells can trigger release of substances by *e.g.* chemical, biological or thermal changes [13–15]. The microspheres are of particular interest for easily hydrolysable active substances, which need to be protected from water contact [12,16]. By using a polyelectrolyte as

* Corresponding author at: Applied Surface Chemistry, Chemical and Biological Engineering, Chalmers University of Technology, SE-41396 Gothenburg, Sweden. Tel.: +46 31 772 2955; fax: +46 31 160062.
E-mail addresses: marina.craig@chalmers.se, marina.craig@molnlycke.com (M. Craig).
[1] Present address: Arboritec AB, Olof Wiks väg 9, SE-44465 Jörlanda, Sweden.

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emulsifier, the layer-by-layer (LbL) assembly technique can be used to create a shell around the oil droplets of water in oil (w/o) emulsions, thus increasing the lifetime of the dispersed system. The LbL technique was pioneered by Decher [17,18], who provided a simple method for creating thin films of different materials. Further, incorporation of microspheres into gel matrices allows for stabilization and partial immobilization of the spheres. Such a formulation does not necessarily have to rely on diffusion inside the gel for drug release. Degradation of the surrounding gel and exposure of the microspheres could be initiated either by an increase in temperature [19], a pH change [20], addition of antibodies [21] or by a combination of triggers [22]. Due to the increased pH and temperature in an infected chronic wound, both these parameters may serve as triggers for degradation of the gel.

In this work the LbL technique was used to make a nanofilm around droplets of an o/w emulsion that contains a bactericidal compound in the core. Microspheres were prepared by the o/w emulsion pathway with a water sensitive bactericide, or a model for such a substance, incorporated into the oily core. The surrounding film, *i.e.* the shell of the microcapsule, was a nanofilm consisting of two biocompatible and biodegradable polypeptides, poly-L-lysine (PLL) and poly(L-glutamic acid) (PLGA). As the endoproteinase V8 (glutamyl endopeptidase) is an exoproduct from *Staphylococcus aureus*, and more so, substrate specific to Glu-X and Asp-X bonds, PLGA was chosen as the terminating layer of the nanofilm. A dispersion of the microspheres was exposed to the V8 enzyme and also to a natural human wound enzyme, and the stability of the microspheres, as well as the release of the incorporated substance, in the two environments was assessed. For the concept to be of practical importance, the microspheres should remain intact when exposed to the human wound enzyme but degrade when exposed to the enzyme from *S. aureus*. The release should be relevant to the bacterial load in the wound, an approach that requires a burst release mechanism when exposed to bacterial enzymes.

2. Materials and methods

2.1. Materials

The oil phase consisted of either corn oil or a 1:1 (per volume) mixture of corn oil and glyceryl trioctanoate (GTO) (Sigma–Aldrich, Sweden). Tetradecyl betainate, *i.e.* the tetradecyl ester of betaine, was kindly provided by Dr. Dan Lundberg, Colloidal Resource (Sweden) and used as active substance (0.1–1.0 wt%) dissolved in the pure GTO oil phase. Nile red (Sigma–Aldrich, Sweden) was used as model substance and was dissolved (0.01 wt%) in the 1:1 mixture of corn oil and GTO. For emulsification and LbL assembly, 1 mg/mL solutions of poly-L-glutamic acid (PLGA) of MW 120 kDa and poly-L-lysine (PLL) of MW 84 kDa were used (Alamanda Polymers, USA). The polypeptides were dissolved in 0.1 mM Tris–HCl buffer (Sigma–Aldrich) with 0.1 M NaCl (Sigma–Aldrich) added. For crosslinking of the multilayer structure 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was used together with N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Sigma–Aldrich) at concentrations of 25 mM/25 mM, 50 mM/25 mM 100 mM/25 mM and 200 mM/50 mM, respectively. For release tests, isopropyl myristate (IPM) (≥98%, Sigma–Aldrich) was used as medium outside a solvent resistant dialysis tube with pore size 8–10 kDa (Spectrum Laboratories, USA). 10 μg/mL glutamyl endopeptidase (V8) of MW 30 kDa originating from *S. aureus* (specific activity = 2.0 U/mg, isoelectric point (IEP) = 5.5) and 5 μg/mL human neutrophil elastase (HNE) of MW 29.5 kDa (specific activity = 30 U/mg, IEP = 9.7–10.5) were purchased from BioCol GmbH (Germany). V8 was diluted into a concentration of approximately 1:50 (enzyme to sample ratio). HNE was diluted into a ratio of 1:8. Both enzymes were used within 15 min of preparation. All water used in this study was Milli-Q water (resistivity ≥ 18 MΩ cm^{-1}).

2.2. o/w emulsion, LbL and crosslinking

The o/w emulsion was made by using a Heidolph Silent Crusher with a high shear homogenizer Heidolph TYP 22 F/M (Heidolph Instruments, Schwabach, Germany), with PLGA as emulsifier. The emulsion was washed three times by centrifugation using Beckman Avanti J-20xp at 10,000 rpm for 1 h (Beckman Coulter, Sweden). The emulsion was added dropwise to a solution of a polypeptide of opposite charge to the previous layer for LbL adsorption and nanofilm build-up. Centrifugation and LbL adsorption were repeated until three or five layers were assembled. The coupling agents, EDC and sulfo-NHS, were added to the emulsion for partial crosslinking and the solution was left overnight at room temperature. The o/w emulsion was inspected with a light microscope (Axio Scope.A1, Zeiss, Germany) after each LbL step and the crosslinked microspheres were inspected by confocal laser scanning microscopy (CLSM) (Leica TCS SP RS, Wetzlar, Germany) using a Plan APO 20× magnification 0.7 NA dry air objective lens. The surface charge was measured after each added layer using a Malvern Zetasizer Nano Range (Malvern Instruments, UK).

2.3. Enzymatic degradation and release studies

V8 enzyme was added to the suspension of microspheres containing Nile red in the oily core and the dispersion was studied with both light microscopy and CLSM at 32 °C. The same procedure was performed for the HNE enzyme. A dialysis membrane with a magnetic clip at the bottom was used for V8-induced release studies during stirring (32 °C). The surrounding release medium was IPM, since the solubility of Nile red in water was extremely low. A standard curve for Nile red in IPM was created using the adsorption peak at 522 nm and the release of Nile red after enzymatic degradation of the shell was then assessed (Agilent 8453, USA).

3. Results and discussion

3.1. PLGA/PLL nanofilms

An overcompensated surface charge enables LbL assembly, *i.e.* a charged polyelectrolyte adsorbed to a surface can act as the substrate for subsequent adsorption of an oppositely charged polyelectrolyte. The procedure can often be repeated many times, giving multilayers with a thickness that is depending on polyelectrolyte type, ionic strength of the solution, temperature, pH, rinsing and adsorption time, as well as the character of the solvent [23,24]. Since both PLL and PLGA are weak polyelectrolytes, for which the charge is pH dependent, it was of utmost importance to control the above parameters. The PLL–PLGA pair forms very dynamic layers, and the two polypeptides intermingle through the film [25]. In solution, the nanofilm stores water in pores and cavities, which makes it swell up to 60–70% compared to the dry state [25–27]. Since no active substance should leak out of the microsphere prior to exposure to bacterial enzymes, the film was assembled under physiological conditions, where the polypeptides are known to assemble tightly in extended β-sheet conformations [28,29]. When distributed intravenously PLL, like many other polycationic molecules, is known to trigger the immune response [30]. However, a recent study showed that PLL when used in combination with PLGA for topical applications is safe from a toxicological point of view [31].