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Stimuli-Triggered Degradation of Nanofilm Shells for Release of Hydrophilic Drugs in the Presence of Bacterial Proteases

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Abstract

Due to an increase in lifestyle diseases in the developed world, the number of chronic wounds increases at a fast pace. Chronic wound infections are common and systemic antibiotics are usually used as treatment. To avoid the overexposure of drugs and development of bacterial resistance, we have produced nanofilms as shells on hollow microcapsules that degrade through the action of a virulence factor from *Pseudomonas aeruginosa*. The shell was assembled by the layer-by-layer (LbL) technique and one of its components was poly-L-lysine. The hollow core was loaded with a model drug. By crosslinking the components in the nanofilm, the film remained intact when exposed to a human wound protease. However, the film was degraded and the model drug released when in contact with *Pseudomonas aeruginosa*'s Lys-X specific Protease IV. The current study can be seen as a contribution to the establishment of a release platform for targeted treatment of wound infections with the aim of minimizing both overexposure of drugs and development of bacterial resistance.

Keywords: Microcapsule, nanofilm, enzymatic degradation, stimuli-responsive, infection

1. Introduction

Controlled release from nanofilms synthesized by the layer-by-layer (LbL) technique has been a popular research area during the last decade. "Intelligent" nanofilms that decompose by some kind of trigger can be used for on-command delivery of drugs to a specific site, thus avoiding unnecessary side effects due to the toxicity of the drug. Decher pioneered the LbL technique [1,2], creating a platform for a self-assembled thin film technology that may be used in many different areas. LbL films as shells built with the template assisted assembly technique can produce capsules in the micro and nano range [3,4], thus creating an opportunity for loading of a drug either into a porous core covered by a nanofilm or, if the template is removed, into the hollow core and possibly also into the shell [5,6]. There are several interesting aspects of using capsules as carriers, such as temporary containment of toxic active substances [7] and protection of sensitive substances such as proteins [8,9] from the surrounding. Within the core or in the shell the substance will remain active until released by some kind of stimulus. The release can be triggered by capsule degradation due

to changes in pH [10,11], temperature [12], magnetic field [13], etc. or when exposed to specific enzymes [14]. In the present communication LbL assembly is employed to tailor nanofilms responding to exoproducts from *Pseudomonas aeruginosa* for release of drugs into infected chronic wounds, including burn wounds.

A chronic wound has, as the name implies, stalled healing. The chronic wound environment differs from that of an acute wound by an increase in protease activity and a decrease in the concentration of protease inhibitors [15-18]. Additionally, the pH of a chronic wound is elevated. Such an environment is favorable for bacterial colonization, which often develops into infection and further complications for the patient [19]. Thus, most chronic wounds have a high amount of bacterial proteases [20-22]. Normal procedures for treatment of infections are systemic treatment with antibiotics or topical disinfectants, *e.g.* wound dressings, creams or gels [23-25]. The drugs are often applied either in excess, in a poorly controlled manner, or over a long period of time, which may lead to bacterial resistance [26-28]. Bacterial biofilms also protect the bacterial colonies, resulting in considerably less efficacy with systemic antibiotics [29-31] compared to the situation with freely moving bacteria [32,33]. Some topical antimicrobial treatments have proven to rupture and inhibit growth of complex biofilms [34-36], especially in combination with wound debridement [37]. Topical administration of the antimicrobial substance triggered by the bacterial load in the wound could help combat the resistance and the exaggerated use. Also, creating a controlled release system that only responds to one specific family of bacteria could streamline the wound treatment and target individual infections, whether administered as a single treatment or combined with systemic antibiotics. Such a system could contribute to a shortened healing time together with substantially lower treatment costs. It may also decrease the patients' suffering.

In this study, bacterial exoproducts from *Pseudomonas aeruginosa* with high substrate specificity have been investigated as release trigger. A nanofilm that was readily degraded by the chosen exoproducts was used as shell of hollow microcapsules, which contained a model drug. Additionally, our group's previous study concerning exoproducts from *Staphylococcus aureus* degrading a specific nanofilm [38] has been extended in this communication. LbL films with antibacterial activity [39,40], studies of antimicrobial and non-biodegradable microcapsules [41] and liposomal vesicles for diagnostics [42] have previously been reported in the literature. However, drug-containing core-shell microcapsules with a polypeptide and/or polysaccharide nanofilm as shell, which degrades and releases a drug only when exposed to *Pseudomonas aeruginosa* exoproducts, seem not to have been described before.

2. Materials and methods

2.1 Materials and techniques for LbL assembly of nanofilms

3 μm porous CaCO_3 spheres ($\approx 2 \text{ g/cm}^3$ in water) from PlasmaChem GmbH (Germany) were used as template (2 wt%) during template assisted assembly. The nanofilm responding to

Pseudomonas aeruginosa exoproducts consisted of hyaluronic acid (HA), MW $\approx 3,8$ MDa (Bohus BioTech, Sweden), and poly-L-lysine (PLL), MW 84 kDa (Alamanda Polymers, USA). The preparation of PAH/PLGA nanofilms (polyallylamine hydrochloride/poly-L-glutamic acid) responding to the *Staphylococcus aureus* infection is described in a previous study [38]. The buffer used was 0.1 mM Tris-HCl (Sigma Aldrich, Sweden) with 0.15 M NaCl (Sigma Aldrich, Sweden). The spheres were washed and separated from the polyelectrolyte solution by centrifugation, 3 times at 3500 rpm (Heraeus Labofuge 200, Fisher Scientific, Sweden), after each layer adsorption. For removal of excess HA, the rate of centrifugation in the first step was increased to 4000 rpm. The ζ -potential was measured with a Malvern Zetasizer nanoseries (Malvern Instruments, UK). All end suspensions and solutions had a pH of 7.4.

2.2 Crosslinking of the nanofilm and dissolving the template core

The buffer was changed to 0.02 M 2-(N-morpholino)ethanesulfonic acid sodium salt (MES) (Sigma Aldrich) and 0.15 M NaCl before crosslinking. Crosslinking agents were N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Sigma Aldrich) in 0.02 M MES and 0.15 M NaCl for 16 hours. EDC was either set to 100 mM or 200 mM, and the concentration of sulfo-NHS was kept constant at 50 mM. Glucono- δ -lactone (GDL) (Sigma Aldrich) was used to dissolve the template core at pH 6.5 in a ratio GDL:CaCO₃ 2:1. For removal of impurities after crosslinking, dialysis with a membrane of 8-10 kDa in pore size was used (Spectrum Laboratories Inc., USA).

2.3 Loading of the microcapsules and enzymatic degradation of the nanofilms

Loading of the hollow capsules was performed after crosslinking and removal of the template core. Model drugs were FITC-dextran (4 kDa) from Sigma, Rhodamine Green (3 kDa) from Fisher Scientific and Vancomycin-BODIPY conjugate (1723 Da), also from Fisher Scientific. For post-loading, the drug was dissolved in MES buffer (0.02 M) with a NaCl concentration that would give a final concentration of either 0.5 M, 0.75 M or 1.25 M. The drug was contacted with the hollow microcapsules for at least 30 minutes. Glutamyl endopeptidase (V8) of MW 30 kDa originating from *Staphylococcus aureus* (specific activity = 2,000 mU/mg, isoelectric point (IEP) = 5.5) and human neutrophil elastase (HNE) of MW 29.5 kDa (specific activity = 30 U/mg, IEP = 9.7-10.5) were from BioCol GmbH (Germany). Protease IV from *Pseudomonas aeruginosa* with a MW of ≈ 27.7 kDa (specific activity unknown, theoretical IEP 6.6 (ExPASy.org) was purchased as rLys-C expressed in *E. coli* from Promega Corporation (Wisconsin, USA). Degradation studies were performed at 32°C at 350 rpm for 16 hours.

2.4 Microscopy techniques

The LbL assembly, crosslinking and hollow capsules were continuously studied in a light microscope (Axio Scope.A1, Zeiss, Germany) and a confocal laser scanning microscope (CLSM) (Leica TCS SP5 II Wetzlar, Germany). Dried microcapsules, hollow and loaded with

drugs were studied in a scanning electron microscope (SEM), which was operated at 5 kV (Ultra 55 FEG SEM, Zeiss, Germany). Microcapsules loaded with drugs and enzymatically degraded microcapsules containing fluorescent probes were studied in a fluorescence microscope (Axiovision with a Photometrics Evolve EMCCD camera, Zeiss, Germany) with x63 optical zoom. The crosslinked microcapsules loaded with 4kD FITC-dextran were studied in a CLSM with objective HCX PL APO CS WATER UV with 63 times magnification and a numerical aperture of 1.20 with optical zoom $\times 4$, $\times 6.5$ and $\times 20$. FITC-dextran was excited by a 488 nm argon laser and the emitted signal was recorded in the wavelength interval 510-550 nm.

3. Results and discussion

3.1 Nanofilm build-up via template assisted LbL assembly

The CaCO_3 template was added to a solution of a polyelectrolyte in Tris buffer. Not only the cationic polyallylamine hydrochloride (PAH) but also the anionic hyaluronic acid (HA) adsorbed at the negatively charged template surface, as can be seen from the ζ -potential measurements shown in Figure 1. Strong adsorption of PAH was expected but it was not obvious that also HA would adsorb well on a negatively charged substrate. However, HA is known to be a sticky macromolecule and its lubricating ability is related to its tendency to interact with biological surfaces of different charges. In order to obtain good coverage at the template surface the first adsorption step with HA was allowed 2 hours while stirring. Each subsequent step, *i.e.* addition of poly(L-lysine) (PLL) followed by HA, then again PLL, *etc.*, were allowed an adsorption time of 1 hour. This procedure was also used for the PAH/PLGA nanofilm fabrication. Both types of nanofilms (HA/PLL and PAH/PLGA) were constructed with 3 bilayers, *i.e.* $(\text{HA/PLL})_3$ and $(\text{PAH/PLGA})_3$. As can be seen in Figure 1, the ζ -potential was measured after each added layer. A light microscope was used to assess the degree of aggregation and the microcapsule structure throughout the LbL assembly.

Figure 1. ζ -potential of CaCO_3 particles after stepwise adsorption of HA and PLL (left) and of PAH and PLGA (right).

As part of the last centrifugation step, the buffer was changed into MES before crosslinking overnight with EDC and sulfo-NHS at pH 6.5. The concentration of EDC was varied between

100 and 200 mM for optimizing the properties of the nanofilm (Figures S1-S4 and S6-S8 in the Supporting Information section). Obviously, a higher degree of crosslinking results in a more rigid nanofilm, thus increasing the stability of the microcapsules. However, when increasing the concentration of EDC to 200 mM, the active substances were not always able to penetrate the film during loading. This is not surprising because the permeability of the nanofilm decreases when the crosslinking density is increased [43]. Increasing the crosslinking degree may also reduce the availability of the substrate for the bacterial enzymes, while simultaneously increasing the possibility of staying intact when exposed to natural wound enzymes. Taking the above parameters into account, 100 mM EDC and 50 mM sulfo-NHS were used for the crosslinking reaction.

The template core was subsequently removed by addition of glucono- δ -lactone (GDL). When exposed to water GDL is slowly hydrolyzed into gluconic acid; thus, GDL is a latent acid which gives a slow and well controlled lowering of the pH. The acidification transformed the suspension of CaCO_3 particles covered by a nanofilm into a suspension of capsules with the nanofilm constituting the capsule walls. This process could be observed by the eye since it resulted in a transition from a turbid to a transparent suspension. The capsule suspension was finally purified from molecules below 10 kDa through dialysis, see Figure 2. Both nanofilm systems could be air-dried and the dried products looked identical to the dialyzed microcapsules in solution apart from a slight elongation of the structures. The vacuum required for SEM, on the other hand, resulted in aggregation of the hollow microcapsules. Large aggregates were also found after the samples had been sputtered with gold in preparation for the SEM studies. It seemed that for the PAH/PLGA microcapsules, formation of aggregates had a positive effect on the strength of the capsules. The SEM images showed that while most of the single capsules had ruptured in vacuum, the majority of the aggregated capsules retained their structure. The HA/PLL microcapsules were more sensitive and almost all such capsules ruptured in vacuum. Figure 3 shows representative SEM images.

Figure 2. Optical microscopy images of microcapsules. Left: $(\text{HA/PLL})_3$ microcapsules in solution. Right: $(\text{PAH/PLGA})_3$ microcapsules in solution. The approximate size of the capsules is $3\mu\text{m}$.

Figure 3. SEM images of hollow microcapsules. Left: (HA/PLL)₃ microcapsule ruptured in vacuum, thus exposing the hollow core (scale bar 200 nm). Right: (PAH/PLGA)₃ microcapsules (scale bar 100 nm). As seen in the micrograph to the right, there was a fusion of the microcapsules when exposed to the SEM environment. The size of dried and hollow capsules in vacuum is 1-2 μm .

3.2 Loading of the microcapsules and nanofilms using model drugs

Before loading the microcapsules with drugs at pH 7 a FITC-dextran 4kDa probe was studied in a fluorescence microscope and in a CLSM. FITC-dextran was believed to be an appropriate probe because its size is comparable to some common polymeric quaternary ammonium compounds, as well as many antibiotic substances. In addition, FITC-dextran is relatively inexpensive. Probes with even closer resemblance in size and charge to actual antibiotics were subsequently employed, *i.e.* Rhodamine Green (cationic, 3 kDa) and a Vancomycin-BODIPY conjugate (VB, neutral, 1.7 kDa). It is known that the ionic strength of a solution can alter the character of the polyelectrolyte nanofilm [44]. The permeability can be increased by increasing the ionic strength as a result of screening of charges in the polyelectrolyte film [45,46]. Previous studies have shown that in a salt-free solution the permeability of similar films will be very small [45,47]. A 2 mg/ml (FITC-dextran, Rhodamine Green) or 1 mM (VB) probe solution was added to an equal volume of capsule suspension, while the ionic strength was kept at either 0.5 M, 0.75 M or 1.25 M before visualization in the fluorescence microscope (Figures S1-S8 in Supporting Information section). As seen in Figures S1 and S7, the images revealed that the microcapsules aggregated severely at 0.75 and 1.25 M NaCl, while at 0.5 M NaCl the capsules remained dispersed, allowing successful loading of the probe. At 100 mM EDC and 0.5 M NaCl, the (PAH/PLGA)₃ microcapsules could be efficiently loaded with all three probes (Figures S1 and S2), *i.e.* FITC-dextran, Rhodamine Green and VB, while with the (HA/PLL)₃ microcapsules the loading was only facile with the cationic Rhodamine green and the neutral VB (Figures S4 and S6). The larger and anionic FITC-dextran required a long loading time due to slow penetration of the film, see Figure S5. This illustrates the fact that the type of nanofilm is crucial for the loading efficacy of a particular drug. The problems experienced in the loading of (HA/PLL)₃ with FITC-dextran may be due to electrostatic repulsion. The anionic component of the (HA/PLL)₃ film, HA, is a very large polyelectrolyte compared with the cationic moiety, PLL. Since the LbL films are composed of diffuse layers with intermingling polymers present throughout the film, it is likely that HA protrudes all the way through the film, generating negative patches on the capsule surface. Based on the results presented in the Supporting Information section an ionic strength of 0.5 M NaCl was used during the incorporation of a model drug for both types of microcapsules.

3.3 Enzymatic degradation of the nanofilm shell and release of the model drug

The microcapsule suspension was placed in a shaker at 32°C to resemble approximate skin temperature. The (HA/PLL)₃ microcapsules were exposed to Protease IV and the (PAH/PLGA)₃ microcapsules were exposed to V8 overnight, before studying the outcome in the CLSM. Both microcapsules were also exposed to the natural wound enzyme, HNE, overnight. In one of our previous studies we showed that nanofilms composed of PAH and PLGA exposed to HNE did not degrade, while V8 destroyed the microcapsule structure and released the model drug [38]. Also the (HA/PLL)₃ nanofilm seemed relatively unaffected by the human wound protease, see Figure 4a. The model drug FITC-dextran remained inside the microcapsules (Figure 4c) although these were partly aggregated in the suspension, see Figures 4b and 4c. HNE carries a net positive charge, which means that one would anticipate repulsion from the likewise positively charged PLL, which is intended to be the outermost surface layer of the microcapsules. However, as discussed above, the large anionic HA may protrude out through the PLL layer and generate negative patches on the surface that can attract HNE.

Figure 4. The optical microscopy images (a) and (b) show (HA/PLL)₃ microcapsules crosslinked using 100 mM EDC and 50 mM sulfo-NHS after HNE exposure. The treatment contributed to aggregation of the (HA/PLL)₃ spheres in certain areas of the suspension; however, the model drug (FITC-dextran) remained inside the microcapsules (CLSM micrograph (c)).

The endoprotease Protease IV is a significant virulence factor in many *Pseudomonas aeruginosa* strains. Protease IV is a substrate specific protease that cleaves lysine bonds [48] and its main purpose is to degrade host tissue to provide nutrients for the *Pseudomonas* bacterium [49]. As the nanofilm structure was composed of the cationic lysine polymer PLL together with HA, Protease IV was able to degrade the film enough to release the model drug (FITC-dextran). As seen in Figures 5a and 5b, the anionic FITC-dextran was mostly found in the nanofilm itself, but released upon exposure to Protease IV (Figure 5c). The increased size of the microcapsule seen in Figure 5b is a sign of the swelling capacity of dextran when exposed to water.

Figure 5. The CLSM micrographs (a) and (b) show one single (HA/PLL)₃ microcapsule crosslinked with 100 mM EDC and 50 mM sulfo-NHS and loaded with FITC-dextran. (a) reveals the full structure of the capsules, while (b) shows the capsule's inner structure after zooming in. (c) shows the loss of fluorescence, and thus the model drug, of the microcapsules after exposure to Protease IV.

The intensity of the fluorescent probe in the two types of microcapsules before and after treatment with bacterial proteases was measured in the CLSM to confirm the release of the probe. For all intensity measurements several sites in one micrograph were measured and then averaged. Due to microcapsule concentration differences and to slight sedimentation, the amounts of measured sites varied between samples, but between three and ten sites were always measured in one CLSM frame. To compare the intensity of the microcapsules with that of the background, the relative intensity was calculated by dividing the internal intensity of a capsule (I_{int}) with the background intensity, *i.e.* the external intensity (I_{ext}). The intensity of the background was set to 1, *i.e.* approximately 1 mg/ml since the added probe volume at 2 mg/ml was equal to the volume of the microcapsule suspension during loading. Microcapsules in 0.5 M NaCl resulted in 50-100% higher intensity than the background, which, if assuming a linear relationship between the probe concentration and the intensity in the micrograph, suggests 2 mg/ml FITC-dextran, see Figure 6. If the model drug was only present in the capsule core, the intensity should theoretically be the same as in the surrounding solution. However, since the nanofilm contained charged components, the model drug is likely to accumulate in the film, thus increasing the intensity as the probe concentration was increased. Intensity measurements were not performed on HNE treated

microcapsules, since the fluorescence still was much higher than that of the background, see Figure 4c. After the treatments with Protease IV and with V8, a substantial loss of fluorescence from the microcapsules was found. As seen in Figure 6, the (PAH/PLGA)₃ microcapsules lost almost four times of their original intensity after exposure to V8, while the (HA/PLL)₃ microcapsules lost half their intensity after Protease IV exposure. The loss of the probe acting as model drug indicated a triggered release after exposure to the enzymes.

Figure 6. The intensity described as the relative intensity compared to the background (external) intensity (set to 1) for the two types of microcapsules. The (PAH/PLL)₃ microcapsules went from about 2 to below 0.5 after exposure to V8. The (HA/PLL)₃ microcapsules went from 1.5 to 0.6 after treatment with Protease IV (rLys-C).

4. Conclusions

The present communication describes enzymatic degradation of microcapsule shells. The enzymes were proteases originating from *Pseudomonas aeruginosa*. The destruction of the nanofilm shells lead to a release of a model drug residing within the shell and in the microcapsule core. However, the nanofilm remained intact when in contact with a human wound protease. Firstly, the crosslinking of the nanofilms was studied for optimization of the enzymatic degradation *versus* capsule stability. Secondly, the loading capacity was compared at different ionic strengths and with probes of different size and charge. An optimal crosslinking was found using 100 mM EDC and 50 mM sulfo-NHS and using an ionic strength during loading of 0.5 M NaCl. (HA/PLL)₃ microcapsules were loaded with anionic, cationic and uncharged model drugs between 1.7 and 4 kDa in size. However, loading of the anionic model drug proved to be slow in the (HA/PLL)₃ microcapsules. Exposure to the human wound elastase HNE caused the (HA/PLL)₃ microcapsules to aggregate; however, the nanofilm stayed intact with the model drug inside the capsule structure. When exposed to Protease IV, a virulence factor originating from *Pseudomonas aeruginosa*, the model drug was released and the spherical structure altered. The enzymatic degradation of the microcapsules was not only monitored visually, but the loss of fluorescence was also quantified by CLSM as loss in intensity. It is known from a previous study [38] that the

substrate specific V8 from *Staphylococcus aureus* degrades a PLGA layer, causing rupture of the (PAH/PLGA)₃ nanofilm, hence releasing the model drug (FITC-dextran). In this work we have now also quantified the loss of fluorescence in the (PAH/PLGA)₃ microcapsules as loss in intensity. In addition, we have studied the loading of the microcapsules with probes having different characteristics.

This study should contribute to the development of controlled release platforms for infected wounds, which only respond to specific pathogenic bacteria. Such a platform would enable targeted treatment using specific antimicrobials, thus avoiding overexposure of drugs, as well as bacterial resistance. Research involving quantification of loading with antimicrobials and their release, as well as antimicrobial efficacy tests on virulent bacteria, is ongoing.

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Appendix A. Supporting Information

Supporting Information presents results when varying the ionic strength during loading. Additionally, fluorescent model drugs with different charge and size were studied. The supporting results clarify the choice of ionic strength during loading, as well as shows which probes were better suited for the loading of the microcapsules. Supplementary data associated with this article can be found in the online version at doi:XXXXX.

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