Smart Biopolymer-Based Multi-Layers Enable Consecutive Drug Release Events on Demand

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All implantation surgeries carry the risk of immediate or recurring infections at the surgical site. To combat this problem, employing implant coatings can be a useful strategy. A multi-layered surface coating that actively responds to two different physiological stimuli with the liberation of two independent drug doses is presented here; the first dose is supposed to overcome an immediate inflammation caused by a contamination of the surgical site whereas the second dose may treat recurring infections. To make this possible, the strategy combines thermoresponsive liposomes with sacrificial layers and a transient condensation of biopolymer layers. Moreover, when the release is completed, the remaining surface coating provides beneficial long-term properties such as improved lubricity. Due to its high specificity toward the two release triggers, its good loading capacity, and excellent release efficiency, the mechanism presented here may pave the way toward the development of a new generation of smart implant coatings that can autonomously combat two temporally separated infection events.

1. Introduction

Physiologically, biopolymers not only exist in solubilized form, that is, as components of different body fluids, but they are often also attached to cell/tissue surfaces. There, they fulfill crucial functions, for example, they act as selective barriers or as mediators for cell signaling. Inspired by this biological role model, surface-immobilized macromolecules are also employed in a variety of biomedical applications.

Compared to bulk modifications, the application of coatings allows for specifically tuning the interaction of a substrate with its environment, while avoiding to compromise its mechanical properties and structural integrity. Most coating strategies share a common goal, that is, they aim at gaining control over molecular/cellular binding toward the coated surface. Depending on the particular application, binding events have to be either promoted, inhibited, or enabled for a selected subset molecules or cells only.

Strongly hydrated polymers, that is, polymers that efficiently bind water molecules, for example, can be used to generate lubricious coatings; such lubricating coatings supply a thin water film on the substrate’s surface thus reducing friction. At the same time, hydrated polymer layers can also help in reducing the unwanted adhesion of proteins, fungi, or bacteria, which are all typical complications associated with implant materials. In contrast, tissue engineering approaches rely—to a large extent—on the ability of a material to support cell adhesion; here, macromolecular coatings have been shown to be a promising and versatile tool to achieve a controlled integration of scaffolds and implants into the body environment.

Moreover, macromolecular coatings can also actively modulate immune reactions to reduce the foreign body response toward artificial materials.

A precise control over the binding of molecules to and subsequent release from coated surfaces has also emerged as an important tool in the field of drug delivery. Drug-loaded coatings are especially interesting for implants: inserting an artificial implant material into the human body always comes with the risk of bacterial infection as it is inevitable that pathogens enter the surgical site. Thus, even though the strategies mentioned above may be able to suppress biofouling events on the implant surface, bacterial infections around the implant still occur—both, immediately after surgery and up to weeks after the operation. In clinical practice, patients therefore typically receive systemic doses of antibiotics to overcome such infections—with all the side effects that go along with such a systemic treatment. As an alternative, bactericidal or drug-loaded implant surfaces and implant materials such as bone cements, scaffolds, or nanoparticles can offer local protection against bacterial infections; however, the drug release process from such systems is typically not well controlled and mostly governed by the diffusive liberation of drugs.

A controlled liberation of previously encapsulated drugs has already been achieved for various applications, especially using nanoparticulate drug carriers. Here, lots of different mechanisms have been proposed in the literature, that can be used to trigger a release event in a controlled manner, for
example, pH changes, enzymatic degradation, or the addition of specific molecules such as oligonucleotides to the environment.\(^\text{[14]}\) Drug delivery systems based on polymeric coatings typically regulate the duration of a release event by either acting as a diffusive barrier or by serving as a degradable drug reservoir.\(^\text{[15]}\) In some recent studies, it was possible to gain better control over the release of pharmaceuticals from such polymer coatings: there, pH-responsive, degradable structures were used, which were disassembled in the presence of bacteria thus triggering the release of an antibacterial agent.\(^\text{[16]}\) However, once the drug load is released, those systems cannot offer any further protection of the surface. In particular, there is no controlled release mechanism yet, which establishes both, short- and long-term protection against bacterial infection by making use of specific trigger events as they occur as part of an infection event.

Here we present a mucin-based, smart multi-layer system that can liberate two independent antibiotic doses to tackle two temporally separated infection. Each of these doses is released in response to two different physiological stimuli as they can occur as consequences of an implantation surgery. The first drug load is liberated as soon as the multi-layer construct is exposed to a physiological NaCl concentration, for example, when the coating is introduced into the human body; therefore, it can deal with early stage infections. In addition, the system includes a second, sealed drug reservoir which is opened only when an increase in temperature occurs, for example, as part of a recurring inflammation response. Compared to existing strategies, where the sequential release of individual doses is either achieved by designing liberation profiles with different kinetics or where the second release event is initiated by the completion of the first, the release events of the two drug doses achieved here are completely decoupled from each other. Finally, we demonstrate that, even after the drug release is completed, the remaining biopolymer layer can provide additional benefits to the coated surface such as improved lubricity.

2. Results and Discussion

2.1. Controlled Release of Antibiotics from Condensed Mucin Layers

To achieve a controlled drug release from macromolecular coatings, our strategy is as follows: First, a surface-bound mucin layer is generated, and drug molecules are added (Figure 1a).
Then, this surface-bound mucin layer is condensed with glycerol (to trap drug molecules) and transiently stabilized with Mg$^{2+}$ ions in this compacted state (Figure 1b). Subsequently, glycerol and excess stabilizing agents are removed, and the compacted layer is stored in distilled water (Figure 1c). Upon exposure to physiological salt concentrations, the condensed mucins return to their original conformation which induces liberation of the trapped drug molecules (Figure 1d).

The idea we follow here, that is, to use small ionic cross-linkers to temporarily stabilize the condensed mucin layer, is motivated by the earlier finding that Ca$^{2+}$ ions can stabilize condensed mucin particles.[17] In a biological context, ions such as Ca$^{2+}$ often play an essential role in physiological regulation mechanisms such as cell signaling or modulating enzymatic activities. However, the literature suggests that—compared to other metal ions—the gradual release of low concentrations of Mg$^{2+}$ into the human body should not influence physiological processes considerably.[18] Also from a biophysical/biochemical point of view, this ion should be well suitable: owing to its small atomic radius, Mg$^{2+}$ can compete well with other divalent ions for binding sites, and Mg$^{2+}$ has already been shown to form ion bridges between negatively charged macromolecules.[19] Indeed, we find that Mg$^{2+}$ ions are suitable for stabilizing a condensed mucin layer after glycerol removal (Figure S1, Supporting Information).

Next, we load the system with a model drug, that is, the broad-spectrum antibiotic tetracycline (TCL), and determine the cumulative TCL release spectrophotometrically after exposing the system to the NaCl solution (Figure 2a). We observe a release of the drug ≈30 min after the salt trigger is added, and the released drug concentration reaches a plateau value after ≈2 h (Figure 2b, full bars). Importantly, the determined release efficiency is >90%, which demonstrates the excellent performance of the NaCl trigger. Furthermore, with the coated surface being 100 mm$^2$, we calculate a TCL loading capacity in the coating of ≈4.5 µg mm$^{-2}$.

Control experiments demonstrate that, in the absence of the NaCl trigger, the mucin layer shows only negligible leakage. If the drug-loaded layer is exposed to dH$_2$O instead of a NaCl solution (this is supposed to simulate storage of the drug-loaded coating), the drug remains well trapped; for the course of several hours, baseline release due to leakage is less than 2% (Figure 2b, empty bars). Furthermore, a control without condensation (Table S1) shows little to no drug release at all—even if it is exposed to the trigger. This can be rationalized by insufficient drug incorporation, which is a consequence of the missing condensation step. The antibiotic we used for drug loading so far exhibits an overall negative net charge at pH = 7.4. Mucin glycoproteins carry both anionic and cationic motifs; at neutral pH, however, anionic residues dominate, which provides the glycoprotein with an overall anionic character.[20] Based on this information, we expect that a drug molecule with an opposite net charge can be loaded into and released from mucin layers as well. Therefore, we tested the antibiotic vancomycin hydrochloride (VAN), which has an overall positive net charge at pH = 7.4 and is widely used in implantation operations to prevent post-operative infections. Indeed, VAN-loaded mucin layers show a similar response as obtained for TCL loading. After exposing the system to the NaCl trigger, VAN is released within ≈30 min and the release completes after ≈2 h (Figure 2c, full bars). Also here, in the absence of the NaCl trigger, drug release is very low (Figure 2c, empty bars). However, the total

Figure 2. Triggered drug release from stabilized mucin layers. a) Mucin layers are generated in transparent cuvettes and the drug release is monitored spectroscopically. Changing the incubation medium from distilled water to 150 mM NaCl solution yields the b) triggerable release of TCL and c) VAN from condensed mucin layers. The error bars represent the standard deviation as obtained from $n$ = 3 independent samples. Asterisks (*) denote statistically significant differences based on a $p$-value of 0.05.

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VAN release efficiency (≈47%) is considerably lower than the corresponding value we determine for TCL. This result can be explained by the polyanionic character of the mucin molecule, which was previously shown to exhibit a higher affinity toward cationic molecules (such as VAN) than toward anionic ones.[21] A control experiment, where we omit the glycerol condensation step (Table S2, Supporting Information), again demonstrates that efficient drug loading is not achieved without mucin layer condensation and—consequently—triggered drug release is not possible.

Together, the results discussed so far show that condensation of surface-bound mucin layers is a very efficient method to entrap drug molecules, and that we can achieve a controlled release of the loaded drugs by using physiological salt solutions as a trigger.

2.2. Controlled Two-Step Release from Mucin Multi-Layer Constructs

So far, the condensed mucin layers allow for releasing a single dose of drug molecules. To efficiently deal with a local infection after surgery, providing a single dose of an antibiotic may, however, not always be enough, for example, when trying to avoid recurring tissue inflammation after implantation surgery.[22] Ideally, a “smart” drug carrier system would provide a second drug dosing when (and only if) required. Thus, for the remainder of this article, we aim at further adjusting our mucin-based release system such that this scenario is made possible. In detail, we introduce a multi-layer approach where two different trigger strategies are combined such that they can lead to two distinct, independent release events. We maintain the NaCl-triggered drug release from a Mg2+-stabilized, condensed mucin layer but relocate this particular mucin layer on top of a mucin-based multi-layer construct (Figure 3a, drug reservoir 1). Below this top mucin layer, we introduce two additional mucin layers, the lower of which is covalently attached to the substrate and also loaded with a drug (Figure 3a, drug reservoir 2). In this configuration, the upper layers need to be removed to initiate drug release from the bottom layer, and this event should only occur if the body requires a second drug dose. Mechanistically, we decided to employ a physiological stimulus associated with an inflammation event to serve as a trigger for this second, optional drug release event. An elevated temperature does not only occur globally in the human body as fever (≈40 °C) but also locally as a typical body reaction to inflammation—and both phenomena can be triggered by an implant-mediated infection.[23] To construct such a temperature-sensitive system, the multi-layer coating is designed in a way that it is disassembled when the local temperature is increased. Such temperature-sensitive systems have previously been introduced for nanoparticles.[24] In our study, we make use

![Figure 3. Assembly of the mucin-based multi-layer construct. a) Schematic representation of mucin-based multi-layers cross-linked with two types of connector molecules, that is, lectin and dopamine. The presence of three distinct mucin layers is verified by fluorescence imaging using different mucin labels for the bottom (red), middle (green) and the top layer (blue). b) The successful formation of the multi-layers, as well as its partial disassembly in the presence of GlcNAc, is demonstrated by QCM-D measurements.](image-url)
of mucin/lectin interactions and thermoresponsive liposomes to construct a thermoresponsive multi-layer surface coating. Previous research showed that lectin-stabilized mucin multilayers can resist a wide range of physiological pH and salt concentrations,[23] but they can be disassembled by exposure to N-acetyl-d-glucosamine (GlcNAc).[26] Here, we incorporate GlcNAc-loaded thermoresponsive liposomes into a central mucin layer, which is sandwiched between the drug-loaded bottom and top mucin layers. The mucin composition is chosen such that the mucin membrane becomes leaky at typical “fever” temperature (Figure S4, Supporting Information).[27]

The central liposome-loaded mucin layer is connected to the bottom mucin layer via lectin cross-links, and to the top mucin layer via dopamine cross-links (Figure 3a). For our purpose, dopamine is a useful cross-linking agent as it can efficiently bind to various substrates; accordingly, it was already successfully employed in layer-by-layer assemblies,[28] and its connection to the neighboring mucin layers is expected to be stable toward both, exposure to NaCl and elevated temperatures.

To verify the formation of a multi-layer construct, we first assemble the system using differently colored mucins for each layer. Microscopy images indeed confirm the presence of all layers when the complete assembly process is conducted (Figure 3a). Moreover, the correct assembly of the components is further supported by QCM-D (Figure 3b). Here, the sequential injection of the different molecules entails a stepwise frequency shift indicating successful adsorption events. The recorded frequency signal shows that mucin layers can be deposited onto the PDMS surface by molecular interconnections using either lectin or dopamine. Interestingly, exposing the multi-layer construct to a physiological NaCl solution induces a slight frequency shift (Figure 3b and Figure S5a, Supporting Information), which can be attributed to a conformational change of mucin molecules from a rather extended state to a more condensed state.[29] However, this event is fully reversible, which demonstrates that the full multi-layer is still present when the NaCl is removed. In contrast, when the system is exposed to a GlcNAc, an irreversible increase in the recorded resonance frequency is detected (Figure 3b and Figure S5b, Supporting Information), which indicates a permanent loss of adsorbed mass. This result is in full agreement with our expectation, that the lectin/mucin cross-links are opened by exposure to GlcNAc, and that the middle and top mucin layers (together with a fraction of the lectin molecules) are successfully detached.

Having verified both, the correct assembly and on-demand disassembly of the multi-layers, we now—in the last step—assess the drug release from distinct layers as a response to two stimuli: a physiological salt solution and a subsequent temperature increase. However, before testing the whole construct, we first assess the correct functionality of the envisioned GlcNAc-mediated multi-layer disassembly and drug release in simplified setups (Figures S6 and S7, Supporting Information). As expected, in both cases we observe TCL release from the multi-layers once the GlcNAc trigger occurs—whether GlcNAc is added externally (Figure S6, Supporting Information) or released from embedded thermoresponsive liposomes upon temperature increase (Figure S7, Supporting Information).

Finally, we initiate the release of two consecutive doses of TCL from a full multi-layer construct. Now, TCL release from the top layer should be triggered as soon as the construct is brought into contact with a physiological NaCl concentration whereas release from the bottom layer is supposed to set in only when a temperature increase occurs. And indeed, the full construct shows the expected behavior: in the absence of the two triggers (Figure 4a, grey diamonds), we detect only little drug leakage from the multi-layer, and the system is stable for 3 days. Moreover, TCL release from the condensed top layer can successfully be initiated by exposing the system to a physiological salt solution, and the release is completed within ≈2 h. Later, the second release event can be triggered by increasing the temperature from 37 to 40 °C (Figure 4a, red data points). The system reaches an overall release efficiency of (64±12)%, which is very good considering its complexity. In a control group, where GlcNAc-free liposomes are embedded into the multi-layer construct, this second release event does not occur (Figure 4a, open circles).

However, compared to the first release event, the TCL dose liberated after the second trigger is rather small in terms of absolute values. We thus improve this second release event by introducing a small modification, that is, by condensing the bottom mucin layer prior to generating the multi-layer system. With this adjusted strategy, we achieve a remarkable increase in the temperature-triggered TCL dose from ≈0.02 to ≈0.12 mg mL⁻¹, while maintaining a similarly high release efficiency of (60±17)% (Figure 4b, full circles). Again, a control group, where GlcNAc-free liposomes are incorporated into the system shows no drug release upon temperature increase (Figure 4b, open circles), and in the absence of the two triggers, TCL leakage is virtually absent (Figure 4b, grey diamonds). Together, these additional results demonstrate that the system we introduce here is very well reproducible, stable, and allows for easily tuning the amount of stored/released drugs.

2.3. Implications for Mucin-Based Coatings on Implants

The mucin-based construct introduced here allows for liberating two independent drug doses on demand. Our engineered mechanism can be especially useful for a local delivery of antibiotics after implant surgery. In this context, the first release event can help to prevent immediate bacterial infections. In contrast, the second antibiotic reservoir would only be opened when the local temperature around the implant increases to fever-like levels; thus, it would help to deal with tissue inflammation occurring at later stages after implantation.

From a different point of view, such programmable multilayers can contribute to reduce the use of systemic drug doses; this, in turn, would minimize the unwanted side effects. For example, a conventional treatment for mild urinary tract infections induced by catheters comprises the systemic use of 500 mg of TCL every 6 h for a duration of 10 days.[30] Typical TCL serum and cervical fluid concentrations achieved with such systemic dosing are 3–4 and 5–12 μg mL⁻¹, respectively.[31] Already with a monolayer coating, we obtain a surface loading capacity of 4.5 μg mm⁻²; thus, for this simple coating, we can estimate a local concentration of liberated drug of ≈180 μg mL⁻¹ within 6 h after exposure to a body fluid. This concentration not
only exceeds the levels achieved by a systemic administration, but is also well above the minimum inhibitory concentrations for common bacterial pathogens responsible for urinary tract infections.\(^{[32]}\)

In addition to serving as a depot for drugs, a covalently coupled mucin layer, which remains after drug release is triggered, can provide further properties beneficial in the context of implant surgery: mucin coatings have been shown to reduce both, protein adsorption, and bacterial adhesion to surfaces,\(^{[33]}\) and they can also reduce friction and tissue damage when an artificial, mucin-coated material slides against a biological surface.\(^{[34]}\) Of course, for these beneficial properties to take effect, the bottom layer needs to still be intact after the drug release is finished. We verify this by analyzing the presence of mucins before and after the release via an enzyme-linked immunosorbent assay (Figure S8a, Supporting Information); and indeed, we obtain virtually identical results for both conditions. Moreover, we also find that the lubricating properties of the mucin coatings are maintained after the drug release is triggered (Figure S8b, Supporting Information). Together, these points indicate the great potential mucin-based multi-layers hold for combining a local, tailored drug-release process with anti-biofouling and lubricating properties. Thus, they can combat different origins of surgery-related tissue inflammations at the same time.

3. Conclusions

The mucin-based multi-layer construct presented here makes use of the fact that mucin glycoproteins can undergo a reversible conformational change by transient exposure to glycerol.\(^{[37]}\) Since this process is attributed to the poor solubility of mucins in glycerol, the same mechanism presented here might be transferable to other (bio)polymers—provided that those polymers can be condensed by similar means. The results presented here suggest that various implant surfaces could benefit from a functional coating that combines several functions, for
example, therapeutical contact lenses that can be triggered to release active agents when placed directly onto the eye surface. Applying a physiologically triggerable coating to tissue engineering constructs could furthermore be a promising strategy to deliver bioactive signals at pre-defined time points, or to influence the local immune response by delivering antigens, adjuvants, cytokines, or chemokines.

4. Experimental Section

Single Layer Coating and Triggered Antibiotic Release Experiments: To determine the drug release of either VAN (positively charged at neutral pH, Applichem, Darmstadt, Germany) or tetracycline hydrochloride (TCL, negatively charged at neutral pH, Applichem) from a mucin-coated surface, a spectroscopic detection method was used. First, the bottom of cuvettes (polystyrene cuvettes, BrandTech macro, VWR, were used for TCL release, and UV transparent cuvettes [VWR] were used for VAN release) was covered with PDMS. To do so, PDMS was mixed and degassed as described above, and 300 µL of the mixture was poured into the cuvette using a displacement pipette. This amount was chosen to ensure that the height of the PDMS layer does not interfere with the light sent into the cuvette by the UV spectrometer (Figure 2a). In the cuvette, the poured PDMS was allowed to cross-link at 60 °C overnight. Afterward, this PDMS layer in the cuvettes was coated with purified mucins as described in the Supporting Information.

Next, a drug solution (TCL or VAN, 0.5 mg mL−1 each) was added to the surface-bound mucin film and incubated at 4 °C for 4 h. Then, a solution containing 30% v/v glycerol was added to condense the mucin layer. Stabilization of the condensed mucin layer was achieved by adding 100 mM MgCl2 (dissolved in a 30% glycerol solution) to the condensed mucin layer. After this addition step, the final Mg2+ concentration was 50 mM, and the system was allowed to incubate for 2 h. Before starting a drug release experiment, excessive glycerol, drug, and ions were removed by washing the cuvettes with ultrapure water.

To initiate the drug release from Mg2+-stabilized mucin layers, 2 mL of a 150 mM NaCl solution was added as a trigger. The release of TCL was tracked spectroscopically with a spectrod210 spectral photometer (Analytikjena, Jena, Germany) at 360 nm. The release of VAN was also tracked spectroscopically, however in the UV range, that is, at 282 nm (for calibration curves, see Figure S5, Supporting Information). During the whole timespan of the experiment, samples were stored at stagnant conditions, that is, without any agitation. By doing so, it was ensured that no additional influences (such as mechanical shear forces) other than the specific triggers employed contributed to the diffusion-mediated release process of the enclosed drug molecules. To determine the release efficiency of the process, Mg2+-stabilized mucin layers were opened by exposing the layer to a trypsin solution (50 µg mL−1) for 12 h. Afterward, the amount of released drug was determined spectroscopically to calculate the maximum loading capacity of the mucin layer.

Multi-Layer Constructs: Mucin/lectin multi-layers were assembled according to Crouzier et al.[25] with small modifications. In brief, mucin-coated PDMS samples were prepared as described in the previous section. Then, a second mucin layer was attached via lectin cross-linking, and the final, top mucin layer was added using dopamine as a connecting layer.

In detail, the covalently mucin-coated samples were incubated in a 0.2 mg mL−1 lectin solution (Trifidum vulgaris lectin, Medicago) for 4 h. After washing twice with HEPES buffer (pH = 7), a second mucin layer was generated by submerging the lectin-coated samples into a mucin solution (1.0 mg mL−1) for 1 h. The top mucin layer was attached to the mucin–lectin–mucin construct using a dip-coating method. Dopamine hydrochloride (4 mg mL−1, H8502, Sigma) was dissolved in 50 mM Tris buffer (pH = 8.5).[19] Samples were incubated in this dopamine solution for 2 h, and then were rinsed thoroughly with ultrapure water to remove any unbound dopamine. Afterward, the dopamine-coated substrates were incubated in a mucin solution (1.0 mg mL−1) for 4 h, and then rinsed thoroughly with ultrapure water again.

The multi-layer construct was visualized by fluorescence microscopy using an Axioskop 2 Mat mot microscope (Zeiss, Oberkochen, Germany) equipped with a 10x lens (EC Epiplan-Neofluar 5x/0.13 HD DIC, Zeiss). To identify the individual layers in the construct, each layer was assembled from a differently labeled mucin: mucins in the bottom layer are labeled in red, mucins in the middle layer are labeled in green, and mucins in the top layer are labeled in blue (see Supporting Information for details). To image the different mucin layers, three different fluorescence filters (corresponding to standard dyes such as DAPI, FITC, and rhodamine, Zeiss) were used. Images were acquired with a digital camera (Orcia R2 C10600, Hamamatsu, Japan) using the software HC Image Live.

The adsorption properties of multi-layers on hydrophobic PDMS surfaces were studied by quartz crystal microbalance with dissipation monitoring (QCM-D) using a qcll T-Q2 platform (3T-Analytik, Tuttlingen, Germany) as described in a previous study.[29] Since QCM-D is a very sensitive technique to detect adsorbed molecules,[30] lower biomolecule concentrations were used here for conducting the coating process in situ: mucin (0.2 mg mL−1) and lectin (0.1 mg mL−1) solutions were prepared in 20 mM HEPES buffer (pH = 7.4). A dopamine solution (14 mg mL−1) was prepared in 50 mM Tris buffer (pH = 8.5). Layers were formed by injecting the test solutions into the sample chamber and keeping them there for 30 min each to allow for adsorption onto PDMS-coated Au chips. Chips were rinsed with HEPES buffer (pH = 7) for 2 min before injecting the next solution. QCM-D measurements were also performed to verify successful multi-layer formation and (partial) disassembly in the presence of N-acetyl-l-glucosamine (GlcNAc) (see Supporting Information).

Antibiotic Release Experiments Triggered by Two Distinct Stimuli: To perform two-step release experiments using two distinct triggers, polystyrene cuvettes were filled with PDMS, coated with mucin and incubated in a TCL solution (0.5 mg mL−1) as described above. Instead of condensing this initial mucin layer, a multi-layer construct as described above was created. Thus, in a next step, the TCL solution was discarded, the cuvettes were filled with a lectin solution (0.2 mg mL−1) and incubated for 1 h. Afterward, the lectin solution was replaced with a mucin solution (1 mg mL−1) containing 4% v/v thermoresponsive liposomes (which were loaded with GlcNAc; empty, unloaded liposomes were used as a control (see Supporting Information) and the samples were again allowed to incubate for 3 h. This step created a second mucin layer loaded with liposomes. Then, a third mucin layer was attached on top as follows: first, a dopamine intermediate layer was generated (see above) and then the samples were incubated again in a mucin solution (0.1% w/v) for 1 h. Then, the samples were covered with a TCL solution for 1 h to load to the uppermost mucin layer with this antibiotic as well. Subsequently, this top mucin layer was condensed with glycerol and with Mg2+ ions as described above.

Before conducting a release experiment, the multi-layer samples were carefully rinsed with ultrapure water to remove any unbound molecules. Then, the samples were stored at physiological body temperature (37 °C). Release from the condensed top mucin layer was initiated by exposing the multi-layer construct to a physiological salt solution (150 mM NaCl), and the liberation of TCL was tracked spectroscopically with a spectral photometer at 360 nm. On the next day, the samples were heated up to 40 °C to mimic an inflammatory scenario, and this elevated temperature level initiated the leakage of the thermoresponsive liposomes, which—in turn—induced disassembly of the multi-layer construct and release of TCL from the bottom mucin layer. Also, this second release cascade was followed using a photo spectrometer. To determine the release efficiency of this process, mucin multi-layers in different drug loading conditions (TCL-loaded top layer; TCL loaded, but uncondensed bottom layer; TCL loaded, condensed bottom layer) were disassembled by exposing them to a cocktail containing 150 mM NaCl, 100 mM GlcNAc, and 50 µg mL−1 trypsin for 12 h. Afterward, the amount of released drug was measured spectroscopically to determine the maximum loading capacity as described above.
**Statistical Analysis:** To detect significant differences between two examined groups, two-sample independent t-tests were conducted. To detect statistical differences between more than two groups, one-way analysis of variances (ANOVA), and for multi-comparison, Tukey post hoc tests were performed. A professional software (Prism 8, GraphPad Software, San Diego, CA, USA) was used to conduct all statistical calculations. The level for significance was set to \( p < 0.05 \), and significant differences were marked with an asterisk in the respective graphs; non-significant differences were indicated by the abbreviation “n.s.” where appropriate.

**Statement of Ethics Approval:** Approval of ethics is not required for the experiments conducted in this manuscript.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

C.K. and B.W. contributed equally to this work. The experiments were designed by the contribution of all authors. C.K., B.W., and J.S. performed the experiments and analyzed data. C.K., B.W., and O.L. wrote the manuscript.

**Keywords**

biopolymers, medical implants, stimuli-responsive delivery

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