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Chitosan based substrates for wound infection detection based on increased lysozyme activity

Gregor Tegl [a], Alexandra Rollett [a,*], Jasmin Dopplinger [a], Clemens Gamerith [b,c], Georg M. Guebitz [a,c]

[a] Institute of Environmental Biotechnology, University of Natural recources and Life Sciences Vienna, Konrad Lorenz Straße 20, 3430 Tulln an der Donau, Austria
[b] Qualizyme GmbH, Neue Stiftingtalstrasse 2, 8010 Graz, Austria
[c] ACIB—Austrian Centre of Industrial Biotechnology, Konrad Lorenz Straße 20, 3430 Tulln, Austria

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ABSTRACT

There is a strong need of point-of-care diagnostics for early detection of wound infection. In this study, substrates based on functionalized chitosan were developed for visual detection of elevated lysozyme activity, an infection biomarker in wound fluids. For efficient hydrolysis by lysozyme, *N*-acetyl chitosan with a final degree of acetylation of around 50% was synthesized. *N*-acetylated chitosan and a chitosan-starch composite were labeled with structurally different dyes resulting in lysozyme-responsive biomaterials. Incubation with lysozyme in buffer and artificial wound fluid lead to a release of colored hydrolysis products already after 2 h incubation. Tests in human wound fluid from infected wounds indicated a clear visual color change after 2.5 h compared to control samples. A higher degree of swelling of the chitosan/starch containing substrate led to faster hydrolysis by lysozyme. This study demonstrates the potential of the lysozyme-responsive materials for diagnosis of wound infection and provides different diagnostic substrates for potential incorporation in point-of-care devices.

1. Introduction

Infections are a major health issue. Traumatic, surgical wounds and accompanying healthcare-associated infections are a major cause of retarded wound healing and of the development of chronic wounds. Life-endangering consequences like sepsis illustrate the importance of effective prevention and treatment strategies. The number of patients suffering from wound infections is going to increase significantly due to an aging population and accompanying diseases (Gottrup, Melling, & Hollander, 2005; Sen et al., 2009).

Wound infection is a consequence of bacterial contamination, often by poly-microbial microflora whose complex networks favor their proliferation and complicate treatment. The variety of microorganisms encompasses aerobic and anaerobic bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa* as the most frequently found representatives (Tegl, Schiffer, Sigl, Heinzle, & Guebitz, 2015). Their interaction with the wound environment can be separated into four phases: contamination, colonization, critical colonization and infection. Despite the still unclear role of individual bacterial species on wound infection, this division allows an assessment whether the wound healing process is impaired or not and if an infection becomes evident. A disruption of the wound healing process is characterized by the interruption of the cell signaling cascade involving growth factors and the extracellular matrix (ECM). Non-healing leads the wound to remain in the inflammation phase and consequently triggers infection. (Howell-Jones et al., 2005; Schultz & Wysocki, 2009)

To date, clinicians still diagnose wound infection using classical signs like redness, heat, swelling and pain. In many cases, like chronic wounds, these indicators are not obvious and consequently lead to false negatives impeding the wound treatment. (Gardner, Frantz, & Doebbeling, 2001; Gardner, Hillis, & Frantz, 2009). To date, no point-of-care (Poc) testing device is available providing an online monitoring of the wound infection status. Nevertheless, the high demand reflects the need for PoC testing devices to facilitate the choice of an appropriate therapy. (Cutting & White, 2005)

More reliable information on infection can be deducted from biomarkers in wound fluids like enzymes or metabolites (Yager,

* Corresponding author.
E-mail addresses: gregor.tegl@boku.ac.at (G. Tegl), alexandra.rollett@boku.ac.at (A. Rollett), jasmin.dopplinger@boku.ac.at (J. Dopplinger), clemens.gamerith@qualizyme.com (C. Gamerith), guebitz@boku.ac.at (G.M. Guebitz).
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Kulina, & Gilman, 2007; Tegl et al., 2015). Elevated activities of certain enzymes in wound fluids directly depict the immune response and consequently give a direct view into the current wound status. Frequently mentioned representatives of enzyme biomarkers are lysozyme, myeloperoxidase and human neutrophil elastase. (World Union of Wound Healing Societies (WUWHS), 2008). Among these, lysozyme takes a special position since it is exclusively produced by the human immune system and thus only reflects the host immune response (Torsteinsdóttir et al., 1999). Its antimicrobial activity was known before the 3-dimensional structure was elucidated. Classified as a glycosidase, the native substrate of lysozyme is the peptidoglycan of bacterial cell walls. It cleaves the glycosidic linkage between units of *N*-acetyl glucosamine and *N*-acetylmuramic acid and thus promotes lysis of the bacterial cell. However lysozyme was found also hydrolyzing chitosan, a biopolymer consisting of β(1,4)-linked units of *N*-acetyl glucosamine and glucosamine (Stokke, Varum, Holme, Hjerde, & Smidsrod, 1995). Due to the different composition compared to peptidoglycan a decreased hydrolysis rate towards chitosan is observed that can be increased via chemical modification of chitosan. Several effects of physico-chemical properties of chitosan have been described to influence its susceptibility towards lysozyme hydrolysis, including the degree of acetylation (DA) and the distribution of acetyl groups along the polymer chain (Han, Nwe, Furuike, Tokura, & Tamura, 2012).

The objective of this work was the synthesis of substrates for fast and visual lysozyme detection in wound fluids. Chitosan was chemically modified ensuring high susceptibility towards lysozyme degradation. Previously processed chitosan was further modified with copolymers and dyes applying two different strategies that resulted in efficient substrates for the visual detection of elevated lysozyme activities in infected wounds. The chitosan based enzyme substrates circumvent the use of bacterial peptidoglycan potentially causing immune reactions and the substrates, differing in chemistry and composition, can be further evaluated for incorporation into diagnostic devices.

2. Material and methods

2.1. Materials

For all experiments, chitosan from shrimp shells was used with a number average molecular weight of 200 kDa (Sigma-Aldrich, Steinheim, Germany) and was purified as described below. All chemicals were from Sigma-Aldrich and used without further purification.

2.2. Purification of chitosan

The commercial chitosan was purified to remove residual proteins and glucans prior to all further modifications. Therefore, 20 g chitosan were dispersed in double distilled water (ddH_2O) prior to acidification with acetic acid to obtain a 1% w/v chitosan solution in 0.05% acetic acid. The solution was stirred overnight and thereafter insoluble material was removed via vacuum filtration using a 30 μm filter. Subsequently, the pH was adjusted to 8 for precipitation of the chitosan. The precipitate was washed with ddH_2O and ethanol until the pH reached 7. The resulting pure chitosan was lyophilized for further use.

2.3. Preparation and characterization of N-acetylated chitosan

The DA of the purified chitosan was adjusted by selective *N*-acetylation ensuring high susceptibility towards lysozyme. The DA was analyzed by ^{1}H NMR spectroscopy and FTIR.

2.4. N-acetylation of chitosan

Purified chitosan was dissolved in 10% acetic acid to obtain a 1% w/v chitosan solution which was then diluted 1:1 with ethanol and stirred for several minutes. Afterwards, 1 mol equivalent of acetic anhydride was added (calculated on the glucosamine units in the polymer) and the mixture was further stirred for 1 h. The pH was adjusted to 8, which led to gel formation which was lyophilized. After subsequent washing with ddH_2O to remove salts, the *N*-acetylated chitosan was lyophilized again.

2.5. Determination of the degree of acetylation (DA)

2.5.1. NMR

Nuclear magnetic resonance 1H and 13C measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequencies 400.13 MHz for 1H and 100.63 MHz for 13C) equipped with a 5 mm observe broadband probe head with z-gradients. D_2O with a drop of 37% DCl was used as NMR solvent if not otherwise specified. The DA was calculated using the integrals of the proton peaks H2 of the deacetylated monomer and the three proton peaks of the acetyl groups:

$$DA\,(\%) = \left(1 - \left(\frac{H2D}{H2D + \frac{HAc}{3}}\right)\right) \times 100$$

where H2D is the integral of H2 proton and HAc is the summation of integrals of the *N*-acetyl group.

2.5.2. FTIR

The degree of acetylation was further confirmed by Fourier transform infrared spectroscopy in attenuated reflection mode (ATR – FTIR) using a Spectrum 100 Perkin Elmer FT-IR spectrometer (Massachusetts, USA). The amide I band at 1640 cm^{-1} and the amide II band at 1560 cm^{-1} were considered and compared with native chitosan. Spectra were normalized considering the peak at 1025 cm^{-1} (C—O stretching virbration) and spectra have been stacked afterwards for better illustration.

2.6. Hydrolysis of N-acetylated chitosan by lysozyme

N-acetylated chitosan (25 mg) was dissolved in 0.1 M sodium acetate buffer (pH 5) or 0.1 M sodium phosphate buffer (pH 6.2) yielding 5 mg/mL chitosan solution. Afterwards lysozyme dissolved in the respective buffer was added resulting in a total concentration of 2 mg/mL (70,000 U/mg) and the samples were incubated at 37 °C and 150 rpm. The progress of hydrolysis was monitored using the DNS method detecting the reducing sugar content in sample withdrawals. Reducing sugars are generated during the hydrolysis of chitosan by lysozyme.

2.6.1. Determination of the reducing sugar content

The amount of reducing sugars present was assessed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). A standard curve was plotted determining the absorbance of different concentrations of *N*-acetyl glucosamine at 540 nm. The relative sugar content is given as mg/mL glucosamine equivalents.

2.6.2. Determination of the degree of polymerization by SEC

The number average molecular mass (M_n) was determined by analytical size exclusion chromatography composed of an Agilent 1100 Series Chromatography system equipped with an Agilent 1200 G1362A refractive index detector. A TSKgel G5000PW_{XL} column was used for analysis (Tosoh Bioscience, Montgomeryville, PA, USA). Calibration was performed using a pullulan standard set (Fluka, Buchs, Switzerland). As mobile phase an acetate buffer