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Enzyme functionalized electrospun chitosan mats for antimicrobial treatment

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ABSTRACT

This work presents electrospun chitosan mats, functionalized with glucose oxidase (GOX) to implement an *in-situ* hydrogen peroxide (H_2O_2) generation system. The as spun CTS-PEO mats exhibited a smooth and homogenous morphology in combination with a high specific surface area (5.4 m^2/g) providing an excellent basis for further functionalization and subsequent glutaraldehyde crosslinking provided them with superior mechanical stability in aqueous environments. GOX was covalently immobilized, as proven by XPS, and resulted in activity recoveries between 20 and 40%. The functional mats generated a steady state concentration of ~60 μM H_2O_2 per cm^2 which resulted in growth inhibition of *E. coli* and of *S. aureus* already after two hours of incubation. Additional cytotoxicity tests of the modified mats against mouse fibroblasts did not show an influence on the viability of the cells which proved it a functional biomaterial of great potential for biomedical applications.

1. Introduction

Chitin is one of the most abundant natural polymers (Cheng, Gao, Wang, & Hu, 2015) and as a by-product of shellfish industry processed cost-efficiently and available in large quantities (Yan & Chen, 2015). Chitosan is the most-important derivative of chitin and shows a wide range of advantageous properties such as it is biodegradable (Kean & Thanou, 2010), non-antigenic (Vandevord et al., 2002) and its cytocompatibility towards various cell types such as osteoblasts, fibroblasts, hepatocytes and neural cells has been demonstrated (Ajalloueian et al., 2014). Furthermore chitosan is known to exhibit a wide range of biological characteristics (Aranaz et al., 2009) including inherent antimicrobial effects (Cunha et al., 2012, chap. 5), haemostatic activity (di Lena, 2014) or analgesic capacity (Okamoto et al., 2002). Therefore chitosan is widely used in wound dressings (Tchemtchoua et al., 2011), drug (Sonia & Sharma, 2011) and gene delivery systems (Köping-Höggård et al., 2001), for hemorrhage-control (Wedmore, McManus, Pusateri, & Holcomb, 2006) as well as in various tissue engineering applications (Croisier & Jérôme, 2013). Furthermore carrying amino- and hydroxyl groups chitosan offers various sites for additional functionalization or crosslinking. Previous studies reported chitosan to be a key player in antimicrobial hydrogel systems for wound treatment (Öhlknecht et al., 2016; Tegl et al., 2016).

The immobilization of enzymes on chitosan as carrier material gained of great interest for multiple applications (Krajewska, 2004). Recently, the approach to augment the inherent antimicrobial activity of chitosan by immobilization of antimicrobial acting enzymes was successfully investigated and proved superior bacterial growth inhibition (Tegl et al., 2016). Several oxidases are known to date generating hydrogen peroxide (H_2O_2) from molecular oxygen in concomitantly to substrate oxidation. Glucose oxidase (GOX) may be the most prominent representative producing H_2O_2 upon oxidation of glucose as substrate. The bactericidal properties of H_2O_2 have been known for long time and concentrated solutions thereof find application for antiseptic wound treatment (Barrett, Brennan, & Patton, 2008; Kanta, 2011). However, the applied high concentrations of H_2O_2 (~3% v/v) were found to show cytotoxic effects towards intact skin tissues, whereas levels above 10 μM H_2O_2 suffice to antimicrobial (Hyslop et al., 1995). Topical wound treatment with H_2O_2 solutions requires overdosing due to the instability of the agent. The same issue holds true for many wound dressings which are directly impregnated with the active antimicrobial agent. Enzymatic H_2O_2 *in-situ* generation systems like GOX could

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overcome this issue since they constantly produce low levels of H_2O_2 for more than 24 h. Incorporation of these enzyme based machineries in wound dressing materials constitutes a promising approach for future wound treatment modalities (Plassmann, 2011; Tegl et al., 2016).

Chitosan can be processed in various forms including films (Tchemtchoua et al., 2011), gels, particles (Tegl et al., 2016) or fibrous mats (Cheng et al., 2015). The advantages of the latter being a high surface to volume ratio, 3D structures mimicking natural extracellular matrix (ECM) (Guex et al., 2012; Nisbet, Forsythe, Shen, Finkelstein, & Horne, 2009) as well as tailored porosity enabling excellent mass transfer (Croisier & Jérôme, 2013). Electrospinning displays a convenient method of producing such fibrous mats using a simple, straight forward setup allowing the systematic design of nano- to micron-sized non-wovens. Addressing electrospinning parameters such as the process conditions (tip to collector distance, applied voltage, flow rate, collector geometry), the solvent system (chemistries, mixing ratio, polymer concentration), the ambient conditions (temperature, relative humidity) and the type of polymer(s) being spun allows to control precisely the physical and biological characteristics of the fibers obtained (Schiffman & Schauer, 2008). Properties such as release and degradation profiles, cellular response or the mechanical stability can be tailored towards specific needs making non-wovens promising candidates for a wide range of biomedical applications (Agarwal, Wendorff, & Greiner, 2008). In case of chitosan based fibrous mats, a tuning of their characteristics is often achieved by blending with other polymers of synthetic or natural origin. An example being poly(ethylene oxide) PEO, a synthetic polymer with good biocompatibility and approved for internal use in food, cosmetics, personal care products and pharmaceuticals (Bhattarai, Edmondson, Veiseh, Matsen, & Zhang, 2005) which reduces the viscosity of chitosan solutions and improves the stability of the spinning process (Pakravan, Heuzey, & Ajji, 2011).

The aim of this study was the development of electrospun chitosan mats suitable for further functionalization with the antimicrobial acting GOX by use of selected reaction routes. Thereby, a thorough investigation of the chitosan fiber mats is presented by use of FT-IR, SEM, krypton physisorption and XPS, including investigation of the crosslinking mechanisms, which is especially important for further GOX immobilization. The mats were tested for clinically relevant H_2O_2 turnover rates, antimicrobial activity (*S.aureus, E.coli*) as well as their cytocompatibility by use of 3T3 mouse fibroblasts. Apart from mimicking natural extracellular matrix and offering tailored release and degradation profiles e-spun mats as novel substrates for the immobilisation of biologically active moieties offer the advantage of spatial controlled activity at the site of application compared *e.g.* to substrate systems based on particles. This can be crucial in order to pass regulatory requirements which are usually less demanding in the case of localized systems (Appendini & Hotchkiss, 2002). The in the course of this study functionalized mats produce H_2O_2 in clinically relevant concentrations and are thus suitable dressing material for wound care applications.

2. Materials and methods

Chitosan with an average molecular weight of 112 kDa, a degree of deacetylation of 86.1% and a viscosity of 32 mPa s (1% in 1% acetic acid) as specified by the supplier was purchased from HMC (Halle (Saale), Germany). PEO with a molecular weight of 600 kDa and glutaraldehyde solution (GA, 50 wt.% in H_2O) were obtained from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were purchased from Sigma Aldrich (Steinheim, Germany) and are of analytical grade and used as received unless stated otherwise. Glucose oxidase from *Aspergillus niger* was purchased from Sigma.

2.1. Polymer solutions

Spinning solutions with a total polymer concentration of 4% w/w and a chitosan/PEO ratio of 95:5 w/w were prepared in 90% w/w acetic acid using ultrapure water (MilliQ, > 18 MΩ cm). The ratio chitosan-PEO of 95:5 was chosen in order to obtain highest possible nitrogen surface concentrations in combination with most stable spinning conditions. Solutions were mixed using a tabletop orbital shaker (3020, GFL, Burgwedel, Germany) at room temperature (RT). After observing complete chitosan dissolution solutions were spun within 48 h in order to avoid aging effects and prevent polymer degradation.

2.2. Solution characterization

Solution viscosity was measured as a function of shear rate (from 0.01 to 500 s^{-1}) on a Physica MCR 300 rheometer with cone–plate geometry (Anton Paar, Buchs, Switzerland). Solutions were homogenized for 30 s by pre-shearing at 50 s^{-1} and equilibrated at 25 °C prior to measurements. Solution conductivity was measured on a 660 conductometer from Metrohm (Herisau, Switzerland) equipped with a Pt 100 dip-type conductometric cell (c = 0.83 cm^{-1}). The results given are the averaged values of triplicates.

2.3. Electrospinning

Electrospinning was performed in a Faraday cage being placed in a mobile fume hood (Captair Flex XLS 392, Erlab, Val de Reuil Cedex, France) located in a climatic cabin in order to control environmental conditions (30 ± 5% RH and 22 ± 0.5 °C). The setup consisted of a 3 mL plastic syringe containing the spinning solution to be ejected by an infusion pump (World precision instruments, Sarasota, FL, USA), maintaining a constant flow rate of 10 μL/min. A 21G × 7/8" blunt needle (Braun, Melsungen, Germany) served as the positive electrode and a custom made rotating drum (Ø 10 cm, axis 15 cm, rotational speed 5 Hz) placed 12 cm apart served as the counter electrode collecting the fibers. Applied voltage was +12 kV and −4 kV respectively, generated by two voltage supply sources (AIP Wild AG, Oberglatt, Switzerland). The electrical field strength was controlled using LabView software (National Instruments, Austin, TX, USA).

2.4. Crosslinking

The spun CTS-PEO fibers readily dissolve in aqueous environments and were therefore crosslinked by means of glutaraldehyde (GA) vapor to allow for subsequent functionalization. Mats exhibiting a thickness of 60 μm were cut into pieces of equal size and weight (660 μg) using a piercer (Ø 15 mm). Crosslinking took place in a desiccator (1 dm^3) containing 4 mL of aqueous GA solution (50 wt.%). After 4 h of crosslinking the mats were removed and dried in a vacuum oven at 40 °C in order to remove any unreacted molecular GA residues. The crosslinked nanofiber mats were dried in a desiccator and stored in plastic bags until further processing.

2.5. Immobilization of GOX on chitosan

GOX was immobilized in a modified approach to the one published by Tegl et al. (2016). A sodium phosphate buffer (50 mM, pH 7.0) was used for glutaraldehyde immobilization, a MES buffer (50 mM, pH 5.7) for EDC coupling and a final concentration of 0.5 mg/mL GOX was. The electrospun chitosan mats were cut into pieces of 0.5 cm^2 prior to the enzyme immobilization. After the functionalization with GOX the mats were stored in the above mentioned buffer at 4 °C.

2.5.1. Glutaraldehyde method

A chitosan mat was suspended in 2 mL sodium phosphate buffer containing glutaraldehyde (2% v/v) before 5 μL of GOX dissolved in the same buffer were added to obtain a final GOX concentration of 0.5 mg/mL. The reaction mixture was incubated for 3 h at 20 °C under stirring (200 rpm) prior removing the chitosan mat from the solution. Afterwards the mat was washed with buffer until no GOX activity in the