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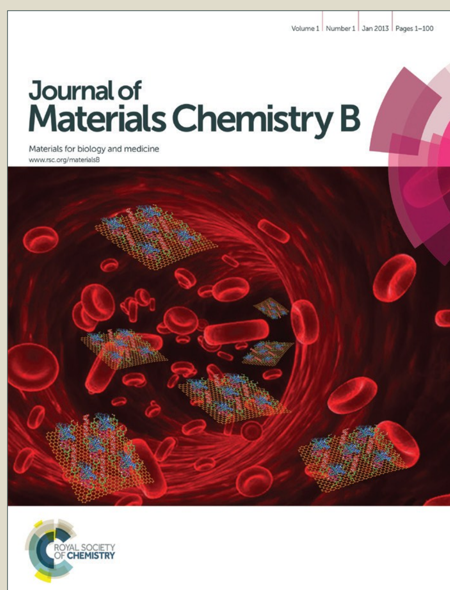

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Ultrasound coating of polydimethylsiloxanes with antimicrobial enzymes

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Abstract

There is an urgent need for antimicrobial functionalization of urinary catheters to prevent their colonization by microbes and biofilm formation. Here, the antimicrobial hydrogen peroxide (H_2O_2) producing enzyme cellobiose dehydrogenase (CDH) was for the first time grafted onto polydimethylsiloxanes (PDMS) using an ultrasound assisted coating method. This resulted in the development of an effective *in-situ* continous H_2O_2 producing system able to continually prevent microbial colonization and biofilm formation on catheters. This enzyme has an added advantage that it uses various oligosaccharides including expolysaccharides (an important part of the bioflim produced by the microbes while colonizing biomaterials) as electron donors to produce H_2O_2. Successful immobilization of active CDH nanoparticles on PDMS was confirmed by ESEM and AFM analysis as well as quantification of H_2O_2. Depending on the initial enzyme concentration, CDH-nanoparticles of varying sizes from 65±17nm to 93±17nm were created by the ultrasonic waves and subsequently deposited on the PDMS surface. PDMS sheets treated for 3 min produced 18 μM of H_2O_2 within 2 hours which was sufficient to significantly reduce the amount of viable *S. aureus* cells as well as the total amount of biomass deposited on the surface. The ultrasound assisted coating of antimicrobial enzymes therefore provides an easy approach to immobilize enzymes and create a surface with antimicrobial properties.

Keywords

Ultrasound assisted coating, polydimethylsiloxane, antimicrobial surface, cellobiose dehydrogenase

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1. Introduction

Microbial biofilms are a major source of infections accounting for >80% of nosocomial infections in hospitals and are the cause of many indwelling medical device associated infections e.g. in the urinary tract[1]. Catheter associated urinary tract infections (CAUTI) are frequently occurring healthcare associated infections which are caused by biofilm forming pathogens colonizing the catheter surfaces [2, 3]. Enzymes are routinely used in medicine, for example for the treatment of lysosomal storage diseases [4],in addition some enzymes are increasingly being exploited for developing antibiofilm and antimicrobial agents[5]. Compared to free enzymes, immobilized enzymes are more stable and enhance their effect in-situ [6, 7]. Consequently, grafting of enzymes to surfaces facilititates their application (continuous processes with enzyme recycling, rapid termination of reactions, and greater variety of bioreactor designs) [8-11]. Moreover, immobilization of enzymes opens new frontiers in their therapeutic applications. Recently, a novel subcutaneous glucose sensor was suggested employing an immobilized enzyme on an electrode [12].There are several techniques for enzyme immobilization including the well-established methods physical adsorption of enzymes, covalent binding, and entrapment of enzymes in a growing polymer [13-15]. The sonochemical process was found to be a very efficient method of synthesizing and simultaneously depositing inorganic nanoparticles (NPs) on a wide variety of surfaces such as fabrics [16], fibers and polymers [17, 18]. Only recently, the sonochemical technique was found to be very efficient in formation of enzymes nanoparticles from their solution [19, 20]. Cellobiose dehydrogenase from *Myriococcum thermophilum* is an oxidoreductase oxidizing cellobiose and a wide variety of structurally different cellooligosaccharides with oxygen as electron acceptor leading to the generation of hydrogen peroxide (H_2O_2) [21, 22]. The strong oxidative properties of H_2O_2 lead to bleaching applications of fibers

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[23] while the antimicrobial effect was recently exploited for the develpoment of wound dressing hydrogels [5, 24, 25]. H_2O_2 in low concentrations is widely used as antimicrobial agent and disinfectant in both medical and food related sectors[26, 27]. One big advantages of using CDH over pure H_2O_2 is the *in-situ* /on-demand production of the oxidative agent which is otherwise known to have a low stability when kept in solution [28]. Immobilization and consequently the possibility of reusing the enzyme and/ or avoid leaching to the process or human body would make it even more interesting for industrial applications. CDH is very temperature stable, with no measurable decrease in enzyme activity up to 70°C. Therefore, CDH is a very suitable enzyme for immobilization using a one-step sonochemical process, as elevation of temperatures is possible during this treatment. The aim of the present study was to investigate antimicrobial functionalisation of PDMS urinary catheters with NPs of CDH grafted with ultrasound.

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2. Experimental

Media components and cellobiose were purchased from Carl ROTH (Karlsruhe, Germany). All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany). The recombinant *Myriococcum thermophilium* cellobiose dehydrogenase (rMtCDH) was optimized for increased oxygen reactivity by a single mutation (N748G) and was produced in *Pichia pastoris* as previously described [21]. Catheters and films were provided by Degania Silicone Ltd (Israel). *Staphylococcus aureus* ATCC 25923 was obtained from the culture collection of the Institute of Environmental Biotechnology (Graz, Austria).

2.1 Immobilization of CDH on PDMS surface

A typical reaction for the preparation of CDH coated PDMS sheets was carried out as follows: Silicone sheet (1x1cm^2) was placed in a beaker containing the enzyme solution (30ml, 1.2mg/ml). The beaker was placed in an ice bath in order to prevent the overheating of the enzyme solution during the sonication process. The solution was irradiated for 3, 10, 15 or 30 minutes using a high intensity ultrasonic Ti-horn (Sonics and Materials, VC-600, 20 kHz, acoustic power 100 W/ cm^2, 750 W at 22% amplitude). The tip of the horn was immersed in the solution to a depth of about 10 mm. Several parameters were tested for their effect on immobilization efficiency including the initial enzyme concentration.

2.2 Characterization

The sonochemically-immobilized CDH - NPs on PDMS were characterized using environmental-scanning electron microscopy (ESEM, Quanta FEG, FEI), and Atomic Force Microscopy (AFM) with a Nanoscope V Multimode scanning probe microscope (Digital instruments, Santa Barbara, CA).

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2.3 Water Contact angle (WCA) measurements

The changes in water contact angle of the ultrasound coated silicone sheets as compared to the untreated silicone sheets was assessed as described by Gindl-Altmutter et al. [29]. Briefly, the WCA measurements were based on the sessile drop method (Scheikl and Dunky 1998) and determined by means of a 2/3″ CCD camera device (Sony 93D model XC-77CE, Tokyo, Japan), which was adjusted against background light. Specimens were fixed on a moveable table and drops applied using a manual dosage system (canula, 1 ml syringe, µm screw driven dosage). Drop shape analysis was performed by the Krüss DSA 1 (Krüss GmbH, Hamburg, Germany) software 20 s after drop application. WCA values mean contact angle of 10 measurements per surface.

2.4 Quantification and determination of enzyme activity of the immobilized enzyme

The enzyme in solution and the enzyme deposited on the silicone surface were quantified using BCA Protein Assay Kit (Pierce, Rockford, USA) with slight modifications. The coated silicone sheets were incubated in 2 ml of working reagent for 30 minutes at 37°C to allow color formation. Then, 200 µl were transferred into 96-well plates and absorbance measured at 562 nm. A calibration curve was recorded using bovine serum albumin (BSA) in concentrations ranging from 0 – 2 mg mL^{-1} as a standard. The obtained values were calculated as mg/plate protein . H_2O_2 production capacity of the immobilized CDH was assessed by the Amplex Red Assay with some modifications using cellobiose as substrate. The CDH - PDMS films were incubated in a solution containing 425 µL 0.25 M sodium phosphate (Na_2HPO4/NaH_2PO4) buffer pH 7.4 and 75 µL 200 mM cellobiose at 37 °C for 2h at 600 rpm to allow H_2O_2 production. After incubation, 197.5 µL of the sample were transferred into a black 96-well plate (Sarstedt, Germany) and 0.5 µL Amplex Red and 2 µL of horseradish peroxidase added. Fluorescence was monitored on a

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microplate reader (Tecan Infinite M200 Pro Platereader, Switzerland) with excitation and emission wavelengths set at 550 and at 587 nm (Ex/Em 550/587 nm), respectively. Fluorescence data were converted to H_2O_2 concentrations by using a calibration curve recoreded with various dilutions of a 30 % H_2O_2 stock solution yielding 0 – 15 µM H_2O_2 in the assay. Pristine PDMS films and films treated only with the immobilization reagents served as references. All experiments were performed in triplicates with sheets deriving from three indepent experiments. The measured values were used to calculate the average and the standard deviation.

2.5 Antimicrobial activity of CDH deposited on PDMS

The antimicrobial activity of CDH coated silicone sheets was investigated by incubation with *S. aureus* in the presence of cellobiose (final concentration 25 mM) in a 24-well plate (Sarstedt, Germany). Briefly, the media containing tryptic soy broth supplemented with 0.5 % (w/w) glucose (TSB+) inoculated with actively growing bacteria (final OD600 0.1). The sheets were incubated for 3 hours at 37 °C without shaking. After incubation, non-attached cells were removed by washing them three times with 0.9% NaCl. Untreated PDMS sheets treated in the same way as the samples served as control. The sheets were stained with the LIVE/DEAD cell viability assay (Life technologies, Carlsbad, USA) for one hour and bacteria visualized using a FluoView 1200 Confocal Laser Scanning microscope (CLSM) from Olympus (Pennsylvania, USA).

3. Results and Discussion

3.1 Characterization of CDH immobilized on PDMS surfaces

The immobilization of the CDH-NPs on PDMS silicone sheets was confirmed by environmental scanning electron microscope (E-SEM) investigations. Figure 1b illustrates the morphology of the PDMS surfaces coated from the low concentration

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solution of 0.13 mg/ml. When the concentration of CDH in the starting solution was increased from 0.13 mg/ml to 0.52 mg/ml, the amount of CDH particles on the surface increased creating a homogenous coating of PDMS with CDH-NPs (Figure 1c). The mean particle size and the distribution of the particle sizes was also affected by changing the initial CDH concentration (Figure 1d, e). Spherical particles of 65±17 nm were formed from 0.13 mg/ml CDH while from 0.52 mg/ml CDH an increase of the particle size to 93±17nm was measured. When 1.2 mg/ml CDH was used and the reaction time reduced from 30 to 3 min to lower the heating effect, particles of 75.2± 10.4 nm were obtained (As seen in the TEM image, Figure 2). These findings are in agreement with previous studies where the size of the nanoparticles formed also depended on the initial enzyme concentration (i.e. higher concentration of enzyme, led to the fomation of larger nanoparticles) [19].

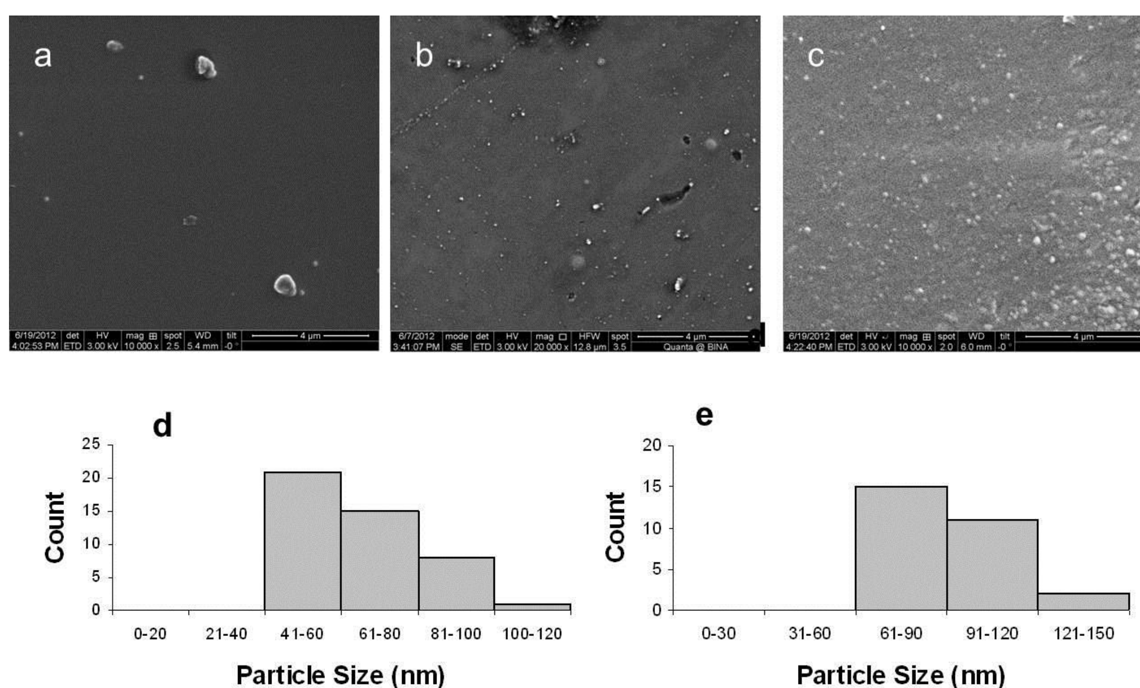

Figure 1: ESEM images of a) pristine PDMS surface, b) PDMS coated with 0.13 mg/ml CDH; c) PDMS coated 0.52 mg/ml CDH; d) and e) histograms of particle size distribution created from 0.13 mg/ml and 0.52 mg/ml CDH, respectively.

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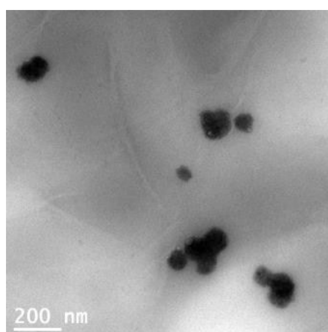
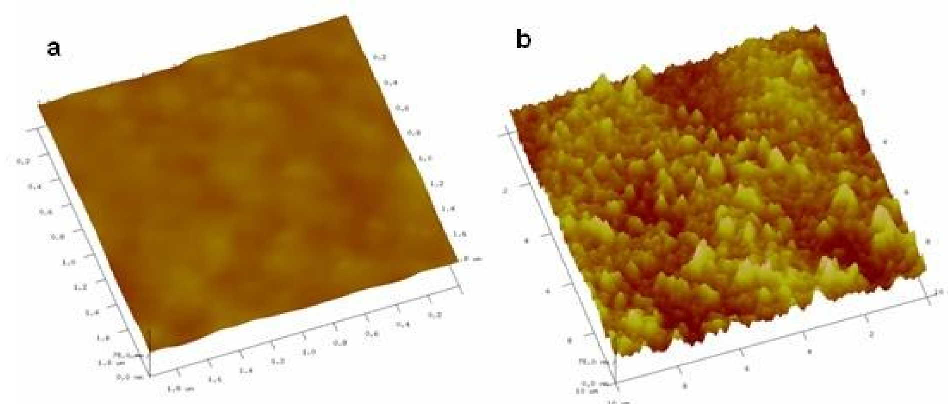


Figure 2: Enzymes NP in solution

AFM inspection of the topography of the PDMS indicates complete coating with enzyme nanoparticles (Figure 3b). The surface roughness at the nanometer scale was significantly altered upon the deposition of enzyme NPs on the PDMS surface. Root Mean Square (RMS) analysis by the nanoscop software revealed a more than 5 fold increase in surface roughness by comparing the pristine and coated surfaces (Table 2).



RMS (Rq)	Uncoated	CDH Coated
Average	2.03 nm	13.8 nm

Figure 3: Atomic force microscopy (AFM) images and roughness of (a) pristine PDMS silicone sheet and (b) ultrasound coated CDH NPs.

Previously, deposition of a large variety of inorganic nanoparticles on different substrates was achieved using the sonochemical method. The sonochemical

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mechanism by which the nanoparticles were deposited on the substrates was discussed and is related to the creation of microjets and shock waves as the after effects of cavitation [16]. In the current case, when high-intensity ultrasound is applied to the aqueous solution of CDH, we hypothesize that the molecules of CDH are adsorbed on the formed acoustic bubbles. When the cavity collapses, the absorbed molecules are exposed to extreme, localized conditions of temperature and pressure. As a consequence, these molecules impact on each other and nanoparticles of the organic compound are produced. Cavitation occurs not only in liquid, but also in a solid-liquid interface, i.e. near polymer surface, due to increased nucleation rate in presence of a solid substrate [30]. After the bubbles collapse, high-speed jets of the liquid transport the generated nanoparticles at high speed toward the PDMS surface where they remain strongly embedded.

3.2 Water contact angle measurements

Contact angle measurements of pristine PDMS and PDMS sheets treated with solutions containing 0.13, 0.52 or 1.2 mg/ml CDH were performed. PDMS is a very hydrophobic material with a contact angle of 106.7 ± 2.1°. When treating the PDMS sheet with a solution containing 0.13 mg/ml of CDH the amount of CDH deposited was not high enough to significantly change the WCA, as it measured to be 108,7±3°. A decrease in WCA was noted for the PDMS films treated with higher concentrations of CDH leading to angles of 92.6 ± 3.4° and 88.3 ± 7.3°, respectively (Figure 4).

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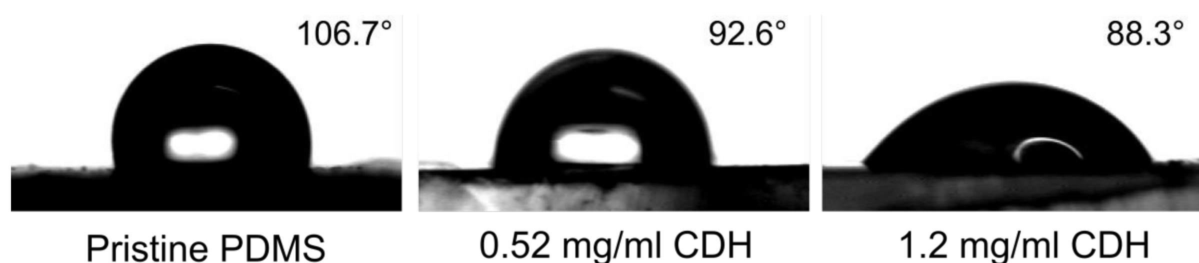

Figure 4: WCA analysis of pristine PDMS films and sonicated in CDH solutions with different concentrations

The WCA measurements demonstrate again that the amount of CDH deposited on PDMS depends on the concentration of the starting solution confirming the findings above and previous data for ultrasound coating of α-amylase nanoparticles on polyethylene films [19]. The biggest advantage of this one step immobilization strategy over conventional immobilization methods is the short amount of time needed to achieve a coated surface as well as the unnecessity of activation of the immobilizeation support and toxic coupling reagents.

3.3 Analysis of immobilized enzyme: Influence of initial concentration and sonication duration on immobilization

Two parameters were found to have an effect on the immobilization efficiency, namely initial protein concentration and sonication duration. It can be stated, that longer sonication times have two consequences: on the one hand, the amount of enzyme deposited on the PDMS surface increases, while on the other hand the enzyme activity of the deposited CDH decreases. In detail, the immobilization efficiency calculated as fraction of the amount of protein intially in solution deposited on the PDSM film increases from 1.4 % after 3 min of sonication to 2.4% after 30 min of sonication, respectively. Interestingly, increasing the sonication time tenfold only lead to about 50% more protein deposited while the enzyme activity almost decreased ten-fold (Figure 5). It is worth mentioning that an attempt was made to follow the leaching of the anzyme off the PDMS surface into water and saline

solution. However, we could not detect any measureable amount. First because the coated amount was small and second the leaching was minimal.

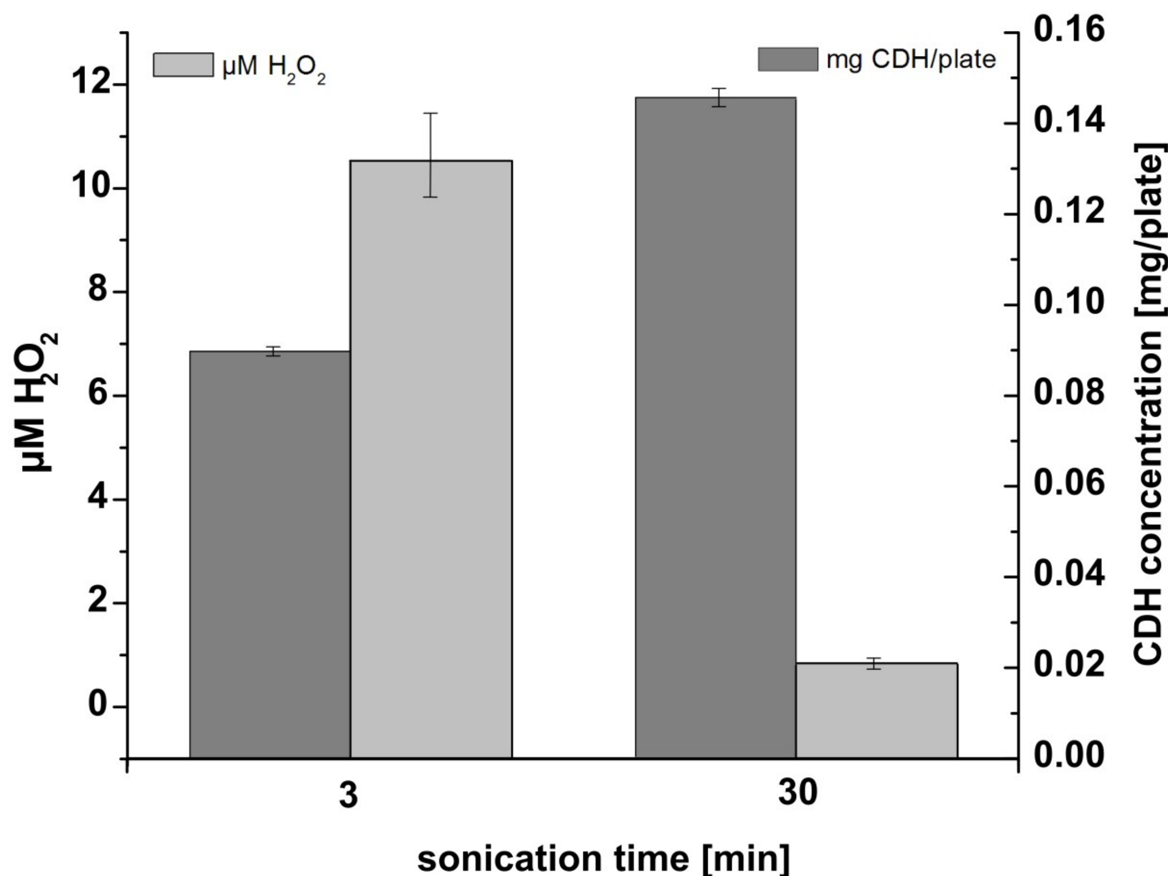

Figure 5: Effect of duration of sonication on amount of protein deposited (dark grey) and resulting enzyme activity given as H_2O_2 production (light grey) when using an initial enzyme concentration of 0.6 mg/ml

These two parameters developing in a contrary fashion over time can have multiple reasons. As temperature in the enzyme-buffer solution increase with time of the sonication treatment it was expected that CDH would lose a certain amount of its activity. Literature also reports that enzymes can undergo conformational changes when exposed to sonochemical treatment thereby decreasing the enzyme activity [31].

In order to yield a surface producing higher amounts of H_2O_2 the effect of the initial enzyme concentration was assessed. Therefore the CDH concentration was increased to 1.2 mg/ml of CDH. Indeed, after 3 minutes of sonication this led to an

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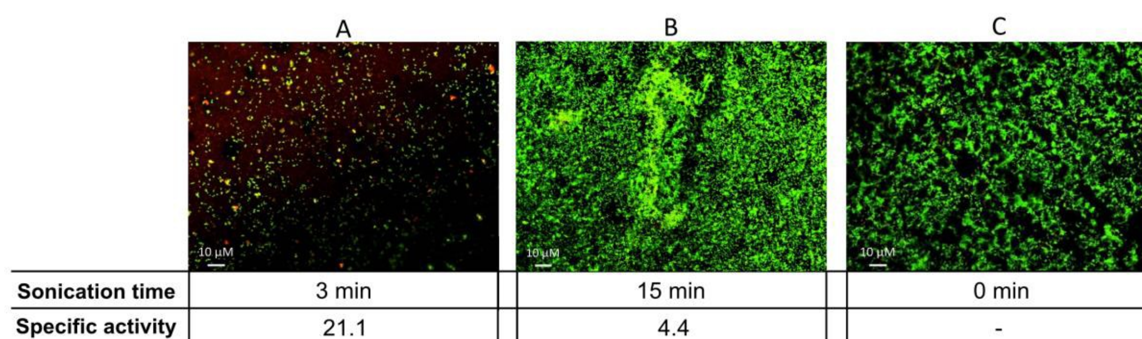
increase of the enzyme activity from 10.0 to 18.4 µM H_2O_2 produced within 2 hours. Again, an increase of the sonication time from 3 to 15 minutes reduced the specific enzyme activity on the plate from 21.1 to 4.4 nmol H_2O_2/min mg protein. In contrast, 3 minutes of sonication did not signficantly change the enzyme activity when compared to the native enzyme (21.2 nmol H_2O_2/min mg protein). Based on the characterization results, the optimal conditions were determined as 3 min of sonication and an initial concentration of 1.2 mg/ml.

3.4 **Antimicrobial activity of CDH deposited on PDMS**

CDH deposited on PDMS was able to produce H_2O_2 in the µM range which, in previous studies, proved to be very effective against a wide range of clinical isolates commonly found in biofilms colonizing urinary catheters[32]. Consequently, LIVE/DEAD staining of *S. aureus* colonies attached to the surface of PDMS was performed and indeed demonstrated that deposited CDH did not only exert its antimicrobial activity but was also able to reduce the amount of cells deposited on the surface (Figure 6).



	A	B	C
Sonication time	3 min	15 min	0 min
Specific activity	21.1	4.4	-

Figure 6: CLSM pictures of LIVE/DEAD stained *S. aureus* colonies attached to silicone sheets after sonochemical grafting of CDH for (A) 3 min, (B) 15 min and (C) positive control – untreated PDMS sheet and the corresponding specific activity of the immobilized enzyme [nmol H_2O_2/min mg protein]

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Expectedly, the amount of alive bacteria (green fluorescence) increases with increasing sonication time which is concomitant to the decrease of enzyme activity and H_2O_2 formation (figures 5 and 6). However, the sample treated for 15 min does not show any difference to the control despite the fact that some H_2O_2 was measured for this sample. A possible reason, for the decreasing antimicrobial activity even though the same amount of H_2O_2 is produced by the samples sonicated for 3 min and 15 min, could be attributed to the inactive/ or only partly active enzyme deposited on the surface. This could help the bacteria with the initial adhesion and therefore cancel out the antimicrobial effect of the H_2O_2 produced.

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4. Conclusion

An enzyme immobilization strategy for the deposition of CDH nanoparticles on PDMS surfaces using a sonochemical approach was successfully developed. ESEM analysis proved the formation of CDH nanoparticles with an average size of 93±17 nm and their deposition on PDMS sheets. Initial enzyme concentration and time of sonication had a strong influence on the amount of enzyme being deposited on the surface and its activity. Longer sonication time lead to higher amounts of enzyme deposited which, however, was partially denatured as reflected by decreasing activity. The amount of H_2O_2 produced by immobilized CDH (18 µM) was sufficient to significantly reduce the amount of viable cells and the total biomass deposited on the surface when tested against *S. aureus*. The method presented here is a fast approach to immobilize enzymes and subsequently produce antimicrobial surfaces.

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Journal of Materials Chemistry B Accepted Manuscript

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