ACS APPLIED MATERIALS & INTERFACES

Research Article

www.acsami.org

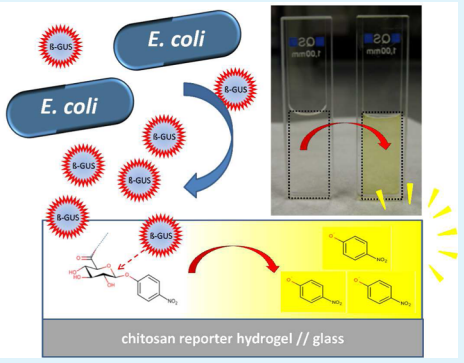
Rapid Detection of *Escherichia coli* via Enzymatically Triggered Reactions in Self-Reporting Chitosan Hydrogels

Mir-Morteza Sadat Ebrahimi, Yvonne Voss, and Holger Schönherr*

Physical Chemistry I, Department of Chemistry and Biology & Research Center of Micro and Nanochemistry and Engineering (Cμ), University of Siegen, Adolf-Reichwein-Strasse 2, 57076 Siegen, Germany

Supporting Information

ABSTRACT: In this work, a self-reporting hydrogel for the rapid in situ detection of bacterial enzymes is reported. To implement the reporting function for the bacterium *Escherichia coli* into a film-based sensing format, chitosan hydrogel films on solid backing supports were equipped with a reporting function for the enzyme β-glucuronidase (β-GUS), which is secreted by >98% of all known *E. coli* strains. Covalent coupling of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide or the complementary chromogenic substrate 4-nitrophenyl-β-D-glucuronide via amide bond formation afforded an attachment that is stable for >24 h under physiological conditions. By contrast, in the presence of β-GUS, the reporter dyes were very rapidly cleaved and produced a signal for the presence of the enzyme, which was detectable by bare eye under appropriate illumination. Detailed investigations of the enzymatic reaction for both types of substrates in neat enzyme solution as well as in bacterial supernatant revealed the apparent reaction kinetics and allowed us to determine the concentration of β-GUS in the supernatant. Under optimized conditions, the 4-methylumbelliferyl-β-D-glucuronide-functionalized hydrogel reported the presence of β-GUS within 15 min with a limit of detection of <1 nM. Finally, the function of the generally applicable hydrogel-film-based sensing approach, which is compatible with polymer-film-based applications, including wound dressings and packaging materials, and is also amenable to address noncultivatable pathogenic bacteria by using appropriate fluorogenic or chromogenic substrates, was demonstrated by direct application with bacterial medium.



KEYWORDS: *hydrogel, biosensor, bacteria detection, E. coli, pathogenic bacteria*

INTRODUCTION

The detection of pathogenic bacteria is an important topic with increasing relevance because of the increasing threat of antibiotic resistance in bacteria. In light of not only an increasing and alarming number of serious outbreaks of infections of multiply-resistant bacteria in hospitals but also in view of food and water safety, simple and rapid early warning systems that signal bacterial infection are highly wanted.

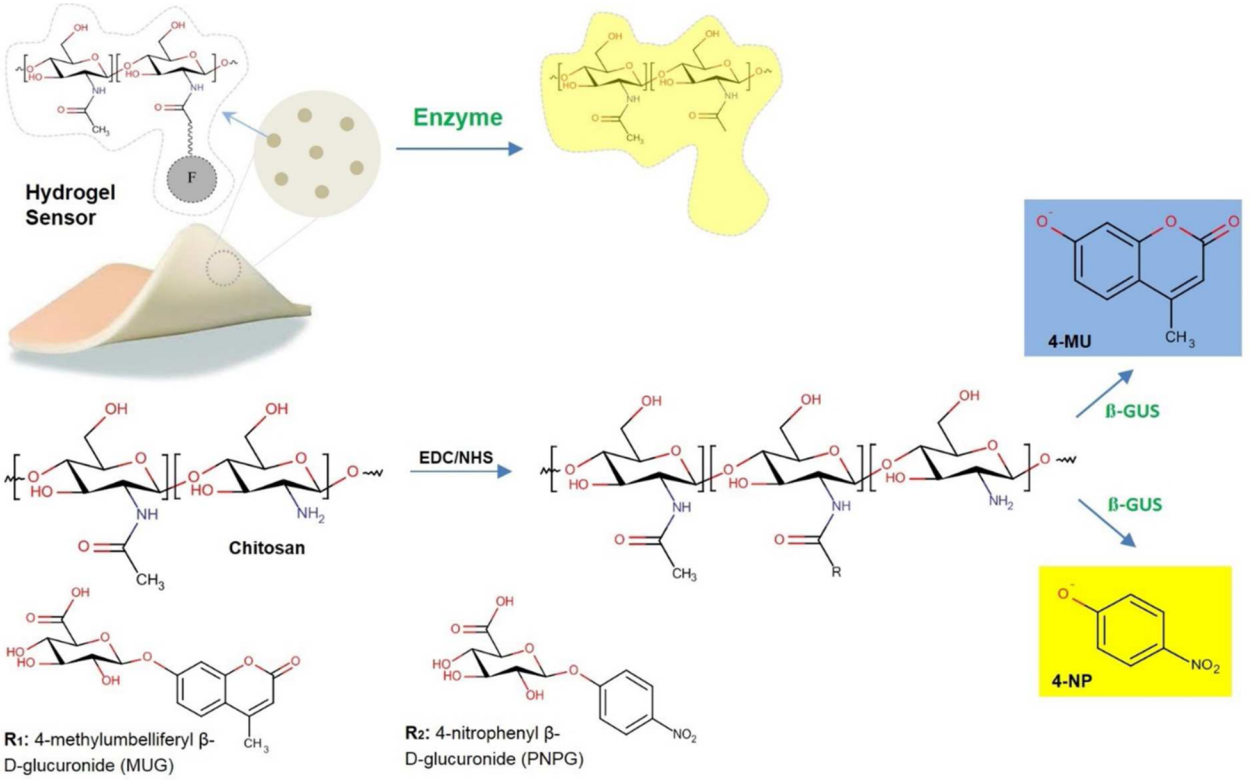
In the case of potentially infected wounds, for example, the detection of infection caused by pathogenic bacteria is typically based on clinical signs, i.e., fever and analysis of laboratory values, such as serum C-reactive protein and counts of white blood cells.[1,2] In most cases, wound swabs are taken, and microorganisms are identified by cultivation in the microbiology laboratories in hospitals. These methods are unfortunately time-consuming and suffer from well-known shortcomings. For instance, the usage of swabs provides information only about the kind of bacteria and may fail for noncultivatable species.[3] For the identification of the bacterial species, the polymerase chain reaction (PCR) or next-generation sequencing are employed to identify specific bacterial genomic elements (16S ribosome) for diagnosis, which are expensive and take at least 3 days for completion.[4,5] Another drawback for the methods based on swab cultures and tissue biopsies is the necessity to take samples from the wound, which requires the removal of the wound dressing. For certain wounds, however, such as second degree burn wounds, advanced dressings must not be removed until the skin is regrown beneath the dressing, hence resulting in an obvious incompatibility. Finally, for applications envisioned in home-care or for a quick assessment, new simple approaches are needed.

Therefore, it is not surprising that approaches for the rapid detection of infection which bypass the necessity for (i) time-consuming isolation procedures prior to bacteria identification and (ii) time-consuming analyses as well as being compatible with the advanced dressings mentioned above have garnered particular attention. Recently, electrochemical biosensors that are based on the recognition of lipopolysaccharide components of the membrane of bacteria have been reported.[6] Other approaches focus on bacterial enzymes or toxins for triggering a response of a sensing device because these are present typically only if bacteria increase in numbers or switch from commensurate to pathogenic. For instance, Tram et al. recently introduced a portable colorimetric sensor based on a bacteria-

Received: June 27, 2015
Accepted: August 19, 2015
Published: August 31, 2015

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

Scheme 1. Schematic of the Enzyme-Sensing Hydrogel Platform and Details of the Modification of Chitosan Hydrogel[a]



[a]Top: schematic of the enzyme-sensing hydrogel platform. Bottom: details of the modification of chitosan hydrogel with the fluorogenic (MUG) and chromogenic (PNPG) substrates and selective enzymatic cleavage by *β*-GUS secreted from *E. coli*, which liberates the reporter dyes 4-methylumbelliferone (4-MU) and 4-nitrophenol (4-NP) into the hydrogel. The deprotonation of the hydroxyl group in 4-MU and 4-NP is responsible for the altered photophysical properties of the dyes utilized for the detection.

specific RNA-cleaving DNAzyme probe as a molecular recognition element, which involves coupling of the enzyme urease to the DNAzyme on magnetic beads.[7] In presence of the target aptazyme, urease is released in the medium that can be used for hydrolyzing urea, hence elevating the pH of the medium which in turn can be detected by litmus paper. In another approach for the detection of infection, Hasmann et al. reported on a sensor material for the detection of human neutrophil elastase and cathepsin G because the amount of these two proteinases are significantly higher in infected wound fluids compared to noninfected ones.[8] In a related report, a peptidoglycan-based device for the rapid detection of lysozyme in wound fluid was introduced.[9]

Among the various approaches explored, concepts for self-reporting infection-sensing wound dressings that exploit nanocapsules have garnered attention recently. Zhou et al. reported on a novel concept based on dye release from phospholipid vesicles as a result of lysing the membrane by toxins/enzymes of the pathogenic bacteria.[10] This concept was expanded to nanoparticles and capsules by the Landfester group,[11] whereas Haas et al.[12] and Tücking et al.[13,14] exploited enzyme-labile vesicles of amphiphilic block copolymers for sensing model proteinases for the proteinase produced by *Pseudomonas aeruginosa* and hyaluronidase produced by *Staphylococcus aureus*.

Because of the wide range of different bacterial species that are important in diverse applications and problems ranging from the medical field via food production and packaging to water safety, a more general platform approach is desirable. In particular, those approaches that allow one to implement the sensitive detection of different bacteria without the need for potentially complex synthetic approaches or time-consuming optimization are wanted. To address this need, we report here on a complementary approach for the rapid in situ detection of bacterial enzymes, which is in principle amenable for transfer to the detection of infection in diagnostic wound dressings, biomedical devices, and food safety monitoring. The approach is a significant extension of our previous report on hydrogel-based enzyme detection.[15] Although this earlier study on the detection of the model enzyme *α*-chymotrypsin established a number of key observations, we demonstrate in this current paper the detection of a relevant bacterial enzyme in buffer and in bacterial supernatant as well as of bacteria in suspension. In addition, two complementary modes of detection, namely, fluorescence and UV−vis spectroscopy, were employed to achieve the main objective, which was to realize the rapid, selective detection of *Escherichia coli*. By exploiting a built-in colorimetric sensor (fluorogenic or chromogenic substrates for particular enzymes produced and excreted by pathogenic bacteria) that is covalently conjugated to a chitosan hydrogel, the detection of infection does not necessarily require advanced equipment or skilled personnel. It can be ultimately envisioned for use in home-care because the detection can be carried out using a hand-held UV lamp or bare-eye observation, depending on the type of liberated dye.[16]

This approach combines the well-established biodegradable and biocompatible polysaccharide chitosan, which is known for its wound-healing activity,[17−19] gene delivery,[20] and tissue

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

engineering applications,[21,22] with enzymatic substrates that are widely used in biochemistry in identification systems.[23,24] The application of neat enzymatic substrates in culture media for microbiology to detect or identify the microorganisms is well-known,[25−27] in particular because of the significantly decreased time and improved accuracy of detection needless of prior isolation procedures.[28−30]

To facilitate the detection of bacteria using the approach shown in Scheme 1, the enzyme β-glucuronidase (β-GUS) is targeted; this enzyme catalyzes the hydrolysis of β-linked D-glucopyranosiduronic acids (β-glucuronides) to glucopyranosiduronic acid and aglycone.[35] The enzyme is secreted by around 98% of all known *E. coli* strains.[27] Other bacteria that may produce the enzyme (among them some strains of *Yersinia*, *Salmonella*, *Shigella*, *A. viridans*, *Bacillus* spp., or *Corynebacterium*)[31,32] are, according to the literature, uncommon in potential applications in wound dressings.[33] There is also evidence that β-GUS production is regulated depending on population density.[34]

In this work, the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) and the chromogenic substrate 4-nitrophenyl-β-D-glucuronide (PNPG) were hence used for the covalent modification of chitosan hydrogels and thereby the rapid detection of β-GUS and the presence of *E. coli*.[35] On the basis of a thorough investigation, the apparent reaction kinetics and the limit of detection (LOD) were determined, which allows one to conclude the general applicability of the platform approach to rapidly detect bacteria, including pathogenic bacteria, in situ within only 15 min in a film-based sensing format with a subnanomolar LOD. In addition to detection by bare eye, β-GUS was also shown to be detectable directly in bacterial suspensions.

EXPERIMENTAL SECTION

Materials. Silicon(100) wafers (P/Boron type, OKMETIC, Finland) and glass slides (ultrathin type, d = 0.1 mm; Menzel Gläser, Braunschweig, Germany) were used as substrates. Chitosan (medium molar mass, 190−310 kDa; 75−85% deacetylated), 4-methylumbelliferyl-β-D-glucuronide hydrate (MUG), 4-methylumbelliferone (4-MU), 4-nitrophenyl-β-D-glucuronide sodium salt (PNPG), 4-nitrophenol (4-NP), β-glucuronidase purified from *E. coli* (β-GUS, E.C.3.2.1.31; type IX-A, lyophilized powder), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC.HCL), *N*-hydroxysuccinimide (NHS), lysogeny broth (LB) (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L, pH 7.2),[36] and phosphate-buffered saline (PBS tablet, pH 7.4) were purchased from Sigma-Aldrich. *Escherichia coli* (strain: Mach1-T1, Invitrogen, California, USA), acetic acid (glacial, J. T. Baker), sulfuric acid (95%, Fischer), hydrogen peroxide (30%, Roth), and sodium hydroxide (99%, Riedel-de Haën) were purchased from the listed suppliers. Milli-Q water from a Millipore Direct Q8 system (Millipore, Schwalbach, Germany) with resistivity of 18.0 MΩ/cm was used for preparation of all aqueous media.

Fluorescence Spectroscopy. Measurements were recorded on a Varian Cary Eclipse spectrometer (Mulgrave, Victoria, Australia) equipped with a temperature-control unit. Spectra were obtained with a resolution of 5.0 nm for both excitation and emission at a scan rate of 600 nm/min, unless otherwise mentioned. The measurements were carried out via the front-face illumination technique in a 0.1 cm path length quartz cell (SUPRASIL, Hellma Analytics, Germany) at an incidence angle of 70°.[15]

UV/Visible Spectroscopy. Measurements were recorded on a Varian Cary 50 Bio spectrophotometer (Mulgrave, Victoria, Australia) equipped with a temperature-control unit in the wavelength range of 300−650 nm at a scan rate of 600 nm/min. The measurements were carried out in a quartz cell with 0.1 cm path length for transmission measurements of coated samples on glass slides. The spectra obtained were baseline-corrected.

Fourier Transform Infrared Spectroscopy. Measurements of chitosan hydrogel films on silicon were recorded on an IFS 66v spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a liquid-nitrogen-cooled MCT detector in transmission mode (1000 scans with a spectral resolution of 4 cm^{-1}). The background spectrum was collected by scanning a freshly Piranha-cleaned silicon wafer.

Ellipsometry. Measurements were carried out in ambient air with an alpha-SE variable angle spectroscopic ellipsometer (J. A. Woolam Co., Lincoln, NE) at three different points in the center area of each sample at three different incidence angles (65, 70, and 75°) with wavelengths between 380−900 nm. The data were fitted using a three-layer model in EASE software: silicon as the substrate, a native silicon oxide layer, and chitosan, using the Cauchy model with a refractive index of 1.512 (at λ = 632.8 nm). The results are reported as the arithmetic mean values with the standard deviation as error.

Modification of Chitosan with Fluorogenic/Chromogenic Substrate. Silicon wafers (cut to 9 × 10 mm^2) and glass slides (cut to 9 × 15 mm^2) were cleaned in Piranha solution (1:3 (v/v) hydrogen peroxide/concentrated sulfuric acid) for 2 min followed by rinsing with copious amounts of Milli-Q water and drying in a nitrogen stream. ***Caution!*** *Piranha solution should be prepared, used and discarded with extreme caution!* Chitosan solutions (0.5% w/v) were prepared in acidified water (using acetic acid, pH ∼5.0); the solution was filtered through Whatman grade 5 qualitative filter paper (VWR, Germany) to remove impurities and particles larger than 2.5 μm. The chitosan hydrogel layer was prepared by depositing 100 μL of chitosan solution on the neat silicon/glass wafers. All coated substrates were annealed in a vacuum oven (125 °C, 0.05 mbar, 4 h) and neutralized by rinsing with NaOH solution (0.1 M) and Milli-Q water several times repeatedly, followed by drying in a nitrogen stream. The thickness of the dried samples was measured by ellipsometry.

Grafting of the Substrates to Chitosan. The fluorogenic (MUG) and chromogenic (PNPG) substrates were grafted to the chitosan hydrogel films according to the literature.[37−39] MUG and PNPG ($COOH/NH_2$ = 0.3:1; mol/mol of glucosamine unit of chitosan) were dissolved in separate buffered solutions (PBS, pH 7.4) in ambient atmosphere; EDC (3 mol/mol of −COOH) was added to the solution while stirring, followed by addition of NHS (3 mol/mol of −COOH). The calculations were carried out on the basis of the deposited dried mass of chitosan hydrogel on each wafer. Details are mentioned for different samples under each graph. The solution was stirred for 60 min; then, the chitosan samples were immersed in the solution for 6 h. The samples were removed and rinsed with Milli-Q water, then were immersed in PBS solution for 2 h (solution replacement every 30 min), rinsed with Milli-Q water, and dried in a nitrogen stream.

Enzymatic Reactions. For enzymatic reactions in solution, 100 μL of MUG/PNPG solution of defined concentration (in PBS, pH 7.4) was added to 10 μL of freshly prepared buffered enzyme solution (PBS, pH 7.4, 0.1 μM) in the quartz cell. The

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

cell was closed with a stopper and immediately inserted into the fluorescence or UV−vis spectrophotometer.[40,41] For enzymatic reactions in the hydrogels, one grafted chitosan sample was inserted inside the quartz cell followed by addition of the buffered enzyme solution (200 μL, PBS, pH 7.4) to cover adequately the surface of the film. Subsequently, the cell was closed with a stopper and inserted into the fluorescence or UV−vis spectrophotometer, respectively.

Bacterial Tests. One single colony (Mach1-T1) was transferred from agar to 100 mL of LB according to Lennox[36] in a sterilized flask and propagated in a shaking incubator (MAXQ 4000, Thermo Scientific) at 37 °C at 220 rpm for 12 h. A 1.0 mL aliquot of the culture was transferred into a 1.5 mL centrifuge tube and centrifuged at 3000 rpm for 5 min at 25 °C to separate the bacteria from the supernatant (Centrifuge: MicroStar 17, VWR International; tubes: Micro Centrifuge Tube, VWR, Germany).[42,43] The supernatant was filtered through an acetate cellulose filter (0.2 μm pore size, VWR, Germany). For enzymatic reactions, one grafted chitosan hydrogel sample was inserted inside the quartz cell followed by the addition of the bacterial supernatant (200 μL) to cover the surface of the film. Subsequently, the cell was closed with a stopper and inserted into the fluorescence or UV−vis spectrometer (37 °C).

Determination of the Limit of Detection for Liberated 4-MU and 4-NP. A buffered solution of 4-methylumbelliferone (4-MU) was prepared (PBS, pH 7.4, 25 °C) and filtered (Whatman grade 5). The concentration was determined by UV−vis spectroscopy ($OD_{365\ nm}$, ε = 16.5 mM^{-1} cm^{-1}). This solution was diluted and mixed with a solution of the substrate (MUG) of predetermined concentrations (Figure S-1a). The fluorescence emission intensity was measured by fluorescence spectroscopy. Each recorded spectrum was deconvoluted into two separate peaks using a Gaussian two-peak fit routine. The integrated surface area under the corresponding peak for the product (4-MU at λ_{em} = 445 nm) was calculated and plotted versus the concentration (Figures S-1b). The intercept from a linear least-squares fit of the data was considered as background. A solution of 4-NP (PBS, pH 7.4, ambient) of predetermined concentration was prepared, diluted, and mixed with the PNPG solution of a chosen concentration (Figure S-2a). The absorption spectra were recorded with the UV−vis spectrometer and each spectrum was deconvoluted into two separate peaks using a Gaussian two-peak fit routine. The integrated surface area under the corresponding peak for the product (4-NP at λ_{max} = 405 nm) was plotted versus the concentration. The calculated intercept from a linear least-squares fit of the data was considered as background. The LOD is defined as background plus three times the standard deviation of the background.[44,45] Hence, this afforded the LOD for the dyes using the detection instrumentation and approach. The LOD for the enzyme (β-GUS) was estimated on the basis of the procedure explained in the Results and Discussion.

Blank Experiments. A series of blank experiments was carried out for MUG-/PNPG-grafted chitosan hydrogels treated with (i) buffer solution (PBS, pH 7.4, 0.5 mL, 25 °C) and (ii) LB medium according to Lennox (0.5 mL, 37 °C) (Figure S-3).[36]

Photography. The optical photographs were taken with a digital camera (A630, Canon, Japan). For fluorogenic substrate-grafted samples, the samples were illuminated by a conventional hand-held UV lamp (type 5361, Heraeus, Hanau, Germany), and chromogenic substrate-grafted samples were photographed under ambient-light illumination.

RESULTS AND DISCUSSION

Modification and Characterization of the Grafted Hydrogels. The self-reporting hydrogel was prepared by an on-film modification of chitosan hydrogel films deposited by solution casting onto silicon or glass supports. The biodegradable and biocompatible polysaccharide chitosan, which is derived by partial deacetylation of the natural biopolymer chitin,[46] forms hydrogels without cross-linking because of physical entanglements of the biomacromolecules with high molar mass.[47] To the hydrogel films, the fluorogenic substrate (MUG) and the chromogenic substrate (PNPG) were conjugated by EDC chemistry via amide bond formation between the carboxyl group in the glucuronide unit of the enzymatic substrates and the free primary amine groups in the glucosamine unit of chitosan (Scheme 1).[15] The modification was verified by FTIR spectroscopy. In the FTIR spectra of MUG-/PNPG-grafted chitosan (Figure 1), the peak at 1598

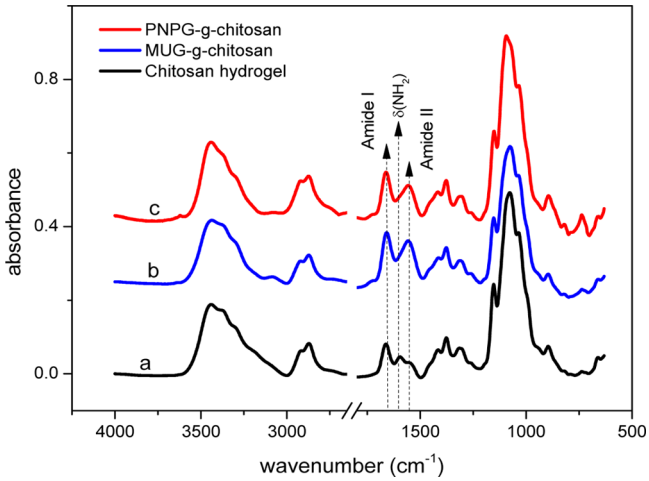


Figure 1. Transmission mode FT-IR spectra of (a) chitosan film, (b) MUG-grafted chitosan, and (c) PNPG-grafted chitosan.

cm^{-1} (primary amine of the glucosamine unit of chitosan) disappeared, whereas the peaks at 1664 and 1556 cm^{-1}, which are attributed to the amide I and II vibrations, respectively, became stronger. This observation is consistent with the formation of new amide bonds in the modified chitosan. The new band at 3078 cm^{-1}, which is assigned to the stretching of the aromatic C−H bonds, confirms additionally the presence of the coupled substrates.[48] The peak at 3442 cm^{-1} in the FTIR spectrum of chitosan is broad because of the presence of several hydroxyl groups in chitosan and also overlaps with the stretching vibration of the primary amine group. The bands observed and the corresponding assignment to molecular vibrations are summarized in Table 1. The procedure afforded a reproducible maximum attainable degree of substitution, which is based on the analysis of the FTIR spectra ~80%. We note that the maximum attainable sensor output corresponding to the maximum possible dye release can be controlled by the hydrogel film thickness, as shown in our previous work.[15]

Enzymatic Reactions in MUG-Grafted Hydrogels. The reporting function of the hydrogels is based on selective enzymatic reactions of the enzyme β-GUS, which is secreted by >98% of all known *E. coli* strains.[27,33] This hydrolytic enzyme

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

Table 1. Peak Assignments for Most Prominent Bands Observed in the FTIR Spectra (Figure 1) of Chitosan and MUG- and PNPG-Grafted Chitosan Hydrogels

vibrations and assignments	chitosan		MUG-grafted chitosan	PNPG-grafted chitosan
	data	ref 48		
ν_s (C–O–C bridge)	899	890–900	899	900
ν_s (C–O–C)	1078	1070–1075	1078	1079
δ_b (C–H)	1377	1375–1382	1377	1378
ν (N–H) amide II	1556	1550–1565	1556	1558
δ_b (NH_2)	1598	1590–1610		
ν (C=O) amide I	1662	1620–1655	1664	1663
ν_s (O–H)	3441	3435–3455	3442	3445
ν $(C–H)_{aromatic}$			3078	3079

with a molar mass of 290 kDa[49] catalyzes the hydrolysis of the β-glucuronides to an acid and an alcohol. Hence, upon hydrolysis of the fluorogenic substrate, the coumarin derivative 4-MU is cleaved from the glucuronide unit. Because of deprotonation in the hydrogel medium, its fluorescence emission is markedly altered: When excited with a wavelength λ_{ex} of 365 nm, the deprotonated free 4-MU can be detected by fluorescence spectroscopy because it emits at an emission wavelength λ_{em} centered at 445 nm (Figure S-4). The kinetics of the enzymatic reaction in the hydrogel films on silicon was recorded in sequential measurements by fluorescence spectroscopy at 37 °C via the front-face illumination technique (Figure 2a,c). The enzymatic reaction was carried out in aqueous buffered solution (PBS, pH 7.4) because the optimal pH for the activity of the enzyme is around 7.[49] The spectra show a monotonous increase in fluorescence emission intensity with reaction time at the same emission wavelength (λ_{max} = 445 nm) that was observed for the enzymatic reaction in aqueous buffered solution (Figure S-4). The sigmoidal curve (Figure 2b) reaches saturation after approximately 3 h under the conditions employed. This temporal evolution of product formation is tentatively attributed to the superposition of enzymatic cleavage that occurs initially in surface near regions of the hydrogel, a concomitant change in hydrogel swelling, and diffusion of the enzymes into the interior of the hydrogel.

By contrast, in enzyme-free buffer, no emission of 4-MU was detected after reaction times of longer than 24 h (Figure S-3b). The emission of the released deprotonated 4-MU can be easily detected by bare eye if the hydrogel is observed under conventional UV illumination (see below).

After having established the enzymatic liberation of the reporter dye in neat buffered enzyme solution, the enzymatic reaction was investigated in bacterial supernatant in a similar manner. The increase in fluorescence emission intensity at λ_{em}

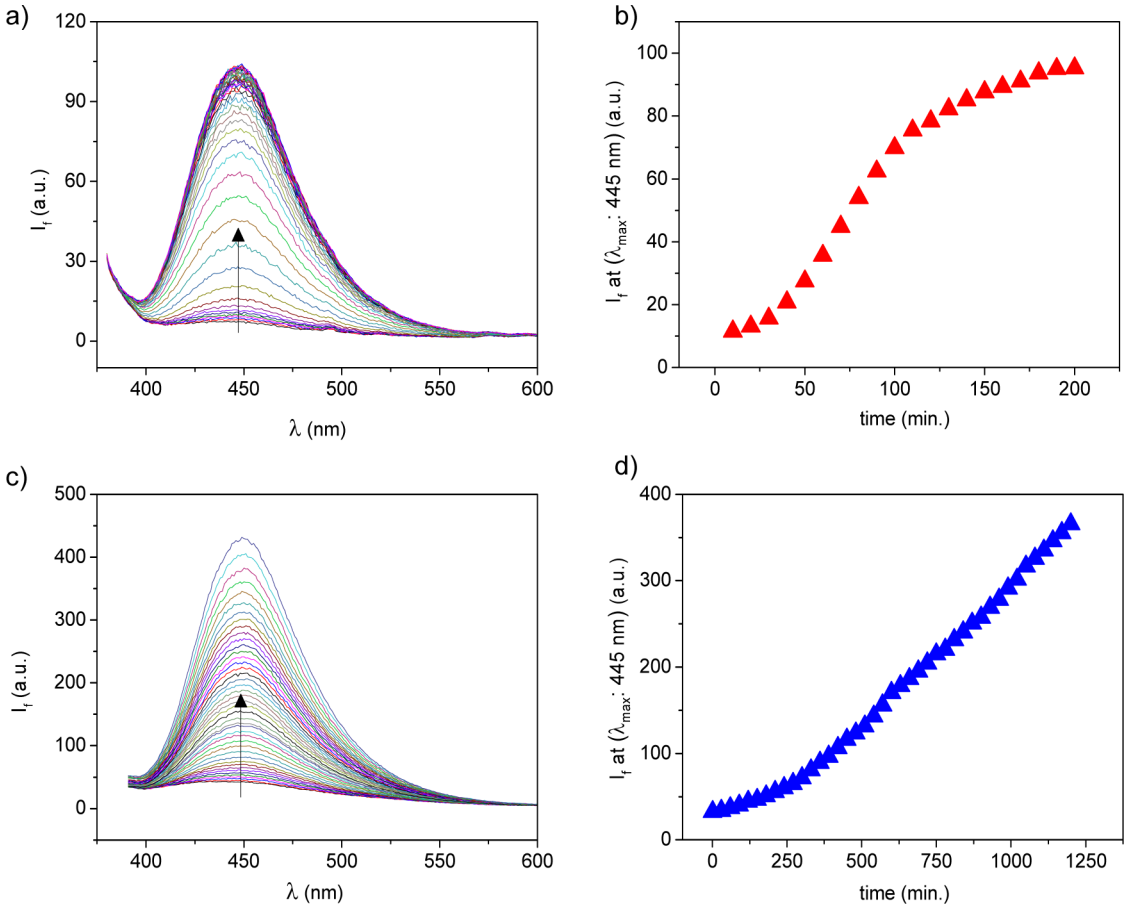


Figure 2. (a) Fluorescence spectra for the enzymatic reaction in MUG-grafted chitosan hydrogels (d_{film} = 2 ± 0.1 μm, $[MUG]_{mod}$ = 0.2 mM, $[\beta\text{-GUS}]_0$ = 0.1 μM, measurement repeat: 10 min, λ_{ex} = 365 nm). (b) Fluorescence emission intensity (I_f) at maximum emission (λ_{max} = 445 nm) of 4-MU in panel a versus time. (c) Fluorescence spectra for enzymatic reaction in MUG-grafted chitosan hydrogels in bacterial supernatant (d_{film} = 4 ± 0.2 μm, $[MUG]_{mod}$ = 0.2 mM, baseline: LB solution, 20 g/L, pH 7.2, $OD_{600\ nm}$ = 3.201, λ_{ex} = 365 nm, measurement repeat: 30 min). (d) Intensity at maximum emission (λ_{max} = 445 nm) of 4-MU in panel c versus time. The spectra shown were not normalized to a blank.

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190–20199

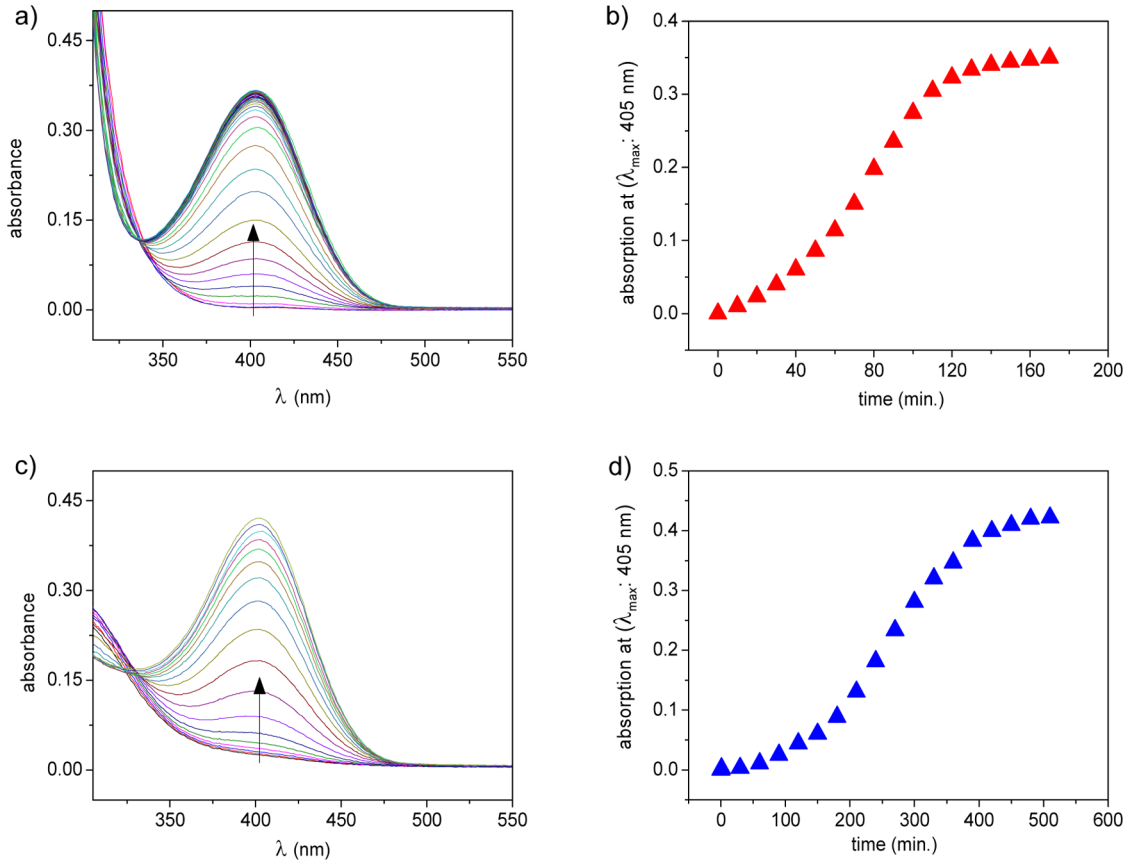


Figure 3. (a) Absorption spectra for the enzymatic reaction in PNPG-grafted chitosan hydrogel on glass (d_{film}= 2 ± 0.1 μm, $[PNPG]_{mod}$ = 0.2 mM, $[\beta\text{-GUS}]_0$ = 0.2 μM; measurement repeat: 10 min). (b) Absorbance at absorption maximum (λ_{max} = 405 nm) for 4-NP in panel a versus time. (c) Absorption spectra for enzymatic reaction in PNPG-grafted chitosan hydrogel on glass (d_{film} = 3 ± 0.1 μm) in bacterial supernatant (LB solution 20 g/L, pH 7.2, $OD_{600\ nm}$ = 2.305, baseline: LB solution in quartz cell, measurement repeat: 30 min). (d) Absorbance at absorption maximum (λ_{max} = 405 nm) for 4-NP in panel c versus time.

= 445 nm was recorded (Figure 2c). The recorded fluorescence emission intensity (I_f) at λ_{max} (Figure 2d) shows qualitatively a temporal evolution similar that of the reaction with neat enzyme. However, because of the lower β-GUS concentration, the reaction proceeds more slowly and did not yet reach completion after 1200 min. We note that the difference in film thickness results in different absolute output signal for long reaction times approaching a complete conversion of the substrates in the hydrogel film.

On the basis of the initial apparent rate in Figure 2b,d, as well as 5b and 6b below, and the assumption that the initial reaction rate (in the first 60 min) is proportional to the concentration of β-GUS, the concentration of the enzyme in the supernatant was estimated to be approximately 60 nM. As shown in Figure 5b below, the apparent rate of the reaction is indeed proportional to the concentration of β-GUS.

Enzymatic Reactions in PNPG-Grafted Hydrogels. The enzymatic cleavage of the chromogenic substrate (PNPG) by β-GUS liberates 4-NP from the glucuronide. The observed color change from colorless to yellow is a result of the deprotonation of 4-NP (Figure S-5). The enzymatic reaction of PNPG-grafted chitosan on glass in aqueous buffered solution (PBS, pH 7.4) at 37 °C studied by sequential measurements in a UV−vis spectrometer leads to a monotonous increase in absorption at λ_{max} = 405 nm (Figure 3a,b). Similar to that of the fluorogenic substrate discussed above, the time evolution of liberated product is sigmoidal in shape. The reaction in bacterial supernatant shows the same qualitative sigmoidal kinetics (Figure 3c,d); however, it proceeds significantly more slowly because of the lower β-GUS concentration. For detection by bare eye, the yellow color was distinguishable after approximately 50−60 min for the reaction in neat buffered enzyme solution and after 220 min for the reaction in bacterial supernatant (Figures 3d and 4c).

Both the fluorogenic and the chromogenic substrate are efficiently and rapidly cleaved off the glucuronides that were conjugated to the chitosan hydrogel. The released reporter dyes can be detected by bare eye, as mentioned above, when appropriate illumination is used (Figure 4a,b). The detection of bacterial enzymes directly in the bacterial suspension is also feasible as shown in Figure 4c, where the following bacterial suspensions are compared: the initially prepared suspension, a suspension after 24 h, and the reacted hydrogel immersed in bacterial suspension after 24 h reaction time. The suspension of the reacted hydrogel appears to be less turbid than the reference sample. This reduced turbidity was observed in this as well as in other conceptually related systems, albeit to different extents. Although we currently have no definite explanation for the observation, it cannot be excluded that the released dye may interfere with the bacteria and therefore may result in a less turbid solution. Because the bacterial medium used here shows inherent fluorescence due to the presence of the yeast extract in the solution, the detection of *E. coli* could not be carried out analogously with the fluorogenic substrate.

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

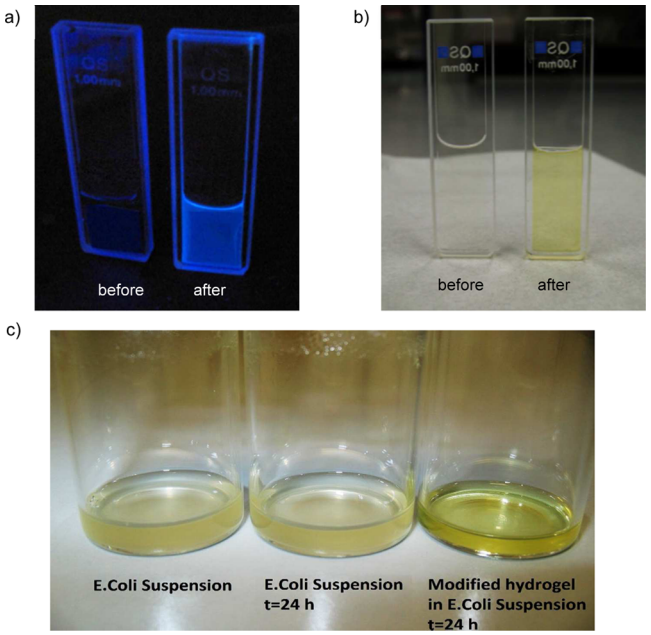


Figure 4. (a) Photograph acquired under UV illumination by a hand-held UV lamp of MUG-grafted chitosan hydrogel before and after completed reaction with β-GUS (Δt = 6 h, d_{film}= 2 ± 0.1 μm, $[MUG]_{mod}$ = 0.2 mM, $[\beta\text{-GUS}]_0$ = 0.1 μM). (b) Photograph acquired under white light illumination of PNPG-grafted chitosan hydrogel before and after completed reaction with β-GUS (Δt = 6 h, d_{film}= 2 ± 0.1 μm, $[PNPG]_{mod}$ = 0.2 mM, $[\beta\text{-GUS}]_0$ = 0.2 μM). (c) Photograph of the *E. coli* suspension before additional incubation, the *E. coli* suspension after additional incubation for 24 h and the detection of β-GUS in bacterial suspension after 24 h. Here the same amount of *E. coli* suspension was added to a dried coated layer of modified chitosan hydrogel on the bottom of vial treated by bacterial suspension (d_{film} = 5 ± 0.3 μm, $[PNPG]_{mod}$ = 0.2 mM, bacterial suspension: LB medium, pH 7.2, $OD_{600\ nm}$ = 2.305). The liberated deprotonated 4-nitrophenol is responsible for the altered photophysical properties of the solution.

Determination of the Limit of Detection. The LOD of the two complementary β-GUS-reporting hydrogel films is of central importance to be able to assess the applicability of the films. As the enzyme present in the medium digests more and more enzyme-labile bonds in the course of the reaction, the concentration of liberated dye increases continuously. If there are no inhibition or deactivation reactions taking place, then a few enzyme molecules may digest all bonds of a given reporter hydrogel if enough time is given. Therefore, it is clear that the value of the LOD depends on the time allowed for the reaction. For the reasons detailed in the introduction, specific and rapid detection methods are desirable. Hence, we focus on detection within time scales less than 1 h. The analysis is based on the facts that the initial apparent rate of the enzymatic reaction is proportional to the enzyme concentration and is independent from film thickness.[15] By definition, fluorescence spectroscopy is more sensitive compared to UV−vis spectroscopy. Hence, we expect the PNPG-grafted system to possess a higher LOD. Therefore, we discuss first the determination of the LOD of the chromogenic substrate, followed by the fluorogenic substrate.

In the following, one set of chitosan films modified with the chromogenic substrate (PNPG) on glass was prepared to investigate how the rate of the enzymatic reaction depends on the initial concentration of β-GUS. The kinetics of enzymatic reaction was recorded by sequential UV−vis measurements. The plot of the absorption of the product 4-NP at λ_{max} = 405 nm versus the reaction time was linear for the first 10 min of the reaction. The slope obtained from a linear least-squares fit was considered as the initial apparent rate of the reaction. The rates observed for different concentrations of β-GUS were plotted versus the initial concentration of the enzyme (Figure 5b). The initial apparent reaction rate was found to increase linearly with initial enzyme concentration, which is in agreement with the Michaelis−Menten kinetics (Figure 5b).[50] To determine the LOD for β-GUS, one first has to determine the LOD for the liberated dye 4-NP using the UV−vis spectrometer according to the literature.[44,45] (See also the details in the Experimental Section.) The LOD of 4-NP in the presence of the conjugated, i.e., unreacted, chromogenic substrates was calculated to be 0.44 μM (Figure S-2). Depending on the concentration of applied enzyme, the minimum detectable signal corresponding to a concentration of 0.44 μM was achieved after different reaction times. Hence, depending on the (arbitrarily chosen) observation time, the LOD for the enzyme differs as shown in Figure 5c. The LOD for β-GUS decreases exponentially with observation time, e.g., for an observation time of 15 min the LOD for β-GUS with a PNPG-grafted hydrogel film is 40 nM. If one waits for 60 min, then the LOD for β-GUS decreases to 15 nM. The latter estimate is consistent with the unequivocal observation of the reaction using bacterial supernatant (Figure 3c,d).

The same measurements and estimates were made for the reporter hydrogels functionalized with the fluorogenic substrate MUG. The kinetics of the enzymatic reaction was recorded by sequential fluorescence emission measurements at λ_{em} = 445 nm. The plot of emission at λ_{max} versus time was linear for the first 12 min of the reaction. From a linear least-squares fit, the initial apparent rate was obtained (Figure 6a). The initial rate was found to depend linearly on the initial β-GUS concentration, which is in agreement with the data for the PNPG-grafted chitosan and the Michaelis−Menten kinetics (Figure 6b).

On the basis of the LOD for the liberated dye 4-MU in the presence of the conjugated, i.e., unreacted, fluorogenic substrates, the LOD for the enzyme could be estimated. The LOD for 4-MU of 4.4 nM was determined according to the literature[44,45] using fluorescence spectroscopy. (See also the details in the Experimental Section and Figure S-1.) The LOD for β-GUS also decreases exponentially with observation time, as shown in Figure 6c. The LOD for β-GUS for an observation time of 15 min is <1 nM. Because of the high signal-to-noise ratio of the fluorescence spectroscopy compared to that of spectrophotometric-based methods, the LOD for liberated 4-MU is more than ca. 40 times less than that of 4-NP.

Our results show that the covalent conjugation of the fluorogenic or chromogenic substrates to chitosan hydrogels does not prevent or significantly hinder the enzymatic reaction. The low LOD values for very short reactions times and the typical β-GUS concentration in bacterial supernatant ca. 60 nM determined here show that the methodology and hydrogel platform for detecting bacteria possesses attractive attributes. Because bacterial counts (cfu/mL) and the production of enzymes often depend on each other in a highly nonlinear fashion, e.g. depending on quorum sensing, available nutrients etc., there is no simple translation to the number of bacteria that could be detected.

Compared to the nanoparticle[11] or nanocapsule-based approaches[10,12−14] the approach discussed here does not need further processes for the immobilization of reporter

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

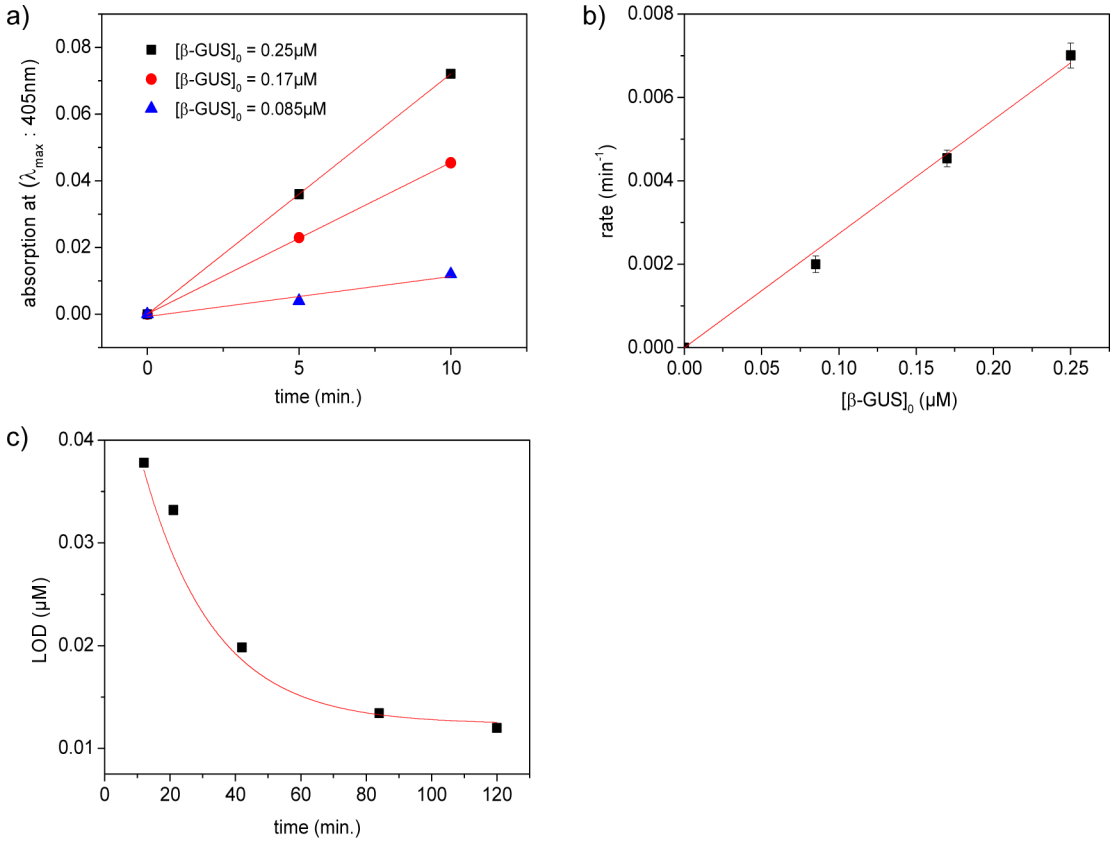


Figure 5. (a) Absorbance at λ_{max} = 405 nm for the enzymatic reaction of modified chitosan hydrogels on glass (d_{film} = 1 ± 0.1 μm, $[PNPG]_{mod}$ = 0.2 mM) with β-GUS in different initial concentrations vs reaction time. (b) Initial apparent rate estimated from the slopes in panel a plotted vs initial enzyme concentration. (c) Plot of the LOD for β-GUS vs reaction time using PNPG-grafted chitosan hydrogels.

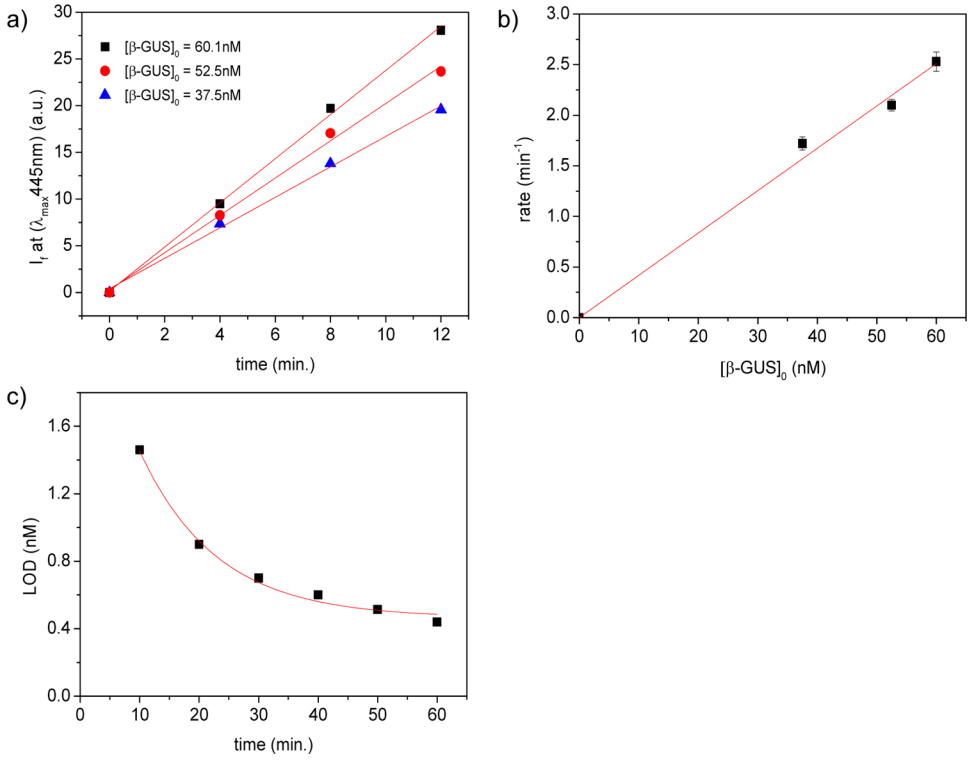


Figure 6. (a) Fluorescence emission intensity at λ_{max} = 445 nm for the enzymatic reaction of three modified chitosan hydrogels on silicon (d_{film} = 2 ± 0.1 μm, $[MUG]_{mod}$ = 0.2 mM) with β-GUS for different initial enzyme concentrations vs time. (b) Initial apparent rate estimated from the slopes in panel a plotted vs initial enzyme concentration. (c) Plot of LOD for β-GUS vs reaction time using MUG-grafted hydrogels.

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

moieties on a typical wound dressing material. Because of the wide variety of known enzymatic substrates and straightforward immobilization chemistries, a broad range of pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, can be selectively detected using this generally applicable detection platform.

For an application of the self-reporting hydrogels, the nature of the released dye is an important issue that needs to be addressed in the further development process. The toxicity of the dyes must be negligible, which is not the case for the model substrates employed in this study. Dye release, which occurs as shown exclusively in the case of an infection, cannot be totally avoided. Possible approaches to eliminate this release into any medium could also include more sophisticated stratified hydrogel layers with a dye-capture layer.

The approach is inherently selective to the enzyme of choice (and for bacteria identification as selective as the enzyme is characteristic for the bacterium of choice) and very rapid, and because of the hydrogel film format, it can be applied to a broad range of potential polymer-film-based applications, including wound dressings and coating materials. Importantly, the direct contact of the hydrogel to the bacteria-containing medium is sufficient, which renders the approach advantageous compared to other schemes that require sampling of wound fluid. Most notably, it avoids in its function as a simple and rapid early warning system that signals bacterial infection the need to (i) analyze genetic material of bacteria with expensive and instrument-intensive methods and (ii) culture bacteria; therefore, it is in principle also amenable to noncultivatable (pathogenic) bacteria.

CONCLUSIONS

We have reported on the development and characterization of a chitosan-based platform for the selective, rapid, and sensitive in situ detection of the enzyme β-GUS, which serves as a marker for the bacterium *Escherichia coli*. The chitosan hydrogels grafted with complementary fluorogenic and chromogenic substrates allow the detection of β-GUS with values of the LOD for the enzyme of 40 and <1 nM for 15 min observation time, respectively. The initial apparent rate of the enzymatic cleavage of reporter dyes is for both substrates linear with enzyme concentration and allowed us to estimate the concentration of β-GUS in bacterial supernatant as ca. 60 nM. Our results further show that the covalent immobilization of the two substrates on the chitosan hydrogels does not hinder the enzymatic reaction for a 290 kDa enzyme. Because the conjugation of other enzymatic substrates for the detection of other species is generally feasible, this self-reporting chitosan hydrogel platform constitutes a promising approach for the in situ detection of pathogenic bacteria that may be potentially exploited in a refined system in medicine as well as food and water safety applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b05746.

Additional spectra of enzymatic reaction in solution, fluorescence and absorption spectra for enzyme blank experiments, and fluorescence and absorption spectra for the calculation of the LOD using fluorescence and UV−vis spectroscopy. (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel.: +49 271 740 2806. Fax: +49 271 740 2805. E-mail: schoenherr@chemie.uni-siegen.de.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. D. Wesner for help with the bacterial tests and inspiring discussions. Financial support by the EU (Project: BacterioSafe, grant no. 245500), the European Research Council (ERC Grant Agreement no. 279202), and the University of Siegen is gratefully acknowledged.

REFERENCES

(1) Rodríguez-Sanjuán, J. C.; Martín-Parra, J. I.; Seco, I.; García-Castrillo, L.; Naranjo, A. C-Reactive Protein and Leukocyte Count in the Diagnosis of Acute Appendicitis in Children. *Dis. Colon Rectum* **1999**, *42*, 1325−1329.

(2) Khan, M. N.; Davie, E.; Irshad, K. The Role of White Cell Count and C-Reactive Protein in the Diagnosis of Acute Appendicitis. *J. Ayub. Med. Coll. Abbottabad* **2004**, *16*, 17−19.

(3) Gübitz, G.; Wehrschütz-Sigl, E.; Hasmann, A.; Binder, B.; Burnett, M.; Schintler, M. Method for Detecting a Wound Infection, Eur. Pat. Appl. WO2010133589A1, 2010.

(4) VanGuilder, H. D.; Vrana, K. E.; Freeman, W. M. Twenty-Five Years of Quantitative PCR for Gene Expression Analysis. *BioTechniques* **2008**, *44 Supplement*, 619−626.

(5) Weisburg, W. G.; Barns, S. M.; Pelletier, D. A.; Lane, D. J. 16S ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.* **1991**, *173*, 697−703.

(6) Giovanni, M.; Setyawati, M. I.; Tay, C. Y.; Qian, H.; Kuan, W. S.; Leong, D. T. Electrochemical Quantification of *Escherichia coli* with DNA Nanostructure. *Adv. Funct. Mater.* **2015**, *25*, 3840−3846.

(7) Tram, K.; Kanda, P.; Salena, B. J.; Huan, S.; Li, Y. Translating Bacterial Detection by DNAzymes into a Litmus Test. *Angew. Chem., Int. Ed.* **2014**, *53*, 12799−12802.

(8) Hasmann, A.; Gewessler, U.; Hulla, E.; Schneider, K. P.; Binder, B.; Francesko, A.; Tzanov, T.; Schintler, M.; Van der Palen, J.; Guebitz, G. M.; Wehrschuetz-Sigl, E. Sensor Materials for the Detection of Human Neutrophil Elastase and Cathepsin G Activity in Wound Fluid. *Exp. Dermatol.* **2011**, *20*, 508−513.

(9) Heinzle, A.; Papen-Botterhuis, N. E.; Schiffer, D.; Schneider, K. P.; Binder, B.; Schintler, M.; Haaksman, I. K.; Lenting, H. B.; Gübitz, G. M.; Sigl, E. Novel Protease-Based Diagnostic Devices for Detection of Wound Infection. *Wound Rep. Reg.* **2013**, *21*, 482−489.

(10) Zhou, J.; Tun, T. N.; Hong, S.-H.; Mercer-Chalmers, J. D.; Laabei, M.; Young, A. E. R.; Jenkins, A. T. A. Development of a Prototype Wound Dressing Technology which can Detect and Report Colonization by Pathogenic Bacteria. *Biosens. Bioelectron.* **2011**, *30*, 67−72.

(11) Baier, G.; Cavallaro, A.; Vasilev, V.; Mailänder, V.; Musyanovych, A.; Landfester, K. Enzyme responsive Hyaluronic Acid Nanocapsules Containing Polyhexanide and their Exposure to Bacteria to Prevent Infection. *Biomacromolecules* **2013**, *14*, 1103−1112.

(12) Haas, S.; Hain, N.; Raoufi, M.; Handschuh-Wang, S.; Wang, T.; Jiang, X.; Schönherr, H. Enzyme Degradable Polymersomes from Hyaluronic Acid-block-poly(ε-caprolactone) Copolymers for the Detection of Enzymes of Pathogenic Bacteria. *Biomacromolecules* **2015**, *16*, 832−841.

(13) Tücking, K. S.; Handschuh-Wang, S.; Schönherr, H. Bacterial Enzyme Responsive Polymersomes: A Closer Look at the Degradation Mechanism of PEG-block-PLA Vesicles. *Aust. J. Chem.* **2014**, *67*, 578−584.

(14) Tücking, K.-S.; Grützner, V.; Unger, R. E.; Schönherr, H. Dual Enzyme-Responsive Capsules of Hyaluronic Acid- block -Poly(Lactic

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199

Acid) for Sensing Bacterial Enzymes. *Macromol. Rapid Commun.* **2015**, *36*, 1248−1254.
(15) Sadat Ebrahimi, M. M.; Schönherr, H. Enzyme-Sensing Chitosan Hydrogels. *Langmuir* **2014**, *30*, 7842−7850.
(16) Poppert, S.; Essig, A.; Stoehr, B.; Steingruber, A.; Wirths, B.; Juretschko, S.; Reischl, U.; Wellinghausen, N. Rapid Diagnosis of Bacterial Meningitis by Real-Time PCR and Fluorescence In Situ Hybridization. *J. Clin. Microbiol.* **2005**, *43*, 3390−3397.
(17) Okamoto, Y.; Minami, S.; Matsuhashi, A.; Sashiwa, H.; Saimoto, H.; Shigemasa, Y.; Tanigawa, T.; Tanaka, Y.; Tokura, S. Polymeric N-acetyl-D-glucosamine (Chitin) induces Histionic Activation in Dogs. *J. Vet. Med. Sci.* **1993**, *55*, 739−742.
(18) Kweon, D. K.; Song, S. B.; Park, Y. Y. Preparation of Water-Soluble Chitosan/Heparin Complex and its Application as Wound Healing Accelerator. *Biomaterials* **2003**, *24*, 1595−1601.
(19) Khor, E.; Lim, L. Implantable Applications of Chitin and Chitosan. *Biomaterials* **2003**, *24*, 2339−2349.
(20) Sato, T.; Ishii, T.; Okahata, Y. In vitro Gene Delivery Mediated by Chitosan. Effect of pH, Serum, and Molecular Mass of Chitosan on the Transfection Efficiency. *Biomaterials* **2001**, *22*, 2075−2080.
(21) Gingras, M.; Paradis, I.; Berthod, F. Nerve Regeneration in a Collagen−Chitosan Tissue-Engineered Skin Transplanted on Nude Mice. *Biomaterials* **2003**, *24*, 1653−1661.
(22) Wang, Y. C.; Lin, M. C.; Wang, D. M.; Hsieh, H. J. Fabrication of a Novel Porous PGA-Chitosan Hybrid Matrix for Tissue Engineering. *Biomaterials* **2003**, *24*, 1047−1057.
(23) Monget, D. Comparison of Enzyme Activity in two Insect Cell Lines: Anthera Eucalypti and Malacosoma Disstria (Lepidoptera). *C. R. Seances Acad. Sci., Ser. D* **1975**, *281*, 651−654.
(24) Tharagonnet, D.; Sisson, P. R.; Roxby, C. M.; Ingham, H. R.; Selkon, J. B. The API ZYM System in the Identification of Gram-Negative Anaerobes. *J. Clin. Pathol.* **1977**, *30*, 505−509.
(25) Perry, J. D.; Freydière, A. M. The Application of Chromogenic Media in Clinical Microbiology. *J. Appl. Microbiol.* **2007**, *103*, 2046−2055.
(26) Perry, J. D.; Archer, L.; Jones, A. L.; James, A. L.; Ford, M.; Gould, F. K. Detection of Carboxypeptidases as Taxonomic Markers for Gram-Negative Bacteria. *Lett. Appl. Microbiol.* **1998**, *26*, 359−362.
(27) Kilian, M.; Bülow, P. Rapid Identification of Enterobacteriaceae. II. Use of a Beta-Glucuronidase Detecting Agar Medium (PGUA Agar) for the Identification of *E. coli* in Primary Cultures of Urine Samples. *Acta Pathol. Microbiol. Scand., Sect. B: Microbiol.* **1979**, *87B*, 271−276.
(28) Orenga, S.; James, A. L.; Manafi, M.; Perry, J. D.; Pincus, D. H. Enzymatic Substrates in Microbiology. *J. Microbiol. Methods* **2009**, *79*, 139−155.
(29) Manafi, M. New Developments in Chromogenic and Fluorogenic Culture Media. *Int. J. Food Microbiol.* **2000**, *60*, 205−218.
(30) Manafi, M. Fluorogenic and Chromogenic Enzyme Substrates in Culture Media and Identification Tests. *Int. J. Food Microbiol.* **1996**, *31*, 45−48.
(31) Molina, F.; López-Acedo, E.; Tabla, R.; Roa, I.; Gómez, A.; Rebollo, J. E. Improved Detection of *Escherichia coli* and Coliform Bacteria by Multiplex PCR. *BMC Biotechnol.* **2015**, *15*, 48.
(32) Fiksdal, L.; Pommepuy, M.; Caprais, M. P.; Midttun, I. Monitoring of Fecal Pollution in Coastal Waters by Use of Rapid Enzymatic Techniques. *Appl. Environ. Microbiol.* **1994**, *60*, 1581−1584.
(33) Watkins, W. D.; Rippey, S. R.; Clavet, C. R.; Kelley-Reitz, D. J.; Burkhardt, W. Novel Compound for Identifying *Escherichia coli*. *Appl. Environ. Microbiol.* **1988**, *54*, 1874−1875.
(34) Leung, J. W.; Liu, Y. Population Density-Dependent Regulation of *E. coli* β-Glucuronidase Gene Expression. *Gastroenterology* **2003**, *124* (Suppl. 4), A247.
(35) Aich, S.; Delbaere, L.; Chen, R. Continuous Spectrophotometric Assay for Beta-Glucuronidase. *BioTechniques* **2001**, *30*, 846−850.
(36) Sezonov, G.; Joseleau-Petit, D.; D'Ari, R. *Escherichia coli* Physiology in Luria-Bertani Broth. *J. Bacteriol.* **2007**, *189*, 8746−8749.
(37) Layek, B.; Singh, J. Amino Acid Grafted Chitosan for High Performance Gene Delivery: Comparison of Amino Acid Hydrophobicityon Vector and Polyplex Characteristics. *Biomacromolecules* **2013**, *14*, 485−494.
(38) Lee, K. Y.; Jo, W. H.; Kwon, I. C.; Kim, Y. H.; Jeong, S. Y. Structural Determination and Interior Polarity of Self-Aggregates Prepared from Deoxycholic Acid-Modified Chitosan in Water. *Macromolecules* **1998**, *31*, 378−383.
(39) Park, S. Y.; Marsh, K. S.; Rhim, J. W. Characteristics of Different Molecular Weight Chitosan Films Affected by the Type of Organic Solvents. *J. Food Sci.* **2002**, *67*, 194−197.
(40) Bergmeyer, H., Ed. *Methods for Enzymatic Analysis*, 2nd ed.; Academic: New York, 1974; pp 929−943.
(41) Tephly, T. R.; Green, M. D.; Coffman, B. L.; King, C.; Cheng, Z.; Rios, G. Metabolism of Endobiotics and Xenobiotics by UDP-Glucuronosyltransferase. *Adv. Pharmacol.* **1997**, *42*, 343−346.
(42) Rice, E.; Allen, M.; Edberg, S. Efficacy of Beta-Glucuronidase Assay for Identification of Escherichia Coli by the Defined-Substrate Technology. *Appl. Environ. Microbiol.* **1990**, *56*, 1203−1205.
(43) Dhende, V. P.; Samanta, S.; Jones, D. M.; Hardin, I. R.; Locklin, J. One-step photochemical synthesis of permanent, nonleaching, ultrathin antimicrobial coatings for textiles and plastics. *ACS Appl. Mater. Interfaces* **2011**, *3*, 2830−2837.
(44) Lévêque, C.; Ferracci, G.; Maulet, Y.; Grand-Masson, C.; Blanchard, M. P.; Seagar, M.; El Far, O. A. Substrate Sensor Chip to Assay the Enzymatic Activity of Botulinum Neurotoxin A. *Biosens. Bioelectron.* **2013**, *49*, 276−281.
(45) Miller, J.; Miller, N. J. *Statistics and Chemometrics for Analytical Chemistry*, 5th ed.; Pearson/Prentice Hall: New York, 2005; pp 121−124.
(46) Manafi, M.; Kneifel, W.; Bascomb, S. Fluorogenic and Chromogenic Substrates Used in Bacterial Diagnostics. *Microbiol. Rev.* **1991**, *55*, 335−348.
(47) Gupta, B.; Agarwal, R.; Alam, M. S. Textile Based Smart Wound Dressings. *Indian J. Fibre Text. Res.* **2010**, *35*, 174−187.
(48) Kasaai, M. R. A Review of Several Reported Procedures to Determine the Degree of N-Acetylation for Chitin and Chitosan Using Infrared Spectroscopy. *Carbohydr. Polym.* **2008**, *71*, 497−508.
(49) Kim, D. H.; Jin, Y. H.; Jung, E. A.; Han, M. J.; Kobashi, K. Purification and Characterization of β-Glucuronidase from Escherichia Coli HGU-3, a Human Intestinal Bacterium K. *Biol. Pharm. Bull.* **1995**, *18*, 1184−1188.
(50) Nelson, D. L.; Cox, M. M., Eds. *Lehninger Principles of Biochemistry*, 4th ed.; W. H. Freeman: New York, 2005; pp 203−208.

DOI: 10.1021/acsami.5b05746
ACS Appl. Mater. Interfaces 2015, 7, 20190−20199