Bioconjugate Chemistry

Communication

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Immobilization of Enzymes via Microcontact Printing and Thiol−Ene Click Chemistry

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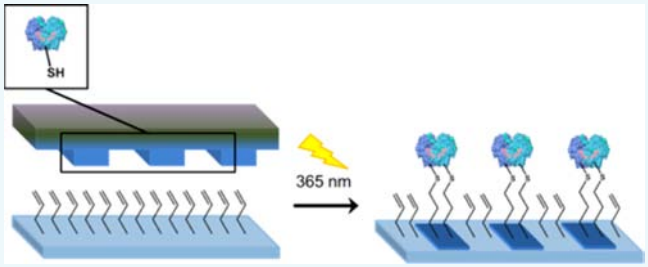
Supporting Information

ABSTRACT: This Communication describes a bioconjugation method for the generation of enzyme microarrays on surfaces using photochemical thiol−ene chemistry in combination with microcontact printing. Glucose oxidase and lactase were readily immobilized (i.e., printing time 2 min) on alkene terminated self-assembled monolayers on glass as demonstrated by X-ray photoelectron spectroscopy and fluorescence microscopy. Furthermore, the activity of both immobilized enzymes was confirmed in single enzyme as well as cascade transformations.



The generation of microstructured surfaces is of great interest for many different applications such as protein or DNA based sensors and microelectronic devices. For the preparation of the latter, photolithography has been widely used. The limitations of this method are the need of resists as well as materials that are compatible with the etchants used. Microcontact printing can bypass such harsh conditions and produce biocompatible substrates.[1] Among others, it was possible to print protein microarrays[2] and to immobilize carbohydrates for the detection of lectins.[3] The covalent attachment of molecules to surfaces via microcontact chemistry[4] is an important improvement since stable covalent bonds prevent desorption of target molecules.

The development of new methods for protein and enzyme immobilization is important for the functionalization of biocompatible surfaces as well as industrial processes. The immobilization of enzymes in commercial applications results in improved procedures due to higher stability, easier separation, and reuse of the enzymes.[5,6] In the following study glucose oxidase (GOx) and lactase (Lac) have been employed. GOx catalyzes the oxidation of glucose to glucono lactone and hydrogen peroxide.[7] GOx is an interesting enzyme because it is inexpensive, extensively investigated in previous studies, well-known for its high stability,[8] and has been employed in medical applications.[9] GOx has been widely used in amperometric sensor arrays for glucose sensing; to this end, it has been immobilized on nanoparticles,[10] polymers,[11] nanogels,[12] and self-assembled monolayers.[13,14] Lac is widely used in industrial and medical processes mainly in the dairy industry due to its valuable enzymatic characteristics.[15] In recent studies it was used as lactose sensor in a polymer matrix.[16] This enzyme catalyzes the cleavage of lactose into galactose and glucose. The hydrolysis of lactose is of great interest for the production of lactose free food products, bearing a solution for the lactose intolerance disease. Other advantages are the improvement of characteristics of lactose containing food as well as the usability of byproducts generated in dairy processes.[17]

In previous studies it has been shown that GOx and Lac can be immobilized by microcontact printing on bare gold surfaces via chemisorption[13,18] or on self-assembled monolayers (SAMs) on gold surfaces by peptide coupling.[13,19] However, since both enzymes have free thiol groups (i.e., cysteines) in their structure,[7,20] they are potentially suitable for immobilization via thiol−ene click chemistry. The photochemical thiol−ene addition is a versatile conjugation reaction, which is fast and occurs under mild and solvent free conditions, without the generation of undesired byproducts.[21] Increasingly, this bioorthogonal "click reaction" finds application in the conjugation of biomolecules to substrates and scaffolds,[22−28] including photochemical microcontact printing.[24,27]

In this Communication we report an easy and fast method to produce enzyme patterns on alkene terminated SAMs on glass surfaces without the need of enzyme modification or coupling reagents (see Figure 1). By using photochemical thiol−ene addition it was possible to form a covalent bond between the alkenes on the substrates and free thiol groups in the native enzymes. The successful immobilization of the enzymes on the alkene functionalized glass supported SAMs was verified via fluorescence microscopy and X-ray photoelectron spectroscopy. Furthermore, the enzyme activity was investigated after the immobilization process to prove that microcontact printing in combination with thiol−ene click chemistry is a suitable method to bind enzymes to surfaces in their active form. The protein modified surfaces were immersed in respective enzyme

Received: May 19, 2015
Revised: May 29, 2015
Published: June 1, 2015

DOI: 10.1021/acs.bioconjchem.5b00282
Bioconjugate Chem. 2015, 26, 1017−1020

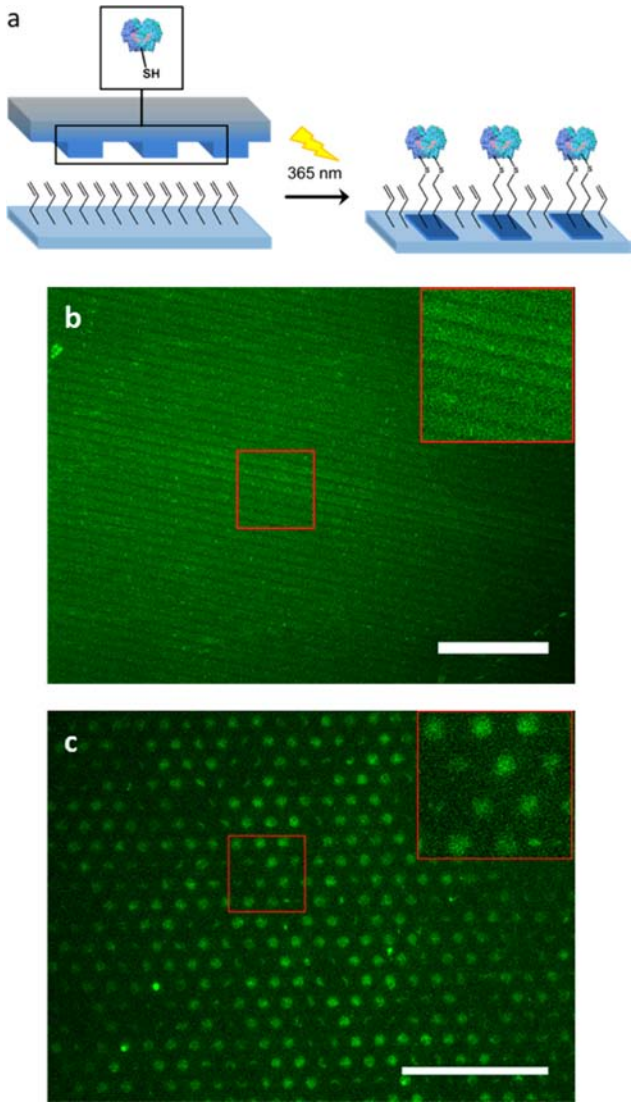


Figure 1. (a) Schematic representation of photochemical microcontact printing. (b) Fluorescence microscopy of Lac-FITC in 10 μm lines spaced by 5 μm. (c) Fluorescence microscopy of GOx-FITC printed in 5 μm dots spaced by 3 μm. Scale bar: 50 μm.

activity test solutions and the catalyzed reaction was monitored in situ via UV/vis spectroscopy.[29]

To exemplify the immobilization scheme the enzymes Lac and GOx were first coupled with fluorescein isothiocyanate (FITC) via free amine groups. The fluorescent enzymes were then immobilized on substrates modified with alkene terminated SAMs by photochemical microcontact printing.[24] The coupling of the dye to the proteins was verified with SDS-PAGE (see Figure S1 in the Supporting Information) and resulted in fluorescent bands for each enzyme. To minimize unspecific binding of enzymes the substrates were incubated with BSA (3 wt %) in HEPES buffer (pH = 7.5) prior to printing. The stamps for the protein patterning were treated with aminopropyltriethoxysilane (APTES) in ethanol overnight to increase the hydrophilicity and the affinity of the enzymes to the stamp. The increased hydrophilicity was visualized by contact angle measurements (see Figure S2 in the Supporting Information). In order to avoid the deactivation of the enzymes by organic solvents, the stamps were first incubated with a solution of the photoinitiator dimethoxyphenylacetophenone (DMPA) in ethanol (50 mM) for 1 min. Afterward, the excess of solution was removed and the stamp was dried carefully in a stream of argon, to avoid interactions between ethanol and the enzymes in the following immobilization step. Subsequently the stamp was incubated with a solution of the enzyme (1 mg/mL) for 1 h in the dark and dried. Finally, the stamp was placed carefully on the substrate and the reaction was initiated by irradiation with UV Light (365 nm) for 2 min. Afterward, the substrates were washed carefully by sonication in the buffer solution and analyzed via fluorescence microscopy.

The successful visualization of surface patterns gives a first proof for the surface modification via microcontact printing (Figure 1). In Figure 1b patterns of Lac-FITC on the modified glass surface are visible. In Figure 1c the surface patterns of immobilized GOx-FITC are shown. The inset shows the high resolution of microcontact printing. The enzymes only reacted with the substrate in the area where the stamp was in contact with the surface. Negative control experiments which were performed analogously but without irradiation and photoinitiator did not show any pattern for any enzyme (see Figure S3 in the Supporting Information).

These results were additionally verified by XPS. For these measurements the enzymes were printed on silicon wafers modified with alkene terminated SAMs using a flat stamp. In comparison with the measurement of an unmodified SAM (Figure 2, red) the appearance of shoulders in the carbon signal

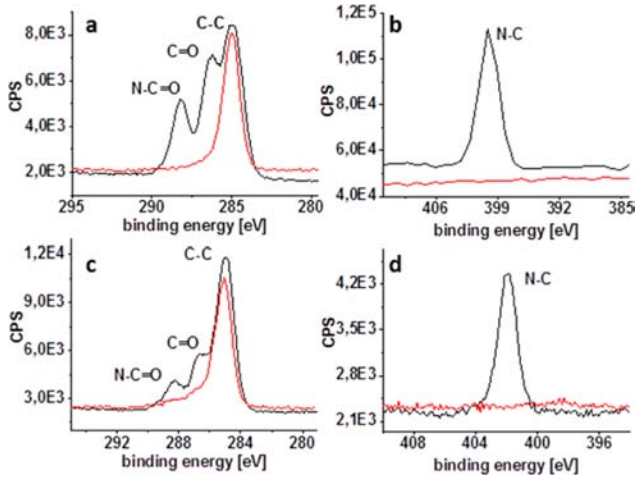


Figure 2. XPS measurements of enzyme modified substrates (black) in comparison with unmodified substrates (red). (a) C 1s and (b) N 1s signal of GOx and (c) C 1s and (d) N 1s signal of Lac.

(C 1s) is clearly visible for the GOx and Lac modified surfaces (Figure 2, black). The additional C 1s peaks are typical of peptide bonds in the immobilized enzymes. Also, the appearance of a pronounced nitrogen signal (N 1s) is additional proof of the immobilization of the enzymes.

XPS measurements were also used to estimate the surface coverage of the enzymes. The enzyme immobilization process was performed as described before. Subsequently the surfaces were functionalized with perfluorodecanethiol from solution. To this end, the surfaces were covered with a solution of perfluorodecanethiol (25 mM) in ethanol with DMPA (50 mM) and irradiated for 10 min with UV light (365 nm). Afterward, the substrates were cleaned by sonication in dichloromethane, acetone, and ethanol for 10 min each. The XPS signal intensity of the resulting fluorine signal (F 1s) was

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compared to the corresponding signals from a substrate that was functionalized analogously only with perfluorodecanethiol (see Table S2 in the Supporting Information). From these values the surface coverage of the different enzymes were calculated to be 69% for GOx and 29% for Lac. It should be noted that GOx is approximately 4 times smaller than Lac and can thus form a more densely packed enzyme layer on the SAM.

For the examination of the surface activity of the immobilized enzymes the modified substrates were immersed in a test solution in a UV-cuvette (see Figure S4 in the Supporting Information) and the activity was monitored by UV/vis spectroscopy. The activity test is based on the formation of a red chinonimine dye from hydrogen peroxide, phenol, and 4-aminoantipyrine.[29] The reaction is catalyzed by horseradish peroxidase (HRP). The required hydrogen peroxide is formed by immobilized GOx. For the investigation of the GOx activity on surfaces, a phosphate buffer (pH = 7) solution was prepared and contained a catalytic amount of HRP, phenol (25 mM), 4-aminoantipyrine (2 mM), and glucose with a concentration of 2.5 wt %. GOx catalyzes the oxidation of glucose to glucono lacton and hydrogen peroxide, which is used for the catalytic reaction of the HRP in the formation of a red chinonimine dye (Figure 3).

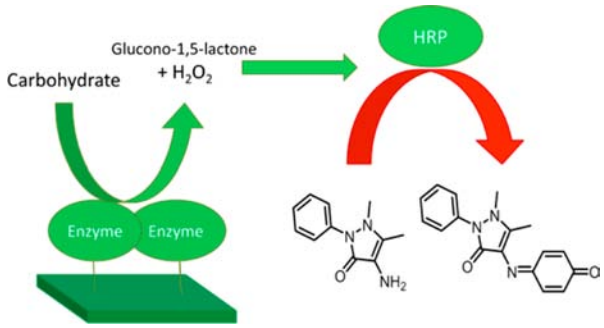

Figure 3. Schematic representation of the enzyme activity test. A carbohydrate is converted to hydrogen peroxide by the respective enzyme(s). Hydrogen peroxide in combination with 4-amino-antipyrine, phenol, and HRP forms the red chinonimine dye.

For verification of the activity of immobilized Lac, the phosphate buffer (pH 7.5) was prepared with a catalytic amount of HRP and GOx, phenol (25 mM), 4-aminoantipyrine (2 mM), and lactose with a concentration of 2.5 wt %. Lactose is cleaved by Lac in glucose and galactose. GOx and HRP catalyze the formation of red chinonimin dye. The UV/vis spectra are shown in Figure 4. The formation of dye by immobilized GOx (Figure 4a) is significantly faster than the formation of dye by immobilized Lac (Figure 4b). This observation may be due to an inherent difference of the enzyme activity in combination with a higher surface coverage with GOx as shown above by XPS analysis. To study an enzyme cascade reaction on the surface, both GOx and Lac were immobilized side by side on a substrate by printing with two flat stamps (14 × 8 mm^2 each) on a glass slide. Phosphate buffer (pH = 7.5) solution was prepared and contained a catalytic amount of HRP, phenol (25 mM), 4-aminoantipyrine (2 mM), and lactose with a concentration of 2.5 wt %. The cascade experiment with both GOx and Lac immobilized (Figure 4c) showed slower activity than the tests with a single enzyme. We note that we can not easily determine the surface coverage of

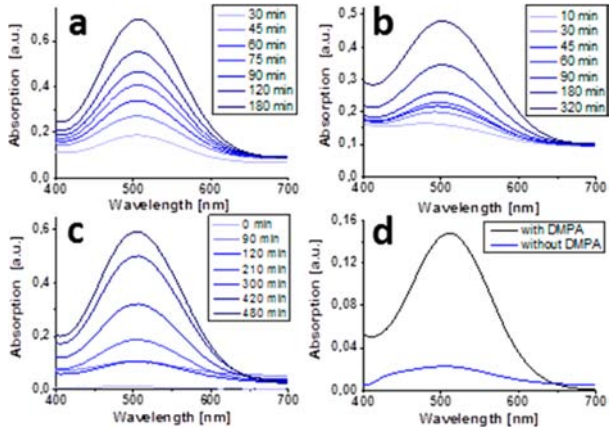

Figure 4. UV spectra of the enzyme activity test with (a) immobilized GOx; (b) immobilized Lac; (c) immobilized GOx and Lac. (d) Reference spectra for a negative test in which GOx was printed without photoinitiator DMPA and without irradiation (t = 30 min).

each enzyme in the dual immobilization. As a negative control experiment, the immobilization and the activity test were carried out as described above but without photoinitiator and irradiation during printing. In that case, the test solution showed no color change within 30 min (Figure 4d). This observation confirms that the enzymes do not physisorb to the surfaces during printing.

In conclusion, we demonstrated that microcontact printing can be combined with photochemical thiol−ene click chemistry to immobilize the native enzymes GOx and Lac on glass substrates. The successful immobilization was verified by XPS and fluorescence microscopy. Additionally, it was possible to detect the enzymatic activity on the surface using suitable test reactions. Finally, both enzymes were immobilized together to form a lactose sensor. This demonstrates the potential of this procedure to conjugate enzymatic sensor arrays on surfaces, which is essential for the generation of biocompatible and bioactive surfaces.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, SDS-PAGE, contact angle measurements, activity test and surface coverage values. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00282.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Alom Ruiz, S., and Chen, C. S. (2007) Microcontact printing: a tool to pattern. *Soft Matter 3*, 168−177.

(2) Bernard, A., Renault, J. P., Michel, B., Bosshard, H. R., and Delamarche, E. (2000) Microcontact printing of proteins. *Adv. Mater. 12*, 1067−1070.

(3) Wendeln, C., Heile, A., Arlinghaus, H. F., and Ravoo, B. J. (2010) Carbohydrate microarrays by microcontact printing. *Langmuir 26*, 4933−4940.

(4) Wendeln, C., and Ravoo, B. J. (2012) Surface patterning by microcontact chemistry. *Langmuir 28*, 5527−5538.

DOI: 10.1021/acs.bioconjchem.5b00282
Bioconjugate Chem. 2015, 26, 1017−1020

(5) Liese, A., and Hilterhaus, L. (2013) Evaluation of immobilized enzymes for industrial applications. *Chem. Soc. Rev. 42*, 6236−6249.
(6) Dicosimo, R., McAuliffe, J., Poulose, A. J., and Bohlmann, G. (2013) Industrial use of immobilized enzymes. *Chem. Soc. Rev. 42*, 6437−6474.
(7) Frederick, K. R., Tung, J., Emerick, R. S., Masiarz, F. R., Vasavada, A., Chamberlain, S. H., Vasavada, A., Rosenberg, S., Chakraborty, S., Schopfer, L. M., et al. (1990) Glucose oxidase from Aspergillus niger. Cloning, gene sequence, secretion from Saccharomyces cerevisiae and kinetic analysis of a yeast-derived enzyme. *J. Biol. Chem. 265*, 3793−3802.
(8) Wilson, R., and Turner, A. P. F. (1992) Glucose oxidase: an ideal enzyme. *Biosens. Bioelectron. 7*, 165−185.
(9) Clark, L., and Lyons, C. (1964) Electrode systems for continuous monitoring in cardiovascular surgery. *Ann. N.Y. Acad. Sci.*, 29−45.
(10) Piao, M.-H., Son, P.-S., Chang, C.-H., and Choi, S.-H. (2010) A glucose biosensor based on deposition of glucose oxidase onto au nanoparticles poly(maleic anhydride)-grafted multiwalled carbon nanotube electrode. *Anal. Sci. Technol. 23*, 165−171.
(11) Sangodkar, H., Sukeerthi, S., Srinivasa, R. S., Lal, R., and Contractor, A. Q. (1996) A biosensor array based on polyaniline. *Anal. Chem. 68*, 779−783.
(12) Cardiel, J. J., Zhao, Y., Tonggu, L., Wang, L., Chung, J., and Shen, A. Q. (2014) Lab on a chip in nonionic micellar nanogels for glucose sensing. *Lab Chip 14*, 3912−3916.
(13) Losic, D., Shapter, J. G., and Gooding, J. J. (2002) Scanning tunneling microscopy studies of glucose oxidase on gold surface. *Langmuir 18*, 5422−5428.
(14) Bai, Y. F., Xu, T. B., Luong, J. H. T., and Cui, H. F. (2014) Direct electron transfer of glucose oxidase-boron doped diamond interface: a new solution for a classical problem. *Anal. Chem. 86*, 4910−4918.
(15) Warmerdam, A., Benjamins, E., de Leeuw, T. F., Broekhuis, T. A., Boom, R. M., and Janssen, A. E. M. (2013) Galacto-oligosaccharide production with immobilized β-galactosidase in a packed-bed reactor vs. free β-galactosidase in a batch reactor. *Food Bioprod. Process. 92*, 383−392.
(16) Jasti, L. S., Dola, S. R., Fadnavis, N. W., Addepally, U., Daniels, S., and Ponrathnam, S. (2014) Co-immobilized glucose oxidase and β-galactosidase on bovine serum albumin coated allyl glycidyl ether (AGE)-ethylene glycol dimethacrylate (EGDM) copolymer as a biosensor for lactose determination in milk. *Enzyme Microb. Technol. 64−65*, 67−73.
(17) Panesar, P. S., Kumari, S., and Panesar, R. (2010) Potential applications of immobilized β-galactosidase in food processing industries. *Enzyme Res.*, 473137.
(18) Casero, E., Petit-Domínguez, M. D., Parra-Alfambra, A. M., Gismera, M. J., Pariente, F., Lorenzo, E., and Vázquez, L. (2010) One-step covalent microcontact printing approach to produce patterns of lactate oxidase. *Phys. Chem. Chem. Phys. 12*, 2830−2837.
(19) Wilhelm, T., and Wittstock, G. (2010) Generation of periodic enzyme patterns by soft lithography and activity imaging by scanning electrochemical microscopy. *Langmuir 18*, 9485−9493.
(20) Craven, G. R., Steers, E., and Anfinsen, C. B. (1965) Purification, composition, and molecular weight of the β-galactosidase of Escherichia coli K12. *J. Biol. Chem. 240*, 2468−2477.
(21) Hoyle, C. E., and Bowman, C. N. (2010) Thiol-ene click chemistry. *Angew. Chem., Int. Ed. 49*, 1540−1573.
(22) DeForest, C. A., Polizzotti, B. D., and Anseth, K. S. (2009) Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments. *Nat. Mater. 8*, 659−664.
(23) Weinrich, D., Kohn, M., Jonkheijm, P., Westerlind, U., Dehmelt, L., Engelkamp, H., Christianen, P. C. M., Kuhlmann, J., Maan, J. C., Nusse, D., et al. (2010) Preparation of biomolecule microstructures and microarrays by thiol-ene photoimmobilization. *ChemBioChem 11*, 235−247.
(24) Wendeln, C., Rinnen, S., Schulz, C., Arlinghaus, H. F., and Ravoo, B. J. (2010) Photochemical microcontact printing by thiol-ene and thiol-yne click chemistry. *Langmuir 26*, 15966−15971.
(25) Gupta, N., Lin, B. F., Campos, L., Dimitriou, M. D., Hikita, S. T., Treat, N. D., Tirrell, M. V., Clegg, D. O., Kramer, E. J., and Hawker, C. J. (2010) A versatile approach to high-throughput microarrays using thiol-ene chemistry. *Nat. Chem. 2*, 138−145.
(26) Dondoni, A., and Marra, A. (2012) Recent applications of thiol-ene coupling as a click process for glycoconjugation. *Chem. Soc. Rev. 41*, 573−586.
(27) Wendeln, C., Rinnen, S., Schulz, C., Kaufmann, T., and Arlinghaus, H. F. (2012) Rapid preparation of multifunctional surfaces for orthogonal ligation by microcontact chemistry. *Chem. Eur. J. 18*, 5880−5888.
(28) Escorihuela, J., Banuls, M. J., Grijalvo, S., Eritja, R., Puchades, R., and Maquieira, A. (2014) Direct covalent attachment of DNA microarrays by rapid thiol-ene "click" chemistry. *Bioconjugate Chem. 25*, 618−627.
(29) Gallati, V. (1977) Aktivitätsbestimmung von Peroxidase Mit Hilfe Des Trinder-Reagens. *J. Clin. Chem. Clin. Biochem. 15*, 699−703.

DOI: 10.1021/acs.bioconjchem.5b00282
Bioconjugate Chem. 2015, 26, 1017−1020