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PAPER

Covalent immobilization of active lysozyme on Si/glass surface using alkoxy Fischer carbene complex on SAM†

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A cross-metathesis reaction between an alkene terminated self-assembled monolayer (SAM) on glass/Si wafer and an alkene tethered Fischer carbene complex yielded a functionalized surface. Rapid aminolysis of the Fischer carbene moieties permit efficient anchoring of amine containing molecules on such a surface. Attachment of 1-pyrenemethylamine was thus monitored by ATR-IR spectroscopy and fluorescence microscopy. Similarly, BSA and lysozyme were individually grafted to such Fischer carbene modified surfaces using their pendant lysine residues. It has been demonstrated that the anchored lysozyme retains its bactericidal property.

Introduction

In recent years, there has been intense research activity into multifarious applications of immobilized enzymes in bioanalytical chemistry.[1] A key step in this research is the immobilization of the enzyme onto the surface in a controlled and reproducible manner such that its activity is not compromised.

There are several protein immobilization strategies that utilise physical, covalent, or bio-affinity interaction for fabrication of protein biochips, and they are continuously being improved.[2] Physical immobilization of proteins or enzymes takes place *via* hydrophobic interactions, intermolecular forces and polar interactions. Consequently, protein molecules across the surface become heterogeneously spread and randomly oriented, since each protein can form many contacts in different orientations with previously adsorbed proteins as well as the surface. Dense packing may also result in blocking of the active sites and thereby affect the catalytic function of the protein. Thus random orientation, weak attachment and poor reproducibility remain major drawbacks of the adsorption mechanism of proteins and enzymes. However, when the protein is covalently attached to a solid support, both shelf-life and reproducibility are considerably improved.

Covalent bond formation takes place between a functional group on the exposed side chains of proteins, and suitable functional groups (such as active esters, carboxylic acids, epoxides, maleimides, vinyl sulfones or aldehydes) on the chemically modified support. Bond formation between the protein and the solid support should ideally proceed under ambient conditions in order to conserve the structure and activity of the biomolecule. In this context we selected the Fischer type alkoxy carbene complex, which is strongly electrophilic, as the reactive functionality on the surface. A Fischer carbene complex is known to readily react with a primary or a secondary amine under mild and aqueous conditions leading to stable aminocarbene complexes,[3] and progress of this reaction can be monitored by the typical shift of M–CO stretch in the region 1900–2010 cm^{-1}, which is transparent to most organic functional groups and water. This reaction was successfully adopted to graft protein molecules onto the surface using the $–NH_2$ group of pendant lysine residues for aminolysis.

We have demonstrated the utility of this approach with reference to two different surfaces. An alkyne tethered Fischer carbene complex was attached to a self-assembled monolayer (SAM) on a silicon wafer or glass using 'click' reactions[4] and then the Fischer carbene coated surface was utilized for grafting proteins. In an earlier study, we grafted alkene tethered Fischer carbene complexes *via* ruthenium catalyzed cross-metathesis[5] to a SAM on colloidal gold nanoparticles in organic solvent.[6]

In this article, we describe the viability of: (a) a Ru-catalysed cross-metathesis reaction to anchor Fischer carbene complexes on glass slides and silicon wafers as solid supports in view of their availability, flatness, rigidity, convenience of chemical modification, non-porosity, mechanical stability and chemical resistance to solvent;[7] (b) covalent anchoring of a model protein BSA by aminolysis reaction with a Fischer carbene function on a SAM; and (c) a similar covalent anchoring of lysozyme enzymes. We observed that the bactericidal property of surface-bound lysozyme was retained.

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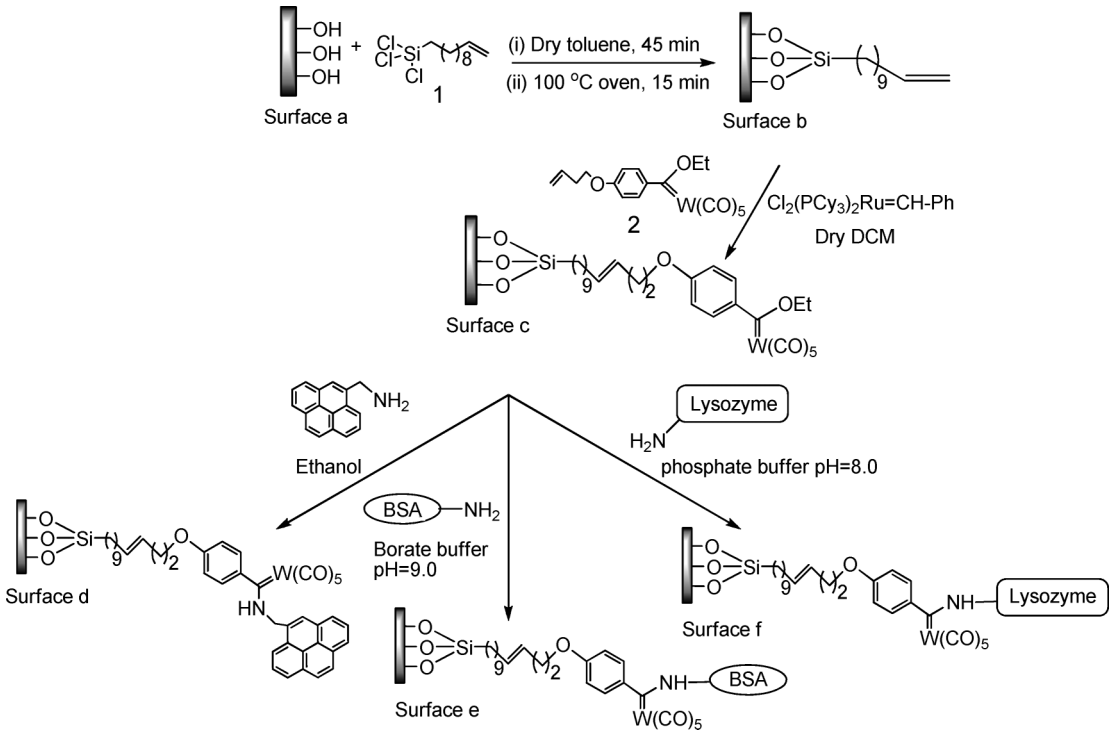
† Electronic supplementary information (ESI) available: experimental details, NMR, ellipsometry, contact angle, IR, AFM details, HRSEM, fluorescence image. See DOI: 10.1039/c0ob00798f



Scheme 1 Grafting of Fischer carbene complexes on a silicon wafer/glass slide followed by anchoring of different proteins.

Results and discussion

Grafting of Fischer carbene complexes

We synthesized the carbene complex **2** that featured a terminal double bond from 4-bromophenol.‡ 4-Bromophenol was at first treated with 4-bromo-1-butene in the presence of small pieces of sodium. The product of etherification was further treated with *n*-BuLi followed by tungsten hexacarbonyl. Subsequent addition of Meerwein's salt afforded the red coloured carbene complex **2**. The carbene complex was characterized by standard spectroscopic analysis. In the ^{13}C NMR spectrum a characteristic carbene carbon peak appeared at 311.01 ppm (see ESI†). The characteristic IR absorptions for W(C–O) appeared at 1936 and 2065 cm^{-1}.

The grafting of Fischer carbene complexes *via* cross-metathesis on a silicon as well as a glass surface to immobilize the proteins is depicted in Scheme 1. Following a standard procedure[8] a silicon/glass slide was activated at first, to allow formation of a self-assembled monolayer of a long chain alkene (Scheme 1, Surface b). The exposed alkene terminus on the SAM was subjected to cross-metathesis with alkene tethered Fischer carbene complex **2** in anhydrous dichloromethane at ambient temperature in the presence of a catalytic amount (5 mol% with respect to carbene complex **2**) of $Cl_2(PCy_3)_2Ru{=}CHPh$, leading to the formation of a Fischer carbene coated surface (Scheme 1, Surface c).§ The change of the functional groups on the surface was monitored by ellipsometry, contact angle measurements and ATR-IR spectrometry. Ellipsometric measurements showed that the thickness of the initially formed undecenyl chain was 1.98 nm and after cross-metathesis reaction the thickness of the SAM changed to 2.54 nm. The observed increase was 0.56 nm. Hydrophobicities of the surfaces change after each modification due to different head groups. This was reflected in the contact angles of the alkene terminated (98.1 ± 2.2) and Fischer carbene terminated (90.2 ± 1.1) monolayers on the silicon wafer. The ATR-IR spectrum of a monolayer of 1-undecene shows two important peaks corresponding to the non-symmetric -$\upsilon_a(CH_2)$- and symmetric -$\upsilon_s(CH_2)$- stretches for methylene groups at 2950 cm^{-1} and 2839 cm^{-1}, respectively. After the cross-metathesis reaction new W–CO bands appeared at 2065 cm^{-1} and 1942 cm^{-1} (Fig. 1A).

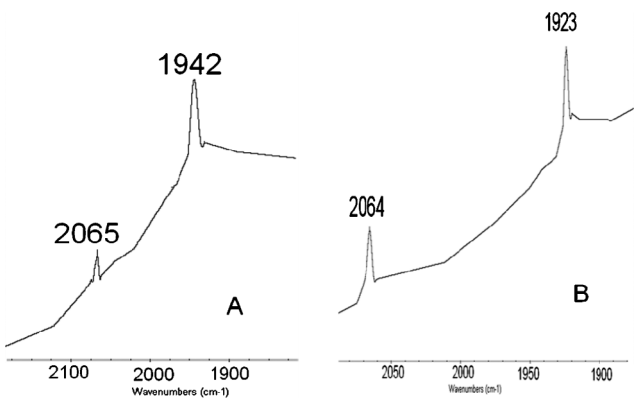


Fig. 1 ATR-IR spectrum of a surface (A) coated with Fischer carbene complexes; (B) coated with 1-pyrenemethylamine.

‡ Initial attempts to use 4-allyloxyphenyllithium failed to furnish the desired carbene complex, presumably due to side reactions involving the allyloxy function. Then we changed over to the homoallyloxy functionality as in structure **2** and the carbene complex was prepared.

§ If we were to use the 'click' reaction, the bromo terminated SAM would have had to be converted to an azido terminated SAM before the reaction. Cross-metathesis does not require this additional step.

Reaction of the terminal Fischer carbene with primary amine

Although the presence of Fischer carbene functionality was evident from spectral data, it was important to map an average distribution of such functionality across the surface, to assess the efficacy of the metathesis reaction on the SAM. In order to do that, the Fischer carbene coated slide was first treated with 1-pyrenemethylamine, a fluorescent probe, in ethanol for 10 min at room temperature (Scheme 1, Surface d). Excess 1-pyrenemethylamine was washed off thoroughly with ethanol. The surface was then studied using ATR-IR spectroscopy and fluorescence microscopy. In the ATR-IR spectrum, a significant shift of the W–CO stretching band to 2064 cm^{-1} and 1923 cm^{-1} was observed (Fig. 1B). This is a direct consequence of the aminolysis reaction, and the changed positions indicate almost complete conversion of alkoxy groups to amino groups in the Fischer carbene complexes. Using fluorescent microscopy, the glass slide shows bright blue fluorescent spots indicating the occurrence of aminolysis reactions whereas the control (alkene terminated slide) shows no such spots (Fig. 2). This image clearly shows that the fluorescent amine remains distributed quite evenly and densely across the surface, clearly indicating the success of both the metathesis and aminolysis reactions on the SAM.

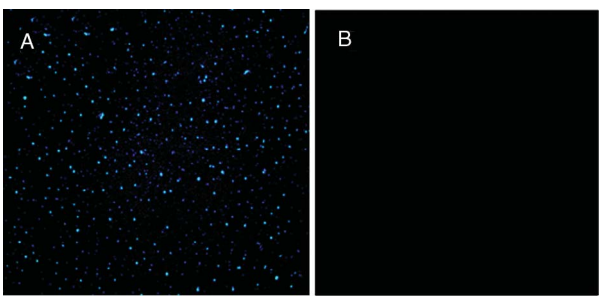


Fig. 2 Fluorescence microscopy image of (A) the glass slide coated with 1-pyrenemethylamine; (B) the control slide (alkene coated surface).

Immobilization of BSA and lysozyme on Si wafer/glass surface *via* aminolysis of Fischer carbene

Encouraged by these observations, we proceeded to immobilize a model protein BSA on the surface (Scheme 1, Surface e) using a similar protocol. The Fischer carbene coated surface was immersed in an aqueous borate buffer solution of BSA at room temperature. We anticipated that the primary amine groups of the pendent lysine residues on BSA would react with the electrophilic carbene carbon to form new C–N bonds and therefore the protein would be covalently attached to the SAM. The surface was rinsed extensively with buffer and milli-Q water and analyzed by ATR-IR and atomic force microscopy (AFM). After aminolysis, the absorption peak for W–CO stretching of the amino carbene complex appeared at 1922 cm^{-1} and 2064 cm^{-1}. Since the protein BSA is a large molecule, it was expected that the surface contour of the Si wafer coated with BSA would have substantial roughness compared to the control surface (alkene SAM dipped in BSA solution). AFM was used in a non contact mode under ambient condition. From Fig. 3A, it is evident that the topography of the BSA attached surface is different from

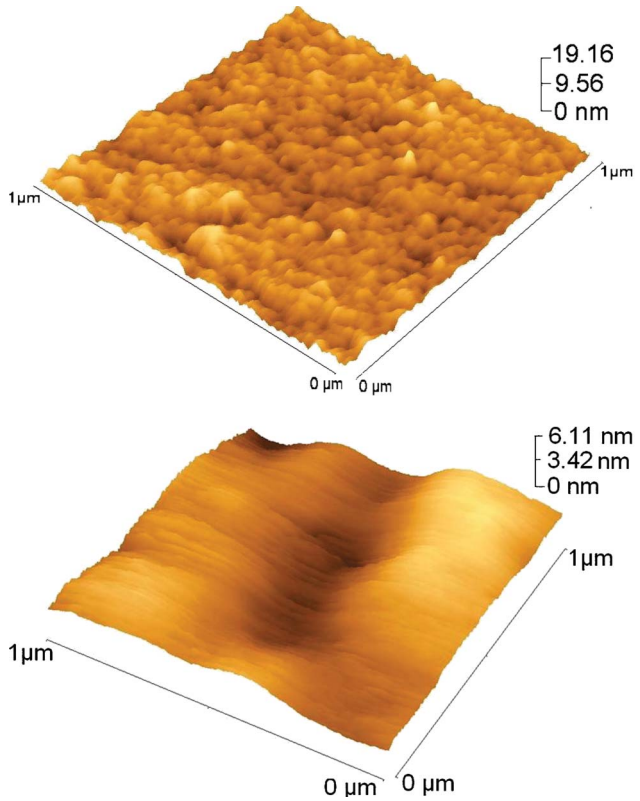


Fig. 3 (A) AFM image of Si wafer coated with BSA; (B) AFM image of control (alkene coated surface).

the alkene terminated silica surface (Fig. 3B). A dense coverage of the protein was observed. The orientation of the protein is random because accessible lysine residues are spread widely across the globular protein surface. In our experiment we obtained an average particle size of 37.77 ± 1.2 nm and an average height of 10.35 nm (average of 20 measurements). We therefore employed a tip-deconvolution calculation to obtain a more accurate value of the size. Considering the spherical geometries for both the tip and the sample the true diameter of the particle was measured from image using the formula:[9] $d_t = d_m^2/8r_{tip}$ where d_t is the true diameter of the particle, d_m is the measured diameter and r_{tip} is the tip radius of curvature. Using the value r_{tip} = 20 nm provided by the supplier and d_m = 37.77 nm, the true diameter of the protein molecule obtained was 8.92 nm. This is in good agreement with the actual size of the BSA molecules.[10] The number of BSA molecules was counted by particle analysis procedure which gave an average density of 385 ± 10 molecules μm^{-2}.

The Fischer carbene coated slide was then used for immobilization of lysozyme[11] (Scheme 1, Surface f). Lysozyme is a globular protein with 129 residues and is stabilized by four disulfide bonds. It can hydrolyze the polysaccharides embedded on the cell wall of many gram positive bacteria.[12] It has many uses in food processing, *e.g.* in food packaging films and in the preservation of meat. Even in the pharmaceutical industry it is used as a drug for treating ulcers and infections. We incubated the Fischer carbene coated slide in an aqueous phosphate buffer containing lysozyme for 10 min at room temperature. The excess enzyme was removed by washing with buffer and milli-Q water. AFM, high resolution scanning electron microscopy and ATR-IR (see ESI†) were employed to

characterize the enzyme layer at the molecular level. High density surface coverage of the enzyme was observed. From Fig. 4 the average particle size obtained was 27.85 ± 3.3 nm and the average height was 6.5 nm (average of 20 measurements). Employing the tip-deconvolution calculation the true diameter of the enzyme molecule obtained was 4.84 nm. This is in good agreement with the actual size of the lysozyme molecules. The average density obtained was 750 ± 10 molecules μm^{-2}.¶ The HRSEM picture (see ESI†) of the lysozyme shows a particle size of about 5 nm which is slightly greater than the actual diameter, probably because the immobilized enzyme has a platinum metal coating.

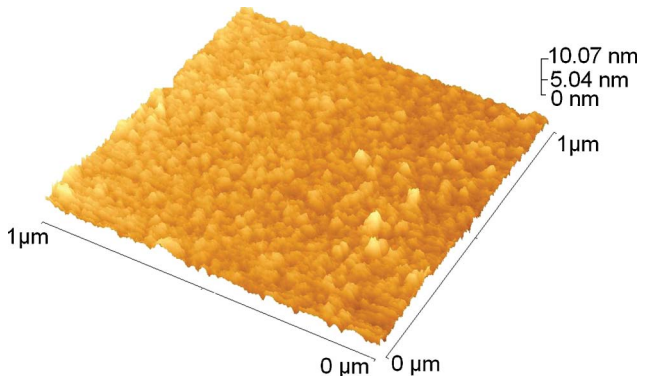


Fig. 4 AFM image of lysozyme on silicon wafer.

The presence of lysozyme on the glass surface immobilized by the same protocol was visualized by labeling the enzyme with a fluorescent tag. The lysozyme coated surface was treated with dansyl chloride in acetonitrile–water at room temperature. After exhaustive washing, the slide was observed under a fluorescence microscope. Widely distributed fluorescent spots were observed whereas the control slide (without the enzyme) exhibited no such fluorescence (Fig. 5). This protocol of grafting, therefore, is mild, reliable and instantaneous.

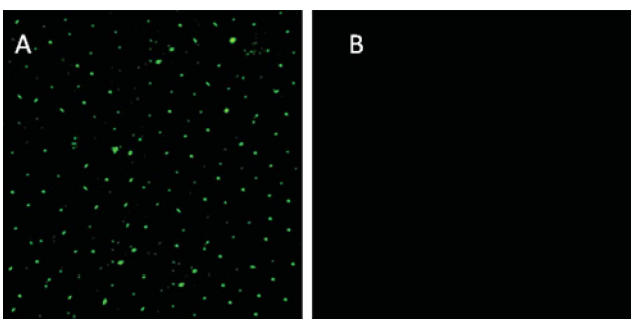


Fig. 5 Fluorescence microscopy image of (A) lysozyme coated surface; (B) control surface after treating with dansyl chloride.

Assay of enzymatic activity of the immobilized lysozyme

Lysozyme is a glycoside hydrolase that cleaves the polysaccharidic component of the cell wall of bacteria, such as *Micrococcus lysodeikticus*, inducing cell lysis. Using three assay methods we showed that the lysozyme immobilized on the SAM retained its lytic activity. An aqueous suspension of bacteria (approximately 10^6 cells ml^{-1}) was spread on the glass slide. After drying for 2 min in air, the slide was placed in a petri dish and 0.7% agar along with bacterial growth medium (1% tryptone, 0.5% yeast and 1% sodium chloride) was added. The petri dish was then closed, sealed and incubated at 25 °C overnight. As seen in Fig. 6, numerous colonies of *M. Lysodiekticus* grew on to the control slide (without lysozyme) whereas no colonies were observed on the lysozyme containing slide.

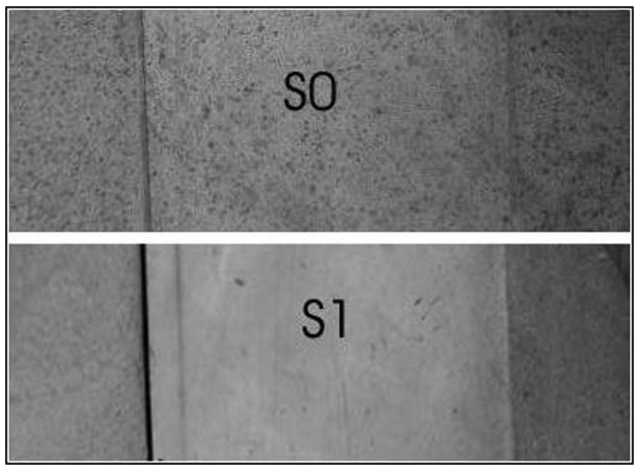


Fig. 6 A portion of petri dish without lysozyme (S0) and with lysozyme immobilized (S1) glass slides. They were spread with ~10^6 cells ml^{-1} of *M. lysodeikticus* and air dried for 2 min. The cells were then incubated in the presence of 0.7% agar in a bacterial growth medium at 25 °C overnight.

The lytic activity of the lysozyme treated slide was also validated by turbidity assay. The lysozyme treated slide was placed in a conical flask covered with 25 ml of a 0.03% (w/v) suspension of the bacteria in phosphate buffer. The flask was placed in an incubator-shaker at 25 °C and the suspensions were sampled every 60 min for 8 h under aseptic conditions, and the absorbance was measured spectroscopically at 450 nm. A control experiment (without lysozyme) was performed to measure the non-ezymatic bacterial lysis. From a plot of the optical density *vs.* time (Fig. 7), we found that with time the absorbance drops more

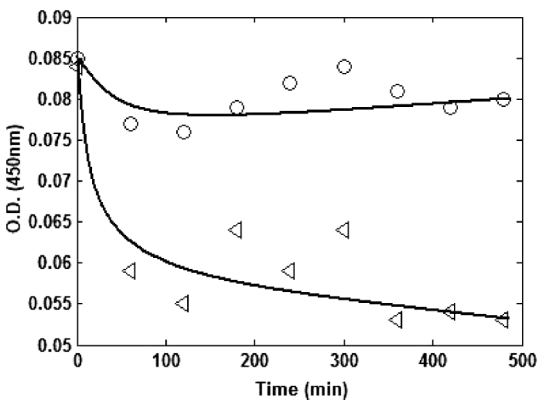


Fig. 7 The kinetics of bacterial cell lysis was monitored as represented by a plot of O.D. at 450 nm *vs.* time (control (○) and immobilized lysozyme (◁)).

¶ The amount of immobilized BSA and lysozyme on the glass slide was 79 ng and 34 ng respectively.

sharply in the case of the lysozyme treated slide. From the absorbance value, the percentage lysis after eight hours was calculated for both the control set and lysozyme treated slide. It was found that in the case of the lysozyme treated slide the lysis of bacterial cells was much greater (36.9%) than the control set (5.9%).‖ The lysozyme treated suspension was recycled twice and the percentage of lysis obtained after the second and third cycle were 36.2% and 35.8%, respectively.

The antimicrobial property of the lysozyme treated glass slide was also investigated using the live/dead two-colour fluorescence method.[13] First, a suspension of bacteria was deposited on the surface and their viability was followed over time by fluorescence microscopy. Green spots represent viable bacteria whereas red spots represent non-viable bacteria. With time the number of red spots increase which is indicative of bacterial cell lysis (Fig. 8), whereas in the control experiment negligible red spots are observed (see ESI†).

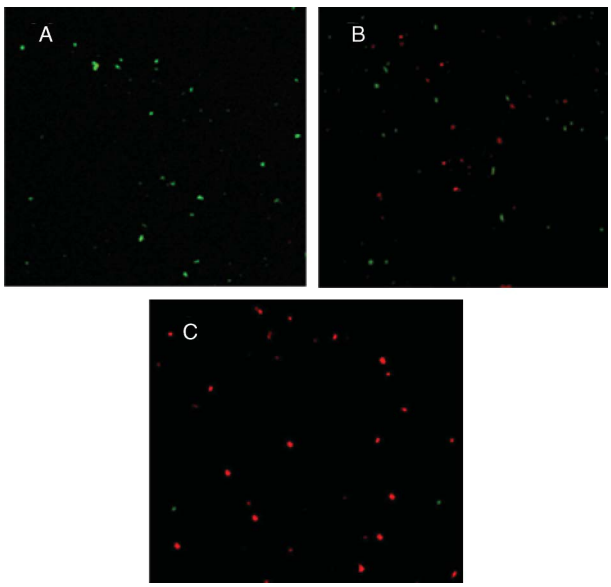


Fig. 8 Fluorescence microscopy image of *M. Lysodeikticus* on lysozyme treated slide stained with live/dead marker after (A) 0 min, (B) 1 h and (C) 2 h.

Conclusion

In conclusion, we successfully utilized a cross-metathesis reaction to decorate glass/silica surfaces with Fischer carbene complexes, an excellent linker for biomolecules. It allowed rapid immobilization of both BSA and lysozyme on the surfaces. The immobilized lysozyme displayed a significant lytic activity and therefore could be a suitable candidate for repetitive batch hydrolysis of microbial cells in the food industry. We believe this procedure is mild, convenient and adaptable to other solid supports with appropriate modifications. We are currently exploring the possibility of anchoring different drugs, antibodies and nucleic acids on surfaces using similar protocol.

‖ The enzymatic activity of the immobilized lysozyme is much lower than the enzyme in solution (91.3%). Though none of the six lysines of lysozyme is included in the catalytic cycle, this partial loss of enzymatic activity on immobilization may be due to the fact that covalent binding to the surface *via* some of these residues may lead to orientations where the enzyme active site is not accessible to substrates due to steric hindrance.

Experimental procedures

General

NMR experiments were carried out on a Bruker AC 200 MHz spectrometer. ATR-IR spectra were recorded on a Nicolet 380 spectrometer. Contact angles were obtained with a digidrop from GBX (Romans sur Isère, France). An ellipsometer (Sentech SE 500) operating at a 70° incidence angle was used to measure the thickness of the SAMs on silicon wafers. Fluorescence microscopy images were recorded on a Leica DM 3000 Upright Trinocular Research Microscope with Leica DFC 425 Scientific digital camera using LAS software. The area covered under the image was 100 μm × 100 μm. High resolution scanning electron microscopy (HRSEM) images of the immobilized enzymes were taken using a JEOL (JSM-6700F) field emission scanning electron microscope. Atomic force microscopy was carried out with AFM (diCP-II) using DI Company SPMlab Analysis software to study the topology of the surface before and after enzyme immobilization. The scanning was performed within 1 μm × 1 μm with scan rate 0.5–0.6 Hz.

Materials

Toluene and dichloromethane were purchased from SRL and dried using standard procedure. Bromoundecene, 4-bromo-1-butene, *p*-bromophenol, tungsten hexacarbonyl, ^{n}BuLi, bovine serum albumin (BSA), chicken egg-white lysozyme, and lyophilized *Micrococcus Lysodeikticus* cells were purchased from Sigma-Aldrich. Reagents were used without further purification. Ethyl acetate and petroleum ether were obtained from SRL and used as reagent grade. Glass slides were purchased from Blue Star. Silicon wafers were gifted by Dr Othman Boloussa.

Trichloro(undec-10-enyl)silane **1** and double bond terminated Fischer carbene complex **2** was synthesized as reported in the literature.[6,14]

Grafting of BSA/lysozyme on glass/silica by cross-metathesis reaction

(A) Silanization of glass/Si surface with 10-undecenetrichlorosilane.

i) Cleaning and activation of Si/glass surface. The glass slides or the silicon wafers were sequentially cleaned with ethanol and double distilled water and then oxidized by a mixture of concentrated H_2SO_4 and 30% aq. H_2O_2 (70 : 30 v/v) at 60 °C, washed with water and dried under N_2 to obtain an activating OH surface group on which the self assembly could take place.

ii) Preparation of SAM with undecenetrichlorosilane. The activated Si wafers or glass slides were incubated in a freshly prepared 1 mmol solution of 10-undecenetrichlorosilane in dry toluene under Ar atmosphere at 10 °C for 45 min. The slides were then rinsed thoroughly with dry toluene, followed by ethanol and baked in oven at 100 °C for 10 min.

(B) Grafting of Fischer carbene complex on surface by cross-metathesis. The slides covered with olefin terminated monolayers

were immersed in a 10 mM solution of Fischer carbene complex **2** in dry dichloromethane in the presence of Ru-catalyst (5 mol%). The reaction was refluxed under nitrogen for 12 h. The slides were then washed with dichloromethane followed by ethanol in order to ensure any physisorbed Fischer carbene complex was washed off. Finally the surfaces were dried under argon flush.

(C) Reaction between grafted Fischer carbene SAM and 1-pyrenemethyl amine. First, 1-pyrenemethylamine hydrochloride salt was neutralized with dil. NaOH solution (10%). Then the amine was extracted with DCM, dried over Na_2SO_4 and concentrated under a vacuum to yield a yellowish white solid compound. The slides coated with Fischer carbene terminated SAMs were then treated with a 10 mM solution of 1-pyrenemethylamine in absolute EtOH for 10 min in the dark. After thorough rinsing with ethanol, the surfaces were dried with argon flush.

(D) Immobilization of BSA/lysozyme on SAM. The Fischer carbene grafted Si wafers or glass slides were then immersed in 1 μM BSA solution in borate buffer (pH 9) or 1 μM lysozyme solution in phosphate buffer (pH 8) for 10 min. They were thoroughly washed with buffer solution (twice), then with milli-Q water (four times) and dried under argon.

(E) Reaction between lysozyme coated surface and dansyl chloride. The lysozyme coated surface was dipped in a 10 mM solution of dansyl chloride in acetonitrile–water (pH 9 by adding sodium bicarbonate) for 45 min at room temperature in the dark. Then the slide was rinsed extensively with milli-Q water and dried under argon.

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