A new bio-active surface for protein immobilisation *via* copper-free 'click' between azido SAM and alkynyl Fischer carbene complex†

Sudeshna Sawoo,[a] Piyali Dutta,[a] Amarnath Chakraborty,[a] Rupa Mukhopadhyay,[b] Othman Bouloussa[c] and Amitabha Sarkar*[a]

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Fischer carbene complex anchored on glass or silicon surface using a Cu-free 'click' reaction allows facile and swift covalent grafting of protein molecules like Bovine Serum Albumin (BSA).

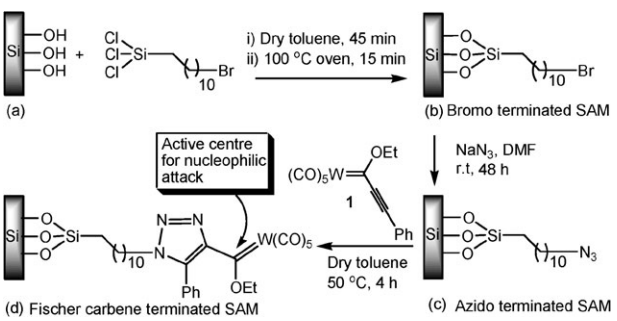
Protein immobilisation on a surface using covalent linkage is vital to proteome research[1] that includes development of microarray- and biochip-based sensors for high-throughput screening of protein structure and function, study of interaction between protein and other biomolecules, multianalyte immunoassay or enzyme assay for clinical diagnostics, *etc.*[2] The linkage between the biomolecule and the inorganic solid support remains a critical variable for optimal performance of a sensor device. A wide range of protein immobilisation techniques have been developed and they are continuously being improved.[3] It is well-recognised that a covalent bond between a biomolecule and a functionalised surface lasts longer and provides greater reproducibility than immobilisation obtained by non-covalent links. The covalent bond formation between the anchored protein and the glass surface must be extremely facile and should proceed under ambient conditions to conserve protein/enzyme structure and activity. Alkoxy Fischer carbene complexes as the linker function for protein to self-assembled monolayers (SAMs) offer three distinct advantages: (a) almost instantaneous reaction with primary amino groups[4] (as in lysine residues of proteins) to form a stable covalent bond; (b) facile detection of immobilised protein by the typical shift in the M–CO stretch in the region 1900–2010 cm^{-1}; and (c) activation of the triple bond of the carbene complex for 'click' chemistry with an azido group on the SAM without the necessity of copper catalysis,[5a] which makes it potentially adaptable for use within a cell.[5b] Herein we report the preliminary results of such a strategy.

The Fischer-type metallocarbene complexes of tungsten with an alkoxy functional group possess a marked electrophilic character at the carbenic carbon and hence are ideally suited for a facile aminolysis leading to stable aminocarbene complex formation. Use of such a strategy in labeling proteins for non-radioactive metalloimmunoassay is precedented.[6]

Among a large variety of solid supports, glass surfaces are often preferred because of ready availability, low cost, high mechanical stability, low intrinsic fluorescence and easy surface modification techniques. We performed the grafting experiments on both glass slides and silicon wafers. We first created a SAM[7] of a long chain bromo-alkylsilane (Scheme 1, surface **b**) on the chosen surface following a standard procedure (see ESI†). The terminal bromide on the monolayer was easily converted to an azide group (Scheme 1, surface **c**) by substitution with sodium azide under usual conditions.[8] This azide function was now ready for a 'click' cycloaddition with a suitably substituted alkyne.[9] The 'click' reaction commonly employs Cu(I) as the catalyst to improve the yield and regioselectivity of the process.[10]

However, exploratory solution studies indicated that the Fischer carbene moiety suffered extensive decomposition in the presence of Cu(I) under the standard conditions.‡ Gratifyingly, the activated triple bond of the complex **1**[11] underwent a facile [3 + 2]-cycloaddition with azide to form the 1,4-regioisomer[12] of the triazolyl carbene complex without the need of a Cu(I) catalyst.§ This reaction was adopted for modification of the SAMs in the present study (see ESI†).

The chemically modified surfaces were characterized by ellipsometry and contact angle measurements. Ellipsometric measurements showed that the initially formed bromo-undecyl monolayer was 1.81 nm thick. After reaction with sodium azide, the thickness was reduced to 1.71 nm. After Cu-free 'click' reaction with the activated alkyne **1**, the thickness of the SAM increased to 2.37 nm. The observed increase of 0.66 nm is consistent with the calculated¶ value of 0.64 nm for the expected



Scheme 1 Introduction of Fischer carbene functions on SAM *via* copper-free 'click' reaction.

[a] *Department of Organic Chemistry, Indian Association for the Cultivation of Science, Kolkata-700032, India. E-mail: ocas@iacs.res.in; Fax: +91332473280; Tel: +913324734971*
[b] *Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata-700032, India*
[c] *Institut Curie, Laboratoire Physico-Chimie Curie (UMR CNRS 168), 26 rue d'Ulm, 75248 Paris cedex 05, France*

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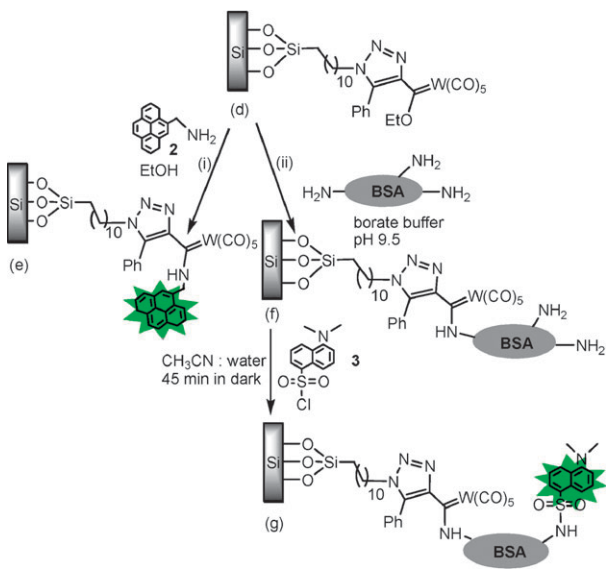
1,4-regioisomer[12] of the 1,2,3-triazole (Scheme 1, surface **d**). This also attests, in a qualitative manner, to a relatively dense packing of the Fischer carbene moieties. Indeed, the hydrophobicity of the surface remains high after different transformations as reflected in the contact angles of bromo-terminated (103.8 ± 3.5°), azido-terminated (94.1 ± 2.2°), and Fischer carbene-terminated (91.1 ± 1.1°) monolayers on silica.

The Fischer carbene-coated SAM was tested for nucleophilic substitution with 1-pyrenemethylamine **2** in ethanol as a fluorescent probe[14] (Scheme 2, surface **e**). After incubation for 10 min, the slide was washed several times with ethanol to remove traces of unreacted pyrenyl compound. The glass slide thus covered with pyrene was observed under fluorescent microscopy (Leica DMLB) and the widely distributed fluorescence spots were recorded at 400× zoom (using a blue filter) under microscope [Fig. 1(A)]. No fluorescence was observed for the control slide (azido-SAM dipped in pyrene solution). Fluorescence spectroscopic measurements‖ of the pyrene-coated glass slide in air showed the characteristic broad emission centred at 480 nm whereas no emission was observed in the control experiment [Fig. 1(B)]. This experiment demonstrates that the Fischer carbene complex was indeed present on the surface for reaction with 1-pyrenemethylamine.

In the protein immobilising experiment, the Fischer carbene-coated SAM was incubated in an aqueous borate buffer (pH 9.5) containing bovine serum albumin (BSA) (1 μM) for 10 min at room temperature. The unreacted protein was removed by washing with buffer and milli-Q water. The slides were then examined by Atomic Force Microscopy (AFM) and High Resolution Scanning Electron Microscopy (HRSEM). AFM imaging was performed using non-contact mode under ambient conditions (see ESI† for details). Fig. 2(A) shows the image of the surface after 10 min of protein incubation. A dense coverage over a large area was observed. No such pattern was observed on the control wafer (azido-SAM exposed to the protein solution) [Fig. 2(B)].



Scheme 2 Grafting of (i) 1-pyrenemethylamine, and (ii) a protein, BSA, modified by dansyl chloride, on a glass surface coated with the Fischer carbene complex.

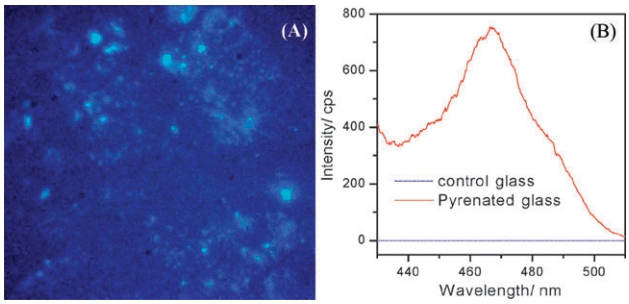


Fig. 1 (A) Fluorescence microscopy of pyrene attached on glass *via* the Fischer carbene; (B) fluorescence spectra obtained from the pyrene-coated glass slide and the control glass slide.

The average particle size obtained from repeated section analysis was 37.9 ± 2.5 nm (from 20 measurements) while the observed average height was 9.14 ± 1.82 nm. The protein size appears larger in the AFM image than the value obtained from crystallography[15] and light scattering experiments[16] due to AFM tip-induced broadening factor.[17] Tip-deconvolution calculations were therefore made. Assuming spherical geometries for both the tip and the sample, the protein diameter obtained after tip deconvolution was as d_t = 8.97 nm using the following expression:[18] $d_t = d_m^2/8r_{tip}$ where d_m is the measured average diameter (37.9 nm), d_t is the calculated diameter of the particle, and r_{tip} is the tip radius of curvature (20 nm as provided by the manufacturer). This value is in good agreement with the measured height of 9.14 nm which includes the

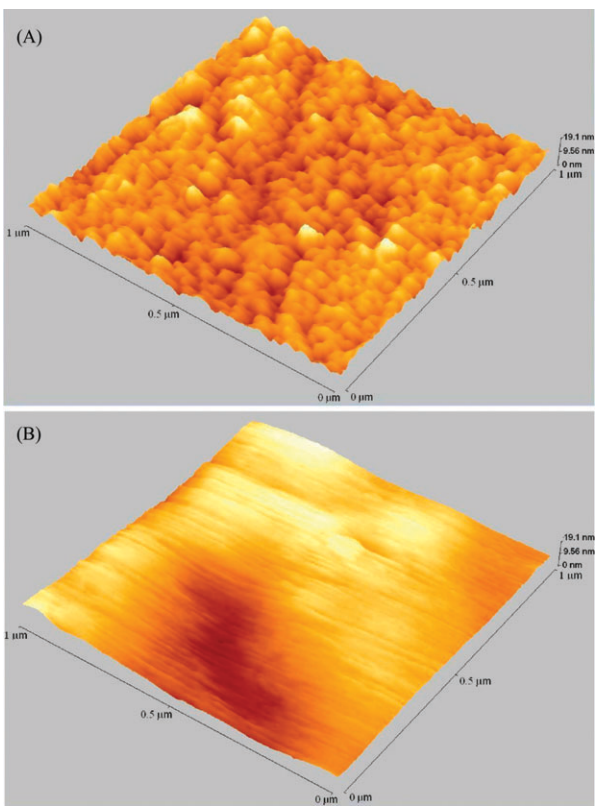


Fig. 2 AFM topographic images of (A) BSA anchored onto a Si surface *via* Fischer carbene complex and (B) the Si surface of control without the Fischer carbene complex.

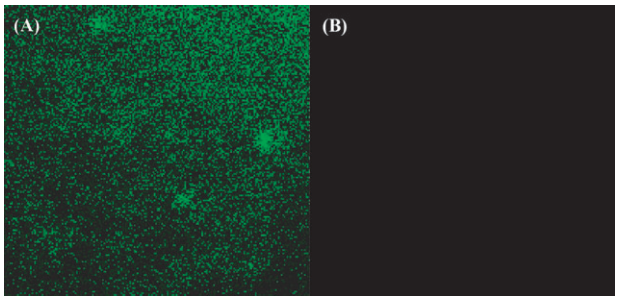


Fig. 3 Fluorescent microscopic picture of (A) dansyl chloride attached to BSA protein and (B) control slide without BSA.

thickness of the Fischer carbene moiety (0.66 nm). The number of BSA molecules was counted by a particle analysis procedure (using DI SPMLab analysis software) which led to an average density of 330 ± 10 molecules μm^{-2}. The SEM picture of the BSA-coated slide (see ESI†) also showed that the average particle size was about 10 nm which was slightly greater than the actual diameter of BSA (9 nm).[15,16]

In another sequence of operations, the BSA-coated glass slide was treated with dansyl chloride **3** (Scheme 2, surface **g**) solution in acetonitrile–water (1 : 4) for 45 min in the dark. After exhaustive washings with acetonitrile–water the slide was observed under a fluorescent microscope [Fig. 3(A)]. The fluorescent spots were found to be uniformly distributed whereas the control slide (without protein) did not exhibit such a fluorescence [Fig. 3(B)].

In summary, we have successfully applied a relatively mild copper-free 'click' chemistry protocol to derivatize a glass/Si surface with a Fischer carbene function that allows rapid immobilisation of proteins on such a surface, leading to the formation of a stable protein adlayer. We believe that this procedure is adaptable with suitable modifications to other metallic or polymeric surfaces. The range of molecules that can, in principle, be immobilised through their amino tethers would include drugs, enzymes antibodies, nucleic acids and others, leading to nanosensors. The present work also widens the scope of applying Fischer carbene complexes in biological and biomedical sciences.

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Notes and references

‡ S. Sawoo, unpublished results.
§ This observation differs from a previous report[13] where the reaction of alkynyl Fischer carbene complexes with azide yielded a β-amino alkenyl carbene complex as the only product. In our reaction, the β-amino alkenyl carbene complex was formed only to the extent of 20% at best. Detailed experimental results of this study will be reported separately in due course.
¶ The calculation is based on Molecular Modelling (MM2) Software.
‖ Fluorescent spectra were recorded at an excitation wavelength of 375 nm on a spectrophotometer (F-4500 Hitachi, Japan).

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