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SPIE.

Event: SPIE BiOS, 2023, San Francisco, California, United States

A new platform for single molecule measurements using the fluorous effect

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ABSTRACT

Irreversible adsorption of biomolecules onto imaging substrates is an impediment to expand the applications of single molecule techniques. Traditional polyethylene glycol (PEG) surfaces are only effective at low concentrations of analytes and their structure prevents their use for interferometric scattering (iSCAT) microscopy. We propose a new platform that virtually eliminates non-specific binding thanks to the omniphobicity of perfluorinated compounds, also known as the fluorous effect. Here, we showcase the anti-fouling properties of these substrates at a single molecule level through iSCAT measurements of a protein mixture. We believe these novel engineered substrates show great promise to study biomachinery processes requiring large analyte concentrations, where other passivation methods are not effective, through iSCAT microscopy and other single molecule techniques.

Keywords: Single-molecule imaging, interferometric scattering microscopy, mass photometry, surface passivation, fluorous effect.

1. INTRODUCTION

A common challenge in single-molecule microscopy is the tendency for biomolecules to irreversibly bind to the imaging substrate.[1,2] This undesirable adsorption not only generates background noise, hindering analysis, it can also alter the experiment by changing the concentration of the sample mixture. To avoid this, great effort has been devoted to the development of passivation methods to prevent fouling on the imaging substrate. Two main strategies have been followed: protein blocking, and polymer grafting. Protein blocking involves populating any available binding sites on the substrate with unlabeled proteins, typically bovine serum albumin (BSA).[3] However, the non-covalent interaction between proteins and the surface results in uneven coatings, and for this reason self-assembled monolayers of synthetic polymers are normally the preferred method.[4] Polyethylene glycol (PEG) is the gold-standard for most single molecule experiments[1,4,5] because of its ability to form strong hydrogen bonds with water molecules. This creates a hydration layer that, together with the steric repulsion caused by the monolayer, prevents the non-specific absorption of biomolecules.[6]

Although many biological studies have been performed using PEG self-assembled monolayers (SAMs), there are several drawbacks that must be taken into consideration. First, traditional PEG SAMs are only effective as anti-fouling substrates at protein concentrations in the nanomolar range, while many biomolecular interactions exhibit dissociation constants in the micromolar range. Increasing the anti-fouling ability of PEG substrates to study biomachinery process

Single Molecule Spectroscopy and Superresolution Imaging XVI, edited by Ingo Gregor, Felix Koberling, Rainer Erdmann, Proc. of SPIE Vol. 12386, 123860A
© 2023 SPIE · 1605-7422 · doi: 10.1117/12.2647953

(e. g. translation, transcription, RNA splicing, etc.), when possible, relies on complex and time-consuming passivation protocols.[7] Furthermore, the coiled structure of PEG polymers excludes them from being used for iSCAT microscopy, a technique that measures the interference of light scattered from subwavelength objects: the conformational mobility of the polymer chains creates too much background noise, preventing the observation of single molecules.[8] The development of iSCAT microscopy as a label-free tool to detect protein mass by Young et al.[9]- referred to as mass photometry - shows great promise to study molecular dynamics of biological process, and therefore it is paramount to develop suitable substrates that will allow full exploitation of its potential.

With this in mind, we propose the use of perfluorinated alkanosilanes as an alternative to traditional passivation methods or bare glass. Perfluorinated chains tend to associate with each other excluding any interactions with other phases. This ability, often called the "fluorous effect", arises from the low polarizability of fluorine atoms, which prevents them from forming instant dipoles and in turn yields weak Van der Wall forces.[11] Thus, the fluorous effect can be described as a highly hydrophobic interaction between perfluorinated moieties, which makes fluorous coatings ideal candidates to reduce adsorption on a variety of substrates.[12,13] In fact, the fluorous effect has been exploited to fabricate anti-fouling coatings for industrial applications and everyday consumer products, such as waterproof textiles or anti-stick cookware. Furthermore, the strong affinity between perfluorinated chains can also be employed to selectively direct fluorous-tagged biomolecules to perfluorinated molecular patterns,[14,15] or for hierarchical assembly of DNA origami structures[16] among other applications.

We recently demonstrated the suitability of perfluoroalkane brushes to monitor adsorption and desorption of single proteins over a prolonged duration via iSCAT microscopy, and their potential to study thermodynamic equilibria between monomeric and multimeric protein components.[8] This work expands on our previous results and presents an optimised protocol for the formation of perfluorinated SAMs on cover glasses, which have been tested with a commercial protein mixture. We believe these results are just the first example of many experimental possibilities that can be unlocked using perfluorinated monolayers for single-molecule studies.

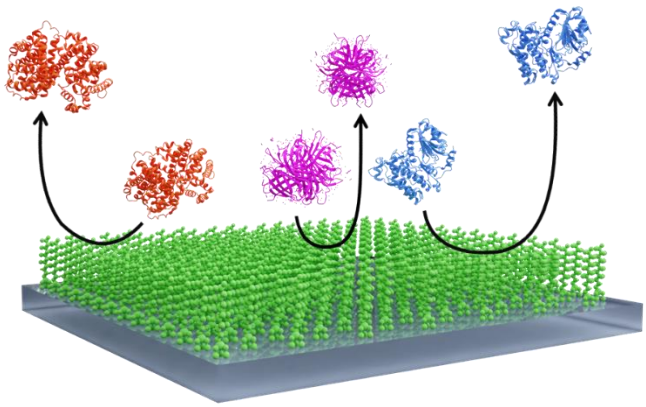

Figure 1: Illustration of the anti-fouling properties of a perfluoroalkane layer in a protein mixture.

2. METHODOLOGY

2.1 Passivation of glass substrates through perfluorinated SAMs

Borosilicate (D 263 M) microscope cover glass (high precision, 24 x 50 mm^2) was purchased from Marienfeld. The slides were ultrasonically cleaned in acetone, isopropyl alcohol (IPA) and reverse osmosis (RO) water for 5 min each, then dried under nitrogen. The substrates were then subjected to oxygen plasma for 5 min at 150 W in an RF PlasmaFab Barrel Asher and immediately placed vertically in an oven-dried glass slide holder, which was then placed inside a glass vacuum desiccator together with 100 µl of (heptadecafluoro-1,1,2,2-tetrahydrodecyl) trimethoxysilane, which was purchased from TCI chemicals and used without further purification. The desiccator was evacuated using a

LABOPORT N86 KT.18 diaphragm vacuum pump which was left on for 3 hours. After this time, the slides were sonicated in methanol for 5 minutes and baked in an N_2-flow oven (Carbolite Gero PF 60) at 90 °C, overnight.

2.2 iSCAT measurements

Fluorous-coated glass slides were rinsed with RO water and IPA 5 times each and dried with nitrogen. A small aliquote of unstained protein standard (NativeMark, Invitrogen) was diluted 200 times in T50 buffer (10 mM Tris–HCl, pH 8.0, and 50 mM NaCl). 10 µl of this dilution was placed on the cover-glass surface. Single adsorption and desorption events were recorded across a 10.8 x 2.9 µm imaging area using a OneMP mass photometer from Refeyn Ltd.

3. RESULTS AND DISCUSSION

3.1 Substrate preparation

Perfluroinated SAMs were generated using a gas-phase deposition method (see section 2.1). It was found that O_2 plasma was essential to ensure the cleanliness of the substrate, and it also reduced the functionalization time by creating active -OH binding sites for the silane groups. Compared to traditional PEG passivation methods, our protocol requires fewer, cheaper reagents and is less time-consuming.

Silanisation under reduced pressure proved to be the best approach to achieve uniform surfaces with minimum background contamination. Wet functionalisation methods are not suitable for perfluoroalkanes because they present traces of bi-products generated during their industrial fabrication. These bi-products are light-emitting and cannot be removed after the functionalisation. Gas-phase methods avoid this issue without compromising the consistency of the coating. Reduced pressure was chosen over heat to evaporate the silane to avoid cross-linking of the silane groups, which can form large structures that anchor the surface irreversibly and create background noise.

The generation of the SAM was confirmed by contact angle (Figure 3b and 3c) of a 5µl water drop. The uncoated glass resulted in an angle of 57.2° which increased to 117.0° after functionalisation because of the higher hydrophobicity of the fluorous layer.

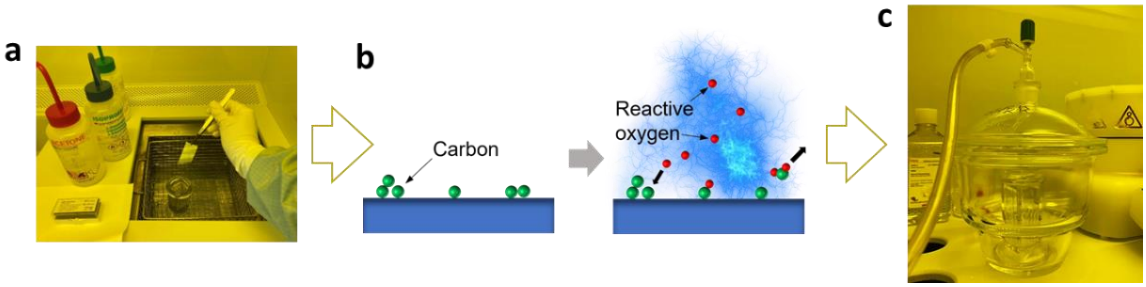

Figure 2: Silanisation workflow: a) solvent cleaning in ultrasonic bath b) scheme of oxygen plasma mechanism c) silanisation under reduced pressure.

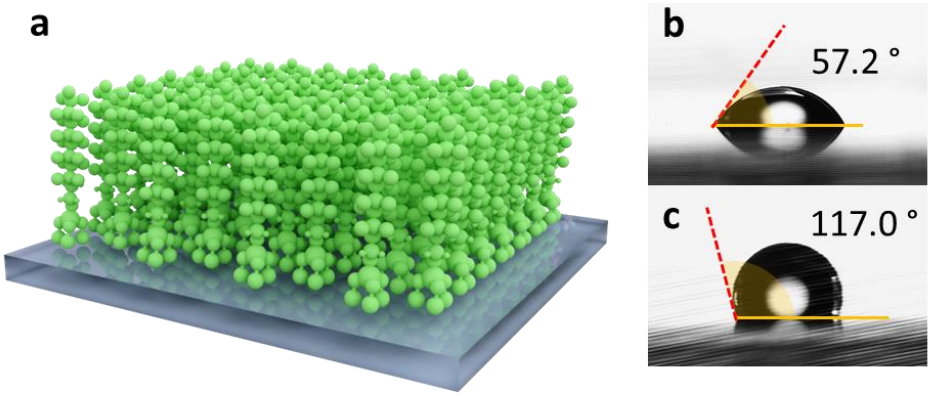

Figure 3: a) Schematic representation of the perfluorinated SAM (modified from ref. 8) b) water droplet on bare glass (57.2°) c) water droplet on silanised glass (117.0°).

3.2 iSCAT

iSCAT microscopy records interferometric images between the light that is reflected from a substrate and the light that is scattered by single-molecule analytes, in this case, proteins. The interference caused by the adsorption of a protein appears as a dark region on the substrate, while desorption events produced the opposite effect. The contrast of these "spots" provides information about the mass of the protein. When using uncoated glass substrates, only a limited number of desorption events are recorded since the proteins bind the glass surface irreversibly. As can be seen on Figure 5, the non-specific adsorption can be prevented by coating the substrate with a fluorous layer. This prolongs the measuring time and prevents changes in the protein concentration that could alter the biological process under study.

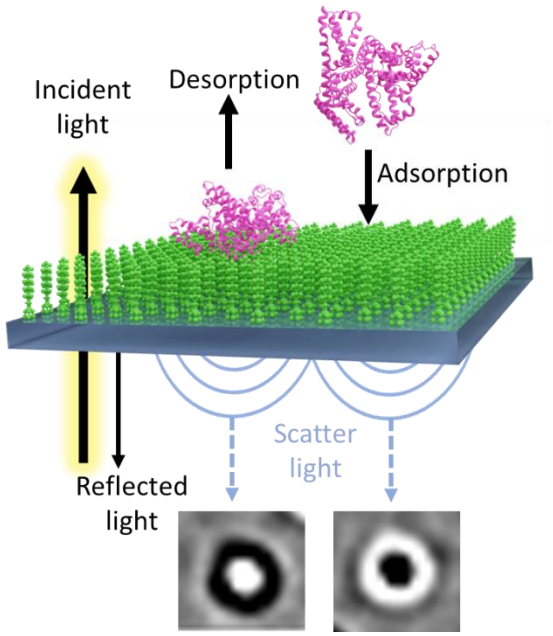

Figure 4: Illustration of the working principle of mass photometry on a perfluorinated-coated glass substrate.

To assess the anti-fouling properties of the fluorous substrate, we used a commercial unstained protein standard, provided by Invitrogen, whose components are unknown. Figure 5 depicts the quantification of measurements recorded over time using this protein mixture when it is introduced to our new fluorous substrate. The measurements yield three distinct adsorption peaks (Figures 5a and 5b), indicating the presence of three proteins within the mixture. We named these protein 1, 2 and 3, in order of their radiometric contrast, this is, their size. Protein 2 results in the most intense peak after 1 minute, which indicates it presents the largest concentration, followed by protein 1 and protein 3. Using glass substrates, these peaks tend to be less intense over time due to the irreversible adsorption of proteins onto the substrate,[8] which limits the time span of the measurements. However, since the perfluorinated SAM prevents irreversible adsorption events, we were able take measurements for several minutes. During the first 11 minutes, the intensity of the adsorption peaks was constant (Figures 5c and 5d), which indicates an equal number of adsorption and desorption events. In other words, during the first 11 minutes our substrate avoided non-specific binding. However, from minute 12 the intensity of the peak corresponding to protein 2 starts to drop at the same time than the peak corresponding to protein 1 increases. Since the total number of counts remains constant, this effect does not correspond to an irreversible adsorption. Rather, we attribute this to the dissociation of protein 2, whose dimers provide a signal that overlaps that of protein 1. This trend has an inflection point at minute 16 after which protein 2 appears to dimerise again.

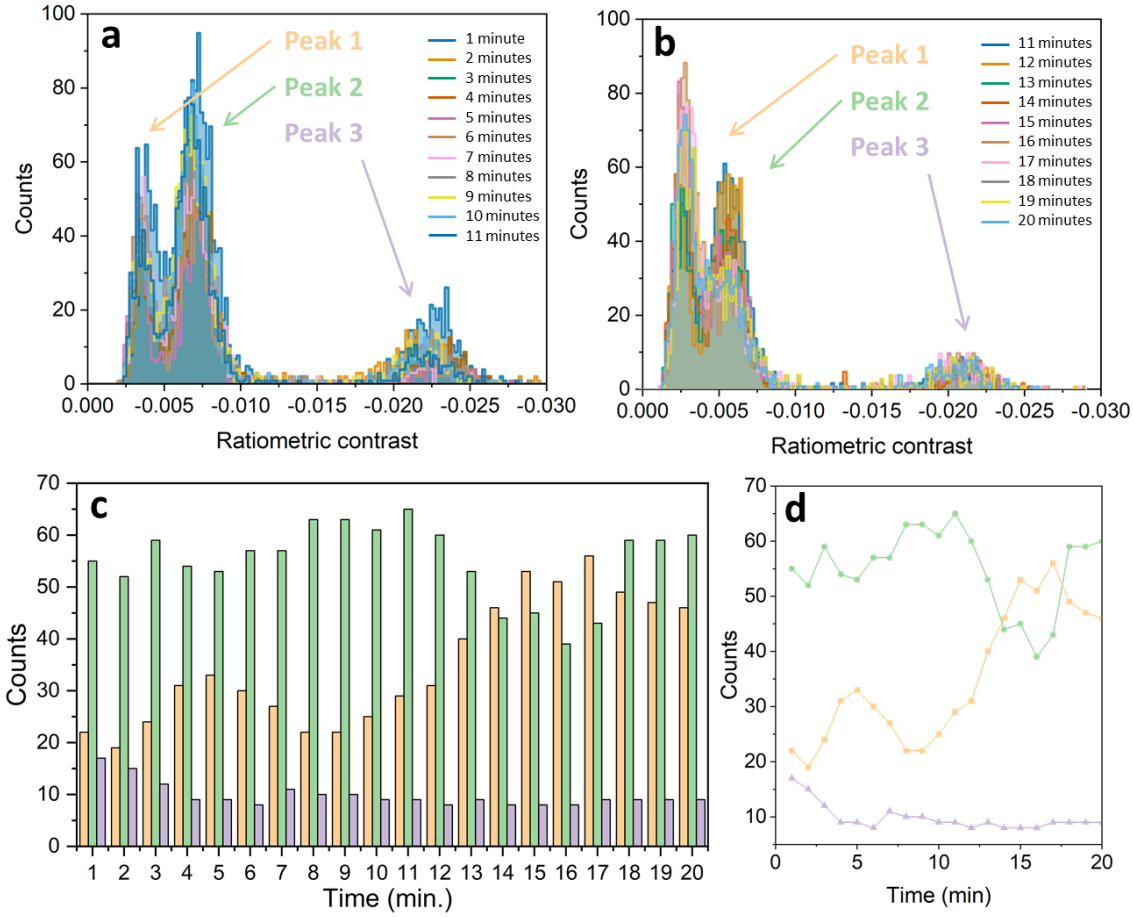

Figure 5: a) and b) Histograms of the frequency of adsorption of NativeMark unstained protein standard from Invitrogen over a) 1-11 minutes and b) 11-20 minutes. c) Cumulative histogram representing the intensity of peak 1 (yellow), peak 2 (green) and peak 3 (purple) observed in a) and b). d) Cumulative adsorption plot of peak 1 (yellow), peak 2 (green) and peak 3 (purple).

4. CONCLUSIONS

The results shown above prove that perfluorinated SAMs prevent non-specific binding events of proteins onto glass substrates, making them excellent candidates for mass photometry studies. The anti-fouling properties of these assemblies, due to the fluorous effect, enable the possibility to record binding and unbinding events for a long period of time, while keeping the concentration of proteins in solution the same. We also observed the dissociation of a protein dimer into its monomers, proving the suitability of perfluorinated substrates to study thermodynamic equilibria of protein complexes. Furthermore, the high specificity of the fluorous effect, together with its outstanding anti-fouling properties, makes our substrates ideal candidates for their use in other single-molecule techniques, such as total internal reflection fluorescence microscopy, potentially allowing for the study of bioprocesses where other passivation methods are ineffective.

REFERENCES

[1] J. Y. Lichtenberg, Y. Ling, and S. Kim, 'Non-Specific Adsorption Reduction Methods in Biosensing', *Sensors*, 19 (2019).

[2] S. Pan, H. Zhang, W. Liu, Y. Wang, W. Pang, and X. Duan, 'Biofouling Removal and Protein Detection Using a Hypersonic Resonator', *ACS Sens.*, 2, 1175–1183 (2017).

[3] R. Ahirwar, S. Bariar, A. Balakrishnan, and P. Nahar, 'BSA blocking in enzyme-linked immunosorbent assays is a non-mandatory step: a perspective study on mechanism of BSA blocking in common ELISA protocols', *RSC Adv.*, 5, 100077–100083, (2015).

[4] F. Cao, Y. Li, J. Wu, W. Liu, and T. Ngai, 'Measurements of interactions between fluorescent molecules and polyethylene glycol self-assembled monolayers', *Soft Matter*, 18, 236–243, (2022)

[5] X. Fu, H. Kaur, M. L. Rodgers, E. J. Montemayor, S. E. Butcher, and A. A. Hoskins, 'Identification of transient intermediates during spliceosome activation by single molecule fluorescence microscopy', *Proceedings of the National Academy of Sciences*, 119, 2206815119 (2022).

[6] S. Lowe, N. M. O'Brien-Simpson, and L. A. Connal, 'Antibiofouling polymer interfaces: poly(ethylene glycol) and other promising candidates', *Polymer Chemistry*, 6, 198–212 (2015).

[7] S. R. Park *et al.*, 'A Single-Molecule Surface-Based Platform to Detect the Assembly and Function of the Human RNA Polymerase II Transcription Machinery', *Structure*, 28, 1337-1343 (2020).

[8] C. J. Bueno-Alejo *et al.*, 'Surface Passivation with a Perfluoroalkane Brush Improves the Precision of Single-Molecule Measurements', *ACS Appl. Mater. Interfaces*, 14, 49604–49616 (2022).

[9] G. Young *et al.*, 'Quantitative mass imaging of single biological macromolecules', *Science*, 360, 423–427 (2018).

[10] A. Sonn-Segev *et al.*, 'Quantifying the heterogeneity of macromolecular machines by mass photometry', *Nat Commun*, 11 (2020).

[11] M. Cametti, B. Crousse, P. Metrangolo, R. Milani, and G. Resnati, 'The fluorous effect in biomolecular applications', *Chem. Soc. Rev.*, 41, 31–42 (2011).

[12] X. Yang *et al.*, 'Surface Fluorination Modification and Anti-Biofouling Study of a pHEMA Hydrogel', *ACS Appl. Bio Mater.*, 4, 523–532, (2021).

[13] X. Shen *et al.*, 'Recycling protein selective adsorption on fluorine-modified surface through fluorine-fluorine interaction', *Colloids and Surfaces B: Biointerfaces*, 214, 112486 (2022).

[14] B.-Y. Li, D. S. Juang, A. K. Adak, K.-C. Hwang, and C.-C. Lin, 'Fabrication of a protein microarray by fluorous-fluorous interactions', *Sci Rep*, 7 (2017).

[15] G. E. Flynn *et al.*, 'Reversible DNA micro-patterning using the fluorous effect', *Chemical Communications*, 53, 3094–3097 (2017).

[16] J. Zou *et al.*, 'Fluorous-Directed Assembly of DNA Origami Nanostructures', *ACS Nano*, 17, 752–759 (2023).