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Deposition of Intact and Active Proteins In Vacuo Using Large Argon Cluster Ion Beams

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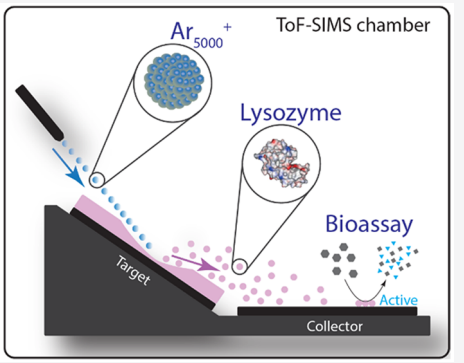
ABSTRACT: Providing inert materials with a biochemical function, for example using proteins, is a cornerstone technology underlying many applications. However, the controlled construction of protein thin films remains a major challenge. Here, an innovative solvent-free approach for protein deposition is reported, using lysozyme as a model. By diverting a time-of-flight secondary ion mass spectrometer (ToF-SIMS) from its standard analytical function, large argon clusters were used to achieve protein transfer. A target consisting of a pool of proteins was bombarded with 10 keV Ar_{5000}^+ ions, and the ejected proteins were collected on a silicon wafer. The ellipsoidal deposition pattern was evidenced by ToF-SIMS analysis, while SDS-PAGE electrophoresis confirmed the presence of intact lysozyme on the collector. Finally, enzymatic activity assays demonstrated the preservation of the three-dimensional structure of the transferred proteins. These results pave the way to well-controlled protein deposition using ion beams and to the investigation of more complex multilayer architectures.



Surface biofunctionalization with proteins is of utmost importance for many applications including biosensing, biocatalysis, drug delivery, and biomaterials.[1−4] The effectiveness of the deposited layers depends on factors such as protein amount, layer composition, protein−surface interactions, and organization of the protein mono- or multilayers. The deposition method must thus ensure a fine control of these parameters. The most straightforward approaches to immobilize proteins rely on spontaneous adsorption from solution.[5] This may however provoke conformational changes of the protein, leading to a loss of bioactivity.[6,7] Moreover, adsorption usually restricts the biofilm to a monolayer, consequently limiting the maximum adsorbed amount.[8] Alternative methods to tackle these limitations include covalent chemisorption, protein trapping in highly hydrated polymer brushes and the layer-by-layer method.[9−11] Despite enhancing some of the performances, these techniques still suffer from drawbacks, such as a lack of control of the protein spatial distribution at interfaces, which relies on patterning approaches[12−14] and potentially decreased performances if the layers are dried.[15,16] Therefore, alternative strategies for protein deposition at interfaces are still highly desired.

Over the years, solvent-free approaches were developed for the deposition of nonvolatile organic and inorganic materials. Gathered under the denomination of preparative mass spectrometry (pMS), these techniques rely on the formation of mass-filtered ion beams which are directed toward surfaces, usually under ultrahigh vacuum (UHV).[17] Promising results regarding the deposition of biomolecules were notably obtained via soft-landing, a subcategory of pMS defined as a deposition process occurring with low translational kinetic energy, precluding the fragmentation of the incident species.[18] 3D structure and activity were preserved for proteins deposited by soft-landing.[19] Yet pMS techniques do not spread widely for surface (bio)coating preparation since the intensity of current generated by the ion sources is too weak to be effective on large scales (cm^2 range). This is inherent to both the instrumental limitation and the very low ionization yield of large biomolecules.[17,20−22]

During the past decades, progress in secondary ion mass spectrometry (SIMS) has resulted in the development of noble gas cluster ion beams (GCIB),[23] which have become a reference as ion sources for organic sample analysis. In comparison with monatomic primary ions, kiloelectronvolt clusters show benefits such as higher sputter yield, higher secondary ion signal and reduced in-depth damage, as they transfer their energy solely in the topmost surface layers.[24,25] In addition, large gas cluster ions of, e.g., argon ($Ar_{500-5000}^+$) favor the emission of molecular versus fragment ions, in an inverse function of the cluster energy per constituent atom

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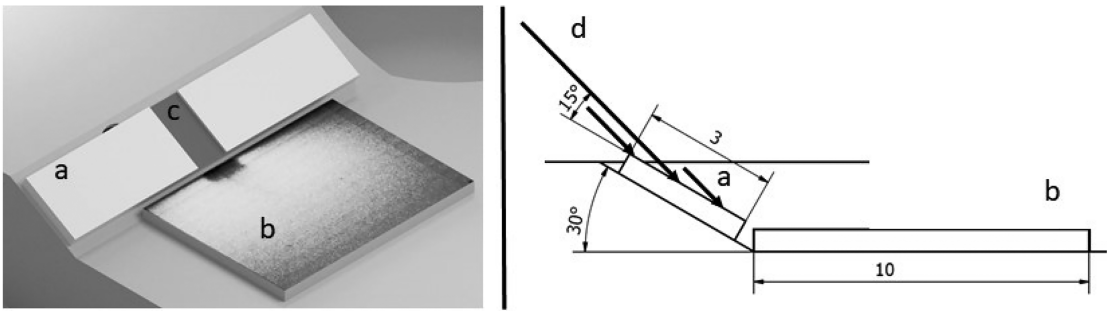

Figure 1. Experimental setup for molecular transfer: (a) target, (b) collector, (c) section of the target where material was removed, and (d) impinging argon cluster ions.

(E/n).[24,26,27] They are therefore excellent for 3D molecular analysis of structured organic and biological samples[28−30] and high-yield detection of large intact (bio)molecules, including proteins.[31,32]

Based on these latest improvements, Lorenz et al. reported a protocol in which the Ar-GCIB of a time-of-flight secondary ion mass spectrometer (ToF-SIMS) is derived from its usual analytical function to be used for the transfer of organic material, here antioxidant molecules, from a solid surface onto another.[33] They showed that a large fraction of the transferred molecules was intact after deposition. Inspired by these results, we propose here an experimental setup that allows the construction of protein mono- and multilayers under UHV. The developed technique enables the deposition of proteins in dry conditions and overcomes the thickness limitation inherent to spontaneous adsorption. In addition, proteins can be transferred regardless of their charge state, enhancing substantially the deposition rate in comparison with ion soft-landing. Hereafter, the first proof-of-concept experiment is reported through the transfer of lysozyme, taken as a model protein. After deposition, the transferred molecules are analyzed by ToF-SIMS and identified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) coupled to mass spectrometry, and their enzymatic activity is measured. Taken together, the data allow the success of the transfer to be established and, in particular to demonstrate that the transferred protein molecules are intact and bioactive.

The experiments were conducted in a commercial ToF-SIMS instrument. However, a homemade sample holder was designed and built to carry out the transfer experiments (Figure 1). The transfer consists in bombarding a pool of proteins (the target) with large Ar cluster ions and collecting the ejected particles onto a clean silicon wafer (the collector). In the current setup, the target is placed with an angle of 30° with respect to the collector (horizontal), so that the Ar cluster ions impinge the target with a grazing angle of 15°(Figure 1 and Figure S-1).

All transfer results reported here were obtained using a primary beam of Ar cluster ions with a size distribution centered around 5000 atoms and an acceleration energy of 10 kiloelectronvolts (10 keV Ar_{5000}^{+}). The size and energy of the clusters were chosen as a compromise between minimizing the energy per atom (2 eV on average), in order to prevent protein fragmentation, while maintaining a sufficient current intensity (≈1 nA). After transfer, mass spectrometric images of the collector are acquired by operating the ToF-SIMS instrument in 2D imaging mode with a highly focused Bi_5^{+} analysis beam. Nonetheless, the molecular ion peak is not detectable with this routine protocol. The maps of the collectors (Figure 2) are *de facto* based on molecular fragment ions characteristic of amino acids.[34] Fragment ions at m/z = 30, 70, and 130 corresponding respectively to CH_4N^+, $C_4H_8N^+$, $C_9H_8N^+$ were selected. The peaks at m/z = 28 (Si^+) and 45 ($SiOH^+$) account for the Si wafer substrate signal. The maps display information about transfer efficiency and deposition pattern, but deliver no relevant information about the integrity of the transferred molecules. Figure 2 (left, protein signal; right, Si substrate signal) compares images of the collector after lysozyme deposition (e and f) with reference images of a clean Si wafer (a and b), and a Si wafer fully covered with lysozyme (c and d). It demonstrates that protein material was transferred efficiently from the target onto the collector. Indeed, the signal of the Si substrate is strongly attenuated or inexistent in an area corresponding to the maximum intensity of the protein

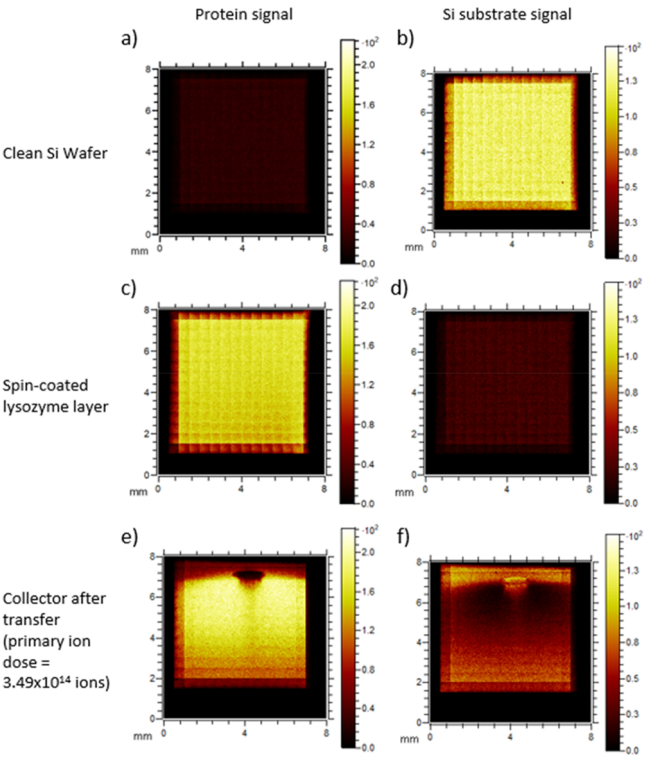

Figure 2. Chemical signal images of the Si wafer collector. A clean Si wafer (a and b) and a Si wafer fully covered with lysozyme (spin-coated sample) (c and d) are shown as references. Sample after transfer of lysozyme with a primary ion dose of 3.49×10^{14} ions is displayed in (e and f). (a, c, and e) Protein signal as the sum of CH_4N^+, $C_4H_8N^+$, and $C_9H_8N^+$ intensities. (b, d, and f) Signal intensity of the Si wafer substrate as the sum of Si^+ and $SiOH^+$.

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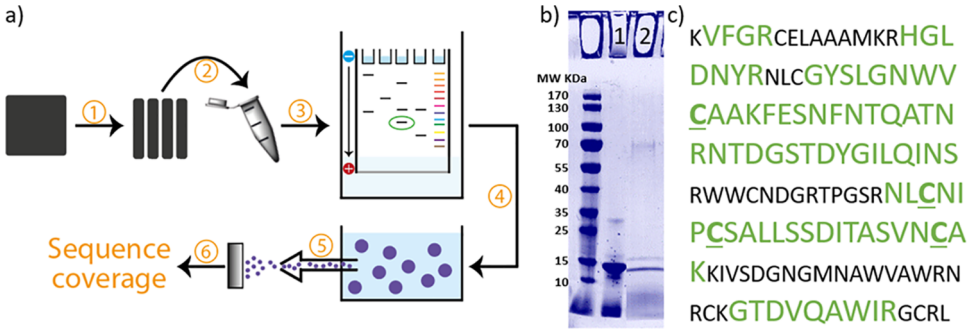

Figure 3. (a) Schematic illustration of the experimental workflow from collector to SDS-PAGE and sequence coverage results. (1) After transfer, collector is cleaved in four smaller pieces. (2) The pieces are mixed in an Eppendorf with SDS-containing Laemmli buffer to desorb the proteins. (3) The solution is placed in a SDS-PAGE gel. (4) The band of the gel believed to be native lysozyme is excised from the gel and mixed in solution with trypsin to be digested. (5) The digested lysozyme solution is analyzed by means of ESI-MS. (6) Data treatment and comparison with database allow the determination of the sequence coverage. (b) SDS-PAGE gel, with reference lysozyme in well 1, and transferred sample in well 2. (c) Amino acid sequence of lysozyme. In green, the part of the sequence identified by mass spectrometry on the trypsin-digested transferred sample, accounting for 60% of the sequence. The underlined Cys are not part of the lysozyme sequence, they appeared because of the carbamidomethylation pretreatment.

fragment ions. A black spot is noticed in the center top of the protein fragments image, corresponding conversely to a brighter spot on the substrate image. This specific mark is due to a part of the incident Ar beam reaching the surface of the collector during transfer, favored by the grazing incidence of the primary ion beam on the target. The surface in this spot is then constantly "cleaned" by Ar clusters and no protein signal is detected. This could however easily be avoided by aiming higher on the target. Proteins are deposited in an ellipsoidal shape pattern mirroring the angular distribution of the ejecta.[33] The substrate image displays a shading with a similar pattern. The substrate signal provides an indirect estimate of the transferred protein thickness variation, since it is expected to decrease exponentially with the overlayer thickness.[34] With the current setup, the layer thickness can only be considered uniform for areas of a few mm^2 in selected spots of the collector. The disappearance of the substrate signal also indicates that the multilayer regime is reached on a relatively large area above a given primary ion dose. Provided that the target is not exhausted, the total amount of transferred material should be directly proportional to the primary ion dose, allowing a fine control of the transferred quantity.

These results demonstrate that protein material was deposited on the collector but do not confirm that the protein is intact. As was previously reported in the literature, multivariate analysis of the ToF-SIMS intensities of amino acid fragments can be successful for revealing the identity, integrity, and orientation of adsorbed proteins.[35,36] However, the outcome of such an analysis seems to be particularly uncertain for proteins randomly oriented and potentially fragmented on the collector. In the best case scenario, it could only provide us with indirect evidence on the protein state. Therefore, a more direct approach was chosen to assess whether the transferred proteins were intact or fragmented and how their 3D structure was affected by the process (cluster impact, desorption in vacuum, and landing on a substrate). Hence, SDS-PAGE electrophoresis, trypsin digestion followed by mass spectrometry, and an enzymatic activity assay were performed on the collectors after lysozyme transfer.

SDS-PAGE is commonly used for the separation and identification of proteins.[37] It delivers information about the integrity of the polypeptide chain length. Figure 3b shows the result of the SDS-PAGE separation carried out with transferred protein material desorbed from the collector. A reference lysozyme sample gives a wide band just below 15 kDa, corresponding to the lysozyme molecular mass. The transferred material reveals a thin band at the same position, indicating the presence of intact lysozyme molecules on the collector. Note that the upper band around 70 kDa and the diffuse area below 10 kDa could not be identified. While the upper band probably arises from a contamination, the diffuse area below 10 kDa most likely results from random fragmentation occurring during transfer. To ensure that the observed band corresponding to intact lysozyme is not an artifact nor a contamination, it was cut from the gel and mixed with trypsin, yielding smaller protein fragments that were then analyzed by mass spectrometry. Four peptides related to lysozyme could be identified, accounting for 40% of the total sequence (Table S-1), confirming that the observed band contains lysozyme. A sketch of the workflow used to obtain these data is represented in Figure 3a. However, trypsin digestion is not optimal on proteins trapped in a gel. To go further, trypsin digestion was also performed directly on a collector after lysozyme transfer. In this case, 60% of the sequence could be identified (Figure 3c). These results clearly demonstrate the presence of intact lysozyme molecules at the surface of the collector.

Confirming the activity of an enzyme provides strong insights regarding its three-dimensional structure, due to the structure−activity relationship. A fluorescence-based enzymatic activity assay was performed at the surface of the collectors.[38] The results suggest that the activity detected on the collectors is roughly equivalent to that of 0.9 μg of fresh lysozyme (Figure 4). This result indicates that at least a fraction of the transferred proteins can be found on the collector in their native functional conformation. However, some protein molecules may unfold to a certain extent upon desorption, and refold once relaxing on the collector surface.[39] Moreover, the enzymatic assay being performed in solution, slightly unfolded proteins might fold back to their native conformation. Both unmodified and refolded proteins contribute to the measured activity. In comparison with the obtained value of 0.9 μg, a monolayer of lysozyme can be approximated to 0.115−0.230 $\mu g/cm^2$ based on its molecular footprint. The collector surface being roughly 2 cm^2, if the proteins were deposited homogeneously, enough material would be trans-

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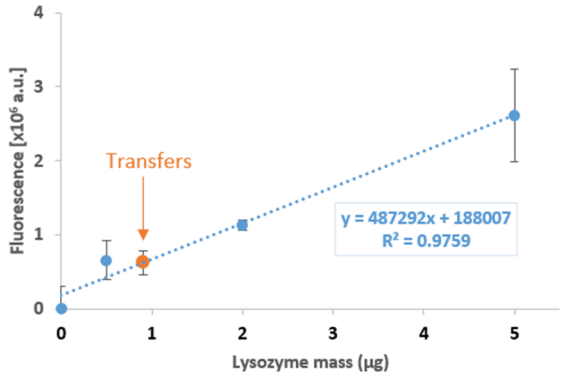

Figure 4. Measured fluorescence/activity as a function of lysozyme mass. In blue, the calibration curve (reference samples prepared with fresh lysozyme). Each data point represents the average of triplicates. The orange dot is the average activity measured on six different collectors. The value obtained for the transfer is significantly different from that of the blank (t test, $p_{value} < 0.01$).

ferred to build two to four molecular layers with the ion dose of 4×10^{14} ions that was used. It is worth emphasizing that the total quantity of transferred material is undoubtedly superior to that obtained with the activity assays, yet it could not be precisely quantified. Two explanations support this claim. The first one is that, with these beam conditions (E/n = 2 eV), some fragmentation is still likely to occur (cf. SDS-PAGE result below 10 kDa). In addition, while the Ar beam distribution is centered on 5000 atoms, it is quite wide, with a full width at half-maximum of about 2000 (Figure S-2). Therefore, the contribution of higher E/n clusters, inducing more bond scissions upon impact, is not negligible. As a result, a significant fraction of proteins should be transferred as nonactive fragments. The second explanation lies in the possibility that the specific activity could differ from reference to transferred lysozyme samples. Indeed, the protein structure might in part be affected by the transfer (see *supra*), and substrate diffusion might be limited when multilayers are deposited. Experiments are currently underway to clarify these hypotheses.

In summary, using lysozyme as a model protein, the efficiency of a novel protein thin film deposition method employing a ToF-SIMS instrument was demonstrated. It is shown that large argon cluster beams (Ar_{5000}^{+}) have the ability to desorb intact proteins from a surface, and that these are later capable of landing on another substrate without fragmentation. The deposited amount should be directly proportional to the primary ion dose, thereby allowing a fine control of the transferred quantity. Enzymatic activity was detected on the newly constructed films, demonstrating that proteins retained their 3D structure even under these harsh conditions. The ultrahigh vacuum inherent to ToF-SIMS allows the production of dry films (in contrast with adsorption methods), and the fact that charged as well as neutral species are deposited largely improves the efficacy in regards with other preparative mass spectrometry techniques. We are confident that the transfer of larger proteins will be possible, since recent molecular dynamic simulations indicate that similar Ar clusters can desorb larger organic molecules (~60 kDa) without fragmentation.[40] It will remain very important to check whether these transferred proteins do keep their activity, since uncertainties remain regarding the potential conformation changes that could occur. In addition, modifications could be implemented in order to construct more homogeneous films (ultimately allowing a fine thickness control, from (sub)mono- to multilayers) or to be able to control the deposition pattern. In addition, the method promises to offer great versatility, especially in terms of substrate nature (target or collector), transferred material (organic or inorganic) and film thickness. These preliminary results pave the way to well-controlled protein deposition using ion beams and to the investigation of more complex multilayer architectures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.0c02510.

Material and methods section containing all the experimental details regarding the target and collector preparation and the transfer experiments in the ToF-SIMS as well as a detailed procedure for the SDS-PAGE gels, trypsin digestion, ESI-MS measurements, and activity assays and a few additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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