Patterning of lactoferrin using functional SAMs of iron complexes†

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A new method has been developed that allows spatially resolved adsorption of lactoferrin on a surface, by means of specific non-covalent interaction between the native protein and a patterned self-assembled monolayer of an iron-containing terpyridine complex.

Patterning of proteins on surfaces is a topic of great interest in wide-ranging areas as biosensing and bioelectronic applications.[1] The formation of a protein pattern implies the co-existence, in spatially contiguous areas of the same surface, of antithetic properties such as binding interaction and resistance to surface adsorption. The preparation of protein-resistant surfaces is a very hard task because each protein system has its own specific surface interaction and behaviour.[2] Typically, this can be achieved by using specific protein-resistant compounds, such as ethylene glycol-based products.[3] As to the anchoring of proteins, it can be performed by means of several approaches[4] including coupling reactions, electrostatic interaction, interaction of a histidine-tagged protein with a metal, *etc.*[5] Most of the anchoring strategies involve the derivatisation of the protein, which can cause more or less pronounced permanent changes in its structure and this, in turn, could affect the biological activity. Hence, the development of methods for anchoring proteins without any functionalisation is highly desirable. Among others, this can be accomplished by exploiting non-covalent binding, *i.e.* specific or unspecific supramolecular interactions of the protein with a suitably functionalised surface.[6]

Micrometric patterning of proteins can be obtained by different techniques such as microcontact printing,[7] ink-jet printing,[8] dip-pen nanolithography,[9] imprint lithography,[10] microfluidic channel networks,[11] and phase separation of polymer blends and block copolymers.[12]

In this paper we present a method for obtaining patterns of non-functionalised proteins by using a combination of top-down and bottom-up techniques, involving the use of focused ion beams (FIB) for "writing" micro- or submicrometre scale patterns[13] combined with a self-assembly strategy to exploit specific non-covalent interaction between a protein and a metal complex-based self-assembled monolayer (SAM). In particular, we have developed a process for the selective adsorption of lactoferrin (LF) onto an iron–terpyridine complex SAM patterned within a hydroxyl-terminated alkanethiol SAM, thought to resist unspecific LF adsorption. The OH-terminated alkanethiol in the present experiment was demonstrated to be satisfactory, even if its wider applicability will depend upon the specific protein features. The idea is to take advantage of the specific non-covalent interaction between LF, an antimicrobial and antiviral glycoprotein component of mammalian milk,[14] and iron ions to obtain a surface anchoring that could resist prolonged rinsing in aqueous media. Indeed, LF is known to have two specific iron-binding sites, respectively belonging to the two identified lobes. The coordination of the iron cations involves four amino acid residues in each lobe, consisting of His253, Tyr92, Tyr192 and Asp60 in the N-lobe and Hys253, Tyr435, Tyr528 and Asp395 in the C-lobe.[15] The synthetic steps involved in the patterning procedure were studied by means of time-of-flight secondary ion mass spectrometry (ToF-SIMS), both in spectroscopic and imaging mode, and by quartz crystal microbalance with dissipation monitoring (QCM-D). The first technique provides a very sensitive, and spatially resolved, chemical characterisation of the surfaces, while the latter allows us to follow *in situ* the kinetics of surface adsorption from liquid phase and provides, at the same time, information on the viscoelastic properties of the adsorbed layer.

In order to establish the shape of the surface pattern, we followed the procedure outlined in Scheme 1.‡ A SAM of 11-mercapto-1-undecanol (henceforth MUO) was prepared on gold. This layer acted as the blackboard where the pattern was written by means of FIB maskless lithography: the MUO SAM was etched with the focused gallium ion beam in order to produce square regions of bare gold. Such regions were "filled" with a SAM of iron–terpyridine complexes, formed *via* a stepwise procedure involving the initial formation of a mixed component SAM that contains terpyridine functionalities, followed by the subsequent reaction with an iron(II) salt that produces the complex. The mixed component SAM consists in a stable and reproducible 1 : 1 assembly of [4′-(4-mercaptophenyl)-2,2′:6′,2″-terpyridine] (MPTP) and mercaptobenzene (MB), which has been already extensively studied in our laboratory.[16] Finally, the patterned substrate was incubated in a LF solution and repeatedly washed, so obtaining the desired spatially resolved LF pattern.

The adsorption behaviour of LF from aqueous media on the surface-anchored iron complex has been studied *in situ* by means of QCM-D.§ Fig. 1 shows the frequency and dissipation curves as a function of adsorption time onto an unpatterned iron-complexed layer and, for comparison, onto a mixed MB–MPTP SAM as well as onto the hydroxyl terminated MUO monolayer. It can be seen that a remarkable lactoferrin adsorption occurs only for the iron-containing MB–MPTP-Fe SAM, with a LF uptake of about 450 ng cm^{-2}, as calculated from the frequency shift. This amount corresponds to 3×10^{12} molecules cm^{-2}, *i.e.* to a monolayer, if we assume that the LF on the MB–MPTP–Fe surfaces keeps its native state dimensions of $15.6 \times 9.7 \times 5.6$ nm.[15b] The saturated layer on the MB–MPTP–Fe surfaces is obtained in about 10 min.

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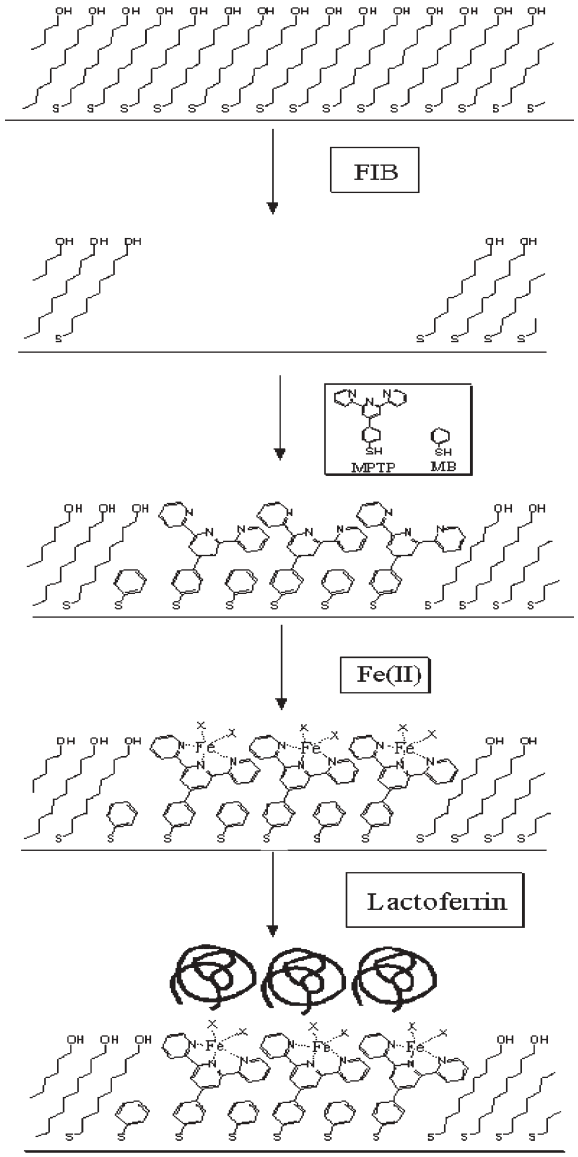

Scheme 1 Patterning method used for spatially selective anchoring of LF.

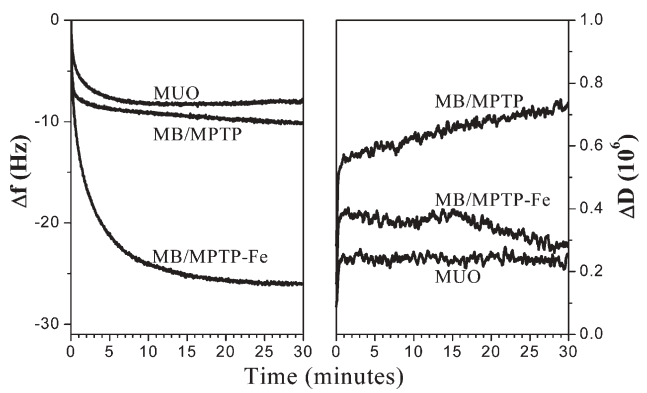

Fig. 1 QCM-D monitoring of LF adsorption onto SAMs of the iron-complex, MB–MPTP and MUO.

At a variance of this, a lower but still detectable adsorption (about 15% of the adsorbed amount observed for MB–MPTP–Fe film) also occurs for the MUO and MB–MPTP surfaces. This result clearly indicates that in the case of the MB–MPTP-–Fe film the specific Fe–LF interaction prompts the massive adsorption, while in the case of the two other films LF is adsorbed by means of unspecific interactions, involving hydrophobic and van der Waals interactions. The comparison among frequency and dissipation curves provides another very important insight into the nature of the basic interactions. In fact, dissipation curves, which depend upon the viscoelastic properties of the films, provide a very sensitive tool to measure the relative strength of the LF linking to the three investigated surfaces. In particular, the behaviour observed for LF adsorbed onto MUO corresponds to the formation of very rigid elastic films, *i.e.*, the small amount of adsorbed LF does not modify in a significant way the rigidity of the combined LF-SAM layer. Also the system MB–MPTP–Fe–LF shows a remarkably higher rigidity, as more important as the adsorbed mass is 5 times that adsorbed on MUO. Such behaviour indicates a very strong complexing bonding of LF to the MB–MPTP–Fe layer. Finally, LF adsorbed onto MB–MPTP monolayers shows a highly viscoelastic behaviour, or, in other words, the small amount of adsorbed LF is very loosely bound to the underlying SAM film, and "slipping" of the adsorbed molecule produces the large viscoelastic response.

The three types of adsorption behaviours above discussed are in full agreement with the assays of relative stability with respect to rinsing the films on the different SAMs. Indeed, LF on Fe-complexed MB–MPTP monolayers was stable, at variance with the two other cases, where the adsorbed LF is completely removed by accurate rinsing of the surfaces, as indicated by ToF-SIMS measurements (see below).

The spatial selectivity of LF adsorption has been demonstrated by using ToF-SIMS chemical imaging.¶ Fig. 2 shows the ToF-SIMS chemical maps of the patterned surface before (Fig. 2a) and after (Fig. 2b) LF adsorption and accurate final rinsing. The figure shows the lateral distribution of the intensities of some relevant

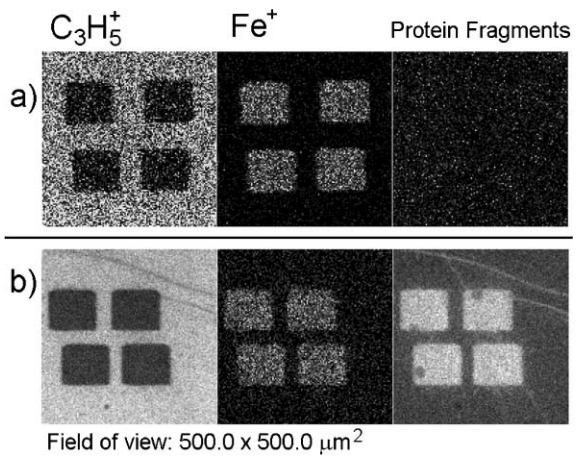

Fig. 2 ToF-SIMS imaging of the pattern (a) before and (b) after lactoferrin adsorption. A lighter colour in each mass resolved image indicates a higher intensity of the pertaining signal. Images (a) and (b) were acquired, respectively, from two different sets of patterns produced on the same sample.

peaks characteristic of the different components present on the patterned surface, namely $C_2H_5^+$ (*m*/*z* 29.039), a fragment typical of MUO, Fe^+ (*m*/*z* 55.935), expected to arise from the iron complex, and the sum of some characteristic protein fragments. The latter fragments, diagnostic of the presence of the protein, have been chosen according to the literature[17] with the exclusion of some peak series (such as $C_xH_yO_z$) that are present also in the spectrum of MUO. In particular, Fig. 2a demonstrates that iron has been selectively fixed on the MB–MPTP SAM areas (the squares in Fig. 2a) whereas the remaining surface, still covered by the MUO SAM, is iron-free. Fig. 2b, and in particular the intensity map of characteristic protein fragments, shows that stable, *i.e.* resisting rinsing, protein adsorption occurred selectively on the iron-containing patterns. It is worth noting that the intensity of the iron signal in Fig. 2b is weaker than the one in the corresponding chemical image of Fig. 2a. Since it is well known that the ToF-SIMS signal mainly arises from the outermost part of the surface, the weakening of the iron signal is due to the fact that the iron complex is covered by a LF layer, further confirming the success of the patterned protein adsorption.

In conclusion, we developed a bottom-up method for preparing surface patterns of LF by specific non-covalent interactions between the native protein and an iron–terpyridine complex-based self assembled mixed monolayer. QCM-D and ToF-SIMS measurements provided information about the relative strength of interaction of LF on the various SAM films and showed that protein monolayers can be obtained, selectively adsorbed on the iron-containing areas, but not on the OH-terminated alkanethiol SAM, which therefore can be used as the blackboard where the patterns are written. As an aside, we observe that the strong affinity of LF to the MB–MPTP-–Fe SAMs could be exploited, at least in principle, in sensors for lactoferrin, as the proposed technique can be easily scaled up to cope with the current microsystem technology. Also, it must be emphasised that, although the experimental results discussed in this paper refer to relatively wide patterns (lateral dimension of 10^{-4} m), the procedure outlined can be used for producing patterns with lateral dimensions down to the submicrometre scale, the limiting factor being the lateral resolution of FIB, which can reach a few nanometres. Finally, in view of the fact that the terpyridine ligand is well known to be able to form coordination compounds with several metal ions of biological interest (such as Fe, Ni, Co, Cu, Zn, *etc.*), the method we propose promises to be applicable, at least in principle, to other proteins having in their structure specific receptors for metal cations.

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Notes and references

‡ SAMs of 11-mercapto-1-undecanol (Sigma-Aldrich) (MUO) were prepared by immersing a gold substrate into a 10^{-3} M thiol solution in ethanol for 24 h and then rinsing in ethanol. Patterning was obtained by rastering a focused $^{69}Ga^+$ continuous beam (produced with the same ion column used for ToF-SIMS analysis, see footnote¶ below) over 10^{-8} m^2 square areas. With a beam current of 10 nA, a single square is produced in about 10 s. AFM measurements of the surface inside and outside the craters shows that ion irradiation produces an increase of average surface roughness (measured on 25 μm^2) from 1.7 nm to 6.7 nm. The etched areas were covered with an iron-complex-containing SAM *via* a multi-step protocol involving: (i) preparation of a mixed component MB–MPTP SAM following a previously established procedure,[13] (ii) rinsing with ethanol and drying in nitrogen flux, (iii) dipping in an Fe^{II} solution ($FeSO_4{\cdot}7H_2O$, Sigma-Aldrich, 5×10^{-4} M, water–ethanol 1 : 1) for 1 min, (iv) rinsing in ethanol and drying. Samples were then incubated for 30 min in a phosphate buffered 10^{-6} g cm^{-3} aqueous solution of human lactoferrin (Sigma-Aldrich) and then consecutively rinsed with a phosphate buffer, Millipore® water, ethanol and then dried in N_2 flux. Surface roughness after lactoferrin deposition is 1.7 nm outside the squares and 5.2 inside the squares.

§ QCM investigation was performed in aqueous medium by means of a quartz crystal microbalance (Q-Sense AB, Sweden). Gold AT-cut crystals with a resonance frequency of about 5×10^6 Hz were employed. Before the QCM-D measurement, the Au surfaces of the crystals were covered respectively with MUO, MB–MPTP, MB–MPTP–Fe monolayers with the same procedure described in the previous footnote.‡ Absorption measurements of LF onto such surfaces from aqueous solution were performed after temperature-stabilisation at 37 $\pm$ 0.1 °C. The frequency-to-mass conversion was obtained by applying the Sauerbrey relation.[18]

¶ ToF-SIMS high mass resolution spectra and images were acquired in "static mode"[19] with a reflector-type spectrometer (ION-TOF TOFSIMS IV), by using a pulsed $^{69}Ga^+$ primary ion beam (25 keV, ~0.1 pA).

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