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An Electrically Reversible Switchable Surface to Control and Study Early Bacterial Adhesion Dynamics in Real-Time **

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Nature provides cleverly concerted mechanisms which are able to control specific and non-specific interactions between cells and biological surfaces in a dynamic environment.^[1] The successful imitation of this ability is of crucial importance for the understanding of cell adhesion to man-made surfaces,^[2] and will aid in making significant impact in the future development and fabrication of new, functional biomaterials,^[3] long-term antifouling

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surfaces^[4] and novel *in vitro* diagnostic tools.^[5] Researchers have previously fabricated surfaces, typically using self-assembled monolayers (SAMs)^[6] in an attempt to control and understand cell adhesion, *via* the introduction of specific terminal moieties to the SAM to elicit specific surface properties.^[7] However, despite the advancements achieved by investigating the interface as a ‘static’ environment, the mechanism behind the cell adhesion process remain unclear^[8] representing one of the biggest challenges for many scientific areas ranging from tissue engineering,^[9] medicine,^[10] cell biology,^[11] immunology^[12] and marine biofouling.^[13] In particular, researchers have tended to use cells to classify surfaces (*i.e.*, bactericidal, repellent) while the ideal approach would be using the surface to understand cell behaviour. Towards this aim, scientists have discovered the possibility to control surface properties by switching their characteristics in response to particular needs.^[1b, 14] Surfaces presenting tuneable moieties have been widely used in the study of specific protein-repellent/adhesion phenomena.^[15] However, there are only a few reports on the control of non-specific cell adhesion^[16] due to the lack of specific targeting on the cell surface, and the difficulties encountered in the interpretation of the outcome of end-point assays.^[17] To date, dynamic control over cell adhesion has been achieved by using “smart” surfaces mostly formed by stimuli-responsive polymers.^[18] For instance, the thermo-responsive polymer poly(*N*-isopropylacrylamide) and the closely related polyacrylamides have been widely investigated for preparing biologically relevant switchable surfaces,^[19] as well as polymeric materials that can tune their properties from bacteriostatic to non-fouling and antimicrobial.^[20] However, compared to such polymer systems, SAMs will allow higher precision of distribution and faster changes in surface properties.^[21] Therefore, utilising such dynamic SAMs, the possibility exists to monitor the first interaction between a cell and a surface in real-time. Additionally, the passage between reversible and non-reversible cell adhesion can be ascertained. SAMs with stimuli-responsive characteristics have been used for the adhesion of smaller analytes such as proteins,^[15a-d] and DNA,^[22] and control specific interactions with cell surface receptors^[23] but there has been no attempt to study non-specific cell adhesion on such smart surfaces.^[24] In this context, the development of a reversible and fast electrochemical switchable surface, based on homogeneously distributed two-component SAMs represents a promising tool for studying cell-surface interactions *in vitro* and in real-time, and is a significant step forward for the fabrication of similar systems *in vivo*. The use of an applied potential for changing the surface properties will allow the study of bacterial interactions without perturbing their natural environment, and therefore their viability (as it may happen by changing the pH and temperature).

We report for the first time a reversibly electrochemical switchable SAM which is able to control the early stages of bacterial cell adhesion by switching between an attractive and a repellent state. This reversibility was achieved by controlling the exposure or concealment of a negatively charged end group (Figure 1), *via* an electrical potential applied to the surface.

The system is based upon the conformational switching of negatively charged 11-mercaptoundecanoic-acid (MUA) tethered to a gold surface in response to an applied electrical potential.^[25] In this system MUA molecules are separated from each other using a

second shorter surfactant, mercaptoethanol (MET) in order to form a homogeneous two-component dynamic SAM. To ensure an optimal spacing of the SAM, a modified literature procedure^[26] was followed. The fabrication of the SAM was achieved by using a bulky group (dendron) which can be successively removed by alkaline hydrolysis allowing the insertion of a shorter backfiller, (Figure 2). This strategy was adopted in order to avoid the phenomenon of phase-segregation in mixed SAMs^[27] that could compromise the efficiency of the switching.

The synthesis of the dendron compound (Figure S1) and SAM fabrication procedure is described in detail in the SI. The SAMs were characterised by contact angle, ellipsometry and X-ray photoelectron spectroscopy (XPS) in order to confirm the formation of a homogeneous surface. The hydrolysis of the dendron end-group was also monitored as shown in **Table 1**.

In particular, the drop in the contact angle values after the alkaline hydrolysis as well as the reduced thickness observed by ellipsometry (reduced by ~ 50%), revealed the removal of the hydrophobic fluorine dendron endgroup. The desired well-spaced MUA/MET SAM was confirmed by analysis of the XPS data. By integrating and comparing the area of the F (1s) and S (2p) peaks for the dendron SAM before hydrolysis, (dendron SAM contains 6 F and 1 S) we were able to confirm the chemisorption of the pure dendron dialkyl disulfide (compound **7** in SI) onto the gold substrate. By repeating the XPS analysis, after KOH hydrolysis and backfilling with MET, we were able to assess the dendron removal (absence of the F (1s) peak) and at the same time we were able to calculate the ratio between MUA and MET. By integrating the S (2p) peak and the C (1s) peak engaged in the carbonyl bond, a ratio of 1 MUA : 3 MET was found (XPS peaks are available in Figure S2, SI).

Prior to performing bacterial adhesion studies it was important to show that SAMs surfaces were stable. To this aim we applied a range of fixed potentials from + 0.25 V to – 0.25 V for 45 min to the SAM-modified Au surfaces. The surfaces were subsequently analysed by XPS and the results confirmed that the SAMs were stable as the Au/S ratio remained constant (Figure S3, SI). The ability of the described SAM to switch its surface properties in response to an applied potential was investigated by monitoring the real-time the adhesion of the Gram negative marine bacterium *Marinobacter hydrocarbonoclasticus* (*M. hydrocarbonoclasticus*, *Mh*). In contrast with previous literature,^[28] where electrical potentials have been used to kill and remove bacteria from surfaces, it is important that our surface technology allows the study of bacterial interactions without perturbing their viability. Thus, in order to demonstrate that we then conducted live/dead bacteria staining assays. Neither of the applied potentials adversely affected cell viability (Figure S4, SI).

In our previous study,^[7b] we employed several SAMs that possessed not only different backbones (i.e., hydrophilic and hydrophobic), but also different terminal functional groups (hydrophilic, hydrophobic, positively charged, negatively charged and neutral) to study in real-time the initial stages of bacterial adhesion to surfaces. As highlighted in the literature,^[29] and demonstrated by our work,^[7b] the bioinertness of the SAM depends on the properties of the bacterial species, with the hydrophobicity of bacteria playing a role. The hydrophobic marine bacterium *M. hydrocarbonoclasticus* exhibited the lowest adhesion on

the most hydrophobic surface, while readily and firmly attaching to both positively and negatively charged surfaces. On the basis of this different bacterial adhesion behaviour, we speculated that the preferential exposure of either positively charged (straight chains with carboxylate anions exposed at the surface) or hydrophobic moieties (bent chains with greasy alkyl chains exposed at the surface) might be used for promoting or inhibiting bacterial adhesion, respectively. To this end, the switchable SAMs were challenged with *M. hydrocarbonoclasticus* and adhesion was monitored by electrochemical surface plasmon resonance (e-SPR) (Figure 3). The bacteria were prepared as described in SI and resuspended in freshly filtered artificial sea water, (ASW, pH = 8.2).

The adhesion of the bacteria to the switchable SAMs was performed at open circuit (OC) conditions and applied positive (+ 0.25 V) and negative (− 0.25 V) potentials for a period of 45 min. As shown in Figure 3a, a high bacterial adsorption (~3000 RU) was observed at OC and − 0.25 V, *i.e.* when the negatively charged end groups were exposed. On the contrary, when a positive potential was applied to the gold electrode and the charged end-groups were therefore concealed, fewer bacteria adhered to the surface (~800 RU), Figure 3a. These findings confirmed that the conformational changes occurred at the gold surface, and that the bacteria sensed the presence or the absence of the carboxylate anionic headgroup.

The SPR chips were also analysed by confocal microscopy at the end of the experiments and enumeration of cells confirmed the results observed by e-SPR (Figure 3c). Furthermore, control samples formed by one-component monolayers of MUA (Figure 3b-d) and MET (Figure S5-S6, SI) exhibited no changes in bacterial adhesion when different potentials were applied to the gold electrodes.

The switching was found to be reversible up to three times within the first 20 min of bacterial adhesion after alternating the potential from negative (− 0.25 V) to positive (+ 0.25 V) (switching cycle) every 3 min (Figure 4a).

However, a decreased ability to switch was observed by increasing the length of exposure between the bacteria and the surface at − 0.25 V. When switching was performed every 5 min during adhesion, the loss of one switching cycle was observed, (Figure 4b), while for adhesion periods as long as 10 min only one switching cycle was accomplished (Figure 4c). Ultimately, for switching carried out every 20 min no changes in the sensorgram traces following a change of potential applied were detected, suggesting that the attachment of bacteria became irreversible (Figure 4d). Observation of the SPR traces obtained also suggested that the adhesion process was almost completely reversible during the first switching cycle after 3 min (Figure 4a, 83% RU drop) and 5 min (Figure 4b, 74% RU drop) of *Mh* adhesion (Figure S7-S8-S9, SI). On the other hand, the possibility to remove the cells attached is diminished after 10-15 min. Microscope observation of the SPR chips after the e-SPR experiment revealed that the % RU drop observed during the switching corresponded to the % of cells removed (Figure S10, SI). The best switching performance was observed for the 5 min switching assay, where the removal of cells between the first and the second cycle was reduced by only 11 % (Figure 4b). Concerning the 3 min switching (Figure 3a) a reduction in switching performance of 23% was observed between the first and the second switching cycles, while a decrease in switching performance of 35% was shown between the

second and the third switching cycles. The least removal was observed for the switch performed every 10 min with only 54% of the cells detached upon the positive applied potential (Figure 4c) during the only switching cycle performed. The overall *Mh* adhesion after 45 min was consistent through all the switching experiments performed with a final RU of $\sim 2000 \pm 200$ RU. Furthermore, no substantial drop in the sensorgram traces was observed after the ASW rinse, indicating a strong non-reversible attachment of the bacteria. Previous studies have highlighted the stepwise process of bacterial attachment^[30] and have measured increased bacterial adhesion strength with time.^[31] Furthermore, the hypothesis of an increased production of the extracellular polymeric substances (EPS), secreted by the bacterial cells during the adhesion process have been also reported.^[32] The EPS are able to form an adhesive layer on the surface and around the cells promoting the formation of the so-called biofilm. Evidence supporting this hypothesis was demonstrated by collecting EPS from bacterial suspension aged in ASW for 0 (T0), 10 (T10), 20 (T20), and 60 (T60) min and evaluating the EPS adsorption on the MUA/MET SAMs for 20 min by SPR (Figure S12). EPS T0 supernatant showed only a small SPR response (~ 40 RU), but this response was progressively greater for supernatants collected at T10, T20, and T60. These data suggest that at T0 the EPS amount is almost negligible, and is thus unlikely to influence the switching performance. On the other hand, at T20, when we observed the irreversible bacterial adhesion, the value of EPS detected was ~ 5 times higher. Therefore, we believe that increased EPS secretion with time, may be responsible for the progressive conditioning of the surface and the decrease in switching efficiency and may represent the key feature in the passage between the reversible and non-reversible adhesion of cells.

In conclusion, we have demonstrated in real-time the passage between reversible and non-reversible non-specific cell attachment using electrochemical responsive SAMs. The adhesion of the bacterium *Mh* to the SAMs was reversible up to 3 times in the first 20 min of adhesion, after which a constant increasing in bacterial adhesion was observed. The measurement was performed by e-SPR using a purpose-built electrochemical cell. Non-specific cell adhesion represents the first step towards the colonisation of a surface, therefore devices that monitor changes in real-time and are able to elucidate the progression of cell adhesion are highly desirable for a number of biomedical applications which range from diagnostic, genetic expression and biomaterials fouling control.^[29a, 33]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of an electrically switchable two-component SAM that is able to reversibly and rapidly switch its molecular conformation in response to an applied potential. The change in molecular conformation induces either bacterial adhesion (anionic head group exposed) or repellence (anionic head group concealed).

Figure 2. Schematic representation of the fabrication of the two-component switchable SAMs: Dendron SAMs lose the dendron end-group upon alkaline hydrolysis while the space between the MUA moieties is simultaneously backfilled with MET.

Figure 3. SPR sensorgram traces showing adhesion of *M. hydrocarbonoclasticus* (*Mh*) to a) MUA/MET switchable SAMs and b) MUA SAMs at three different applied electrical potentials (-0.25 V, OC and $+0.25$ V); Confocal microscope micrographs and cell count of c) MUA/MET SAMs and d) MUA SAMs SPR chips that were taken at the end of the 45 min e-SPR adhesion assay.

Figure 4.

SPR sensorgrams traces showing the binding of *Mh* to the switchable surface when opposite potential changes (from -0.25 V ; to $+0.25\text{ V}$, switching cycle) are applied every a) 3 min, b) 5 min, c) 10 min and d) 20 min. As the switching cycles became prolonged the possibility to observe the reversible bacterial adhesion is decreased with 20 min being the limit after which no switch is observed.

Table 1

Characterisation of the dendron SAM before and after hydrolysis: thickness measurement (nm); advancing and receding contact angle ($^{\circ}$) and XPS element atomic ratio (X/S).

Characterisation	Before KOH hydrolysis			After KOH hydrolysis		
Thickness (nm)	1.35 $\pm$ 0.23			0.62 $\pm$ 0.15		
Contact angle(θ°)	<i>Advancing</i>		<i>Receding</i>	<i>Advancing</i>		<i>Receding</i>
	91 $^{\circ}\pm$ 3 $^{\circ}$		71 $^{\circ}\pm$ 2 $^{\circ}$	35 $^{\circ}\pm$ 2 $^{\circ}$		18 $^{\circ}\pm$ 2 $^{\circ}$
XPS atomic ratio	Element	Found	Expected	Element	Found	Expected
	F/S	6.2	6	F/S	0	0
	C/S(C=O)*	1.3	1	C/S(C=O)*	0.3	0.3