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Stereochemical effects of chiral monolayers on enhancing the resistance to mammalian cell adhesion†

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This work describes the different durations of surface confinement of adhered mammalian cells by monolayers comprised of enantiomers of bio-inert polyol-terminated alkanethiols. Enhanced resistance to protein adsorption and cell adhesion is obtained on monolayers formed by a racemic mixture of the enantiomeric alkanethiols.

Biofoulings, due to protein adsorption,[1] mammalian cell adhesion[2] and biofilm formation,[3] occurs ubiquitously on a wide variety of surfaces. Developing effective anti-fouling surface chemistries requires the understanding of fundamental science that governs the interaction of surfaces with proteins, mammalian cells and microbes. Self-assembled monolayers (SAMs) on surfaces,[4–6,7] particularly on gold films, have provided a chemically and structurally well-defined platform for studying both protein adsorption and mammalian cell adhesion. In this work, we show that the resistance of mammalian cell (3T3 fibroblasts) adhesion is different on monolayers comprised of chiral polyol-terminated alkanethiols on gold films, and that the anti-biofouling chemistry can be enhanced by using monolayers formed by a racemic mixture of the enantiomeric alkanethiols.

Antifouling chemistry has been long sought after beca use of the intriguing fundamental science and applications that impacts multiple industries. Among different bioinert surfaces, sugar and sugar derivatized SAMs[5,8–10] have been shown to be highly effective at resisting all aspects of biofouling (protein adsorption, mammalian cell adhesion and biofilm formation). However, the rich stereochemistry of sugar derivatives and the mechanism of their antifouling chemistry remains unexplored. Here, we synthesized two pairs of enantiomers[11] of alkanethiols, D-gulitol (**1**) and L-gulitol (**1′**), and D-mannonamide (**2**) and L-mannonamide (**2′**), and two diastereomeric alkanethiols, D-gulonamide (**5**), D-gluconamide (**6**) and examined their effectiveness at resisting mammalian cell adhesion, and at confining cells adhered in micrometre-scale patterns.

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We created patterned monolayers by microcontact printing, in which circular regions of pentadecanethiol (135 μm in diameter) are surrounded by SAMs formed by **1**, **1′** or SAMs formed by racemic mixture of the enantiomers (Fig. 1). The patterned SAMs were cultured with Swiss albino 3T3 fibroblasts, and monitored using optical microscopy. Cell adhesion confined to circular patterns was observed for SAMs presenting the enantiomeric alkanethiols, **1** and **1′**, and the racemic mixture of the enantiomers. Surprisingly, the D-gulitol-terminated monolayers were more resistant to cell adhesion than the L-gulitol-terminated monolayers. While D-gulitol-terminated monolayers confined cell adhesion in circular patterns up to 19 days, L-gulitol-terminated monolayers failed at 13 days. This observation demonstrated that mammalian cells have a higher propensity to overcome the bioinertness of a surface formed by one enantiomer over the other. Interestingly, SAMs formed by the racemic mixture of the enantiomers, **1** and **1′**, confined cell adhesion up to 23 days, longer than SAMs formed by either of the enantiomers (Fig. 2). This result suggests an enhanced anti-fouling surface chemistry by using a racemic mixture of chiral molecules that can form bioinert monolayers.

Mammalian cell adhesion on surface is mediated by selective molecular binding including cell surface receptor integrin binding to a small tripeptide Arg-Gly-Asp (RGD) on extra cellular matrix (ECM) proteins fibronectin or

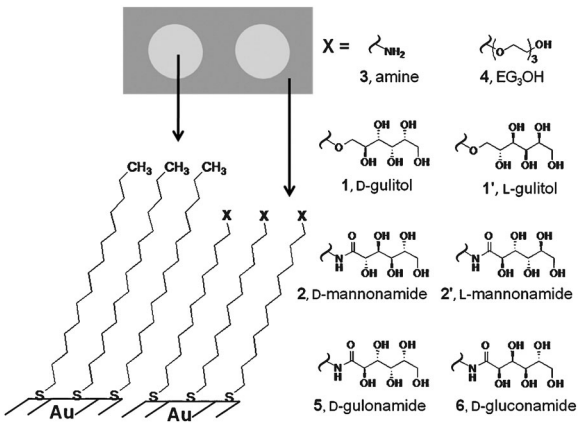

Fig. 1 Schematic representation of patterned SAMs of $HS(CH_2)_{14}CH_3$, surrounded by the chiral polyol- or amine- or EG_3OH-terminated SAMs on gold films.

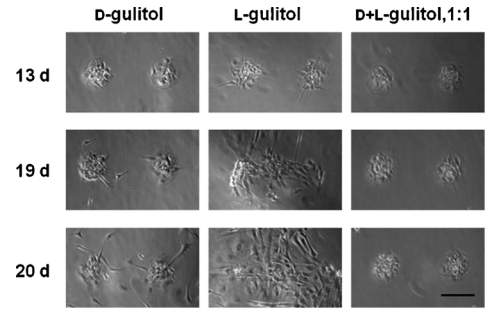

Fig. 2 Optical micrographs for the adhesion of Swiss albino 3T3 fibroblast on circular patterns (135 μm in diameter) of $HS(CH_2)_{14}CH_3$, surrounded by alditol-terminated SAMs on gold films. The number of days the substrates were in the culture is indicated to the left. Scale bar = 152 μm.

vitronectin,[12] which is the key event for initiating vital cell activities.[13] Interestingly, Addadi and co-workers have shown that adhesion of epithelial cells (A6 *Xenopus laevis*) on (S,S)-tartrate tetrahydrate crystal was slow and mediated by RGD containing adhesive proteins, but attachment and spreading on the (R,R) crystal was fast, RGD-independent and led to cell death.[14] Recently, macrophages were reported to have reduced adhesion with round morphology on a monolayer of *N*-isobutyryl-D-cysteine on gold surface, but with a larger number of cells and well-spread morphology on a monolayer of *N*-isobutyryl-L-cysteine.[7] In contrast to these findings, the different ability of enantiomeric monolayers to resist cell adhesion suggests that cells can distinguish chirality of the surface, even when the surface chemistry is resistant to cell adhesion.

We also examined diastereomeric polyol-terminated monolayers, for which the polyols are linked by amide bonds instead of ether bonds. We observed that mannonamide-terminated monolayers, **2** and **2′** failed to resist cell adhesion, but D-gulonamide, **5**, and D-gluconamide, **6**, confined cell adhesion up to 2 days and 5 days, respectively (Fig. 3). Interestingly, while monolayers presenting either D-mannonamide (**2**) or L-mannonamide (**2′**) were not adhesion-resistant, monolayers formed by a racemic mixture of the enantiomers (50/50 : **2**/**2′**) were adhesion-resistant and confined adhered cell up to 10 days (Fig. 3 and supporting information†).

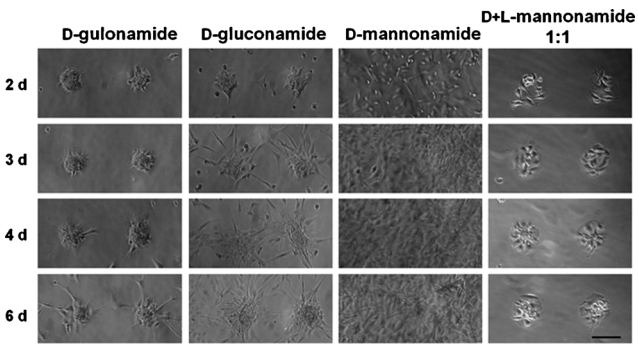

Fig. 3 Optical micrographs for the adhesion of Swiss albino 3T3 fibroblast on circular patterns (135 μm in diameter) of $HS(CH_2)_{14}CH_3$, surrounded by aldonamide-terminated SAMs on gold films. The number of days the substrates were in the culture is indicated to the left. Scale bar = 152 μm.

These results enforce the observed racemic enhancement effect on bioinertness.

Because protein adsorption is intimately accompanied with, and sometimes a prerequisite for,[4,13] cell adhesion, we used surface plasmon resonance (SPR)[4] to examine protein adsorption, and the resistance of which, by SAMs presenting D-mannonamide, L-mannonamide and racemic mixture of the enantiomers, and compared them to SAMs presenting methyl groups. Fig. 4 shows the data for adsorption of bovine serum albumin (BSA) on the four SAMs formed on a SPR array chip (16 spot per chip). Buffer (1 × PBS, pH 7.42) was first introduced to the SAMs, followed by PBS containing BSA (0.5 mg mL^{-1}). Pure PBS was introduced again to observe the irreversibly adsorbed protein on SAMs. The percentage of the monolayer covered by the adsorbed proteins (%ML) was compared to the amount of protein adsorbed on the methyl-terminated SAMs, and was estimated by $\%ML = (\Delta PIU_{mannonamide}/\Delta PIU_{methyl}) \times 100$, for which ΔPIU is the difference in the pixel intensity units before and after the surfaces were exposed to the protein solution.[8,15]

We observed that SAMs presenting D-mannonamide and L-mannonamide were both protein-resistant, supporting about 21.18 ± 7.13% and 6.95 ± 0.71% of monolayer (%ML), respectively. Interestingly, SAMs presenting the racemic mixture of the two enantiomers exhibited little protein adsorption (%ML = 1.11 ± 0.42) that was within the noise of the SPR signals. This result suggests that SAMs presenting racemic mixture of enantiomeric alkanethiols is likely more resistant to protein adsorption than SAMs presenting either of the enantiomers.

The mechanism of an antifouling chemistry is not trivial; hydrophilic surfaces are not necessarily beneficial for achieving bioinertness. Proteins are known to adsorb on surfaces presenting hydrophilic amine groups.[16] An interesting theoretical study by Sharp and co-workers suggests that similar water organization is induced by methyl and amino moieties.[17] To examine the effect of potentially protein-denaturing, yet hydrophilic surface on mammalian cell adhesion, we prepared patterned squares (600 μm-wide) of $HS(CH_2)_{14}CH_3$ surrounded by SAMs presenting amine group,[3,18] and tri(ethylene glycol) (EG_3OH).[4,19] At the early stage, cell adhesion was onlycell adhesion was only seen on

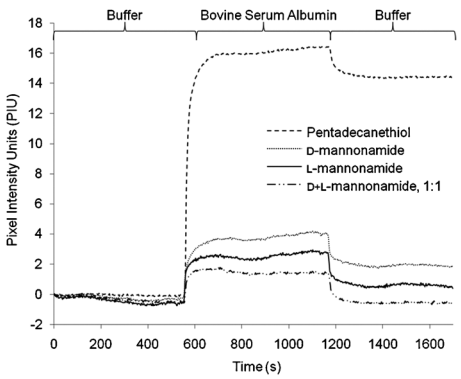

Fig. 4 Intensity of surface plasmon resonance (SPR) for adsorption of bovine serum albumin (0.5 mg mL^{-1} in 1 × PBS, pH 7.42) over time on four SAMs presenting methyl groups, D-mannonamide; L-mannonamide; or a racemic mixture of D & L-mannonamide alkanethiols.

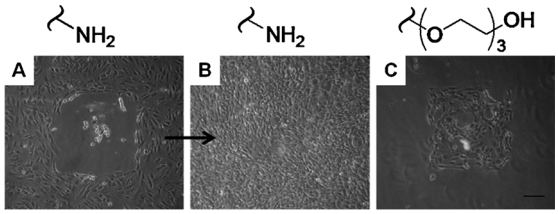

Fig. 5 Optical micrographs for the adhesion of Swiss albino 3T3 fibroblast on 600 μm-wide squares of pentadecanethiols, surrounded by (A) amine-terminated monolayer, **3**, for 1 day, (B) for 3 days, and (C) EG_3OH-terminated monolayer, **4**, for 1 day. Scale bar = 152 μm.

amine-terminated SAMs, and little cells were seen inside the methyl-terminated squares, giving a "reversed" pattern (Fig. 5A). Over a short period of 3 days, a confluent monolayer of cells was observed, covering the entire monolayer (Fig. 5B). In contrast, EG_3OH-terminated monolayer resisted cell adhesion from the beginning of cell culture (Fig. 5C). These results support the notion that amine groups are likely chaotropic in nature, and supported rapid cell adhesion.

Several theories exist for bioinert chemistries that resist protein adsorption.[8,9,15,20,21,28] To understand the bioinert surface chemistry, we note that polyols are similar in structure to glycerol and sucrose that are known to stabilize protein structure.[22] Other small molecules such as urea and guanidinium are known to denature proteins.[23–25] While the stabilizing or destabilizing effects of added ions or molecules on protein folding may be due to direct[25,26] or indirect[24,25] interactions between the additives and the proteins, the stabilizing effect by nonionic organic osmolytes on protein structure is likely indirect.[25] Together, these results are consistent with the notion that organic molecules that stabilize protein structure can also resist biofouling when immobilized on a surface.[9,27] One plausible mechanism for such antifouling chemistry is an extensively hydrogen bonded water network on the monolayer, which prevents biological entities from contacting or attaching to the surface.[9,27,28]

In summary, SAMs presenting chiral polyols that have the same chemical composition have different abilities to resist mammalian cell adhesion. While cells can distinguish chiral surfaces that support adhesion,[7,14] our results show that cells can also distinguish chirality of the surface when the surface chemistry is to resist cell adhesion. We observed enhanced bioinertness on monolayer formed by racemic mixtures of *both* gulitol- and mannonamide-terminated alkanethiols, suggesting that this racemic effect on bioinertness is likely general for other bioinert chiral monolayers. These results suggest an approach for potentially enhancing antifouling chemistry on materials beyond gold films. We believe that this bioinert chemistry is influenced strongly by the water solvation and organization at the interface.[20,29]

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