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Nano-scale Superhydrophobicity: Suppression of Protein Adsorption and Promotion of Flow-Induced Detachment

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Abstract

Wall adsorption is a common problem in microfluidic devices, particularly when proteins are used. Here we show how superhydrophobic surfaces can be used to reduce protein adsorption and to promote desorption. Hydrophobic surfaces, both smooth and having high surface roughness of varying length scales (to generate superhydrophobicity), were incubated in protein solution. The samples were then exposed to flow shear in a device designed to simulate a microfluidic environment. Results show that a similar amount of protein adsorbed onto smooth and nanometer-scale rough surfaces, although a greater amount was found to adsorb onto superhydrophobic surfaces with micrometer scale roughness. Exposure to flow shear removed a considerably larger proportion of adsorbed protein from the superhydrophobic surfaces than from the smooth ones, with almost all of the protein being removed from some nanoscale surfaces. This type of surface may therefore be useful in environments, such as microfluidics, where protein sticking is a problem and fluid flow is present. Possible mechanisms that explain the behaviour are discussed, including decreased contact between protein and surface and greater shear stress due to interfacial slip between the superhydrophobic surface and the liquid.

Keywords: Superhydrophobic; Protein Adsorption; Slip; Anti-fouling, Topography

Introduction

Superhydrophobicity defines a combination of roughness and hydrophobic chemistry that render a surface extremely water repellent, in some cases causing water drops to roll off removing particulate contamination[1]. Superhydrophobic surfaces have been explored for various applications including self-cleaning and anti-mist/fog surfaces, power-on-demand batteries and electrostatically controllable liquid optics. The basic correlations between surface roughness and water repellency were originally defined by Wenzel[2] and Cassie and Baxter[3]. In the simplest case, the Wenzel state, liquid conforms to the roughness; increasing its interfacial contact area. In contrast, the Cassie-Baxter bridging state involves the liquid sitting on top of the roughness with gas bubbles in the hollows, giving potentially a lower solid-liquid contact area, (Figure 1a). Surfaces in the bridging state presenting low interfacial areas allow water to slide or roll off very easily and are therefore of the most interest. A large number of techniques have been developed to produce different superhydrophobic surfaces for study[4], a small number have been developed into products.[5,6]

Protein adsorption is the first stage in biological contamination of surfaces, with cells binding to a pre-adsorbed protein layer before proliferating and spreading. Surfaces that hinder or obstruct this early adsorption process would reduce cell growth. Anti-fouling surfaces that show low protein adsorption are important in many areas, especially for surfaces that cannot be cleaned for extended periods such as boat hulls and some biomedical devices. Biofouling of boat hulls and some pipes considerably increases energy consumption, making reduction of the effect important. Some medical devices also benefit from antifouling coatings, as pathogens can attach to the adsorbed protein. Another area where protein adsorption is problematic is in enzyme catalysed reactions, where enzyme adsorption reduces the rate of reaction. This is particularly evident as the scale of a reaction environment is reduced and the surface area-to-volume ratio increases, with microfluidic devices often experiencing serious problems.

Reducing protein adhesion has been approached in several ways in the past, including chemically coating the surfaces with hydrophilic groups such as poly(ethylene glycol)[7,8], filling the surface sites with other molecules[9] and attaching proteolytic enzymes to surfaces[10]. Surfaces that employ flow shear removal are used in a small number of applications, typically fast boats as the shear rate required is high and the material used can easily become damaged.[11]

Adhesion of proteins is usually rapid on flat hydrophobic surfaces and proteins that bind to hydrophobic surfaces are probably altered by the interaction[12]. Highly hydrophilic surfaces are usually used to reduce protein fouling.[7,8] It has, however, been suggested that superhydrophobic surfaces could reduce the extent of protein adsorption due to the reduction in solid surface area at the liquid interface (Cassie-Baxter bridging case only)[13,14,15]. A recent publication highlights the possibilities of superhydrophobic coatings but also shows how little work has been undertaken in this area.[16] Proteins dissolved in water do adhere to superhydrophobic surfaces[17], although often less rapidly than on flat surfaces[17,18]. The reduction in rate may be due to a requirement for conformational changes prior to adsorption or the hydrophilicity of an adsorbed protein layer driving the solvent front into the surface structure allowing water and protein to penetrate.[13,19] This suggests that it will not be possible to prevent protein adhesion entirely, but it may be possible to reduce the binding strength and therefore allow easy removal by flow shear or other methods.

The current study demonstrates the effect of superhydrophobic surface roughness dimensions and surface chemistry on static protein adsorption and efficacy of protein removal under flow. The hypothesis is that proteins will probably adhere to superhydrophobic surfaces, but several additional factors may contribute to their effective removal under flow, particularly if micro-metre scale roughness is replaced with nano-metre scale roughness. Interfacial slip between the liquid and solid would cause an increase in liquid flow rate near the surface[20,21,22]; adsorbed molecules would then experience greater shear forces and are therefore more likely to be swept away. On nano-scale roughness the curvature of the surface approaches protein molecular dimensions, reducing the contact area unless the protein molecules deform.[23,24] The smallest scale roughness used here is similar to the dimensions of the protein used so this effect may play a role. It is important to note that, although the materials are porous, only the surface of the material is exposed to solution as water cannot enter the hydrophobic pores at the pressures used. Nano-scale superhydrophobic surfaces remain in the Cassie-Baxter bridging state under much higher hydrostatic pressure compared to micro-structured surfaces so would be of more use in a real microfluidic system where back pressure can be high.

Reduction of protein adsorption due to flow shear has previously been reported on nano-wires[25] and polymer brushes[23].

Experimental

Four types of roughness were used, untreated glass slides (smooth), two sol-gel materials[26,27] with typical roughness dimensions of (~800 nm particle size, ~4 µm pore size) and (~4 µm particle, ~20 µm pore) and copper oxide filaments with flat ends that are around 60 nm wide and 10 nm thick (Fig. 1d). More detailed micrographs of these materials are available elsewhere.[28] Copper coated slides were used to check that the underlying metal had no effect on protein attachment.

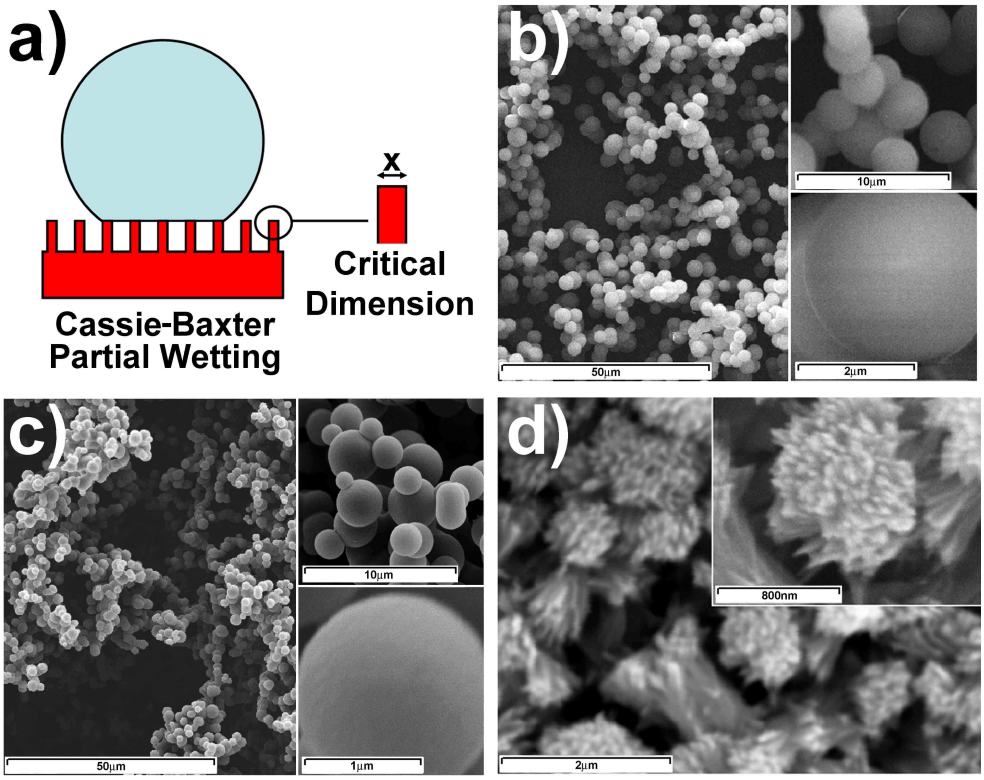

Fig. 1 (a) Diagram showing Cassie-Baxter superhydrophobicity and the critical dimension used to define surfaces here. Electron micrographs of (b) the larger scale sol-gel material, (c) the smaller scale sol-gel and (d) the copper oxide nano-pillars.

All surfaces were subsequently chemically modified to afford hydrocarbon or fluorocarbon surface chemistry; giving water contact angles of 169° for micron and 152° for nano-rough fluorinated surfaces. Fluorocarbon terminated superhydrophobic surfaces are more resistant than hydrocarbon ones to ingress of liquid into the structure under the action of surfactants (such as proteins).

Standard microscope slides (Sail Brand, China) were used for most of the samples; these were sonicated in ethanol (Haymans, absolute, UK) before use. Five types of samples were prepared and used immediately after surface modification:

1. Untreated glass slides
2. Slides sputter coated with 200 nm of copper (Goodfellow, 99.95 %, UK) on 5 nm of titanium.
3. Large grained silica sol-gel on slides[29]
4. Small grained silica sol-gel on slides
5. Copper oxide nanoneedles on copper sheet (Goodfellow, 99.95 %, UK).

The surfaces were coated using one of the following treatments:

1. To deposit a hydrocarbon terminated layer samples were placed in a glass slide holder and immersed in 2 % vol. octyltriethoxysilane (ABCR, 95 %, DE) in toluene (Fisher, low sulphur, UK) for 24 h, rinsed in toluene and air dried before use.
2. To deposit a fluorocarbon terminated layer samples were placed in 'Grangers Wash-in' solution (Grangers, UK) diluted 1:9 with distilled water for 10 min., rinsed thoroughly with distilled water and blown dry before being heated to 100 °C for 1 h in a vacuum oven.

Sol-gel preparation

Sol-gel films were prepared as previously reported[27] by mixing methyltriethoxysilane (Alfa Aesar, 98 %, UK), aqueous HCl (diluted to 0.37 % from Fisher 37 %, UK) and a solvent (2:3:2). The mixture was stirred for 1 hr and then ammonia solution (Fisher, 35 % diluted with

deionised water) was added (1:4). The large grained sol-gel was prepared using ethylene glycol (Fisher, 99+ %) as the solvent and 0.9 M ammonia solution; the small grained material used dimethylformamide (Acros, 99 %, UK) as solvent and 3.6 M ammonia solution. A 0.4 ml aliquot of the gel solution was then cast between two glass slides using cover slips as spacers. The top slide was hydrophobised with Grangers Wash-in solution to allow its removal after around 6 h without damaging the hardened sol-gel film. The larger grained material was scraped to remove a top layer of smaller particles. Films were heated at 2 °C min^{-1} to 500 °C for 1 h in a furnace and allowed to cool slowly before surface treatment, as above.

Copper oxide nanoneedle preparation

Copper foil was cut to the size of standard microscope slides (76 × 25 mm) and sonicated in ethanol. The samples were then placed into 36 mM ammonia solution in a slide holder and placed in a refrigerator at 4°C until they became uniformly black. The time required for this varied with the number of samples and the volume of the solution (2-8 days). The samples were then heated in a furnace at 1 °C min^{-1} to 180 °C in air and held for 3 h. After cooling they were chemically treated as above.

Protein adsorption and removal

Bovine serum albumin (BSA) (Fluka, 98 %, HPCE) was chosen as a model protein since it is known to adhere well to surfaces. Moreover, it is important in various biological applications such as PCR, found in high abundance in serum and is commonly used as a surface blocking agent due to its binding characteristics. This protein is of the order of 15 nm in size, but is known to deform when strongly adhered.[24]

The experiment was performed by incubating the substrates in 3 mg ml^{-1} BSA in 200 mM phosphate buffer at pH 7.4 for 1 h. A portion of the samples were then placed in a flow cell with a 1500 × 650 µm cross section, 65 mm long. Buffer solution was flowed over at 20 ml min^{-1} for 30 min. The flow rate was chosen to generate flow shear similar to that which may be encountered in microfluidic devices; equivalent to a flow rate of 3 µL min^{-1} in a channel of 500 × 50 µm, such as that used by Kim *et. al*.[30] Protein measurements were made over the centre section where the flow pattern was expected to be constant.

Measuring protein on surfaces

A fluorimetric assay was used to quantify protein remaining adsorbed and that adsorbed on the slides not exposed to flow. This technique has previously been demonstrated to quantify small amounts of protein on surfaces; considerably less than a monolayer on a surface of a few cm^2 and is fully described in another publication.[13] Briefly, the surfaces were rinsed in three sequential wash cycles of ethanol (Haymans, absolute) and distilled water to detach adsorbed protein. All washings were carefully collected and reduced to dryness by vacuum centrifugation before being re-dissolved in a known volume of fluorescent probe solution. The molecular probe used becomes highly fluorescent when bound to protein allowing quantification of very small amounts of protein. Reference samples of each type were used to as background standards, giving a zero reading for the fluorimetric assay.

Results and discussion

Flat copper reference samples showed identical BSA adhesion properties, in both static adsorption and after flow, to flat glass samples with the same coatings. This indicates that the chemical coatings and not the underlying copper influenced the adsorption process.

None of the superhydrophobic surfaces used were penetrated by the protein solution or the flowing buffer, so they behaved as rough surfaces not as porous substrates. The ethanol used to wash the samples penetrated into the structure of some of them; this transition could be observed by a change in colour of the samples and the amount of protein adsorbed would be expected to increase massively if penetration occurred, due to the extremely high internal surface areas of the materials.

Under static conditions similar amounts of albumin were observed to adsorb to flat glass and the nano-structured copper oxide surfaces with both sol-gel superhydrophobic surfaces showing much higher adsorption (Fig. 2). The small sized sol-gel surface (~800 nm particle size, ~4 µm pore size) had a lower degree of protein adsorption compared to the larger sized (~4 µm particle, ~20 µm pore) material. This indicates that the pressure in the system combined with the surfactant nature of the protein used is sufficient to wet more of the larger pored material, allowing a larger available surface area for adsorption[13], although full penetration into the pores still does not occur.

Greater adsorption was observed on fluorinated flat glass and copper oxide needle surfaces compared to the corresponding methylated surfaces. This may be due to the greater hydrophobicity of these surfaces[31], increasing the binding strength of hydrophobic interactions between BSA and the surface. It is also possible that the fluorocarbon waterproofing agent generates some small scale roughness and thus increases the area available for adsorption, although results using silane coupling agents were similar to these (data not shown).

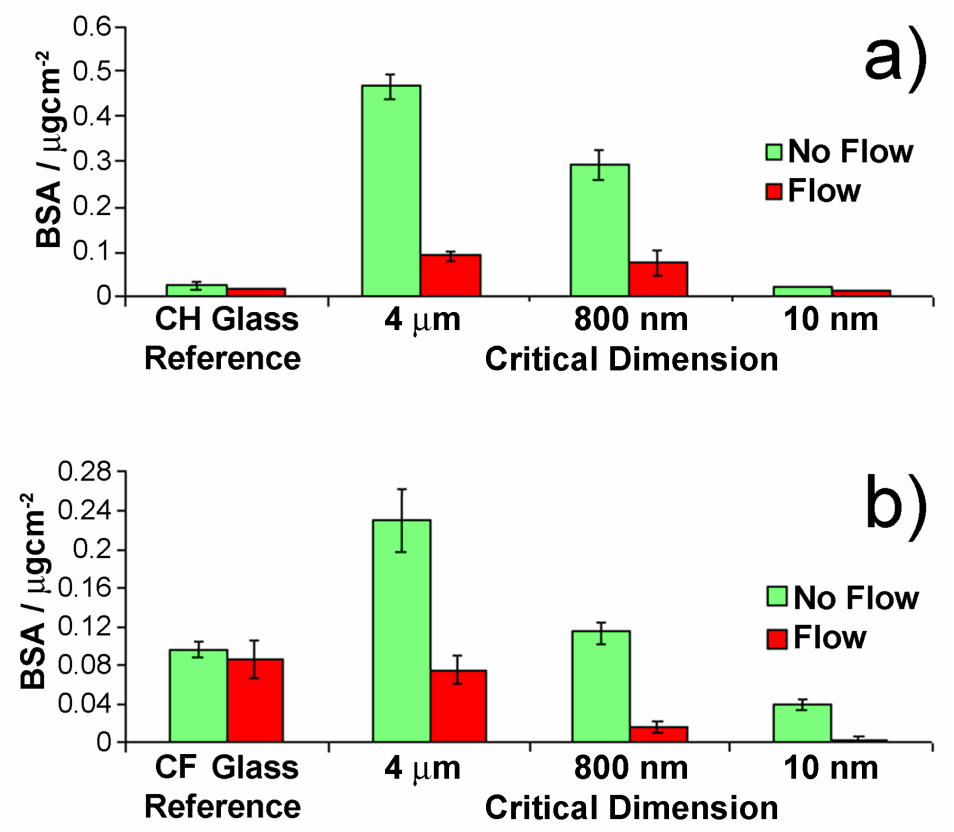

Fig. 2 Albumin adsorption onto micro-scale and nano-scale surfaces: (a) hydrocarbon terminated and (b) fluorocarbon terminated under static conditions and after subsequent flow of buffer.

The structured fluorinated surfaces showed considerably lower protein adsorption than the equivalent hydrocarbon terminated surfaces. On fluorocarbon surfaces the increased hydrophobicity will result in a lower interfacial surface area available for protein adsorption. The nano-structured surfaces with both coatings were the most resistant to protein adhesion

under static conditions. It has previously been reported that proteins and peptides are affected by nano-structures of similar size to those used here[24,32,33].

A proportion of the adsorbed protein was removed from all surfaces under flow conditions. Considerably greater amounts of protein, however, were lost from the superhydrophobic sol-gel surfaces than from flat surfaces, with the amount remaining being lower on successively smaller structured surfaces (Fig. 2). This suggests that micro-structures, despite being very large compared to the protein molecules, have a strong effect on protein retention under flow. Interfacial slip, if present, would create high shear-fields around the edges of contact areas, which would induce protein desorption. Our results demonstrate that on fluorocarbon terminated surfaces a higher degree of desorption was found on smaller structured surfaces, where higher shear fields would be expected (Fig. 2b). This trend was also generally observed on the hydrocarbon surfaces, although a relatively large proportion of the protein was lost from the larger scale sol-gel surfaces. The large loss of adsorbed material from the rough surfaces supports our view that the pores were not penetrated by the protein solution as internal protein would not be removed very easily by flow.

The nano-structured copper oxide surfaces showed similar adsorption to flat surfaces under static conditions, but also showed greater losses after exposure to flow. The fluorinated surfaces show slightly lower levels of adsorption than flat surfaces under static conditions, but after flow were clear of protein within the detection limits of the measurement ~3 ng cm^{-2}. This continues the trends observed on the sol-gel surfaces.

The amount of protein adsorbed onto superhydrophobic surfaces in the absence of flow was similar to or greater than that onto flat reference samples, except for fluorocarbon terminated nano-structured surfaces with critical dimension of *ca*. 10 nm. However, when buffer was flowed over the sample surfaces, more protein was removed from the superhydrophobic surfaces than flat ones. Fluorinated nano-structured surfaces became almost completely *clear* of protein where equivalent flat surfaces only lost around 10-20 % of their protein. It is not clear from these measurements if the enhanced effect at nano-structured surfaces is due to reduced distance of any point from an area of slipping fluid or to reduced contact area between protein

molecule and surface due to the small size of the tips of the nano-pillars and their roughness. Reduced binding strength has previously been reported for BSA on high curvature surfaces[24].

Conclusions

Although adsorption of BSA from solution was not reduced by using superhydrophobic surfaces under static conditions the ease by which it could be removed was, particularly on nanostructured surfaces with a fluorocarbon coating, where complete removal was observed within measurement error.

The almost complete removal of protein films from some superhydrophobic surfaces under flow conditions shown here is likely to be of significant interest in applications where flow is already present, such as in micro- and nano-fluidics. The flow shear experienced in such devices is often similar to that used here so similar results may be achieved, although the effect of the higher pressure in such systems was not tested here. Different proteins may prove to be less affected; the size, shape and alignment of protein molecules on the structures will affect how much force the liquid can exert even when it is slipping.

The effect demonstrated here is of particular use in micro-/nano-fluidics, where the surface area to volume ratio favours reagent/product loss from solution. In addition to this, surfaces that hinder or prevent protein adsorption are also sought after for use in many industries including biomedical, optical, electronics and engineering, where devices are prone to contamination.

Here we have shown how nano-scale superhydrophobic surfaces can be used, firstly to obstruct adsorption taking place in the absence of fluid flow, but mainly to reduce the amount of adsorbed protein under flow conditions by increasing the desorption rate. We have also shown that larger scale superhydrophobic surfaces can have the opposite effect, causing increased adsorption; which goes some way to explaining the mixed results achieved by other studies. The effect of varying feature size and chemistry was consistent with the hypothesis that flow slip over the superhydrophobic surfaces causes the difference in removal under flow.

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