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Self-assembly of a tripeptide into a functional coating that resist fouling

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5 **This paper describes the self-assembly of a tripeptide into a functional coating that resist biofouling. Using this peptide-based coating we were able to prevent protein adsorption and interrupt biofilm formation. This coating can be applied on numerous substrates and therefore can serve in applications 10 related to health care, marine and water treatment.**

Biofouling is an undesirable process that results in the accumulation of organisms and their by-products on surfaces. The process initiates with the adsorption of bioorganic molecules onto a substrate, and proceeds with the attachment of organisms 15 to this bioorganic layer.[1] In the case of bacteria, this process leads to the formation of a well-defined bacterial network, termed biofilm, which provides the bacteria with superior survival properties under exposure to antibiotics.[2] Biofilm formation on medical devices and implants may lead to severe infection.[3] This 20 phenomenon termed hospital-acquired infection is of major concern today for the health care system. In addition, biofouling has an enormous impact on the marine industry. This is due to the attachment of marine organisms such as barnacles and marine mussels to ships and other marine devices.[4] The thick heavy 25 layer formed by these organisms causes delay in transportation, and additional consumption of fuel.[5] In addition, the colonization of ship hulls has been linked to two major environmental pollutions, which are emission of gases into the atmosphere and invasive species to marine habitats.[6]

30 Antifouling materials alter the physical and/or chemical properties of the surface in order to prevent the accumulation of the organisms on the substrate.[1-4,7] The need for nontoxic antifouling materials led to the development of numerous approaches. These strategies include enzymatic degradation, 35 sonication and chemical modification of the substrates.[1-4, 7] Each approach has some disadvantages. These include low stability, short-term activity, limitation to specific surfaces and complicated and expensive synthesis or fabrication procedures.[8]

This paper presents a new rationally designed antifouling 40 material formed by the self-assembly of a low-molecular weight peptide into a supramolecular coating. The peptide is a synthetic tripeptide that interferes with the first step of biofouling. We chose a peptide backbone for the design of this material since peptides are diverse, biocompatible, stable, and can 45 spontaneously form ordered structures by self-assembly.[9] There are numerous reports in the literature on antibacterial peptides, however very few on peptides with antifouling activity. This includes the ultra-low fouling natural peptides composed of negatively and positively charged residues in the form of either 50 alternating or randomly mixed charge.[10] These peptides,

however, are longer than the peptide proposed herein and require an alkanethiol for adhesion to gold.

The peptide sequence contains three elements that enable i) its self-assembly into a coating, ii) its adsorption onto any substrate 55 and iii) its antifouling activity (Fig. 1).
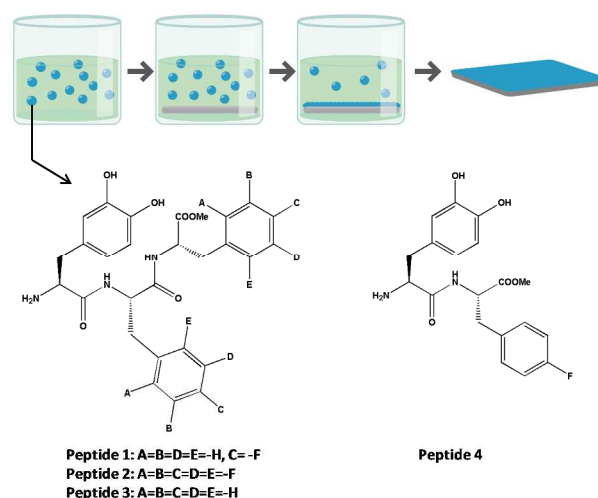

Fig. 1. The illustration at the upper panel schemes the formation of a coating on a substrate by dip coating. The lower panel illustrates the molecular structures of the studied peptides.

60 The element that we chose to direct the assembly of the peptide comprises two adjacent fluorinated phenylalanine residues. Due to aromatic interactions, the dipeptide diphenyl-alanine and its fluorinated analogues can self-assemble into highly ordered structures such as fibers and tubes.[9,11] We 65 assumed that this motif will promote molecular recognition and direct the self-assembly of the peptide into a film. In addition, we expected that the carbon-fluorine bond of the fluorinated aromatic ring would lead to the formation of a "Teflon-like" material that will prevent the attachment of proteins to the surface and 70 therefore will act as the antifouling motif. We chose to explore two variations of the peptide: one contains only one fluorine atom on each of the benzene rings (Peptide **1**) and the other contains five (Peptide **2**). The third amino acid of the peptide is 3,4-dihydroxy-L-phenylalanine (DOPA), the main constitute of 75 mussel adhesive proteins (MAPs), the glue proteins of marine mussels.[12] These proteins can adhere to almost any substrate and survive harsh conditions such as tide and high salt concentration.[12] DOPA itself can adhere to various surfaces.[13] We assumed that the insertion of the amino acid DOPA into the

peptide sequence would allow its function as glue and immobilize the peptide on different substrates.

We compared the physical properties and antifouling activity of peptide **1** and **2** to two additional peptides: Peptide **3**, which does
5 not contain any fluorine atoms and a dipeptide (peptide **4**) that comprise the amino acid DOPA and one fluorinated phenyl-alanine residue (Fig 1.). To coat a substrate (e.g. gold, silicon, titanium, glass or stainless steel) with the peptide, we dipped bare substrates in the peptide solution. To determine if peptide **1**
10 indeed generated a "Teflon-like" layer on the different substrates we measured their contact angle. As we assumed, the modified surfaces exhibited an increase in the contact angle indicating an increase in the substrate hydrophobicity (Fig. 2c-j**)**. Similarly, peptides **2-4** followed the same trend (Fig. S1, ESI†): peptide **2**
15 which contains more fluorine atoms increased the contact angle of titanium to 83°. Due to its hydrophobic nature peptide **3** increased the contact angle of the substrate, however this increase was lower than the increase resulted from peptide **1**. The dipeptide, peptide **4** increased the contact angle similarly to
20 peptide 1 to 67°. We also found a correlation between the angle size and the concentration of the peptide solution, as the peptide concentration increased, the contact angle was larger (Fig. S2, ESI†). Due to the hydrophobic moieties of the peptides, water could not be used as a solvent system despite its high polarity.
25 We used methanol as the solvent since it dissolved the peptide completely and at the same time allowed it to adhere the substrate. Since methanol is a toxic solvent, we also examined other solvents with different polarities.
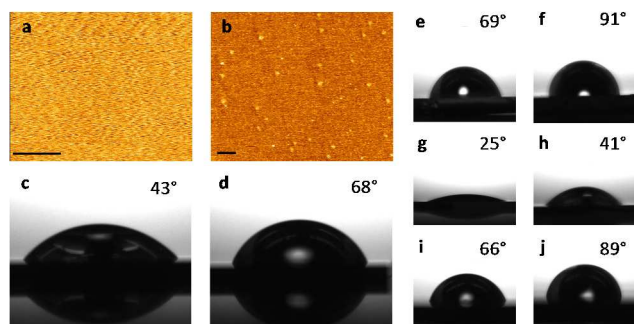

Fig. 2. Surface characterization of bare substrates *versus* substrates coated
30 with peptide **1**. AFM topography images of (a) bare mica, (b) coated mica**,** the scale bars represent 500 nm. Contact angle measurements of (c) bare titanium, (d) coated titanium (e) bare gold (f) coated gold, (g) bare silicon, (h) coated silicon, (i) bare stainless steel , (j) coated stainless steel .

35 When we used solvents, such as acetone, ethanol and isopropanol, with polarities that resemble the polarity of methanol, the peptide-based coating self-assembled in a similar manner as it did in the methanol solvent system (Fig. S3 and S4, ESI†). However, in solvents with high polarity, such as
40 dimethylsulfoxide (DMSO) or 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) the peptide dissolved but did not adhere to the substrate (Fig.S3, ESI†).

To characterize the topography of the modified surfaces we performed atomic force microscopy (AFM) analysis to mica
45 surfaces coated with the different peptides. AFM analysis clearly showed that the topography of the mica coated with peptide **1** was different from the topography of the bare mica. In addition, some spherical aggregates appeared on the coated substrate (Fig. 2). We detected similar topography for surfaces modified with

50 peptides **2-4** (Fig. S5, ESI†). However, peptides **3** and **4** formed more aggregates on the surface than peptides **1** and **2**.

Using attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy, we studied if the peptides are indeed present on the substrate and what their configuration on the
55 substrate is.[14] The bare titanium substrate has a typical peak at 880-870 cm^{-1} (Fig. 3a). This peak did not appear when the substrate was coated with peptide **1** and peptide **2** indicated the formation of a coating on the substrate. Another informative IR frequency range is 3500–3200 cm^{-1} since a narrow peak at this
60 frequency corresponds to the N–H stretching vibrations of the peptide. In addition, a broad peak at this range results from the vibration signal of the native oxide of the titanium.[15] In our experiments, a narrow peak occurred at 3330 cm^{-1} when peptide **1** coated the titanium surface. This IR peak suggests the binding of
65 the peptide to the substrate (Fig. 3a). Similarly, a narrow N-H stretching band occurred between 3305cm^{-1} to 3322cm^{-1} for the surfaces modified with peptides **2-4** (Fig. S6, ESI†). In this case the peak was not as significant as in the spectrum of peptide **1**.Additional peaks in the region 1310-1000 cm^{-1} appeared in the
70 spectra of all peptides. This region is characteristic of the C-F stretching band, however it overlaps with the region 1200-1330 cm^{-1} which is typical for amide III (Fig. S6, ESI†).[16] Therefore, the peaks in this region were also expected for peptide **3**.
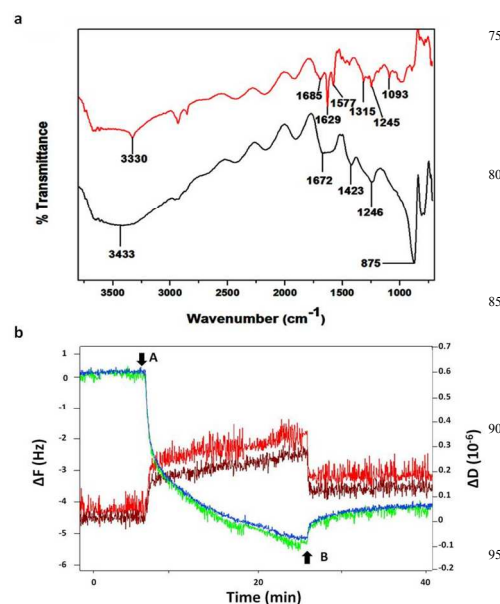

Fig. 3. (a) ATR-FTIR spectra of bare titanium (black) and titanium coated with peptide **1** (red)**.** (b) Real-time QCM-D measurements of
100 peptide **1**. Frequency overtones 5 and 7 are presented in green and blue respectively. Dissipation overtones 5 and 7 are presented in red and maroon respectively. Arrow A, indicates peptide addition, arrow B, washing with methanol.

The IR region 1800 cm^{-1}-1500 cm^{-1} is related to the stretching
105 band of amide I and can indicate on the secondary structure of the peptides. The ATR-FTIR spectrum of a substrate coated with peptide **1** comprises two peaks at 1685 cm^{-1} and 1629 cm^{-1} indicating an anti parallel beta-sheet secondary structure.[17] For peptide **2** the amide I peak appeared at 1678 cm^{-1} and 1608 cm^{-1}
110 indicating the same type of peptide secondary structures on the substrate (Fig. S6, ESI†).[17-18] The IR spectrum of peptide **4** had a peak at 1620 cm^{-1} which indicated a beta sheet structure. The

higher peak, however, shifted to 1696 cm^{-1}, and another peak at 1655 cm^{-1} appeared. This peak may suggest alpha helical structure for the supramolecular structure. (Fig. S6, ESI†) Overall, this spectrum indicate that the coating formed by peptide 5 4 is less organized than the one formed by peptide **1**. An additional indication for the formation of a less homogenous and organized coating by peptide **4** is the low intensity of peaks, and low signal to noise ratio of the spectrum, when compared to the spectra of peptides **1** and **2**. Similarly, the peak related to titanium 10 substrate was also present in the ATR-IR spectrum of peptide **3.** The other regions of the spectrum had low signal to noise ratio and significant peaks could not be detected. This is probably due to the tendency of this peptide to form spherical aggregates rather than a homogenous coating on the substrate. This prevented a 15 good contact with the ATR crystal which is needed in order to obtain a good signal (Fig. S5, ESI†).

Using quartz crystal microbalance with dissipation monitoring (QCM-D) we studied the real-time adhesion of the peptides to titanium substrates. Each peptide was injected into a flow cell 20 containing a titanium coated sensor. The injection of peptide **1** resulted in changes both in frequency (ΔF) and dissipation (ΔD). This suggests that the peptide bonded to the titanium sensor. Upon washing with MeOH, we only observed small changes in frequency and dissipation; this indicates the formation of a stable 25 film on the surface (Fig. 3b). Peptide **2** followed a similar trend, however, the frequency change was lower (Fig. S7, ESI†). This implies that the number of fluorine atoms had an effect on the adhesion of the peptide to the substrate. In the case peptide **3,** the change in frequency was even lower. In addition, upon washing, 30 the frequency changed again significantly, resulting in a total change of 0.5 Hz. This indicates that most of the peptide did not adsorb to the sensor. Since QCM-D is a mass dependent technique, we expected a change in frequency that would be linearly dependent in mass. Because the mass ratio of peptide **1** 35 and **4** is 0.7, we expected that the change in frequency for peptide **4** would be 3.5 Hz (Fig. 3b and Fig. S7, ESI†). However, the change in frequency was lower and equaled 1.5 Hz. In addition, the change in frequency for peptide **4** reached a plateau after only ~ 10 minutes, while peptides **1-3** did not attain a plateau in 40 frequency even after 25 minutes (Fig. S7, ESI†). These QCM-D results along with the information obtained from the ATR-FTIR and AFM analysis further imply that the formation of a film by peptide **3** and **4** is less favorable under these conditions when compared with peptides **1**. The magnitudes of changes in 45 dissipation and frequency are also very informative. The changes in dissipation observed for all peptides were around 0.1x10^{6}, characteristic of a rigid film.[19] This allowed us to use the Sauerbrey relation in order to calculate the mass adsorbed to the sensor (Fig. S8, ESI†). The loaded mass/area were found to be 50 72.1±0.4 ng/cm^2,56±2 ng/cm^2, 14±3 ng/cm^2 and 13±2 ng/cm^2 for peptides **1-4** respectively. These calculations can serve as a quantitative basis for our speculations regarding the poor coverage of peptides **2-4** in comparison to peptide **1**. It should be noted that the QCM-D experiments lasted 40 minutes and 55 therefore measured only the beginning of the coating process.

Using X-ray photoelectron spectroscopy (XPS) analysis we characterized surfaces that underwent a prolonged incubation with the peptide to ensure the complete modification of the titanium substrates. When compared with a bare titanium, the 60 signals resulted from the substrates coated by peptide **1-2** and **4** indicated the presence of fluorine (Fig. S9, ESI†). These signals indicated the deposition of the peptides on the surface. As expected, no fluorine signal was obtained from peptide **3**, however, the intensity of carbon and nitrogen signals was higher 65 than the intensity of the signals obtained from bare titanium substrates. This indicated the deposition of peptide **3** on the surface (Fig. S9, ESI†). The average thickness of the peptide layer evaluated by the XPS analysis was 3.9±0.1 nm, 4.2±0.1 nm, 4.0±0.1 nm, and 3.82±0.04 nm for peptides **1-4** respectively. We 70 also determined the thickness of the coating using ellipsometry. By fitting the measurement to Cauchy film model,[20] which is suitable for organic coatings. We evaluated a thickness of 3.41±0.05 nm, 5.2±0.1, 4.61±0.08 nm, and 3.66±0.04 nm for peptides **1-4** respectively. These findings are with agreement with 75 the results obtained by XPS analysis.

The formation of a biofouling depends on the initial adsorption of bioorganic matter which mediates the subsequent attachment of organisms.[1] We, therefore, investigated the resistance of the peptide-based coating to protein adsorption. A 80 bare titanium surface and a coated titanium substrate were incubated in a protein solution (either bovine serum albumin (BSA), or lysozyme at a concentration of 150 μM for two hours at 37ºC. To determine the adsorbed amounts of the proteins on the substrates we used the non-interfering protein assay™ kit. 85 The plot in Fig. 4a summarizes the adsorbed amounts of proteins to bare and coated titanium substrastes. Both BSA and lysozyme adsorbed to the bare titanuim substrate and in similar amounts to the substrate coated with peptide **3** (Fig. 4a).
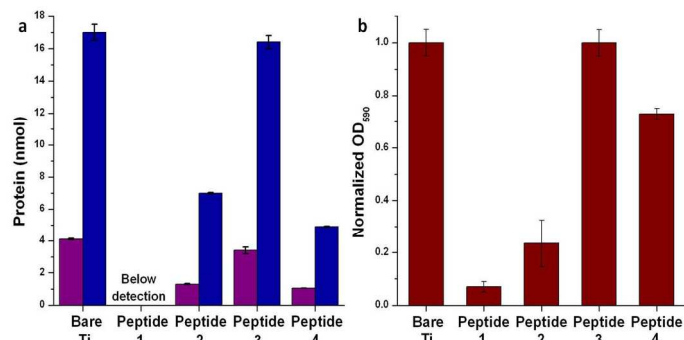

Fig. 4. Assessment of the antifouling activity of the studied peptides**.** 90 Adsorbed amounts of (a) BSA (violet) and lysozyme (blue) to bare and coated titanium substrates with peptide **1-4**. (b) Optical density quantification of the accumulated *P.aeruginosa*, on bare and coated titanium substrates with peptides **1-4**. Error bars represent standard deviations (n=9).

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Both proteins also adsorbed to substrates coated with peptide **2** or **4**. The amounts of proteins adsorbed to these substrates were smaller than those that adsorbed to the bare substrate. However, the amount of proteins, either BSA or lysozyme, adsorbed to a 100 titanium substrate coated with peptide **1** were negligible and below the detection limit of the kit (Fig. 4a). Overall these results demonstrate that in order to achive antifouling activity the peptide must contain fluorinated phenylalnine residues. In addition, the best configuration is of peptide **1**. 105 To assess the bacterial attachment to the surface, bare and peptide coated substrates were incubated in inoculums of

Pseudomonas aeruginosa and *Escherichia coli* for 9 and 96 hours respectively. These incubation times allowed the formation of a biofilm by the different pathogenic bacterial strains. After incubation we stained the substrates with 2%(w/w) Crystal Violet (CV).[21] Using an optical microscope we observed a thick and dense purple layer on the bare titanium surface which indicated a thick bacterial coverage of the substrate, while on the coated titanium we only detected sparse bacteria (Fig. S9, ESI†). To quantify these results, we extracted the CV dye from the bacteria and measured its absorbance.[22] The absorbance of the CV is proportional to the number of bacteria attached to the surface. The plot in Fig. 4b shows that the best antifouling activity is achived by peptide **1**. For surfaces coated with peptide **1** and incubated with *P.aeruginosa* we observed a reduction of 93% in the amount of CV on a coated substrate when compared to a bare substrate (Fig. 4b). For surfaces coated with peptide **1** and incubated with *E.coli*, a reduction of 74% in the amount of CV was detected (Fig. S10, ESI†). As our results on the nature of the coating indicate that the best coating is formed by this specific peptide when compared to peptides **2-4,** we indeed expect that its antifouling activity would be the most sufficient.

In summary, we suggest a new antifouling material based on the self-assembly of a tripeptide. The peptide design includes the amino acid DOPA as the adsorption motif, the diphenylalanine as the element that directs the self-assembly process and fluorine atoms as the antifouling agents. The application of the peptide on the surface is very simple as it occurs spontaneously. We demonstrated the formation of a coating on various substrates and presented its ability to interrupt with the process of biofouling. The peptide presented here can be useful in a large variety of applications. Its coating on medical devices or hospital equipment can lead to a reduction in the number of hospital-acquired infections. In addition, it may be also useful in preventing the adsorption of aqueous and marine organisms and assist in dealing with water treatment and marine fouling.

Notes and references

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† Electronic Supplementary Information (ESI) available: Peptide synthesis, experimental details and additional figures.
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