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Filamentous Phages Displaying Multivalent Peptide Motives with Specific Affinity to Anodic Alumina Surfaces

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Abstract

In this work, we report for the first time on the successful selection and identification of peptide motives that exhibit a specific affinity to anodic alumina surfaces when multivalently displayed on a filamentous phage. It was also demonstrated that, for a selected phage clone, a chemical functionalisation (biotinylation) of the bacteriophage does not deteriorate its specific affinity to anodic alumina. Moreover, such biotinylated bacteriophages, after being immobilised onto an anodic alumina surface, have been shown to allow for the quantitative detection of streptavidine using an ELISA protocol. These results are believed to pave the way for shifting the surface design of integrated biosensing devices from traditional, chemically modified synthetic surfaces, like silane-based self-assembled monolayers, towards molecular linkers based on genetically engineered polypeptides.

Keywords: Bacteriophage; Peptide; Anodic alumina

Introduction

Surface design for biologically oriented micro- and nano-electronic devices is currently shifting from chemically modified synthetic surfaces towards interfacial layers of genetically engineered biomaterials, like polypeptides, nucleic acids or other macromolecules [1]. This new approach is motivated by the fact that traditional molecular linkers, like thiol- or silane-based self-assembled monolayers, are relatively non-specific in their reactivity with the underlying inorganic substrate, and generally do not convey specific biorecognition properties to the synthetic micro- and nanoscale devices [2]. On the contrary, genetically engineered polypeptides have recently been shown to exhibit specific affinity to several Si-based inorganic surfaces [3,4], on top of their specific molecular recognition and genetic activity [5].

In this respect, the aim of the current work is to explore the affinity of metallic oxide surfaces, namely anodic Al_2O_3, for specific multivalent peptides. Anodic alumina has recently been shown to hold great promise for integrated biosensing devices, based on interdigitated Al/Al_2O_3 micro-electrodes [6]. As compared to metallic or silicon oxide thin films, it provides for a more reliable passivating encapsulation of the device in the corroding biological media that are typically encountered in diagnostic applications. Using the well established phage display technology [7], we report for the first time on the successful selection and identification of peptide motives that exhibit a specific affinity to anodic alumina surfaces when multivalently displayed on the filamentous phage. We also demonstrate, for one of the selected phage clones, that a chemical functionalisation (biotinylation) of the bacteriophages does not deteriorate their specific affinity to anodic alumina. Moreover, such biotinylated bacteriophages, after being immobilised onto an anodic alumina surface, have been shown to be able to recognize and quantify streptavidine using an ELISA protocol.

Materials and Methods

Materials

Anodic Al_2O_3 surfaces were obtained by anodising 400 nm thick pure aluminum thin films, which were evaporated onto oxidised 3" silicon wafers. Thin film anodising was carried out galvanostatically according to previously described procedures [8] in 0.01 M ammonium octahydrate pentaborate at 7 mA/cm^2, leading to a 250 nm thick dense anodic Al_2O_3 film. As compared to other, high temperature oxidation methods, Al anodising is well-known to produce relatively smooth amorphous oxide films, without any crystalline features [8]. Therefore, its bio-affinity (if any) can be expected to be governed by chemical, rather than microstructural features.

Peptide selection

As to the selection of specific peptides, a combinatorial library of M13 bacteriophages displaying random octapeptides fused to the N-terminus of the pVIII major coat protein was used (courtesy of V.A. Petrenko [9]). The phages of this library are called landscape phages because each one is displaying approximately 4000 copies of the foreign peptide fused to the 4000 copies of the pVIII coat protein that are assembled along the rod of the filamentous particle. Before starting the bio-panning protocol, which has already been described in detail elsewhere [10], anodic alumina surfaces were washed three times with 1 ml of Tris-HCl buffered saline (TBS). A round of selection consisted of incubating an equal amount of phages (1.5×10^{12} cfu) in a TBST buffer (0.14 M NaCl, 0.1 M Tris-HCl, 0.1% v/v of Tween 20, pH=7.25) for 1 hour. The non-bound phages were then collected, and the anodic alumina surfaces were washed 10 times with TBST. An elution solution (0.2 M glycine-HCl, 1 g/l BSA, pH 2.2) was then added and incubated for 8 min in order to recover bound phages, followed by neutralisation in 1 M Tris-HCl at pH 9. This elution procedure was repeated twice. Eluted phages were then amplified in *E. coli* for the next round of

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biopanning. A total of six biopanning rounds have been performed independently on two anodic Al_2O_3 surfaces. After the sixth round, individual phage clones were sampled randomly from each surface to identify peptide sequences.

Phage biotinylation

Chemical functionalisation of phage clones with a specifically selected peptide sequence was performed with varying concentrations (0-100 µM) of NHS biotin (Thermo Scientific). A phage purification protocol using cesium chloride centrifugation was first carried out. The phage suspension was diluted with TBS to reach a final volume of 12 ml, and then 5.4 g of CsCl were dissolved. After centrifugation, the phage-containing fraction was collected, and the harvested solutions dialysed overnight against TBS. PEG (20 vol. %) was then added to 2 ml of the purified phage suspension. After incubation for 1 h on ice and centrifugation, the supernatant was discarded and the pellet resuspended in 1.2 ml of buffer solution (NaCl 100 mM/$NaHCO_3$ 100 mM, pH 8). The final volume was then divided in 6 aliquots of 200 µL, to which 2 µL of NHS-biotin were added to obtain different final concentrations (0, 0.1, 1, 10 and 100 µM). After 1.5 h of room temperature incubation, 20 vol.% of PEG was added again, incubated for 15 min on ice and centrifuged. The PEG-procedure was repeated 3 times. The pellet was finally resuspended in 100 µL of TBS.

Streptavidine recognition

Streptavidine recognition of biotinylated bacteriophages was tested using a classical ELISA protocol. Anodic Al_2O_3 surfaces were first placed in the wells of a 24 wells plate (Nunclon Surface), and washed 10 times with 1 ml TBS 1X + 0.5% TWEEN 20. Then, 50 µl of the biotinylated phage suspension and 450 µl of TBS 1X + 0,5% TWEEN 20 was added to the wells, and incubated for 1 h at room temperature. After discarding the solution, the wells were washed 5 times with 1 ml of TBS 1X + 0,5% TWEEN 20 under shaking, 3 times with 1 ml PBS 1X + 0,05% TWEEN 20, and 3 times with 1 ml PBS 1X. HRP-streptavidine, diluted 1:200 in 2% MPBS, was added (400 µl/well), and incubated for 1 h at room temperature. After washing, 400 µl of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma T-8665) solution was added to each well and incubated at room temperature for 10 min in the dark. The ELISA reaction was then quenched by adding 400 µl of H_2SO_4 1 M, and the absorbance recorded at 450 nm.

Results and Discussion

Peptide selection

Biopanning experiments can be considered successful when the phage binding yield increases from one round of selection enrichment to another [11]. As seen in Figure 1, significant enrichment was indeed observed in our experiments during the first three biopanning rounds. During the next three rounds, the yield of phage binding stabilised, resulting in a final 50,000-fold increase between the first and sixth round. Both the increase in the initial stage and the stabilisation in the later stage are encouraging first indications for significant peptide affinity towards anodic alumina surfaces.

In order to further identify the phages that showed affinity towards anodic alumina, DNA analysis was carried after the sixth round. Peptide sequences of 19 selectants randomly isolated from the two anodic Al_2O_3 surfaces are shown in Table 1. Over the 19 sequences, the following clones were picked several times: D-P-S-K-P-G-S-S (3x), E-N-T-P-R-G-V-Q (2x) and E-P-P-N-K-P-G-T (6x). This indicates that the diversity of the library is now greatly reduced. In order to verify

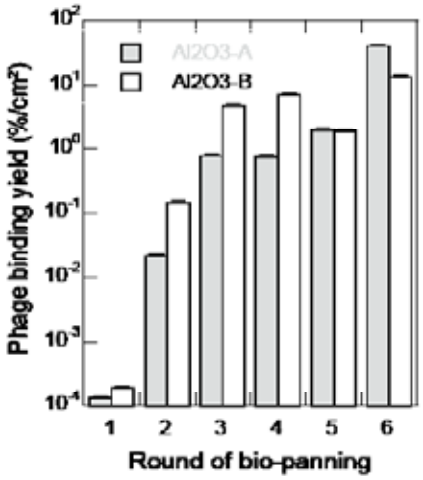

Figure 1: Evolution of phage binding yield during 6 rounds of biopanning on two identical anodic alumina surfaces.

whether the phage clones identified in Table 1 indeed selectively bind to anodic Al_2O_3, the analytical binding yield for each one was determined from single round biopanning experiments on fresh anodic Al_2O_3 surfaces, and compared to results from blank experiments without any Al_2O_3 surface in solution. Based on the thus obtained yield ratio's, shown in the last column of Table 1, only phage clones #1, 2 and 3 are exhibiting specific affinity to the anodic alumina surfaces, their analytical biopanning yield exceeding the blank result by 2-3 orders of magnitude. Note that some variation is evidenced from the 3 replica experiments on the 3 identical phage clones #1, but this inherent to any biopanning protocol [12]. Our results also clearly indicate the need for complementary analytical biopanning protocols on blanks, as the phage clone present with the highest frequency (# 4, present 6 times out of 19 random selectants) is not necessarily the one showing a specific affinity.

Based on the obtained yield ratios, which allow distinguishing specific and non-specific binders, we then tried to identify some distinct amino acid consensus throughout the selected peptide motives, in order to reveal the origin of the specific affinity towards anodic Al_2O_3. As to the position of the acidic, negatively charged amino acids aspartate (D) and glutamate (E), these are found systematically at the first position of the peptide sequence. Moreover, enhanced levels of positively charged, basic lysine (K) and arginine (R) are found systematically on the fourth or fifth position. However, since these features appear systematically for both specifically and non-specifically bonded phages, they are rather indicative for a preferred tolerance within the filamentous phage structure or for a hidden *in vivo* selection happening during the culture step necessary to amplify the phages eluted from the surface. More interestingly, for the phage clones # 1, 2 and 3, i.e. the ones identified as showing specific affinity towards anodic Al_2O_3, the systematic presence of the polar, hydroxyl-containing amino acids serine (S) or threonine (T) at the third position within the peptide motive is striking. Moreover, as this feature is not present for any other selectant phage clones, this is believed to be an essential aspect governing their affinity towards anodic alumina surfaces. In fact, it seems to indicate that the specific affinity of the selected amino acid sequences towards anodic Al_2O_3 can, at least partially, be accounted for by interactions between polar groups. Indeed, based on published values for the point of zero charge of Al_2O_3 (PZC ≅ 9) [13], it is to be expected that, at the pH of the

Phage clone	Amino acid sequence								Analytical bio-panning yield [%/cm²]	Yield ratio vs. blank [/]
1 (3x)	D	P	S	K	P	G	S	S	0.10 0.05 0.22	931 ± 413 193 ± 55 166 ± 25
2 (2x)	E	N	T	P	R	G	V	Q	1.09	922 ± 114
3	E	P	S	K	A	A	G	T	1.64	1457 ± 206
4 (6x)	E	P	P	N	K	P	G	T	0.01	2.4 ± 0.4
5	D	P	A	A	R	T	Q	V	0.12	1.5 ± 0.3
6	E	P	V	Q	K	A	G	S	0.66	1.9 ± 0.6
7	E	P	I	K	G	G	G	S	1.10	3.3 ± 1.0
8	D	P	G	P	K	P	S	T	0.40	3.8 ± 0.8
9	D	V	T	K	A	T	A	Q	0.52	4.5 ± 0.6
10	D	P	A	K	S	P	S	S	2.58	2.4 ± 0.3
11	E	P	G	K	A	S	G	S	4.06	2.2 ± 0.2

Table 1: Amino acid sequences for successfully analysed selectant phage clones, their analytical biopanning yield after single round biopanning experiments on anodic Al_2O_3, and the yield ratio with respect to a reagent blank.

NHS-biotin concentration [µM]	Analytical bio-panning yield [%/cm²]	Yield ratio vs. blank [/]
0	0.17	5634 ± 338
0.1	0.18	5892 ± 177
1	0.23	7556 ± 907
10	0.21	7024 ± 1545
100	0.67	22429 ± 2467

Table 2: Analytical biopanning yield on anodic Al_2O_3 and yield ratio with respect to a reagent blank of phage clone 1 (D-P-S-K-P-G-S-S) after biotinylation to different concentrations.

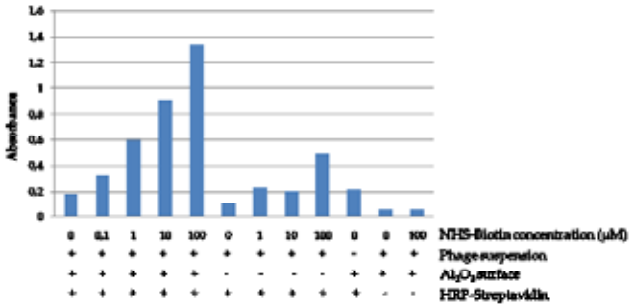

Figure 2: ELISA protocol for the detection of streptavidine by phage clone 1 (D-P-S-K-P-G-S-S) after biotinylation to different concentrations.

selection medium (pH=7.25), the alumina surface becomes protonated. Therefore, one reasonable hypothesis is that it will preferentially be attracting peptide sequences offering hydroxyl-containing groups, i.e. S or T.

Phage functionalisation

In order for bacteriophages to be a successful molecular linker for biosensing devices, its specific affinity to the anodic alumina substrate should not be affected by their chemical or genetic modification. Such phage functionalisation is generally needed to convey to the selected phage clone specific recognition towards targeted biological analytes in solution. In this respect, for demonstration purposes, we performed a chemical functionalisation with NHS-biotyn of one of the successfully selected phage clones (D-P-S-K-P-G-S-S, i.e. phage clone #1 in Table 1), and verified its analytical biopanning yield on anodic Al_2O_3. Table 2 shows the obtained results after biotinylation to different concentrations in the range 0-100 µM, expressed both in absolute value

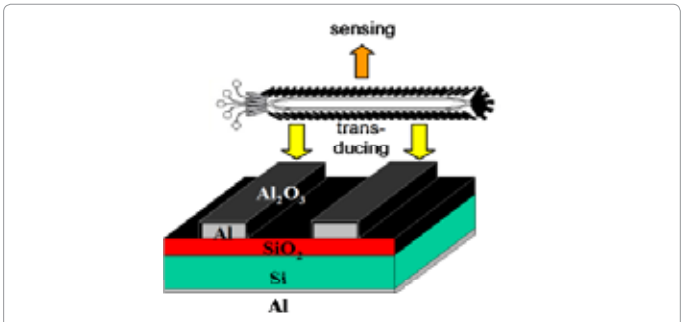

Figure 3: Concept illustration of an integrated capacitive bio-sensor, based on filamentous phages displaying multivalent peptide motives with specific affinity to anodic alumina surfaces.

and in terms of a yield ratio with respect to a reagent blank (similarly as in Table 1). It is clearly observed that for the selected phage clone, biotinylation of the bacteriophages does not prevent their binding to anodic alumina. Also note the one order of magnitude increase vs. Table 1 in the yield ratio vs. the reagent blank, which we believe can be attributed to the ultra-purification protocol preceding the biotinylation procedure.

Finally, such biotinylated bacteriophages, after successful binding to anodic alumina surfaces, have also been tested for their ability to recognize streptavidine, using a classical ELISA protocol. Besides confirming that the biotinylation protocol was indeed performed correctly, the main purpose of these experiments was to demonstrate the bio-recognition capability of functionalised bacteriophages after their successful immobilisation on anodic alumina surfaces. Results are summarised in Figure 2, including several control experiments. It is clearly seen that, when the concentration in biotin is increased for a same concentration in streptavidine, the recognition signal, expressed in units absorbance, increases proportionally. On the other hand, without any anodic alumina surface, there is no quantitative streptavidine recognition. Moreover, the same blank result was confirmed for the three other control experiments shown in the last three columns of Figure 2. These results are also encouraging for further exploring the use of such isolated octopeptide motives in the elaboration of integrated biosensing devices, based for instance on the interdigitated Al/Al_2O_3 microelectrodes already used in [6]. A concept illustration of such an integrated capacitive bio-sensor, based on filamentous phages

displaying multivalent peptide motives with specific affinity to anodic alumina surfaces, is given in Figure 3.

Conclusions

In conclusion, we have successfully used the phage display technique to select and identify phages multivalently expressing octapeptides that exhibit a specific affinity to anodic alumina surfaces. We also showed, for a selected phage clone, that biotinylation does not deteriorate its specific affinity to anodic alumina and that biotinylated bacteriophages, after being immobilised onto an anodic alumina surface, are able to recognize and quantify streptavidine.

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