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Selection and characterization of peptides binding to diamond-like carbon

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ABSTRACT

Phage display was used to find peptides specific for amorphous diamond-like carbon (DLC). A set of putative binders was analyzed in detail and one sequence was found that functioned both as a peptide fused to the pIII protein in M13 phage and as a peptide fused to the enzyme alkaline phosphatase (AP). The dissociation constant of the peptide–AP fusion on DLC was 63 nM and the maximum binding capacity was 6.8 pmol/cm^2. Multiple ways of analysis, including phage titer, enzyme-linked immunosorbent assay, and ellipsometry were used to analyze binding and to exclude possible false positive results. DLC binding peptides can be useful for self-assembling coatings for modifying DLC in specific ways.

1. Introduction

Interactions between biomolecules and inorganic material surfaces are essential for the formation and properties of biological composite materials such as nacre, bone, and spiculi [1,2]. For example, proteins and peptides control the nucleation, growth, and assembly of the mineral phase via specific molecular recognition [2]. Although interactions of proteins and peptides with solid surfaces are very common in nature there is only limited knowledge of how proteins specifically recognize solid materials and how this process can be controlled.

Understanding material specific molecular binding is important for the engineering of new self-assembled systems that can be utilized for example in biomimetic materials, biomedical materials, and biosensors [3–5]. Selecting novel material specific peptides from combinatorial peptide libraries (such as phage display libraries) peptide–surface interaction factors can be identified [6–9]. Due to their relative simplicity, peptides serve as good model systems for the study of molecular recognition, surface binding, and self-assembly at interfaces. Furthermore, the short, functional peptides offer many possibilities for the formation of hierarchical assemblies [10–13].

Material specific peptides can be identified through peptide display or directed evolution systems, thereby overcoming difficulties in rationally designing peptides with predicted functions [14]. In this approach, a combinatorial peptide library usually with equal length but randomized amino acid sequence is displayed for example on the surface of filamentous phages or bacterial cells. Surface displayed peptides with a desired function can be identified by selecting or screening for a particular binding function. Phage display and cell surface display have been used to select peptides that bind to various solid materials like metals [15–17], metal oxides [18], semiconductors [19], minerals [20], carbon materials [21–23], polymers [24]. The short functional peptides can be further engineered using recombinant DNA technology to create mutations, recombinations, and tandem repeats. The modifications can improve binding properties of the peptides and tailor their function for a particular application. Furthermore, engineered material specific peptides can be fused with other biomolecules thus creating multifunctional molecules [22,25].

In this study we focused on diamond like carbon (DLC) which is an amorphous carbon material that is used as an extremely wear-resistant coating material. DLC coatings are chemically stable, optically transparent and have good mechanical properties due to their hardness, low friction coefficient, high wear and corrosion

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resistance, and smoothness. Depending on the fabrication process, amorphous DLC can be formed with different carbon-sp^3/carbon-sp^2 hybridization ratio, incorporated with hydrogen, or can be doped with other elements in order to tune its properties. All DLC types combine some of the superior properties of diamond [26].

DLC has been widely used as a coating material for many applications such as electronics, optics, and mechanical and biomedical devices [27,28]. Understanding and controlling the surface properties of DLC is a necessity for the use of DLC in applications such as biomedical implants, sensors, and lubrication [29–31]. Development of biomolecules that would be able to recognize DLC and bind to it strongly would enable targeted and well-defined modification and functionalization of DLC coatings for various applications. Moreover, surface modification through self-assembly of biomolecules can be advantageous compared to covalent modifications which have drawbacks such as the need to use harsh conditions, limited number of available protocols, cost and time consuming and low yield reactions. The reaction conditions in forming covalent linkages are often not biocompatible which limit usefulness in some biomedical applications [32]. The hydrogenated amorphous DLC (a-C:H) is well studied with respect to its biocompatibility properties [33], and several groups have studied adsorption of human blood proteins (HSA, fibrinogen) to DLC coatings [34–36]. However, a detailed view of the protein adsorption mechanism as well as binding affinities is still lacking. To our knowledge there are no previous studies on developing DLC binding peptides. These peptides could be used as simple model systems for understanding protein interactions with DLC and DLC biocompatibility in general.

In this paper we describe the development and characterization of peptides that bind to DLC (a-C:H) coating. Phage display was used to select DLC binding peptides from a 12-mer peptide library. The peptides were studied as fusion constructs where they were linked to either phage particles or alkaline phosphatase (AP) enzyme. Using DLC binding peptides that were genetically fused to an enzyme we could show the targeted functionalization of DLC surfaces.

2. Materials and methods

2.1. Preparation of DLC surface

DLC coating with the commercial name BALINIT® DLC (Oerlikon Balzers, Liechtenstein) was applied on blocks of stainless steel (martensitic AISI440B quenched). BALINIT® DLC is an amorphous hydrogen containing (a-C:H) type of DLC prepared by plasma-assisted chemical vapor deposition (PACVD) process. The hydrogen content of the coating was between 15 and 20% and its thickness around 2 μm. In biopanning and binding studies of selected phage clones, blocks having rounded depressions to contain the liquid (approx. 0.5 ml capacity) were used. In binding experiments with alkaline phosphatase fusion proteins, similarly coated steel rods with a diameter 3 mm, length 20 mm, and rounded edges were used as binding surfaces. For ellipsometry measurements flat blocks were used. All DLC surfaces were cleaned before the binding experiments with 2% Hellmanex®II (Hellma GmbH & Co. KG, Germany), rinsed with MilliQ – water and ethanol, and finally dried under nitrogen.

2.2. Biopanning with a phage display library

Peptides binding to DLC were selected from a M13 bacteriophage library displaying random 12-mer peptides (Ph.D.™-12 phage display library, New England Biolabs, MA, USA) using the phage display method [37], and according to the suppliers instructions. The phage library stock (reported complexity of 10^9 independent clones) was diluted in TBST-0.1% buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% Tween 20) to a concentration of 2×10^{12} pfu/ml of which 100 μl was used as the input library for the biopanning. The target surface was incubated with the phage library for 1 h at room temperature with gentle agitation, washed 10 times with TBST-0.1% buffer, and subsequently bound phage were eluted by incubating the substrate for 15 min with 0.2 M glycine–HCl buffer at pH 2.2 containing 1 mg/ml bovine serum albumin (BSA) (Sigma–Aldrich, UK). The eluate was then neutralized with 1 M Tris–HCl pH 9.5 buffer. An aliquot of eluted phage solution was used for titering and the rest of the eluted phages were amplified by infecting *E. coli* (ER2738). Titering, amplification, purification, and sequencing of amplified phage were performed according to instructions provided by the supplier. The amplified phage pool was then used for a subsequent round of biopanning. Three cycles of biopanning were done with increasing Tween 20 concentration in rounds two (0.25%) and three (0.5%). After the third round of biopanning the peptide sequences of randomly picked clones were determined by DNA sequencing using an automatic DNA sequencer (Applied Biosystems, CA, USA).

2.3. Quantification of phage particle binding by titering

Analysis of phage binding to DLC was performed using 2×10^{11} pfu of amplified single phage clones in TBST-0.5% and placed in the wells coated with DLC film and incubated for 1 h at room temperature with gentle agitation. After washing 10 times with TBST-0.5% the bound phages were eluted, neutralized and titered as described above. Wild type phages (without displayed peptides) were used as negative controls.

2.4. Quantification of phage particle binding by ELISA

Amplified phage particles were diluted to a concentration of 1×10^{12} pfu/ml in TBST-0.5% and incubated with the substrate (DLC coated wells) for 1 h. After washing the wells 10 times using TBST-0.5% a 5000-fold dilution of Anti-M13 Monoclonal antibody conjugated to horse radish peroxidase (HRP) (GE Healthcare, UK) in TBST-0.5% containing 5 mg/ml BSA, was incubated with the phage particles bound to the DLC for 1 h. Subsequently the substrate surface was washed 6 times with TBST-0.5% and an HRP substrate solution of 0.4 mM 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma–Aldrich, UK) and 0.05% H_2O_2 (Merck, Germany) in 50 mM sodium citrate was added. After 10 min the solution was transferred from the DLC coated well to a 96-well plate and the absorbance at $\lambda = 405$ nm was measured using the Varioskan Flash Multimode Reader (Thermo Scientific, MA, USA).

2.5. Construction of fusion proteins of peptides and alkaline phosphatase

For analyzing the binding properties of peptides outside of the phage particle context, fusion proteins were made in which selected peptides were linked to the enzyme alkaline phosphatase (AP) from *E. coli* by recombinant DNA techniques. The AP thus functioned as a reporter enzyme. Peptides were fused to the N-terminus of AP with a short linker segment (GGGPTSGGGS) inserted in between. DNA constructs for fusing peptides with AP were prepared using the "Golden gate" cloning method [38] which is a one-step cloning reaction that allows the simultaneous insertion of several DNA fragments into a vector. The 12-mer peptide encoding constructs were assembled using sense and antisense synthetic oligonucleotides (Sigma–Aldrich, UK). The inserts encoding the longer peptides, and the bacterial AP gene [39] with a