Colloids and Surfaces B: Biointerfaces 120 (2014) 102–109

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Molecular engineering of avidin and hydrophobin for functional self-assembling interfaces☆

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ARTICLE INFO

Article history:
Received 18 November 2013
Received in revised form 3 February 2014
Accepted 11 May 2014
Available online 22 May 2014

Keywords:
Hydrophobin
Avidin
Protein engineering
Nanomaterial
Biofunctional surface

ABSTRACT

Control over the functionality of interfaces through biomolecular engineering is a central tool for nanoscale technology as well as many current applications of biology. In this work we designed fusion proteins that combined the surface adhesion and interfacial activity of a hydrophobin–protein together with the high affinity biotin-binding capability of an avidin–protein. We found that an overall architecture that was based on a circularly permuted version of avidin, dual-chain avidin, and hydrophobin gave a highly functional combination. The protein was produced in the filamentous fungus *Trichoderma reesei* and was efficiently purified using an aqueous two-phase partitioning procedure. The surface adhesive properties were widely different compared to wild-type avidin. Functional characterization showed that the protein assembled on hydrophobic surfaces as a thin layer even at very low concentrations and efficiently bound a biotinylated compound. The work shows how the challenge of creating a fusion protein with proteins that form multimers can be solved by structural design and how protein self-assembly can be used to efficiently functionalize interfaces.

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1. Introduction

Molecular-level control of the structure and function of interfaces is of key importance in numerous biological systems, ranging from adhesion and signal transduction to the building of larger structural assemblies. Similarly, new technological development is focused toward the use of controlled interfaces for nanostructured systems. Ideally molecular self-assembly can be used for full control of interfaces, orienting molecules in a specific way and determining how they interact with other molecules in their surroundings. Such nanostructure-controlled systems are used for example to find new ways of detecting signals and for making materials with new functional properties. This leads to a new base for technologies for biosensors, the compatibility of implantable devices, and the construction of high-performance biomimetic nanomaterials [1,2].

Examples show that biological systems provide several potential solutions for achieving molecularly well-defined systems. DNA assemblies are very promising because they are extremely versatile for creating structural assemblies [3]. However, in nature proteins are the primary molecules that provide chemical and physical functions. Proteins have the advantage that they show an astounding variety of functions, but we are still in the very early stages in being able to use first principles in designing new functions in protein-based systems. Although there are substantial difficulties in designing new proteins, we can in many cases use natural structures as components and fuse them together into chimeric variants that combine desired functions. The challenge herein is to design the overall architecture of the system so that the desired functions can be utilized. In this work we link together the interfacial assembly functionality of a hydrophobin protein with the affinity binding functionality of an avidin protein.

Hydrophobins are extracellular proteins produced by filamentous fungi [4–6]. In fungal growth they have a multitude of roles that involve controlling interfaces in different ways, for example allowing aerial growth through reducing surface tension or

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http://dx.doi.org/10.1016/j.colsurfb.2014.05.010
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functioning as adhesives. Through structural analysis it is understood that the basis for the function of hydrophobins is their amphiphilic structure, which is due to a relatively large hydrophobic patch on the surface of the protein. This structure causes the hydrophobins to assemble at interfaces and form adhesive films on hydrophobic surfaces [7–9]. Hydrophobins have a tendency to aggregate and exhibit complicated self-assembly features. Based on the occurrence of hydrophobic and hydrophilic amino acids in the amino acid sequence, the hydrophobins have been divided in two classes, class I and class II [10]. Generally the class I hydrophobins form insoluble aggregates, whereas many of the class II hydrophobins have been found to be soluble by formation of dimers, tetramers or even higher types of oligomers [11]. These properties have led to the use of hydrophobins in advanced material applications such as the functionalization of nanomaterials such as carbon nanotubes and graphene [12,13].

Chicken avidin is a tetrameric egg-white protein that binds the small molecule D-biotin (vitamin H) with an exceptionally high ($K_d \sim 10^{-15}$ M) affinity [14]. Like avidin, streptavidin which is of bacterial origin is also a widely used tool in biotechnology applications, and the term (strept)avidin collectively refers to chicken avidin and streptavidin [15,16]. The wild-type avidin is a tetrameric protein in which four identical chains come together to form the functional assembly. Therefore the wild-type avidin binds four biotin molecules. The subunits are not functional unless assembled as a tetramer [17,18]. The very high affinity, specificity and multivalency, has made the (strept)avidin–biotin technology widely used in applications of life sciences and nanotechnology [19–21]. The popularity of the biotin–avidin system for functionalization is supported by the very easily performed coupling reactions in which biotin activated with different reactive groups can be chemically bound to a variety of compounds. Thousands of biotin-functionalized molecules are readily available commercially.

The insight that hydrophobins show very promising features for modification and control of interfaces lead us to explore how fusion proteins should be constructed in order to utilize this property in a wider and more functional way. The aim of this work was to construct a fusion protein that could combine the surface-assembling properties of hydrophobins with the biotin-binding functionality of avidin. We used the class II hydrophobin HFBI from *Trichoderma reesei* because it was expected to be efficiently purified by two-phase extraction and can be handled without irreversible aggregation [10]. The produced fusion protein was anticipated to readily assemble on interfaces and provide controlled and oriented biotin-mediated linking functionality. In addition to the functionality of the engineered fusion protein we were interested in the most efficient molecular design of the chimeric protein. This was addressed by fusing the hydrophobins to either the subunit of the avidin tetramer or a modified dual-chain avidin unit. The dual-chain avidin is a result of duplication and fusion of two circularly permuted avidin subunit chains, resulting in a structure in which two monomer chains have been fused into one polypeptide chain [22]. In the dual-chain avidin (dcAvd), the functional quaternary structure is formed by two peptide chains, but retaining the overall native four-binding site arrangement, i.e. forming a pseudotetramer. The designed constructs HFBI–Avd and HFBI–dcAvd result in different stoichiometry of hydrophobin units and the functional quaternary structure of avidin (Fig. 1).

2. Materials and methods

2.1. Cloning of HFBI–Avd and HFBI–dcAvd

The Avi and dcAvd cassettes were released from the plasmid pGemT-easy(attL1-ompA-AVD-attL2) and pGemT-easy(attL1-ompA-dcAvd-attL2) [23] (named p2768 (Avd) and p2769 (dcAvd)) with *Kpn*I and *Nde*I (New England Biolabs, Ipswich, USA). The cassette was subcloned into the corresponding restriction sites of a shrimp alkaline phosphatase treated fungal expression vector pTNS29 carrying a cbhI promoter and HFBI as an N-terminal fusion partner. The resulting plasmids pTNS47 (containing the Avd gene) and pTNS48 (containing the dcAvd gene) were transformed into DH5α *Escherichia coli* strain by electroporation.

The expression cassettes containing the HFBI–Avd or HFBI–dcAvd inserts were released with *Sph*I (New England Biolabs, Ipswich, USA) from the plasmids and co-transformed with pTNS48/7 and the selection plasmid pToC202 (acetamide resistance (Amd+)) in *T. reesei* strain Rut-C30 Δ*hfb2* VTT D-99676 as described previously [24]. The obtained Amd+ transformants were tested for high HFBI–Avd or HFBI–dcAvd expression in microtiter plate and shake flask cultivations and analyzed for hydrophobin by Western blot analysis.

2.2. Production and purification of HFBI–Avd and HFBI–dcAvd

The *T. reesei* strains VTT D-051057 (HFBI–Avd) VTT D-051059 (HFBI–dcAvd) were cultivated in a bioreactor for 72 h in media containing 40.0 g/l lactose, 4.0 g/l peptone, 1.0 g/l yeast extract, 4.0 g/l

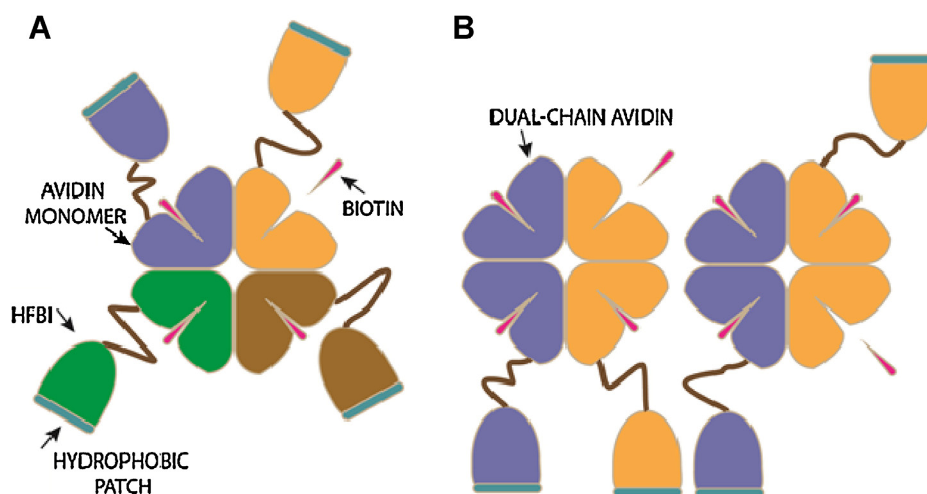

Fig. 1. (a) Schematic presentation of HFBI–Avd construct. (b) Schematic representation of the HFBI–dcAvd construct, which contains avidin assembly (pseudotetramer) resembling the native tetramer. Hydrophobins are also known to form multimers in solution through interaction of the hydrophobic patches.

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