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Introducing a combinatorial DNA-toolbox platform constituting defined protein-based biohybrid-materials

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ABSTRACT

The access to defined protein-based material systems is a major challenge in bionanotechnology and regenerative medicine. Exact control over sequence composition and modification is an important requirement for the intentional design of structure and function. Herein structural- and matrix proteins provide a great potential, but their large repetitive sequences pose a major challenge in their assembly. Here we introduce an integrative "one-vector-toolbox-platform" (OVTP) approach which is fast, efficient and reliable. The OVTP allows for the assembly, multimerization, intentional arrangement and direct translation of defined molecular DNA-tecton libraries, in combination with the selective functionalization of the yielded protein-tecton libraries. The diversity of the generated tectons ranges from elastine-, resilin, silk- to epitope sequence elements. OVTP comprises the expandability of modular biohybrid-materials via the assembly of defined multi-block domain genes and genetically encoded unnatural amino acids (UAA) for site-selective chemical modification. Thus, allowing for the modular combination of the protein-tecton library components and their functional expansion with chemical libraries via UAA functional groups with bioorthogonal reactivity. OVTP enables access to multitudes of defined protein-based biohybrid-materials for self-assembled superstructures such as nanoreactors and nanobiomaterials, e.g. for approaches in biotechnology and individualized regenerative medicine.

1. Introduction

Challenging applications in material science, (bio)nanotechnology & regenerative medicine require molecular materials with defined but adjustable properties ranging from the nano- to the macroscale. Controlling order, structure and function over this large dimensional space requires access to functionally & structurally programmable building blocks (tectons) [1–3].

Abbreviation: 2D, two-dimensional; 3D, three-dimensional; AA, amino acid; CL, cyclic-ligation; EbM, elastin based materials; ELP, elastin-like-protein; ECM, extracellular matrix; GdmCl, guanidinium chloride; hMSC, human mesenchymal stroma or stem cells; NCAM, neural cell adhesion molecule; NHDF, normal human dermal fibroblast; Ni-NTA, nickel - nitrilotriacetic acid; mEGFP, monomeric enhanced green fluorescent protein; pAzF, para-Azido-L-phenylalanin; PBO, protein-based vesicular organelle; PBS, phosphate buffered saline; OEPCR, overlap-extension polymerase chain reaction; OERCA, overlap-extension rolling circle amplification; ORI, origin of replication; OVTP, one-vector-toolbox platform; RCA, rolling circle amplification; SDL, sequential-directional ligation; SDS, sodium dodecylsulphate; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; SEM, standard error of the measurement; TEM, transmission electron microscopy; TCEP, tris(2-carboxyethyl)phosphine; THPTA, Tris-[1-(3-hydroxypropyl)-1H-[1,2,3]triazol-4-yl)methyl]amine; UAA, unnatural amino acid.

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Protein-based tectons constitute the most defined, efficient, reliable, flexible and functional molecular material toolbox in nature. Protein-based nanobiomaterials include the advantages of designability and precisely accessible molecules via blueprint-based biosynthesis. Standard synthetic methods in chemistry and biology are limited in accessing defined complex molecules, especially with large alternating repeating units and macromolecules with site-selective chemical modifications. Bio- and nanotechnology approaches have to overcome these restrictions [1,4], allowing to develop advanced hybrid-materials [5,6].

Structural proteins such as silk [7,8], resilin [9,10] and elastine [11–13] are suited for the engineering of materials with defined properties because of the strength, biointeractivity, resiliance or elasticity of their native archetypes [8,14]. In order to fine tune and access new protein properties the sequence, the spatial position of hydrophobic and hydrophilic protein sequence blocks and the selective introduction of interaction sites, need to be encoded. Contemplatable sequences comprise homorepetitive and highly asymmetric sequence motives for the controlled formation of supramolecular structures. The allocation of a large variety of defined but tunable sequences as combinatorial libraries effectively provides adjustable biomaterials. Examples are protein-based vesicular organelles (PBOs) for drug formulation & synthetic biology, 3D-scaffolds with tunable elasticity and epitope presentation mimicking the extracellular matrix (ECM) for regenerative medicine approaches.

Current recombinant DNA-technology is optimized to access individual proteins for the intentional and rational design of new functional and structural materials [15,12,5]. Known strategies for the rational design of protein polymers via concatemerisation and recursive ligation techniques [16–21] providing defined sequences, but are limited if access to large numbers of diversified long sequences is required. Current combinatorial approaches using PCR based amplification strategies (e.g. OEPCR) or RCA (e.g. OERCA) [22,23] were developed with the focus on DNA-libraries with a statistical pool of structurally homogeneous sequences but different length. Thus, resulting repetitive genes can not achieve defined individual heterogeneous sequences required for the intentional design of new material libraries.

We present an approach creating complex but defined protein polymers in one unified system. Inspired from natures principle of modularity [24] we developed the modular OVTP (Fig. 1A). This platform facilitates the generation of DNA-template entities (DNA-tectons) from the *de novo* synthesis, unimpaired by the grade of repetitive sequence motives. The facile implementation of structural and functional DNA-tectons derived from natural sequences and their free spatial arrangement allows to assemble defined composite sequences with the potential to create combinatorial libraries (Fig. 1B).

Several hundred DNA-tectons are generated so far. They are serving as DNA-templates for protein-tectons and subsequently as structural or functional building blocks for higher order structures *in vitro* or *in vivo* [1,3]. Furthermore, the OVTP permits the direct expression of all DNA-tectons and the integration of post-translational elements for subsequential combination or modification of the yielded tectons. Site-selective incorporation of unnatural amino acids (UAA) [25] enables the supplementation of protein-tectons specifically with synthetic molecules (chemical-tectons) (Fig. 1A & B). Thus, a protein-tecton-library can be further modified with chemical-tectons towards protein-hybrid tecton libraries (Fig. 1B, Supplementary Fig. 1).

Challenging applications for resulting complex protein-hybrid nanomaterials include individualized ECM-mimicry that is presented below. They could be used as environment for the differentiation of multipotent primary cells, a major challenge in personalized medical approaches, or for the formation of complex vesicular assemblies with site-selective chemical functionalizations for biopharmaceutical and biotechnological applications. The OVTP could fill an apparent gap between the bioderived and the chemically synthesized biomaterial world. We believe that it will play an important role in the reliable and efficient rational production of defined, programmable composite 2D and 3D protein biomaterials for biomedical and pharmaceutical applications in future.

2. Methods and materials

2.1. Vectors and linker-regions for the modular one-vector-toolbox platform (OVTP)

Major components of the modular OVTP are vectors consisting of an adapted vector-backbone and a defined linker-region. Within the linker-regions the localization of the recognition sequences of the type IIS restriction enzymes EarI (NEB, New England Biolabs) and BspQI (NEB) and the restriction enzyme SacI (NEB) as well as the generated overhangs are precisely defined (compare Fig. 2A). Preserving these sequences for all platform vectors will permit their compatibility to each other. The type IIS restriction enzymes are cutting next to their recognition sequences and therefore allow for seamless cloning of constructs into the linker regions. The configuration of the flanking sequences between the NcoI site upstream and the MfeI site downstream is variable and characteristic for each of the different linker-regions (MCL, NMCL, NMCysL, NMBL, NMBXL, (TAG)NMBL, NMCysL (TAG); Supplementary Table 3). It is crucial for the OVTP that the restriction enzyme recognition sequences in the linker-module were absent in the vector backbone. Therefore, we removed all redundant restriction enzyme recognition sequences by site-directed mutagenesis [26] from the vector backbones of the OVTP vectors (Table 1A, Supplementary Table 1A).

As vector backbones we adapt a set of former expression vectors: the IPTG inducible pET28-vector backbone (Novagen), the constitutively expressing pIBHisC-vector backbone (Invitrogen/Life Technologies) and a vector backbone consisting of a completely reconstituted pEVOL vector [27] with an arabinose inducible promoter, a chloramphenicol resistance and the 15A ORI (Supplementary Materials section in Supplementary Data). The pET28-MCL vectors (or linker variants) are used for all cloning purposes (insertion, multimerization and rearrangement) and IPTG inducible protein expression in *Escherichia coli*. The pIB-NMCL vectors are used for cloning purposes in *E. coli* and for protein expression in insect cells (Sf9, HighFive; Invitrogene). The pBAD15a-NMBL vector is used for all cloning purposes and protein expression in *E. coli*. Because of its different ori it can be co-expressed with pET28-based OVTP vectors (Supplementary Table 1A).

2.2. DNA-tecton, protein-tecton, chemical-tecton

In synthetic biology basic units starting from natural amino acids up to synthetic molecules are linked together to built programmable tectons. These tectons can self-assemble to higher order units and beyond to form complex functional and structural assemblies [3]. Based on this specification we denominate the modular functional & structural programmable building blocks of the OVTP as DNA-tectons (DNA-template units of different length, complexity and determination), protein-tectons (protein units from the respective DNA-tectons, which could be further assembled to higher order structures via non-covalent or covalent interactions) and chemical-tectons (chemically synthesized (macro)molecules that could be conjugated to protein-tectons with UAA via bioorthogonal reactivities). The OVTP vectors are DNA-tecton harbouring vectors with a special integrated linker-region (Fig. 2A, Supplementary Table 3).

2.3. Cells and host organisms for the cloning of DNA-tectons and the expression of protein-tectons

All cloning steps such as monomer-integration, multimerization and arrangement of DNA-tectons were performed in *E. coli* DH5α, XL1-blue, TOP10 strains (Invitrogene/Life Technologies). For the expression of DNA-tectons in pET28-vectors the OVTP vectors have to be transformed into *E. coli* BL21(DE3), BLR (from Novagen) or ER2566 (NEB) cells. DNA-tectons integrated in the pIB-vector could be expressed in lepidopteran insect cells (HiFive or SF9; Invitrogen/Life Technologies). DNA-tectons in pMAV4rc-vector were expressed in *physcomitrella patens* or *arabidopsis thaliana* protoplasts. pBAD15a-vector constructs were expressed in *E. coli* DH5α, BL21(DE3), BLR and ER2566 cells.

2.4. DNA- and amino acid sequences of selected DNA- and protein-tectons

The constructs used in this study are presented in Table 1. For some vectors with individual DNA-tectons the overall sequences are displayed in the Supplementary Materials section of Supplementary Data.

For the *de novo* synthesis and multimerisation of elastin-like-protein (ELP) variants we ordered chemically synthesized short single-stranded oligonucleotides from Invitrogen/Life Technology. The single-stranded oligonucleotides for monomers of DNA-tectons used for *de novo* generation of multimers via CL and SDL process are the following: