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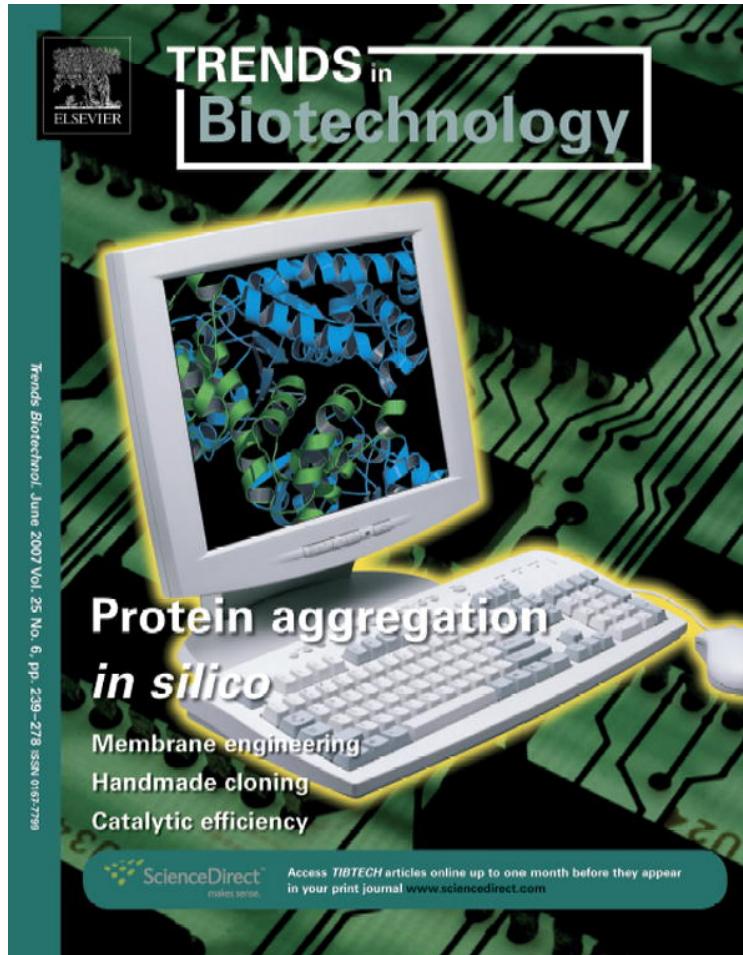
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Brave new (strept)avidins in biotechnology

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Avidin and streptavidin are widely used in (strept)avidin–biotin technology, which is based on their tight biotin-binding capability. These techniques are exceptionally diverse, ranging from simple purification and labeling methods to sophisticated drug pre-targeting and nanostructure-building approaches. Improvements in protein engineering have provided new possibilities to develop tailored protein tools. The (strept)avidin scaffold has been engineered to extend the existing range of applications and to develop new ones. Modifications to (strept)avidins – such as simple amino acid substitutions to reduce biotin binding and alter physico-chemical characters – have recently developed into more sophisticated changes, including chimeric (strept)avidins, topology rearrangements and stitching of non-natural amino acids into the active sites. In this review, we highlight the current status in genetically engineered (strept)avidins and illustrate their versatility as advanced tools in the multiple fields of modern bioscience, medicine and nanotechnology.

Introduction

The genetic engineering of proteins to modify their properties is one of the most important approaches to improve them as tools in different fields of science and technology. There are numerous examples of successful improvements in the stability and specificity, as well as the reaction kinetics, of industrially valuable enzymes, the modification of the pharmacokinetics of protein drugs, and the development of efficient probing and labeling tools for *in vitro* and *in vivo* diagnostics. Although ample examples of the improvement of individual proteins exist, a more interesting class comprises proteins that provide scaffolds for multi-faceted purposes. Antibodies can be considered as an example of such a protein class because, in addition to epitope recognition, enzymatic forms of antibodies have been created by protein engineering [1]. In this review article, we show how chicken avidin and bacterial streptavidin from *Streptomyces avidinii* provide a flexible protein scaffold that can be adjusted for different approaches by protein engineering.

Avidin and streptavidin, collectively (strept)avidin, are structurally and functionally [2] analogous proteins and prominent by reason of their extraordinarily high affinity

toward biotin ($K_d \approx 10^{-14}$ – 10^{-16} M) (Figure 1; Box 1). In addition, they are also unusually stable against heat, denaturants, extremes of pH and to the activity of proteolytic enzymes [2]. These properties have rendered (strept)avidins an outstanding tool for the different disciplines of biosciences, biomedicine and, recently, for other fields of natural science and technology. Such applications include labeling, separation and targeting, constructing self-assembling nanostructures and their use as biopesticides. Collectively, the methods based on the (strept)avidin–biotin interaction are known as (strept)avidin–biotin technology [2]. To extend the range of the existing applications, as well as to develop completely new ones, avidin and streptavidin have been engineered, both chemically and genetically.

Owing to its potency as a biotechnical tool, this relatively simple protein scaffold has been selected as the target of various manipulations. Here, we summarize and discuss the potential of genetically improved (strept)avidin proteins as observed in recent studies. Our aim is to give an overview of the strategies already applied and simultaneously point the way to applications of these protein engineering strategies for other proteins.

Genetically designed (strept)avidins

Streptavidin and avidin can be considered extreme examples of tight ligand-binding. For this reason, numerous studies have been made using site-directed or rational mutagenesis to study the role of the residues participating in biotin binding. These studies are conclusively reviewed elsewhere [3]; therefore, they are not the main focus of this article.

However, some biotechnologically invaluable studies are summarized, to give an insight into the strategies for improving (strept)avidin as a tool. For example, a simple point mutation, Y33H, in avidin is an interesting modification, leading to pH-adjustable biotin binding in avidin [4]. In another case, Ting and co-workers [5] constructed a streptavidin that binds biotin as tightly as wild-type streptavidin, but in monovalent fashion. This was done to avoid the cross-linking caused by the tetravalency of wild-type (strept)avidin applications, such as site-specific protein biotinylation and streptavidin labeling using quantum dot conjugates [6]. The actual monovalent protein was created by first destroying its biotin-binding activity with three point mutations (N23A, S27D and

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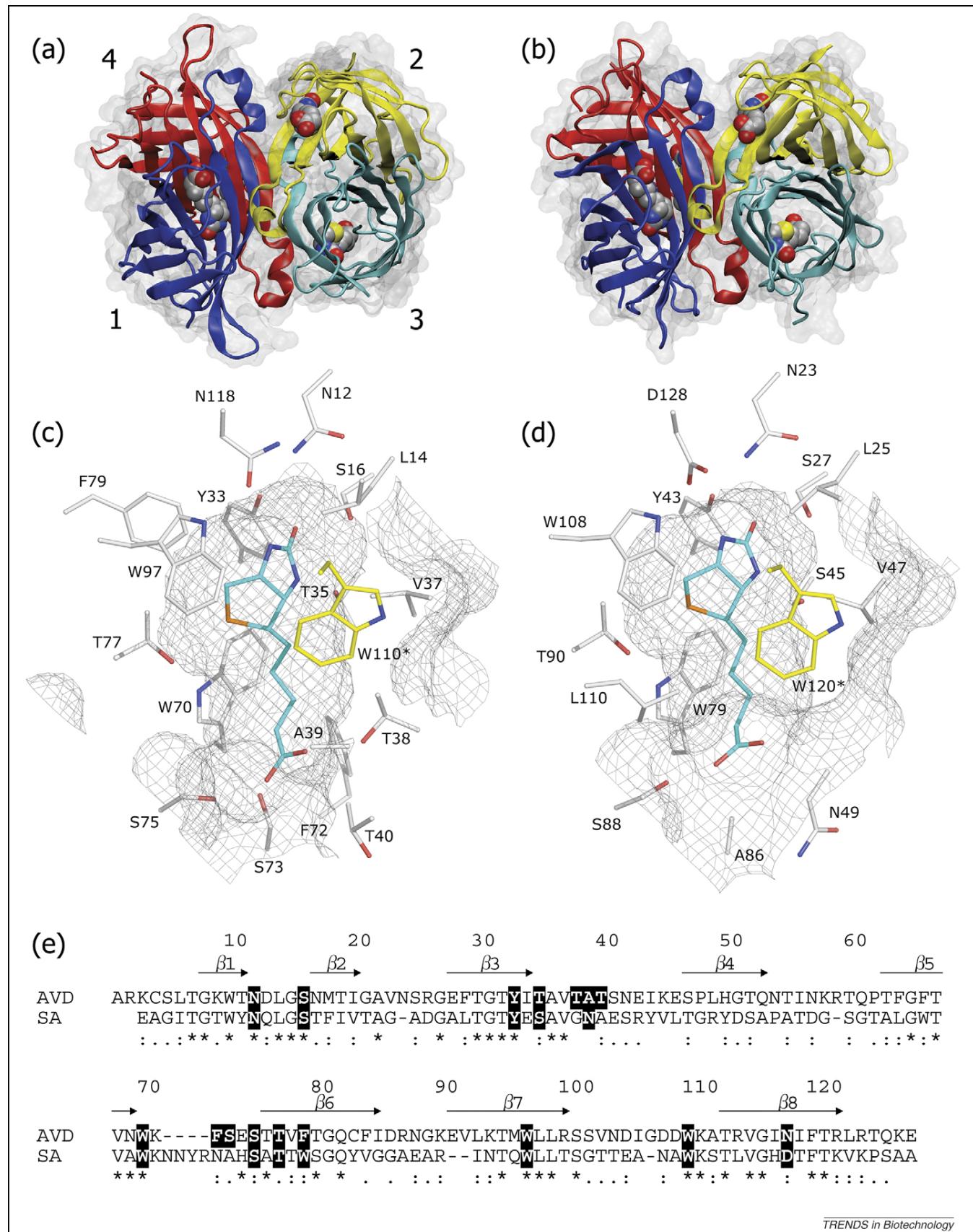


Figure 1. Overview of the structural properties of (strept)avidin. X-ray crystallography structures of (a) avidin (PDB 2avi) and (b) streptavidin (PDB 1mk5) tetramers. The molecular surface (1.4 Å probe) is shown in transparent grey, the secondary-structure elements are indicated as cartoons and bound ligands. Four biotin molecules are shown in van der Waal's spheres. The shape of the biotin-binding site in avidin (c) and streptavidin (d). The ligand-binding cavity in the proteins is visualized by grey mesh, and the side chains of residues within 4 Å from the bound biotin are shown as sticks; carbon atoms of biotin are shown in cyan; the W110/W120 'functional interplay

Box 1. Structural characteristics of (strept)avidin.

Although the primary structures of avidin and streptavidin are only moderately conserved, their secondary, tertiary and quaternary structures are surprisingly similar (Figure 1). Each subunit of (strept)avidin is made of eight antiparallel β -strands, which fold to an up-and-down β -barrel, and their quaternary structures contain four identical barrels (Figure 1). Each barrel has one biotin-binding site; therefore, the (strept)avidin tetramer can bind four biotin molecules (Figure 1). This has important implications for (strept)avidin applications and technology. Residues directly contacting biotin are much better conserved in (strept)avidin compared with other residues (Figure 1e), and both proteins possess two means of forming the biotin-binding site: first, the hydrophilic contact residues form hydrogen bonds with biotin; and second, the aromatic residues create the hydrophobic environment or pocket for the relatively hydrophobic but water-soluble ligand.

The subunits of (strept)avidin are not independent. In the ligand-binding process, two subunits make contact with each ligand. Thus, functional interplay (between subunits 1 and 2, and identically between subunits 3 and 4) prevails owing to the conserved W110 of avidin or W120 in streptavidin (Figure 1). This residue forms part of the biotin-binding pocket of the neighboring subunit (Figure 1), and subunits 1 and 2 (3 and 4) can therefore be regarded as a 'functional dimer'. However, structurally, the interface between monomers 1 and 4 (or 2 and 3) is more extensive, and these pairs can be called 'structural dimers' (Figure 1). In conclusion, as a structural entity, the (strept)avidin tetramer is essentially a dimer comprising two kinds of dimers.

S45A), and then denaturing the wild-type and mutant proteins in a 1:3 ratio, followed by renaturation of the mixture and purification of the desired form, based on its His-tag quantity. The biotin-binding affinity of the resultant monovalent streptavidin was indistinguishable from that of the wild-type protein and it was successfully used to label cell-surface proteins without cross-linking problems.

An interesting approach to adjust the streptavidin–biotin interaction more precisely is site-specific conjugation of stimuli-responsive polymers to streptavidin by mutating the desired linking residues into cysteines or lysines [7]. These 'smart' polymers respond to environmental stimuli, such as changes in temperature, pH and light, by altering their conformation, concomitantly affecting the biotin-binding activity of the polymer-conjugated streptavidin (Figure 2). Applications for these molecular switches can be found in separation and diagnostic methods. In particular, specialized uses for smart (strept)avidin polymers are found in biomedical applications, ranging from targeted drug delivery, controlled enzyme function and gene delivery or expression to cell-adhesion mediators [8]. For example, release of an internalized antibody from the endosomal system into the cytoplasm due to the acidic environment in lysosomes has been demonstrated using a pH-sensitive streptavidin–polymer conjugate [7].

Stability, oligomeric assembly and biotin-binding modifications through interface engineering

The oligomeric nature of (strept)avidin makes it possible to use this protein as a linker between two biotinylated

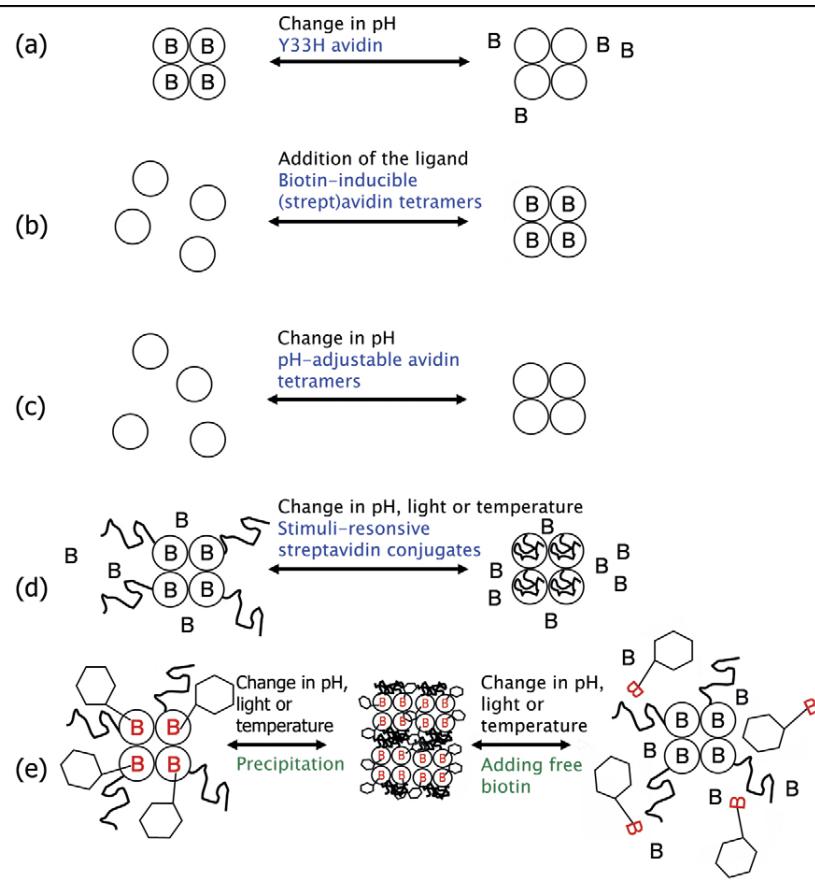
molecules, leading to signal amplification; however, it can also be a cause of aggregation. For this reason, the quaternary structure of (strept)avidin has also been the target of engineering. The contact patterns between the (strept)avidin subunits are structurally well defined, which facilitates their engineering. Indeed, monomeric and dimeric forms of (strept)avidin have been created by breaking these interactions in the tetramers. Even more interestingly, some of these engineered proteins oligomerize in an adjustable fashion (Figure 2). This helps to envisage the potential for environment-sensitive (strept)avidin oligomers as building blocks in a nanoscale device, providing a novel molecular switch. Furthermore, the stability of (strept)avidin has been improved by enhancing and optimizing its interface contacts, which might be vital for applications requiring extreme durability, for example, high-temperature conditions or in the presence of strong denaturants.

To enhance the stability of the avidin tetramer, His117 has been mutated to Cys, to form inter-monomeric disulfide bridges; this increased the melting point (T_m) from 85.5 °C to 98.6 °C [9]. These stabilized avidins are likely to find a use in applications requiring extreme durability and where extreme stability is required.

Weakening of the quaternary structure of avidin has been achieved by mutating residues M96, V115 and I117, which are located in the one-to-three interface – between subunits one and three (Figure 1) – in addition to residues N54 and N69, which are located in the one-to-four interface (between subunits one and four) [10]. Mutants bearing alanine substitutions in all the one-to-three interface residues and either N54A alone or both N54A and N69A were completely monomeric in the absence of biotin, but the presence of biotin induced their tetramerization and stabilization (Figure 2). Because many cellular processes, such as signal transductions, are mediated through inducible protein–protein interactions, this kind of ligand-inducible oligomerization could, possibly, be used to build an artificial signal transduction pathway.

A fully monomeric avidin form has been produced by two interface mutations: W110K in the one-to-two interface, and N54A in the one-to-four interface [11]. Monoavidin binds biotin in a rapidly reversible manner ($K_d \sim 10^{-8}$ M) and might, therefore, prove useful in catch-and-release applications, such as reversible immunoprecipitation or ligand–receptor interaction and reversible binding of biotinylated drugs to an appropriate carrier, both *in vitro* and *in vivo*. To obtain the same result – weakening of the quaternary assembly – residues at the streptavidin subunit interfaces have been changed from non-polar to polar and from polar to charged, to generate electrostatic repulsion and steric hindrance between subunits [12]. The mutations T76R and V125R were adequate for the monomerization of streptavidin; to further improve the usefulness of this monomeric streptavidin, a quadruple mutant (T76R, V125R, V55T and L109T) was constructed to increase its solubility [12].

residue' from the neighboring subunit is shown in yellow. The figures were generated using the PyMOL (<http://pymol.sourceforge.net/>) and VMD [37]. Alignment of avidin (AVD) and streptavidin (SA) primary sequences (e). The secondary-structure elements are indicated by arrows, and the residues forming direct contacts with biotin are shown against a black background (compare with (c) and (d)). The numbering follows the mature avidin sequence. Conserved residues are indicated with an asterisk below the alignment; strongly similar amino acid residues are indicated with a colon; and weaker group similarity is indicated with a full stop.



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Figure 2. Adjustable (strept)avidins. Some (strept)avidin forms have been modified so that their biotin binding or oligomerization can be adjusted by changing environmental parameters or by the presence of the ligand. **(a)** Change of pH alters the biotin-binding kinetics of the Y33H avidin mutant. **(b)** Addition of biotin turns some monomeric and dimeric (strept)avidin mutants into tetramers. **(c)** Adjustment of pH can be used to change monomeric avidins to tetramers, or vice versa. Conjugation of stimuli-responsive polymers to streptavidin can be used to block its biotin-binding activity when the polymers are in collapsed state **(d)** or alternatively, for example, to precipitate streptavidin bound to biotinylated molecules, after which reverting the polymer to extended confirmation solubilizes the precipitate, and the biotin-conjugated molecules can be released by free biotin **(e)**. The latter approach can be used in separation and purification methods instead of column-based techniques.

(Strept)avidin-fusion proteins have been used for different purposes. Affinity purification of such fusion proteins in recombinant protein expression approaches is a direct way to use them. In some cases, the tetrameric quaternary structure can be a problematic owing to the oligomerization tendency of the fusion partner, which can lead to aggregation problems [13]. In such cases, dimeric or monomeric (strept)avidins can be beneficial compared with tetrameric ones, and the increased binding kinetics, as well as the milder elution conditions, connected to these dimeric and monomeric forms is also a possible advantage.

An avidin–biotin pair has been used as a model system to develop protein self-assembly techniques. However, the tetrameric structure of this pair can cause structural constraints, as pointed out by Wagner and co-workers [14]. They showed a dihydrofolate reductase–dimeric methotrexate pair to be more flexible in such circumstances. In this sense, monomeric (strept)avidins can provide a potential alternative to relying on widely used avidin–biotin techniques.

Beyond weakening or strengthening of the interfaces, the ability to control avidin oligomerization by adjusting the pH has been achieved. When residues M96, V115, I117 were individually replaced with histidines, in the

one-to-three interface, and in combination with W110H in the one-to-two interface, avidin forms showing pH-dependent oligomerization were obtained [15]. M96H and V115H alone, in the one-to-three interface, rendered the subunit associations of these mutants pH-dependent. W110H replacement mostly affected the biotin-binding properties of the mutants, in particular the reversibility of binding, and the protein–ligand complex formation of the double mutants was pH-sensitive. For example, M96H was found to be a tetramer at pH 7.2, both in the absence and in the presence of biotin. Interestingly, it was a monomer at pH 4 in the absence of biotin, but the presence of biotin induced its tetramerization.

To summarize, it is possible to regulate the quaternary structure assembly of engineered avidin, either by changing the pH in the absence of biotin or by adding biotin to the protein solution at a low pH (Figure 2). For example, pH-sensitive reporters can be constructed, based on these engineered avidins fused to different fluorescent proteins, thus making it possible to measure local pH using fluorescence resonance energy transfer (FRET). These structurally adjustable (strept)avidins provide alternative options for stimuli-responsive polymer approaches.

Chimeric, (pseudo)catalytic and enantioselective (strept)avidins

The genetic modification of (strept)avidins is not limited to point mutations; more complex molecular rearrangements can also be achieved. The biotechnologically valuable properties of (strept)avidin have been successfully improved by transferring structural components from an analogous protein through chimeragenesis. An example of this approach is avidin stabilization. The chicken avidin family includes other members, known as the avidin-related proteins (AVRs) [16], of which AVR4 is the most heat-stable, wild-type biotin-binding protein characterized thus far, with a T_m of 106.4 °C in the absence of biotin [17]. To study the molecular origin of the high thermal stability of AVR4, its most variable region (compared with avidin), a 21 amino acid residues section, was genetically transferred to the corresponding region in avidin [18]. This chimeric avidin (ChiAVD) protein was more stable (T_m = 96.5 °C) than wild-type avidin (T_m = 83.5 °C). For further tuning of the chimeric AVD–AVR4 protein, a point mutation, I117Y, was introduced onto the subunit interface. By this means, a ChiAVD(I117Y) protein was produced with an even higher thermal stability (T_m = 111.1 °C). These molecules should be valuable in applications performed under extreme conditions and for the extended storage of ChiAVD-based reagents. In addition, better performance could be obtained in purification protocols and diagnostic applications, and completely new settings could be used for product manufacturing, formulation and regeneration.

Avidin is known to be capable of enhancing the hydrolysis of biotinyl *p*-nitrophenyl ester [19]. To move this property to streptavidin (which is not naturally capable of catalyzing hydrolysis reactions), a chimeric protein was created by transferring structural elements from avidin to streptavidin [20]. In the mutant M1, the loop between β-strands three and four in streptavidin [residues 48–52, (GNAES)] was replaced with the corresponding loop from avidin [residues 38–45 (TATSNEIK)]. A further mutation, L124R, was introduced to the M2 streptavidin sequence in accord with the avidin sequence. As presumed, both modifications increased the hydrolytic activity of streptavidin and their combination resulted in full avidin-like activity [20]. One possible application for this would be a pro-drug treatment using a biotin-conjugated inactive drug, which would become active after the hydrolysis. In this approach, the avidin-based drug activator could be delivered to the required site using the pre-targeting approach, where biotinylated or other specific binders are used to target a biotinylated effector molecule (e.g. a drug or a radionuclide) to the site of treatment using a (strept)avidin bridge [21], and the ligand binding could be controlled with stimuli-responsive polymers.

Enantioselective catalysis is used for the synthesis of pure stereoisomeric compounds; proteins can be used to catalyze the reaction favoring synthesis of certain stereoisomers [22]. To increase the range and efficiency of the enantioselective catalytic potential of (strept)avidin, it has been modified by rational point mutations. For example, enantioselective hydrogenation to apply a biotinylated catalytic moiety [biotin connected to amino-diphosphine moiety with arylamino acid spacer (biotin-4^{ortho}-2)]

combined to streptavidin mutant V47G favored production of the *R* enantiomer [44% enantiomeric excess (ee)], whereas wild-type protein favored production of the *S* enantiomer (28% ee) [23]. In future, (strept)avidin might be a tool in the industrial production of valuable enantioselectively pure components.

Recently, mutagenesis at position S112 was used to fine-tune the enantioselectivity of streptavidin. The most efficient enantioselectivities were obtained with aromatic residues in this position (S112F and S112Y), favoring *R* enantiomers or with cationic residues (S112R and S112K), favoring *S* enantiomers in the hydrogenation reactions [24].

Chicken avidin has been successfully used in affinity capillary electrophoresis (ACE), where a recognition site in the protein molecule works as a chiral selector. In ACE, separation of enantiomers is based on the differential affinity of the immobilized protein to the individual enantiomers [25]. Avidin has also been used for the separation of chiral isomers of ketoprofen [26] and other chemical compounds. In this method, organic solvents, typically 0–30% methanol, are applied to the liquid phase to improve the resolution [26]. Therefore, improved avidins can offer benefits in terms of stability of the columns and, furthermore, novel selective properties can potentially be developed using genetic engineering tools.

New topological designs

(Strept)avidins with different properties in neighboring subunits provide a novel application when nanometer-scale resolution is required [27]. Because (strept)avidin is a homotetrameric protein, the modification of only one or some subunits of the tetramer is troublesome, requiring the mixing of different denatured subunits, followed by renaturation and purification of the correctly folded and assembled protein [5]. An obvious solution to this problem is a (strep)avidin fusion, in which the tetramer-like structure is composed of one or two polypeptide chain(s) instead of the four chains in wild-type proteins. However, in the quaternary structure of (strept)avidin the termini of the different subunits are far away from each other; therefore, a simple fusion strategy would probably fail. An alternative option is to change the structural topology of (strept)avidin by a circular permutation approach, to create building blocks suitable for subunit fusion.

To use this approach for generating avidin with independently modifiable subunits, two distinct circularly permuted forms of avidin were designed, with the aim of constructing a fusion avidin with two subunits as domains in one polypeptide [28]. The natural termini of wild-type avidin were connected by a short linker, and the new termini were introduced into two different loops (Figure 3). This ‘structure shuffling’ enabled the creation of the fusion construct using a short linker peptide between the two different circularly permuted subunits. The resulting pseudotetrameric dual-chain avidin (dcAvd) showed conserved biotin binding and also displayed structural characteristics similar to those of wild-type avidin [28].

The dcAvd scaffold was used to generate avidins where the neighboring biotin-binding sites have different

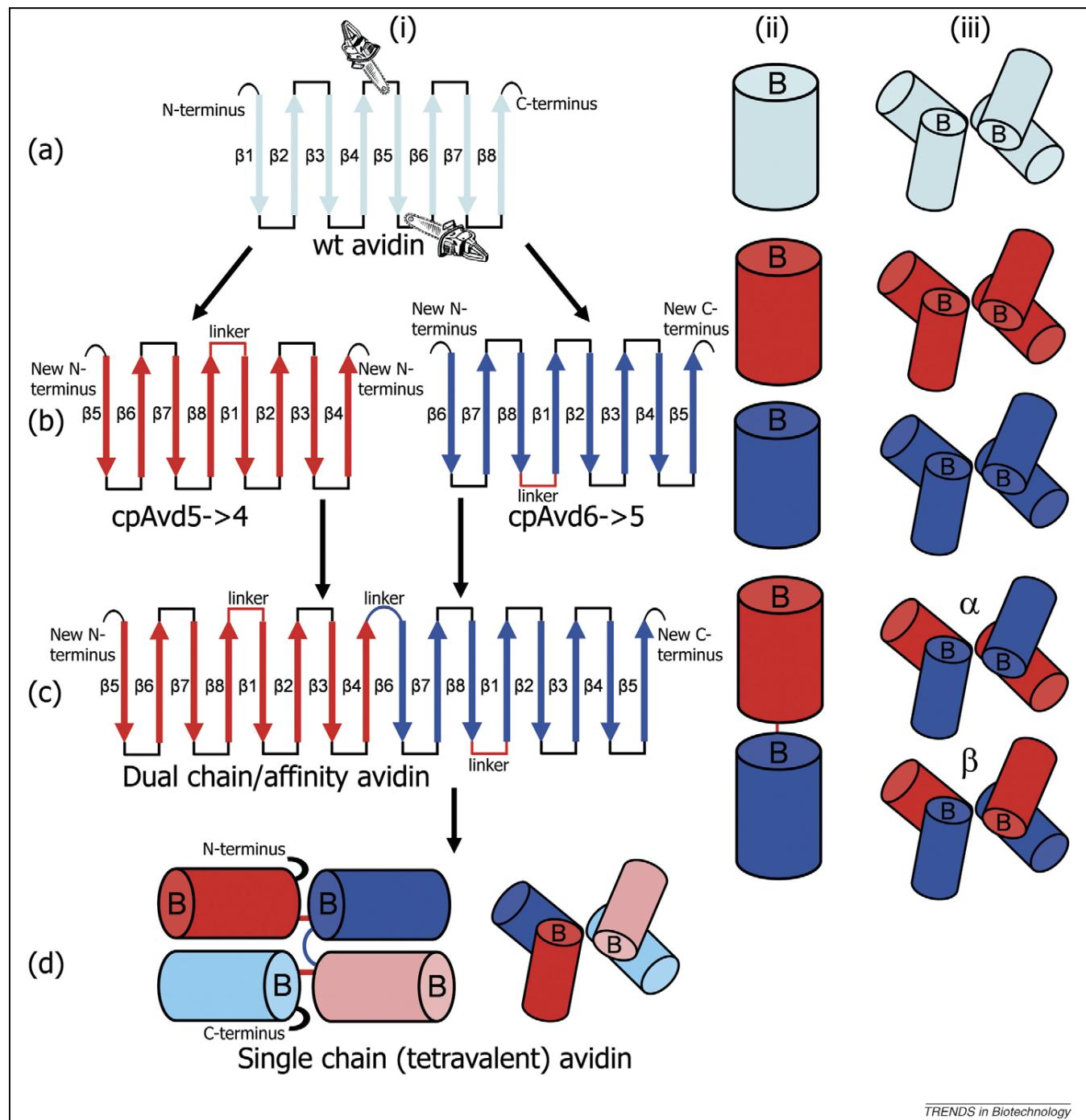


Figure 3. Topology engineering strategy to generate dual-chain or dual-affinity and single-chain avidins. **(a)** Topology of wild-type avidin (i), its tertiary (ii) and quaternary structure (iii). **(b)** Initially, two different circularly permuted mutants were generated: one where the new termini were introduced in the avidin polypeptide between β-strands 4 and 5 (upper chain saw, circularly permuted avidin (cpAvd)5→4); and in another between β-strands 5 and 6 (lower saw, cpAvd6→5). Both circularly permuted mutants have a quaternary structure composed of four identical subunits. In the next phase, the C-terminus of cpAvd5→4 was joined to the N-terminus of cpAvd6→5 (c) to generate dual-chain avidin (dcAvd) [28]. Owing to the fixing of the interface between subunits 1–4 in dcAvd, however, two different quaternary structure outcomes are possible [29]. Dual-chain avidin can be modified to have subunits with different properties by modifying only one of its domains [4]. Finally, two dual-chain avidins were fused together to generate a single-chain avidin (d). Single-chain avidin consists of four domains (corresponding to subunits in wild-type avidin), which can be independently modified to introduce different ligand-binding and/or other properties [30].

affinities for biotin [4]. Mutations S16A, Y33H and T35A were induced separately in one of the binding domains of dcAvd to weaken its biotin-binding affinity. The pseudotetramer (a dimer of the modified dcAvds) had two higher (wild-type-like) and two lower (modified) affinity biotin-binding sites. These dual-affinity dcAvd molecules (dadcAvd) should provide totally new possibilities in

avidin-biotin technology, where they might have uses as novel bioseparation tools, carrier proteins or nanoscale adapters. The feasibility of dadcAvd was demonstrated in a system in which the protein was immobilized to a biotinylated matrix by its high-affinity sites, and the modified sites were used to isolate biotinylated alkaline phosphatase, which was then eluted by pH change [4].

In a more recent study, the quaternary structure assembly of dcAvd was affected by introducing a cysteine residue (I117C) to one domain and by mutating the residues at the one-to-three interface of the second domain [29]. A mutant dcAvd form, with a I117C mutation in one domain and V115H in the other, displayed only one, disulfide-locked, quaternary structure assembly (Figure 3a). This dcAvd derivative, with a defined quaternary structure, facilitates the development of even more versatile protein tools than those already possible with the original dcAvd.

Furthermore, a single-chain avidin (scAvd) with four biotin-binding domains in a polypeptide chain was constructed by a fusion of two dcAvds [30]. This scAvd showed biotin-binding and thermal stability properties similar to wild-type avidin. This avidin design enables each of the four avidin domains to be modified independently. The scAvd scaffold can thus be used to construct spatially and stoichiometrically defined avidin molecules, with different domain characteristics in each pseudotetramer (Figure 3d).

Streptavidin has also been modified with the aim of having two binding sites in one polypeptide chain [31]. This novel streptavidin was further modified by point mutations. Phage-displayed mutated streptavidins were then subjected to panning with biotinylated beads. A surprising result of this study was that these novel streptavidins displayed high affinity towards biotin-4-fluorescein conjugate ($K_d = 10^{-10}-10^{-11}$ M). By contrast, the affinity to biotin was radically reduced ($K_d = 10^{-5}-10^{-6}$ M) compared with wild-type streptavidin. These proteins also possess another interesting feature: they showed both dimeric (pseudotetrameric) and monomeric (pseudodimeric) quaternary structures; furthermore, the oligomerization process was biotin-dependent (Figure 2), which somewhat restricts its use as a defined scaffold in new applications. However, the capacity to bind biotin derivatives with higher affinity than biotin makes these proteins interesting candidates for novel applications.

(Strept)avidin has been widely used as a building block in nanotechnology, with the aim of constructing self-assembling structures. An example of this is DNA tracks

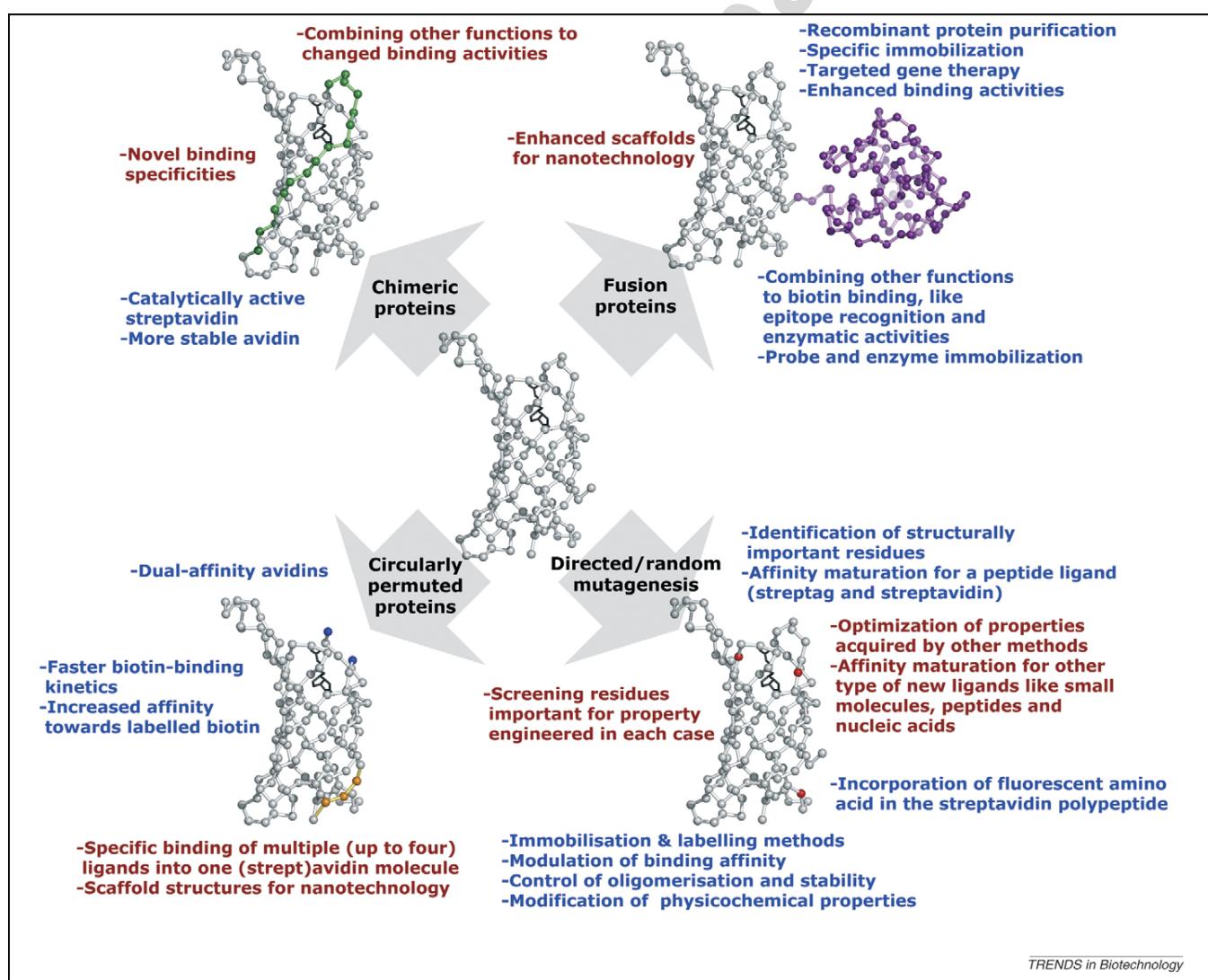


Figure 4. Engineering approaches conducted (blue text) and possible future (strept)avidin modifications (red text) and their applications. An imaginary fusion protein between avidin (PDB 2AVI) and calponin homology domain from human β -spectrin (PDB 1BKR) is presented in the right upper-corner.

functionalized with streptavidin [32]. (Strept)avidins with binding sites for multiple ligands would be promising tools for such purposes, making it possible to fabricate more sophisticated structures and to increase the functionality of the generated objects.

Conclusions

Several modified forms of (strept)avidin have been described, and since the development of efficient production systems for recombinant (strept)avidins [33–36], an increase in commercialized (strept)avidin products should be expected in the near future. Engineering of (strept)avidin has until recently been limited to the homotetrameric approach because different types of subunits have been obtained in the same protein only by mixing the different denatured proteins, thus limiting the quality and the yield of the final product. In this respect, dual-chain and single-chain (strept)avidins [28,30,31] offer powerful scaffolds to further develop (strept)avidin technology for novel applications that demand improved and fully controlled proteins, as exemplified by dual-affinity avidins [4].

In summary, many pivotal properties of (strept)avidin have been successfully engineered to improve their value as biotechnological tools: their binding affinity and specificity have been modulated; their oligomeric and topological structure features have been fundamentally altered; their physicochemical and pharmacokinetic characteristics have been improved and adjusted; and they have been used as scaffolds to generate novel enzymatic activities. Although there have been numerous successful attempts to engineer (strept)avidin, the enormous potential of this protein scaffold has not yet been realized (Figure 4). This simple protein–ligand interaction can be extended and modified for use in methods ranging from the life sciences to chemistry and physics, providing a versatile scaffold for novel applications. The knowledge obtained from this protein–ligand pair and the molecular engineering strategies (Figure 4) can also be used in the context of other molecular designs. Genetically engineered (strept)avidins can open the way to the next-generation of protein tools, and it will be intriguing to see what kind of (strept)avidins we will have in our toolbox within the next ten years.

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