Designing repeat proteins for biosensors and medical imaging

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Abstract

Advances in protein engineering tools, both computational and experimental, has afforded many new protein structures and functions. Here, we present a snapshot of repeat-protein engineering efforts towards new, versatile, alternative binding scaffolds for use in analytical sensors and as imaging agents. Analytical assays, sensors and imaging agents based on the direct binding of analyte are increasingly important for research and diagnostics in medicine, food safety, and national security.

Repeat proteins as alternative scaffolds

The sensitivity and specificity of biosensors and imaging agents are decidedly dependent upon high-affinity, specific molecular recognition between the recognition element, i.e. affinity probe and desired target (Figure 1A). Traditionally, the commonly used affinity probes are antibodies. However, a 2008 study by Berglund et al. [1], showed that fewer than half of approximately 6000 routinely used commercial antibodies were specific for only their intended target [2]. This lack of specificity is especially exaggerated for detection of analytes that are not highly immunogenic, e.g. small molecules, peptidoglycans or nucleic acids and for which high-quality monoclonal antibodies are not available. As a result, many medically and environmentally important analytes still lack high affinity, specific recognition elements. However, the emergence of protein and peptide engineering techniques, such as synthetic libraries and selection and evolution technologies, has allowed substantial progress towards a new generation of affinity probes based on alternative protein scaffolds. Advantages of such probes are: (a) small size for ease of handling and reduced non-specific interactions; (b) the absence of aggregation, which is essential for sensitivity; (c) high chemical, thermal and proteolytic stability; (d) ease of chemical coupling to transducer elements such as fluorophores, nanoparticles and solid support; (e) cost-efficient production by chemical synthesis or recombinant means [3]. Additionally, alternative scaffolds can be easily amended to enhance pharmacokinetic properties such as length of half-life and clearance of probes used as imaging agents [4]. Furthermore, multifunctional scaffolds, i.e. multiple independent binding sites for the same or different targets, can be used to greatly enhance selectivity of sensors through avidity. These advantages have been demonstrated in a number of alternative scaffold designs for therapeutics, diagnostics and imagining devices (Figure 1B) [3–8].

The modular architecture and extended non-globular structure of repeat proteins position them as increasingly successful alternative scaffolds [3]. Designed ankyrin repeat proteins (DARPins), tetratricopeptide repeats (TPRs), armadillo repeat proteins (ARMs) and leucine rich repeat proteins (LRRs) have all been used for development of high-affinity binders [9–13]. For this reason, designed repeat proteins that have been successfully used for imaging and/or biosensing applications will remain the focus of this review.

Methods for generation of new protein scaffolds

A defining feature of repeat protein structure is that both the individual repeats and their positions relative to each other are the same regardless of the protein in which they occur [14]. Upon ligand binding, there is typically little, if any, conformational change. Thus repeat proteins are regarded as constant frameworks that are 'decorated' with a binding site. It is therefore not surprising that consensus sequence proteins are common starting points for the design of functional repeat proteins. Additionally, recent computational designs endeavoured to create repeat protein scaffolds with specific shape complementary to that of the target ligand [15,16]. For instances where the desired analyte is another protein, this approach can potentially increase affinity and selectivity due to the shape complementarity of the binder and the target. Parmeggiani et al. [17] demonstrated a general computational approach to designing repeat proteins that resulted in well expressed, stable scaffolds for ankyrins, TPRs, LRRs, ARMs, HEAT repeats (huntingtin, elongation factor 3, protein phosphatase 2A, and the lipid kinase TOR (target

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Abbreviations: ARM, armadillo repeat protein; DARPin, designed ankyrin repeat protein; HEAT repeats (huntingtin, elongation factor 3, protein phosphatase 2A, and the lipid kinase TOR (target of rapamycin)); HER2, human epidermal growth factor receptor-2; LRR, leucine rich repeat protein; MDP, muramyl dipeptide; NLR, nod-like receptor; Puf, pumilio and fem-3 binding factor protein; PPR, pentatricopeptide repeat; TAL, transcription activator-like effectors; TPR, tetratricopeptide repeat; TRAP, tetratricopeptide repeat affinity protein; VLR, variable lymphocyte receptor.

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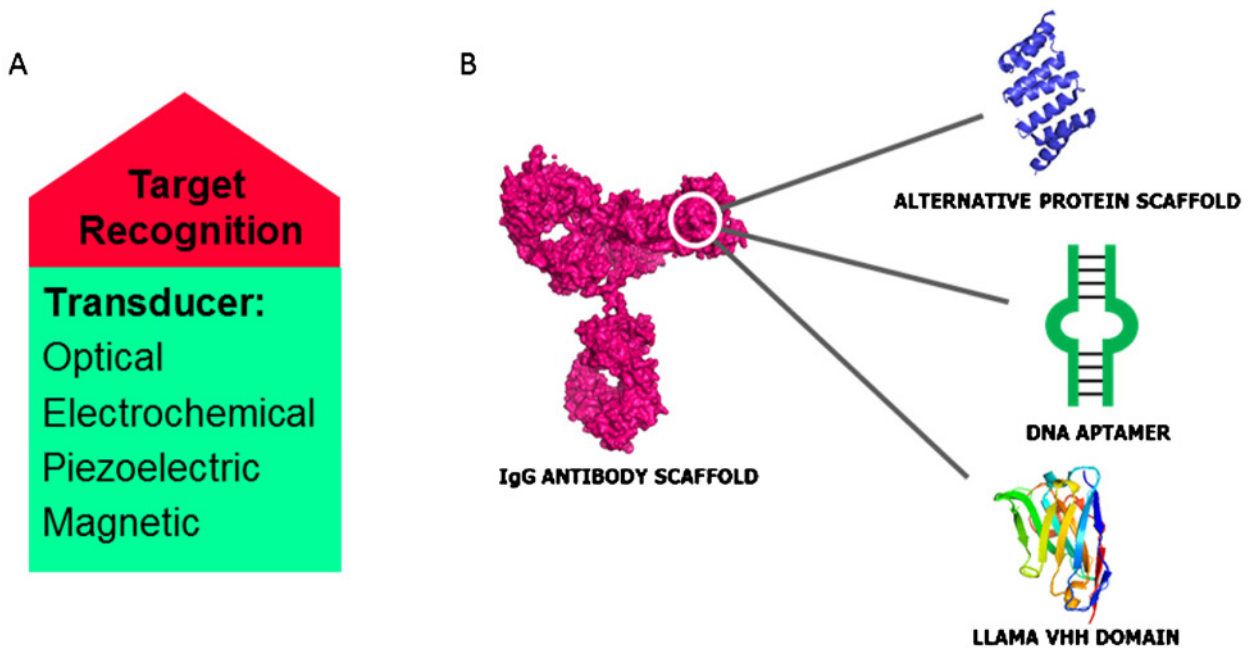
Figure 1 | **Biosensor architecture and recognition elements**

(**A**) The architecture of a biosensor consists of two main components: 1. Target recognition that is required for specific and sensitive analyte detection and 2. A transducer element needed to convert target recognition into a readable signal. (**B**) Many alternatives to the Igg antibody scaffold have been proposed including protein scaffolds, DNA aptamers and single chain antibody domains. The binding site of the IgG scaffold (PDB: 1HZH), indicated by the white circle is analogous to that of the consensus tetratricopeptide repeat 3 (CTPR3) alternative scaffold (PDB: 1NAO), DNA aptamer and llama VHH domain (PDB: 1I3V).



of rapamycin)) and WD40 repeats. The importance of shape complementarity has also been the focus of computational design efforts as highlighted in the work of Park et al. [15] and Ramisch et al. [16] where novel design approaches are used to create LRRs with a predetermined curvature.

Experimentally, the design of new binders requires methods that can rapidly and robustly assemble and screen protein libraries that contain many millions of unique sequences. Recently, Speltz et al. [18] described a new cost-effective and efficient method for library creation. This strategy utilizes the fluorescence from GFP to determine colonies that contain the desired insert after ligation. This 'white and green screen' is not limited to GFP and can be easily implemented with any fluorescent protein.

Protein display methods, such as yeast, phage and ribosome display, have been successfully employed for generation of high-affinity binders. Libraries of LRRs, TPRs and DARPins have been screened to identify proteins with affinities for desired targets that are equivalent to and even surpassing that of antibodies [19–22]. Additionally, *in vivo* screening strategies, such as the split-GFP reassembly, have been used to screen TPR libraries [23]. TPR binders, T-mods, were then successfully used to modulate protein-protein interactions *in vivo* [24].

Scaffolds for recognition of protein and peptide targets

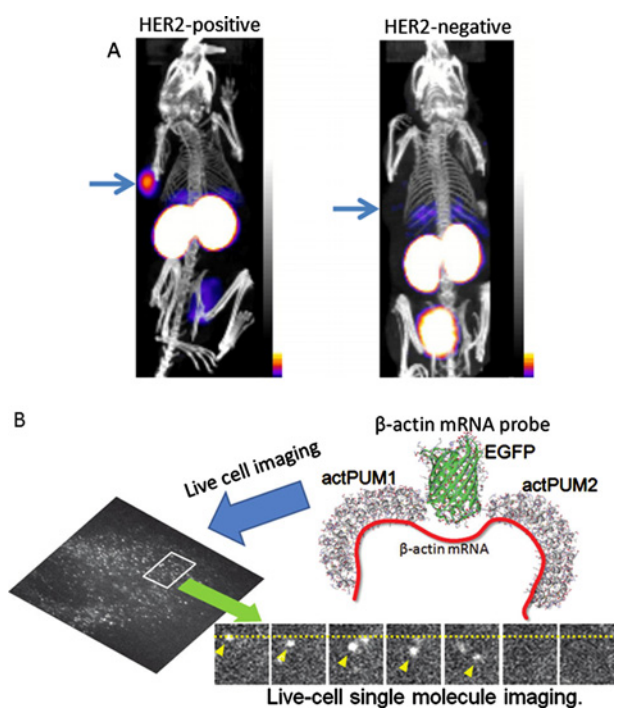
DARPin G3 designed to specifically bind human epidermal growth factor receptor-2 (HER2) was used to image HER2 overexpression on breast cancer cells *in vivo* via selective attachment of ^{111}In-DOTA to a C-terminal cysteine residue (Figure 2A) [25]. This labelling strategy is generally applicable to other repeat-protein scaffolds and thus holds promise for future clinical use.

Design of binders through engineering repeating units and adding or removing repeats to create binding scaffolds increases their utility as alternative molecular recognition modules [14]. The modular recognition would allow for efficient generation of a virtually unlimited repertoire of binders and remove the need for evolution of binding affinity to individual targets. Plueckthun and colleagues [11] used this strategy to design ARMs that selectively bind the peptide, neurotensin. This work illustrates the potential of modular peptide binders from ARM repeats based on the binding interaction of a repeat per dipeptide.

In an analogous strategy, design of TPR modules was used for detection of phosphopeptide–protein interaction in *Escherichia coli* using split mCherry assembly as a readout

Figure 2 | Examples of designed repeat protein binders for imaging applications

(**A**) microSPECT/CT scan of radiolabelled DARPin G3 in severe combined immunodeficiency-beige mice bearing: a HER2-positive human breast tumour and HER2-negative human breast tumour (MDA-MB-468). Tumours, indicated by arrows, were analysed at same sensitivity level. Reproduced from [25]: Goldstein, R., Sosabowski, J., Livanos, M., Leyton, J., Vigor, K., Bhavsar, G., Nagy-Davidescu, G., Rashid, M., Miranda, E., Yeung. J. et al. (2015) Development of the designed ankyrin repeat protein (darpin) g3 for her2 molecular imaging. Eur. J. Nucl. Med. Mol. Imaging **42**, 288–301. (**B**) A pumilio homology domain (PUM-HD) was designed by Yoshimura et al. [36] to recognize the sequence of β-actin mRNA and subsequently developed into an mRNA probe. The probe, consisting of two PUM-HD mutants flanking full-length EGFP, successfully and specifically labelled β-actin mRNA in the cytosol as seen through fluorescence microscopy with the probe in living cells. Fluorescent spots from the probe were co-localized with microtubules and moved directionally in living cells indicating the potential for visualization of β-actin mRNA localization and dynamics in living cells. Reproduced from [36]: Yoshimura, H., Inaguma, A., Yamada, T. and Ozawa, T. (2012) Fluorescent probes for imaging endogenous beta-actin mRNA in living cells using fluorescent protein-tagged pumilio. ACS Chem. Biol. **7**, 999-1005.



[26]. The TPR affinity protein (TRAP) was designed to selectively recognize phosphorylated compared with non-phosphorylated peptides in a sequence-dependent manner. TRAP binding to the peptide is modular: amino acid 'pockets' are designed to bind specific amino acid sequences in the target peptide. Thus, through mixing and matching of TRAP pockets virtually any peptide sequence can be recognized.

Another repeat protein scaffold, HEAT, has been used as a promising universal scaffold that can bind a variety of structurally and functionally diverse protein targets with micromolar to nanomolar affinities [27]. In this study, a library of HEAT proteins was created to bind four pre-defined protein targets with specific secondary structure demonstrating the ability to distinguish between proteins with differing characteristics [27].

Scaffolds for nucleic acid recognition

Recognition of nucleic acid targets has been an area of growing interest due to the potential for control of cellular transcription and translation [28]. Although not imaging and biosensing tools in the strict sense, these binding modules are important for systems biology research. The majority of DNA- and RNA-binding modules are based on the re-design of three classes of endogenous nucleic acid binders: transcription activator-like effectors (TAL effectors), pumilio and fem-3-binding factor proteins (Puf) and pentatricopeptide repeats (PPRs). Vibrant research into better understanding of natural ligand recognition properties of these proteins has resulted in novel nucleic acid recognition modules [29–33].

TAL effectors are 33–35 amino acids repeat containing proteins implicated in host gene expression. Two hypervariable amino acid residues in each repeat interact with one base pair in the target DNA. Several *de novo* TAL proteins were designed computationally and have demonstrated the ability to target specific DNA sequences resulting in selective control of gene transcription [34,35].

Puf proteins have drawn much interest due to their RNA recognition properties [28]. Eight consecutive Puf repeats comprise a three-helix bundle that in turn contacts a single RNA base through 'tripartite recognition motif' of three amino acids [28]. This modular recognition suggests great potential engineering affinity for specific RNA sequences. Yoshimura et al. [36] demonstrated this in an example where they design a fluorescently-tagged pumilio module for specific binding and imaging of β-actin mRNA *in vivo* (Figure 2B).

PPRs are essential proteins for the regulation of many facets of RNA metabolism in eukaryotic cells; however, understanding of their natural function has been hindered by poor solubility and limited success of computational methods. Using a consensus-based approach, Coquille et al. [37] designed a synthetic, stable PPR scaffold for RNA binding. This study confirmed that PPRs bind RNA according to the 'PPR code' where amino acids at positions 4 and 34 determine specificity for certain nts [37]. Advances such as this will enable further elucidation and exploitation of PPR–RNA recognition capabilities.

In parallel, the ever versatile DARPin framework was recently used for selective and specific DNA binding. Scholz et al. [38] described the redesign of DARPins for selective recognition of human telomere G-quadruplex DNA with nanomolar affinity.

Other biomolecular targets

More than 100 different post translational protein modifications act as an on/off switch of function, activity and stability. Detection of these events, such as phosphorylation and glycosylation, is thus biomedically important. Several carbohydrate-binding lectins and mammalian antibodies have been investigated for specific binding to glycosylated proteins; however, these binders generally suffer from low affinity and broad specificity, thus limiting their clinical utility [39–42]. Variable lymphocyte receptors (VLRs), which are LRRs involved in the adaptive immunity of jawless vertebrates, have been successfully engineered for recognition of several glycosylated proteins [12,43,44]. For example, Hong et al. [33] describe a yeast surface display screen of VLRs from lamprey that enabled the selection of binders for a number of glycan targets with nanomolar affinities.

Our group endeavours to engineer repeat protein modules for use in whole-cell pathogen sensing. For that we are drawing inspiration from the pattern recognition receptors, such as Nod-like receptors (NLRs) in the innate immune system. Analogous to man-made sensors, NLR proteins have an architecture where the recognition and transducer elements are easily identifiable [45]. Previous studies have shown that these proteins utilize LRR domains to bind a large repertoire of chemically diverse ligands including bacterial cell wall peptidoglycans, glycolipids and bacterial RNA. We have recently used consensus sequence approach to create an artificial LRR, consenus leucine rich repeat 2 (CLRR2), with micromolar binding affinity to muramyl dipeptide (MDP), a bacterial cell wall component [13]. Surprisingly, CLRR2 is a specific binder for the physiologically relevant isomer L-D MDP and does not bind D-D MDP or N-glycolyl MDP. This strict discrimination between similar ligands suggests that CLRR scaffolds are promising candidates for further design to specifically bind other biomolecules.

Summary

Here we have presented a snapshot of protein engineering efforts for design of modular-binding scaffolds for analytical assays, sensors and imaging applications. The approaches described here for design of scaffolds are applicable for a variety of ligands. Assays and sensors based on direct analyte recognition have important applications not only in medical research and diagnostics but also in food industry, environmental science, forensic science and national security.

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