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Engineered Repeat Protein Hybrids: The New Horizon for Biologic Medicines and Diagnostic Tools

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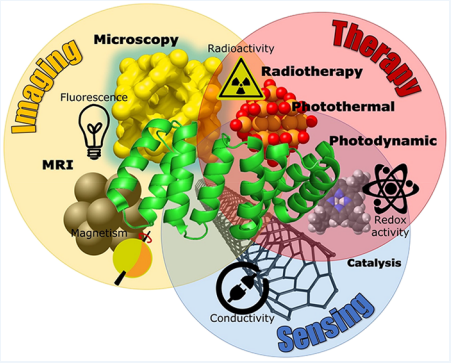
CONSPECTUS: The last decades have witnessed unprecedented scientific breakthroughs in all the fields of knowledge, from basic sciences to translational research, resulting in the drastic improvement of the lifespan and overall quality of life. However, despite these great advances, the treatment and diagnosis of some diseases remain a challenge. Inspired by nature, scientists have been exploring biomolecules and their derivatives as novel therapeutic/diagnostic agents. Among biomolecules, proteins raise much interest due to their high versatility, biocompatibility, and biodegradability.



Protein binders (binders) are proteins that bind other proteins, in certain cases, inhibiting or modulating their action. Given their therapeutic potential, binders are emerging as the next generation of biopharmaceuticals. The most well-known example of binders are antibodies, and inspired by them researchers have developed alternative binders using protein design approaches. Protein design can be based on naturally occurring proteins in which, by means of rational design or combinatorial approaches, new binding interfaces can be engineered to obtain specific functions or based on *de novo* proteins emerging from state-of-the-art computational methodologies.

Among the novel designed proteins, a class of engineered repeat proteins, the consensus tetratricopeptide repeat (CTPR) proteins, stand out due to their stability and robustness. The CTPR unit is a helix-turn-helix motif constituted of 34 amino acids, of which only 8 are essential to ensure correct folding of the structure. The small number of conserved residues of CTPR proteins leaves plenty of freedom for functional mutations, making them a base scaffold that can be easily and reproducibly tailored to endow desired functions to the protein. For example, the introduction of metal-binding residues (e.g., histidines, cysteines) drives the coordination of metal ions and the subsequent formation of nanomaterials. Additionally, the CTPR unit can be conjugated with other peptides/proteins or repeated in tandem to encode larger CTPR proteins with superhelical structures. These properties allow for the design of both binder and nanomaterial-coordination modules as well as their combination within the same molecule, making the CTPR proteins, as we have demonstrated in several recent examples, the ideal platform to develop protein−nanomaterial hybrids. Generally, the fusion of two distinct materials exploits the best properties of each; however, in protein−nanomaterial hybrids, the fusion takes on a new dimension as new properties arise.

These hybrids have ushered the use of protein-based nanomaterials as biopharmaceuticals beyond their original therapeutic scope and paved the way for their use as theranostic agents. Despite several reports of protein-stabilized nanomaterials found in the literature, these systems offer limited control in the synthesis and properties of the grown nanomaterials, as the protein acts just as a stabilizing agent with no significant functional contribution. Therefore, the rational design of protein-based nanomaterials as true theranostic agents is still incipient. In this context, CTPR proteins have emerged as promising scaffolds to hold simultaneously therapeutic and diagnostic functions through protein engineering, as it has been recently demonstrated in pioneering *in vitro* and *in vivo* examples.

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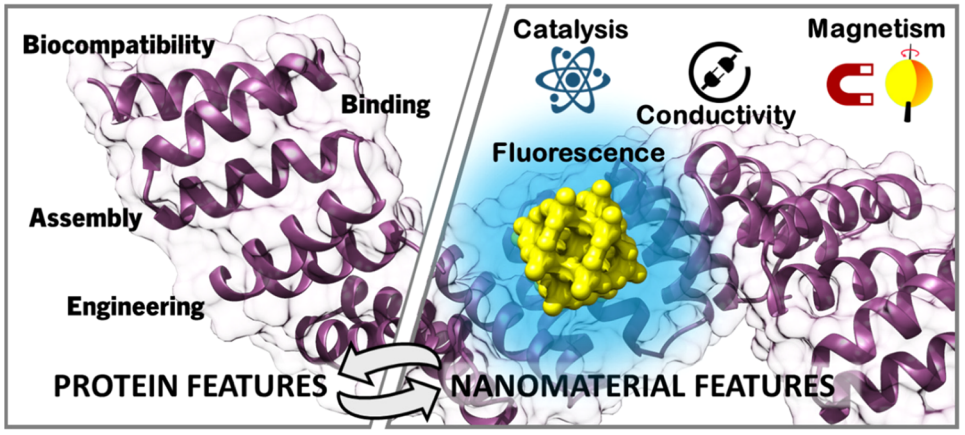

Figure 1. Scheme of engineered protein−nanomaterial hybrids. The protein is not only a scaffold for different functional nanomaterials but also serves as an engineering platform for adding biologically relevant features such as molecular recognition and inhibition. The nanomaterials expand the theranostic capabilities of the proteins by means of their particular physicochemical properties. The combination of both approaches allows the development of tailored protein hybrids for a wide range of biomedical applications.

module, with specific binding capability, stabilized fluorescent gold nanoclusters. This multifunctional hybrid nanosensor reported on the specific ligand recognition of the CTPR module by the change in the fluorescence of the nanoclusters.

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INTRODUCTION

Despite the significant recent advances in the diagnosis and treatment of prevalent diseases, we are still far from reducing their health and economic burden. Therefore, there is a need to continue exploring novel technologies and strategies to overcome the limitations and disadvantages of conventional therapeutic and diagnostic approaches.

The development of new nanotechnological tools may offer innovative ways of approaching the remaining challenges in biomedicine. Nature has often served scientists as inspiration, making the design, control, and modification of biomolecules the main source to expand the biomedical toolbox. Among biomolecules, proteins are highly versatile and responsible for a wide variety of functions in cells: transport, regulatory, structural, enzymatic, *etc.* Therefore, they are optimal candidates to build biological medicines and diagnostic tools.[5] Over the last years, the advances made in gene sequencing, crystallography, and electron microscopy, as well as the development of computational biology, have boosted the potential of engineered proteins in a wide range of biomedical applications.[6]

Protein biologics have emerged as a highly effective drug group and, currently, engineered monoclonal antibodies (mAbs) have taken a central role in both targeted therapy and diagnosis.[7] These highly specific proteins are extremely useful tools for different biomedical applications. However, the use of engineered proteins can go beyond the possibilities of antibodies, as their sequence and structure can be easily modified to modulate their targeting and inhibitory capabilities. For example, engineered protein domains, inspired by natural binding proteins, such as tetratricopeptide repeats (TPR), ankyrin repeats, and other repeat protein scaffolds, have shown great promise as the next generation of therapeutic biologics.[8−10] Furthermore, modular design strategies can be used to incorporate functional modules and combine different functionalities. In this way, the development of protein-based hybrids by the fusion of therapeutic engineered proteins with nanomaterials/organic molecules gives rise to cooperative entities with new functionalities able to overcome the limitations of current applications or even develop novel solutions (Figure 1). We have extensively applied this strategy for the engineering of protein-based hybrids to open unexplored routes for the synthesis of the next generation of advanced multifunctional therapeutic agents.

This Account provides an overview of recent developments in the field of protein hybrids, focusing on the use of engineered repeat proteins as scaffolds, and their potential use as tools for biomedical applications. In particular, it emphasizes

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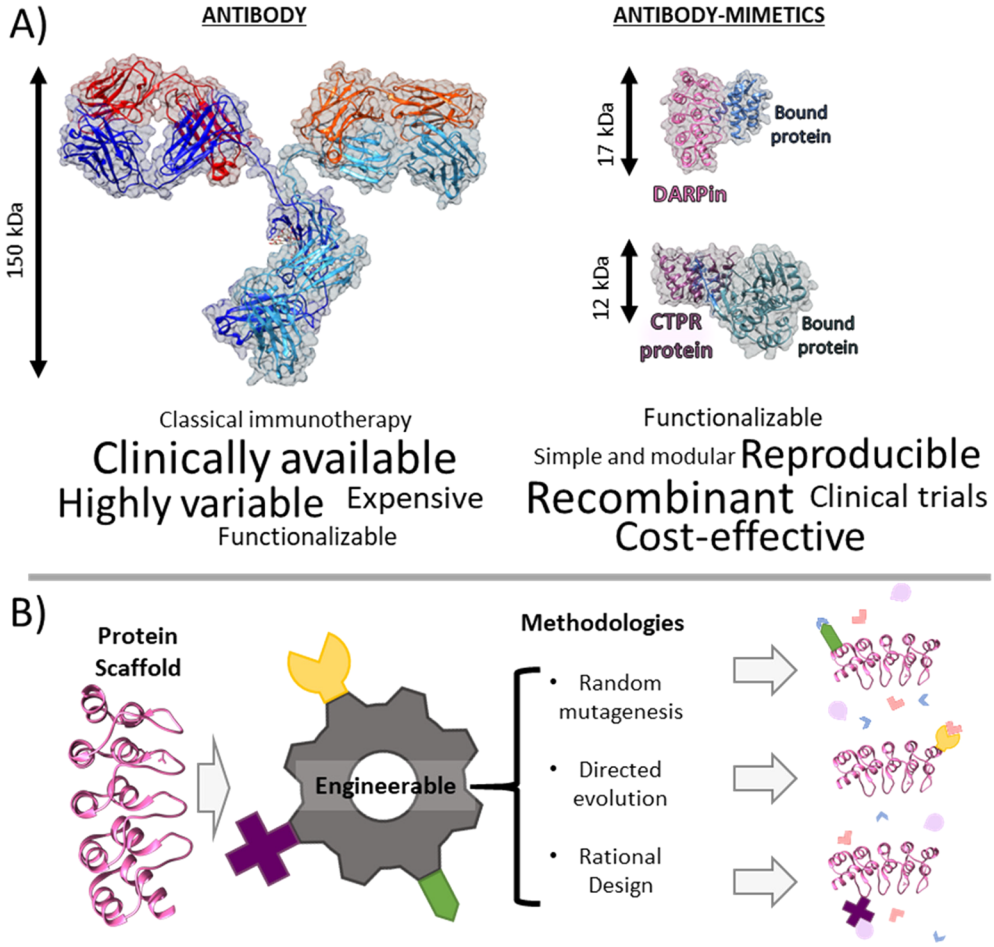

Figure 2. (A) Comparison between conventional antibodies (Ab) and engineered protein-based antibody mimetics. Scale structural models of Ab and target bounded DARPin and CTPR proteins show their differences in size and complexity. The word-clouds indicate the main features of each approach. (B) Scheme of the workflow of antibody mimetics modification by different methodologies for the development of new binders. PDB entries: DARPin in complex with IL-13 (5KNH); IgG antibody (1IGT); and engineered CTPR Hsp90 binder (CTPR390) (3KD7) modeled with a Hsp90 fragment.

the advances made using CTPR scaffolds as example that aims to walk the reader through the process from the design to the application.

DESIGNED PROTEIN BINDERS AS BIOLOGIC DRUGS

Protein binders or simply binders are a class of designed proteins that have brought to the protein design field an exciting new avenue of research and development. In general terms, binders are bioengineered scaffolds designed for the molecular recognition of their counterparts. For a significant subset of binders generated to exert a therapeutic effect, the binding results in target inhibition or modulation.[11,12] This effect may impact downstream effectors and ultimately alter key biochemical processes. However, the biological effects and applications of binders are broader, including their usage as biorecognition elements in diagnostics.[13,14] After intensive research carried out over the last decades, a totally new market has emerged around them, and those with biopharmaceuticals capabilities have been positioned as the next generation drugs that could take over from the already established chemical products.

Currently, antibodies (Abs) are the reference system in new binder design. Abs are protein complexes naturally synthesized to interact with recognizable molecules/antigens (Figure 2). This process occurs in the antibody binding site or paratope and, depending on the structure and composition of the epitope, the affinity of the interaction can reach the picomolar range.

From the diagnostic/therapeutic perspective, mass production of Abs has marked a before and after in the management of diverse health conditions, as disease diagnosis or novel anticancer immunomodulatory therapies. Therefore, the demand of these therapeutically active biologics is high and the market is still growing unstoppable.[7] However, their large size (>150 kDa), their complex quaternary structure stabilized by disulfide bridges, and the post-translational modifications required to become fully functional inherently involve some disadvantages, including limited stability and intracellular usage and high production and storage costs.[13] Also, the use of polyclonal antibodies, mainly through animal immunization, results in batch to batch reproducibility problems.[15] These drawbacks have led to the search for better solutions. Synthetic antibody fragments (Fab', scFv, etc.) and more recently discovered single chain antibodies (single VHH domain or nanobodies) are examples of these tools with improved characteristics, mostly stemming from the simplification of the systems.[16]

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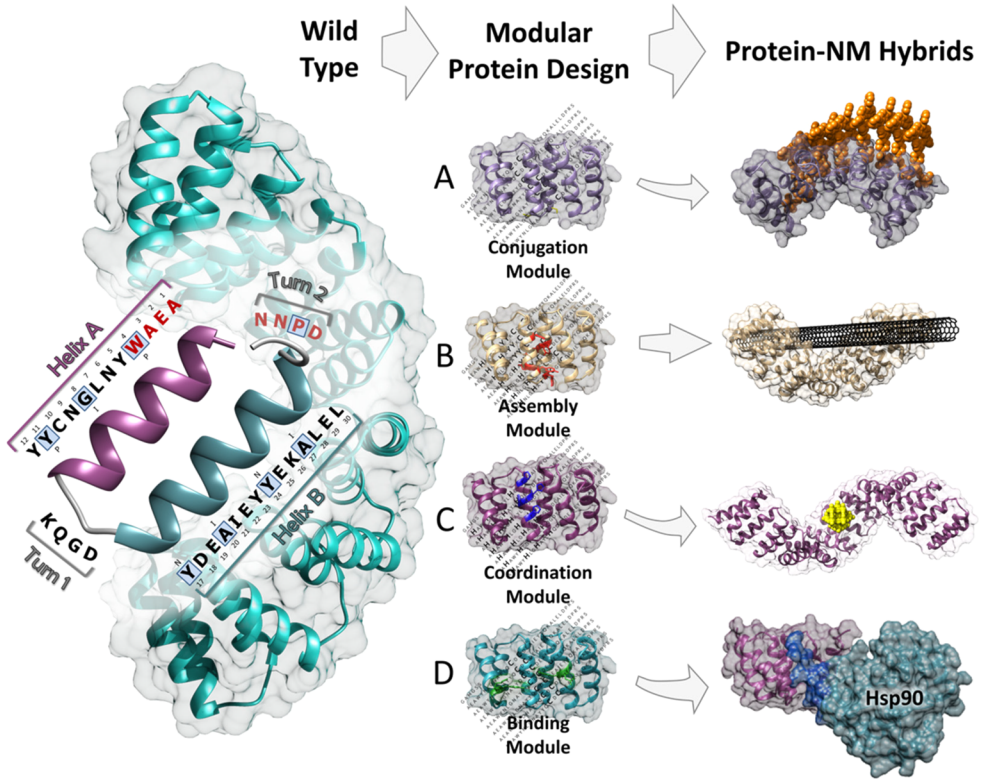

Figure 3. CTPR protein structure and protein-nanomaterial hybrid design. On the left, a CTPR model of eight repeat modules with the sequence of the consensus TPR unit shown over the inner concave face of the CTPR domain. The start and end sequences of the repeat are shown in red (AEAW and DPNN), the structural amino acids are boxed in blue, and their spatial position is indicated with I, intrarepeat interface; P and N, inter-repeat interface with the previous repeat (R−-1) and with the next repeat (R+1), respectively. Right panel: versatility of the modular protein design that can be tuned to host different nanomaterials by the control of reactive residues (A), aromatic residues (B), metal-coordinating residues (C), and protein binding sites (D).

Alternatively, to overcome the aforementioned limitations of antibody-based technologies, designed antibody-mimetic proteins started to attract interest in recent years. These bioengineered "blocks" have been developed as protein binders starting from small and very stable protein scaffolds and where devised molecular recognition interfaces are designed for diverse targets, analogously to Abs.[13] The paratope implementation is usually attempted by directed protein evolution, rational and computational design, or a combination of those approaches.[17] A cornerstone benefit in comparison to the classical antibodies is that these mimetics are fully recombinant, sequence defined, and large-scale producible units. Consequently, some representatives of this group, for instance, affibodies, nanobodies, affimers, or anticalins, are already commercially available and applicable in a wide range of areas, including in-clinic.[14]

Some examples of functional protein binders emerge from state-of-the-art computational approaches to protein design, such as Rosetta, including the development of *de novo* proteins, which, as well as structures, can incorporate complex functional motifs.[18] The development of these computational tools paved the way for the design of vaccines by creating protein scaffolds that mimic the viral epitope exposure.[19,20] Additionally, protein docking has been successfully implemented for the study of protein inhibition and for the design of ligand binding sites, including the protein−protein docking to develop inhibitors or modulators.[21,22]

Among the emerging designed proteins, DARPins (Designed Ankyrin Repeat Proteins) require special mention, as a key example in the field. DARPins consist of the repetition of a 33 amino acid consensus sequence. The concatenation of a number of these repeats allows for the formation of highly stable scaffolds, which have evolved for their implementation in diverse areas of application.[8,23] The concave region of these scaffolds has been exploited as an epitope-interacting surface (Figure 2), presenting low nanomolar affinities as binders with special interest for their use in biomedicine.[12] The re-engineering of the interacting surface by the addition of extra loops (Loop DARPins) has extended the paratope surface and the binder capabilities.[24] In addition, the fusion of different epitope-interacting DARPins in the same chimeric protein has allowed the development of multivalent and multispecific antibody-mimetic proteins, such as the clinically tested antitumor MP0250.[25]

For its robustness, the consensus tetratricopeptide repeat (CTPR) protein platform has also been chosen as protein scaffold for antibody-mimetic development. Analogously to DARPins, CTPR proteins can form an extremely stable scaffold with a concave surface for ligand binding (Figure 2). Indeed, using a combination of rational and combinatorial design of the binding surface together with *in vivo* functional screening methods, several antibody mimetics against cancer key targets have been generated.[11,26,27] Moreover, CTPRs have been exploited to display short linear binding motifs (SLiMs) onto the loops between repeats to engineer mono- and multivalent binders, which expand even further the potential of the CTPR platform to assemble synthetic binding molecules with built-in multivalent capabilities and precise preprog-

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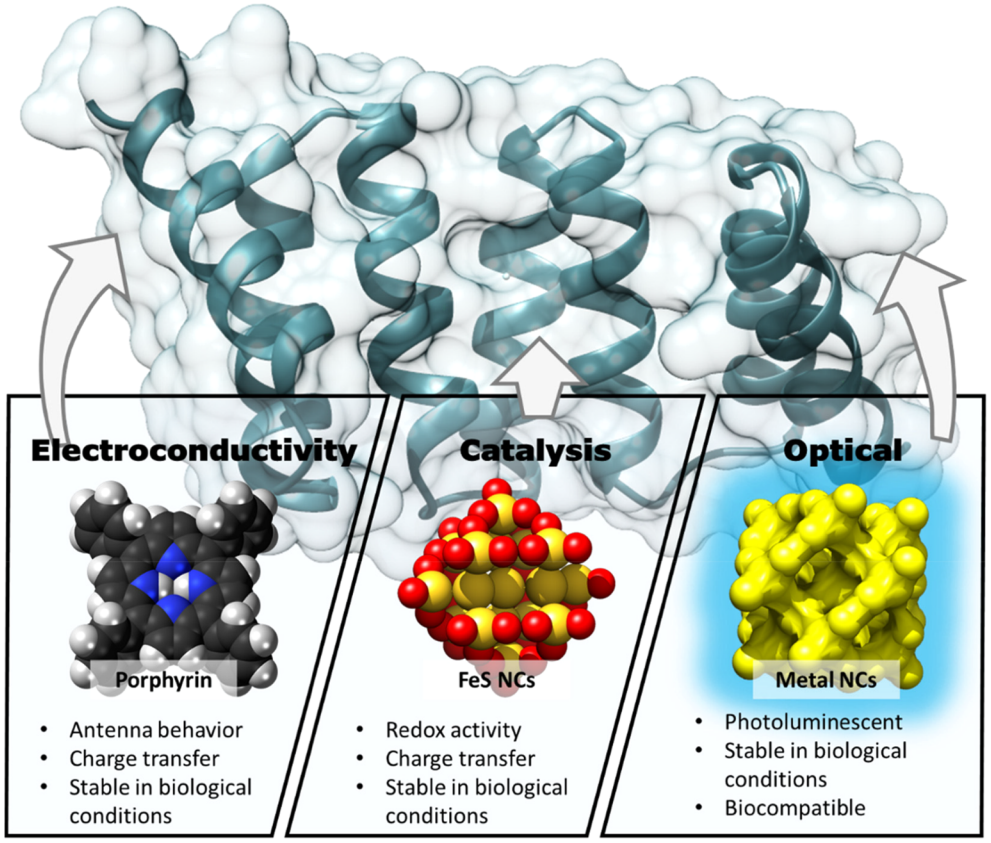

Figure 4. CTPR proteins have been engineered to conjugate or grow many different nanomaterials and functional elements leading to new hybrid systems. As an example, hosting a nanocluster within the designed pocket of a CTPR confers stability in physiological conditions to the NCs while providing fluorescence to the protein for tracking. Other examples of the many different properties that the hybrids could address in the biomedical field are electroconductivity by conjugation of synthetic molecules such as porphyrins and catalysis mediated by iron−sulfur NCs.

rammed geometries.[28] The following section will describe in detail the CTPR platform and its design potential toward the fabrication of hybrid systems.

CTPR REPEAT SYSTEM AS A VERSATILE SCAFFOLD FOR PROTEIN DESIGN

Although the complexity of protein design has been compared with the design of airplanes and the importance of the advances in computation for the progress of both of them acknowledged, the key role of physicochemical intuition in protein design has also been highlighted.[29] This intuition starts with the knowledge of the structures of the proteins. The natural propensities of the amino acids in proteins of the same family, meaning those with similar 3D structure, provide information on the role and importance of the amino acids within the protein structure and function. In 1990, the analysis of repeat motifs gave rise to the discovery of the tetratricopeptide repeat.[30,31] Each TPR repeat is composed of 34 amino acids that encode a helix−turn−helix motif. The stack of the helices triggers the formation of a characteristic right-handed superhelical tertiary structure.[32] The TPR motif is present in many other proteins with a wide range of functions, identifying the role of the TPR as mediator in protein−protein interactions.[33] The current CTPR was established in 2003 by analyzing 1837 TPRs of 107 different proteins (Figure 3)[34] which gave rise to the first designed TPRs.

More analytical protein design started with the application of simple models such as the amphipathic helices and contact maps. These models provide further guidelines in the allowance of amino acids with certain properties at given positions, but there is still plenty of room for intuition. For example, the amphipathic helices model shows where polar and nonpolar residues must be placed to keep the structure. Contact maps show the short-range character of the interactions in TPR, unlike globular proteins which require long-range interactions. These graphs allowed the understanding of which residues were involved in only intrahelical interactions and which were responsible for interhelical stability.[32] Additionally, simple 1D Ising models have been employed to understand different features of these proteins such as the thermodynamic stability and the effect of the number of repeats.[35,36]

TPRs have been designed to carry out different functions following physicochemical intuition and some design rules derived from the understanding of the natural structures. In the CTPR, only 8 of the 34 residues have structural significance, meaning that only these must be preserved to keep the tertiary structure, leaving plenty of space for functional mutations (Figure 3).[34] In-depth analysis of the crystal structures allowed for the understanding of the role of different amino acids in their structure but also in their function. This is the case of the aforementioned design of modules with specific binding capabilities[11,27] or the most recently developed TRAPs (Tetratricopeptide Repeat Affinity Proteins)−peptide interaction pairs that are functional *in vivo* and applicable as tools for super-resolution microscopy.[37,38] The natural ability of TPRs to mediate protein−protein interactions has also been

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exploited to build complex supramolecular architectures, expanding the scope of these proteins to applications in the materials field. Since the intrinsic ability of TPRs to self-assemble by forming highly ordered films through deposition on a surface was demonstrated, we have expanded this property further through design.[39] This has been the case, for example, of CTPR self-assembled monolayers on gold surfaces through cysteine-directed immobilization,[40] the formation of nanofibers by the assembly of CTPR units with cysteines on both edges,[41] and the supramolecular assembly of two CTPR into tubes with different intermolecular forces.[42]

Despite the simplicity and versatility of exploiting the robustness of the structure-based design, this approach limits the protein toolbox to existing natural motifs and to modifications that do not disrupt the natural scaffold. Computational methods have shown great success in overcoming this limit through the design of *de novo* proteins, expanding drastically the protein toolbox. For example, the use of the Rosetta algorithm to study the conformational space of repeat proteins shed light on the versatility that these modular proteins can reach far beyond the natural examples.[43]

ENGINEERING MULTIFUNCTIONAL PROTEIN HYBRIDS BASED ON CTPR SCAFFOLDS

Recently, we have made several efforts to bring proteins to another level of complexity by combining them with nanomaterials. The versatility that these hybrids bring to the biomedical field supports their continuous development and improvement. The possibility of engineering novel functional protein−hybrid nanomaterials in which the resulting structures and functions could be precisely tailored by the protein template has attracted tremendous interest in the last years and continues growing. In this context, the highly designable modular CTPR unit represents an ideal scaffold for the templating of different functional elements (Figure 4). For instance, we designed CTPRs to arrange porphyrin arrays by bioconjugation through engineered cysteine residues along the superhelix. The same scaffold was later designed to bind single-walled carbon nanotubes (SWCNTs), exploiting the presence of aromatic tyrosines and adding histidines to the core to make the CTPR wrap around the tubes,[44] giving rise to photoconductive donor−acceptor conjugates. Additionally, we developed other promising combinations with metallic clusters to give rise to bioinorganic hybrid materials. On these materials, the proteins are designed to control metal coordination and template the formation of nanoclusters (NCs), enhancing the control over the NC properties while incorporating a biomolecule that can mediate their application to biological systems. The shape of the CTPR and the protein/metal ratio can be used to control the size of Au and Ag nanoprisms and, hence, their plasmonic properties.[45] Furthermore, the strong binding of the CTPR with the clusters confers to the latter plasmonic ellipticity. Later, the effect that the inclusion of single cysteine residues for metal coordination had on the formation and properties of blue-emitting Au-NC was also assessed.[1] Overall, these hybrids have been built through modifying the CTPRs to include metal-binding amino acids through chemical intuition.[2,3,46,47] The lack of examples using computational approaches for the design of these hybrids suggests a limitation of the methods to deal with ions beyond standard metalloproteins. The potential of these nanomaterials shown in the following sections would encourage the development of computational design methodologies that can boost their applicability.

ENGINEERING PROTEIN−NANOMATERIAL HYBRIDS BASED ON CTPR SCAFFOLDS

In nature, several biological systems control the growth of inorganic materials whose structures and crystal phases are precisely dictated by different biomolecules, including proteins and peptides. The importance of molecular recognition in the nucleation of inorganic elements within living organisms and the envisioned technological potential derived from understanding and controlling the interactions between biomolecules and inorganic composites has been highlighted.[48] Inspired by these biomineralization processes in nature, the biotemplated synthesis of nanomaterials has recently flourished as a field. Protein-based templates enable the control over nanomaterial size, shape, and structure, barely achievable using standard synthetic procedures. In addition, these protein-directed synthetic procedures are performed under mild conditions, decreasing the environmental impact of nanomaterials while increasing their biocompatibility and stability under a wide range of pH and ionic forces, making them ideal for biological applications.[49]

The advances in protein design and recombinant techniques expanded the protein set found in nature and encouraged researchers to look for a variety of novel protein templates for the synthesis of multifunctional inorganic nanomaterials. The need for finding new metal-binding protein moieties and peptide sequences fueled the expansion of combinatorial approaches in biomimetics, such as phage and cell-surface display.[50] Other types of biological 3D assemblies were used for the templating of complex inorganic structures, such as virus crystals, bacterial protein crystals, amyloid fibers, or virus-based nanowires.[51]

Among all protein-metal hybrids, protein-stabilized noble metal NCs have attracted considerable interest due to their strong photoluminescence, size-dependent fluorescence properties, photostability, and biocompatibility. Attending to AuNCs, the most used method for their synthesis on proteins consists in the coordination of Au(III) ions on reactive amino acids, such as cysteines and histidines, followed by its reduction to Au(0) at a high pH or by the addition of strong reducing agents such as NaOH or $NaBH_4$.[52] Some other milder reductants such as ascorbic acid have also been used to achieve partial or total metal reduction. Inspired by previous pioneering works,[53] during the last years, we have explored the potential of designed repeat proteins as templates for the synthesis and stabilization of metal NCs. We first assessed the capability of CTPR proteins to act as templates for the synthesis and stabilization of fluorescent AuNCs using a single cysteine residue.[1] Next, we tackled the challenge of engineering metal-coordination sites into the protein template as a simple and general approach for the green synthesis of protein-metal NC hybrids to expand their applications. This work demonstrated that the grafting of a $[i, i + 4]$ bis-histidine motif on the CTPR α-helices created a metal-binding site that enabled the synthesis of protein−metal NC hybrids of different metal composition (Au, Ag, and Cu). Their excellent blue-emitting fluorescent properties (Φ = 6.3−9.5%), photostability, biocompatibility, and *in vitro* cell internalization made a great impact in the field.[3] The further introduction of strong gold binding sites, based on cysteines, into the CTPR scaffolds allowed the synthesis of blue-emitting protein−AuNC

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hybrids with a Φ of 10%[4] using a previously reported four Cys coordination site.[2] When the scaffold was extended to a 16 Cys coordination pocket, a mixture of blue- and red-emitting AuNCs was obtained with a remarkable record of Φ = 20%.[47]

Quantum dots (QDs) are another type of inorganic nanomaterials that have been intensively studied for their use as fluorescent reporters for biomedical applications due to their excellent fluorescence quantum yields, photostability, resistance to photobleaching, and large Stokes shifts. However, traditional chemical synthesis of QDs requires extreme reaction conditions, organic solvents, toxic reagents, and postsynthetic steps that hinder their biological application, and greener protein-based approaches produced QDs with low Φ.[54,55] To address this challenge, we recently investigated the design of a metal-coordination site based on four histidines into a CTPR repeat module and generated CTPR scaffolds with different numbers of units for the sustainable synthesis and stabilization of CdS QDs with improved fluorescent properties, photostability, and biocompatibility.[56] These protein−metal QDs hybrids were able to enter into living cells, showing great cell labeling capacity at very low doses, making them useful tools for biomedical applications.

Finally, magnetic nanoparticles (MNP) are inorganic nanomaterials with a wide range of potential applications, from contrast agents for magnetic resonance imaging (MRI) or tracer materials for magnetic particle imaging (MPI), to bioseparation, biosensing, or therapeutic approaches. Recently, the need to improve the uniformity and biocompatibility of the MNPs has motivated the development of reliable, sustainable, and reproducible methods. In this sense, some incipient works applied protein design to modify protein cages and scaffolds for the synthesis and stabilization of MNP with controlled features.[57] Currently, we are exploring the design of engineered CTPR protein scaffolds, presenting different sizes and iron-binding sites, for the sustainable synthesis of protein−IONP (iron oxide nanoparticles) hybrids controlling the size, morphology, and composition of the resulting IONP.

The aforementioned recent works on CTPR-based hybrids illustrate the potential of proteins as scaffolds for the tailored fabrication of nanomaterials through protein engineering approaches.

PROTEIN−NANOMATERIAL HYBRIDS TOWARD BIOMEDICAL APPLICATIONS

One of the aims of the development of protein hybrids is to create new therapeutic and detection agents. Despite protein−polymer or protein−dye conjugates being considered hybrids and widely used in biomedicine, we aim to focus this section on how proteins and nanomaterials can be combined to develop detection, therapeutic, and theranostic agents. While the protein counterparts can act as stabilizing scaffolds, drug reservoirs, and targeting agents, the properties of the nanomaterials can be exploited for detection and additional therapeutic modalities. In this synergy, therapeutic proteins can be fused to engineered scaffolds that host a nanomaterial, leading to a more versatile platform that upgrades the actual treatments or detection methods. Several publications have demonstrated the biocompatibility and viability of these systems *in vitro* and *in vivo*.[58] Although there are still scarce examples of biomedical applications using engineered protein hybrids, their native counterparts have proved to be extremely powerful.

Imaging is probably the most implemented application with these hybrids, based on either the fluorescence, radioactivity, or magnetic properties of the nanomaterials. In the case of fluorescence imaging, those that can overcome the tissue light absorption are especially interesting.[59] The introduction of magnetic or radioactive nuclei within the hybrid system greatly expanded the imaging performance by means of multimodal imaging.[60] Moreover, triple-modal imaging (NIRF, near infrared fluorescence/CT, X-ray computed tomography/MRI) has been achieved with BSA-AuNC and Gd(III) coordinated to the protein, showing an effective and highly accurate localization in mice bearing tumor xenografts without any toxicity.[61] From the therapeutic perspective, the presence of metals in the hybrids allows them to be adapted to a wide range of applications, such as secondary electrons emitting radiotherapy agents, reactive oxygen species (ROS) or O_2 generators for photodynamic therapy (PDT), or photothermal therapy (PTT) agents.[62−64] It is worth noting that in all these examples the protein scaffold acts as a bare template to protect and stabilize the AuNCs, without having any predefined role in defining the properties of the nanomaterial.

One step beyond is the development of theranostic agents capable of being tracked through different physiological barriers, detecting their accumulation in diseased tissues, and delivering an effective therapy. A remarkable work carried out by Yang and co-workers demonstrated how α-lactalbumin can host AuNCs and keep its ability to form an anticancer lipoprotein complex to deliver its therapeutic effect. This theranostic hybrid could be detected by NIRF, X-ray computed tomography, and MRI along with the molecular mechanisms of the anticancer AuNC−lipoprotein nanocomplex.[65] This collection of applications brings to light the opportunities and benefits of the protein-nanomaterial hybrids in biomedicine.

BIOMEDICAL APPLICATIONS OF ENGINEERED PROTEIN HYBRID BASED ON CTPRs

The aforementioned biomedical applications of protein−nanocluster hybrids have been generally restricted to natural proteins [BSA (bovine serum albumin), HRP (horseradish peroxidase), and lysozime]. To date, protein engineering in hybrid nanomaterials has been applied to unveil the mechanistic insights of NC formation and properties[66] or for single-molecule imaging in electron microscopy (EM),[67] but the biomedical applications remain mainly unexplored. This fact hinders the achievement of the aforementioned challenges of theranostics, which currently relies mainly on the use of antibodies. The ability to target, both extracellular and intracellularly, and to combine different functionalities takes the antibody-mimetic proteins beyond the antibody capabilities.

In this context, repeat proteins as the CTPRs present an exceptional framework for multidomain protein engineering and the development of theranostic multifunctional nanomaterials, adding a whole series of benefits to the design of protein binders. The path we chose with CTPRs in the development of chimeric proteins has exceeded the concept of protein-mimetics and has been focused on the search of multifunctional complex nanomaterials with novel attributes. CTPRs and other repeat proteins enable the engineering of multidomain proteins based on block architectures. Thus, by fusing discrete CTPR sequences with unique functions as building blocks, multifunctional proteins can be obtained. In

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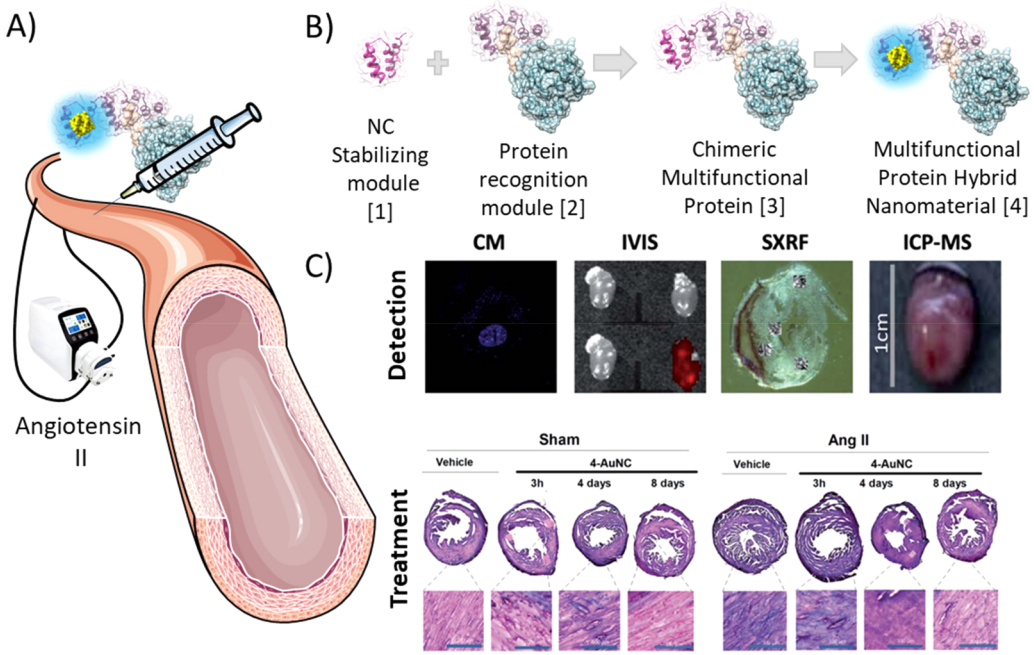

Figure 5. CTPR–AuNC hybrid for the treatment and monitoring of cardiac fibrosis. (A) Representation of the approach followed for the development of myocardial fibrosis (Angiotensin II treatment) and its treatment with the theranostic agent. (B) Scheme of the design and fabrication of a multifunctional protein hybrid. (C) Detection of the AuNCs in the diseased tissues and fibrosis evaluation. Detection: CM, confocal microscopy; IVIS, *in vivo* imaging system; SXRF, synchrotron X-ray fluorescence; ICP-MS, inductively coupled plasma mass spectrometry. Treatment: purple color corresponds to highly fibrotic tissue, which decreases when the protein hybrid is administrated. Adapted with permission from ref 4. Copyright 2021 The Royal Society of Chemistry.

this aspect, the potential benefits of a new generation of multifunctional hybrid metalloproteins have been presented in our recent proof of concept study.

Our group has recently demonstrated the concept of multimodal hybrid systems by the fusion of the CTPR390 binder[10,11] with a nanocluster stabilizing module, which enabled merging of synergistically therapeutics with diagnostics/monitoring (Figure 5). Among the CTPR designed binders, CTPR390 is an antibody-mimetic protein built on a three block CTPR scaffold (15 kDa) in which several mutations enabled binding to the C-terminal region of the eukaryotic chaperone Hsp90 with moderate affinity (~200 μM) but high specificity, inhibiting it.[11] Since Hsp90 is responsible for the folding of oncogenic proteins, its inhibition by a CTPR390 variant with higher affinity (1 μM) resulted in the reduction of HER2 protein production, responsible for cancer cell proliferation.[11] Additionally, the inhibition of Hsp90 has been reported to block the transforming growth factor beta (TGFβ) cascade and the reduction of the profibrotic effects of TGFβ in cardiac fibrosis, with no effect on its foldase function, probably due to the moderate affinity and low concentration of the CTPR390 used in this application.[10,69] Both *in vitro* and *in vivo* experimentation resulted in a concentration- and time-dependent down-regulation of Hsp90 clients involved in cancer or myocardial fibrosis, thus endorsing its exploration as new routes for the development of antifibrotic and anticancer therapies.[10,11] It is interesting to note that analogously to CTPR390, a new class of "C-terminal" Hsp90 chemical inhibitors has been proved effective as anticancers and devoid of the adverse effects arose in the past from the inhibition of ATPase activity.[68] On the other hand, the metal NC stabilizing module was engineered with four Cys residues as metal coordination site in the CTPR concave surface in which a AuNC was grown through a mild reducing process, with no effect on the Hsp90 binding affinity.[4] The presence of fluorescent AuNCs allowed the tracking of the binder *in vitro* and *ex vivo* by fluorescence-based techniques and by "detecting" the metal in therapeutically treated *in vivo* samples by synchrotron X-ray fluorescence at micrometer scale.[4] When studied in a cardiac fibrosis mouse model, the protein-hybrid effectively reduced the myocardial fibrosis and heart hypertrophy via the Hsp90 inhibition function. After 8 days of a single dose administration, heart cross sections from fibrotic animals were comparable to those of the control group, due to a drastic reduction of fibrotic areas after the treatment both phenotypically and at the molecular level. Moreover, on account of the multimodality character of the hybrid antifibrotic, it was possible to conclude that the therapeutic effect was linked to its accumulation in the fibrotic tissue. Thus, this comprehensive study entails the first engineered protein–nanocluster hybrid drug as theranostic tool and the beginning of a new generation of genetically encoded multimodal nanomaterials.[4]

■ CONCLUSIONS AND PERSPECTIVES

The escalating number of approvals of protein-based biopharmaceuticals by the regulatory agencies and the onset of their mainstream use in the clinical practice have drawn the attention of key players that are now focused on the development of the next generation of pharmaceuticals: protein-based theranostics. Leaving aside the development of new antibodies and their derivatives, designed protein binders emerge as alternative candidates. Many of them exert their therapeutic function by modulating the structure and function of their target, as antibodies do, but are more efficient in terms of reproducibility, stability, biocompatibility, and cost of

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production. Moreover, designed protein binders can be finely tailored to present a high-affinity toward target molecules. In this context, repeat proteins offer an optimized and sturdy platform. Repeat proteins arise from the repetition of simple sequences, in which few amino acids have structural roles, while mutations in the others impart specific functionalities to the protein. Moreover, repeats can be considered as independent building blocks that can be assembled to generate multifunctional proteins comprised of different domains, including nanomaterial stabilizing units. Since the properties of nanomaterials are highly dependent on their size, shape, and structures and protein-based templates enable control over those features, the design and development of templates to coordinate nanomaterials will be essential to produce the next generation of functional hybrids. In this context, in addition to the rational protein design, the development of new computational paradigms that boost improvements or help in the materialization of advanced protein design will define future advances.

In particular, we have extensively explored the CTPR protein platform looking for innovative solutions to numerous applications, from protein−protein recognition to protein−nanomaterial stabilization. In this Account, we emphasized the potential of new protein-hybrid nanomaterials in the biomedicine field. Through rational design of the CTPR scaffolding modules, complex hybrid materials have been formed by tailoring the interactions between the different components of the hybrid material and defining binding sites. These highly tunable protein hybrids stand at the frontier of the development of advanced nanomaterials. This approach has made possible the production of hybrid materials with, for example, noble metal nanoclusters, QDs, or magnetic nanoparticles. All of these hybrids display unique capabilities that allow for their implementation in clinical imaging and/or therapeutics. But more interestingly, these protein−nanomaterial hybrids not only bring together what both components can do separately but also exhibit new specific properties arising from their hybridization. Recently, a pioneering example of protein-hybrid nanomaterials comprising a chimeric fusion of an effective binder and protein-stabilized AuNCs was tested *in vivo*, paving the way for the use of protein−nanomaterial hybrids as therapeutic and diagnostic agents. Therefore, this sheds light on the myriad of potential applications that these hybrid nanomaterials can tackle, namely, the development of protein-based theranostic agents by the incorporation of traceable nanomaterials into the protein scaffold. The optimization for a complete adaptation to the preclinical research stage or the search for new and unexplored niches of opportunity will be some of the future challenges that these protein−nanomaterial hybrids will have to face.

In perspective, a tantalizing area of research has been newly established around protein−nanomaterial hybrids, which has only just begun to give a glimpse into its opportunities. Hybrids generated from novel protein functionalities as well as combinations with improved nanomaterials will lead the world of theranostics toward previously unattainable goals.

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■ ABBREVIATIONS

Ab, antibody; BSA, bovine serum albumin; CM, confocal microscopy; CTPR, consensus tetratricopeptide repeat; CT, computed tomography; DARPin, designed ankyrin repeat proteins; EM, electron microscopy; Fab', antigen binding fragment; HER2, human epidermal growth factor 2; HRP, horseradish peroxidase; Hsp90, heat-shock protein 90; HSA, human serum albumin; ICP-MS, inductively coupled plasma mass spectrometry; IONP, iron oxide nanoparticle; IVIS, in vivo imaging system; mAb, monoclonal antibody; MNP, magnetic nanoparticle; MPI, magnetic particle imaging; MRI, magnetic resonance imaging; NC, nanocluster; NIR, near-infrared; NIRF, near-infrared fluorescence; PDT, photodynamic therapy; PTT, photothermal therapy; QD, quantum dot; Φ, quantum yield; ROS, reactive oxygen species; scFv, single-chain variable fragment; SXRF, synchrotron X-ray fluorescence; SWCNT, single-walled carbon nanotube; TGFβ, transforming growth factor beta; TPR, tetratricopeptide repeat; TRAP, tetratricopeptide repeat affinity proteins; VHH, single variable domain on a heavy chain.

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