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Protein scaffolds in human clinics

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ABSTRACT

Fundamental clinical areas such as drug delivery and regenerative medicine require biocompatible materials as mechanically stable scaffolds or as nanoscale drug carriers. Among the wide set of emerging biomaterials, polypeptides offer enticing properties over alternative polymers, including full biocompatibility, biodegradability, precise interactivity, structural stability and conformational and functional versatility, all of them tunable by conventional protein engineering. However, proteins from non-human sources elicit immunotoxicities that might bottleneck further development and narrow their clinical applicability. In this context, selecting human proteins or developing humanized protein versions as building blocks is a strict demand to design non-immunogenic protein materials. We review here the expanding catalogue of human or humanized proteins tailored to execute different levels of scaffolding functions and how they can be engineered as self-assembling materials in form of oligomers, polymers or complex networks. In particular, we emphasize those that are under clinical development, revising their fields of applicability and how they have been adapted to offer, apart from mere mechanical support, highly refined functions and precise molecular interactions.

1. Introduction

Structure supports life. Then, living beings are mainly composed by soft matter (cells and tissues). Being highly hydrated and with a primary gel-like cell organization, those materials require supporting structures to acquire sufficient mechanical stability to endure environmental pressures (Dalby et al., 2014; Guimarães et al., 2020) and to interact with the media through bi-directional mechanical signals (Kumar, 2014). From lower to higher organization levels, cytoskeleton, membranous systems, cell walls, extracellular matrices, cartilage, bones, xylem and exoskeletons sustain, at different levels of stiffness, the complex functional dynamism required for life (Brule et al., 2016; Chen and Ingber, 1999; Deville and Cordes, 2019). Some of these scaffolds recruit inorganic components for tailored functionalities. Combined with cells and organic protein matrices, the resulting composites are particularly robust platforms. In higher animals, bones are representative of such hybrid materials, in which approximately 70 % of the mass is provided by mineral-based complexes (Boskey, 2013). Mainly formed by calcium and phosphorus, the resulting trabecular structures support the biomechanical profiling of the whole body and the complex functions related to both physical resting and movement (LeVeau and Bernhardt, 1984).

Organic scaffolds include membranous systems and proteins. Among them, proteins provide multidimensional structural stability and a notable tensegrity (tensional integrity), required for cell sensing, migration and morphogenesis, and to keep the geometry of mature cells against deformation forces (Chen et al., 2010; De Santis et al., 2011; Volokh, 2011; Volokh et al., 2002). These properties are combined with an unusually dynamic functional versatility, in part mediated by the capability of proteins to respond to external stimuli by conformational changes (Boehr et al., 2018; Huck, 2008; Orellana, 2019; Shah et al., 2018; Ulijn and Lampel, 2020). Such a blend of mechanical stability and responsiveness is not observed in any other organic or inorganic material. Protein scaffolds usually consist of combinations of several

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polypeptides or multiple copies of a single protein species, that form supramolecular structures supported by self-assembling domains. Found in many polypeptides, cross-interacting protein motives allow generating a wide spectrum of protein materials, in form of fibers, particles, cages, matrices or layers (Corchero et al., 2014). These elements sustain subcellular structures such as amyloids, viral capsids and vaults, but also whole cells, tissues and organs. Furthermore, protein materials show supportive roles out of the body. Tools involved in biofilm formation in single-cell organisms (pili and related bacterial structures (Epler Barbercheck et al., 2018)), adhesive pads in climbing plants (Burris et al., 2018) and animals (Hallahan et al., 2009), viscoelastic gels in marine invertebrates (Smith, 2002), adhesive worm secretions (Corrales-Ureña et al., 2017), silks in spiders (Yarger et al., 2018) and underwater adhesive fibers of mussels (Park et al., 2019) among others, are based on particular protein species often found as nanostructured entities.

A significant part of industries relies on the fabrication of materials with supportive roles. When looking for application in biological interfaces, apart from the needed geometry and mechanical properties, biocompatibility and biodegradability are required to avoid the often observed toxicity linked to xenobiotic substances (Raftis and Miller, 2019; Yuan et al., 2019). Because of the above-mentioned properties of natural proteins and their intrinsic biocompatibility, they emerge as ideal biomaterials in clinics. Apart from the mimicry of natural protein functions in synthetic constructs (Hansen and Khare, 2020; Wang et al., 2020; Xu et al., 2020) the capability of de novo designing polypeptides expand the spectrum of application. This would cover functionally inert entities useful as plain scaffolds but also bioactive materials that combine mechanical properties and refined functionalities. The spectrum of those protein-based platforms (Fig. 1) spans from single polypeptidic chains as mere drug carriers or stabilizers (such as human serum albumin, HSA, in the paclitaxel nanoscale formulation called Abraxane (Ma and Mumper, 2013), Table 1) to complex macromolecular entities such as protein-based matrices, hydrogels or related architectures. Mimicking the extracellular matrix (Bhattacharjee et al., 2017; Wu et al., 2018), these later offer mechanical support in regenerative medicine (Fig. 1, Table 2). In an intermediate concept, relatively simple nanoscale oligomers (Cespedes et al., 2018a; Molino and Wang, 2014) (including virus-like particles (Hill et al., 2018)) are highly convenient as selective carriers for targeted drug delivery and theragnosis (Fig. 1, Table 1). Most of these materials result from the biological fabrication of recombinant building blocks in cell factories followed by spontaneous or induced assembly. The recombinant production approach makes thus possible a genetic tuning of the amino acid sequence of such polypeptides (Corchero et al., 2013). Aiming at enhancing biocompatibility, humanizing the resulting materials, mostly based on non-human proteins, is a particularly challenging task. In the next sections, the complexity of such clinically-oriented protein materials is exemplified by three main categories, namely plain monomeric polypeptides, nanoscale oligomers and complex protein networks.

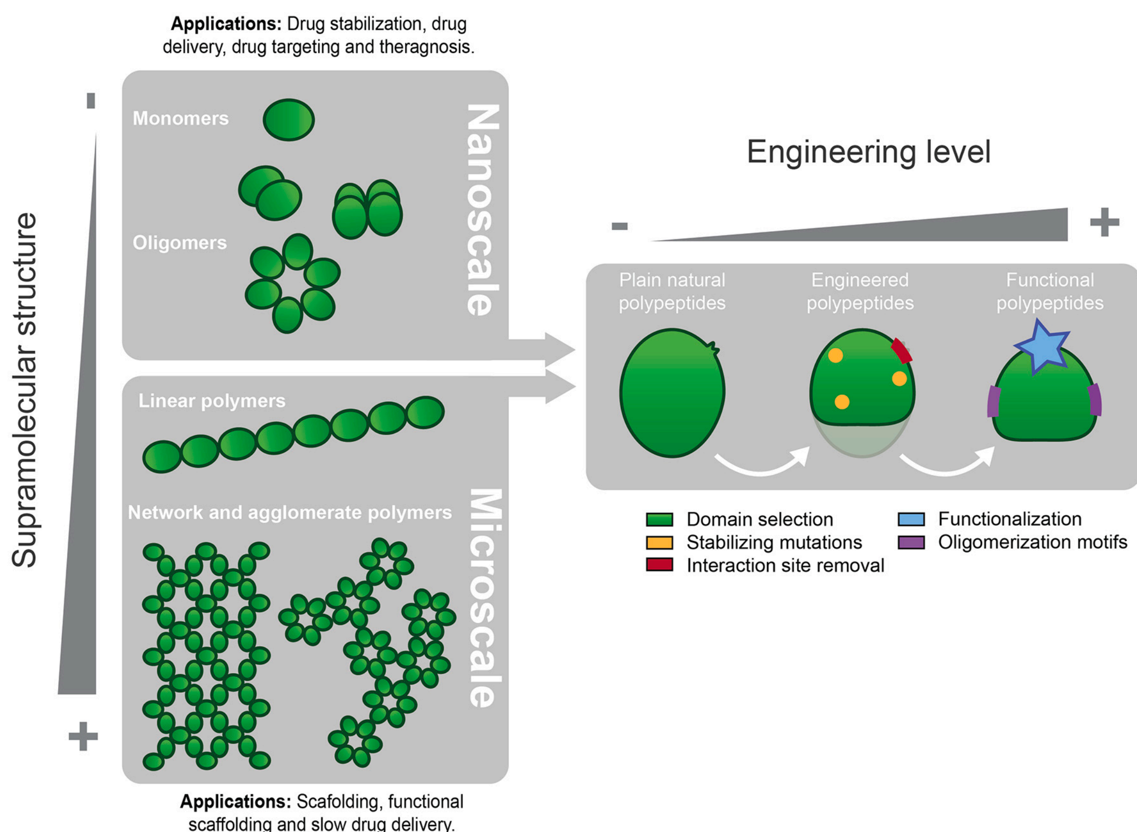

Fig. 1. Engineering and application of protein scaffolds in biomedicine. In the vertical axis, different protein oligomerization levels are presented, spanning from plain monomeric forms to progressively complex structures. Two main sets of applications are indicated; while monomeric or nanoscale protein complexes have been mainly used as carriers for drug delivery (upper section), more complex polymers or networks (usually within the microscale) are essentially tailored as scaffolds in the context of tissue engineering (bottom section). On the other hand, the horizontal axis indicates the extent of protein engineering. It starts from plain recombinant versions of natural proteins and moves towards conferring advanced functions, including oligomerization (by engineering protein-protein contacts) or the incorporation of functional domains such as toxins, growth factors or hormones, by their genetic fusion to the building blocks. The abolition of undesired interactivity of these proteins with cell or body components and their de-immunization or humanization is in general required when intended for systemic administration.

Table 1
Representative examples of polypeptides used as scaffolds in drug delivery.

Protein	Origin	Form	Application or target tissue	Reference
Ferritin	Human	Nanocages	MRI contrast	(Klem et al., 2008)
			Cancer therapy	(Zhen et al., 2013)
Nidogen G2	Human	Nanoparticles	Cancer therapy	(Alamo et al., 2021a)
Stefin A	Human	Unassembled	Peptide display	(Woodman et al., 2005)
		Nanoparticles	Cancer therapy	(Serna et al., 2022)
CTP	Human	Nanoparticles	Cancer therapy	(Serna et al., 2022)
Albumin	Human	Unassembled, clustered	Cancer therapy	(Gradishar, 2006)
		Nanoparticles	Bioimaging	(Chen et al., 2016)
		Nanoparticles	Ocular drug delivery	(Tiwari et al., 2021)
Elastin	Human	Nanoparticles	Cancer therapy	(Hu et al., 2015)
Fibrin	Human	Matrix	Nerve regeneration	(Taylor and Sakiyama-Elbert, 2006)
Chaperonin 10	Human	Nanoparticle	Multivalent peptide display and targeting.	(Alsultan et al., 2016)
FN3	Human	Unassembled	Peptide display and targeting	(Bloom and Calabro, 2009; Getmanova et al., 2006)
Centyrin	Human	Unassembled	Targeted drug delivery	(Goldberg et al., 2016; Klein et al., 2021)
		Unassembled	Targeted imaging	(Klein et al., 2021)
mCH3	Human	Unassembled	Cancer therapy	(Shen et al., 2019)
Silk	Non-human	Hydrogel	Drug delivery	(Kundu et al., 2012)
Keratin	Non-human	Sponge	Drug delivery	(Rouse and Van Dyke, 2010)
GFP	Non-human	Nanoparticles	Cancer therapy	(Cespedes et al., 2018b)

Table 2
Representative examples of polypeptides used as scaffolds in regenerative medicine.

Protein	Origin	Form	Application or target tissue	Reference
Collagen	Human	Self-assembling lattices	Vascular	(Achilli et al., 2012)
		Cross-linked neoglycopolymer	Cornea	(Merrett et al., 2009)
Fibrin	Human	Sealant	Hemostat and wound repair	(Spotnitz, 2014)
		Hydrogel	Bone regeneration	(Kneser et al., 2005; Peretti et al., 2006)
Silk	Non-human	Hydrogel	Angiogenesis	(Arkudas et al., 2007)
		Fibroin	Bone	(Ding et al., 2021)
Gelatin	Non-human	Gel	Wound dressing	(Suzuki et al., 2013)
Keratin	Human	Hydrogel	Nerve regeneration	(Sierpinski et al., 2008)
		Hydrogel	Hemostat	(Aboushwareb et al., 2009)
		Cation formed film	Wound dressing	(Fujii and Ide, 2004)
Albumin	Human	Protein cross-linking	Proof-of-concept (bone)	(Li et al., 2014)
Soy protein	Non-human	Freeze-dried	Proof-of-concept	(Chien et al., 2013)
		Nanofiber	Proof-of-concept	(Ramji and Shah, 2014)
		3D bioprinting	Proof-of-concept	Chien et al., 2013
FGF-2	Human	Inclusion bodies	Wound repair	(Seras-Franzoso et al., 2014)
		Artificial inclusion bodies	Wound repair	(Serna et al., 2020)
Lipoxygenase	Non-Human	Inclusion bodies	Wound repair	(Stamm et al., 2018)
Elastin	Human	Hydrogel	Tissue engineering	(Fernández-Colino et al., 2018)

2. Results and discussion

2.1. Single polypeptides as plain drug carriers

Some proteins themselves are drugs, which do not need additional structural support apart from the appropriate formulation. Enzymes used in enzyme-replacement therapies, (Li, 2018; Marchetti et al., 2022) antibodies or antibody fragments in oncotherapy (Duan and Luo, 2021) have an intrinsic dual role as drug and structural agent. For instance, antibody-only drugs can suppress tumor growth, either by inhibiting signaling from specific receptors in cancer cells to induce their death (Trastuzumab, Cetuximab), by blocking endothelial receptors to inhibit tumor angiogenesis (Bevacizumab), or instead by blocking immune checkpoints to activate the immune system against the tumor (Pembrolizumab, Nivolumab, Ipilimumab) (Wong et al., 2021; Zahavi and Weiner, 2020). Antibody-drug nanoconjugates (ADCs), such as Gemtuzumab Ozogamicin (Jen et al., 2018), Trastuzumab Deruxtecan (Keam, 2020), Brentuximab Vedotin (Richardson et al., 2019) and others (Kadkhoda et al., 2021; Lu et al., 2021; Serna et al., 2018) use the antibody scaffold to achieve targeting to specific cancer cells to subsequently trigger the release of conjugated conventional drugs in their cytosol. Similarly, immunotoxins (ITs), such as Denileukin diftitox, Tagraxofusp-erzs or Moxetumomab pasudotox (Khirehgesh et al., 2021; Kreitman and Pastan, 2021) also use antibody scaffolds as targeted carriers to transport a bacterial toxin, either covalently linked or genetically fused, to be internalized triggering cytotoxic activity in cancer cells. Both types of antibody-based materials must be administered at their maximal tolerated dose to be effective, a procedure that often associates with severe side effects. Therefore, their clinical use is usually limited to a single cancer type that lacks an effective therapy (Jen et al., 2020; Serna et al., 2018). The side effects induced by the ADCs and ITs could be due to the induction of cytotoxicity through the release of the transported drug on normal cells that express the targeted receptor (on-target effect). Moreover, the adverse effects could also derive form the lack of biological neutrality of the antibody scaffold that interacts with Fc receptors expressed on normal cells (off-target effect) (Criscitiello et al., 2021; Wilkinson et al., 2021). In addition, both particular nanomedical approaches have been extensively reviewed, also by us (Sanchez-Garcia et al., 2016; Serna et al., 2018), and are out of the scope of the present review that focuses on the plain scaffolding uses of proteins in clinics.

On the other hand, different categories of recombinant, single-chain polypeptides have been exploited, as natural or engineered forms, to be used as convenient partners for drug delivery. They are intended for stabilizing the drug or for increasing the size of the whole pharmacological complex, thus preventing renal clearance. In advanced constructs, the carrier might also confer cell type selectivity in the delivery process. The human serum albumin (HSA) was the first FDA-approved human protein scaffold for drug delivery (in form of Abraxane (Gradishar, 2006)). Its structure is well known, stable, and non-immunogenic, exhibiting high biocompatibility and long plasma

circulation time. In contrast, HSA presents complex post-translational modifications and multiple interactions with receptors in several body tissues (Merlot et al., 2014), what impedes biological neutrality and aborts any potential for receptor-targeted delivery. In this sense, a plain HSA has been widely used as human scaffold for several nanomedical applications (An and Zhang, 2017) in absence of precise engineering addressed to improve its performance. Interestingly, alternative human scaffolds have also attracted attention as tools for drug delivery. Still under development, they exhibit some superior properties to those shown by HSA, including high manipulability and less molecular interactivity. In contrast to HSA, these alternative scaffolds have been engineered at different levels of complexity, paying special attention to strategies to oligomerize them in form of multimeric materials, by a proteomic control of cross-molecular contacts and self-assembling. The views and concepts underlying these strategies are illustrated in the next section through representative examples.

2.2. Nanoscale protein oligomers in drug delivery

In the context of drug delivery, Stefin A-derived proteins represent a paradigmatic example of rational design of human protein scaffolds for nanomedical applications (Woodman et al., 2005). Stefin A is an intracellular small single-chain, single-domain protease inhibitor with high structural stability and no post-translational modifications. Its structure has been also completely solved and solvent-exposed regions that are suitable for drug conjugation or peptide presentation have been identified (Jenko et al., 2003). On this basis, a structurally stable variant called STM (Stefin A triple mutant) was created so that it abolished the natural interactivity of the protein. This version is based on two selected amino acid substitutions involved in protein-protein interactions plus an additional modification in a solvent exposed loop to allow peptide insertion for surface presentation (Woodman et al., 2005). Through this engineered accommodation site, STM has been successfully used as a scaffold to display peptide libraries (Woodman et al., 2005) and to perform specific target protein interactions (Evans et al., 2008). Also, and by engineering its self-assembling through short end-terminal architectonic peptides, STM-based oligomeric nanoparticles have been used to selectively deliver the ultra-potent anti-mitotic drug monomethyl Auristatin E in acute myeloid leukemia models (Serna et al., 2022). Later, the versatility of the scaffold has been expanded by introducing additional multiple peptide insertion sites (SQM, Stefin A quadruple mutant) (Hoffmann et al., 2010). A trivalent SQM version has proved to be successful in the detection of target proteins (Song et al., 2011) and in simultaneous peptide presentation (Hoffmann et al., 2010). Starting from such trivalent SQM version, a new variant has been generated called SQT (stefin A quadruple mutant-Tracy), with significantly improved structural stability and higher tolerance to multiple peptide presentation (Stadler et al., 2011). SQT has been used for the presentation of BH3 domains for interaction with pro-apoptotic effectors (Stadler et al., 2014). Recently, it has been shown that SQT with AU1 and Myc peptide insertions (called SQT-1C) spontaneously oligomerized into dimers and tetramers by a loop-mediated domain swapping process (Zalar et al., 2019). The slightly minimized stability of this mutant was recovered by introducing a disulfide bond that locked the monomeric state (Zalar and Golovanov, 2019).

As a further example, the human nidogen is a multi-domain structural protein from the basement membranes that naturally binds collagen IV, perlecan and laminin (Takagi et al., 2003). Interestingly, the G2 domain of nidogen shows a stable β-barrel structure identical to *Aequorea Victoria* Green fluorescent protein (Hopf et al., 2001), which has already proved to be structurally robust and stable in blood circulation when used in oligomeric vehicles for drug delivery (Cespedes et al., 2018b; Pallares et al., 2020). Moreover, since this protein naturally lacks post-translational modifications, its easy production in bacterial cell factories is feasible. Then, the G2 domain of the human nidogen has been recently engineered to abolish its natural binding to extracellular matrix components and thus, to generate a GFP-like non-fluorescent human scaffold, called HSNBT (Alamo et al., 2021a). This was achieved by applying *in silico*-predicted mutations of four residues implicated in the interaction with collagen IV and perlecan, that resulted into a biologically neutral, novel protein scaffold successfully validated in drug delivery (Alamo et al., 2021a).

The human chaperonin 10 (hCpn10) is an intracellular homo-oligomeric protein composed by 7 subunits that assist protein folding or re-folding. It is a stable beta-barrel core that does not undergo post-translational modifications, shows low immunogenicity and it has been safely tested in clinical trials (Alsultan et al., 2016; Broadley et al., 2009). The loop responsible for the natural interactivity of Cpn60 has been successfully substituted for different target-specific ligands, thus preventing the native binding and conferring multivalent ligand display with different clinical applications. Molecular dynamic modelling was used to design and insert linkers at the junctures of the mobile loop to prevent interferences of inserted ligands with the subunit interface and to maintain the heptameric structure that allows multivalent peptide presentation (Alsultan et al., 2016).

The human 10th fibronectin type III domain (FN3) is a structurally stable small protein with a beta-sandwich fold similar to the variable domain of antibodies. It shows no post-translational modifications and consequently it has been successfully produced in a variety of eukaryotic and prokaryotic expression systems. Moreover, its high levels in extracellular body fluids permit to envisage minimal immunogenicity for any FN3-based scaffold (Chandler and Buckle, 2020; Koide et al., 1998). FN3 naturally binds multiple targets involved in the formation of the extracellular matrix, and natural ligand-binding loops have been successfully engineered to confer new affinities for alternative therapeutic targets. For that, the human FN3 has been used as a scaffold to display peptide libraries in randomized loops in directed protein evolution studies and to generate new target-specific proteins, called monobodies (Bloom and Calabro, 2009; Getmanova et al., 2006). A derived scaffold based on two different proteins has been generated by a consensus sequence approach of different FN3 domains within human Fibronectin and Tenascin-C (Centyrins). Additional rational design was then applied to still increase the structural stability of the consensus FN3 domain. This complex approach resulted in a very stable scaffold that was robustly expressed in prokaryotic systems such as *E. coli* and was also able to display functional peptides accommodated in its exposed loops (Jacobs et al., 2012). Later, an EGFR-targeted FN3 consensus scaffold has been further engineered to introduce site-specific Cys residues to be used for drug conjugation, devoted to generate a receptor-targeted drug delivery platform. In this sense, centyrins have been then successfully conjugated with MMAF (Goldberg et al., 2016) or siRNA (Klein et al., 2021) molecules to be specifically delivered into EGFR^{+} cells. The same EGFR-targeted FN3 scaffold has been also used for fluorescent dye conjugation and in vivo fluorescence-guided surgery applications (Mahalingam et al., 2017). In a similar approach, the antimicrotubule agent DM1 has been conjugated in a Cys-functionalized consensus FN3 scaffold (Shi et al., 2018).

Finally, the human Ig-G constant domain (Fc) is a homodimer protein composed by one glycosylated CH2 and non-glycosylated CH3 domains that show long half-live in plasma but no interaction with the target antigen due to the missing variable region. However, the Fc domain still naturally binds different effectors such as Fcɣ receptors, the FcRn receptor and the complement factor C1q. In this sense, several mutations that inhibit the interaction with most of those receptors have already been described (Davis et al., 2007; Glaesner et al., 2010). Other mutations have also been reported to minimize the interaction with the FcRn receptor (Jain et al., 2007). To generate a less complex scaffold, several mutations have been introduced that generate highly stable monomeric Fc versions, that can be efficiently produced as non-glycosylated forms in *E. coli* (Ying et al., 2012). Also, an unglycosylated CH2 domain has been further isolated and produced in bacteria resulting in a smaller protein scaffold with conserved structure

(Prabakaran et al., 2008). Its lower stability and aggregation tendency has been further corrected by the deletion of several critical residues (Gong et al., 2013). Similar strategies have been used to develop an isolated monomeric CH3 domain scaffold by introducing four punctual mutations. Very interestingly, the new mCH3 scaffold shows weaker FcRn receptor binding than Fc and a complete loss of Fcɣ and C1q receptor binding. Moreover, the incorporation of an additional disulfide bond within the protein structure has considerably improved its stability (Ying et al., 2013; Ying et al., 2014). In this sense, the mutated mCH3 scaffold has been successfully used to improve the pharmacokinetics of a genetically fused immunomodulatory Tα1 peptide by significantly increasing its circulation half-life (up to 47h) and its therapeutic activity in tumor xenograft models (Shen et al., 2019). Other representative examples are summarized in Table 1.

Of course, the engineering of human proteins for functional gains or to confer new interactions or higher stability, even limited to specific and usually short regions, might generate new non-natural epitopes with potential to trigger an inappropriate immune response upon administration in humans. Even plausible, such effects have not been generically described (Fleishman and Mariuzza, 2022; Narayanan et al., 2021) even using non site-directed approaches, such as molecular evolution (Liu et al., 2019), and protein engineering in the context of biopharmaceuticals has proved to be successful and safe (Lagasse et al., 2017; Narayanan et al., 2021; Radziwon and Weeks, 2021). Importantly, the analyses of remote effects of mutations on protein structure and stability offer a limited but important catalogue of alternative mutation targets in a protein engineering process (Wilding et al., 2019). Finally, it must be noted that the risk for immunotoxicities of modified human proteins should globally remain much lower than when straightforward using non-human proteins.

2.3. *Protein scaffolds for complex materials in tissue engineering and regeneration*

Tissue engineering recruits a set of strategies aimed to regenerate damaged tissues assisted by biomimetic platforms, which combining cells, scaffolds and bioactive factors should bring an appropriate environment for regeneration or improvement of tissue function (Fig. 2). The scaffold moiety plays a critical role in presenting biochemical, structural and mechanical properties similar to those of the extracellular matrix (Hussey et al., 2018), and it is expected to offer sufficient plasticity, biocompatibility and biodegradability in a range appropriate for given applications (Boekhoven and Stupp, 2014). In this context, protein polymers are exploited as scaffolds in cartilage regeneration, wound healing, vascular grafts and tracheal splints. Synthetic polymers such as poly-glycolic acid (PLG) and poly-lactic acid (PLA) show relatively high tunability (through the modification of the molecular weight and crosslinking process) but relatively low capacity to induce appropriate cell responses through mechanical and biological stimuli (Elmowafy et al., 2019). In addition, their capacity to affect the performance and activities of the immune system (through a recognized immunomodulation potential) (Dobrovolskaia et al., 2016; Khademi et al., 2018) represents a matter of concern when used for non-vaccine purposes. Natural human and non human protein polymers such as collagen, elastin and silk offer optimal biochemical signals for communication with cells (Costa et al., 2018). In this sense, generating protein-only scaffolds exclusively made of human proteins is feasible, and a design based on a modular repetitive pattern would allow the ordered construction of complex, humanized supramolecular structures (Corchero et al., 2014). This type of design should be based on repeating structural units of the natural protein components of human extracellular matrix that include collagen, elastin and fibrillin, among others. These building blocks self-assemble into hierarchical structures such as peptide fibrils that form the structural backbone of the biomaterial. The predominant format to formulate these proteins is the hydrogel, which permits to incorporate cells in an aqueous media suitable for proliferation and differentiation. Those hydrogels also provide the possibility of biological interactions for cell adhesion and degradability (Lee and Kim, 2018), and they are used as a glue in many surgical uses (Ahmed et al., 2008; Apel et al., 2008).

In addition, further protein engineering allows the incorporation of a set of biologically active proteins for enhanced functionalities (Bichara et al., 2012; Lorentz et al., 2011) (Fig. 1). The integrin RGD sequence either naturally present in keratin or added in elastin by protein engineering increases cell adhesion and growth on scaffold surfaces (Daamen et al., 2007; Mogosanu et al., 2014). The use of the heparin-binding site A from fibrin permits capturing growth factors for their slow release (Noh et al., 2015; Yang et al., 2010; Yang et al., 2011). Cross-linking scaffolds are also under development towards more stable sponge-like surface in which cells could adhere but at the same time allowing free

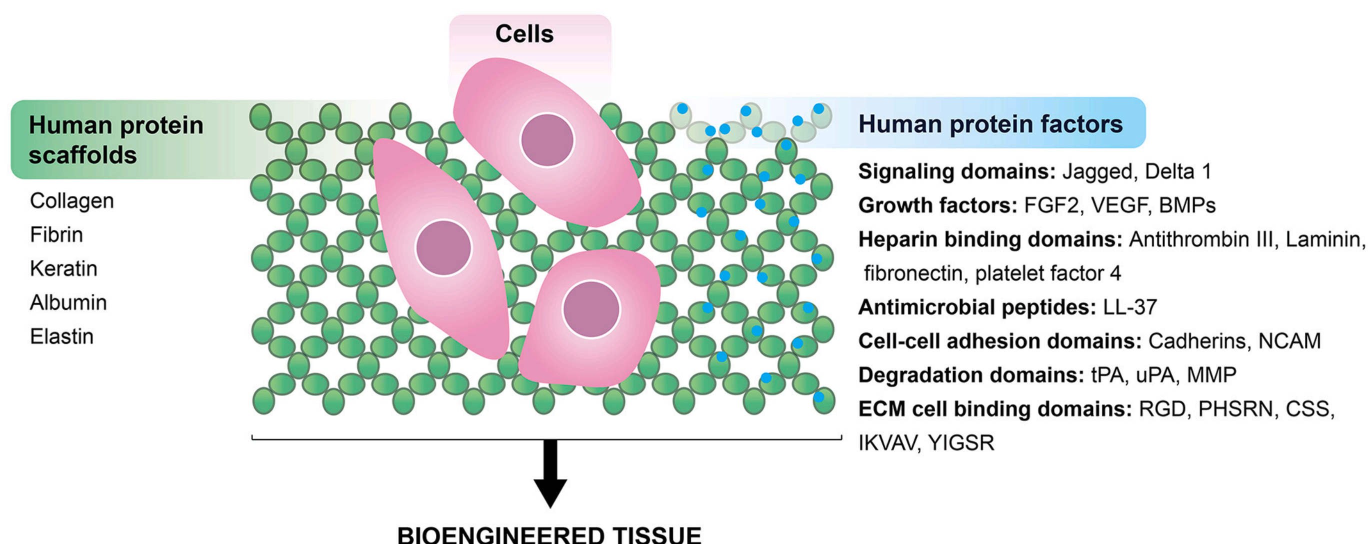

Fig. 2. Paradigm of human protein-based materials for tissue engineering. In tissue engineering, plain scaffolds (left) can be functionalized or combined with functional agents (right) for multifaceted interactions with cells (center). FGF2: Fibroblast growth factor 2; VEGF: Vascular endothelial growth factor; BMPs: Bone morphogenetic proteins; NCAM: Neural cell adhesion molecule; tPA: tissue plasminogen activator; uPA: urokinase-type plasminogen activator; MMP: Matrix metallopeptidases; RGD: arginylglycylaspartic acid motif is the integrin recognition domain found in fibronectin; PHSRN: Fibronectin motif; CS5: residues 90-109 of the type III connecting segment region of fibronectin. IKVAV and YIGSR: Laminin motifs.

movement of water and nutrients. These structures can be polymerized by protein–carbohydrate interactions (Merrett et al., 2009) and covalent proteins bonds (Li et al., 2014), among others.

From a different point of view, bacterial inclusion bodies are self-assembling secretory amyloids in between the nano- and micro-scales (de Marco et al., 2019), that spontaneously formed in recombinant bacteria provide mechanical and biological stimuli when decorating surfaces for cell growth, motility and differentiation (Martinez-Miguel et al., 2020; Seras-Franzoso et al., 2015; Tatkiewicz et al., 2018). Inclusion bodies formed by growth factors such as the human FGF-2, the Mexican axolotl lipoxygenase and others offer a combination of mechanical and biological stimuli favoring among other effects, wound healing (Seras-Franzoso et al., 2014; Stamm et al., 2018). This is because the forming protein is slowly released under physiological conditions through a slow self-disintegrating process (Cespedes et al., 2020; Sánchez et al., 2022), and therefore, the functional building blocks are available for biological activities. Synthetic versions of inclusion bodies, fabricated *in vitro* from pure protein (Alamo et al., 2021b; López-Laguna et al., 2021; Sanchez et al., 2020), offer regulatory friendly versions of such functional topographies (Serna et al., 2020). These and other examples are summarized in Table 2.

2.4. De novo design of protein scaffolds

Apart from exploiting well-known proteins from nature, constructing *de novo* protein scaffolds for clinical applications is perfectly plausible (Fig. 3). Generically, the main traits of an ideal protein building block intended for supportive roles should include those generically contributing to protein stability and solubility, apart from a lack of undesired interactivity. A robust candidate should contain properly arranged segments of secondary structure connected by short loops. Also, the hydrophobic residues should be tightly packed at its core and most of the polar residues being surface-exposed or having satisfied its hydrogen bonding potential (Worth and Blundell, 2009). While listing the desired traits of a protein might be trivial, coming across a sequence that folds appropriately to satisfy such needs is not straightforward.

In this regard, two different approaches can be pursued to design new building blocks for protein scaffolds. On the one hand, conventional protein engineering has been the source of new constructs from already existing proteins in nature, selecting stable human globular domains to be used directly, or upon mutagenesis through directed evolution. The incorporation of mutations looks for robustness and lack of interactivity of the building block by introducing energetically-favorable residues toward a proper folding, removing the affinity towards other molecules or tightening the tertiary structure by means of disulfide bridges, that act as molecular-level staples (Bhardwaj et al., 2016; Silva et al., 2018). In this regard, aside from the finite catalogue of available proteins, there is a limitation in the number of mutations that can be introduced to a protein without making it lose its native structure. Proteins have evolved to operate efficiently in specific cellular environments and temperature ranges, sometimes at the expense of unfavorable backbone angles (Herzberg and Moult, 1991) and often trading off stability for specific functionality (Beadle and Shoichet, 2002; Schreiber et al., 1994). Because of this compromise, an excessive number of stabilizing mutations leaves the protein at the risk of exhibiting a folded state that diverges too much from the native conformation. This could make, even a human protein, susceptible to activate an immunogenic response towards unfamiliar epitopes. In this same context, aggregation-prone proteins are known to easily trigger undesired immune responses (Sauerborn et al., 2010; van Beers et al., 2010). Then, a high solubility of a target protein is vital to secure its success as a scaffold or scaffold component.

On the other hand, the emergence of computational *de novo* protein design has revolutionized the protein engineering field, making it possible to design a virtually infinite amount of sequences with unique structures not found in nature (Huang et al., 2016). The process starts with the backbone design. Fragment assembly is generally the most successful method. It embraces the use of well-known structural motifs (5–50 residues) that can be chained in iteration to construct new proteins with either globular or non-globular architectures, depending on the type and number of repeating units acting as components (Brunette et al., 2015; Parmeggiani and Huang, 2017). Alternative and analogous approaches employ curated secondary structures of known proteins to create new tertiary structures (Jacobs et al., 2016), or fully mathematical approaches (i.e. parametric design) to build arrangements of secondary structures (Schafmeister et al., 1997). Several methods for backbone design often require a final generation of loops to connect the projected secondary structures (Canutescu and Dunbrack Jr., 2003; Stein and Kortemme, 2013). Next, the precise amino acid sequence is to be optimized based on the side chain interactions that best minimize the

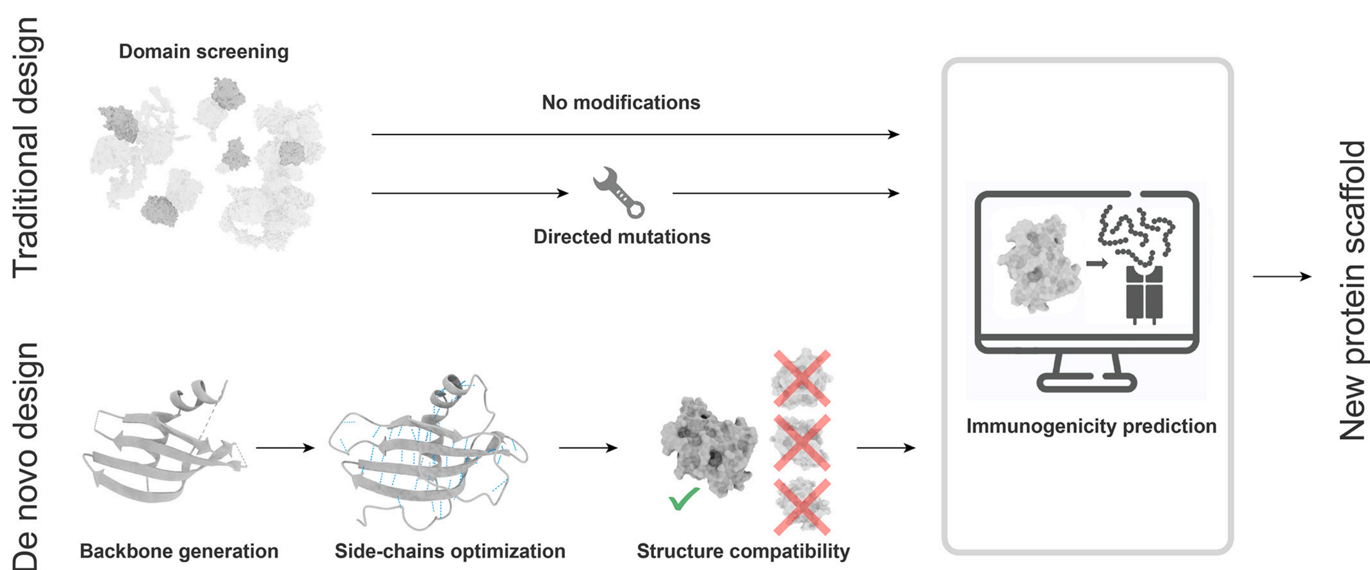

Fig. 3. Simplified overview of the main strategies to generate novel protein scaffolds. The traditional approach (top) involves screening known human proteins for stable and soluble globular domains, assuming the convenience to introduce stabilizing mutations. Alternatively, de novo design (bottom) involves three main steps; defining the protein backbone, optimizing the amino acid sequence to promote favorable side chain interactions, and testing the folding compatibility of the resulting candidate. Immunogenicity prediction can be observed as a generic common and convenient step.

energy function of the chosen backbone. This process is often interspersed with the steps of backbone perturbation and minimization, in a way that more diverse lower energy structures can ultimately be achieved compared to fixed-backbone approaches (Murphy et al., 2012). Finally, the compatibility between sequence and structure must be evaluated to ensure that no other folding state is favored over the predicted design. Working towards the stabilization of one sequence is considered positive design, while intentionally building the protein in a way that alternative states are destabilized is known as negative design. Ideally, both strategies should be simultaneously applied although the latter is not always implementable since it is computationally more intense.

Pressed by the need of highly stable and soluble proteins to avoid unwanted immune responses, many *de novo* design approaches often resulted in proteins with highly regular secondary structure patterns with compact cores (Bhardwaj et al., 2016; Kuhlman et al., 2003). This is setting the best possible scenario even for non-human protein constructs or for fully *de novo* designed materials with no specific source. Recent studies with small hyper stable peptides in murine models showed close to no-immune response in intranasal, intraperitoneal and intravenous administration (Chevalier et al., 2017; Silva et al., 2019). Despite a handful of examples cannot be extrapolated to a general trend, these promising results set the stage for the evaluation of upcoming *a la carte* design of protein scaffolds or scaffold components.

Regardless the selected approach to design a novel scaffold protein, current *in silico* methods allow a very efficient search for antigenic epitopes. Such tools can serve as a last quality check before proteins are tested experimentally and can then provide a temporary assumption of safety. Predictors of either lineal (continuous) or conformational (discontinuous) epitopes have achieved significant progresses in the last decade (Sanchez-Trincado et al., 2017). This is even despite the intrinsic difficulties particularly inherent to conformational antigenic determinants, which require molecular dynamics simulations limited to only infer the tested interactivity.

To assess whether a lineal antigen will be able to trigger an immune response, its likelihood to be presented by the major histocompatibility complex, and hence, potentially recognizable by T-lymphocytes, is typically evaluated. State-of-the-art predictors have embraced the use of machine learning strategies to enhance the prediction performance and they are mostly available as web servers (Chen et al., 2019; O'Donnell et al., 2020; Reynisson et al., 2020; Venkatesh et al., 2020). In contrast, conformational epitopes are exclusively recognized by B-lymphocytes and its prediction has lagged behind of those of linear nature due to requiring knowledge of the protein 3D structure and being hard to isolate for study. Coincidently, such isolation is typically achieved through epitope grafting of suitable scaffolds. Despite these setbacks, several methods have shown better-than-random discrimination capacity and are publically available (Liang et al., 2010; Rubinstein et al., 2009), even without recurring to machine learning (Ponomarenko et al., 2008; Sun et al., 2009; Sweredoski and Baldi, 2008).

3. Conclusions

Pivotal areas in human medicine such as drug delivery or regenerative medicine require biocompatible agents to perform structural functions, with null or regulatable interactivity with components of the body. Being natural macromolecules sustaining life and because of their structural plasticity and suitability for tailoring through genetic engineering, proteins are excellent building blocks to perform scaffolding actions. Proteins can be used as scaffolds as they are in their original sources or upon targeted modifications addressed to improve stability and solubility, remove undesired interactions or gaining self-assembling capabilities. Many natural proteins have been incorporated to the expanding catalogue of clinically oriented scaffolds, either as single polypeptides or as building blocks of more complex oligomers or polymers, upon promoting self-assembling. Protein modification can be also useful to combine several desired functions in a single polypeptide chain by domain recruiting, conferring additional biological activities beyond a plain structural role. In addition, protein materials used in human clinics should be non-immunogenic. In this regard, immunoreactivities, mainly associated to non-human proteins, can be minimized by site-directed mutagenesis allowing foreign proteins to be 'humanized' and then usable in human medicine. On the other hand, advanced computational methods permit to design, fully de novo, non-natural polypeptides that fulfill precise structural requirements. Ensuring high solubility and structural stability of these constructs, that do not have a natural origin, will in turn results in non-immunogenic materials. Even in early stages, this approach complements and is expected to largely expand, in a close future, the catalogue of protein-based materials with medical usability.

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