~~Emerging~~ Recombinant protein materials for bioengineering and nanomedicine

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Abstract

Proteins are essential macromolecules supporting life. Being efficient catalysers and offering specific cross-molecular contacts, proteins are largely exploited in biotechnology and biomedicine as therapeutics, in industrial catalysis or as molecular reagents. Recombinant enzymes, hormones, immunogens and antibodies are produced aiming to different applications, on the basis of their ability to interact with or modify substrates or biological targets. In nature, proteins also perform task-specific architectonic roles, and they can organize in supramolecular complexes with intriguing physical properties such as elasticity and adhesiveness, and with regulatable stiffness, flexibility and mechanical strength. Proteins have recently gained interest as materials for bioengineering and nanomedicine as they can combine these features with functionality, biocompatibility and degradability in unusually versatile composites. We revise here the fundamental properties of the diverse categories of emerging protein materials resulting from biological synthesis and how they can be genetically re-designed to engineer the interplay between mechanical and biological properties in a medically-oriented exploitable way.

Keywords

Structural proteins; Recombinant proteins; Biomaterials; Mechanical properties; Bioengineering

Introduction

Structural and functional proteins are found combined in nature to support all activities that maintain life. While functional soluble proteins (namely enzymes, hormones and ligands) have been largely exploited as agents for catalysis and in medicine and research, those with relevant mechanical properties are progressively observed as plastic biocompatible materials. Proteins as materials are especially appealing since they can be produced as recombinant versions in bacteria, yeasts, insect and animals cells, and in a variety of emerging alternative hosts that offer metabolic advantages [1,2] ~~(1,2)~~, through cost-effective processes. In addition, even structural proteins display biological activities that enrich the natural functionalities of the material. Also, conventional DNA manipulation and more than 30 years of accumulated experience in protein design and engineering permit tuning, adapting and combining protein structure and performance under rational, semi-rational or combinatorial approaches. Protein materials are usually bio-inspired. Many natural proteins are appealing because of their peculiar mechanical properties such as elasticity, strength, adhesiveness of responsiveness to external *stimuli* including temperature and pH (~~Table~~ Appendix 1). Collagen, silks, elastins, keratin, resilins and adhesive proteins fall within this category [3] ~~(3)~~. On the other hand, the supramolecular organization that proteins might adopt, might be *per se*, of interest for biomedical applications (Figure 1). In this regard, tubulin, flagelin, amyloidogenic proteins and structural proteins from viruses and microcompartments, among others, offer architectures and mechanical abilities than once combined, empower the material to perform very complex activities (~~Table~~ Appendix 1). If those structural proteins are in addition bioactive, smart materials might result from the combination. For instance, viral capsids are nanocompartments evolutionarily designed to hold, protect and deliver to the cell nucleus their cargo DNA in a cell-receptor mediated process, what ensures not only stability but also targeting and compartment-linked cargo release.

In this context, biofabrication of protein materials tends to straightforward reproduce natural composites, or to adapt them to specific functions of relevance for a given application, for instance, engineering the tropism of a recombinant virus-like particle (VLP) by replacing the natural cell-receptor ligand on the capsid surface. In addition, modest attempts are also found in the literature addressing the fully *de novo* design of self-assembling protein-based entities at both nano and micro scales. Being biocompatible, biodegradable and tunable, any ordered, supramolecular protein assemblage might have a potential in biomedicine as biomaterial. We revise here the most important mechanical properties of protein materials exemplified by a few specific

proteins as well as the main eye-catching hierarchical organizations of protein materials that attract the engineers' attention from the expanding fields of bioengineering and nanomedicine.

Properties of protein materials

Elasticity and temperature responsiveness: elastin

Elastin is a natural protein that plays a key role in the extracellular matrix of tissues with specific requirements of elasticity such as blood vessels, cartilage, ligaments, skin and lungs [4][4]. This protein not only confers rubber-like elasticity but it also modulates cell behaviour and promotes tissue repair [5][5]. The primary sequence of elastin, composed of conserved repeat motifs (VPGG, VPGVG, APGVG, and VGVAPG), allows deformation without breaking under stress conditions (mainly temperature) and it is able to return to the original conformation [6][6]. Since valine and alanine, being both hydrophobic residues, are prevalent in these repetitive motifs, insoluble elastin fibers cross-interact by hydrophobic contacts resulting in the formation of native elastin fiber networks [7][7].

As most natural proteins, large-scale isolation of elastin from natural sources for applications in bioengineering is not affordable. Thus, elastin-like polypeptides (ELPs) have being developed by genetic engineering as recombinant alternatives to natural elastin. As the immune system is unable to discern between recombinant and natural elastin forms, ELPs and derived materials show an extraordinary biocompatibility than enables *in vivo* application. ELPs are usually composed by repeats of the (VPGXG) pentapeptide, one of the motifs of elastin primary sequence [8][8]. X is the so-called ELP guest residue, being any amino acid except proline [6][6].

One of the most important hallmarks of ELPs is their structural responsiveness to temperature. Different ELPs have a characteristic reversible phase transition known as transition temperature (Tt) [9][9]. More specifically, in aqueous solution and below Tt, these thermosensitive biopolymers remain hydrated [10][10]. On the contrary, above Tt, water organization disappears and in consequence, hydrophobic domains interact to drive self-assembling into highly-ordered structures [11][11] . Besides temperature, other factors such as protein concentration, ionic strength and pH determine the specific Tt, what needs to be considered when designing ELP polymers for specific applications [12][12]. Interestingly, this dependence on environmental factors remains unaltered when

fusing ELP to other proteins (ELPylated proteins) [13]. Also, the amino acid sequence has a strong impact on both thermal and mechanical properties of ELPs [8], and the polarity of amino acid X has a direct influence on Tt [6]. Similarly, the substitution of glycine by alanine (GXGVP to AXGVP) alters the elastic properties of the biopolymer that becomes more plastic [14].

In general, *Escherichia coli* is the most used system for the biofabrication of ELPs [9], although it also presents some limitations. First, the repetitive nature of the ELPs sequences can have a negative impact on the overall translation process efficiency. In this context, the addition of specific amino acids to the culture can notably improve the ELP expression levels [15], as well as the use of engineered *E. coli* with increased pools of specific aminoacyl tRNAs [16]. Other expression systems such as yeast and plants are also used as ELP factories [17,18], essentially to prevent the presence of endotoxins in the final product. Initially, chimerical ELP constructs were designed as tags to facilitate recombinant protein purification [19], taking advantage of the environmental responsiveness of ELP tags. However, a large number of other applications have been reported. For example, ELP material is used to construct scaffolds for the regeneration of dermal, vascular, cardiac and cartilage, among other tissues (~~Table~~ Appendix 1), or to coat other materials (eg metallic surfaces [20]) that are too hydrophobic or negatively charged to support cell growth, improving cell adhesion and function [5].

On the other hand, some ELPs form temperature-dependent arrangements [21] that are used as carriers for drug delivery (~~Table~~ Appendix 1). Chimeric ELP-based biopolymers incorporate bioactive motifs, thus improving the uptake of the conjugated drug in the target tissue [21,13]. The delivery of DNA [22], of chemotherapeutic agents for cancer therapy [6,8] and of growth factors in wound healing [6] exemplify the potential of thermoresponsive ELPs as delivery platforms. ELPs with a Tt higher than the body temperature (Tb) are good soluble carriers. Alternatively, ELPs with a Tt between 37ºC and 42ºC are used in combination of local hyperthermia. There, ELPs aggregate in the tumor, increasing the effective concentration of the cargo and, consequently, their efficiency [8,22].

Although ELPs are gaining importance as promising biomaterials, there is a consensus in which their functional versatility can be further extended by appropriate genetic engineering, especially for drug delivery purposes. Active research is currently done in this direction.

Adhesiveness and flexibility; mussel adhesive proteins

Some proteins from mussel and other marine organisms such as barnacle and tubeworm show extraordinarily strong and flexible adhesive properties, with the capacity to bind to different substrates such as plastic, glass, metal, Teflon, organic tissues, etc. They displace water and thus adhere in aqueous media, while no conventional synthetic (chemical based) adhesives can be applied in aqueous environments. Being biodegradable, they have been proved to be safe for humans [23] ~~[23]~~. So far, nine unique proteins have been found in mussel byssus, among which six locate in the adhesive plate [24]~~[24]~~ (named fp1-6). fp-3, fp-4 are cationic and rich in arginines, lysines or histidines; fp-2, fp-6 are rich in cysteines; fp-1, fp5 are repeats of amino acids with post-translational modifications, such as 3,4-dihydroxyphenyl-L-alanine (DOPA), hydroxyproline, dihydroxyproline, 4-hydroxyarginine, O-phosphoserine and glycosylation [25,26]~~[25,26]~~. In particular, DOPA, the product of tyrosine hydroxylation, has a side chain able to form strong hydrogen bonds with metals and semimetals [27]~~[27]~~, and in fact, the closest to the adhesion interface the highest content in DOPA residues the proteins have [28]~~[28]~~. On the other hand, fp-1 is covering the adhesive plaque; fp-3 and fp-5 are mostly hydrophilic proteins located at the adhesion-sustratum interface, and promote water-substrate bond displacement to subsequently spread the adhesive for substrate attachment [24]~~[24]~~. Fp-2 and fp-4 form the bulk of the adhesion plaque, the first one playing a role in plaque stability, and the second one, rich in histidines, lysines and arginines is useful to couple the thread of the byssus to the adhesive plaque. The role of other residues in mussel adhesion is not yet known.

Obtaining mussel adhesive proteins by chemical extraction is inefficient and it results in highly contaminated material [29]~~[29]~~. Currently, the only commercial mussel proteins are Cell-Tak (BD Bioscience Clontech), which is an extracted mixture of fp-1 and fp-2, and MAP (Swedish BioScience Lab.), an fp-1 extract. ~~On the other hand, the production of recombinant adhesion proteins in expression systems such as *E. coli*, yeast and plants, generally failed because of low levels of expression, toxicity, low purification yield [30], insolubility upon purification [30], and diminution of adhesive properties [31].~~ On the other hand, the production of recombinant adhesion proteins in expression systems such as *E. coli*, yeast and plants, generally failed because of different reasons including low levels of expression, toxicity, low purification yield [30], insolubility upon purification [30], and loss or reduction of the adhesive properties [31]. ~~In this context~~To address these issues, Prof. Cha's group designed hybrid proteins combining fp 1 and 5 (fp151) and fp5 and fp3A (fp353), which are produced at high yields and keep the adhesive

properties [30,32][30,32]. Moreover, these proteins can be fused to other domains, such as RGD, that provide cell recognition properties [30][30].

Mussel adhesive proteins are gaining interest in material sciences (~~Table~~ Appendix 1) taking advantage of optimized production processes [33][33], *in vivo* posttranslational modifications [34][34], a better knowledge of the molecular mechanisms driving mussel protein adhesion [35][35], and the possibility to directly coat surfaces with mussel adhesive proteins through glycosaminoglycan functionalization [36][36]. In this context, mussel adhesion proteins may behave like extracellular matrix when fused to functional peptides [37][37] serve as scaffolds for bone regeneration [36][36], inspire multiple-interaction for the synthesis of nanoparticles [38][38] and also inspire the construction of polydopamine thin films [39][39] used in re-endothelialization of artificial vessels [40][40].

Mechanical strength; silk

Silk proteins from silkworms and spiders are appealing due to their unique mechanical properties, namely high strength and excellent elasticity [41][41]. Fibrous silk proteins are polymers of repetitive sequences rich in glycine, alanine and serine for silkworm silk, and also glutamic acid, proline and arginine for spider silk, which are arranged in β-sheets due to the hydrophobic domains [42][42]. It is now well established the processing of silk cocoons by physical or chemical treatments. They are improved to eliminate the antigenic protein sericin and to get the silk fibroins in different formats [41][41], namely as sutures used in surgery and for reconstruction of tendons and ligaments, or as highly rough mats on which different cell lineages can grow, like connective tissue and blood vessels [43][43]. Films can be also obtained from the aqueous silk fibroin solution that could be blended with other polymers that promote adhesion of fibroblasts (with results comparable to collagen [44][44]), as antithrombogenic (when combined to S-carboxymethyl kerateine (SCMK) [45][45]), for the regeneration of skin wounds and as a bone formation agent (see ~~Table~~ Appendix 1). Hydrogels processed also from aqueous silk solution promote cell proliferation and are useful in the regeneration of cartilage and bone [46][46]. When combined with elastin, they generate the silk-elastin-like protein polymers (SELPs), useful for the release of small molecular drugs like theophylline, vitamin B12 and cytochrome C [47][47] and also for the slow release of expressible DNA [48][48]. These materials have with multiple applications in bone and cartilage engineering as well as for wound protection [49][49]. Also, silk proteins can be functionalized by different methods [49][49] namely adsorption, taking advantage of silk hydrophobicity (e.g. HRP), by the covalent linking of functional peptides like RGD, BMP-2, by covalent bonding of inorganic compounds (such as hydroxyapatite) and by constructing chimeric

recombinant proteins with additional functional domains. These modifications have allowed to improved cell adhesion and the differentiation of hMSCs into bone cells (BMP- 2) (~~Table~~ Appendix 1).

In a strict sense, silk proteins should be considered non-biodegradable materials as they maintain tensile strength for more than 60 days *in vivo* [50]~~[50]~~. However, they are targets for proteases *in vivo*, losing tensile strength at year 1 and disappearing after 2 years. Interestingly, the degradability varies according to parameters of the production process and the morphology and mechanical and biological conditions of the implantation site. As silk proteins may be sterilized by autoclaving, gamma -radiation or treatment with 70 % ethanol without changing their morphology or secondary structure, they are therefore fully suitable for *in vivo* uses.

Large scale spider silk production has not yet been achieved, representing an engineering problem that needs to be addressed.~~Large-scale production of recombinant spider silk protein has not been achieved yet~~. At laboratory scale, it has been reported in transgenic tobacco and tomato [51]~~[51]~~, and in *E. coli* where the protein self-assembles into nanoparticles [52]~~. Since recombinant spider silk proteins do not assemble into fibers, transgenic silkworms with chimeric silkworm/spider silk genes have been generated to produce modified proteins with improved mechanical properties~~ [53]~~.~~[52]. Since at difference from their natural counterparts recombinant spider silk proteins do not assemble into fibers, transgenic silkworms with chimeric silkworm/spider silk genes have been generated to produce modified proteins with improved mechanical properties [53]. Different processing of spider silk recombinant proteins for tuning the biomaterial properties has been recently revised elsewhere [54] ~~[54]~~. Finally, the recently characterized silk from honeybees appears as a very promising biomaterial, as the recombinant versions appear as easy to produce and refold from aggregates into their native soluble conformation [55]~~[55]~~.

Supramolecular organization of protein materials

Protein polymers and films

Protein polymers can be formed by functionalized self-assembling peptides (SAPs), which form well-defined nanostructures stabilized *via* non-covalent interactions, mostly hydrophobic interactions and hydrogen bonds. One of the most popular SAP is RADA-

16, a 16-residue peptide composed of alternating hydrophilic arginine, hydrophobic alanine, and hydrophilic aspartic acid. RADA16-like SAPs are composed of natural amino acids that spontaneously fold, under physiologic conditions, into antiparallel β-sheets, and self-assemble as nano- and microfibers that mimic the extracellular matrix (ECM) architecture [56]. RADA16-I peptide scaffolds were developed by Zhang and coauthors [57] and are now commercially available as PuraMatrix™. RADA16-I has been shown to induce osteoblast proliferation, differentiation, and migration [58]. A RADA16-I peptide hydrogel can also facilitate wound healing and it arrests bleeding in a few seconds [59]. On the other hand, fusion of heterologous motifs to peptide RADA16-I did not significantly inhibit self-assembling properties and nanofiber formation, and simulations have been performed to demonstrate the motifs are displayed on the surface of the nanofibers [60].

Several SAPs have successfully been used for neural cell culture [61]. Furthermore, it has been demonstrated that a peptide nanofiber scaffold can repair hamster brain lesions by reconnecting nerve fibers [62]. Proteins structured in the form of biofilms have been also explored as emerging protein-based materials. In this context, whey proteins (also known as milk serum proteins) provide great potential as controlled release systems [63]. In this regard, whey proteins form biofilms in the presence of plasticizers. These components, generally polyols, can modify the physico-chemical and mechanical properties and permeation of films, and their adhesion strength, which in turn can affect the release properties of embedded drugs. It has been recently shown that whey-based biofilms can be used for bioencapsulation and as delivery systems. Soy proteins can also form biofilms used as extracellular cell culture matrices for tissue engineering [64]. According to these results, protein-based biofilms could address the lack of connective tissue when engineering human epithelial (epidermis, oral mucosa) structures. Soy protein isolate has also been investigated as a matrix for wound-dressing applications. Such soy protein-based wound dressings were loaded with gentamicin for bacterial inhibition. The films could effectively inhibit *S. aureus* and *S. albus* infections for at least 2 weeks and *P. aeruginosa* for 3 days [65].

Hydrogels

Hydrogels have high water content and are rigid, three-dimensional, crosslinked networks formed by polymers that offer promise in tissue engineering, wound dressing, cell therapy, and drug delivery as they can act as both, scaffolds and releasing matrices of therapeutically active substances [66]. Natural hydrogel-forming polymers are

polysaccharides (alginate, agarose, dextran, chitosan) and proteins (collagen, gelatin, and fibrin). Diverse peptides self-assemble into hydrogels. Sol-to-gel transition involves self-assembly of soluble monomeric peptides triggered by a perturbation in the solution conditions, like thermal cycling [67], or pH switch [68]. The high water content of hydrogels, regulatable swelling properties, pore size, morphology and mechanical properties, and the underlying chemistry offers applications in tissue engineering and sustained drug delivery [66], for instance, as regulated by temperature [69]. In this context, pH-responsive hydrogels composed of PEG-containing ionic networks have been applied for the oral delivery of proteins (~~Table~~ Appendix 1). Therapeutics can be directly encapsulated into the network by triggering self-assembly in the presence of the drug. Drugs can be also attached by specific interactions in non-covalent or covalent binding via degradable linkers to be released afterwards via diffusion, degradation, swelling or a by specific trigger such as temperature, pH, etc. Non-covalent binding may be by electrostatic interactions with charged polymers, by affinity through heparin sulphate to immobilize proteins or through specific protein interactions. Covalent binding can take advantage of the reactive amine and thiol groups, through interaction with polymer functional groups like hydroxyl, amine, carboxyl, etc.

Hydrogels have been applied to deliver proteins such as insulin, epidermal growth factor (EGF) and platelet-derived growth factor BB (PDGF-BB) among others [66]. In tissue engineering, cells can be encapsulated during self-assembly, rendering network hydrogels with cells homogeneously distributed. Mesenchymal stem cells encapsulated within the peptide MAX8 hydrogel for *in situ* release retain viability during and after delivery and the gel remains localized at the injection site [70]. Three-dimensional cell culture of mesenchymal stem, neural cells and venous endothelial cells has been successfully achieved in hydrogels [66]. Hydrogel biofunctionality may be tailored by changing hydrogel's chemical and mechanical properties to direct cell fate in tissue engineering [71].

One of the challenges associated to the use of hydrogels is to improve their injectability. Traditional hydrogels are predominantly designed to be injected in their soluble state and to solidify within the body. The most significant weakness of this approach is that response time is slow, allowing the low-viscosity polymer solution leaking into surrounding tissues during gelation. Moreover, block copolymers have the risk of syringe clogging during injection if conditions are not properly controlled or if polymer concentration is too high. Thus, development of fast-acting hydrogels are necessary [70]. On the other hand, *ex vivo* gelled polymers do not show such problems

but they require surgical implantation. Such polymers are usually covalently cross-linked hydrogels, exhibiting good mechanical properties. However, most of the cross linkers used are either toxic, their fate in the body is unknown and/or there is a lack of data about their biocompatibility. This issue must be addressed by performing additional purification steps before hydrogel administration. Until safe covalent cross linkers with good biocompatibility are available, alternative hydrogels, such as ionically cross-linked hydrogels are to be preferred, as they are generally well-tolerated. However, their main disadvantages are the possible lack of mechanical stability and the risk of dissolution of the system, due to a highly pH-sensitive swelling [72].

Control over degradation rates that facilitate appropriate clearance of the scaffold or delivery vehicle from the body is also an important issue. These properties depend highly on the desired application. For instance, scaffolds loaded with cells or drugs may require slowly degrading materials for long-term applications. Finally, peptide hydrogels fail to recreate molecular level spatial organization of natural extracellular matrix. Hence, another goal for current research in regenerative medicine involves control over 3D spatial organization of components because this would enable mimicking the highly complex microarchitecture of the extracellular matrix [73].

Fibers and ~~A~~amyloids

~~A diversity of non-amyloid, nanoscale protein fibers has been generated in an attempt to reproduce the topography with which mammalian cells interact [72]~~. Some of these materials are formed by peptide amphiphiles, that

~~Although the biological production of short peptides has been for long a complex issue, difference approaches now under development will hopefully enable their large scale recombinant production [73].~~

A diversity of non-amyloid, nanoscale peptide-based fibers has been developed in an attempt to reproduce the topography with which mammalian cells interact [74]. In this regard, peptide amphiphiles, resulting from chemical conjugation between an hydrophilic peptide moiety and a non peptide hydrophobic molecule [75], organize as 1D fibrils and into more complex 3D nanofiber networks with multiple biomedical applications but especially in regenerative medicine [76].Peptide-only fibers (and more complex 3D-derived networks and nanoparticles) have been also developed by using specific amino acid stretches such as the RAD motif [77], the ABA motif [78] and a spectrum of short peptides with defined conformations such as β-sheet or β-hairpins [79]. Although the biological production of short peptides has been for long a complex

issue, different engineering approaches will hopefully enable their large-scale recombinant production [80,81].

On the other hand, aAmyloid fibrils are nanostructures formed by the ordered deposition of β-sheet rich proteins or peptides following a seeding-driven process. Although amyloid assemblies are related to some progressive neurodegenerative diseases, they have been also detected in normal cellular processes [82][72]. The amyloidogenic ability of peptides has been determined to be contained in short stretches as short as five amino acids. The common characteristic of these peptides is the presence of aromatic amino acids which play a central role in the self-assembling process suggesting an important role of stacking in ATP-independent intermolecular interactions mediated by multiple hydrogen bonding [83][73]. Based on this molecular principle, amyloydogenic peptides have been explored in a bottom-up strategy for the development of biocompatible nanostructures to be used in novel biosensing devices, in surface decoration, as drug-delivery systems and for tissue engineering among others.

Conducting nanowires incorporate metallic ions in the amyloid fibers through cysteine residues resulting in nanocomponents of electrochemical biosensors for the detection of nuclceic acids and metabolites [84][74]. In addition, amyloid nanofibers can be accommodated in polymeric networks as components of hidrogels, improving sensitivity and specificity of immunoassays [85][75]. Amyloids can be also adapted to the delivery of petide-based drugs. This concept orbits around the possibility to increase the bioavailability of the drug after administration. In that sense, it is crucial to regulate the effective release of the soluble peptide from the fibrils and ensure the absence of cross-seeding with amyloid-related host proteins [86][76]. The development of such strategy could be used as a platform for the administration of therapeutic peptides fused to aggregation prone peptides. Also is of relevance to note the use of decorated surfaces with amyloid fibrils to enhance cell adhesion in regenerative medicine applications [87][77]. Finally, as the toxicity of the amyloid aggregation seems to be related to soluble oligomers, seeding amyloid hyidrogels able to capture these soluble oligomers in neurodegenerative diseases are been developed [88][78].

Very recently, bacterial amyloids formed by recombinant proteins (inclusion bodies, IBs) are being adapted to the slow release of the forming protein administered as entities in suspension (top-down) for protein replacement therapies [89][79] or adsorbed to two-dimensional or three-dimensional substrates (bottom-up) in tissue engineering [90]

~~(80)~~. These pseudo-spherical submicron protein particles, being mechanically stable and with a stiffness and Z potential appropriate for cell adhesion (91)~~(81)~~, are also used as topographies for substrate colonization and cell differentiation (92)~~(82)~~ or as agents for cell guidance in proto-neuron cell culture (93)~~(83)~~. The tuneability of bacterial amyloids by genetic approaches and the easy biofabrication makes them extremely versatile materials regarding biological and mechanical profiles and biomedical applicability (94)~~(84)~~ (~~Table~~ Appendix 1). Of course, the heterogeneity in composition and the eventual presence of potentially endotoxic bacterial contaminants are important concerns that need to be fully addressed before considering *in vivo* biomedical applicability.

Microcompartments

The use of protein cages of non-viral origin as nanomaterials provides a number of unique advantages in biomedicine and biotechnology. Their biological origin makes them both amenable to genetic modification and large-scale production. Genetic modification enables the site-specific introduction of chemical and/or structural functionality onto highly symmetric protein cage platforms. By either chemical and/or genetic subunit alterations, it is feasible to simultaneously add new functions to different particle surfaces to direct cage assembly, encapsulation of a synthetic cargo, or targeting to a specific surface or cell.

Many bacterial species contain intracellular nano- and micro-compartments consisting of self-assembling proteins that form protein-only shells (95)~~(85)~~. Protein-based microcompartments are large macromolecular complexes consisting of metabolic enzymes encapsulated within multiprotein, polyhedral shells, reminiscent of viral capsids. A common feature of such bacterial microcompartments (BMC) is a thin shell, primarily composed by a few thousand protein subunits, which encapsulates enzymes while allowing transport of substrates and products. BMC were first isolated in 1973 and determined to contain the CO_2-fixing enzyme RuBisCO (96)~~(86)~~. They were named carboxysomes, and are recognized as the first member of a diverse group of microcompartments. BMC proteins were later found to be also encoded in the propanediol utilization operon (pdu operon) of *Salmonella* and by an operon for metabolizing ethanolamine (eut operon) in enteric bacterial species, including *Salmonella* and *Escherichia coli* (95)~~(85)~~.

BMC can spontaneously self-assemble in the absence of native interior enzymes offering appealing opportunities to fill them with therapeutic molecules. In this context, mechanisms directing enzyme encapsulation within BMC have been studied and

revealed during the last years. In some cases, a stretch of a few (~15-20) amino acids at the N-terminus of the inner cargo protein directs and binds it to the inner surface of the shell protein. When such directing peptide is not present, the alternate strategy is to synthesize the cargo protein together with the shell-forming domain from one unique gene. As an example of the first strategy, Fan and colleagues [97][87] demonstrated that a short N-terminal peptide is necessary and sufficient for packaging enzymes into the Pdu microcompartment. Support for the existence of the second strategy is provided by *Pyrococcus furiosus*, where a Flp coding sequence (without any targeting sequence directing its encapsulation by physical interaction with BMC proteins) is fused in frame with an encapsulin gene [98][88]. In this situation, cargo and encapsulin proteins are synthesized as a fusion that further self-assembles to form a nano-cage containing the cargo protein. The discovery and understanding of signal sequences able to direct enzyme encapsulation into BMC and the underlying mechanisms of such process are key milestones in our understanding of BMC assembly, and leads the way for the development of these bacterial organelles toward biotechnological and biomedical applications.

Vaults, another example of protein-based intracellular microcompartments, are found in nearly all eukaryotic cells [95][85]. Vault particles were first observed in 1986 as contaminants in preparations of clathrin-coated vesicles from rat liver. There are between 10^4 and 10^6 vault particles in the cytoplasm of most eukaryotic cells, being the largest ribonucleoprotein particles described (as 13-MDa ribonucleoprotein complexes) to date [99][89]. Vault particles display a complex barrel-shaped morphology (Figure 1), organized in two identical moieties, with two protruding caps and an invaginated waist, a structure that results from the self-assembly of multiple copies of three proteins: the major vault protein (MVP), the telomerase-associated protein 1 (TEP1) and the poly-(ADP ribose)-polymerase (VPARP) [99][89]. It has been possible to obtain recombinant vaults from only its most abundant protein (the 97 kDa MVP). *In vitro*, expression of MVP alone in Sf9 insect cells employing a baculovirus expression system resulted in the production of particles with the characteristic vault morphology [100][90]. The central cavity of vaults can be used to encapsulate proteins simply by fusing the cargo protein to a vault-targeting peptide [101][91]. Furthermore, studies have revealed that vaults are non-immunogenic, and immunogenic proteins can be then encapsulated to generate vaccines [102][92]. Studies on the conditions for reversible vault disassembly and reassembly could enable their application in drug delivery. In this context, vault dissociation triggered by low pH may become useful for delivery within cellular systems given that endosomes and lysosomes are normally maintained at acidic pH.

Nanocompartmens

Viral capsids and recombinant virus-like particles (VLPs) are nanometric size protein cages made of one or few different proteins and that able to accommodate therapeutic cargos [103][93]. The forces governing viral capsid assembly have been evolutionally conserved and include electrostatic interactions and the burying of hydrophobic residues at the intermolecular interface [104][94]. These dynamic structures undergo conformational transitions in the interior of host cells releasing the internalized cargo at specific cell compartments [105][95]. More interestingly, assembled VLPs produced by recombinant methods can be readily purified and loaded with unrelated nucleic acids or drugs using scalable disassembly-reassembly *in vitro* protocols, making this type of nanostructured material a versatile tool for the specific delivery of therapeutic molecules [106][96]. On the other hand, the possibility to obtain VLPs offer great promises for vaccinology in the prevention of viral diseases [107][97]. However, modification of solvent exposed loops to include cell receptor ligands drives to the development of chimeric nanoparticles with the desired tropism, while the addition of antigenic peptides will maintain their immunogenic potential on the nanoparticle surface [108][98]. However, since the cros-protein interactions in viral capsid are complex and not completely understood, such engineering is not done under rational rules and it might affect the stability of the nanoparticles. The success in these modifications is then still a matter of trial and error.

Nanoparticles

The rational control of protein self-assembling is becoming a major issue in biomaterials science [109][99]. Assembling of full-length soluble proteins as nanoparticles of defined size and morphology is progressively reached by the straightforward engineering and fusion of oligomerization domains from natural oligomers [110][100]. In more sophisticated approaches, the *in silico*-assisted engineering of cross-molecular protein-protein contacts [111][101] or the design of disulfide bridges [112][102] permits the controlled assembly of protein building blocks as nanostructures of regular size. On the other hand, the fusion of assembly-promoting peptides (namely a cationic peptide and a poly-histidine) at both the N and C-terminus of a core protein respectively enables its oligomerization irrespective of the protein amino acid sequence [113][103]. The end-terminal peptides of one molecule interact with the opposite peptides of the neighbouring protein molecule forming arrays of several protein molecules that can be stacked in supramolecular structures forming rod-shaped or disk-shaped structures [114]

[104]. This type of protein nanoparticles accommodate expressible nucleic acids and could be also loaded with therapeutic drugs [114][104]. The formation of the protein nanoparticles depends on the cationic nature of the peptides located at the N-terminus of the protein, in a configuration that enables the direct contact with the polyhistidine tag located at the C-terminus of the protein. The system is presented as a platform to shelter therapeutic molecules to be delivered into target tissues [115][105], in a conformation that is fully stable *in vivo* and allows escaping renal filtration [116][106]. In addition, the modular nature of the recombinant building block allows the replacement of both cationic tag and protein core [116][106], and the introduction of selected functional modules including the specific interaction with the target cell receptor, the endosomal escape ability and the control of cell compartment release of therapeutic molecule [117][107].

Future perspective

The comprehension of how protein arrange and self-organize for specific structural functions will allow the identification of a growing amount of natural protein polymers with mechanical properties of biomedical interest, to be produced by biological synthesis. Genetic engineering will be the driving force in the adaptation of such emerging protein materials to specific tasks in bioengineering and nanomedicine, by combining mechanical and biological activities in biocompatible composites. As the plasticity and versatility in protein manipulation is essentially unlimited the spectrum of tuneable functional protein materials is expected to grow exponentially in the next years. Degradability, full biological compatibility and morphological adaptability of protein materials will fulfil the demands of new targeted drugs, sustained drug delivery platforms and functional scaffolds in tissue engineering and nanomedicine.

Executive summary

Properties of protein materials

Elasticity, adhesiveness, strength and mechanical stability can be found in natural proteins that can be further engineered and produced as recombinant versions to

perform task-specific activities, particularly in tissue engineering but also in other biomedical fields.

Most of these proteins can be produced by cost-effective process as recombinant versions.

Supramolecular organization of protein materials

Supramolecular protein complexes adopt diverse forms, including biofilms, hydrogels, micro- and nanocompartmens, nanoparticles and submicron structures such as regular clusters and fibres.

Most of these arrangements are not linked to a specific amino acid sequence but they can be reached by diverse protein species with converging structural traits.

Those architectonic patterns are especially appealing in targeted and/or sustained drug delivery.

Self-assembling of proteins as hierarchical nano- and micro-entities of biomedical values is progressively reachable by conventional protein engineering.

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~~Table~~ Appendix 1. Main applications of protein materials in bioengineering and nanomedicine, illustrated by representative examples.

Application	Material	Organization	Materials' relevant property	Mode of action	Representative references
Sutures in surgery	Twisted silk fibers purified from silkworm	Silk cords	Strength and elasticity, biodegradable, soluble	Strong sutures absorbed after 2 years	(118)~~(108)~~
Tendons and ligaments reconstruction	Twisted silk fibers RGD-modified	Fibers	Strenght and ealsticity; stiffness	Bone marrow stromal cell ligament engineer.	(119)~~(109)~~
Antithrombogenic	Aqueous silk fibroin sol. combined to SCMK	Films	Change in the polarity of the surface	Dependent on treatment temperature and solvent.	(120)~~(110)~~
Topographies for tissue engineering	Bacterial amyloids	Submicron spherical particles; recombinant	Size, stiffness, and Z potential in the cell's sensing range; adhesiveness	Adhesion; mechanotrasduction is suspected	(90)~~(80)~~
	Self-assembling peptides	Hydrogel	Enrichment of neural populations and formation of 3D scaffold.	Support tissue growth and enhanced cell migration	(121)~~(111)~~
	Elastin-like proteins	Tissue engineered vascular conduit	Reduction of fibrinogen and immunoglobulin adsorption and decrease of release of proinflammatory cytokines by monocytes	Improve the blood compatibility of cardiovascular devices	(122)~~(112)~~
	Elastin-like proteins	Modified polystyrene dishes	Differentiation of ESCs into cardiomyocytes	ESCs differentiation is promoted by IGFBP4 (Thy) immobilized on dishes using an elastin-like polypeptide	(123)~~(113)~~
	Elastin-like proteins	RGD-ELPs decorating polystyrene plates	Enhancement of cell attachment, migration and differentiation	(RGD) sequence improves cell behavior being an integrin-binding ligand	(22)~~(22)~~
	Silkworm silk proteins	Non- woven mats	Strength, roughness, elasticity.	Facilitates growth of different cell lineages	(124)~~(114)~~

	Silkworm silk proteins	Films	Permeable to gases, strength and elasticity	Bone formation and wound regeneration	(125)~~(115)~~
	Silkworm silk proteins	Hidrogels	In aqueous solution swells but not dissolves	Regeneration of cartilage and bone	(46)~~(46)~~
	Silkworm silk proteins	Porous sponges	Tunable physical properties according to the purification method	Bone and cartilage regeneration	(126)~~(116)~~
	Polydopamine (PDAM) inspired on mussel adhesive proteins	Coating stainless steel stents	Adhesiveness, protective effect, elasticity	Re-endothelialization of artificial vessels	(40)~~(40)~~
	Mussel adhesive proteins	Coating of 3D scaffolds by dipping	Attachment, proliferation	Bone regeneration	(36)~~(36)~~
Cell adhesion	Mussel adhesive proteins fused to biofunctional peptides	Coating suspension	Cell adhesion, proliferation, differentiation	Behaves like extracellular matrix	(37)~~(37)~~
	Amyloid-like proteins	Amyloid fibril networks	Formation of controlable topographies	Promotion of focal adhesions	(87)~~(77)~~
	Aqueous silk fibroin sol.	Films	Permeable to oxygen and water vapor	Dependent on sericin protein proportion	(44)~~(44)~~
Growth factor release	Hydrogels	3-D hydrogels formed by amphiphile peptides and bFGF	Can be delivered to living tissues by simply injecting a, aqueous solution	Prolonged release of bFGF; enhanced vascularization	(127)~~(117)~~
	Bacterial amyloids	Functional submicron spherical particles; recombinant	**Porosity**	*In situ* protein refolding and release from amyloids	(128)~~(118)~~
	Self-assembling peptides	Nanofibers	Amphiphilic nature (potential to bind other proteins through weak molecular interactions)	Nanofibers decrease rapid diffusion of proteins away from the injection site, and serve as a reservoir for slow release of PDGF.	(129)~~(119)~~
Targeted drug delivery	Nanoparticles	Pentamer-based oligomers; recombinant	Functionality, *in vivo* stability, large size to	Cell surface receptor binding and endosome-	(116)~~(106)~~

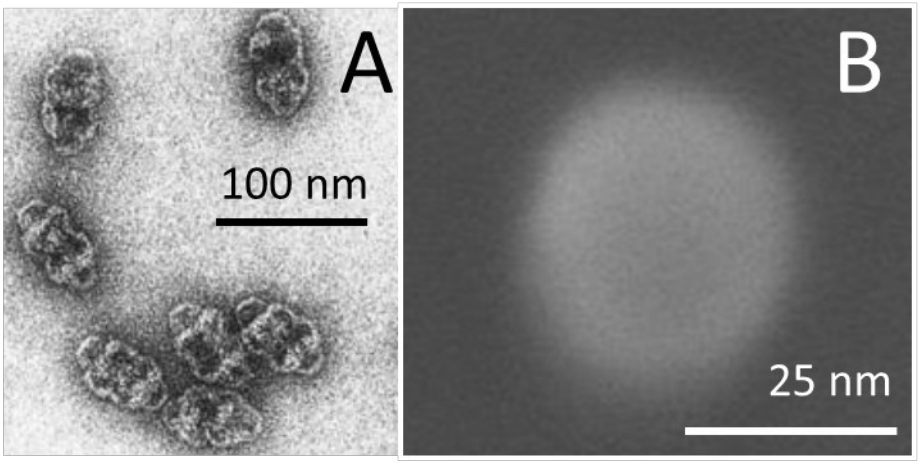
			scape renal filtration, self-assembling	based internalization	
	Eukaryotic vaults	Barrel-shaped, organized in two identical moieties; recombinant	Cell targeting and endosomal escape	Fusing a membrane lytic peptide derived to the major vault protein	(130)~~(120)~~
	Elastin-like proteins	Viscous, gel-like coacervate phase	Thermally sensitive biomaterial (soluble-insoluble phase transition)	Reduce the growth of tumors through the delivery of cancer therapeutics	(131)~~(121)~~
	Elastin-like proteins	Cell penetrating peptides-Elastin-like proteins micelles	Efficient internalization of a wide variety of cargo in diverse cell types	Internalization through cell penetrating peptides	(13)~~(13)~~
Slow drug release	Soy proteins	Biofilms	High tensile strength and modulus; good ductility	Antibiotic encapsulation and slow, sustained release	(65)~~(65)~~
	Hydrogels from aqueous silk solution combined with elastin	Silk-elastin-like protein polymers	Thermally responsive reversible phase transition	Controlled delivery dependent on swelling	(47)~~(47)~~
	Amyloid-like peptides	Fibrils	Improved stability of protein-based drugs	Slow releases of soluble therapeutic protein	(132)~~(122)~~
Vaccination	**Eukaryotic vaults**	Barrel-shaped, organized in two identical moieties	In vitro stability, non-immunogenicity.	Acting as “smart adjuvant”, and reduction of the inflammatory response.	(102)~~(92)~~
	Virus-like proteins	Protein nanocages	Stable epitope-displaying protein structures	Elicit humoral immune response	(107)~~(97)~~
Gene therapy	Nanoparticles	Pentamer-based oligomers	Flexible design and functionalization; self-assembling	Targeted cell penetrability	(114)~~(104)~~
	Elastin-like proteins	DNA-ELPs nano-sized polyplexes	Cationic thermosensitive biomaterial; endosomal	Gene delivery through cationic ELP domains	(133)~~(123)~~

			escape enhancement		
	Virus-like proteins	Protein nanocages; recombinant	Stable DNA-containing protein cages	Tumor specific delivery of expressible DNA using viral functions	(134)~~(124)~~
Anticorrosive protection	FP-1 mussel protein	FP-1 mussel protein composite film with ceria layers	**Dissipation monitoring, quantitative nanomechanical mapping**	High strength coating	(135)~~(125)~~
	Fusion of nacre, mussel adhesive protein and leaf lotus	Coating multifunctional composite grapheme paper	Superhydrophobicity, self-cleaning, anti-corrosion, and remarkable mechanical properties underwater.	Adhesive property and reducing character	(136)~~(126)~~
Wound protection	**Silworm silk proteins**	Porous sponges	Tunable properties	Absorbed exudates and accelerates healing	(137)~~(127)~~
Water dispersible nanoparticles	Mussel based Poly-DOPA + PEI + PEG	Nanoparticles with multiple interaction ligands	Size, stability, biodistribution	Confers stability to other nanoparticles	(38)~~(38)~~
Diagnostics	**Virus-like proteins**	Protein nanocages; recombinant	Mimic antigenic structure of viruses	Quantification of antibody titters	(138)~~(128)~~
	Mussel adhesive proteins	Mussel adhesive prot. coating a cell biosensor	Stability, reusability, sensitivity, microscopy	Immobilizing linker that increases the stability of cells	(139)~~(129)~~
	Amyloid-like protein	Nanofibers	Repeated and ordered disposition of antigenic peptides	Detection of antibodies	(85)~~(75)~~

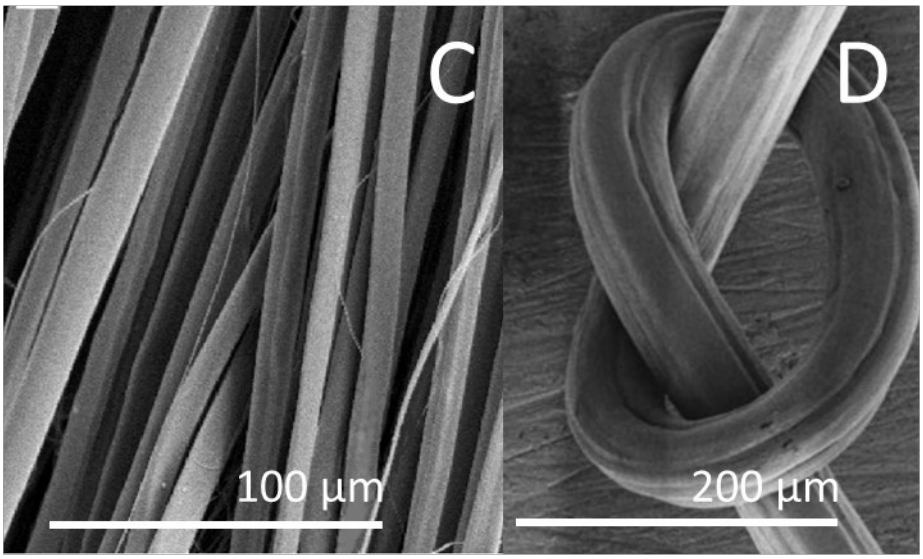
SCMK= S- carboxymethyl kerateine
PEI= polyethyleneimine
PEG= poly (ethylene glycol)

Figure 1. Architectonic and morphologic organization of representative protein materials with potential uses in biomedicine. See ~~Table~~ Appendix 1 for examples of applicability. A) Purified vault particles from rat liver (modified from [100]~~[90]~~). B) Toroid nanoparticles formed by the self-assembling of protein building blocks empowered with end-terminal cationic tags (modified from [116]~~[106]~~). C) Sericin-free silk fiber extracted from *B. mori* (modified from [43]~~[43]~~). D) Recombinant honeybee silk fiber (modified from [55]~~[55]~~). **E)** RADA16-I peptide fibres forming complex matrices ~~Elastin-derived electrospun fibers (left) and hydrogel (right)~~ (modified from [140]~~[5]~~**).** F) Porous silk sponges with pore gradient (modified from [41]). All images were ~~are~~ adapted with permission from the references above indicated. Copyright (2012, 2014, 2003, 2013,2005 and 2007) by American Chemical Society, Wiley and Elsevier respectively. ~~reproduced with permission~~.

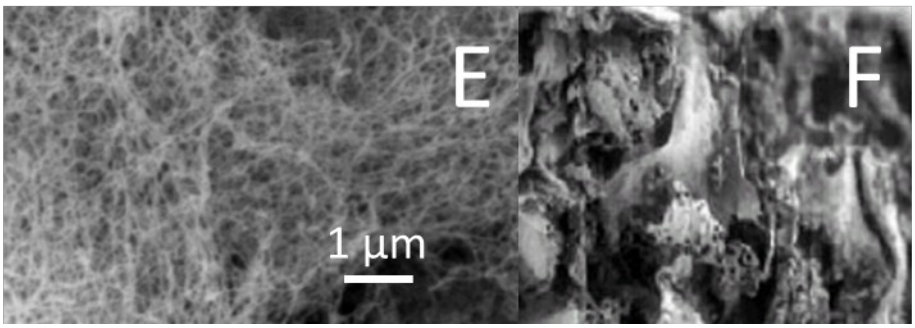
Nano and micro particles



Fibrils, ribbons and tapes



Three-dimensional matrices



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