Advanced Review

Ordered and disordered proteins as nanomaterial building blocks

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Proteins possess a number of attractive properties that have contributed to their recent emergence as nanoscale building blocks for biomaterials and bioinspired materials. For instance, the amino acid sequence of a protein can be precisely controlled and manipulated via recombinant DNA technology, and proteins can be biosynthesized with very high purity and virtually perfect monodispersity. Most importantly, protein-based biomaterials offer the possibility of technologically harnessing the vast array of functions that these biopolymers serve in nature. In this review, we discuss recent progress in the field of protein-based biomaterials, with an overall theme of relating protein structure to material properties. We begin by discussing materials based on proteins that have well-defined three-dimensional structures, focusing specifically on elastin- and silk-like peptides. We then explore the newer field of materials based on intrinsically disordered proteins, using nucleoporin and neurofilament proteins as case studies. A key theme throughout the review is that specific environmental stimuli can trigger protein conformational changes, which in turn can alter macroscopic material properties and function.

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INTRODUCTION

Proteins play a critical structural and mechanical role in both the intracellular and extracellular environments. Within eukaryotic cells, networks of cytoskeletal polymers—classically, actin filaments, microtubules, and intermediate filaments—define cell shape, polarity, and viscoelastic properties. For example, it has been proposed that cellular structure is stabilized in part by a mechanical balance between tensile loads on intermediate filaments and actin, and compressive loads on microtubules.[1] Cytoskeletal networks thereby enable cells to deform in a controlled manner in response to forces imposed by other cells and the extracellular environment. Furthermore, dynamic interactions between cytoskeletal polymers and motor proteins enable directional cell motility and trafficking of cargo within the cell. Outside the cell, the extracellular matrix (ECM) is a solid-state, protein-rich scaffold often composed of network-forming proteins such as collagen, fibronectin, and laminin. The ECM has historically been regarded as a passive scaffold that enables tissues to resist mechanical loads. However, current paradigms view the ECM as being rich in biochemical information with the ability to transduce signals to cells by engaging specific transmembrane receptors on the cell surface, which can in turn lead to cell proliferation, death, and differentiation. The ECM can immobilize and present a variety of growth and differentiation factors to cells as solid-state inputs, which can dramatically influence these molecules' functions.[2–6] It has recently become widely recognized that the biophysical properties of the ECM, such as topography, geometry, elasticity, and dimensionality, can also deliver instructive signals to cells.[7–12] Thus, the cytoskeleton and ECM together represent a continuum of biopolymer networks whose structural, functional, and mechanical properties are determined by the composition and architecture of their respective molecular components.

Critical to the function of these and other protein networks is their ability to self-assemble in defined and reversible ways, respond to specific environmental stimuli, and adapt to mechanical loads. Given the technological desirability of these properties, there

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has been a significant effort to develop biomaterials that mimic core 'design principles' of cytoskeletal and ECM proteins for a variety of engineering applications. For example, a tissue engineering scaffold must provide architecture and mechanical support, and potentially be able to deliver instructional cues to cells; drug delivery carriers must release their cargo in a controlled fashion and ideally respond quickly to external stimuli; indwelling medical implants must provide mechanical support where required and be capable of integration with the surrounding tissue without evoking an inflammatory response. For these reasons, a variety of strategies have been developed to 'tune' physical properties of the biomaterial to achieve specific functions. These include varying material composition,[13–16] imparting micro- or nanoscale texture,[17–19] varying material topography,[20–22] and incorporating biochemical cues.[23–27]

Efforts to develop biomaterials that mimic the unique functional characteristics of native proteins can be broadly divided into two categories: synthetic polymer-based approaches and engineered protein-based approaches. The use of synthetic polymers as biomimetic materials has been explored for many decades (see Refs 28 and 29 for a detailed review). Synthetic polymers capture one or more aspects of structural proteins, including biocompatibility, biodegradability, stimulus-responsiveness, and mechanical strength, with the advantage of almost limitless chemical diversity. However, an important drawback of these systems is that unlike proteins, synthetic polymers in general do not adopt the well-defined secondary and tertiary structures that may be required for maximal bioactive presentation of ligands to cells. Moreover, despite advances in synthesis and purification strategies, polydispersity (i.e., variation in molecular weight) remains a significant restriction on the development of synthetic polymers as biomimetic materials. By contrast, solid-phase synthesis of peptides or generation of recombinant proteins in bacteria (or other expression systems) routinely yields products with a precise amino acid sequence and molecular weight. In principle, it should be possible to confer protein-based scaffolds with desired mechanical and biochemical properties through judicious tailoring of the amino acid sequence. This could be accomplished by incorporating domains from two or more naturally occurring proteins, *de novo* sequence design (i.e., as predicted by computational models), or identification of optimal sequences by directed evolution or other selection/amplification approaches. In all of these cases, protein synthesis using recombinant DNA technology in hosts such as yeast, mammalian, insect cells or more commonly in bacterial systems such as *Escherichia coli* allows for unparalleled control over sequence and hence biochemical properties. Recombinant DNA technology greatly facilitates a modular approach to design in which individual material parameters may be orthogonally manipulated. For instance, increasing the number of domains involved in cross-linking without changing the number of domains providing the ligand (biochemical cues) would be expected to affect scaffold rigidity without affecting ligand density and vice versa.[23] For these reasons, researchers are increasingly exploring engineered peptides and proteins as building blocks for biomimetic materials. These efforts have included the development of semisynthetic scaffolds with protein domains that elicit specific cellular responses, self-assembling peptides, and unstructured stimulus-responsive protein domains.

In this review, we highlight recent progress made in the field of protein-based bioinspired materials. We begin by discussing recent advances in the use of proteins that adopt well-defined secondary and tertiary structures, focusing specifically on the use of silk- and elastin-like proteins in semisynthetic scaffolds. We then discuss the use of unstructured proteins as engineered biomaterials, which share many common physical features of synthetic polymers and represent a largely untapped resource for the creation of new stimulus-responsive biological macromolecules.

ORDERED PROTEINS IN BIOMATERIALS

Proteins are traditionally understood to derive function from their ability to adopt a stable and well-defined three-dimensional structure, which is typically described in terms of various levels of hierarchy. The primary structure is defined as the linear sequence of amino acids, secondary structure (e.g., α-helices, β-sheets) is formed as a result of hydrogen bonding between peptide backbone atoms, and tertiary structure refers to the spatial association of specific secondary structural elements. Many biomaterials have been developed for tissue engineering and drug-delivery applications that harness folded proteins or protein domains to perform a variety of functions, such as conferring elasticity,[30] promoting cross-linking,[23] facilitating material degradation,[23] and fostering biomineralization.[31] In some cases, these materials have included peptides or proteins that undergo conformational changes in response to environmental stimuli, such as pH-induced helix-coil transitions that give rise to macroscale sol–gel transitions[32,33] or large-scale conformational changes triggered by ligand binding.[34,35]

Structural changes modulated by temperature have been observed in elastin-like proteins (ELPs).[30,36] Elastin is a fibrous unit formed by many monomers of its precursor tropoelastin, which is comprised of alternating repeats of hydrophobic and cross-linking domains. The cross-linking domain is comprised of Ala and Lys residues, while the hydrophobic domain is composed of repeats of Val-Pro-Gly-Val-Gly or Ala-Pro-Gly-Val-Gly-Val motifs. The hydrophobic domains drive amphiphilic self-assembly, resulting in a structure that has a hydrophobic core and is stabilized by cross-linking of the lysine residues in the hydrophilic exterior. Biosynthetic ELPs are modeled with repeating units of Val-Pro-Gly-X-Gly, where X is any amino acid except proline. ELPs are soluble in aqueous solution, but at temperatures higher than the transition temperature, ELPs form insoluble particles with β-spiral structure that coalesces upon further incubation and form highly organized filamentous structures. This 'inverse phase transition'[30] is reversible, with subsequent cooling resulting in resolubilization of the aggregates. Both the identity of the 'wobble' residue and the length and number of the repeating hydrophobic unit have a profound effect on the temperature at which the phase transition occurs. These properties make ELPs highly tunable and therefore an attractive medium for drug delivery, tissue engineering, and wound healing applications.

The ability of ELPs to transition between soluble and insoluble phases has been exploited in tumor targeting (Figure 1). In a recent study, the tendency of ELPs to aggregate at higher temperatures was utilized by injecting soluble peptides intravenously into mice. ELPs accumulated to a greater extent in implanted, subcutaneous tumors exogenously heated to 42°C in comparison with tumors maintained at physiological body temperature of mice.[37] Furthermore, *in vitro* studies on heated tumor cells also reveal enhanced accumulation of ELPs than cells maintained at 37°C.[38] It has been suggested that the temperature-dependent uptake of ELPs could be exploited for the targeted delivery of chemotherapeutic agents to tumors. Furgeson et al. anchored doxorubicin to an ELP via a hydrozone linker and showed that at acidic pH, representative of endosomes and lysosomes, the hydrosome linker is cleaved and the free drug is released.[39] Studies have suggested that it is essential for ELPs to remain as particulates for efficient uptake and delivery of therapeutics, which has led much attention to be placed on tuning the size and monodispersity of ELP-based particles. In a recent study, hollow spheres of ELPs were generated by a template-based technique in which ELPs were allowed to self-assemble on the surfaces of polystyrene beads

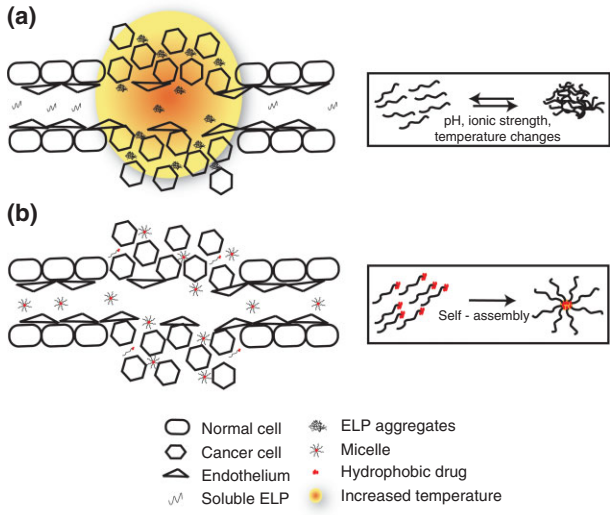

FIGURE 1 | Schematic of two common drug delivery strategies using elastin-like proteins (ELPs). (a) Thermally triggered aggregation of soluble ELPs in tumors, which typically have higher temperatures than surrounding healthy tissues (Inset: aggregation triggered by temperature, pH, or ionic strength changes). (b) Hydrophobic drugs attached to ELPs trigger self-assembly into micelles, which then preferentially accumulate in tumors by passive diffusion (Inset: self-assembly triggered by drug attachment).

and then stabilized using a cross-linking agent.[40] The polystyrene beads were then dissolved using an organic solvent leaving behind a spherical shell of ELPs. These spheres exhibited higher loading efficiencies of plasmid DNA than nontemplated, self-assembled ELP particles and displayed controlled release upon proteolytic degradation. Loading efficiencies were further improved by complexing the DNA with a polymer.[41] The spheres also supported higher transfection and expression efficiencies than either nontemplated ELPs or naked plasmid DNA as observed by the expression of the luciferase reporter gene. These results indicate that ELP hollow spheres could potentially be employed as gene delivery machines as well.

ELPs have also found applications in therapies designed to foster wound healing, illustrating their versatility. For example, Koria et al. created a recombinant fusion protein of ELP and keratinocyte growth factor (KGF).[42] KGF, an FGF-family growth factor important for epidermal morphogenesis and wound healing, is secreted at high levels after injury. In diabetic patients, however, the secretion is reduced, contributing to poor wound healing.[43] An ELP-KGF fusion protein was expressed in *E. coli* and found to self-assemble into nanoparticles when reconstituted in solution. Culture studies performed on A431 epithelial cells[44] demonstrated that the ELP-KGF nanoparticles

were capable of inducing cell proliferation similar to free KGF. When applied to wounds in diabetic mice, the nanoparticles induced re-epithelialization, granulation, and enhanced dermal/epidermal regeneration. Granulation induced by the fusion protein was just enough to allow for migration of keratinocytes in the wound but not excessive enough to cause fibrosis or scarring. These results suggest that self-assembled ELP nanoparticles may be used to promote wound healing.

While elastin-based biomaterials offer improved water solubility and flexibility, it is possible to introduce crystallinity in a controlled fashion within the matrix using silk-like proteins (SLP), which confer thermal and chemical stability. These two materials represent the two extremes of coupling between structure and function; silk-like domains can spontaneously form crystalline β sheets, while elastin-like domains decrease crystallinity (comparative properties summarized in Table 1). Similar to ELPs, SLPs represent a particularly instructive example of microscale structural properties that modulate macroscale material properties.

Silk is a high molecular weight protein that contains a high percentage of alanine and glycine residues occurring in a repeating motif. Silk proteins form highly fibrous structures that possess high strength, toughness, and ductile elongation. Long fibers produced from a recombinant spidroin protein expressed in *E. coli* have higher tensile strength than mammalian bones and tendons.[45,46] Observations such as these have inspired efforts to engineer proteins that have similar mechanical properties. For example, an engineered polypeptide (SLP4) consisting of alanine-glycine repeats (Gly-Ala-Gly-Ala-Gly-Ser) was found by wide angle X-ray scattering to have a crystalline structure very similar to silk.[47] The crystallinity, however, depended strongly on the presence and composition of 'interruption sequences' between the silk-like blocks; for instance, incorporation of a Gly-Ala-Ala-Gly-Tyr block containing a bulky tyrosine residue decreased crystallinity.[48] An SLP interspersed with the Arg-Gly-Asp (RGD) cell-binding domain of fibronectin was found to self-assemble into a semicrystalline structure of a uniform width whose value depended on the length of the silk domain.[49] To address the ability of SLPs to support cell adhesion, Wang et al.[50] produced recombinant spider silk protein with an RGD motif and showed that these scaffolds could support fibroblast adhesion, growth, and secretion of FGF-2, which plays key roles in fibroblast-mediated wound healing and tissue repair.

TABLE 1 | Comparison of the Properties of Silk-Like Proteins and Elastin-Like Proteins

Properties	SLP	ELP
Domain sequence	GAGAGS	VPGXG X ≠ Pro
Structural characteristics	Crystalline	Disordered below transition temperature but forms β-spirals above transition temperature
Tunability	Chain length	Temperature
	Fusion with other proteins	pH
		Ionic strength
		Chain length
		Fusion with other proteins
Applications	Tissue engineering scaffolds	Tissue engineering scaffolds
		Drug delivery
		Protein purification

Because of the ability of silk and SLPs to assemble into fibers and sheets with high structural integrity and well-defined supramolecular order, these materials have been explored as implant coatings that might promote better integration with surrounding tissue. Gomes et al.[51] created a fusion protein of a hexameric SLP (termed '6mer' in the study) and bone sialoprotein (BSP), which plays a significant role in the calcium phosphate deposition that is needed to form hydroxyapatite and support collagen binding in bone tissue. The SLP-BSP protein was cast as thin films and the film morphology and mechanical properties were characterized.[52] These studies revealed that the 6mer-BSP fusion protein maintained the ability of the SLP domain to self-assemble and fold into β-sheets. AFM studies indicated that the fusion protein and 6mer films exhibited very different self-assembly and mechanical properties. The fusion protein assembled into stiff elongated sheets as well as spherical particles, whereas the 6mer alone formed relatively soft, globular, and amorphous structures. The authors speculated that these differences might be due to the high glutamic acid content of BSP and subsequently postulated that these residues could also be coordinated and ionically cross-linked by Ca^{2+} to induce supramolecular structure.[52] Similar to full length BSP, the 6mer-BSP film was capable of inducing calcium phosphate deposition and supported human mesenchymal stem cell proliferation and differentiation into an osteogenic lineage. Scanning electron microscopic analysis of the morphology of calcium phosphate deposited on the fusion protein film indicated that upon adhesion, cells induced controlled mineralization resulting in a globular structure (Figure 2).[51] This study demonstrates the potential application of SLPs

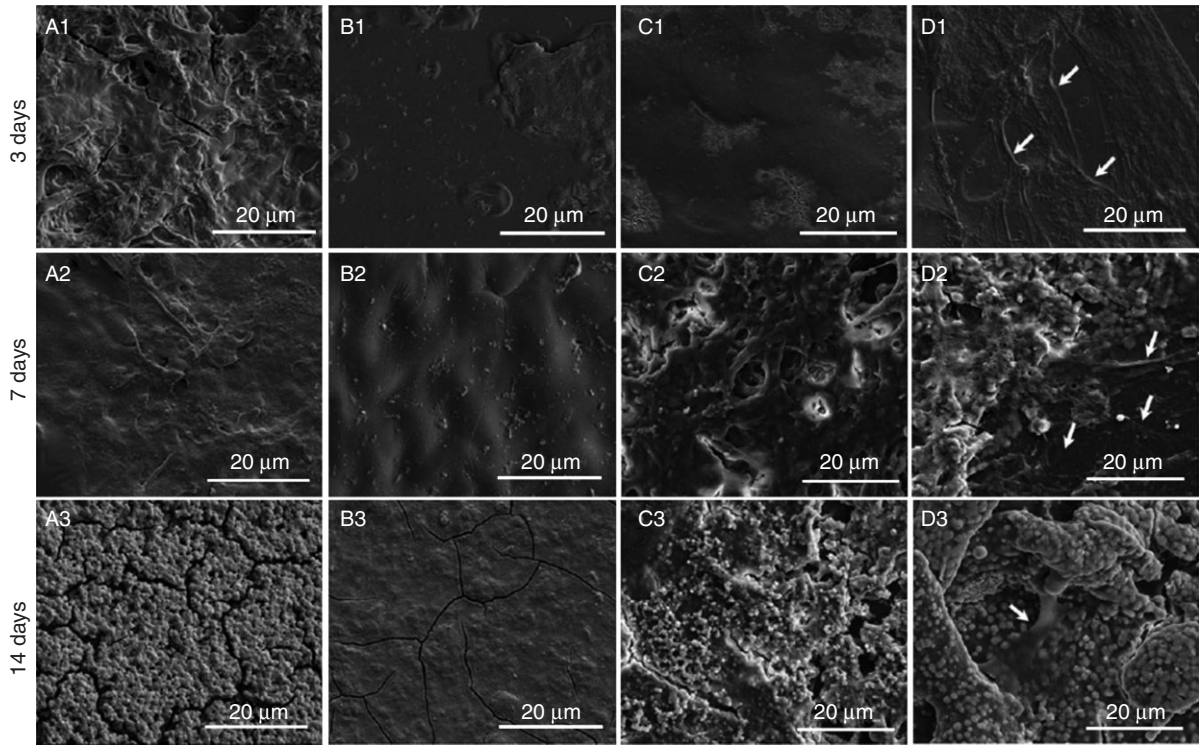

FIGURE 2 | Stem cell adhesion and differentiation on silk-like protein (SLP)-based materials. Morphology of hexameric SLP (6mer) and 6mer-BSP films seeded with human mesenchymal stem cells and cultured for 3, 7, and 14 days. A1–A3 and C1–C3 represent 6mer film with and without cells, respectively. B1–B3 and D1–D3 represent 6mer+BSP film with and without cells, respectively. Calcium phosphate film on 6mer+BSP film seeded with cells (D3) appears globular while those without cells appear flat (B3). Arrows indicate cells with osteoblastic morphology. (Reprinted with permission from Ref 51. Copyright 2011 RSC Publishing)

as a scaffold for bone regeneration and validates the use of chimeric systems with functional domains as a new class of biomaterials. Along similar lines, human antimicrobial proteins were expressed as fusion proteins with spider SLP, with the goal of producing films that were highly resistant to bacterial adhesion.[53] These chimeric proteins possessed silk-like properties (self-assembling into β-sheets) as well as antimicrobial properties against *E. coli* and *Staphylococcus aureus*. These scaffolds exhibited low toxicity to mammalian cells and could also sustain proliferation of the human osteosarcoma cell line (SaOs-2).

In addition to developing new biomaterials consisting entirely of SLPs, SLPs can also be used to further optimize the mechanical properties of existing scaffolds. For example, gelatin methacrylate (gelMA) hydrogels, which have been shown to promote cell proliferation, migration, and spreading, are used as tissue engineering scaffolds because of their natural cell adhesivity, biodegradability, and compatibility with microfabrication techniques.[54–56] However, these gels are degraded rapidly and are intrinsically quite soft, rendering them inappropriate for applications requiring rigid materials. To address these shortcomings, Khademhosseini and coworkers recently developed an interpenetrating network of silk fibroin and GelMA.[57] Photocrosslinking GelMA followed by methanol-activated crystallization of the silk fibroin resulted in a gel with an interpenetrating, crystalline β-sheet silk structure that functioned as a reinforcement by increasing the compressive modulus of the gel.[57] As expected, increasing the silk concentration resulted in stiffer and less porous gels. With increasing silk concentration, the hydrogel displayed resistance to degradation by collagenase probably due to the decreased diffusion of the enzyme through the interpenetrating network. Cell attachment studies indicated that an optimal silk-to-GelMA ratio exists at which cells spread and proliferate rapidly. Increasing the silk concentration beyond this limit resulted in decreased cell proliferation. Therefore, by altering the silk-to-GelMA ratio, it is possible to optimize the physical and biological properties of the matrix to reflect the desired cell proliferation properties.

The powerful features of the two structural components described here (silk-like and elastin-like domains) have been harnessed in a single biomaterial

by synthesizing fusion proteins referred to as silk elastin-like proteins (SELPs). In general, the physical properties of SELPs may be tightly controlled by varying the relative fractions of SLP and ELP blocks in the polymer. For instance, selected polymers from the SELP family, usually with more elastin-like blocks, form hydrogels spontaneously from aqueous solution.[58] Another significant property, the rate of resorption of SELP implants, was found to depend on the length of the silk-like blocks, such that polymers with less than eight repeating silk-like units degraded and resorbed faster than those with more than eight repeating silk-like units. By carefully tailoring the sequence of amino acids within each block, it is possible to program 'smartness' into the polymers.[59] Since SELPs have many traits frequently valued in drug delivery vehicles, recent efforts have been directed toward understanding the ability of the matrix to incorporate and release small molecules, proteins, and DNA. Studies have also been undertaken to address biocompatibility and optimize the rate of biodegradation under physiological conditions. The study of SELPs, ELPs, and SLPs are rapidly maturing fields, and extensive literature describing properties and characterization of each system is available.[30,36,48,60,61]

Silk- and elastin-like domains function as structural components whose well-defined conformational properties largely determine bulk mechanical properties and are highly sensitive to environmental cues. Although these structured proteins have many attractive qualities exemplified in the studies discussed above, protein domains lacking inherent structure are also emerging as a novel starting material for developing biomaterials. The following section describes recent advances in using intrinsically disordered proteins (IDPs) as building blocks.

INTRINSICALLY DISORDERED PROTEINS AS BIOMATERIALS

Unlike proteins that have a well-defined three-dimensional structure that dictates their function, IDPs have little secondary structure and assume a highly flexible, extended conformation.[62,63] IDPs play a key regulatory role in a wide variety of cellular processes and have been classified into many broad categories based on their functional attributes.[64,65] For the purpose of discussing biomimetic materials, we focus on a group of IDPs that function as entropic chains in the form of springs and bristles. To maximize conformational entropy, the degree of disorder increases: In the case of springs, the system returns to its original, tightly coiled state upon removal of deforming forces while bristles function primarily by excluding a region of repulsion due to random, thermal motion of the protrusion. Entropy-based function is a niche that IDPs can uniquely fill due to the inherent flexibility conferred by their disordered state. The entropic role of IDPs is evident in a variety of systems, such as the projection domain of the microtubule associated protein 2 (MAP2) and the sidearm domain of the neurofilament medium and heavy proteins. Intact neurofilaments (NFs) and microtubule-bound MAPs share similar structural characteristics: They both form long filamentous structures with protrusions extending away from a rod-like backbone, giving them a bottle-brush like appearance. The projections exert long-range repulsive forces that lead to mutual steric exclusions and thereby maintain interfilament spacing in the cytoskeleton.

Before discussing IDPs as entropic bristles, we first review the incorporation of short peptides into synthetic polymer brushes, which are structures that form due to entropic repulsion when certain polymers are grafted to surfaces at very high densities. Polymer brushes have been characterized extensively and have found wide applications in nonbiomedical fields as lubricants,[66,67] colloidal stabilizers,[68,69] and adhesives.[70] More recently, in the realm of biomaterials, polymer brushes have been explored for use as protein and cell adhesion-resistant, nonfouling coatings for biomaterials. This is especially important for *in vivo* applications such as biomedical implants where protein absorption can either disrupt bioactivity or trigger a variety of undesirable events such as thrombus formation and fibrosis. In recent years, polymer brushes have increasingly been used to precisely regulate cell adhesion and prevent nonspecific interactions. In one approach, the ECM protein fibronectin was adsorbed onto polymer brushes grafted with a linear molecular weight and/or density gradient, with the goal of allowing cells to adhere to the fibronectin layer while reducing nonspecific protein absorption via the underlying brush layer.[71] However, the nonfouling property of the underlying brush layer was somewhat unstable due to the noncovalent nature of protein immobilization on the brushes, and in an attempt to rectify this problem, Harris et al.[72] covalently conjugated fibronectin to the polymer brushes. In this case, the ligand density gradient was maintained by varying the brush density. Using photopolymerization, the grafting density was increased along the length of substrate without altering the molecular weight such that the thickness of the grafted brush layer increased linearly. With increasing brush density, an increasing RGD ligand density gradient was established. Interestingly, much higher surface RGD concentrations were achieved using this approach than through a

variety of other traditional protein immobilization strategies such as passive adsorption of proteins or assembly of peptide-modified self-assembled monolayers. The flexibility of the chains was postulated to allow for maximum receptor occupancy and ligand clustering.

As an extension to this study, Raynor and colleagues recently modified polymer brushes with peptides capable of controlling cell adhesion and adhesion-dependent differentiation, with the goal of coupling these materials to titanium implants to enhance integration and osteogenesis.[73] Specifically, they developed modified poly (oligoethyleneglycol) methacrylate (OEGMA) brushes terminated with either an adhesion-promoting peptide (RGD) alone or a recombinant fibronectin fragment containing both the RGD sequence and the synergy site. The modification to OEGMA played a crucial part in anchoring the protein to the brush and led to a sevenfold improvement in the tethering of the signaling peptide to the modified brushes in comparison to unmodified OEGMA brushes. This allowed control of cell adhesion on protein functionalized brushes over a background of nonfouling, unmodified brushes. Integrin blocking assays (using antibodies specific to β_3 and α_5 subunits) confirmed that $\alpha_v\beta_3$ and α_5 β_1 integrins were required for cell adhesions to substrates presenting the RGD peptide and the fibronectin fragment, respectively. When the adhesion of cells seeded on the fibronectin fragment-tethered substrate was inhibited by treatment with antibodies against $\alpha_5\beta_1$ integrin, focal adhesion kinase (FAK) phosphorylation decreased, indicating that the surface-immobilized signaling sequence may not only increase cell adhesion but may also regulate downstream signaling. Tyrosine phosphorylation of FAK (important in osteogenic differentiation) increased seven days after seeding the cells on substrates with tethered fibronectin fragments, which correlated with increased osteoblast differentiation. Furthermore, fibronectin-tethered brushes triggered expression of markers associated with osteoblast differentiation in rat bone marrow stromal cells as evidenced by elevated levels of transcription factor for bone formation and late osteoblastic markers. *In vivo* studies in rat using clinical grade titanium implants coated with modified polymer brushes tethered to fibronectin fragments have revealed successful integration with the surrounding bone and reduced dislocation of the implant.[74]

The above results illustrate the power of incorporating peptides into synthetic polymer brushes. Additional recent work has focused on the development of polymer brushes composed entirely of peptides and proteins, which might be expected to exhibit even higher biocompatibility. Such materials would also be ideally positioned to take advantage of recombinant DNA technology, which readily enables engineering proteins with desired properties such as responsiveness to specific environmental stimuli. A classic example involves a polyglutamate peptide brush grafted on to a porous polymer membrane that is capable of pH gating.[75] The highly ionizable peptide can undergo a conformational change from a compact, helical structure at low pH to a swollen, disordered state at high pH, thereby controlling permeation of water and ions across the membrane. At high pH, the electrostatic repulsion between the glutamate side groups causes the chains to be extended. These extended chains cover the pores and reduce the rate of water permeation. The coiled conformation at low pH reduces steric obstruction and maintains the porosity of the membrane to allow for higher permeation rates.

One of the main considerations in forming brushes using proteins is that the three-dimensional structure of proteins may preclude the high grafting densities needed for effective brush formation. More specifically, the protein must be able to maintain an extended structure at high density without collapsing or aggregating. For this reason, IDPs have emerged as attractive building blocks for constructing polymer brushes. They often have fairly simple sequences comprised of repeating units of charged amino acids that aid in the formation of brushes.[62–65] In addition, IDPs can be designed to undergo rapid conformational change (e.g., from random coil to extended state) when exposed to external stimuli such as changes in pH, ionic strength, or phosphorylation state. Of the many IDPs that have been identified recently, we discuss two specific examples that have received particularly intense interest: the phenylalanine-glycine repeat domain of nucleoporins and the sidearm domain of NFs.

Nucleopore complexes are gaps in the nuclear envelope that enable transport of molecular cargo into and out of the nucleus. The central pore of the complex is an hourglass shaped channel that is responsible for gating. While small ions and small molecules such as water permeate freely through the channel, large molecules such as proteins require the presence of a nuclear localization signal to pass through the pore. Proteins that do not harbor the signal sequence or those that are not anchored to a transporter protein are prevented from transport across the membrane irrespective of their size. It is believed that natively unfolded phenylalanine-glycine domains within nucleoporins play a significant role in defining the gating mechanism, models for which have been reviewed elsewhere.[76] In a recent study

aimed at delineating the mechanism behind gating, the phenylalanine-glycine domains were expressed in *E. coli*, purified, and anchored on gold nanodot surface arrays of approximately 100 nm diameter and 1 μm interparticle spacing.[76] At sufficiently high grafting densities, the thermally mobile protein molecules were expected to repel each other, extend out from the surface and acquire a brush-like conformation. AFM force measurements indicated that the brush layer exerted a long-range repulsive force that decays exponentially, consistent with an Alexander-deGennes polymer brush.[77] Furthermore, the brush collapsed when exposed to a less polar solvent and swelled again when the initial solvent was restored. These results were interpreted as supportive of the entropic barrier model, wherein a barrier is formed by the brush-like conformation of the nucleoporin complex. Under this model, cargo not destined for transport into the nucleus would be repelled by the brush, whereas a molecule bound to a transport protein or a receptor would be trapped due to attractive forces (hydrophobic interactions) between the receptor and the phenylalanine-glycine domains. Similar brush-like properties of different phenylalanine-glycine domain-containing nucleoporins such as NSP1 from yeast have been observed, and their interaction with transport receptors have been modeled.[78,79] On the basis of these studies, artificial nanopores that mimic nucleopore complexes have been constructed. One such system consists of a perforated membrane sputter-coated with gold on one face to which thiol-terminated phenylalanine-glycine domains of the nucleoporins were adsorbed (Figure 3).[80] This membrane was shown to function as a 'nanoselective filter' that allows passage of transport factors and transport factor bound cargo that bind nucleoporins, while hindering transport of proteins that are not bound to transport factors. Specific and reversible accumulation of nuclear transport factor protein on the functionalized membranes is reminiscent of the reversible, transient binding between nucleoporins and transport factors that exists *in vivo*.[81] The transient binding of transport factor and the accompanying change in the conformation greatly enhanced the inhibition of transport of 'noncargo' proteins. The selectivity of the channel was found to be dependent on the pore diameter and the length of the pore that was coated with the nucleoporins. Consistent with results from Lim et al.[76], introduction of a less polar solvent completely abolished the selectivity of the membrane indicating that the maintenance of the brush structure was essential to the proper functioning of the channel. Biomimetic systems constructed using other nucleoporin proteins (Nup98 and Nup153) also exhibit similar transport

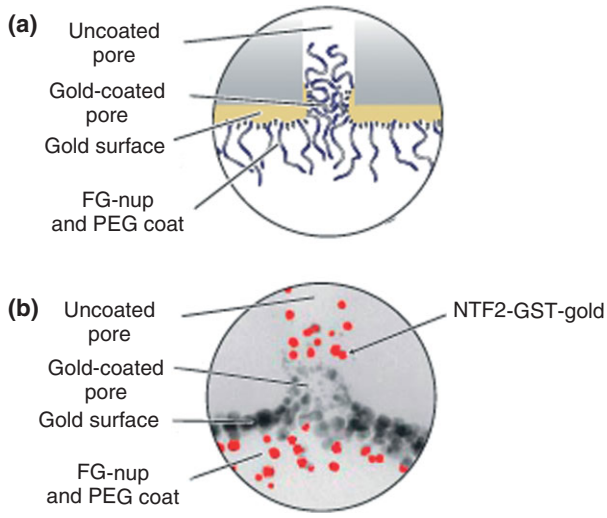

FIGURE 3 | Gating nanopores with synthetic polymer brush. (a) Schematic of a single pore in a polycarbonate membrane coated with gold and functionalized with nucleoporins. A PEG layer was used to block any remaining exposed gold surface. (b) Transmission electron micrograph of a single pore on the functionalized membrane, incubated with receptor protein-bound cargo (pseudocolored red). Receptor protein-bound cargo binds to the nucleoporin layer on the gold surface and transits through the pore. (Reprinted with permission from Ref 80. Copyright 2009 Macmillan Publishers)

properties.[82] On the basis of ionic current measurements, it was demonstrated that the barrier's effectiveness in screening out cargo not intended for transport through the pores was primarily due to the intrinsic difference in amino acid sequence of the proteins.[82]

Whereas the FG repeats of nucleoporins represent an IDP that functions by regulating transport, NFs represent an example of an IDP that mediates the assembly and mechanics of a cellular structural network (See Table 2 for a comparison of nucleoporins and NFs). NFs are type IV intermediate filaments that are the most abundant cytoskeletal component of many axons, including large myelinated axons. Mammalian NFs are composed of three proteins, namely the light (61 kDa), medium (90 kDa), and heavy (110 kDa) subunits. The rod-like N-terminal domains of the three proteins interact to form the core of the filament while the disordered C-terminal sidearm domain of the medium and heavy proteins extend out from the backbone of the filament to form protrusions.[83] The sidearms contain repeats of lysine-serine-proline (KSP) motif in which the serines are strongly subject to phosphorylation. The nonrandom arrangement of NFs within the axon suggests that the filaments physically interact with one another, and while the sidearms are believed to modulate these interactions, the details of this interaction have remained controversial. In one model, NFs are held

TABLE 2 | Comparison Between Neurofilaments and Nucleoporin Proteins

Properties	Nucleoporin Proteins	Neurofilament Proteins
Structural features	Proteins of the nuclear pore complex that form an eightfold symmetric central framework encompassing a central pore	Filaments comprised of neurofilament light, medium, and heavy chains. Unstructured sidearm domains extend from the filament backbone
In vitro function	Regulate transport across nuclear membrane	Mediate assembly and mechanics of cytoskeletal networks
Unstructured domain	Phenylalanine-glycine domain of nucleoporin proteins such as Nup 153 and NSP1	Sidearm domain of neurofilament medium and heavy chains
Molecular weight of unstructured domain	≈ 60 kDa	≈ 70 kDa
Tunability	Solvent polarity	pH Ionic strength Phosphorylation Subunit composition
Potential applications	Gated artificial nanopores	Protein-based environmentally sensitive polymer brushes

together by noncovalent crossbridges between neighboring sidearms.[84] This is supported by studies in which NFs form gels with parallel arrays of filament bundles that appear cross-linked in the presence of specific solutes, such as divalent cations.[84] Further support for this model was provided by studies in which the cross-linking and gelation kinetics could be modulated by the addition of synthetic peptides that mimicked the sidearm domain.[85] More recently, a second model suggesting repulsive interaction between NFs has gained momentum (Figure 4).[77] The Brownian motion of the disordered sidearms is proposed to produce a zone of exclusion wherein repulsive forces of entropic origin dominate. This causes the sidearms to protrude away from the backbone in an extended fashion resulting in a structure approximating a cylindrical polymer brush.[77,86] Both computational studies[87] and *in vitro* measurements[88] strongly suggest that altering the electrostatics of the sidearm by phosphorylating and dephosphorylating the KSP repeats can further modulate the repulsive interaction. Subsequent studies have suggested that this model may also hold for microtubule associated proteins, which form a brush-like layer around microtubules.[89] The polymer brush-like sidearms have been postulated to provide the axon with mechanical stability by protecting it from compression, which could in turn help preserve electrical conduction and transport. The Brownian motion of the unstructured sidearms can potentially allow them to function as springs, resist compression and radially stiffen the axon. It is interesting to note that NF gels have comparatively large elastic moduli, and as a result resist stresses and strains much more effectively than gels composed of vimentin, microtubules, or actin filaments.[84] In fact, even when external pressure (osmotic stress in this study) was increased by two orders of magnitude, the interfilament spacing did not change significantly.[90,91] Theoretical studies indicate that the interaction between NF-medium and NF-heavy proteins is weaker than the interactions between NF-medium and NF-light or NF-heavy and NF-light proteins.[92] The length of their tails, however, is important in dictating NF thickness and compressibility.[92]

Another important property of NF gels is their tunability; for example, the gel's mechanical properties and responsiveness to external stimuli can be regulated by altering subunit stoichiometry. Gels composed of NFs containing all three subunits undergo an abrupt transformation from an expanded to a condensed state when osmotic pressure is applied,[90] and this transition depends very weakly on salt concentration. Gels composed of NFs formed from the heavy and light chains only showed a similar transition but with steeper salt dependencies. Gels composed of NFs formed from light and medium chains displayed a less abrupt transition. Further control of interfilament spacing is afforded by the phosphorylation state of the sidearm domains. Calculations indicate that extensive phosphorylation of the NF-medium and NF-heavy protein sidearms leads to an increase in brush size, which would be expected to increase interfilament spacing.[93] Similarly, dynamic light scattering studies show that upon

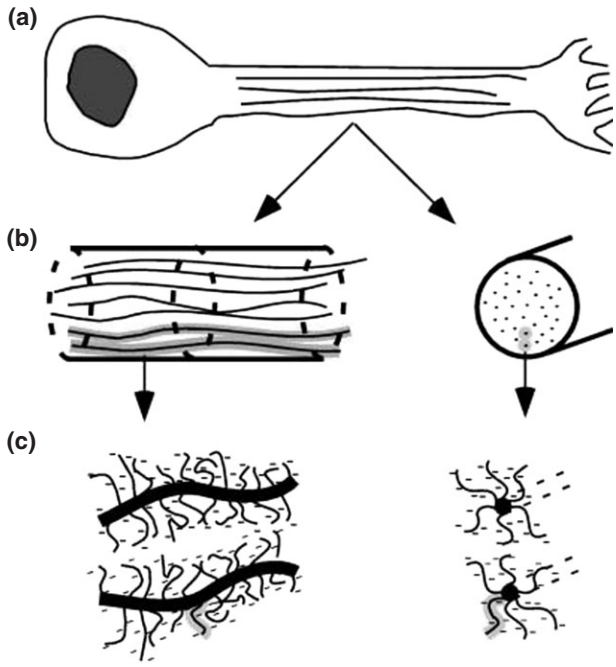

FIGURE 4 | Regulation of neurofilament (NF) architecture by intrinsically disordered proteins (IDPs). (a) NFs are arranged in a parallel fashion along the length of the axon. (b) Cross-sectional views showing the nonrandom spatial arrangement of NFs. (c) Individual NFs with a structured backbone and IDP domains extending away from it. Steric repulsive forces mediated by the IDPs generate a zone of exclusion around each NF core, represented in gray. (Reprinted with permission from Ref 88. Copyright 2009 Elsevier Inc.)

phosphorylation the hydrodynamic radius of the sidearm domain increases by as much as 50 nm, indicating expansion of the sidearm.[94] NF gels have also been shown to respond strongly to variations in pH and ionic strength.[91,95] NF gel volume has been found to increase with increasing ionic strength over a wide range of pH. This effect is further magnified when the sidearms are phosphorylated.[88] In summary, the unstructured NF sidearm domain enables the structure and mechanics of NF gels to be tuned over a wide range through alterations in phosphorylation and solvent conditions. Future studies should reveal whether this IDP could serve as a template for the construction of synthetic IDP-based materials.

FUTURE PERSPECTIVES AND CONCLUSIONS

Our goal has been to provide an overview of the emerging field of peptide- and protein-based biomaterials, with an emphasis on the relationship between protein self-assembly, three-dimensional structure (or lack thereof) and macroscopic properties. A recurring theme is that recombinant DNA technology may be leveraged to generate molecules with great diversity and potential for optimization toward specific functions by inclusion of domains that confer tailored responsiveness and biochemical cues (e.g., bioactive ligands, cross-linking). As the examples in this review illustrate, these materials have found extensive use both in fundamental studies of biological processes, new microdevices that mimic these processes, and technologies targeted toward specific applications, including clinical applications.

One of the most attractive properties of protein-based biomaterials, as exemplified by our discussion of ELPs and SLPs, is their ability to self-assemble into higher-order structures with desirable material properties. In a sense, then, protein-based biomaterials can be regarded as nanoscale biological machines, and a major challenge and opportunity for the field will be to learn how to incorporate these machines into synthetic micro- and nanodevices. Recent work with microtubule-based devices offers an early glimpse at how this might be accomplished. Microtubules are cylindrical cytoskeletal polymers that serve many critical cellular functions, including establishing cell polarity and serving as tracks for the directional trafficking of intracellular cargo. In an attempt to capture the latter function, microtubules have been incorporated into a variety of microscale transport devices. For example, a variety of microdevices have been developed that mimic microtubule-kinesin intracellular transport machinery,[96,97] including a microtechnological platform consisting of a network of oriented microtubules along which kinesin-coated beads could move in a directional fashion.[96] In a more recent effort, microtubule-actin hybrid nanostructures were fabricated in an effort to harness the transport functions of kinesin and myosin along microtubules and actin, respectively.[97] Microtubules have even served as the basis of new scaffolds for bone regeneration. For example, calcium phosphate nanocrystals were grown on RGD functionalized microtubule-based structures assembled from a bolaform amphiphile. The coated microtubules were found to be biocompatible and supported cell-attachment and proliferation of mouse embryonic fibroblasts, suggesting that this system may eventually find use in orthopedic and dental applications.[98]

A second major challenge for the field is to expand the use of IDPs, which have been explored on a very limited basis, but could represent an important untapped design concept in the development of new 'smart' biomaterials. As a relatively modest starting point, there are several basic-science

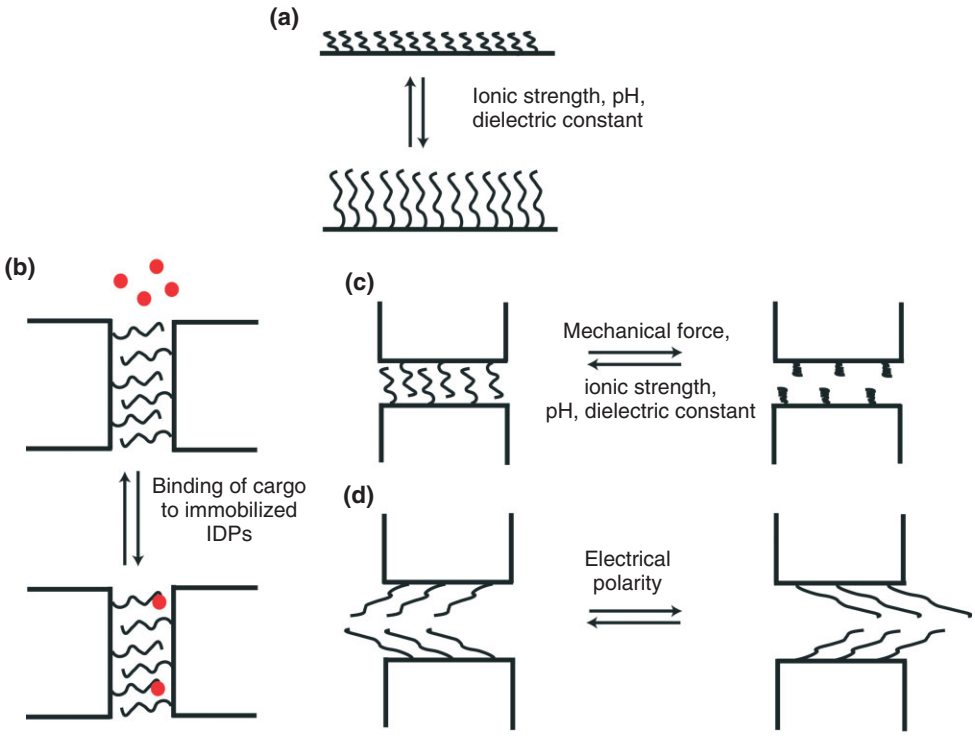

FIGURE 5 | Potential engineering applications of intrinsically disordered proteins (IDPs). (a) Environmentally sensitive IDPs immobilized on solid support. (b) A setup that mimics *in vivo* gating mechanism via an entropic barrier formed by an immobilized brush layer. Cargo is translocated through the pore due to attractive interactions between the cargo and the IDP. (c) A setup mimicking mechanotransduction, in which mechanical stimuli induce conformational changes in IDPs. Such changes can also be triggered by varying solvent conditions. (d) Response of charged IDPs immobilized on electrodes to changes in electrical polarity.

and technological settings in which synthetic polymer brushes could potentially be replaced by IDP-based brushes (Figure 5). In one such study aimed at recapitulating mechanical–electrical coupling in mechanosensitive ion channels,[99] it was shown that the application of mechanical forces (compression) to the brush layer triggered changes in the chemical forces (effective ionic strength) within the brush.[100] A major challenge in developing these systems into mechanotransduction devices is the use of flexible substrates on which the polymer brush layer is grafted. A flexible support, such as a porous membrane that can deform, stretch, and bend can alter the degree of lateral forces and transfer the stimulus to the brush, thereby mimicking its biological counterpart. IDPs represent an elegant way to further improve the biological content of such systems, as exemplified by recent efforts in which a polypeptide brush was successfully grafted on to a porous membrane.[75,80] Moreover, using IDP domains as brushes might offer a broader dynamic range due to the larger conformational changes often associated with protein chains. Similarly, IDP domains could conceivably replace DNA in the DNA-based artificial nanotube ion channel[101] and offer much more design flexibility. IDPs also present a new approach to tackle problems associated with fouling of biomaterial surfaces. As described earlier, synthetic polymer brushes can be conjugated both to antiadhesive/antimicrobial and cell-adhesive peptides, thus minimizing bacterial adhesion and nonspecific adsorption while still allowing specific cells to adhere. One can envision replacing the synthetic polymer brush with an IDP-based brush, thereby enabling one-step synthesis of a single polypeptide with very high monodispersity and purity. The prospect of these and other applications suggest that IDPs will offer many rich avenues for exploration as new biomaterial building blocks.

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