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PROTEIN ENGINEERING APPROACHES for BIOMATERIALS EVOLUTION

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Abstract:
Engineering proteins for diverse applications by using evolutionary strategies is currently an active research area. Two broad classifications of protein engineering strategies to develop new or enhanced proteins, include rational and directed evolution approaches. Rational engineering of proteins has found its own limitations of insufficient knowledge about parent proteins, whereas directed evolution finds difficulty in screening, though it does not depend on prior information about the parent proteins. These protein engineering approaches, results in the creation of novel with various desired features. Protein biomaterials are created with a combination of natural/synthetic polymers or engineered proteins/protein domains for its use in biomedical field. Both protein engineering and biomaterials have evolved over time. This review mainly serves to discuss the engineered proteins/protein domains/a combination of natural proteins with synthetic polymers in creating protein biomaterials of enhanced properties for biomedical applications like scaffolds for tissue engineering, wound healing etc. Machine learning is seen as an emerging computational technique that is being explored for use in evolving proteins.

Keywords: Protein engineering, biomaterials, rational design, directed evolution.

INTRODUCTION

Few decades ago, protein engineering worked with primitive techniques, using approaches that integrated protein crystal structure and chemistry, with artificial synthesis of genes to create novel proteins [1]. Modification of individual protein sequences allows us to go beyond nature's evolution and results in entirely new features or functions. This process of modifying proteins is termed as protein engineering and the modified protein variants can be used to enhance the life of humans or contribute to medical/industrial/environmental applications. Proteins are modified with the aim of obtaining a protein variant more appropriate for a particular targeted application than the native wild type protein. Rational engineering is one of the earliest protein engineering strategy, that redesigns proteins only with prior information about the protein structure and topology [2]. Whereas, directed evolution is another strategy followed in protein engineering which follows 'random mutagenesis and selection', without any structural information about the parent proteins [3]. *De novo* protein design was then established that followed *in silico* based screening of protein libraries [4], which led to *de novo* enzyme engineering [5, 6].

The classical rational methodology has been limited due to the lack of complete information on existing proteins and some contradictory results in engineering stable proteins. On the other hand directed evolution along with a powerful screening method, allows us to randomly mutate without any constraints, resulting in a large library of variants among which positive desirable variants are ensured[7, 8]. The applications of protein engineering are extensive in

industries and therapeutics, and it has been broadening with time and new research endeavors [9, 10].

In an attempt to mimic the natural biological functions, the characteristics and design criteria of biomaterials have observed a paradigm transformation. Several setbacks in the contemporary medicine/healthcare industry has led to the improvement in the field of tissue engineering and regenerative medicine. Engineered proteins with desirable features and ability to be crosslinked into biomaterials like hydrogels are becoming significant in the present scenario, for biomedical application like drug delivery, wound healing, scaffold materials [11-14], etc. The biomaterials that are available are custom prepared particularly based on the type of application and requirements.

This review focuses on understanding the evolution or history of protein engineering strategies and biomaterials, followed by the application of the engineered proteins by rational or directed evolution approaches for biomaterial generation. Several progresses and setbacks in constituting these engineered proteins for biomaterial formation are addressed, along with the potential proteins/protein domains suitable for biomaterial construction which possesses various specific applications in human healthcare.

EVOLUTION OF PROTEIN ENGINEERING

Natural evolutionary process has forged the proteins available in nature with functions and properties in such a way they contribute to favorable phenotype in life forms. These beneficial functions/properties are only a fraction of those biologically achievable ones. Modification of individual protein sequences allows us to go beyond nature's evolution and results in entirely new features or functions. This process of modifying proteins is termed as protein engineering and the modified protein variants can be used to enhance the life of humans or contribute to medical/industrial/environmental applications. Proteins are modified with the aim of obtaining a protein variant more appropriate for a particular targeted application than the native wild type protein. During the early phase of protein engineering, primitive techniques such as X-ray crystallography, DNA chemical synthesis, were mostly practiced for the creation of desirable protein variants[1]. Rise of molecular biology techniques and recombinant technology has aided the evolution of protein engineering. With the advent of recombinant DNA technology, amino acid modifications for protein engineering have become simpler. Development in recombinant DNA technology was witnessed during the early 1970s, following the discovery of two kinds of enzymes, the restriction endonucleases [15] that cuts the DNA molecules selectively and specifically, and the DNA ligases [16, 17] that links polynucleotides by forming phosphodiester bonds. Also, assembly of recombinant DNA followed by transformation of *E.coli* were initiated for the first time with the construction of several cloning vectors and the development of calcium dependent[18] transformation method. The advent of molecular cloning has aided in protein engineering.

In general protein engineering works by three main steps, where the first step involves choosing the protein engineering strategy to perform the changes, followed by implementation of those changes or mutations in the targeted parent gene and screening of the resulting protein variants for improved properties. The best choice of method is ruled by boundaries like information intensity/prior knowledge on three-dimensional structure, mutagenesis technique and screening tools. Different strategies have been used to obtain improved proteins, out of which most of the strategies have ultimately resulted in proteins with enhanced or desirable properties. Protein engineering strategies can be mainly divided

into three categories, namely, rational design, *de novo* design and directed evolution. Rational design is the most conventional method in protein engineering that usually introduces mutations in the parent proteins by site-directed mutagenesis (Arnold, 1993). Site-directed mutagenesis involves modification of specific amino acids in the parent gene. Gutte *et al* initiated the rational protein design with the solid phase synthesis of a peptide analog of ribonuclease S[2]. This rational design can be used only when prior knowledge about the structure and function of targeted protein are available. The inadequacy of these information about targeted proteins led to the establishment of an evolutionary method called the directed evolution. Directed evolution of proteins, introduces mutations by random mutagenesis method along with a reliable selection/screening technique to analyze the modified protein variants[3]. This method of directed protein evolution allows engineering of proteins randomly without any information about structure or function, and results in a very large library of variants, screening of which is a bottleneck in directed evolution. Whereas the *de novo* protein design was introduced to replace the manual way of analyzing the protein designs by a completely automated computational algorithm that could screen several amino acid sequences compatible with the targeted protein design[4]. *De novo* method is more like a computational rational design, where protein is rationally created from the scratch and not a modified form of parent proteins. These three methods rational design, directed evolution and de novo design are represented in the figure 1. Also the rational and directed evolution strategies have been integrated for their benefits, by introducing random mutations to localized or specific domains to design proteins, which is known to be semi-rational protein engineering [5, 6]. Apart from these, machine learning assisted protein evolution[19] is an upcoming protein engineering strategy which localizes the region to be experimentally mutated or performs screening in silico, with the help of computational algorithms/models devised based on repository protein data. This is resource intensive, which works well with the availability of targeted or similar protein data.

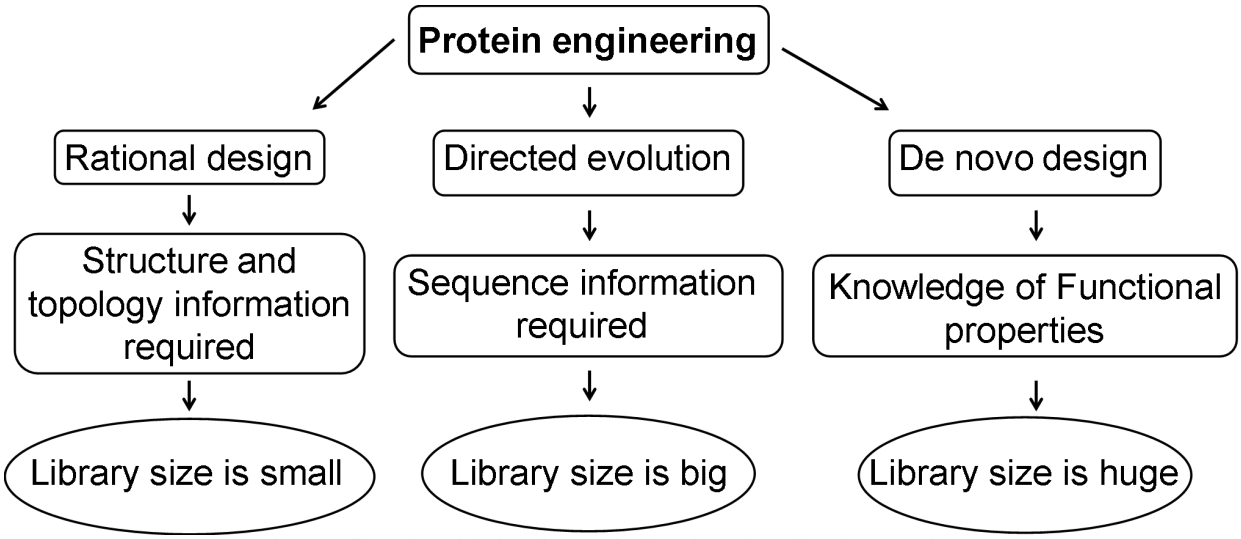

Figure 1: Illustration of the broad categorization of protein engineering strategies

EVOLUTION OF BIOMATERIALS

Biomaterials have been evolving since the olden times for the benefit of humankind. During the period of 1960s to 1970s, the first generation biomaterials was designed to maintain balance between physical properties and mechanical strength along with minimal toxic effects [20]. Table 1 summarizes the various generations of biomaterials, with its key features and limitations that paved way for the development of successive generations. The first-generation biomaterials were bio-inert, mainly fabricated for use inside the human body

called prostheses laid foundation for the bottom up construction of biomaterials. The basic principle followed to produce these biomaterials was to maintain biological inertness and to reduce the immunological response to the foreign body [21]. Vanadium steel plate, the first alloy was developed in 1912 exclusively for medical use in humans, as bone fracture plates called Sherman plates [22]. But vanadium steel is currently not in use as implants because of its insufficient corrosion resistance within the human body [23]. In 1931, stainless steel was developed to design the first femoral neck fracture fixation nail which was later changed to Vitallium in 1936 [22]. Circulatory assist devices like heart valves, blood vessel replacement made of cloth, intra-aortic balloon pumping were also developed during 1950s to 1960s [24]. Examples of first generation biomaterials include metals (Titanium and its alloys, stainless steel, cobalt-chrome alloys), ceramics (Alumina and Zirconia) and polymers (silicone rubber, acrylic resins). When these inert materials were placed inside the body as implants, it remained isolated from the surrounding host tissues.

Table 1: Evolution of various generations of biomaterials.

Generations	Key Features	Limitations	Examples
First	Bio-inert	Absence of interaction of biomaterial (implant) with host tissues	Titanium and its alloys, alumina, zirconia, acrylic resins
Second	Bio-active or bio-resorbable	Limited shelf life as they are artificial/man-made	Bioglass, hydroxyapatite, poly-lactic acid, poly-glycolic acid
Third	Cellular and gene activating (bio-active and bio-resorbable)	Has no control on electrical signals of cells	Hybrid (natural+synthetic polymers/domains) like polycaprolactone+collagen, recombinant protein polymers (silk-elastin like peptides)
Fourth	Manipulates and monitors bio-electrical signals of cells	Complete understanding of link between electrophysiology of cells and specific tissue regeneration	Polypyrrole, polyaniline, peptide self-assemblies

The limitations of the first-generation materials necessitated the need for second generation biomaterials emerged. As a result, a shift started from biologically inert reactions with tissues to the formation of bioactive agents that induces a regulated response in physiological environment [25]. Under this category, different forms of ceramics/glass ceramics, bioactive glasses and composites were added to clinical testing. These bioactive biomaterials found various clinical applications in orthopedics and dentistry by 1980s [21]. Bioactive fixation was initiated using synthetic hydroxyapatite as powdered fillings and coatings on porous and metal prostheses [26] [27] [28]. These hydroxyapatite coatings led to the formation of bone, called osteoconduction, which formed a mechanically strong association along the coating. The initial bioglass composition was improved that resulted in bioactive glasses and glass ceramics [29]. These bioactive materials were used in middle ear as implants to restore auditory ossicles and hearing loss [30]. Biocomposite materials were incorporated with bioactive agents and usually composed of two or more materials to attain combined properties comparable to the mechanical strength of the host bone [31]. Further, a novel composite made up of a polymeric matrix of polyethylene distributed with bioactive hydroxyapatite particles, trademarked as Hapex, was used in medical devices. Later it was improved to another new kind of bioactive material, Silicon-substituted hydroxyapatite, that emerged as an excellent bone graft material [32]. Another set of materials that were established as second generation biomaterials were resorbable, in which foreign material or the implant was replaced by resorbable or biodegradable materials. This includes fixing cracked bones with plates and screws that are resorbable [33]. A biodegradable suture made up of a combination of polylactide and polyglycolide was developed, that undergoes hydrolytic decomposition resulting CO_2 and H_2O, which are readily processed [20]. By 1984, these biodegradable polymers, had found regular clinical use as sutures. These second generation biomaterials also found their applications in drug delivery systems with controlled release of

therapeutics [34]. The advancements in first- and second-generation biomaterials are, however, limited mostly due to the limited lifetime of these biomaterials.

With advancements in genomics and proteomics in 1990s and 2000s, significant development of biomaterials occurred, and third generation of biomaterials emerged. Advent of third generation biomaterials began with the shift towards a more biologically based method that elicits distinct cell responses at microscopic level [21] to restore or regenerate tissues. Third generation biomaterials use a combination of both biologically active and bioresorbable materials which are able to induce genes that trigger recreation of living tissues. There are two ways of using these biomaterials, namely, tissue engineering and in-situ tissue regeneration. Tissue engineering comprises formation of tissue-engineered constructs by deposition of precursor/stem cells upon the bioresorbable scaffold, leading to cell growth and differentiation that mimics the natural tissues. Implantation of such constructs into individuals assisted in restoring the injured tissues. These scaffolds were absorbed and restored by host tissues with time. It has the ability to form an artificial immune-tolerant organ and tissue substitutes that can develop inside the host body. Thereby it provides a durable solution to the injured organ or tissue with no other additional remedies, thus forming as an economical treatment long term [35]. Scaffold materials were reported to be fabricated from natural and synthetic inorganic ceramic materials like hydroxyapatite, tricalcium phosphate etc, for bone tissue engineering [36], synthetic polymers including aliphatic polyesters such as polyglycolide, polylactide, their copolymers and polycaprolactone [37-39] and natural polymers including proteins (collagen) and polysaccharides (chitosan) for various tissue engineering scaffold applications. In situ tissue regeneration approach is concerned with the use of these biomaterials in the form of solutions, powders or coated micro or nanoparticles that elicits repair of local tissues. Both the approaches offer regenerative medicine where tissue repair process is genetically controlled, and the consequence is almost same as restored natural host tissue. Evidence supporting this hypothesis is that several families of genes responsible for transcription, cell cycle regulation, cell attachment etc, were up regulated to different folds by the use of bioactive glasses. These bioactive glasses disintegrates into ionic products and acts on the deposited human/host cells [40, 41]. Molecular level modifications of the microenvironment of biomaterials resulted in specific cellular response and tissue regeneration. Fourth generation of biomaterials is now emerging with adequate information about electrophysiological role of cells and tissues that can aid in tissue regeneration. The bioelectrical signals from the activation or deactivation of ion channels and pumps are reported to control cellular activities, cell apoptosis/proliferation, cell migration, position and differentiation [42, 43]. These smart electronic biomaterials are designed with the aim to alter the cellular bioelectrical signals for specific tissue regeneration and also to check for the cellular reactions and interactions with host tissues according to these signals. For example, in a recent research, hybrid hydrogels using polypyrrole, a conducting polymer and alginate, a natural polysaccharide resulted in a conductive soft biomaterial which might enable us to study about the electrophysiological behavior of stem cells/ neural cells and further could be developed into neural tissue engineered scaffolds [44].

It is necessary to study the history/evolution of any strategy or developments like biomaterials, to make further progress in that field. With the knowledge about the evolution of protein engineering and biomaterials, this review focusses on bringing both together for useful applications.

BRIDGING THE GAP BETWEEN PROTEIN ENGINEERING AND BIOMATERIAL FORMATION

With the evolution of protein engineering and biomaterials, it has been evident that protein engineering that includes rationally designed proteins and randomly evolved proteins are used in biomaterial construction. Here this review gives a brief account on various biomaterials constructed using proteins, designed rationally categorized as rational design of protein biomaterials and those evolved randomly known as directed evolution assisted protein biomaterials.

Rational design of proteins for biomaterial formation

With the prior information about the existing proteins, either natural or recombinant ones are combined in enhancing the properties of new proteins, that can subsequently be taken for biomaterial formation. Large scale isolation of proteins from natural sources have always been a tedious task, for which genetic engineering comes to the rescue. *Genetic engineering* is known to assist in the rational engineering of proteins by incorporation/deletion of amino acids, unnatural amino acids, functional sequences and combination of unique peptide domains resulting in novel protein combinatorial designs and stimuli-induced biomaterials.

Rational engineering of modular protein biomaterials or hybrid materials, typically involves choosing the required peptide domains with required property/functionality, structural designing of the selected peptide domains (illustrated in figure 2), sequence design with appropriate amino acid residues and corresponding DNA sequences, subsequently followed by cloning and protein expression and purification in appropriate host organism [45]. This method of rationally engineering modular protein biomaterials with the prior knowledge of its structure and function, thus promises to meet the requirements of specific application, that cannot be achieved by native or synthetic materials [46]. Hybrid biomaterials include the combination of elastin protein with polymers like polycaprolactone [47], collagen [48] and silk [49] that finds application in engineering vascular grafts [50], bone repair [51], hydrogels [52] and delivery of therapeutics [53]. Recombinant technology has been applied to combine the two proteins, silk-like-proteins and elastin-like-proteins, resulting in the formation of silk-elastin-like-proteins. These silk-elastin-like-proteins are reported to act as a stimuli-responsive system, that respond to various properties like ionic strength, pH, enzymatic stimuli, and can potentially be applied in controlled drug delivery [54]. These rationally engineered protein materials are proven to exhibit tunable structure-function features[55], improved mechanical and elastic property, biocompatibility, bioresorbability and target specificity.

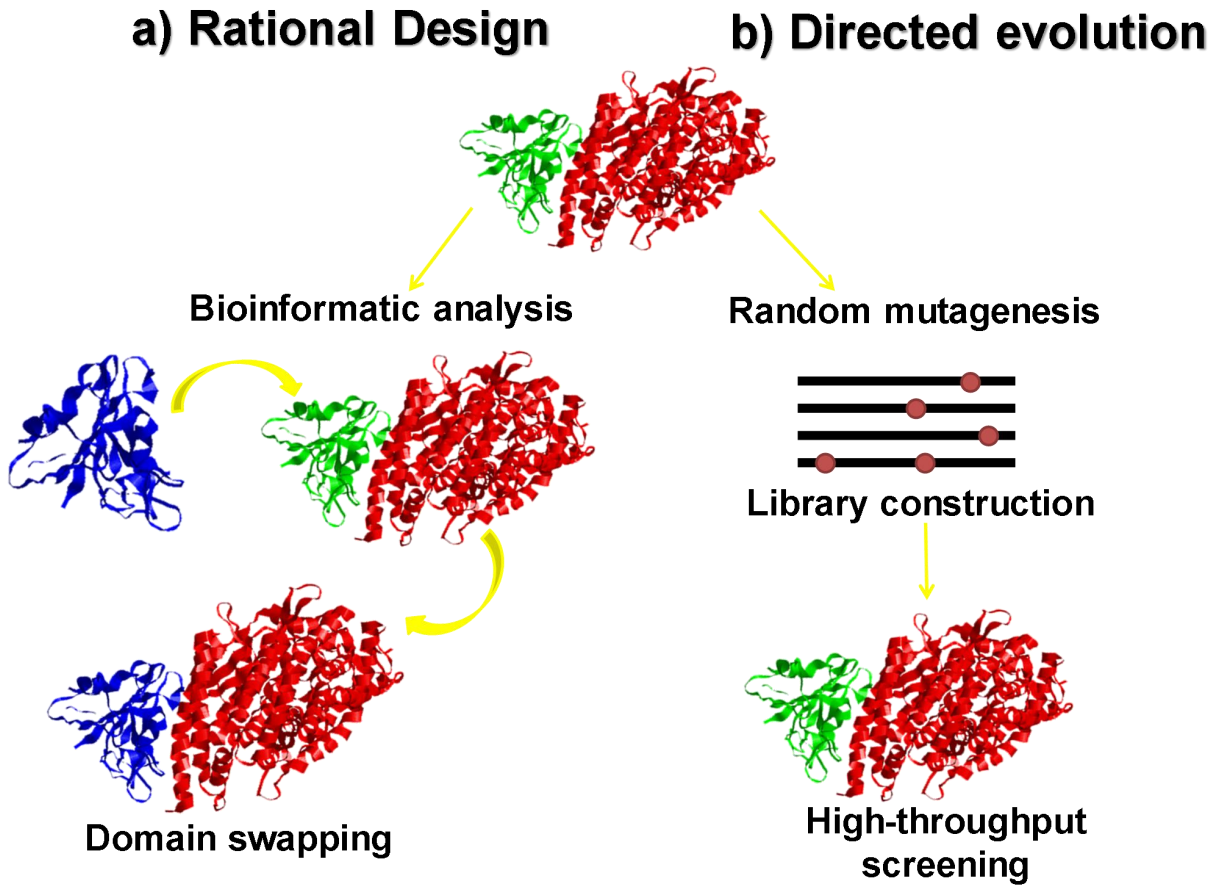

Figure 2: Illustration of the two protein engineering strategies: a) Rational Design involving rational domain swapping by bioinformatic analysis to design novel proteins; b) Directed Evolution following random mutagenesis, library construction and high-throughput screening to create novel proteins

Wound healing materials

Numerous wound dressing materials are continuously being developed to satisfy the needs of treatment of different types of wounds. Recent findings reported development of nanofibrous matrices from a non-mulberry silk protein sericin, a polysaccharide chitosan and polyvinyl alcohol, which was found to possess structure similar to that of the extracellular matrices[56]. These nanofibrous matrices were shown to help in the keratinocytes growth *in vitro* and exhibited wound healing properties, when tested *in vivo* along with epithelial cellular growth and angiogenesis. Currently, chronic wounds facing failure of normal healing process have been treated with the help of biomaterials seeded with mesenchymal stem cells. A combination of soy protein and chitin was used to obtain a biomaterial with high moisture retaining ability, which were seeded with human adipose tissue derived mesenchymal stem cells, to help in wound healing by promoting anti-inflammatory effect with the formation of indoleamine 2,3-dioxygenase [57]. Several collagen protein based blends [58] with the polysaccharide chitosan[59] are also reported to aid in wound dressing applications due to their biocompatible nature. Gelatin based films containing carboxy methylated guar gum and curcumin antimicrobial compound are shown to be a nearly ideal wound healing biomaterial[60]. Other wound dressing biomaterials obtained from the proteins like collagen, silk proteins sericin/fibroin blended with chitosan have been improved with the addition of anti-inflammatories, antibiotics, nanoparticles, antiseptics and other biologically active agents[61, 62]. Plasma treated collagen is one other novel development towards wound healing, especially in diabetic patients and those with immunodeficiency[63]. These plasma treated

collagen materials have been shown to speed up the angiogenesis rate along with wound healing.

Suture materials are the most used biomaterials in surgeries for the purpose of aiding in wound healing process by enclosing the wounded area with sutures. It is therefore necessary that these suture materials ought to be biocompatible with desired mechanical strength and no toxicity. Sutures are typically of two kinds: absorbable and non-absorbable sutures. Rational engineering of silk proteins for the development of biodegradable/absorbable sutures are reported[64]. Other than this, biodegradable metal[65] and biopolymeric sutures[66] are also used for generation of sutures for application in soft and hard tissues. Another interesting study has reported the use of in situ photochemical deposition technology to coat silver over the rationally designed silk sericin-poly(lactic-co-glycolic acid) suture material [67].

Biological or tissue adhesives/sealants are yet another kind of wound dressings applicable for wound closure, alternatives to sutures or staples in surgeries, attachment of dental or corneal onlays or inlays and alleviation of post-surgical adhesions [68, 69]. The proteins albumin and gelatin are the widely available tissue adhesives with desirable mechanical properties and less toxicity. Gelatin adhesives with alkaline treatment were shown to possess high porosity, promoting cell adhesion and angiogenesis [70]. Another significant finding includes a novel adhesive referred to as "BCD" developed from, bovine serum albumin (BSA), citrate acid (CA) and dopamine[71] which showed 10 fold higher adhesion stress than that of the fibrin glue (commercial tissue adhesive) within a time period of 30 minutes.

Functional domains for biomaterials

Rational engineering of the well-studied peptide domains in two or more combinations are recombinantly obtained, crosslinked into protein materials of different mechanical/functional properties which possess different potential applications in tissue engineering. Tissue engineering in general involves two main approaches, namely *in vitro* cell culture on scaffold materials and *in situ* regeneration of tissues. To attain the goal of cell interactions and specific cell proliferation, specific growth factors either in soluble form or matrix bound proteins have been used. Significant peptide domains that include growth factors and structural/functional peptide domains for cell adhesion are mentioned in the table 2 along with specific references for further reading.

For selective adhesion to nerve cells, laminin-derived peptides like YIGSR, CDPGYIGSR, IKVAV, etc., were reported, while cell adhesion mediated by $\alpha 5\beta 1$ integrin have been promoted by using fibronectin-derived peptide KQAGDV[72]. Growth factors, stromal cells, platelets rich in plasma and fibrin, have been analyzed and are used for wound healing and tissue engineering applications in health care[73, 74]. The preparations of platelets can be used in a personalized way for individual needs of the patients, in which specific cellular pathways get activated upon the release of growth factors from the platelet preparations, thereby aiding in specific tissue repair. Wound healing has been established by means of re-epithelialization[75] using platelet growth factors that involves homo and heterodimeric polypeptide dimers. Moreover, platelet lysates of humans are also used as additives in stem cell therapy, which shortens the culture time and promotes tissue regeneration[76, 77]. Blood vessel growth and re-epithelialisation are promoted by vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D)[78] [79] and transforming growth factor [TGF-β] respectively[80]. Also, there are other significant structural protein domains such as, elastin like polypeptides[81] and resilin like polypeptides[82], that are crosslinked using different

methods [83] resulting in hydrogels or other kinds of biomaterials with improved mechanical properties.

Another significant finding involves a rationally engineered recombinant protein obtained from a combination of 'B1 immunoglobulin binding domain of streptococcal protein G' (GB1) [84] and resilin (R) protein domain, with superior mechanical and elastic properties respectively. These two protein domains were combined together in different random combinations using recombinant techniques that yielded artificial elastomeric proteins [85]. Photochemical crosslinking[86] of tandem repeats of GB1 and resilin, of combinations, $(GR)_4$, GRG_5RG_4R, GRG_5R , GRG_9R , G_8, resulted in rational protein hydrogel biomaterials. Each of the hydrogels obtained were shown to possess different mechanical properties. In particular, the ensembles $(GR)_4$ and GRG_5RG_4R formed hydrogels with mechanical strength equivalent to that of the muscle proteins 'titin' of humans and could be exploited as scaffolds/matrices for muscle tissue regeneration. Rational engineering of protein-based biomaterials can thus be obtained with ease if prior information about structure of the parent proteins exists.

Proteins used in scaffold biomaterials

Collagen proteins are known for its mechanical properties and the interwoven fibres, which makes them suitable scaffold candidates for various tissue regeneration including skin[87, 88], vascular tissues [89], bones [90] and also drug delivery [91] and wound healing applications[92]. Collagen are rationally combined with specific synthetic (like polycaprolactone)/natural polymers to generate biomaterials with improved mechanical strength and degradable properties[93]. Silk proteins are another significant fibrous proteins with good mechanical features and self-assembling property, naturally suitable for scaffold formations[94]. Silk fibroins possessing superior mechanical strength, biodegradability, biocompatibility are reported to be more suitable for musculoskeletal tissue engineering[95, 96]. Scaffolds formed from electrospinning nanofibers of natural proteins like collagen and silk along with mechanically strong polycaprolactone have been proven to be useful in urethral reconstruction, apart from providing improved mechanical properties[97]. Polyanilines have been rationally combined with collagen fibres in generating materials possessing conductivity that aided in proliferation of cells [98]. Yet another significant protein, keratins with α-helical coiled coil dimers[99, 100], are structural proteins that promotes cell adhesion with its cell binding domain (LDV or Leu-Asp-Val) making it a promising scaffold candidate for tissue engineering [101, 102]. Nerve regeneration have been reported to be successful using keratin hydrogel scaffolds[103]. The ability of calcium phosphate ceramics to act as scaffold material for the bone regeneration by osteogenesis[104] have been utilized by combining it with natural polymers like collagen fibres[105, 106], for use in bone tissue engineering applications. Various other functional protein molecules such as laminin, heparin, and bone morphogenetic proteins[107, 108], are reported to be used along with the calcium phosphate composites/hydroxyapatites[109].

Directed evolution of proteins and biomaterial formation

Directed evolution helps in the laboratory-based evolution of RNA, proteins, various metabolic/signaling pathways, gene circuits and hence the entire cell by means of a repetitive approach to attain the features not available in nature. Here we discuss briefly about the strategy behind directed evolution followed by its potential to be used in biomaterial construction.

Directed evolution of proteins

Typically, directed evolution of proteins is performed to obtain new proteins with same or enhanced properties relative to the properties of parent proteins. It has been usually used in chemical or pharmaceutical industries to get proteins or enzymes of desirable properties. The strategy behind directed evolution assisted protein engineering generally follows four important steps, as follows:

i. Choosing the desirable parent sequences
ii. Creation of mutation in parent sequences by any one of the several methodologies resulting in mutant library formation
iii. Screening the library of mutant proteins for improved/required properties
iv. Repetition of the mutations again and again until desirable novel protein mutant are formed with the required properties

Mutant libraries are created by either random mutagenesis or recombination techniques, though many other methodologies have been emerging continuously. The commonly used method of directed evolution include site saturation mutagenesis, DNA/gene shuffling[110] and error prone PCR[111]. Also, several high throughput screening techniques are also reported for the purpose of reducing the repetitive tedious experimental work in obtaining the desired mutant proteins with required properties as shown in figure 2[112-114]. In this protein engineering method, inherent properties of enzymes/proteins have been enhanced though there is lack of prior knowledge about the protein structure, folding, mode of action of the enzymes and its expression[115]. Several features of enzymes that are targeted using directed evolution includes enzyme activity, selectivity of enzymes, scope of the substrate and its stability for use as pharmacological intermediates or fine industrial chemicals [116]. For instance, enhanced thermostability has been achieved in enzymes like laccases, cellobiohydrolases and α-amylases[117] by following directed evolution strategy with subsequent screening of the mutants. Though direct evolution doesnot require prior information of the parent proteins for creating desirable mutant proteins, screening of the vast library of mutant have always been reported to be a tough task.

A notable finding has been reported that uses directed evolution based DNA shuffling of secondary structures of two similar proteins, ‘immunoglobulin domain 27 of titin’ (I27) and ‘immunoglobulin domain 32 of titin’ (I32) to create novel proteins with superior mechanical proteins[118] (Figure 3). Out of 13 tandem repeats of protein hybrids, 6 protein hybrids were proved to be stable by studying the unfolding events using single molecule atomic force microscopy, showing contour length of 28 nm, which is almost same as that of the wild type parents, I27 and I32[119]. These studies involved the use of a well-known protein GB1, ‘B1 immunoglobulin binding domain of streptococcal protein G’, as a standard with contour length of 18 nm [120, 121] and the hybrids (i.e. $[GB1\text{-}hybrid]_4$) [118]. Three protein hybrids exhibited dual nature possessing both weaker mechanical properties unfolding at undetectable limits as well as properties as same as the parents. And, four of the protein hybrids were categorized to be mechanically labile. This study shows the significance of amino acid residues adjacent to the secondary structures in protein folding. Computational methods like SCHEMA [122, 123] has been reported to be useful in designing proteins by DNA shuffling based recombination strategies, to attain success. SCHEMA[123], works by predicting the fragments of parent proteins homologous to each other which can be recombined without affecting the structure or folding ability of the proteins. It locates compact polypeptides with more intra-block interactions which can be swapped or recombined. The schemata or the resulting compact polypeptide fragments are then taken for experimental recombination.

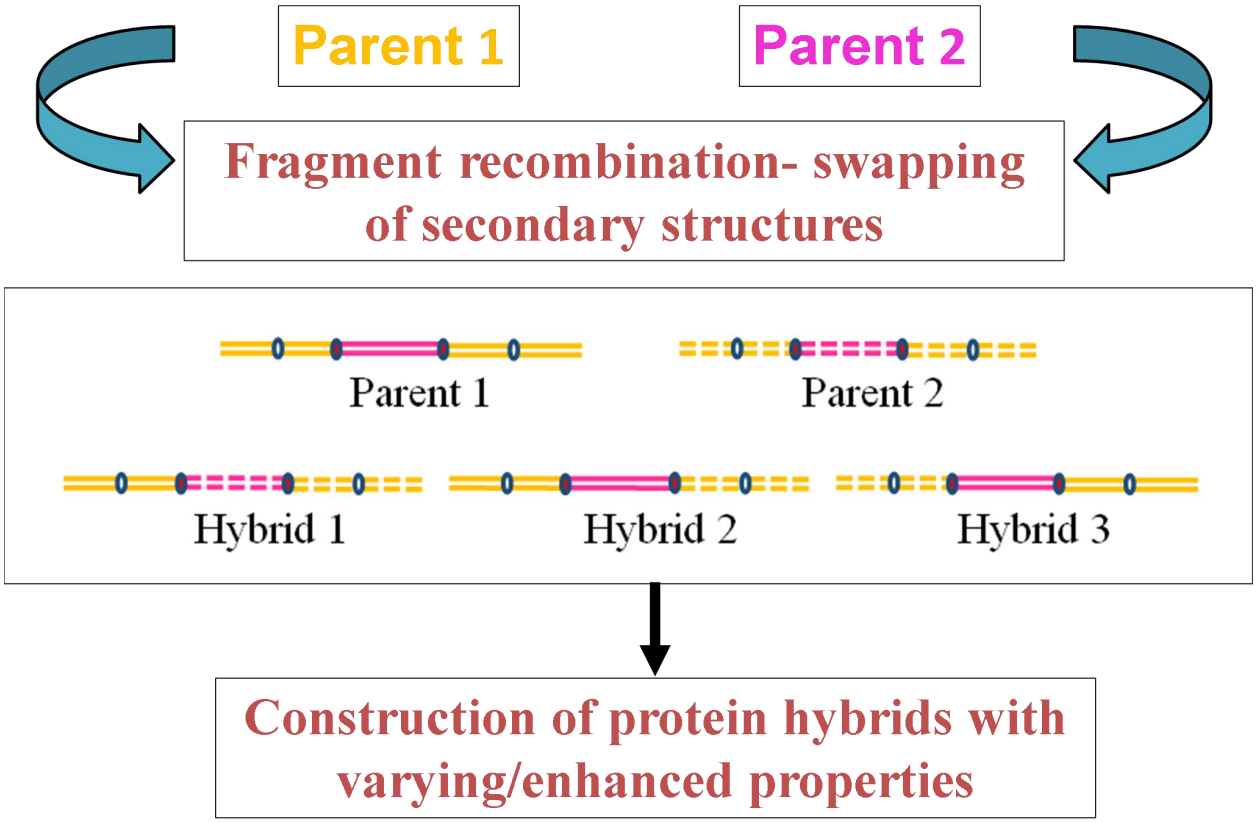

Figure 3: Illustration showing fragment recombination method of protein engineering between two homologous parent proteins resulting in several protein hybrids

Directed evolution based proteins for biomaterial formation

Like the strategies mentioned above, novel protein mutants obtained using directed evolution approaches possessing desirable properties can be taken to the next stage of crosslinking for fabrication of biomaterials including hydrogels, based on their features. Till now there has been no successful reports in literature for directed evolution based protein biomaterial construction, but attempts are made in creating several new proteins with properties that doesn't exist in nature using the directed evolution strategies. As mentioned in the previous sub-section, several hybrid proteins have been created from immunoglobulin domain 27 of titin (I27) and immunoglobulin domain 32 of titin (I32)[118] using directed evolution approach. Few of those hybrids were found to be similar to their parents in the aspects of mechanical properties and were recommended to be crosslinked for fabrication of protein biomaterials. This kind of directed evolution based protein biomaterials can be constructed without complications if the limitations in screening the mutant library is overcomes with simple handy screening universal and effective to all kinds of proteins and their respective properties to be screened. This proposed approach of directed evolution in protein biomaterial construction has been illustrated in figure 4 for better understanding.

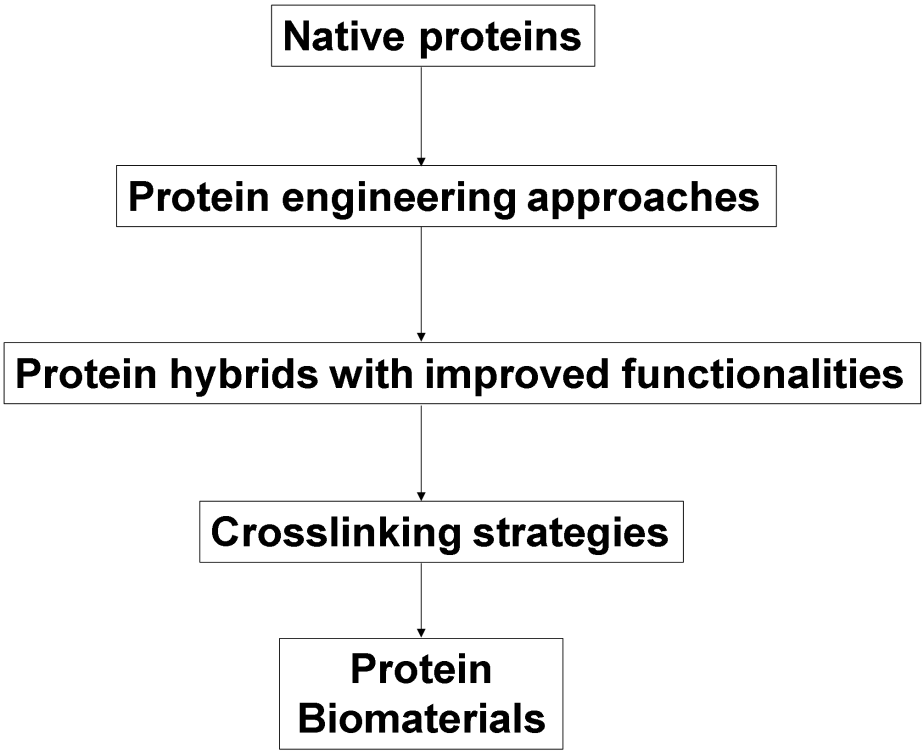

Figure 4: Schematics showing the steps involved in generation of protein biomaterials

EMERGING MACHINE LEARNING-ASSISTED PROTEIN EVOLUTION

Machine learning belongs to the field of artificial intelligence, that works with statistical algorithms and tools for analysis of scientific research models. These methods are used for dealing with intricate problems with 'larger combinatorial spaces or nonlinear processes', which cannot be solved using conventional methods or that requires huge amount for computation[124]. This technique could be used for the evolution of a larger number of proteins for diverse applications. This works by collecting data that are appropriately represented using suitable models. These models are then trained with existing or training data.

In general directed evolution involves generation of library of variants from parent proteins, followed by screening of desirable properties, and the best variant is chosen in the first round of mutation, and made as the parent for the next set of mutations until the best variant with desirable features are obtained. Machine learning is employed in protein evolution by feeding the sequences and screening information of the library of variants to train a set of 'models' like kernel, linear, neural network and ensemble methods. Here, the accurate models are chosen and used for screening the variants, known as *in silico* evolution. Restricted library of variants are obtained by ranking the screened variants based on scores or fitnesses followed by experimental validation of these restricted variants [125]. Similar to the conventional directed evolution strategy, the best variant is chosen in the first round of mutation and made as parents for the next round of new mutations, investigating the whole combinatorial space with new mutations and screening until desirable variants are obtained. Recently Wu et al , reported the effect of mutations of GB1 proteins of humans on its ability to bind antibody[126], by using *in silico* screening employing machine learning techniques and the collected datasets[127]. There are other recent reports that has used machine learning in protein engineering to improve enzyme activity and protein stability [19]. More applications including biomaterial fabrication using machine learning in protein engineering are yet to be explored.

FUTURE SCOPE

Protein engineering strategies, with the broad categorization of rational design and directed evolution along with the emerging machine learning techniques are all utilized for creating new proteins with required properties for use in various applications. Rational engineering of proteins have been well explored in various fields of applications, in particular biomaterial construction using the well-studied proteins are found to be useful in wound healing, scaffold materials etc. However, it is necessary to understand the molecular basis of all the protein engineering successes along with experimentally validated hypothesis. On accumulation of such success reports and hypotheses would potentially be helpful in further redesigning proteins. Also, to make directed evolution assisted protein engineering a success for biomaterial construction and bridge the gap, more simpler screening tools/techniques are to be developed, in order to select the protein variants with desirable features. Machined learning assisted protein engineering, in particular directed evolution of proteins ought to be explored further. There are several proteins and protein domains with significant properties/functionalities, which can be more and more be exploited to meet all the needs of humans without causing toxic/immune responses. Bridging the gap between protein engineering and biomaterial construction will thus immensely help the humankind.

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