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Review article

Protein materials as sustainable non- and minimally invasive strategies for biomedical applications

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ABSTRACT

Protein-based materials have found applications in a wide range of biomedical fields because of their biocompatibility, biodegradability and great versatility. Materials of different physical forms including particles, hydrogels, films, fibers and microneedles have been fabricated e.g. as carriers for drug delivery, factors to promote wound healing and as structural support for the generation of new tissue. This review aims at providing an overview of the current scientific knowledge on protein-based materials, and selected preclinical and clinical studies will be reviewed in depth as examples of the latest progress within the field of protein-based materials, specifically focusing on non- and minimally invasive strategies mainly for topical application.

1. Introduction

Proteins are the building blocks of life. The complexity of proteins allows them to catalyze reactions and play vital roles in transport and storage of natural molecules, and furthermore proteins make up the main structural elements of cells and tissue in the body [1]. Through evolution, proteins have gained specialized properties encoded in their primary amino acid sequence that allow them to perform their biological tasks with high specificity and potency. The rational use of proteins has advanced several fields within medical and health sciences. They are for example a source for vital nutrition [2,3], biopharmaceuticals for the treatment of severe and often chronic diseases [4,5], and constituents of innovative biomaterials; the latter being the focus of this review. Materials of different physical forms including particles [6–8], hydrogels [9–11], films [12–14], fibers [15–17] and microneedles [18–20] can be fabricated from protein (examples are depicted in Fig. 1) to perform tasks inspired by those functions that proteins naturally perform e.g. as carriers for drug delivery, factors to promote wound healing and as structural support for the generation of new tissue. Accordingly, protein-based materials have attracted great attention in many scientific areas including tissue engineering, material- and pharmaceutical sciences.

Proteins are polymers consisting of a linear chain of amino acids linked together by peptide bonds (amide bonds), which makes up the primary structure of the protein. Each amino acid consists of a repeating part, which makes up the backbone of the protein [1]. The variable part of amino acids, i.e. the side chains, contains a wide range of functional groups that determine the physical and chemical properties of the protein. The sequence of amino acids dictates the folding of the protein into more or less ordered secondary structures e.g. α-helix, β-sheet and random coils etc. [1] Three-dimensional arrangement of the secondary structure elements (i.e. tertiary structure of the protein) is dictated mostly by hydrophilic hydrogen and ionic bonding on the exterior part and internal hydrophobic interactions between nonpolar side chains, when the protein is exposed to an aqueous environment [1]. Quaternary structures arise when multiple chains of amino acids, polypeptides, come together as individual subunits to make up the protein [1]. The specific three-dimensional structure determines the biological function of the protein.

The great versatility of proteins is a key feature that makes these macromolecules highly interesting as the basis for new and innovative biomaterials. Molecular weight [18,21], charge [22], hydrophilicity/hydrophobicity [23–26], mechanical strength [14] and functionality [6,27] of the protein affect the properties of the material, but also provide the possibility to tune the materials based on the choice of the specific protein and the conditions under which the materials are formed. Furthermore, chemical modification or cross-linking of functional groups of the amino acid side chains of the protein provide yet another possibility to change or enhance certain properties within the

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protein-based material [24,28,29]. Taking inspiration by nature and the great versatility of proteins, it is evident that the number of materials that can be made from proteins is virtually infinite, but from an application point of view, this number is often limited to proteins of broad availability and low cost. It is also possible to design protein-based materials made by a combination of multiple proteins [14,30] or proteins blended with natural or synthetic polymers [12,24,31–33]. These materials benefit from the specific properties of several components within one material or from new properties emerging from the interaction of multiple components. Protein-based materials made by rational design and through the appropriate choice of components could be the solution to meet many of the significant challenges faced within health and medical science, i.e. creating biocompatible materials that interact with the body in a safe, convenient and efficient manner. Accordingly, protein-based materials are evaluated for a plethora of medical applications and routes of administrations, with one of the most evaluated routes of administration for materials based on proteins being topical administration. This route of administration is most often employed for local treatment of dermal or mucosal conditions in order to avoid exposure of the systemic circulation and limit off-target side effects. In addition, transdermal or transmucosal absorption of drugs has recently attracted great attention by employing non-invasive or minimally invasive strategies e.g. film- and fiber-based patches or microneedles, to circumvent the permeation barrier of skin and mucosa. Also, treatment by topical administration may improve patient compliance as an alternative to oral administration, which can be challenging for some patient groups with difficulties swallowing i.e. the young or elderly [34]. Indeed, the physical barriers of the skin and mucosae of the human body serves as the first line of defense against entry of foreign entities such as bacteria, viruses and fungi, and thus also comprise major hindrances for the absorption of most drug molecules especially hydrophilic drugs of high molecular weight [35]. Overcoming the biological barriers of skin and mucosae to achieve sufficient drug absorption is one of the major challenges for drug delivery by these routes [16,36]. In addition, much research is conducted to advance treatment of topical lesions that compromise the integrity of skin and mucosal barriers. This consists in the realization of dressings for wound healing [15], drug delivery systems for treatment of dermal and mucosal infections [14,24,37] and grafts for tissue engineering for regeneration of skin and mucosal tissue [38,39]. Recombinant proteins comprise a group of highly specific and potent pharmaceuticals, and the use of the active pharmaceutical protein as part of the drug delivery system e.g. as self-assembled protein particles or protein microneedles may be a strategy to enhance or tune the delivery to the specific application [36,40,41].

This review aims at providing an overview of the current scientific knowledge on protein-based materials, and, from a biomedical perspective, highlight the cutting edge research in the context of some of the most promising scientific findings. Selected preclinical and clinical studies will be reviewed in depth as examples of the latest progress within the field of protein-based materials, specifically focusing on non- and minimally invasive strategies mainly for topical applications. The unique characteristics that make proteins highly suitable building blocks for safe and patient-friendly biomaterials such as their great sustainability, biocompatibility and high versatility will be discussed in detail to elucidate the growing biomedical potential of protein-based materials. Special attention will be given to protein-based materials including particles, hydrogels, films, fibers and microneedles. Finally, opinions on the current development from a pharmaceutical perspective will be provided to underline the need for biocompatible and sustainable biomedical solutions.

2. Proteins as biocompatible and versatile building blocks of biomaterials for biomedical applications

Proteins can be of either plant [42] or animal origin [43,44], they are abundant in nature, biodegradable and can often be tuned into materials via eco-friendly processes and under mild conditions [45–47]. Being cost-effective, resulting in low emissions of greenhouse gases and

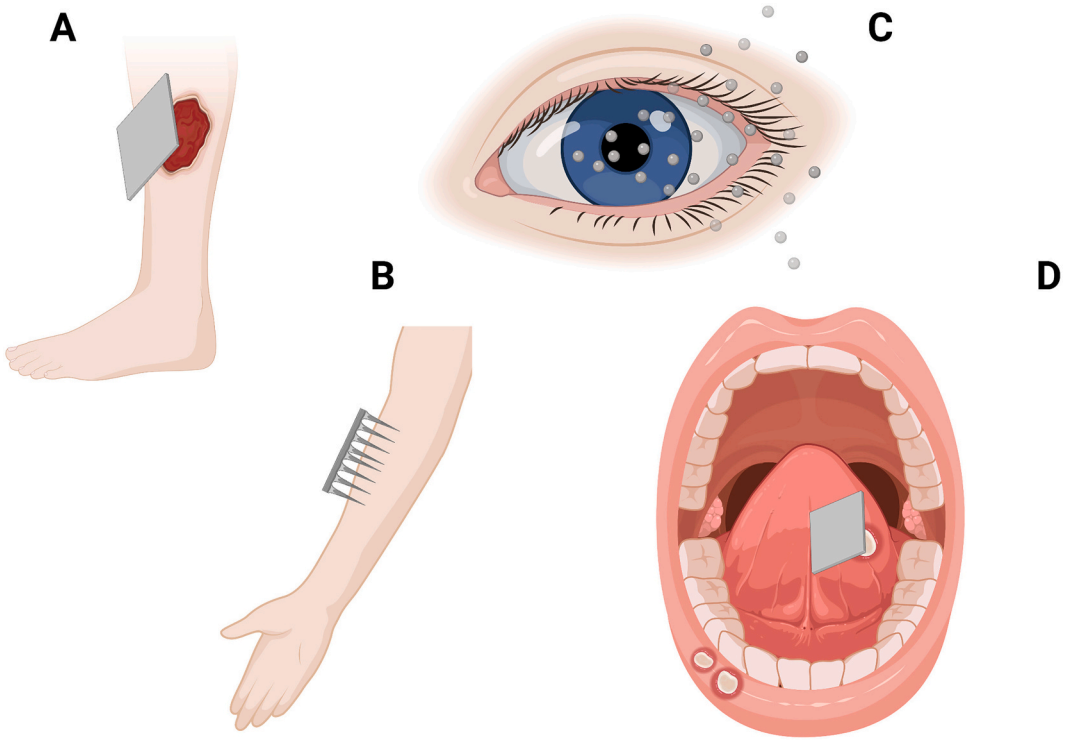

Fig. 1. Examples of protein-based materials as non- and minimally invasive strategies for biomedical applications. A) Protein-based patches/films for wound healing. B) Protein-based microneedles for skin delivery. C) Protein-based particles for ocular drug delivery. D) Protein-based patches/films for the treatment of local infections (e.g. ulcers in the oral cavity). Image created by Biorender.com.

deriving from renewable sources, proteins of plant origin can be produced in large quantities and represent a green and sustainable polymer source [48–50]. Furthermore, proteins derived from plants are in general not associated with animal-borne diseases and can provide a solution for individuals, who avoid consuming animal-based products due to religious or ethical beliefs or personal preferences [51,52]. Although often more costly than plant proteins, proteins derived from animals sources can also be obtained from alternative sources or as side streams of the agricultural or food industry, which also makes proteins of animal origin ecologically friendly and cost-effective alternatives for biomedical applications [53–56]. For example, collagen from marine waste products can be an alternative to mammalian collagen [53], and osteoinductive biocomposite scaffolds for bone tissue engineering have been prepared from among other, jellyfish collagen [56]. Also, keratin can be isolated from biological byproducts of the poultry industry or human hair to be used as building blocks for biomaterials [54,56,57]. Currently, awareness is raised for the need of greener, safer, eco-friendlier and more sustainable technologies and a circular bio-economy [58]. The actual environmental status calls for redesigning the entire material life cycle in terms of both raw materials and production/processes/technologies in order to reduce the environmental impact by 2030 [58]. Because of the aforementioned characteristics of proteins, several key principles of green chemistry are addressed by the use of proteins for the production of biomaterials such as use of renewable and degradable building blocks, limited generation of waste and restricted use of limited raw resources [59].

In general, biomaterials made from proteins derived from animal sources show superior mechanical strength as compared to that of materials based on proteins derived from plants. Furthermore, the majority of plant proteins have a limited range of compatible solvents, which can render the production of biomaterials of plant origin more challenging [60,61]. Approaches to further increase the versatility and applicability of animal and plant proteins in diverse applications include choosing the appropriate solvent [25,26,28,30,32,39,62,63], cross-linking [24,28,29,45,46,63–66] and blending with synthetic or natural biopolymers such as polysaccharides or other proteins [14,30,32,33,45,67,68].

2.1. Proteins as biocompatible and biodegradable building blocks for biomaterials

Because of their natural origin, proteins are in general categorized as biocompatible and biodegradable, which makes them highly suitable as sustainable and safe building blocks for materials intended for diverse pharmaceutical applications. Proteins are degraded by proteolytic enzymes, and their rate of degradation can be highly important for the performance of protein-based biomaterials *in vivo*. Different proteases show affinity for specific recognition motifs that can render some proteins more or less susceptible to proteolytic degradation by some proteases depending on their primary amino acid sequence [69]. Lack of access to recognition motifs may render a protein more resistant to degradation by some proteases, and protein folding i.e. secondary and tertiary structures may bury recognition motifs, making these regions of the protein less accessible to proteolytic cleavage. Thus, the degradation behavior of a protein-based material depends on the protein, but also the type of material can influence the degradation rate [69,70]; Müller-Herrmann and Scheibel [70] investigated the degradation behavior of different types of materials, i.e. nanoparticles, films and electrospun nanofibers, made of (recombinant) spider silk protein (eADF4(C16)) [70]. Silk-based nanoparticles were more susceptible to proteolytic degradation by the proteases bacterial model enzymes protease type XIV from *Streptomyces griseus* (PXIV) and collagenase type IA from *Clostridium histolyticum* (CHC) than films and nanofibers, which showed most resistance to proteolytic cleavage [70]. Cross-linking of the proteins that make up the materials can also improve the resistance to proteolytic degradation and tune the degradation behavior [70]. Furthermore, exposure to solvents such as methanol or formic acid during material fabrication can change the secondary structure and crystallinity of the proteins, making the protein-based materials less prone to degradation [70–72]. For example, swelling and degradation of silk fibroin/polyvinyl alcohol (PVA) microneedles by PXIV was shown to be dependent on the silk to PVA ratio, and the β-sheet content of silk, which was increased by exposure to methanol during fabrication [72]. Different applications may require specific degradation kinetics, and the proteolytic environment that the protein-based material will encounter *in vivo* will affect the rate of material degradation. A thorough assessment of the degradation behavior of new protein-based materials is therefore an important step to predict their performance *in vivo*.

Noteworthy, biodegradable proteins may in many cases be able to substitute synthetic polymers and may in some circumstances not only be a greener but also a safer choice because of their biodegradability [13,18]. Proteins and peptide/protein isolates have already obtained GRAS-status (generally recognized as safe) by the United States Food and Drug Administration (FDA) (Table 1) and companies continuously file for granting of GRAS authorization of new peptide/protein or protein-based products [73].

Approval of peptides and proteins as safe excipients is an important stepping-stone for clinical approval of biomaterials based hereof. Certain companies have achieved FDA authorization for marketing of protein-based delivery systems. Abraxane® is an injectable albumin-bound paclitaxel delivery system for the treatment of various cancer types [77]. Furthermore, gelatin-based gebapentin capsules are marketed under the tradename NEURONTIN® for the treatment of post-herpetic neuralgia and partial onset seizures [78]. For the case of protein-based materials for topical administration several candidates have entered clinical trials (ClinicalTrials.gov identification numbers NCT01993030, NCT04743375, NCT05122130 [79–81]). For example, the safety and effectiveness of a silk fibroin film, HQ® Matrix Medical Wound Dressing, was clinically accessed and compared to a commercial dressing in a randomized, active-controlled, single-blind, parallel two-group clinical trial for the treatment of donor site wound, conducted for 71 patients (ClinicalTrials.gov identification number NCT01993030 [80]). Interestingly, by application of the waterproof, non-porous and gas-permeable silk fibroin film the wound healing process was faster and the incidence of adverse events was lower compared to the commercial product (ClinicalTrials.gov identification number NCT01993030, [79,80]). Furthermore, a silk sericin dressing with collagen was developed for wound healing to provide moisture and bioactive ingredients to the wound bed (ClinicalTrials.gov identification number NCT04743375 [81]). Currently, the dressing is compared with a commercial dressing, Bactigras®, in a randomized, double-blind, parallel clinical trial to access its effectiveness and safety (ClinicalTrials.gov identification number NCT04743375 [81]). Moreover, a gelatin-based melatonin-loaded

Table 1
Relevant peptides/proteins, protein derivatives and isolates hereof approved as 'generally recognized as safe' (GRAS) by the United States Food and Drug Administration (FDA) [74–76].

Protein/peptide	Section in Code of Federal Regulations (CFR)/CAS Reg. No.
Bromelein	184.1024
Casein	182.90, 9000-71-9 (enzymatically hydrolyzed)
Catalase	184.1034 (bovine liver)
Ficin	184.1316
Gelatin	90000–70-8
Lipase	184.1415 (animal), 184.1420 (Rhizopus niveus)
Soy protein isolate	977076–84-8
Pancreatin	184.1583
Papain	184.1585
Pepsin	184.1595
Peptones	184.1553
Rennet	184.1685
Trypsin	184.1914
Zein	184.1984

sponge aimed for palatal wound-healing after harvesting of palatal graft is currently recruiting patients for a Phase 2 clinical trial (ClinicalTrials.gov identification number NCT05122130 [82]).

Noteworthy, several companies are currently commercializing materials made from proteins as alternatives to already existing materials utilized for diverse applications. For example, Xampla, a spinoff company from the University of Cambridge, has untangled pea protein and reassembled the protein into a structure that resembles the properties of spider silk protein [83]. Strong and flexible materials entirely derived from plant-protein e.g. microcapsules, films and coatings for diverse applications such as personal care products can be made by tuning the pea-derived protein [83]. Furthermore, a product of Gelatex, GelaCell™ employs biobased and ecofriendly cross-linked gelatin and corn-based zein to produce nanofibrous three-dimentional scaffolds that resemble the extracellular matrix for use in e.g. wound management and tissue engineering [84]. Moreover, SERI®, currently owned by Sofregen Medical Inc. is a silk-based scaffold utilized for surgical applications in the US [85]. Also, WounDres is a collagen hydrogel by Coloplast that assists the healing process of wounds [86]. Finally, Matriderm® Dermal Matrix, a product of Medskin solutions, is a non-chemically cross-linked collagen-elastin-template, which is used as a dermal replacement scaffold for the treatment of e.g. burns, poorly healing wounds and dermatological diseases [87].

2.2. The great versatility of proteins and protein-based materials

The wide range of functions, the ability to change conformation as a response to specific triggers or to be part of several reactions are the most prominent strengths of the use of protein as building blocks for the fabrication of new biomaterials [14,31,88,89]. Many proteins have specific properties from which the biomaterials may benefit, some of which are related to the natural roles of the proteins e.g. as carriers of other molecules, which can be beneficial for drug delivery purposes, or as structural elements with superior mechanical properties, which can be valuable in fields such as wound healing and tissue engineering [14,31,89–91]. For example, albumin, which is a natural carrier of molecules, has been used in albumin-based particles as a carrier of small molecules and biopharmaceuticals [90,91]. Furthermore, materials, such as films intended for wound healing applications and nanofibers for tissue regeneration, were made from silk to benefit of high mechanical strength [14,89]. Moreover, gelatin can be added to materials such as hydrogels and films to improve their gelation properties and provide hemostatic properties [14,31]. Numerous studies have demonstrated that the intrinsic properties of the specific protein are often conserved in the biomaterials. Some proteins e.g. zein and whey proteins such as alpha-lactalbumin and lactoferrin have been shown to display antibacterial [60], anticancer [92,93] and antiviral [94] properties, respectively. Others, including elastin and collagen, have the ability to enhance cell proliferation and have shown enhanced tissue regeneration potential [39,95]. The strength, elasticity, modulus and degradation behavior of the material should be tailored to the specific biomedical application, and matched to the human tissue that the material interacts with. Certainly, proteins are not only inert polymers, but can contribute favorably by transferring their properties (e.g. resistance to harsh conditions, elasticity, hydrophobicity) or a specific biological activity to the produced materials. Matching the protein with the appropriate application is thus key to achieve a synergistic effect within the formed biomaterials.

Within the scientific community, materials made from a short list of proteins, which includes silk [10,18,96], collagen [28,38], elastin [11,39] and zein [23,97,98] have been given the most attention. This is partially because of the great abundance of these specific proteins in nature and consequently their good commercial availability. Recently, also other proteins including alpha-lactalbumin, lactoferrin, and fibrin have been exploited as constituents of biomaterials because of their properties [16,24,32,99–101]. Compared to single purified proteins, isolates of proteins, such as whey, potato and soy proteins isolates, [33,62,102–104] which include a mixture of proteins and other constituents e.g. carbohydrates and lipids, are often cheaper to obtain. Nevertheless, their use can be more challenging as the physicochemical properties, their biological activity and shelf life may vary significantly from one to another as the isolates often have varying content of the individual peptides/proteins, purity, and overall composition due to factors such as origin and purification method [105,106].

The amino acid sequence of a protein, together with the surrounding environment e.g. solvent, pH, ionic strength, dictates the inter- and intra-molecular interactions and thus the resulting three-dimensional tertiary and quaternary structure of proteins [1]. Some proteins display good solubility in water due to a high density of hydrophilic amino acid side groups being displayed on their outer surface [1]. In contrast, some fibrous proteins such as collagen, silk and elastin are made by repetitive amino acid sequences, and their surfaces are covered by non-polar hydrophobic amino acids, which makes them insoluble in aqueous solutions [25,26]. Although, water is per se the most environmentally friendly and safe solvent, some applications require biomaterials with stability in an aqueous environment. For these applications, hydrophobic proteins can provide structural hierarchy, protective encapsulation, mechanical support and slower degradation rates compared to hydrophilic proteins [26]. Organic solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) [32,39] and formic acid [30,104] are often used to solubilize water-insoluble proteins, but the use of organic solvents is not ideal as it can increase the environmental impact and toxicity, and compromise the biocompatibility of the resulting biomaterial. It should be noted that some organic solvents e.g. acetic acid that has been used to solubilize e.g. collagen and elastin display lower toxicity and is GRAS approved [28,107]. Pharmaceutical companies are frequently updating their solvent selection guides to encourage use of solvents with lower environmental, safety and health (ESH) impact [108].

Protein modification is a general approach to enhance the potential of a protein-based material for example by conjugation to other molecules e.g. poly(ethylene) glycol (PEG) or by cross-linking of the proteins within the materials toimprove the stability. For example, conjugation of proteins with PEG can potentially decrease the risk of an immunogenic response and provide a prolonged half-life *in vivo* [109]. PEG-modified silk- and gelatin-based films loaded with ciprofloxacin resulted in faster wound healing compared to a non-modified films [14]. The faster wound healing was attributed to PEG resulting in a reduction in the speed of degradation of the film and maintaining the action of ciprofloxacin for a prolonged period by evading macrophages [14]. Proteins can also be conjugated to scaffolds of synthetic origin to provide a specific bioactivity [110,111].

Chemical, physical or enzymatic cross-linking are strategies often employed to improve the mechanical properties and stability of the material in an aqueous environment [24,112]. Cross-linking can make protein-based materials less susceptible to instabilities by exploiting the diverse functional groups of proteins [113]. Currently, chemical cross-linking that primarily leads to formation of covalent bonds is applied extensively due to the high cross-linking efficiency. Thus, chemical agents such as small chain aldehydes, in particular glutaraldehyde (GTA), are widely applied chemical cross-linkers, being readily available, relatively inexpensive and giving a high cross-linking degree [39,64]. Nevertheless, the use of aldehydes raises concerns related to their negative health impact [112,114]. Alternatively, cross-linkers including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with or without N-hydroxysuccinimide (NHS) and genipin have been exploited for better biocompatibility and safety [9,28,29,38]. In general, physical cross-linking generates mainly noncovalent bonds by ionic or hydrophobic interactions and H-bonds, thus leading to a low cross-linking degree and mechanically weaker materials compared to chemical cross-linking methods [24]. It should however be noted that via physical cross-linking, addition of potentially toxic compounds in the

polymer network is avoided and thus cytotoxic effects are limited.

Approaches for physical cross-linking include thermal or plasma treatment or electron beam irradiation [24,66,115]. In addition, UV treatment of riboflavin-loaded, protein-based biomaterials has shown to induce cross-linking by the generation of reactive oxygen species (ROS) [116]. Moreover, by physical cross-linking e.g. by γ-irradiation, it is possible to achieve material sterilization simultaneously with the cross-linking reaction [117]. Finally, enzymatic cross-linking of protein and peptide-based materials by e.g. the enzyme transglutaminase (TGase) is an attractive alternative due to the specificity of enzymes and the occurrence of mild cross-linking reactions [45,63]. Alternatively, choline salts were recently studied as a physical cross-linking approach for the cross-linking of collagen-based biomaterials [46]. It should be noted that the majority of studies on the cytocompatibility of cross-linked biomaterials describes relatively short-term results, and hence the potential long-term toxic effects of the cross-linked materials and products of their degradation are often not considered. Furthermore, chemical cross-linkers may not only react with the protein constituents but also with the active pharmaceutical ingredient (API) to be loaded into the material.

Plastisizers, polysaccharides or a combination of proteins are often included in protein-based materials to add or enhance specific properties such as mucoadhesiveness, flexibility and water retention ability, which consequently broaden the potential functionalities of protein-based materials [13,33,45,54,67,68]. For example, addition of hyaluronic acid and carboxylated chitosan to human-like collagen resulted in hydrogels with enhanced mechanical properties, imroved resistance to deformation and better cell adherence [45]. Furthermore, incorporation of chondroitin sulfate and hyaluronic acid in silk fibroin scaffolds enhanced the pore interconnection and water retention, which are important characteristics for cell survival and proliferation [68]. Similarly, an inner layer of electrospun gelatin/keratin nanofibers and an outer layer of a commercial polyurethane wound dressing were combined to achieve the collective benefits of gelatin and keratin and thus enhance wound repair [30]. Cell adhesion studies demonstrated deeper cell spread and migration inside the gelatin/keratin nanofibers as opposed to gelatin nanofibers, and animal studies on wound re-epithelization also indicated more uniform, complete and thicker re-epithelization of the wounds treated with the gelatin-keratin-polyurethane membrane compared to those treated with a gelatin-polyurethane membrane only, a gauze or a commercial wound dressing [30].

3. Protein-based biomaterials as non- and minimally invasive strategies for biomedical applications

Starting from specific protein building blocks, a broad spectrum of materials with unique physical forms can be fabricated by employing different methods of preparation (Fig. 2). The intrinsic properties of the protein such as bioactivity can be conserved in the material. For other applications, it is the chemical and physical properties of the protein that are exploited to produce materials with specialized properties. Appropriate choice of the specific protein and complete control over processing parameters are crucial to achieve materials with optimized and specific properties. Table 2 lists examples of protein-based materials that will be covered in more detail in the following sections. The broad range of applications truly highlights the great versatility of protein-based materials.

3.1. Protein-based particle systems

Acting as natural carriers of other molecules including ions, small molecules and other macromolecules is one of the important roles of proteins *in vivo*. For this reason, protein materials have been explored as potential carriers of small molecular drugs and biopharmaceuticals. Particles can be fabricated from proteins by multiple techniques, which include physical aggregation/self-assembly [22,126], desolvation [7,127], electrospraying [102], and/or by cross-linking to stabilize the colloidal system [90,127]. For example, nanoparticles made from gliadin, a water-insoluble mixture of proteins extracted from gluten by ethanol, have been used to encapsulate all-*trans*-retinoic acid for the potential treatment of skin diseases such as acne, psoriasis, hyperkeratosis, ichthyosis and malignant skin tumors [7]. The nanoparticles were prepared by desolvation, and particles of a reproducible size and high drug loading (up to 97%) were achieved [7]. Interestingly, these protein-based nanoparticles displayed zero-order sustained release of retinoic acid over at least 3 h after an initial short burst release (20%) of the drug seen within 15 min [7]. Furthermore, the mucoadhesive properties of gliadin nanoparticles have been demonstrated *in vitro* and *ex vivo*, which indeed makes gliadin-based nanoparticles promising drug carriers for mucosal applications [6].

Nanoparticles of 40–70 nm in size based on lactoferrin were loaded with the antimicrobial-spermicidal curcumin and/or anti-HIV Efavirenz [99]. Compared to soluble curcumin and Efavirenz, drug absorption was lowered and inflammation was also decreased in vaginal tissue and plasma for rats *in vivo* administered with lactoferrin-based nanoparticles

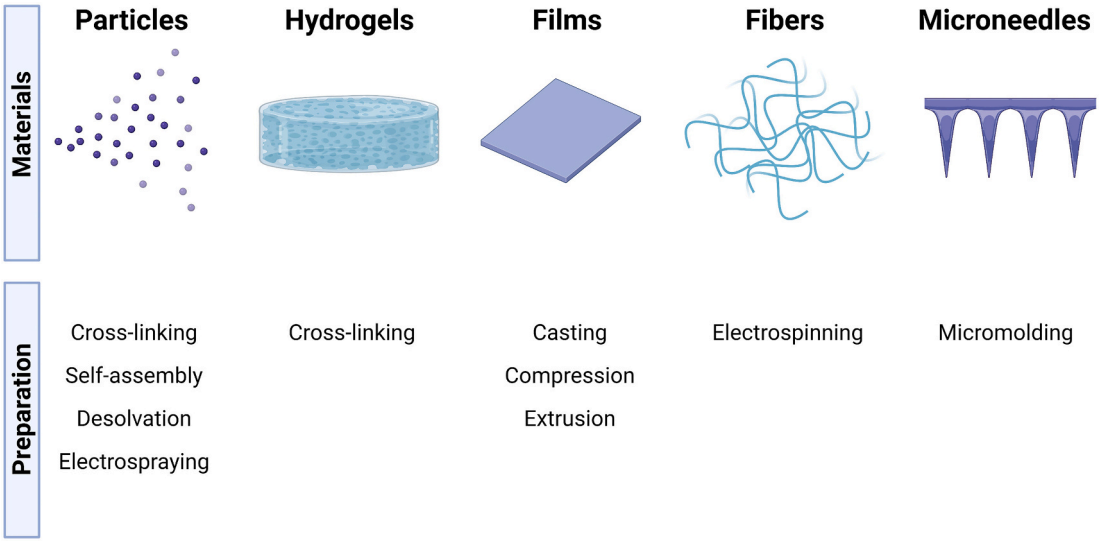

Fig. 2. Examples of protein-based materials that are covered in the main text and the most common methods of their preparation. Image created by Biorender.com.

Table 2
Examples of protein-based materials for non- and minimally invasive strategies and their specific applications as discussed in the main text of the review. The list is not exhaustive but specifically includes literature on thorough pre-clinical and clinical evaluation of the protein-based material.

Material	Protein	Application
Nanoparticles	Gliadin	For encapsulation of all-*trans*-retinoic acid for the potential treatment of skin diseases [7]. As drug carriers for mucosal applications [6].
	Lactoferrin	Vaginal microbicide with curcumin and efavirenz [99].
	Serum albumin	For decontamination of nerve agents used as tools for assassination [8]. For ocular delivery of aspirin for treatment of diabetic retinopathy [88]. For delivery of bevacizumab for treatment of proliferative (neovascular) eye diseases [90].
	Zein	For encapsulation of a variety of drugs from small molecules to proteins for topical applications [118].
Hydrogels	Collagen	As composite with chitosan as corneal implants [9].
	Silk fibroin	For delivery of curcumin-loaded nanoparticles for the treatment of psoriasis [119].
	Gelatin	As wound dressings with hyaluronic acid [120]. As in situ forming hydrogels with oxidized alginate for wound healing [31].
	Keratin	As wound dressings with polyvinyl alcohol (PVA) and poly(ethylene imine) for accelerated wound closing [57].
Films	Silk	For delivery of ciprofloxacin by silk/gelatin films coated with polyethylene glycol (PEG) for wound healing purposes [14].
	Gelatin	For encapsulation of Thymol/β-cyclodextrin in mucoadhesive gelatin-based films for treatment of oromucosal infections [37]. As mucoadhesive films based on chitosan and gelatin for delivery of sumatriptan succinate for buccal administration [12].
	Collagen	For sustained release of human growth hormone intended for wound healing purposes [13].
Nanofibers	Alpha-lactalbumin	For encapsulation of ampicillin for topical treatment [24]. For oromucosal delivery of nicotine as a nicotine replacement therapy [16]. To accelerate wound healing [17].
	Collagen	As skin grafts [38].
	Elastin	For tissue regeneration [39].
	Silk	To promote wound-healing [89].
Microneedles	Gelatin	Formulated with calcium sulfate [121] or sodium carboxymethyl cellulose [122,123] or cross-linked with genipin [40] for insulin delivery. As a glucose-responsive insulin release system with gold nanoclusters (AuNC) [41]. For delivery of inactivated polio vaccine [124]. For induction of lipolysis and suppression of lipogenesis to reduce subcutaneous adipose tissue [27].
	Silk	For sustained release of levonorgestrel over several months [18]. For delivery of insulin [19,125]. For delivery of vaccines [20].
	Insulin	For delivery of insulin [36].

[99]. The protein-based nanoparticles displayed lower toxicity by histopathological analysis of vaginal tissue and improved pharmacokinetics profiles compared to soluble curcumin and Efavirenz [99]. As stated by the authors, the safety and low inflammatory response of lactoferrin-based nanoparticles might be due to the natural abundance of the protein in vaginal secretion [99].

Recently, bovine serum albumin (BSA) was chemically linked to polymers such as DMAEMA to create particles of 100–400 nm in size for the purpose of decontamination of nerve agents used as tools for assassination [8]. BSA and the polymers alone or in physical mixture do not display any significant decontamination activity, but when combined into a nanocomposite, the decontamination effect of the BSA protein linked to polymer was comparable or even enhanced compared to traditional decontamination materials [8]. *In vivo*, using a poisoning rat model with the nerve agent Soman, particles based on BSA and polymer showed better decontamination of Soman on skin than the traditional decontamination material $NaCO_3$ [8]. Rats treated with traditional decontamination materials such as NaOH and $NaCO_3$ showed irritation on the skin. In contrast, rats treated with decontaminating nanocomposite particles based on BSA showed no injuries on the skin after treatment *in vivo*, thus representing a potential safer alternative [8]. Finally, the authors show that the decontamination effect is not dependent on the specific protein but on the globular nature of the proteins in the nanocomposite as globular proteins such as BSA show better decontaminating efficiencies than fibrous proteins [8].

Albumin is a natural carrier of various molecules in the blood stream, the protein contains multiple binding sites, and thus albumin-based materials have been exploited for drug delivery [88,90,128]. Nanoparticles based on albumin have been evaluated for drug delivery of both small molecular drugs and biopharmaceuticals of high molecular weight such as antibodies [88,90]. For example, nanoparticles based on albumin intended for ocular delivery have been used for encapsulation of aspirin (180 Da) [88] and the monoclonal antibody bevacizumab (149 kDa) [90]. BSA nanoparticles for ocular delivery of aspirin for treatment of diabetic retinopathy were < 200 nm, monodisperse, with a high drug loading (81%) and displayed sustained release of aspirin in biorelevant media (e.g. artificial tear solution) [88]. To be administrated topically as eye drops for the treatment of proliferative (neovascular) eye diseases, bevacizumab was loaded into nanoparticles based on human serum albumin (HSA) by desolvation and the necessity of cross-linking with GTA was evaluated [90]. Stabilization of the particles by cross-linking with GTA was only necessary for particles of low antibody to HSA ratio (e.g. 0.01) [90]. Particles of high antibody to HSA ratio (310 nm in size and a zeta potential of −36 mV) were stable without cross-linking and displayed a burst release of antibody (>30% in 5 min) followed by sustained drug release over many hours [90]. Post-administration of the protein-based nanoparticles as eye-drops to rats *in vivo*, showed that radio-labeled bevacizumab in HSA particles retained in the eye for longer time (≥ 4 h) compared to radiolabeled HSA diluted in a commercial collyrium (retention time ≤ 1 h), which might be due to the mucoadhesive nature of the particles versus free protein [90]. These examples indeed demonstrate the versatility of albumin-based nanoparticles as carriers for a broad variety of drugs.

Formulations of protein particles have also been patented and licensed. Specifically, for topical administration, protein nanoparticles based primarily on zein were patented (US 2012/0195947 A1, [118]) and later licensed by the research company Transderm Solutions. Zein, a protein extracted from corn, is approved by the US Food and Drug Administration (FDA) as generally recognized as safe (GRAS), and thus of great interest for medical applications (Table 1). Drug encapsulation/absorption of a variety of drugs with different physicochemical properties in zein-based particles for topical administration was demonstrated including retinol, the anticancer drug 5-fluorouracil, the model proteins BSA and platelet rich plasma, and the fluorophores rhodamine 123 and FITC [118].

3.2. Protein-based hydrogels

Hydrogels are water-retaining polymeric three-dimensional networks that are stabilized by physical and chemical cross-linking that keep them insoluble in aqueous phases. Hydrogels can absorb from 10% to several thousand percent of their weight in water [129]. The high water content of hydrogels confers to the biomimetic properties of the material making them similar to natural tissue [129]. In general, hydrophilic amino acids e.g. containing amino ($-NH_2$), acid (-COOH) or

amide groups (-CONH) on their side chains contribute to the hydrophilicity of the hydrogels and their swelling potential [129]. It is worth noting that recently ultralight protein-based solid materials were proposed to be formed by replacing the water component in hydrogels with air [130–133]. These aerogels are formed by using an amyloid fibrils gel as template and may constitute a new class of highly stable 3D structures. Their enhanced retaining properties may enlarge the range of accessible *applications [130–133].

Protein hydrogels can be produced via non-covalent physical cross-linking i.e. by hydrogen bonding, hydrophobic or electrostatic interactions, or by chemical cross-linking creating covalent bonds [9,10,134]. Formation of protein-based hydrogels by physical cross-linking is driven by supramolecular assembly induced by destabilizing conditions. When protein destabilization is induced, some molecular regions such as hydrophobic regions or free SH-groups become accessible to new intermolecular interactions, thus leading to the formation of supramolecular structures through non-covalent (electrostatic, van der walls, H-bonds, hydrophobic) and disulfide bonds. Intermolecular association can also occur via the formation of intermolecular β-sheets but also α-helical coiled-coil domains, which are stabilized by a regular pattern of H-bonds conferring high stability to the structure [135]. Different structures can be modulated by regulating the hierarchy of supramolecular interaction controlling solvent conditions by changing physicochemical parameters e.g. pH, temperature or ionic strength [134,136,137]. Hydrogels formed by physical cross-linking are generally characterized by lower stability and mechanical strength than those produced by chemical cross-linking [134]. For example, thermally induced hydrogels usually lack the ability to swell to their original volume after being dehydrated [134]. It is also possible to exploit the combined properties of proteins with other materials to create synergistic effects. For example, to improve the absorption of water, acrylic acid can be added via chemical modification or graft copolymerization to introduce a higher density of negative charges and creates superabsorbent materials [138,139]. However, organic solvents or other cross-linking agents used for chemical cross-linking of proteins for the formation of hydrogels are often toxic, which limits their use in biomedical applications.

In the field of tissue engineering, tunable hydrogels based on collagen [9], silk fibroin [10] and elastin [11] have been produced to closely mimic the extracellular matrix. For example, collagen/chitosan composite hydrogels (hybrid polymer networks – HPN) have been developed for the use as corneal implants [9]. The hydrogels were stabilized by standard EDC/NHS cross-linking (HPN-1) or hybrid cross-linking with polyethylene glycol dibutyraldehyde (PEG-DBA) and EDC/NHS (HPN-2), both of which led to covalent amine and amide bonds [9]. The rationale for the development of the HPNs was to achieve a synergistic effect by the different components of the hydrogels for improved biocompatibility, physiological relevance and enhanced mechanical properties [9]. The scaffolds demonstrated enhanced mechanical properties compared to control scaffolds (collagen cross-linked by EDC/NHS), and interestingly, HPN-2 demonstrated enhancement of not only the elasticity of the scaffold but also the tensile strength because of the combined use of both short- and long-range cross-linking agents [9]. Moreover, the HPN scaffolds were shown to be 100% suturable when trephined and sutured on human corneal rims, and the scaffolds were more optically transparent than rabbit and human corneas at visible light wavelengths [9]. Remarkably, the stability of HPN-2 was similar to human cornea (HC) during *in vivo* biodegradation studies in rats [9]. Finally, implantation of HPN-2 in porcine cornea for 12 months resulted in successful graft integration and regeneration of corneal epithelium, stroma and nerves, which thus underlies the potential of collagen hydrogels for corneal tissue engineering [9].

Silk fibroin hydrogels were explored as a delivery system for curcumin-loaded nanoparticles for the treatment of psoriasis [10]. To achieve a sufficient level of skin hydration and a high curcumin penetration through the thickened stratum corneum due to psoriasis, curcumin was loaded into RRR-α-tocopheryl succinate-grafted-ε-polylysine conjugate (VES-g-ε-PLL) and then subsequently incorporated into silk fibroin-based hydrogels to prolong the retention time of the particles on the skin [10]. Inhibition of the expression of inflammatory cytokines was achieved to a greater extend by hydrogel-loaded curcumin VES-g-ε-PLL compared to the VES-g-ε-PLL nanoparticles alone in an imiquimod-induced psoriatic mice model *in vivo* [10].

Hydrogels have also been explored for wound-healing purposes [120]. Wound dressings based on gelatin and hyaluronic acid were prepared and cross-linked with EDC in different ratios and evaluated in *in vitro* and *in vivo* wound healing models [120]. The potential use of biocompatible gelatin/hyaluronic acid hydrogels as wound dressings was demonstrated by improved cell proliferation *in vitro* compared to control and a promotion of wound healing *in vivo* [120]. In another study, *in situ*-forming hydrogels based on gelatin and oxidized alginate were evaluated as a formulation for wound healing [31]. Cross-linking of gelatin was achieved in the wound as periodate oxidized alginate rapidly cross-links the proteins in the hydrogel in the presence of minute amount of borax [31]. Hydrogels were also prepared from keratin extracted from discarded human hair or wool in the presence of PVA and poly(ethylene imine) and crossed-linked by electron beam irradiation [57]. Treatment of wounds with keratin-based hydrogels accelerated wound closing compared to control groups (blank or hydrocolloid wound dressings) *in vivo,* most probably due to production of new collagen [57].

3.3. Protein-based films

Thin films are especially suitable for topical administration as they can make close contact with the surface of the skin or mucosa. Protein-based films can be prepared by methods equivalent to the preparation procedure of films based on traditional polymers, which include casting [14], compression [140], and extrusion [141]. Films made from neat protein are often rigid and brittle [142,143], which restricts the flexibility of the protein-based material and may limit their ease of handling and ability to bend to the curved surfaces of the body. Different strategies have been evaluated with the purpose of enhancing of the mechanical properties of protein-based films. Plasticizer such as glycerol and PEG can be added to increase chain mobility and the free volume to improve the mechanical properties of protein-based materials such as films [142,143]. Improvement of the mechanical properties of a protein-based film was also achieved by combining proteins with different physicochemical characteristics into one material. For example, films made from a composite of silk and gelatin displayed the strong mechanical properties of silk and the advantageous gelation properties of gelatin [14]. Silk/gelatin films with the antibiotic ciprofloxacin were prepared by casting, coated with PEG and explored for their wound healing properties [14]. PEGylated silk/gelatin films facilitated fast healing of wounds within 7 days *in vivo*, and the authors attribute the beneficial results to the presence of sericin in silk, and the natural healing properties of gelatin, which are known to improve cell attachment and proliferation [14]. Silk/gelatin films are indeed an interesting material for wound healing purposes as previously reported [144,145]. Wound dressings based on silk/gelatin were evaluated in a randomized clinical trial for split-thickness skin graft against commercial paraffin gauze dressings (Bactigras®) [145]. The safety and efficacy of the protein-based wound dressing were confirmed, and interestingly, donor sites treated with the bilayered silk/gelatin wound dressing had more rapid skin functional barrier recovery than sites treated with Bactigras® [145]. The biological process of wound healing is complex, and materials intended as wound dressings must meet several requirements to support natural healing and minimize scarring. The materials must display suitable mechanical properties to be able to bend to the curved surfaces of the skin and act as protection against mechanical stress. The material must be porous enough to allow exchange of oxygen and moist but at the same time be able to absorb exudates from the wound. Indeed,

one of the most studied potential roles of protein-based materials are their ability to promote wound healing. Protein-based materials in the form of films are no exception, and films intended as wound dressings have been prepared from a broad range of proteins also including soy protein isolate [146] and keratin [63]. Proteins are biocompatible and may promote tissue repair by acting on cellular processes involved in cell attachment and cell proliferation as exemplified above [14]. Protein-based films may also act as delivery systems for bioactive compounds such as antibiotics to prevent infections [37]. For example, recently, antimicrobial patches with thymol/β-cyclodextrin encapsulated in mucoadhesive gelatin-based films, made by solvent evaporation, have been proposed for treatment of oral infections [37].

Proteins may also be added to materials based on synthetic or other types of biopolymers to achieve specific properties. For example, mucoadhesive films made from chitosan and gelatin was evaluated as a delivery system for sumatriptan succinate for buccal administration [12]. By mixing polymers, plasticizer and protein in a specific ratio prior to casting, films with suitable properties for buccal delivery could be fabricated. Proteins may in some cases be better alternatives over synthetic polymers. For example, films based on collagen were developed for sustained release of human growth hormone intended for wound healing purposes [13]. The authors argue that films made from the protein collagen display true benefits over films made from polymers such as chitosan or poly(lactic-co-glycolic acid) (PLGA)/polycaprolactone (PCL) [13]. It is stated that the limited solubility of chitosan may require that the drug is to be loaded onto the films by absorption, which most likely will result in an undesirable burst release of drug upon administration [13]. Alternatively, PLGA and PCL are well-known polymers often included in drug delivery systems aiming for sustained drug release. However, processing of PLGA often involve dispersion of the polymer in toxic organic solvents and treatment by heat. These potentially harming conditions are unnecessary for the preparation of gelatin films, which can be fabricated by casting or by lyophilization of an aqueous-based solution of protein.

3.4. Protein-based electrospun fibers

Electrospinning is technique to produce nanofiber-based materials including scaffolds and patches for topical administration. By the technique of electrospinning, thin fibers in an intertwined network making up the dry formulation can be produced by the means of a strong electric field. The electrospinning technique operates under mild condition i.e. at room temperature and at atmospheric pressure with short processing times but requires appropriate conditions that favor intermolecular entanglement, solvent evaporation and smooth fiber formation. Proteins can be challenging to electrospin in their native form as their limited intermolecular entanglement limits the formation of fibers. Protein denaturation by exposure to elevated temperatures or to extreme pH can improve the intermolecular entanglement of the proteins and facilitate electrospinning into fibers. However, denaturation may compromise the activity of the protein, and exposure to extreme conditions may also impair the activity of an encapsulated drug. Electrospinning in organic solvents with good volatility is a well-known strategy to enhance fiber formation during electrospinning and has also been demonstrated as a suitable strategy for electrospinning of several proteins among others gelatin, collagen, elastin, and silk [147]. However, the use of organic solvents not only compromises the environmental impact of the protein-based materials and complicates the production scheme in an industrial setting, but also compromises the biocompatibility of the protein-based materials as trace amount of toxic solvent can be deposited on the fibers despite solvent evaporation. Accordingly, water-borne electrospinning that is electrospinning from aqueous-based solution has attracted great attention in the pharmaceutical area of the electrospinning field. To achieve electrospinning of native proteins, hydrophilic polymers such as polyethylene oxide (PEO) and PVA that increase the solution viscosity and improve intermolecular entanglement can be added to facilitate the electrospinning of proteins. This strategy has been shown to be very versatile and has let to water-borne electrospinning of a variety of proteins among others alpha-lactalbumin [24,16], silk [96] and BSA [148].

Fig. 3 demonstrates the versatility of protein-based materials as exemplified by electrospun nanofibers based on alpha-lactalbumin. The electrospun protein-based nanofibers have shown potential as both a drug delivery system for small molecules but also as a bandage for wound-healing. Alpha-lactalbumin, a significant component of whey, was electrospun in water with a minimum amount of PEO (16% w/w) to achieve biocompatible and tuneable nanofibers for drug delivery purposes [24]. The antibiotic ampicillin was encapsulated in the alpha-lactalbumin-based nanofibers that were cross-linked with GTA to achieve fiber mats stable in water [24]. In a zone-inhibition assay simulating topical administration, release of ampicillin from the nanofibers inhibited several gram-negative bacteria *in vitro* [24]. In another study, the water-soluble alpha-lactalbumin-based nanofibers were explored for oromucosal delivery of nicotine as a nicotine replacement therapy (Fig. 3A-C) [16]. Due to the fast and local release of nicotine from the electrospun protein-based nanofibers, nicotine permeated significantly faster through *ex vivo* porcine buccal mucosa compared to a solution with ten times the content of nicotine compared to the electrospun patch [16] (Fig. 3A-C).

The extracellular matrix that supports the generation of new tissue is made mainly from proteins such as collagen, elastin, fibronectin and laminin [149]. The three-dimensional fibrous architecture of nanofibers resembles the extracellular matrix, and nanofibers made by electrospinning of the aforementioned group of proteins have been explored for their ability to support cell attachment and proliferation to aid the generation of new tissue. Matthews *et al.* [150] were the first to successfully electrospin collagen and demonstrate that the fibers promote cell growth and penetration of the cells into the fibrous matrix. Since, electrospun collagen nanofibers have been extensively evaluated both *in vitro* and *in vivo* as for example skin grafts [38]. With a similar application in mind, elastin was also electrospun into nanofibers in combination with collagen and PCL to achieve the appropriate mechanical properties and promote tissue regeneration [39]. The presence of elastin in the nanofibers improved the elasticity of the scaffold and enhanced cell infiltration [39]. Preclinical studies in mice showed keratinocyte and fibroblast proliferation, tissue integration and accelerated early-stage angiogenesis after application of the scaffolds to wounds *in vivo* [39]. Importantly, the scaffolds displayed good biocompatibility and only mild foreign body reaction that is an important property to consider for implantation of materials [39]. It should be noted that due to the low aqueous solubility of the extracellular matrix protein collagen and elastin, nanofibers hereof are often produced in highly volatile and often toxic solvents such as HFIP. The number of studies covering electrospinning of the water-soluble proteins fibronectin and laminin are fewer in comparison to collagen and elastin, which is most likely due to the lower availability and higher commercial price of fibronectin and laminin. Most studies therefore involve surface functionalization of electrospun nanofiber-based materials made from synthetic polymers such as PCL with fibronectin [110] and laminin [111]. Nanofibers made from synthetic polymers lack the bioactivity of the proteins and surface functionalization with one or multiple bioactive proteins is a strategy to achieve nanofibers with specific properties that, for example, favor specific interaction with cells. One of the most studied potential roles of protein-based electrospun nanofibers are indeed their ability to enhance wound healing often in combination with antimicrobial agents, which was recently reviewed elsewhere (see Akhmetova and Heinz 2021 [15]). Nanofibers as wound dressings to aid healing of skin and mucosae have been based on both plant proteins e.g. pea, soy and zein and animal proteins such as alpha-lactalbumin, casein, keratin and silk [15]. For example, electrospun nanofibers based on silk fibrinogen [89] or alpha-lactalbumin [17] were shown to accelerate wound healing and also prevent scar formation in the oral mucosa and skin in *in vivo* murine

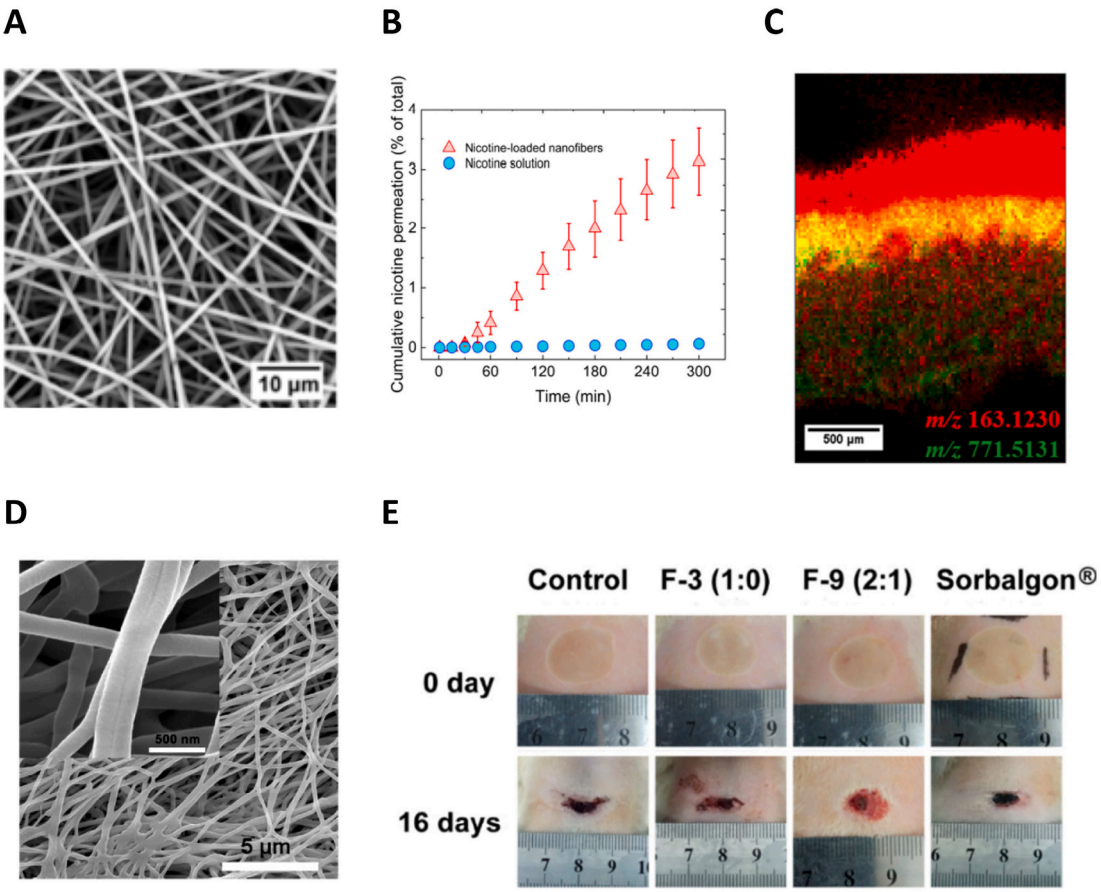

Fig. 3. Protein-based (alpha-lactalbumin) electrospun nanofibers for topical administration. A) Scanning electron microscopy image of electrospun alpha-lactalbumin/PEO nanofibers. B) Permeation of nicotine through *ex vivo* porcine buccal mucosa from nicotine solution and nicotine-loaded nanofibers. C) Representative image obtained by MALDI MS imaging of *ex vivo* porcine buccal mucosa exposed to nicotine-loaded nanofibers for 1 h. MALDI MS overlay image of nicotine (red) and an epithelium tissue marker (green). Red: nicotine, *m/z* 163.12297 [M + H]+. Green: epithelial marker PG (34:1), m/z 771.5131 [M + Na]+. Spatial resolution: 20 µm. From Kalouta *et al.,* 2020 [16]. D) Scanning electron microscopy image of electrospun alpha-lactalbumin/PCL nanofibers. E) Representative macroscopic images of various experimental groups treated with electrospun nanofibers mats (F-3 and F-9, PCL:alpha-lactalbumin ratio) and a commercial dressing (positive control). From Guo *et al.,* 2020 [17]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

models, respectively **(**Fig. 3D-E, for alpha-lactalbumin**)**.

3.5. Protein-based microneedles

One of the greatest challenges faced in the field of drug delivery today is to overcome major biological barriers that include the skin and mucosal membranes. This is especially a challenge for the absorption of drugs with a high molecular weight and of hydrophilic nature such as therapeutic peptides and proteins. Drugs with poor permeability and thus low bioavailability are therefore most often administered by injections, which can be associated with discomfort, pain and low patient compliance. Microneedles are needles often smaller than a few mm in height that have gained increasing attention for their ability to overcome some of the main obstacles faced by traditional injections. Microneedles are in contrast to hypodermic needles minimally invasive and only penetrate the outermost and most significant barrier of skin and mucosae [151]. Application of patches of an array of microneedles causes less tissue damage and is in general associated with less pain compared to hypodermic needles because of their smaller size [152,153]. Several types of microneedles exist including solid microneedles, dissolving microneedles, coated microneedles, hollow microneedles and hydrogel-forming microneedles [151] (Fig. 4A). Microneedles from protein are most often prepared by micromolding namely solvent casting using centrifugation, vacuum or pressure to fill the microstructure of the molds followed by solvent drying to create the needles [151]. Proteins are very suitable for preparation of microneedles because of their excellent biocompatibility and biodegradability. The biocompatibility of the microneedles but also degradation products hereof are key to minimize inflammatory responses *in vivo*. Microneedles have been fabricated based on proteins, mainly silk [18,20,125,154,155] and gelatin [27,41,121,123,124,156]. Other protein-based microneedles systems include zein-based microneedles for vaccine delivery [97] or anticancer treatment [98], and kanamycin-loaded squid suckerin microneedles for antibacterial treatment [157].

Sufficient mechanical strength is required to penetrate the tough layers of the keratinized epithelium that make up the stratum corneum of the skin. Silk possess mechanical properties suitable for fabrication of strong microneedles. Controlled release of FITC-Dextran [158], rhodamin-B [159], BSA [154], levonorgestrel [18], and horse radish peroxidase [155] have been demonstrated from microneedles made from silk fibroin *in vitro*. Control of the drug release kinetics can be achieved by tuning the shape and number of needles [154], addition of surfactants and solubility enhancers [18], by tuning the molecular weight and concentration of silk fibroin [18], tuning the secondary structure of silk [155] or the degree of cross-linking [40]. Interestingly, sustained release of levonorgestrel from silk fibroin-based microneedles was achieved over several months [18]. Thus, microneedles for sustained drug release based on proteins such as silk fibroin are indeed an

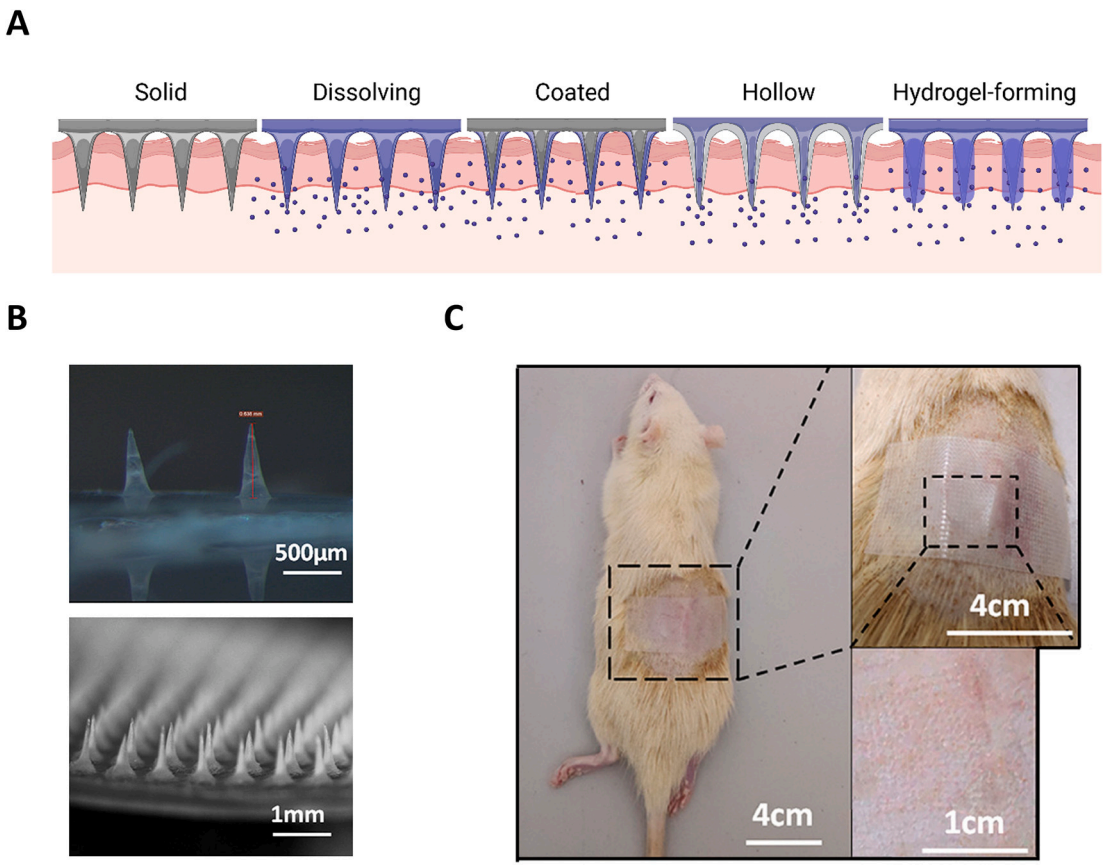

Fig. 4. A) Several types of microneedles exist including (from left to right) solid microneedles, dissolving microneedles, coated microneedles, hollow microneedles and hydrogel-forming microneedles. Image created by Biorender.com. B) A single microneedle under the microscope with a height of about 600 µm (top). A microneedle patch array visualized under the microscope (bottom). C) *In vivo* experiments with insulin-loaded microneedles in rats: Photographs of rats treated with insulin-loaded microneedles and surface of rats after microneedles were removed. From Zhu *et al.,* 2020 [125].

interesting alternative to microneedles based on synthetic polymers such as PLGA. Microneedles made from silk fibroin were also employed for the delivery insulin [19,125] and vaccines *in vivo* [20]. Insulin was released fast (<2 h) from the silk-based microneedles, and the relative bioavailability of insulin after administration of insulin-loaded silk-based microneedles was 98.8 ± 12.6% [125] (Fig. 4B-C). Furthermore, insulin released from the protein-based microneedles displayed a more sustained effect compared to intraperitoneal injection *in vivo* [125]. In another study, the efficacy of antigens derived from influenza, *Clostridium difficile*, and *Shigella* as potential vaccines delivered transdermally by the means of silk fibroin-based microneedles was evaluated *in vivo* [20]. Silk fibroin microneedles did not display a significant inflammatory response compared to antigen-loaded needles, thus demonstrating the potential of silk-based microneedles as means for biocompatible transdermal vaccine delivery [20].

In contrast to microneedles made form silk fibroin, gelatin possesses reduced mechanical strength and shape stability to make microneedles of high quality. Blending gelatin with starch and gold nanoclusters (AuNC) have been shown to improve the quality of the fabricated microneedles [41]. The AuNC not only enhanced the mechanical properties of the microneedles but also functioned as a glucose-responsive insulin release systems [41]. Blood glucose levels in normoglycemic ranges was maintained for 1–2 days *in vivo*, and diabetic symptoms in type 1 diabetic mice were alleviated after administration of insulin in AuNC formulated in gelatin/starch microneedles [41]. With the same motivations, gelatin-based microneedles formulated with calcium sulfate [121] or sodium carboxymethyl cellulose [122,123] or cross-linked with genipin [40] were explored for the delivery of insulin *in vitro, ex vivo* (porcine or human) and *in vivo* (rats or mice).

In a study in *rhesus macaque*, inactivated polio vaccine were administrated by gelatin/sucrose microneedles [124]. Compared to intramuscular injection, neutralizing antibody titers were equivalent among monkeys vaccinated using protein-based microneedle patches with inactivated poliovirus 1 and 2; however, serologic response to inactivated poliovirus type 3 vaccination was weaker after vaccination with protein-based microneedles compared to injection (potential artifact form the analytical method) [124]. Nevertheless, the study demonstrates the benefits of a simple alternative method based on gelatin/sucrose microneedles for inactivated poliovirus vaccination [124].

Dissolving microneedles are partly or solely composed of the API. For example, dissolvable microneedles based on gelatin were used to induce lipolysis and suppress lipogenesis to reduce subcutaneous adipose tissue [27]. Out of the natural polymers tested, which also included hyaluronic acid and collagen, gelatin displayed the best lipolytic activity e.g. by down-regulating adipogenesis-associated gene expression via regulation of their transcription factors *in vitro* and *in vivo* and furthermore reduced subcutaneuous adipose tissue *in vivo* [27]. Recently, using microneedles composed of therapeutic protein and polyvinylpyrrolidon (PVP) as a binder, 1 mg of human insulin or human growth hormone was delivered by the buccal route within 30 s in an *in vivo* porcine model [36] (Fig. 4B-D). In addition, microneedles applied to the palate and buccal mucosa delivered similar amounts of therapeutic protein i.e. human insulin and human growth hormone compared to subcutaneous injection [36]. In the same study, needles made from sorbitol were tested in clinical study with 100 human volunteers. Interestingly, 95% of subjects reported that they would choose the presented microneedle-based technology over conventional injections with hypodermic needles [36].

4. Concluding remarks

The versatility of proteins in nature is remarkable. Because of the extraordinary ability of proteins to be tuned according to the specific application, it is not surprising that protein-based materials have found applications in a wide range of fields as textiles, food packaging, wound dressings and drug delivery systems etc. Proteins are highly suitable candidates as building blocks for materials for applications within health and medical sciences, as proteins possess superior properties over many synthetic polymers by being biocompatible and biodegradable and a sustainable resource. Proteins furthermore benefit from often complex and peculiar physicochemical properties that can be exploited in the fabrication of biomaterials as specific structural features on a molecular level. Proteins also comprise a group of highly specific and potent pharmaceuticals, and the use of the protein API as the significant part of the drug delivery system e.g. as self-assembled protein particles, protein microneedles, is an intriguing approach. The field of protein-based materials is highly interdisciplinary and great attention has been given to a thorough characterization of the physical, chemical and mechanical properties of new and innovative materials based on proteins. Understanding and exploiting the deep knowledge generated to tailor the materials to the specific medical applications are needed to elucidate the full potential of protein materials. Furthermore, preclinical and clinical research evaluating protein-based materials is still required to advance the field of protein-based materials to develop technologies that can eventually improve the life of patients. Nevertheless, much research within the field has proven the success of protein-based materials in many relevant clinical settings already, and the prospects for materials based on proteins to advance a wide range of fields are indeed bright.

CRediT authorship contribution statement

Mai Bay Stie: Conceptualization, Formal analysis, Investigation, Project administration, Writing – original draft. **Kleopatra Kalouta:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Valeria Vetri:** Conceptualization, Validation, Funding acquisition, Resources, Writing – review & editing. **Vito Foderà:** Conceptualization, Project administration, Validation, Resources, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interest.

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