Stimuli-Responsive Hydrogels for Protein Delivery

Subjects: Polymer Science | Materials Science, Biomaterials

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Proteins and peptides are potential therapeutic agents, but their physiochemical properties make their use as drug substances challenging. Hydrogels are hydrophilic polymeric networks that can swell and retain high amounts of water or biological fluids without being dissolved. Due to their biocompatibility, their porous structure, which enables the transport of various peptides and proteins, and their protective effect against degradation, hydrogels have gained prominence as ideal carriers for these molecules' delivery. Particularly, stimuli-responsive hydrogels exhibit physicochemical transitions in response to subtle modifications in the surrounding environment, leading to the controlled release of entrapped proteins or peptides.

Keywords: stimuli-responsive hydrogels ; proteins ; peptides ; protein delivery

1. Introduction

Peptides and proteins perform vital functions in the human body during almost all biochemical processes, having received growing attention as drug candidates in recent years [1][2]. However, their physicochemical properties render them difficult to use as drug substances. Particularly, peptides and proteins are not ideal for oral administration, mostly because they lack stability in the gastrointestinal tract (GIT), and their hydrophilicity and size result in poor oral bioavailability [3][4][5]. There are also some disadvantages associated with other routes of administration, including intravenous injection, which may not be enough to achieve optimal therapeutic effects since various peptides and proteins have a short half-life [3][6][7]. Accordingly, significant effort has been devoted to developing drug delivery systems that allow peptides and proteins to reach their target sites more effectively.

Hydrogels have enduring popularity in protein delivery due to their suitable features, such as biocompatibility, porous structure, which enables the transport of various peptides and proteins, and protective effect against degradation [8][9]

2. Therapeutic Proteins

2.1. Characteristics

Peptides and proteins are essential biological macromolecules that have a central role inside cells during enzyme catalysis, transportation, signal transduction, gene regulation, and immunity-related functions [10]. These compounds are also involved in several pathological conditions, including cancer, diabetes, and hypertension. Therefore, considering their diversity of functions and participation in the control of various diseases, proteins and peptides are promising therapeutic agents [11][12].

Since the approval of the first protein used as an active substance—human recombinant insulin, Humulin®—in 1982 by the U.S. Food and Drug Administration (FDA), several therapeutic proteins have been approved for clinical usage, and others are in the process of development [1][2].

Therapeutic proteins can be used as drugs to (i) substitute a protein that is abnormal or deficient, (ii) increase an existing pathway, (iii) provide a new function or activity, and (iv) interfere with a molecule or organism [13].

Peptides and proteins consist of amino acid units joined together by peptide bonds. Whereas peptides contain two to fifty amino acids, macromolecules with more than fifty amino acids are known as proteins. The sequence of amino acids in their structure is designated as the primary structure [14]. Following the interaction and folding of amino acid chains, higher levels of organization arise, namely secondary, tertiary, and quaternary structures [15]. The functional characteristics of proteins rely on their three-dimensional (3D) conformation. As the 3D structure depends on the primary structure, any difference in the latter may produce a protein that is unable to perform its function [10].

Therapeutic proteins include molecules ranging in size from 1 to 50 kDa to much larger proteins like monoclonal antibodies (mAbs) with around 150 kDa; thus, even the smallest of these molecules exceed in size the so-called conventional drugs, such as aspirin (**Figure 1**) [16][17][18].

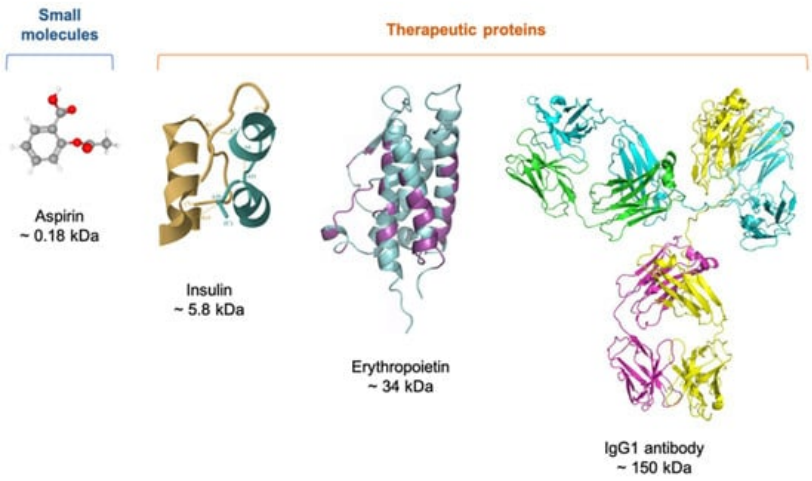

Figure 1. Comparison between the complexity of small molecules and therapeutic proteins.

The higher molecular weight of peptides and proteins impedes them from crossing the intestine mucosa [19] and other membranes. In addition, most proteins and peptides are hydrophilic and may have groups with charges that further reduce their translocation ability through the cell membrane and are absorbed by the systemic circulation. The lipophilic nature of these membranes thus hampers the passive diffusion of relatively high hydrophilic molecules [20].

Generally, the lower the molecular weight of a peptide or protein, the higher the metabolism and, in turn, the shorter the half-life. Likewise, proteins or peptides with higher molecular weight are related to minor metabolism and longer half-lives [5]. Proteins and peptides are sensitive to environmental changes, such as pH. By disrupting structural, noncovalent interactions, these changes can alter the native 3D structure of proteins and peptides, with loss or change in the biological activity being the outcome [12].

2.2. Delivery of Therapeutic Proteins

2.2.1. Parenteral Route

Therapeutic peptides and proteins are mostly administered by intravenous (IV), subcutaneous (SC), and intramuscular (IM) routes [21][22]. Although medicines can be given intravenously as a bolus, proteins are frequently administered as an infusion [23]. With the IV route, it is possible to achieve an immediate physiological response due to the complete delivery of the administrated proteins to the systemic circulation, avoiding the first-pass metabolism [24]. Notwithstanding its high bioavailability, IV administration is invasive and often painful. Moreover, treatment with high doses, as in the case of antibodies, requires infusion and, thus, visits to the hospital, which increases the overall cost of intravenously given drugs [23].

For some polypeptides and proteins, SC administration poses an alternative to the IV route while also bypassing the first-pass metabolism. Furthermore, as the SC approach could allow patients to self-administer proteins [23], patient preference and adherence are improved, resulting in overall cost savings. Regardless of the benefits of SC-administrated proteins, it still represents an invasive route and demands patients know how to take their medication safely. Additionally, SC injection is restricted to the maximum volume of 2.0 mL because higher volumes would cause rapid changes in the hydrostatic pressure that are perceived as painful [25]. Although such a volume is usually adequate for administering peptides due to their potency, high concentrations are often necessary if proteins are the case. For instance, some antibody solutions at higher concentrations exhibit high viscosity, which might increase injection time and discomfort at the site of injection, with a negative impact on patient compliance [26].

2.2.2. Oral Route

The preference for the oral route for drug delivery can be attributed to its ease of administration and noninvasive nature [27]. Nevertheless, delivering peptides and proteins by the oral route is very challenging.

The GIT contains large quantities of several enzymes, such as pepsin, trypsin, and chymotrypsin, and bile salts, which may elicit premature leakage and degradation of therapeutic proteins [28]. Moreover, the pH values in the GIT vary

considerably from highly acidic (pH 2.0–4.0) in the stomach to pH ~5.5 in the duodenum, ~6.0 in the jejunum, 7.2–8.0 in the ileum, and ~6.5 in the colon, also adding difficulty for oral delivery [29].

Besides lubricating and protecting the cell layer, the thick mucus layer covering the intestinal epithelium acts as a physical barrier to the absorption of drugs, hindering contact with epithelial cells and, thus, drug transport [30]. Molecules can be electrostatically trapped in mucus by virtue of its mucin proteins and proteolytic enzymes in abundance [31]. In addition to the mucus layer, the intestinal epithelium represents a second physical barrier, consisting of a continuous monolayer of epithelial cells, such as enterocytes, goblet cells, Paneth cells, and microfold cells [28][31]. This cellular barrier regulates the transport of nutrients and proteins across the gut lumen and the bloodstream or lymphatic system [31]. The permeation of proteins and peptides between adjacent intestinal cells, designated paracellular transport, is prevented by tight junctions, having an estimated average pore radius of 8–13 Å [31][32].

There are only a few commercially available therapeutic proteins for administration via oral route. One of the first peptide drugs approved by FDA for oral delivery is linaclotide (Linzess®), approved in 2012, which is both acid- and pepsin-resistant and used to treat patients with irritable bowel syndrome and chronic constipation. In 2017, semaglutide (Rybelsus®) was the first oral glucagon-like peptide-1 (GLP-1) approved for type 2 diabetes treatment [30].

2.2.3. Nasal Route

In general, the nasal route is best suited for drug delivery as it is noninvasive and the nasal mucosa is easily accessible, considering that the epithelial barrier is thin, porous, and highly vascularized [1]. Since the nasal venous system provides direct access to the systemic circulation, the loss of drug by the hepatic first-pass metabolism can be prevented [33].

Like the intestinal epithelium, the nasal epithelium is the main physical obstacle to the passage of proteins and peptides due to their low membrane permeability [34]. It is noteworthy that nasal mucociliary clearance is a primary defense mechanism of the lungs, in which mucus and its foreign, potentially harmful substances are removed from the respiratory tract. Knowing that the mucus layer is renewed every 15–30 min, the contact time between the protein or peptide and the nasal epithelium is thus limited [35].

2.2.4. Pulmonary Route

In addition to noninvasiveness and hepatic first-pass metabolism avoidance, other advantages of the pulmonary route for drug delivery that merit attention and intensive research include (i) the large surface area of lungs, (ii) a very thin alveolar epithelium, and (iii) a rich vascular supply, allowing for rapid systemic absorption [1][34].

However, some factors affect the delivery efficacy of inhaled proteins and peptides, with the primary barrier for inhaled particle deposition being the highly branching structure of the lung [16]. The rate and extent of this process depend significantly on the physicochemical properties of aerosol particles, especially the diameter of a particle in airflow, referred to as aerodynamic diameter [34][36]. Whereas particles with aerodynamic diameters ranging from 1 to 5 μm are deposited in the lower respiratory tract, those with diameters greater than 10 μm are deposited in the oropharyngeal region [16]. Particles exhaled during tidal breathing are under 1 μm [37].

After their deposition in the lungs, therapeutic proteins can be removed by either mucociliary clearance or alveolar macrophage uptake via pinocytosis [16][38]. The latter is size-dependent and becomes more relevant to large proteins (≥40 kDa) owing to their slower transport and absorption across the alveolo-capillary barrier. Alveolar macrophage uptake may not have such an impact on small proteins and peptides (≤25 kDa) as they are readily absorbed from airspaces [38]. Therapeutic proteins also encounter enzymes in the lungs but undergo less degradation compared to the GIT [39]. It is established that proteins and peptides with molecular weights around 6–50 kDa have good bioavailability following inhalation [1][16][40].

2.2.5. Ocular Route

It is the route of choice to deliver drugs directly to the ocular tissue [23]. Bearing in mind how accessible the front of the eye is, it comes as no surprise that topical instillation of eyedrops is often selected to treat diseases affecting the anterior segment of the eye, including the cornea, conjunctiva, aqueous humor, iris, ciliary body, and lens [41]. Nevertheless, less than 5% of a topically applied drug reaches deeper ocular tissues because reflex blinking and increased tear turnover collectively lead to poor drug retention and permeation [42][43]. The nasolacrimal duct drains the excess volume into the systemic circulation [42]. The rest of the protein or peptide faces the corneal epithelial barrier, formed by five to seven cell layers, also limiting its penetration [44].

2.2.6. Transdermal Route

Skin delivery of proteins and peptides may be efficient since it bypasses the liver, allows for sustained-release effect, and has less proteolytic activity than other mucosal routes [45]. Sustained release may overcome the need for frequent injections if the protein or peptide has a short in vivo half-life [46]. Seeing that the primary function of the skin is to protect the body against exogenous substances, achieving the permeation of protein molecules through the skin is undoubtedly a challenge [34]. Acting as the first and principal barrier to the transdermal route, the topmost layer of the skin, designated stratum corneum, consists of keratinocytes embedded in a lipid matrix, highly organized in a “brick-and-mortar” formation [1][45].

3. Hydrogels

3.1. Definition

Hydrogels are 3D, hydrophilic polymeric networks that can swell and retain significant amounts of water or biological fluids without being dissolved [47].

Over the last few decades, hydrogels have been widely used as tissue engineering scaffolds, wound dressings, medical adhesives, and contact lenses. Additionally, hydrogels are becoming increasingly attractive as vehicles for protein delivery due to their desirable properties. Hydrogels are similar in structure to the natural extracellular matrix and enable the physical incorporation of peptides and proteins [8][48]. The crosslinked nature of hydrogels is beneficial for transporting peptides and proteins, as it prevents large foreign molecules from interacting with the encapsulated proteins, thus promoting their retention in circulation without immune rejection.

3.2. Stimuli-Responsive Polymers

Stimuli-responsive hydrogels exhibit rapid physicochemical transitions in response to subtle variations in the surrounding environment, leading to the release of the entrapped molecules in a controlled manner [49]. Also termed “smart” polymers, stimuli-responsive polymers respond to external stimuli with reversible changes as they return to their original state after the stimulus is removed [50]. As illustrated in **Figure 2**, their macroscopic response can be a change in solubility, swelling/shrinking, or switching between hydrophilic/lipophilic, depending on whether the “smart” polymer chains are dissolved in an aqueous solvent (sol state), crosslinked forming a hydrogel, or grafted onto/bound to a surface [51].

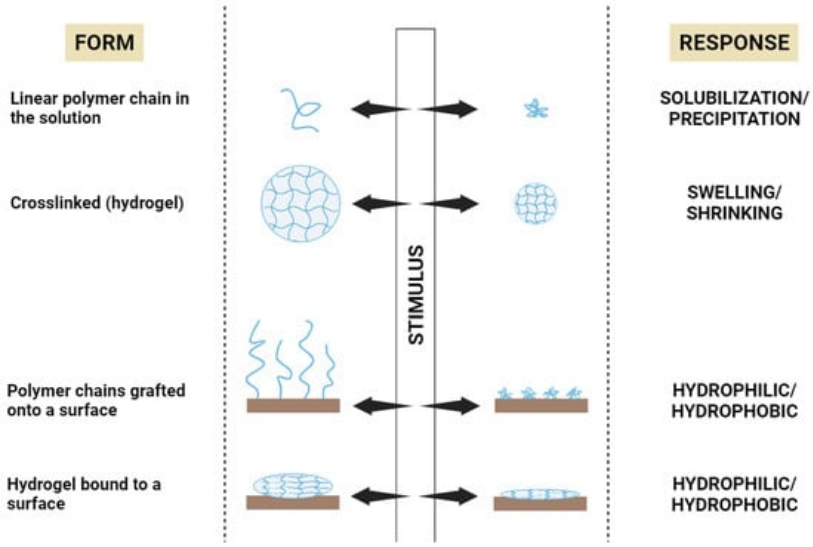

Figure 2. The macroscopic response of different forms of “smart” polymers [51]. Created with BioRender.com.

3.2.1. Temperature-Responsive Polymers

By shifting from ambient to body temperature, some temperature-responsive (or thermoresponsive) polymers undergo a sol–gel phase transition [52]. The ideal thermoresponsive polymer-based system is a free-flowing liquid at room temperature and only transforms into a gel once administered to the body [53].

Thermoresponsive polymers that form a gel with the elevation of the temperature have a lower critical solution temperature (LCST). At temperatures below the LCST, these polymers are miscible with water [54]. An upper critical solution temperature (UCST)-type behavior is identified when thermoresponsive polymers yield a gel below the critical temperature and return to the sol state above it [55].

3.2.2. pH-Responsive Polymers

The use of pH-responsive polymers in drug delivery systems takes into consideration that pH differences exist in the human body under normal or pathological conditions. For instance, as previously mentioned, the pH of the GIT varies greatly, with the stomach being strongly acidic and the intestine alkaline. Therefore, some pH-responsive polymers can be used to prevent gastric degradation and premature release in the stomach upon reaching the intestine [56]. pH-responsive polymers have also found applications in cancer-targeting strategies that capitalize on the acidic environment of the tumor (pH 5–6), as opposed to a normal physiological pH of 7.4 [57].

pH-responsive polymers have acidic (carboxyl) or basic (amine, imine) ionizable groups attached to the hydrophobic backbone, thus being considered polyacids (anionic) or polybases (cationic). These pendant groups can either donate or accept protons, depending on their pK_a and the environmental pH value [58]. Cationic hydrogels swell at a low pH (pH < pK_a), and anionic hydrogels, on the other hand, swell at a higher pH (pH > pKa) due to the protonation of amino/imine groups and ionization of the acidic groups, respectively. As a result, electrostatic repulsion between charges leads to polymer chain expansion and impels the hydrogel to imbibe larger quantities of water [59][60].

3.2.3. Ionic Strength-Responsive Polymers

Gelation can occur as a response to alterations in the ionic content of the surrounding medium if ionic strength-responsive polymers are involved [61]. It is suggested that high salt concentrations reduce the repulsive electrostatic strength of the polymer, followed by an increase in hydrophobic interactions and, in turn, network precipitation [62]. Also, hydrogels made from these polymers swell differently in water and in an electrolytic solution [63]. Besides inducing hydrogelation, ionic strength is an effective way to improve mechanical and transport properties [64].

3.2.4. Biomolecule-Responsive Polymers

Biomolecule-responsive hydrogels can undergo structural transition in response to specific target biomolecules, such as glucose, proteins, nucleic acids, and polypeptides [65].

Glucose-responsive hydrogels can be suitable materials for diabetes management based on the glucose levels in the bloodstream. To achieve a self-regulated delivery of insulin, glucose-responsive moieties, such as glucose oxidase, lectin (concanavalin A, Con A), and phenylboronic acid (PBA), are incorporated into the hydrogel system [66].

The first approach is possible upon immobilizing glucose oxidase in a pH-responsive hydrogel enclosing a saturated insulin solution. At high glucose concentrations, glucose diffuses into the hydrogel and is oxidized to gluconic acid, prompting mesh expansion and release of previously entrapped insulin to the medium. As a result, sugar levels drop, causing a rise in pH that prevents further insulin release [67][68]. A different strategy takes advantage of the competitive binding of Con A to glucose and glycosylated insulin. Since Con A has a greater affinity for glucose, increased levels of glucose trigger the displacement and release of glycosylated insulin by diffusion across the hydrogel matrix [69].

In the case of antigen-responsive hydrogels, the ability to undergo volume or structural changes relies on antigen–antibody interactions. This group of bio-responsive hydrogels can be prepared by the (i) immobilization of antigens or antibodies within the hydrogel structure, (ii) chemical conjugation of the polymer to antigens or antibodies, and (iii) copolymerization with the antigen-binding fragment of the antibody [70]. To illustrate, grafting the polymer network with an antigen and its corresponding antibody enables a hydrogel to form upon an antigen–antibody binding.

3.2.5. Enzyme-Responsive Polymers

Enzymes are increasingly used as stimuli to trigger structural transformations in hydrogels. To understand this, one should acknowledge that many medical conditions are associated with altered expression of proteins, more precisely overexpressed enzymes in diseased tissues [71].

In general, the design of enzyme-responsive hydrogels has three basic requirements. First, the hydrogel system must have substrate mimics or other elements that only enzymes can recognize [72]. For proteolytic enzymes, common recognition elements could be peptide chains/linkers or polymer–peptide conjugates with specific amino acid sequences that determine enzyme–substrate specificity [73].

3.2.6. Dual and Multiple Stimuli-Responsive Polymers

On some occasions, polymer materials with a single responsiveness may not fully serve the therapeutic purpose in a complex physiological or pathological microenvironment [74]. Therefore, polymer materials that respond to various physical or chemical stimuli are in high demand for biomedical applications.

Dual stimuli-responsive polymers respond to two stimuli combined (pH/temperature, ionic strength/pH, ionic strength/temperature, temperature/enzyme, etc.). As regards multiple stimuli-responsive polymers, more than two stimuli, such as temperature/pH/redox, temperature/pH/biomolecule, or temperature/redox/biomolecule, will trigger a response [75][76].

Applying polymers with pH and temperature responsiveness is a growing trend for anticancer agents' delivery since many tumors display elevated temperature and low pH compared to healthy tissues. The most investigated thermoresponsive polymer is pNIPAAm with an LCST of 32 °C in water; the polymer network collapses above the LCST, and the corresponding hydrogel shrinks at body temperature (37 °C). In the aforementioned context of cancer treatment, combinations of pNIPAAm and pH-responsive polymers, such as polyacrylamide and polyacrylic acid, also provide valuable options to generate dual responsiveness [77].

4. Stimuli-Responsive Hydrogels for Protein Delivery

In a strategy to deal with the problems of protein delivery in the stomach, Lima et al. [78] chose alginate as the hydrogel matrix and bovine serum albumin (BSA) as a model protein. The resulting hydrogel showed biocompatibility and pH-dependent BSA release and swelling profile, reaching the highest value of swelling at pH 7.4. The overall results suggested that the performance of this alginate-based hydrogel as an oral drug delivery system would be excellent.

Knowing that keratinocyte growth factor (KGF) repairs potently epithelial tissue, Xu et al. [79] proposed a thermoresponsive heparin-modified poloxamer hydrogel containing KGF to prevent intrauterine adhesion, the main cause of infertility and recurrent pregnancy loss in women with reproductive capacity. In vitro studies showed a sustained release of KGF from the hydrogel. On the seventh day after injection into the intrauterine cavity, the authors observed endometrial epithelial cell growth and angiogenesis in the injured uterus of a rat model.

After evaluating a series of thermoresponsive hydrogels, Dutta et al. [80] selected a poly(lactide-co-glycolide)-b-poly(ethylene glycol)-b-poly(lactide-co-glycolide) hydrogel for encapsulating glucagon-like peptide (peptide A) and modified insulin analogs. When treating diabetic mice with a single SC administration of peptide A-loaded hydrogel, their blood glucose level decreased and was below 50–65% of the initial values over two to three days. For self-regulation of insulin delivery by SC injection, alginate was grafted with a temperature/glucose dual-responsive copolymer consisting of N-isopropylacrylamide and 3-acrylamidophenylboronic acid monomers, maintaining good biocompatibility [81]. It was found that insulin can be dispersed uniformly in a cold copolymer solution (10 °C), which turns into a gel in situ by raising the temperature to 37 °C. Diabetic nephropathy is a complication of type 1 and type 2 diabetes related to the progressive reduction of kidney function [82].

Antimicrobial peptides (AMPs) are essential components of the innate immune defense in multicellular organisms and are currently under development as novel anti-infective drugs [83]. While most AMPs kill microbial pathogens directly, others act indirectly by regulating the host's defensive system [84]. Since an ideal skin wound dressing should have antibacterial activity against antibiotic-resistant bacteria, Rezaei et al. [85] prepared thermoresponsive chitosan hydrogels loaded with different concentrations of AMPs (4, 8 and 16 µg/mL).

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