Journal of Drug Delivery Science and Technology 89 (2023) 105066

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Review article

Pullulan hydrogels as drug release platforms in biomedicine

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ARTICLE INFO

Keyworks:
Biodegradable hydrogel
Pullulan polymer
Drug delivery platforms
Controlled release

ABSTRACT

It is increasingly urgent to develop new therapeutic systems to combat the spreading and evolution of various pathologies globally. Nonspecific therapies and/or insufficient medication biodistribution might hinder the patient's recovery. In this sense, a targeted and controlled delivery of various biomolecules allows overcoming the limitations of conventional delivery systems, taking the user one step closer to the successful treatment of a disease. Hydrogels have been highlighted for their drug delivery abilities, particularly for their tunable properties, like hydration capacity, biodegradability, release kinetics, etc., that can be adjusted to the desired needs. Additionally, they can be produced from either natural and/or synthetic polymers, with natural-origin sources providing exceptional features like biodegradation and acceptable integration in biological systems. One of those polymers is pullulan, a biodegradable, biocompatible and hemocompatible material, with multiple uses in biomedicine. Investigations into pullulan-based hydrogels have progressively increased over the last few decades. This review addresses the uses of pullulan in biomedical engineering, emphasizing its exceptional properties for drug delivery and its processing into hydrogel systems, either in its original or derivative forms.

1. General introduction

Along with the evolution of humans and technology, diseases and pandemics that are difficult to control have emerged. In the last years, the COVID-19 pandemic highlighted the urgency for research investment into more effective strategies for fighting pathogenic microorganisms [1]. In many disease treatments, a limited efficacy is still observed, along with insufficient biodistribution and a marked lack of selectivity introduced by the conventional administration of drugs/-bioactive agents [2]. In fact, to stimulate the intended therapeutic effect, high dosages and/or consecutive administration of drugs are often necessary. Although it may work at first, this approach remains linked to a reduce overall effectiveness of the treatment and possible systemic toxicity [3].

Scaffolds are porous solid biomaterials, with a three-dimensional structure, known to be good vehicles for delivering therapeutic substances into the body. Depending on the structural organization, the size and interconnectivity of the pores and the chemical composition, these structures have the ability to absorb and immobilize various substances and consequently to control and trigger their release at a specific time and place. In addition, they are described as structures that allow the transport of body fluids and gases [4]. Hydrogels, three-dimensional (3D) structures, have introduced new insights into the prevention and combat of several diseases. Indeed, because of their physicochemical properties, hydrogels have been used in many branches of medicine and tissue engineering, including oncology, immunology, wound healing, cardiology, pain control, tendon and ligament repair, and cartilage replacement [3,5]. Depending on the origin of the polymers composing the hydrogel, they can be constructed with exceptional biocompatibility and biodegradability, porous structure, flexibility and adaptability, good mechanical properties and even thermal stability. By means of natural polymers, biodegradable, non-toxic hydrogels can be produced at reduced cost [6,7]. From a drug delivery perspective, hydrogels can be produced in such a way as to have certain characteristics that allow to protect, target and evenly distribute the drugs at the desired site in a controlled and gradual manner [3]. Also, in wound healing, hydrogels can facilitate skin regeneration by balancing local moisture and absorb exudates and assist with infection control. Here, the pullulan biopolymer can be highlighted because of its biocompatible and biodegradable nature, having anti-inflammatory, antimicrobial, adhesive and non-immunogenic activity. Furthermore, it has excellent flexibility, high water-absorbing capability, and good solubility. Due to the

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https://doi.org/10.1016/j.jddst.2023.105066
Received 18 July 2023; Received in revised form 27 September 2023; Accepted 12 October 2023
Available online 18 October 2023

unique properties of pullulan, this polysaccharide is extremely interesting for the production of hydrogels with good properties [8,9]. Pullulan hydrogels can incorporate various bioactive and immunomodulatory agents, namely natural extracts, growth factors, antibiotics, proteins and peptides, within their structure to increment their biological activity [10–12], or serve as platforms for cell immobilization [13].

This review exposes the main characteristics of pullulan-based hydrogels developed in recent decades, with emphasis on hydrogels for drug controlled release systems and biomedicine purposes. A detailed discussion of the chemical alterations that may be conducted on the pullulan natural polymer to improve its characteristics is also presented.

2. Methodology: PRISMA protocol

The preparation of this review article on pullulan hydrogels as drug release platforms in biomedicine was done following the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). This systematic review aimed at understanding the current impact of pullulan hydrogels as drug delivery systems. Initially, an exhaustive search was carried out in several databases [e.g., MedLine (PubMed), Web of Science and Embase (Scopus)], using a comprehensive set of keywords related to the topic, such as "hydrogels", "polymers", "drug release", "applications in biomedicine". This initial process resulted in an extensive list of approximately 3950 articles. We then proceeded to screen this large number of articles, applying previously defined inclusion and exclusion criteria. These criteria assessed the relevance of the title and abstract, availability in English, and fit within the research topic. As a result of the screening, the number of articles was reduced to around 1000. To assist with this screening, the keywords "pullulan hydrogels", "pullulan polymer" and "drug delivery platforms" were used. To ensure the quality and relevance of the studies included in the final review, we carried out a detailed analysis of these 300 articles, closely examining their contents and methods. After, 178 articles were considered the most pertinent for this review work. This selection ensured that the data and information presented in the article were comprehensive, reliable, and representative of the current state of the research on pullulan hydrogels with applications in drug delivery.

3. Hydrogels

Hydrogels are three-dimensional (3D) hydrophilic polymer organizations capable of absorbing and retaining considerable amounts of fluids [7], adapting to the demands of the surrounding environment by interchangeable sol-gel conditions. These polymeric structures were first produced by Wicherle and Lim in 1960. Since then, they have distinguished themselves in a wide spectrum of applications, including smart drug delivery systems [14], wound healing [15] and cartilage and bone tissue engineering [16,17], and food packaging [18]. In the last, hydrogels have shown potential as detection matrices for hazardous substances presence in food (stimuli-responsive hydrogels that can act as sensors) [19]. On the other hand, in biomedical formulations, because of their similarity with the extracellular matrix, they can be easily recognized by the human body and integrate human tissues without any constraints (Fig. 1).

The classification of hydrogels is not fixed; in fact, it dependents on different parameters, including origin, polymer load, polymer composition, size [macrogels (mm to cm), microgels (μm) and nanogels (nm)], degradability (biodegradable or non-biodegradable), and physical properties (conventional or smart) [7,21]. Regarding the physical properties, conventional hydrogels are not sensitive to environmental changes. Yet, smart hydrogels are known to respond to changes in their external environment (e.g., pH, temperature, ionic strength, humidity and light), triggering alterations in their physical structure and chemical properties [14]. According to Hu et al., the pH-responsive agarose/succinoglycan hydrogel incorporating ciprofloxacin shows a different release kinetics depending on the pH, with a cumulative release of approximately 41% in 35 h at pH = 1.2 and a complete release at pH = 7.4 for the same period of time [22].

Crosslinking approaches define the hydrogel's characteristics [23]. Physical and/or chemical methods are used to crosslink the polymers to form a hydrogel. Physical crosslinking (e.g., ionic interaction, hydrogen bonding, hydrophobic interaction, and host-guest interaction) is very dynamic, with structural stability being highly dependent on external environments (e.g., temperature and pH), since these hydrogels have low mechanical strength and are often unstable. Furthermore, these hydrogels are structurally weak and their gelation is reversible. On the other hand, with chemical crosslinking (e.g., photopolymerization, enzymatic crosslinking, crosslinking molecules and polymer–polymer

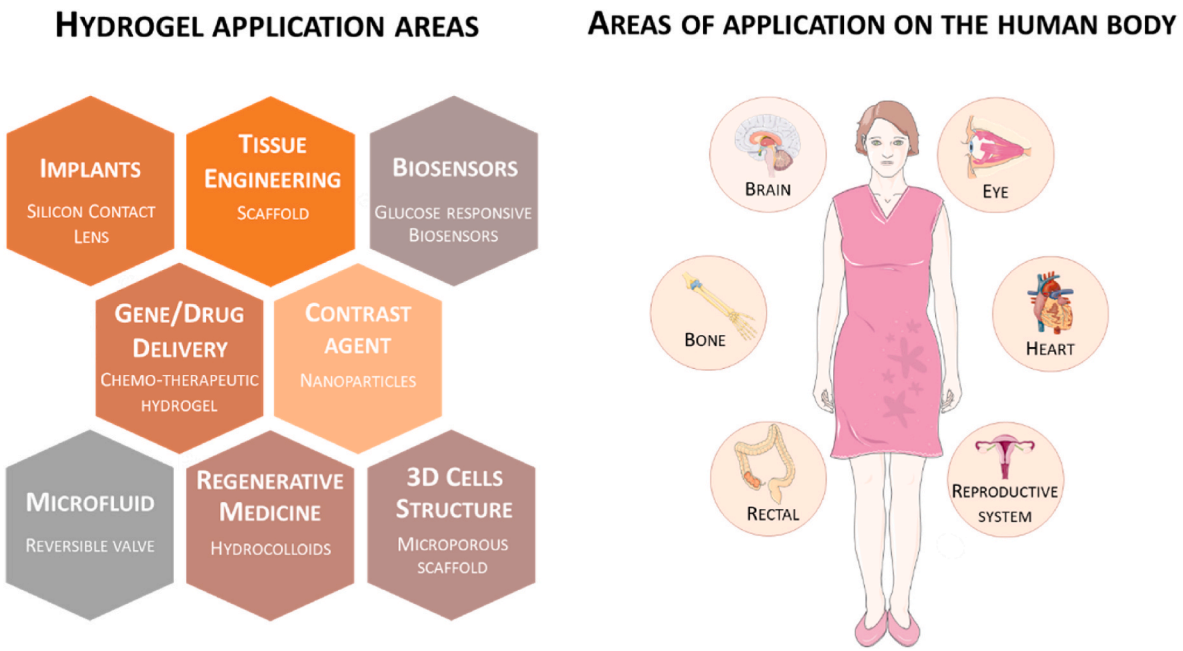


Fig. 1. Potential applications of hydrogels in the human body. Adapted from Ref. [20] with CC BY 4.0 permission.

crosslinking) polymer networks gain permanent junctions (irreversible bonds), raising their stability and structural integrity (increased degradation time) [7,24,25]. However, they still exhibit low fracture strength and extensibility and many crosslinkers (e.g., glutaraldehyde and epichlorydrin) are toxic, causing adverse side effects [7,26]. As alternative, natural-origin crosslinkers, such as ethylene glycol diglycidyl ether (EGDE) [27], citric acid [28], and genipin [29], with a less harmful profile, are being employed as alternatives, while demonstrating limited effectiveness that is confined to particular types of polymers [5].

Hydrogels can be produced from synthetic and natural polymers or from the combination of both (hybrid) [6,30]. Based on their composition, hydrogels with certain features are obtained. Hydrogels derived from natural polymers have molecular recognition sites (improved biocompatibility) and are biodegradable, which make them appealing for medical uses [7]. Particularly, hydrogels obtained from polysaccharides (e.g., pullulan, alginate and chitosan) have been widely used in biomedicine due to their excellent hydrophilicity, biocompatibility and low toxicity [6,31,32]. However, their stability in aqueous media is reduced, they exhibit poor mechanical properties, and are associated to a rapid degradation profile [33]. Hydrogels composed of synthetic polymers [e.g., poly (vinyl alcohol) (PVA), polycaprolactone (PCL) and poly (vinylpyrrolidone)] guarantee reproducibility of physical properties and excellent mechanical features. However, these tend to display low biocompatibility and, hence, poor cell affinity [5,7]. To improve their biological features, certain bioactive molecules are often incorporated into these hydrogels. For instance, synthetic hydrogels made of PVA have been modified with tea polyphenol for improving the scaffold cytocompatibility and antimicrobial capacity and, this way, ensure an effective wound dressing performance [34]. Mixing natural and synthetic polymers or combining different structures, like fibers [35,36] or nanoparticles [37,38] within hydrogels to generate composites, may be other options of improving the biological attributes of the scaffolds or even overcome limitations of thermal and mechanical stability [7,39].

There are several factors [e.g., porous structure, similarity to native soft tissue extracellular matrix (ECM) and remarkable biocompatibility] that make hydrogels versatile and useful in a variety of applications [40–44]. Hydrogels can be administered in various ways, including orally, topically, by implantation or by injection [25]. Depending on the administration route, physical characteristics must be controlled, namely the gelation capacity (solidification time). For example, in injectable hydrogels, it is important that the gelation time of the hydrogel is long enough so that it can be injected before completely solidifying. However, although this system must have a slow gelation to the point of not clotting in the needle, it has to be fast enough to prevent dilution in the body fluids [3]. It is also described that hydrogels have high water absorption capacity (in part due to the presence of polar/-charged functional groups) [7,30], being able to absorb up to thousands of times their equivalent weight in water until the process reaches an equilibrium [45]. Hydrogels can be used as support materials for cells during tissue regeneration, thereby allowing the diffusion of nutrients, metabolites and growth factors [46]. For example, with regard to their application in wound therapies, it is known that hydrogels assist with tissue granulation or epithelialization and stimulate autolytic debridement in the presence of necrotic or torn tissue [41]. Additionally, it has been described that these constructs maintain a moisture balance at the wounded site [7]. Their usual transparent appearance allows monitoring of the wound evolution without to the removal of the dressing from place [41], reducing tissue damage and patient discomfort. Also, they protect the wound bed against the invasion of pathogens, preventing contamination and proliferation of microorganisms, aside from impact and abrasion [47]. Recently, Pan et al. demonstrated the potential of a tannic acid-based hydrogel crosslinked with quaternized chitosan in the healing of diabetic wounds by showing that the hydrogel promoted blood clotting, suppressed inflammation and accelerated collagen deposition. The incorporation of tannic acid was also shown to play an antibacterial role and an inhibitory action on reactive oxygen species (ROS) [48].

Drug delivery systems using hydrogels have gained increasing prominence in biomedicine. Their inherent degradation rate remains an important aspect on the control of the incorporated drugs release [14,24,45]. This is frequently defined by the type of polymer used, level of porosity, and location and size of drug molecules. By locally delivering their payload, large drug doses (associated with drug loss by enzymatic degradation) can be avoided and healthy tissue-related side effects (associated with non-specific absorption by other tissues) can be reduced [49]. There are several studies that describe the potential of hydrogels as controlled drug delivery systems in the treatment of different types of cancer [50–53]. In particular, Gao et al. highlighted that the hydrogel composed of poly-(D,L-lactic acid-*co*-glycolic acid), polyethylene glycol and poly-(D,L-lactic acid-*co*-glycolic acid) (PLGA--PEG-PLGA) loaded with docetaxel could be an effective tool in treating lung cancer (subcutaneous use) [54]. Lin et al. indicated that incorporating with paclitaxel (a hydrophobic anticancer agent) could provide a moderate inhibition of breast cancer [51]. Lima-Sousa et al., on the other hand, explored an injectable thermo-responsive hydrogel based on chitosan-agarose modified with reduced graphene oxide (photothermal nanoagent) and loaded with the combination of drugs doxorubicin: ibuprofen (DOX:IBU), which showed to be effective as a therapy against breast cancer cells (*in situ*). This hydrogel demonstrated a good swelling capacity (maximum swelling, about 24%, after 45 min of incubation) and adequate degradability (after 14 days, weight loss of about 56%) for the intended biomedical application. Furthermore, the authors also found that the incorporation of DOX:IBU into the hydrogel reduced the cell viability (MCF-7 cells) from 60% to 34%, thus introducing a significant chemophotothermal effect [53]. Other studies indicate that the incorporation of antimicrobial peptides in hydrogels can make their action more effective [10,55]. According to Atefyekta et al., the covalent binding of the antimicrobial peptide (AMP) RRP9W4N in the developed amphiphilic hydrogel contributed to the increase in the stability of AMP in human serum, thus allowing them to maintain their antibacterial action for up to 48 h [55].

Another possibility to control drug release from hydrogels is by incorporating nanoparticles loaded with the desired drugs. This way bioavailability and controlled release is improved to prevent overdosage. The combination of these two structures enables the formation of a structure with better mechanical strength compared to hydrogels alone and with greater retention of payload compared to nanoparticles alone [56]. The study developed by Wu et al. shows the differences in the release rate of a drug when incorporated in nanoparticles loaded in a directly incorporated in a hydrogel. In this study, the authors developed a composite with the function of a controlled temporal release system to be used in clinical scenarios of combined therapy. This composite was developed to release two drugs, cisplatin (CDDP) and irinotecan (IRN). CDDP was incorporated directly into the hydrogel and IRN was incorporated into alginate nanoparticles (AlgNP; loaded onto the hydrogel). The analysis of the release profiles of the hydrogel with CDDP and the hydrogel with AlgNP/IRN showed that the IRN had an adjustable release (about 11.3% released in 9 h) due to its incorporation in the nanoparticles while the CDDP presented a fast release (about 45% in 9 h). Furthermore, the authors also studied the release profiles of both drugs when combined in the same composite. In the first 9 h the presence or absence of AlgNP/IRN had no impact on the percentage of released CDDP (rapid release in both). However, after 6 days the presence of a higher AlgNP/IRN content (0.6 wt%) reduced the percentage of CDDP released (from 75% to 65%). Regarding IRN, in the first 9 h the presence of CDDP reduced the percentage of released IRN (to values below 7%). Furthermore, after 6 days the authors verified that the presence of a higher percentage of AlgNP/IRN (0.6 wt%) and in the presence of CDDP there is a lower release of IRN (from 60.7% to 20.5%) [57]. Afshar et al. described the production of a sodium alginate (SA)/PVA-based hydrogel incorporating chitosan nanoparticles loaded with the drug rosuvastatin. In this study, the authors verified that the SA/PVA hydrogel allowed a

sustained release of the drug for 24 h (time required for full release), and that the chitosan nanoparticles played a crucial role in the release behavior. More specifically, in this study the authors suggest that the slow release observed in the first hours (13% in 4 h) may be associated with the low water solubility of the nanoparticles. In the following 4 h, a high rosuvastatin release rate (67%) could have been triggered by the gradual dissolution of the drug at the surface of the nanoparticles. A slow release in the last hours could be justified by a longer diffusion from the inner parts of the particles to the release medium [58].

4. Pullulan and its derivatives

Numerous interesting characteristics make polymers excellent biomaterials for hydrogel production, particularly those of natural origin [6,7]. The natural-origin polymer pullulan was initially discovered by Bernier in 1958 from the fungus *Aureobasidium pullulans* (producer fungus). Yet, it was in 1959 that Bender et al., studying this novel polysaccharide, named it pullulan. In 1960s, the structure of pullulan was resolved [59,60] and in 1976 the Hayashibara Company Ltd. (Okayama, Japan) was the first to commercialize the pullulan product [61]. It is known that the mentioned fungus can use different carbon sources (e.g., glucose and lactose) for pullulan synthesis and that the pH of the culture medium can influence the efficiency of pullulan production [8]. The polymer is a linear, neutral and non-ionic glucose homopolysaccharide, composed of maltotriose units that are interconnected by α-1,6 and α-1,4 glycosidic bonds (co-existence of these two bonds) having the chemical formula $(C_6H_{10}O_5)_n$ (Fig. 2). This polymer is a unique polysaccharide, which, due to its physicochemical properties, offers important advantages (such as biodegradability, elasticity, thermal stability and high barrier properties toward oxygen and carbon dioxide) in various fields [62].

Pullulan biosynthesis is a multistep biological reaction undertaken by *A. pullulans*. Due to the complex properties of the pullulan-generating microorganism, the specific method for the pullulan biosynthetic pathway has not yet been fully clarified [63,64]. Fig. 3 shows the biosynthetic pathways for pullulan formation know to this moment [64,65].

Pullulan's distinctive structural arrangement is responsible for its amorphous and randomly ordered character, as well as its slightly slimy appearance compared to other polysaccharides [66]. During production, the carbon sources used for its synthesis define its lower (high concentration of glucose) or higher (high concentration of sucrose) production yield. In addition, the pH of the culture medium, temperature and nitrogen source also influence the efficiency of pullulan synthesis [8,67]. For pullulan production, the ideal pH range should be between 5.5 and 7.5; under conditions of reduced pH, production of insoluble glucan is stimulated but pullulan synthesis is suppressed [68]. Regarding temperature, higher ranges (32 °C) supports cell growth, while lower temperatures (26 °C) promote pullulan formation [69].

Nuclear magnetic resonance of proton and carbon (^1H, ^{13}C NMR), 2D techniques and Raman spectroscopy have been used to determine the pullulan structure [70,71]. The molecular weight of pullulan is highly influenced by production conditions, culture and microbial strain used, ranging from 45 to 600 kDa [61]. A high molecular weight can be obtained by fermenting agro-food residues and is in higher demand for commercial purposes [72], whereas a low molecular weight is obtained

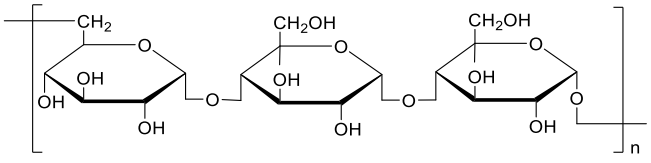


Fig. 2. Chemical structure of pullulan.

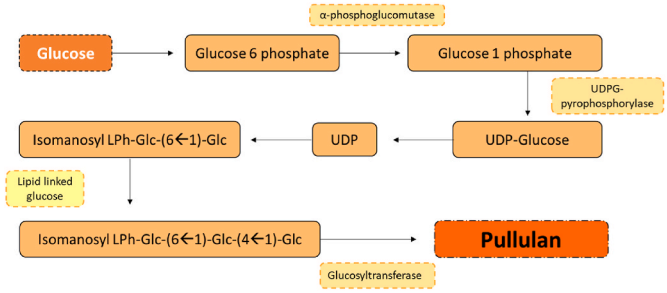


Fig. 3. Schematic mechanism of pullulan biosynthesis [64,65].

from synthetic media [73]. The major limitation to the generalized use of pullulan relates to cost, as it is three times more expensive than other polysaccharides [74]. Pullulan presents good structural flexibility (due to the regular alternation of glycosidic bonds), elastic properties and a thermal stability that allows it to withstand high temperatures (≥250 °C; melts in a range of 250–300 °C) and optical rotation of 192° in 1 g/dL solution [67]. Because of its unique bond shape, pullulan is highly soluble in both cold and hot water, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF). Additionally, it is insoluble in organic solvents (acetone, ethanol, isopropanol and methanol) and exhibits distinctive structural flexibility and non-hygroscopic features in nature [67,75,76]. Curiously, heat, pH variations, and the presence of most metal ions have no effect on the viscosity of a pullulan solution [77].

The degradation of pullulan can occur due to the action of enzymes that can hydrolyze glycosidic bonds of polysaccharides. These enzymes are divided into two major groups: pullulanases (types I and II) and pullulan hydrolases (types I, II and III) (Table 1) [78].

Pullulan has an adhesive white powder with film-forming properties, is non-carcinogenic, non-ionic, non-irritant, non-immunogenic, non-hygroscopic, non-toxic, hemocompatible, biodegradable, hydrophilic, odorless and tasteless, and possesses a non-mutagenic edible nature [61,73]. It is currently used in a wide range of fields, including the textile, food packaging and pharmaceutical industries. For pharmacology purposes it has been linked to antibacterial, anticancer, anticoagulant, anti-inflammatory, antifungal, antitumor, antiviral and antithrombotic activities, and has also been used as a carrier for nasal, oral and lung transmucosal drug delivery systems and wound-dressings [8,67,79], in coatings for capsules [80], granules, tablets, and pills [81], or for topical formulation in the form of gels [9] and oral films [82]. In the United States, pullulan has been deemed safe for human uses by the Food and Drug Administration (FDA; since 2002) for numerous applications in the food and cosmetic industries. In cosmetics, it has been used, among

Table 1
Mechanisms of action of pullulanases and pullulan hydrolases on the pullulan polymer [75,78].

Enzymes	Type	EC number	Bonds and Products
Pullulanases	I	EC 3.2.1.41	Hydrolyzes α-1,6 glucosidic bonds in pullulan to produce maltotriose and linear oligosaccharides
	II	EC 3.2.1.1/41	Acts on both α-1,4 and α-1,6 glucosidic bonds in starch and other polysaccharide and is divided into two subgroups (i) amylopullulanase and (ii) α-amylase-pullulanase
Pullulan hydrolases	I	Neopullulanase EC 3.2.1.1.35	Acts on α-1,4 glucosidic bonds of pullulan to form panose
	II	Isopullulanase EC 3.2.1.57	Cleaves α-1,4 glucosidic bonds of pullulan to produce isopanose
	III	3.2.1.-	Attacks α-1,4 and α-1,6 glucosidic bonds in pullulan and forms panose, maltotriose, maltose and glucose.

others, for producing body lotion, eye and skin care creams, facial packs, face powders and shampoos. Listerine, for instance, was the first commercial product to use pullulan, and is currently one of the most used breath fresheners in the market [83].

Among the various polysaccharides, pullulan has attracted much attention for its processing versatility, which include nanoparticles [84], films [85], fibers [66] and hydrogels [60]. Pullulan-based nanoparticles are reported to have efficient antibacterial activities against a variety of pathogens. Indeed, Kanmani and Lim synthesized silver nanoparticles (AgNPs) using pullulan biopolymer as reducing and stabilizing agent and established its antibacterial, antifungal, and antibiofilm properties *in vitro* [86]. Additionally, pullulan scaffolds have been shown to protect the wound from infection [87]. Several pullulan-based dressings have been explored in the form of films and fibers, with recognizable transparency and uniformity, flexibility and excellent mechanical resilience [85]. The pullulan hydrogels, particularly those processed in the form of microgels and nanogels, have been reported as exceptional drug delivery systems [88]. This biopolymer has also been shown to accelerate fibroblast proliferation, collagen synthesis and maturation and epithelialization, thus playing an active role in promoting wound closure and shortening the healing period [60].

4.1. Derivatives: modification approaches

The pullulan molecule contains nine hydroxyl groups on its pyranose rings per maltotriose unit and can be chemically modified with different functional groups (changed structures and properties) to change many native properties of pullulan, including reducing or preventing its solubility in water. Depending on the solvents polarity, the reactivity of the pullulan groups can be significantly altered to generate a polymer that is either partially soluble or fully insoluble in water or to add pH sensitivity [89]. Pullulan's esterification or etherification tend to reduce its solubility in water. On the other hand, hydrogenation increases its thermal stability, and carboxylation increases its solubility in cold water [90].

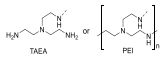
Several studies report the modification of pullulan (Table 2) with chemical reactions including amidification, co-polymerization, chlorination, etherification (alkyl esters [91], carboxymethylation [92] and cationization [93]), esterification (acetylation [94], succinylation [95], alkylation [96]), oxidation/periodate oxidation [97], sulfation [98], or the introduction/incorporation of groups such as chloroformate [99], cholesterol [100], isocyanates [101], PEG [102] or phosphate [103]. Cholesterol, hexadecanol, vitamin H or fatty acids are examples of molecules that can be linked to the pullulan structure to obtain micelles in aqueous solution, as well as used to stabilize fatty emulsions [104,105]. The production of pullulan derivatives also makes it possible to increase its crosslinking capacity, because the replacement of pullulan's hydroxyl groups with certain functional groups facilitates the binding of the polymer with other compounds that would not establish a link with native pullulan [106]. For example, pullulan is easily modified with aldehyde functional groups, increasing its cross-linker potential. This happens because aldehyde functional groups react easily with polymers containing amino groups through the Schiff's base reaction [107].

5. Pullulan-based hydrogel formulations: new combinations of polymers and structures

As described previously, pullulan is a polymer with numerous advantageous features, including its biodegradability, biocompatibility and hemocompatibility, and, when dissolved in water, it exhibits superior adhesiveness [125]. These endow pullulan with desirable properties for tissue engineering applications, namely in the form of 3D-structures, like hydrogels. Pullulan can be processed into hydrogels alone [26,60] or in combination with other water-soluble organic polymers [32,121]. Because of their properties, hydrogels made of pullulan can find uses in a wide range of areas, from water treatment to biomedicine (such as drug and/or gene delivery systems) [126–128]. Priya et al. showed that a 10 wt% pullulan hydrogel (hydrogel formed by dissolving 10 g of pullulan in 100 mL of distilled water) can display strong adhesive properties (≈14% more than commercially available cyanoacrylate tissue adhesives), allied to an active role in instigating wound (impact on collagen synthesis and maturation). Indeed the hydrogel was seen to increase wound breaking strength and shrinkage temperature [more than 25% when compared to control (untreated wounds)] and to exhibit superior tensile strength (3.63 MPa), all critical for an effective wound healing [60]. Bang et al. described the potential of hydrogels formed of pullulan and modified with tyramine in preventing the formation of postoperative peritoneal adhesions. A hydrogel with 30 wt% or greater concentration of pullulan has physical properties suitable for use as a post-operative anti-adherent agent. This polymeric concentration has therefore an ideal viscosity with the potential for secure coverage of the wound surface without flow down. Altogether, the results demonstrated that the hydrogel production parameters conditioned the final properties of the structure. In particular, the stiffness of the modified pullulan hydrogel seems to increase with the increased concentrations of HRP (enzyme used for crosslinking) [126]. In another study, a methacrylathed pullulan hydrogel produced from multiscale light assisted 3D printing showed enhanced cell viability against HEK293 and human mesenchymal stem cell (MSC) cultures. Cell adhesion properties were only verified in the presence/or after functionalization of fibronectin. Thus, with proper functionalization, these hydrogel structures can effectively serve as support substrates for cell growth [129].

Combination of pullulan with other polymers (natural or synthetic) has been shown to improve its properties (e.g., inflammation, mechanical strength, and degradation kinetics), and consequently broaden its areas of application [77]. Numerous natural polymers have been combined with pullulan for developing hydrogels, namely chondroitin sulfate [130], collagen [131], dextran [132] and gelatin [133]. In a study conducted by Chen et al., the authors produced an injectable hydrogel of carboxymethylated pullulan/chondroitin sulfate (both modified with tyramine) enzymatically crosslinked. At a morphological level, they observed that the combination of chondroitin sulfate caused alterations in the hydrogel structure, namely increasing pore diameter. Furthermore, a faster hydrogel degradation rate was associated with the electrostatic repulsion triggered by the negative charges of the chondroitin sulfate-tyramine. From a biological stand point, the hydrogel improved the deposition of cartilaginous ECM and promoted cell proliferation, thus evidencing a prominent role in the regeneration of cartilaginous tissue [130]. Similarly, Li et al. tested the combination of pullulan with the polysaccharide chondroitin sulfate, verifying that the mechanical properties, gelation time, swelling equilibrium, degradation behavior and network morphology were intrinsically dependent on the weight ratio and concentration of the polymers. Here, pullulan was oxidized and chondroitin sulfate was functionalized resorting to adipic dihydrazide. In this way, the authors avoided extra crosslinking agents for hydrogel formation and promoted self-crosslinking (reaction between hydrazide groups of chondroitin sulfate functionalized with adipic dihydrazide and aldehyde groups of oxidized pullulan) [134]. In another study, Baron et al. explored the addition of dopamine to the periodate-oxidized pullulan polymer, observing the production of a hydrogel with greater network stability, due to the interaction of dopamine with the aldehyde groups [97]. Alternatively, Iswariya et al. found that by adding piscine collagen to pullulan, it enhanced the hydrogel mechanical stability, thermal stability and biocompatibility. They also demonstrated that this polymer mixture accelerates wound repair, stimulating tissue granulation and subsequent regeneration and the formation of new blood vessels, highly beneficial in wound healing [135]. Wong et al., using the same polymer combination, developed a pullulan/collagen hydrogel (biomimetic scaffolding) with the ability to accelerate normal wound healing. It was observed that the hydrogel stimulated the formation of granulation tissue within 72 h post-treatment. In addition, this structure promoted the migration of

Table 2
Examples of the possible chemical modifications that pullulan can undergo.

Type of reaction		Substituted compound (Chemical structure represented at the end of the table*)	Characteristics	Potential Applications	Ref.
Etherification	Alkyl esters	R]R^1 = CO-CH_{n-2}-CH_3 (n = 2–14)	- Decomposition temperatures of pullulan esters were higher than the neat polymer; - Tg decreased with increasing DS values or with chain length (acyl carbon number).	Unspecified application	[91, 108]
	Carboxymethylation	R]H R^1]CH_2COONa or $CH_2CONH(CH_2)_3N(CH_3)_2$ or $CH_2CONHCH_2CH_2C_6H_4OH$ or $NH(CH(CH_3)CH_2O)_Y(CH2CH2O)_XCH_3$	- Both functions (anionic and cationic charges) present a similar DS of 0.35, which is characteristic of an ampholytic polymer with possible zwitterionic-type properties.	Drug delivery	[109, 110]
	Cationization	R]R^1 = H or 	- Pullulan-TAEA and pullulan-PEI showed a 6- and 37.8-fold growth in TS, a 5- and 13.3-fold augment in dynamic viscosity, and a 72- and 120-fold increase in muco-adhesion time compared to neat pullulan; - Reduced mass flux was obtained with pullulan-PEI which was 10.4-fold smaller than the unmodified polymer.	Mucosal drug delivery	[111]
Esterification	Acetylation	R]H R^1]$COCH_3$	- Pullulan-Acetate (pullulan-Ac) showed a higher decomposition temperature (306-363 °C, that increased with increasing DS) than original PU (295 °C), and exhibited a Tg which decreased with increasing DS in the range 1.0–2.4; - The pullulan-Ac with DS 3.0 formed a semi-clear gel in organic solvents such as DMSO, DMF, NMP and 1,4-dioxan. The DS was varied by altering the feed ratio of acetyl chloride to one glucose unit of pullulan.	Unspecified application	[112]
			- The size of the pullulan Ac nanoparticles increased from 185.7 nm to 423.0 nm with the degree of acetylation increasing from 2.71 to 3.0; - Drug entrapment increased with the increasing DS of Pullulan-Ac; - A superior cytotoxicity against KB cells (a human nasopharyngeal epidermal carcinoma cell line) was found for epirubicin-loaded pullulan-Ac nanoparticles in comparison with free epirubicin.	Chemotherapy	[113, 114]
			- All pullulan films showed lower TS and elongation at break with increasing DS compared to natural pullulan; - Pure pullulan films exhibited higher water vapor permeability (3.718×10^{-11} g/m s Pa) than the films prepared with pullulan esters, and PLBu (DS = 3), with the highest water contact angle (93.5°), achieved the highest oxygen (0.625×10^{-3} g/m^2 s) and water vapor barrier properties (0.792×10^{-11} g/m s Pa)	Packaging materials	[94]
	Succinylation	R]H R^1]$COCH_2CH_2COOH$ or *n*-octenyl succinic anhydride	- Pullulan-succinate (pullulan-Suc) was converted into the pullulan succinate sodium salt form (pullulan-Suc-Na); - The sorbent pullulan-Suc-Na showed that more than 94% and 90% Cd is removed in the first 15 min from distilled water and ground water solution, respectively; - The sodic form of the sorbent pullulan-Suc-Na showed high Cd-uptake (94%) due to an ion exchange mechanism, whereas the acidic form of the sorbent PU-Suc did not show significant Cd-uptake (13.2%); - The thermal stability testing indicated that pullulan-Suc-Na is more stable than pullulan-Suc.	Sorbent for cadmium-uptake from spiked high-hardness ground water	[115]
			- The pullulan-Suc films (yellow color) showed a significant diminution in TS (50%) and extensibility, as well as a minor rate of water vapor permeability (30%) than pullulan films; - A decrease in TS was observed with increasing DS. Therefore, pullulan-Suc can be a good substitute for an emulsion-based edible coating formulation.	Edible coating on fruits	[116]
			- The biocompatibility of the curcumin-hyaluronic acid grafted succinylated pullulan (Cur-HA-pullulan) polymer was confirmed by skin irritation, cytotoxicity, and hemolysis testing. It also exhibited bactericidal activity against *Escherichia coli* and *Staphylococcus aureus*;	Wound dressing	[117]

(*continued on next page*)

Table 2 (*continued*)

Type of reaction	Substituted compound (Chemical structure represented at the end of the table*)	Characteristics	Potential Applications	Ref.
		- Cur-HA-pullulan film displayed higher swelling ratio than the succinylated pullulan (pullulan-suc) film and put in evidence its potential for accelerating wound healing and assisting in infection control.		
Alkylation	R]R^1 = H or $CH_2CH(OH)CH_2N(CH_3)_3^+$	- Pullulan containing quaternary ammonium groups (AP) was produce by alkylation with glycidyltrimethylammonium chloride (GTMAC); - This derivative has the capability to condense with each other, forming nanoparticles capable of associate a model protein (BSA); - This nanoparticle showed adequate size for drug delivery applications and low *in vitro* cytotoxicity upon delivering the protein to a respiratory cell line; - BSA was successfully associated to a pullulan bearing quaternary ammonium group nanoparticles with DS of 2; - Great candidates to produce pullulan-based nano-carriers by polyelectrolyte complexation.	Nanocarriers produced by polyelectrolyte complexation	[98]
		- The alkylated pullulan was able to interact with miRNA and form stable polyplexes that were characterized regarding size, zeta potential and morphology; - Cationic pullulan did not show toxicity up to 72 h of incubation with increasing concentrations of QA-pullulan until 1000 μg/mL when compared to non-modified pullulan; - Negative zeta potential values of QA-pullulan/miRNA polyplexes (−12±5 mV) as compared to zeta potential of cationic pullulan solution (31±6 mV), suggested the presence of additional negative charges at the surface due to miRNA presence in the complex; - *In vitro* tests did not evidence any toxic effect of the QA-pullulan/miRNA complexes after 1 day of incubation up to a maximum tested dose of 200 μg/mL of polyplexes.	miRNA delivery nanosystems	[96]
Cholesteroyl group	R]H R^1 = cholesterol group	- Epirubicin-containing nanoparticles prepared by cholesterol-modified pullulan showed enhanced drug stability with a long half-life (2.8-fold longer than of epirubicin, 19.33 h), higher blood plasma concentration, and comparatively less drug toxicity; - The size of the nanoparticles increased when the epirubicin was loaded with cholesterol-modified pullulan. On the other hand, with mitoxantrone drug resulted in targeted drug delivery and enhanced stability, permeability, retention effect, efficacy, and reduced drug update by normal tissues; - The hydrophobically modified pullulan nanoparticles with mitoxantrone were prepared with average size of 166.9 nm; - The size particle and zeta potential of the particle were found out with 0, 3, 6, 9, 12, 15, and 18 h. From 0 to 12 h, the average particle size increased from 89.91 to 144.4 nm, from 12 to 18 h, the particle size remained stable (145 nm); - The zeta potential gradually changed from −15 (0 h) to −3 mV (18 h).	Drug delivery	[100, 118, 119]
Isocyanates compounds	R]H R^1]OCONH-R^2	- Introduction of N-phenyl or N-hexyl urethane groups in the pullulan structure, with good solubility and water resistance; - The addition of phenyl and hexyl isocyanate to pullulan increased its solubility in ethanol or in acetone and water, respectively; - The degree of addition of phenyl and hexyl isocyanate changed the properties of pullulan, leading to a decrease in the Tg temperature and TS of pullulan.	Unspecified application	[101]
Oxidation/Periodate oxidation	R]H R^1]CHO	- Hexamethylenediamine (HMDA)-pullulan with sodium alginate (SA) showed the light transmittance in visible region was 20% (8:2, v/v) and TS value of 34 MPa (2:8, v/v), the twice that of HMDA-OxPullulan film; - Diethylenetriamine (DETA)-pullulan with SA showed that the light transmittance in the visible	Antibacterial composite films	[120]

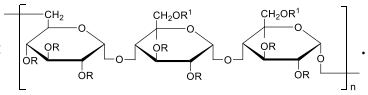
(*continued on next page*)

Table 2 (*continued*)

Type of reaction	Substituted compound (Chemical structure represented at the end of the table*)	Characteristics	Potential Applications	Ref.
		region was 11% (8:2, v/v) and the TS value was 31 MPa (2:8, v/v) 1.8 times greater than that of the DETA-OxPullulan film); - Antibacterial experiments showed that the composite film had an inhibitory effect on the growth of *E. coli* and *S. aureus* and exhibited an excellent film-forming ability and water vapor barrier;		
		- Periodate oxidized pullulan has the highest porosity (80.41%) followed by dialdehyde pullulan–dopamine cryogels (72.76% and 55.43%); - The network density is the greatest for the dialdehyde pullulan–dopamine cryogels (47.1%), followed by periodate oxidized pullulan (40.4%) that also exhibited the lowest adsorption efficiency, 31.41%; - The gel showed yield stress of ≈220 Pa and the value of the zero-shear viscosity was determined as 5144 Pa s.	Wound dressings	[97]
		- The resultant pullulan dialdehyde crosslinked gelatin hydrogels showed very high mechanical strength, manifested in the achieved optimal compressive stress of 5.80 MPa at 80% strain, which is up to 152 times higher than pure gelatin hydrogel; - The MTT assay demonstrated that these hydrogels were non-cytotoxic against the MC3T3 cells with high RGR values.	Macromolecular crosslinker	[121]
	R]H R^1]COOH	- The presence of some carboxyl groups and hydroxyl in the PVA/pullulan-ox hydrogels facilitated the water penetration into the porous network and conferred a very hydrophilic character to the hydrogels; - PVA/pullulan-ox hydrogels presented an enhanced swelling rate in the first 20 min, reaching equilibrium at the 30 min mark.	Wound dressing applications	[122, 123]
Phosphate	R]H R^1]PO_3H	- Pullulan-phosphorylated/β-tricalcium phosphate composites exhibited excellent mechanical properties, including shear bond strength of 13–16 MPa and compressive strength of 56–76 MPa; - The compressive force of the composite tested as high as ≈ 75 MPa. It was also deemed biocompatible and induced excellent bone formation, even in a load bearing vertebroplasty model.	Bone engineering	[103]
PEG incorporation	R]R^1 = H or PEG	- The DS (0.02–0.2) of the products increased with the amount of PEG added; - With increasing DS, the intensity of the ester carbonyl group (1733 cm^1) and the methylene group (2800–2950 cm^1) bands also increased; - Unlike pullulan that is soluble only in water and DMSO, the solubility of the PEG incorporated pullulan in organic solvents was improved, becoming slightly soluble in acetone or THF and soluble in MeOH.	Solubility in organic solvents	[102]
Sulfation agent	R]R^1 = H or SO_3Na	- Pullulan-sulfates were obtained with DS ranging from 0.17 to 1.99 and MW between 15 and 250 kDa; - Their activity not only improved with increasing DS and MW, but also with sulfate groups in positions 2, 3 and 4; - The anticoagulant activity of the new pullulan-sulfates changed not only quantitatively but also qualitatively according to their individual structure as reflected by the ratio between thrombin time to the activated partial thromboplastin.	Anticoagulant effect	[124]

Abbreviations: BSA – Bovine Serum Albumin; Cd – cadmium; DMF - dimethylformamide; DMSO – dimethyl sulfoxide; DS – degree of substitution; MeOH – methanol; miRNAs – microRNAs; MTT - 3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium bromide (MTT 3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium bromide; MW – molecular weight; Na – sodium; NMP - N-methyl-3-pyrrolidone; PEI – polyethyleneimine; PLBu – pullulan butylate; PVA – poly(vinyl alcohol); QA-Pullulan – quaternized ammonium-pullulan; RGR – relative growth rate; SA – sodium alginate; Suc – succinate; TAEA – tris(2-aminoethyl)amine; Tg – glass transition temperature; THF – tetrahydrofuran; TS – tensile strength.

*Chemical structure of pullulan and its substitution groups:



keratinocytes along the wound, contrary to what happened in untreated excisional wounds in which keratinocytes remained on the surface of the lesion [136]. The effectiveness of collagen/pullulan hydrogel (TWD) over two commercial dressings, Promogran™ (55% collagen and 45% oxidized regenerated cellulose) and Fibracol® Plus (90% collagen and 10% alginate) was described as having a role in wound area reduction and resolution. The engineered hydrogels were found to preserve the anatomical structures of the skin and to induce no rejection/response by the living tissues. After 14 days, the TWD hydrogel showed the ability to accelerate healing and to promote the formation of a scar tissue with an organized collagen fiber architecture (less dense, more randomly aligned and shorter). Data also demonstrated that the hydrogel significantly decreased the infiltration of macrophages, lymphocytes and the general tissue response, which consequently facilitated the normal healing cascade [131].

Hydrogels can also be produced with a unique set characteristics derived from the combination of pullulan with synthetic polymers, like PVA [32,122]. Pullulan/PVA composite hydrogels have been prepared through chemical (with sodium trimethaphosphate; STMP) and physical crosslinking (via freeze-thaw technique) with exceptional swelling capacities, good biocompatibility and excellent stability in water and phosphate buffer saline solution (PBS; pH = 7 at 4-37 °C) [32]. Bercea et al. also prepared a hydrogel from PVA and 7.5% oxidized pullulan (OxP), giving rise to a structure with high crystallinity (75%; compared to pure PVA hydrogel), high gel strength (verified by elastic modulus or loss tangent) and self-healing abilities [122]. They also demonstrated the potential for the pullulan/PVA hydrogel to release bovine serum albumin protein (BSA) and tripeptide reduced glutathione (GSH) in a controlled manner [137]. Kamoun et al. explored the properties of hydroxyethyl methacrylate-derivative pullulan (pullulan-HEMA) hydrogel crosslinked by a three-component photoinitiation system (carboxylated camphorquinone, folic acid, and an iodonium salt) under visible light. The obtained results demonstrated that the increase in the degree of substitution (DS) of the copolymer together with the increase of folic acid concentration improved the properties of the hydrogel, namely the crosslinking density, glass transition temperature (Tg) and degree of conversion. Even though a decrease in water absorption was observed, it did not compromise the hydrogel potential for biomedicine uses [138].

In many studies, crosslinkers are not employed [60]; yet, the use of a crosslinking agent may change the elastic properties of the polymer, reduce its viscosity, increase its thermal stability and improve its strength and toughness. The elevated water solubility of pullulan can be an issue for many applications, this way requiring crosslinkers for improving their stability overtime [139]. The amount of crosslinking agent used defines the properties of the hydrogel, namely its degree of dilation [140]. There is a wide variety of crosslinking methods available for the formation of hydrogels and to improve the stability of a pullulan-based hydrogel [26,32,79]. In regard to the chemical crosslinking options, STMP, diglycerols [141], $NaIO_4$ [77] and glutaraldehyde have been identified as very effective for treating the pullulan polysaccharide [32,142]. More recently, pullulan dialdehyde has also been regarded as an efficient hydrogel stabilizer [121]. STMP is a known non-toxic crosslinker, it is biocompatible and safe for human uses. However, the STMP crosslinking reaction requires alkaline conditions (pH $\geq$ 9.5), which may limit its use in some applications [26,143], and gives rise to a secondary byproduct, an inorganic pyrophosphate (PPi) that can be easily eliminated [144]. Although its mechanism of action is still not entirely clear, under alkaline conditions the STMP crosslinker reacts with: (1) pullulan, giving rise to a grafted sodium tripolyphosphate (STPPg; active species that leads to the formation of a crosslinked chain (Pc) or a grafted chain (Pg)); and (2) with NaOH giving rise to sodium tripolyphosphate (STPP; byproduct of the STMP degradation reaction in alkaline medium) [143]. On its turn, glutaraldehyde is a low molecular weight reagent that guarantees polymer structural stability and has the ability to change the physical properties of a hydrogel. Emam et al. resorted to glutaraldehyde as crosslinker for establishing a link between the –COOH groups of carboxymethyl pullulan (CMP) and the –OH groups of the polysaccharide pectin, thus allowing the formation of a hydrogel [142]. Nonetheless, it is dangerous or toxic to humans, limiting its use [121,145]. Enzymatic reactions (chemical crosslinking) can be used to produce pullulan hydrogels via eco-friendly approaches, safer towards human cells. Hadrich et al., through a coupling reaction mediated by carbodiimidazole, conjugated pullulan with ferulic acid (pullulan-FA) by means of the enzyme laccase (prepared in citrate-phosphate buffer; 0.1 M, pH = 5.5). This enzyme was used as a crosslinker since, through oxidation, it induces the biomimetic dimerization of the grafted portions of FA. This crosslinker has no effect on the final mechanical characteristics of the gels, acting only as a gelling catalyst [145]. Alternatively, the enzyme horseradish peroxidase (HRP) can also be used. This enzyme is known for its remarkable substrate specificity and efficiency, mild reaction conditions, programmable reaction rate and excellent cytocompatibility, being frequently used in biomedical applications [130]. Li et al. and Chen et al. used the HRP enzyme to produce a silk fibroin/pullulan (SF/pullulan) and carboxymethylated pullulan/chondroitin sulfate hydrogels, respectively. In both cases, the composite hydrogels were proven biocompatible and possessed adjustable mechanical properties [92,130]. Also noteworthy is the work conducted by Bae et al., in which cell-responsive three-dimensional (3D) tissues were grown from a pullulan methacrylate (PulMA)/gelatin methacrylate (GelMA) hydrogel. In this research, the replacement of the methacrylate groups by the hydroxyl groups of pullulan (forming PulMA) allowed the pullulan to be crosslinkable by ultraviolet light (UV) in the presence of a photoinitiator (2-hydroxy-1-(4-(hydrox-yethoxy) phenyl)-2-methyl-1-propanone) [146]. There are many studies where physical methods have been employed to produce pullulan hydrogels. Freeze/thawing method is a widely explored method with pullulan [122,147], in which the construct mechanical properties are dependent on the time and number of freezing and thawing cycles performed [7]. Regardless, this method can still be combined with chemical crosslinkers to improve the hydrogel stability, namely with the agent STMP [32].

In recent years, new research has emerged and demonstrated the biomedical potential of pullulan-based hydrogels as main or co-adjuvant therapies for different clinical contexts. Table 3 explores this further, highlighting possible polymer and crosslinker combinations, the incorporation of bioactive agents, and the main properties acquired from such conjugations.

Several structures (e.g., fibers and nanoparticles) can be incorporated into hydrogels to improve their mechanical and biological features [37,38,152]. Even though the incorporation of fiber- or nanoparticle-based structures into pullulan hydrogels is not yet as explored as the combination of pullulan with other polymers, there are already studies on this. Cutiongco et al. developed a composite scaffold by incorporating chitosan/alginate fibers into a pullulan/dextran hydrogel, with the aim of improving the functionality of the biologically compatible scaffold [153]. In turn, Su et al. successfully developed a pullulan hydrogel (PHG) with incorporated polydopamine microfibers (PDA). Here the hydrogel was prepared through chemical crosslinking using the agent poly (ethylene glycol) diglycidyl ehther (PEGDGE) and NaOH. Having varied the composition of the hydrogel in terms of PDA and NaOH content, characteristics such as mechanical performance, viscoelastic characteristic, mesh sizes and swelling/deswelling properties could be tuned. At a morphological level, for example, when adding PDA fibers to the PHG hydrogel, it changed from a compact and highly ordered structure to a rough and disorganized architecture, with increased average pore size. The authors also reported that water retention in the absence of fibers was greater, most likely because PHG with a compacted porous structure conditioned the release of water to the outside [125]. Amrita et al. developed a triple composite formed of a pullulan hydrogel reinforced with nano-crystalline hydroxyapatite (nHAp) and poly (3-hydroxybutyrate) (PHB) fibers (3 wt% fibers in

Table 3
Examples of pullulan-based hydrogel systems, respective crosslinking approaches employed and main properties acquired in light of the foreseen applications.

Polymers	Hydrogel composition	Crosslinking process	Properties	Application Perspective	Ref.
Only pullulan	Modified pullulan (tyramine grafted pullulan)	Enzymatic oxidative coupling reaction with HRP and H_2O_2	- Pullulan has been successfully modified with tyramine; - TEMPO and EDC reagents were used to introduce carboxyl and phenyl groups as crosslinking sites, in pullulan and tyramine, respectively; - The amount of H_2O_2 used in hydrogel production slightly affects cell viability; however, cell growth progresses gradually; - Pullulan hydrogel has good injectability properties, being easily released from the syringe, and binds easily to abdominal wound defects.	Prevention of postoperative tissue adhesion	[126]
	CMP	Chemical crosslinking with jeffamines (ED-600 and ED-2003)	- Successful production of a thermo-associative and pH-sensitive hydrogel with incorporated lutein (antioxidant), lysozyme and BSA (proteins); - Increasing the proportion of crosslinking agent decreases the hydrophilicity of hydrogels (ED-600 makes hydrogels more hydrophilic than ED-2003); - Samples with a lower degree of crosslinking are able to retain greater amounts of lutein (in part by hydrophobic forces); - Compared to lysozyme, smaller amounts of BSA are retained in hydrogels; - Less crosslinked hydrogels (with a higher amount of non-crosslinked crosslinker) retain higher amounts of BSA.	Drug delivery	[148]
	Pullulan	Chemical crosslinking with STMP	- Hydrogel is completely enzymatically degraded within 180 minutes of incubation with pullulanase (44 U/mL) at 37°C; - Crosslinked pullulan hydrogel did not show cytotoxicity; - Adhesion, dissemination and proliferation of smooth muscle cells promoted by the hydrogel were observed.	Smooth muscle cell culture	[149]
Combinations with natural-origin polymers	Pullulan/dextran (80/20 wt.%); Cationized pullulan/ pullulan/dextran (30/ 50/20% w/w/w)	Chemical crosslinking with phosphorus oxychloride under alkaline conditions	- Hydrogel was produced in the form of a tubular structure; - Dextran at a weight ratio of 20% enhanced the stiffness of the hydrogel; - Only the cationized pullulan tubes showed high affinity (capacity to retain) towards the plasmid DNA due to the amine content and amine type.	Implantable biomaterial for arterial gene therapy	[127]
	Pullulan/piscine collagen	Chemical crosslinking with STMP	- Swelling studies revealed the good water absorption properties of the hydrogel, with swelling rates of up to 320%; - A porous structure with interconnected pores was obtained; - Biocompatibility assays with NIH3T3 fibroblast cell lines confirmed the hydrogels biocompatibility and their ability to induce cell adhesion and proliferation; - In an excision wound model, treatment with the pullulan/collagen hydrogel promoted contraction and re-epithelialization of the wound in 11±2 days, triggering 96% of wound closure compared to the 49% observed in the control group.	Wound healing	[135]
	CMP-tyramine (CMP-TA)/chondroitin sulfate-TA	Enzymatic oxidative coupling reaction with HRP and H_2O_2	- Hydrogels with higher chondroitin sulfate-TA content show an increase in pore diameter; - Increasing CMP-TA content decreases fracture stress (the critical stress); - The hydrogel that provided the best host tissue mimetic of the microenvironment was the hydrogel with the highest amount of CMP-TA (CMP-TA/ chondroitin sulfate -TA weight ratio of 3/1); - Hydrogels at CMP-TA/chondroitin sulfate-TA weight ratios of 1/3 were completely degraded after 7 days, while hydrogels in the weight ratios of 0/1 were completely degraded within 14 days.	Cartilage tissue engineering	[130]
	Pullulan/collagen	Chemical crosslinking with STMP	- Average pore size was dependent on collagen content (average pore size 75.60 mm in the absence of collagen and 15.70 mm when the percentage of collagen was 5%); - Scaffold with lower porosity (68.8% ± 1.8%) were attained when 10% collagen was added; - The 5% collagen-pullulan hydrogel exhibited good water holding capacity and was flexible;	Wound healing	[136]

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Table 3 (*continued*)

Polymers	Hydrogel composition	Crosslinking process	Properties	Application Perspective	Ref.
			- Wounds treated with the engineered hydrogel showed greater wound closure compared to the untreated wounds.		
	Pullulan/CMC	Enzymatic oxidative coupling reaction with HRP and H_20_2	- Pullulan/CMC hydrogels with higher concentrations of HRP displayed a slower rate of degradation; - Hydrogels with high concentrations of H_2O_2 (3-7 μL/10 g) presented weight losses of about 60-50%. - The developed hydrogel was shown to decrease cell proliferation without cytotoxicity; - Blocked tissue adhesion and improved wound healing when applied CMC/pullulan hydrogel was confirmed by animal testing.	Anti-adhesive barrier for laparoscopy and normal surgeries	[150]
	Pullulan/dextran	Chemical crosslinking with STMP	- A higher dextran content increased the hydrogel swelling ratio (hydrogels formed of 100% pullulan presented the lowest swelling ratios); - Superior Young's modulus were observed in pullulan/dextran hydrogels compared to pullulan hydrogels; - There were no significant differences in degradation between the tested hydrogels; - Hydrogels with high pullulan content seemed to induce an increase in the inflammatory reaction (compared to 100% dextran hydrogels and 75% dextran/25% pullulan hydrogels); - Increasing the pullulan content in the hydrogel caused a thickness reduction of the fibrotic capsule (with values smaller than 300 μm) compared to hydrogels with more dextran (100% dextran reached values of 599 μm).	Implantable devices, drug delivery systems and tissue engineering applications	[132]
	Pullulan/gelatin	Solvent casting with particulate leaching and freeze-drying methodology	- Pullulan/gelatin hydrogel presented a mean pore size of 61.69 ± 2.76 μm, with a pore size range of 20 to 200 μm; - Degradation rates of 50% in 20 min were obtained with microbial pullulanase; - The pullulan-gelatin material's elastic modulus was of 22.43 ± 4.54 kPa; - Hydrogel swelling varied between 1758% (in fibroblast media) and 1812% (in PBS), depending on the medium in which they were incubated; - Effective incorporation of human fibroblasts and keratinocytes into pullulan/gelatin hydrogel (cellular hydrogel) was attained; - Macrophage infiltration was significantly reduced, while angiogenesis was enhanced in cellularized pullulan/gelatin hydrogel; - Excisional wounds treated with the cellularized pullulan/gelatin hydrogel showed a thicker skin formation (204.00 ± 19.65 μm; compared to acellular hydrogels, 115.63 ± 9.83 μm, and controls, 123.77 ± 21.37 μm), with a higher proportion of cells in the proliferative phase and incorporation of human cells compared to acellular hydrogels (hydrogel without cells) or the control group;	Skin regeneration	[133]
	Pullulan/HLC	Chemical crosslinking with $NaIO_4$	- Hydrogels composed only of pullulan (with different molecular weights, MW; 100, 170 and 530 g/mol) and pullulan/HLC (100, 170 and 530 g/mol of pullulan) were produced and tested; - Pullulan/HCL hydrogels exhibited lower swelling rates than 100% pullulan hydrogels (in both ddH_20 and PBS); - The elasticity modulus was directly proportional to the crosslinking density (higher in pullulan/HCL hydrogels (MW 170 and 530 g/mol)); - Pullulan hydrogels display pores with larger diameters than pullulan/HCL hydrogels; - With the decrease in pullulan MW, a linear increase in porosity was observed; - Pullulan hydrogels were degraded by the enzyme pullulanase and collagenase I after 30 and 50 h of contact, respectively. Pullulan/HCL hydrogels presented lower degradation rates, with no complete degradation; - Pullulan/HCL hydrogels were less cytotoxic than pullulan hydrogels.	Tissue engineering and *in vivo* applications	[77]

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Table 3 (*continued*)

Polymers	Hydrogel composition	Crosslinking process	Properties	Application Perspective	Ref.
	PulMA/GelMA	Radiation crosslinking with UV/visible light irradiation	- Increasing the degree of methacrylation of PulMA causes a significant increase in the modulus of compression (e.g., with a degree of methacrylation of 16.5% they obtained a compression modulus greater than 75 kPa); - Both the decrease in the degree of methacrylation (constant polymer concentration) and the presence of smaller amounts of PulMA polymer in the hydrogel led to an increase in the mass swelling ratio; - Excellent viability was demonstrated by cells encapsulated in PulMA;	Cell-responsive microtissues	[146]
	Pullulan/*Artemisia sphaerocephala Krasch.* polysaccharide (ASKP)	Chemical crosslinking with ferric ions	- The gel strength of the ASKP hydrogel was significantly improved by the incorporation of pullulan; - ASKP hydrogel gel strength was also higher in the presence of more Fe^{2+}; - Compared to the ASKP hydrogel, the ASKP/pullulan hydrogel displayed a lower swelling capacity (the network is more stable and denser, which limits the incorporation of water); - Tg increased with the addition of pullulan to the hydrogels; - Incorporation of pullulan into the ASKP hydrogel improved the structure compatibility.	New gelling material/ or structure for ferric ions delivery	[151]
Combinations between natural- and synthetic-origin polymers	PulMA/PEGDA	Printed by multiscale light assisted 3D printing technique	- Pullulan hydrogel was successfully functionalized with the glycoprotein fibronectin; - Adhesion of HEK293 cells was only observed in PulMA/PEGDA hydrogels with the addition of fibronectin; - A higher PEGDA content was found to lead to an increase in the elastic modulus and a decrease in the percentage of water absorption of the hydrogel.	Tissue engineering and regenerative medicine	[129]
	OxP/PVA	Physical crosslinking via freeze/thawing method	- OxP/PVA hydrogels with a porous network structure and pore sizes ranging from 14 μm to 36 μm (hydrogel pores increase with increasing oxidized pullulan content) were produced; - The amount of OxP played a significant role on the swelling behavior of the hydrogels, with the maximum swelling increasing from 620% (for the sample with 0.5% OxP) to 1020% for the hydrogels with 15% OxP; - The sample with 7.5% OxP showed optimal cytocompatibility. After 72 h of exposure, cell viability was maintained above 90%.	Tissue engineering	[122]
	PVA/pullulan/Poly-L-Lysine/gelatin (P/pullulan/L/G)	Physical crosslinking via freeze/thawing method	- The appearance of the hydrogels was transparent and clear in the absence of gelatin. Hydrogels with gelatin in their composition exhibited an opaque yellowish color and a rough surface; - Hydrogels maintained their integrity and did not degrade when incubated in water for 24 h; - The PVA hydrogel exhibited a water absorption of 91.7 mg/cm^2. Incorporation of pullulan reduced the hydrogel water absorption capacity (49.7 mg/cm^2). P/pullulan/L/G hydrogels presented the highest water absorption capacity (117.1 mg/cm^2); - The incorporation of pullulan into PVA caused a reduction of about 36.2% in the adsorption of BSA protein on the P/pullulan hydrogel. However, the incorporation of Poly-L-lysine and gelatin led to an increase in the absorption of BSA; - After 24 h, significant cell proliferation and cell migration were observed in the presence of P/pullulan/L, P/pullulan/G and P/pullulan/L/G hydrogels;	Wound healing	[147]
	OxP/PVA	Physical crosslinking via freeze/thawing method	- PVA/OxC hydrogels were successfully produced; - With increasing OxP content in PVA hydrogels, an increase in swelling was observed. However, PVA/OxC hydrogels presented superior swelling ratios than PVA/OxP; - At shear stresses of up to 30 Pa, a high hydrogel elasticity was observed; - The PVA/OxP hydrogels did not present cytotoxicity and maintained the metabolic activity of the cells over time. After 72 h, the 10% PVA/OxP	Wound healing	[123]

(*continued on next page*)

Table 3 (*continued*)

Polymers	Hydrogel composition	Crosslinking process	Properties	Application Perspective	Ref.
			hydrogel was the one that exhibited the best cytocompatibility (cell viability over 90%); - Low amounts of OxP (0.5%) are more easily dispersed in the PVA matrix for hydrogel production; - Increasing the OxP content in the PVA matrix caused an increase in the optical density and, consequently, a gradual decrease in the L-arginine load; - The addition of OxP to the PVA matrix allowed the hydrogel to sustain a better molecule release overtime compared to single PVA hydrogels; - The OxP (0.5%)-L-arginine sample (lower amount of OxP) was the one that promoted the longest release overtime (90.21 ± 0.15% over a period of 8 h).		

Abbreviations: BSA – bovine serum albumin; CMC – carboxymethyl cellulose; CMP – carboxymethyl pullulan; ddH_20 – deionized water; EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GelMA – gelatin methacrylate; HDE – 1–6-Hexanediol diglycidyl ether; HLC – human-like collagen; H_2O_2 – hydrogen peroxide; HRP – horseradish peroxidase; MW – molecular weight; OxC – oxidized cellulose; OxP – oxidized pullulan; PDA – polydopamine; PEGDA – poly(ethylene glycol) diacrylate; PulMA – pullulan methacrylate; PVA – poly(vinyl alcohol); STMP – sodium trimethaphosphate; TEMPO – 2,2,6,6- tetramethyl-1-piperidinyloxy.

hydrogel; fibers that physically mimicked collagen) containing nano-crystalline hydroxyapatite (nHAp; 5 wt% nHAp in hydrogel) for prospective applications in bone tissue engineering, namely bone graft substitutes. The addition of these structures to the hydrogel improved its mechanical properties (in some cases a 10-fold increase in the compressive modulus of pullulan scaffolds was evidenced) as well as allowed it to mimic the native hierarchical composite structure of the bone (due to the presence of the reinforcements). Additionally, it was also proved that the triple composite scaffold had an interconnected porous architecture, with porosity being around 68% and most of the pores being in the size range of 100–300 μm. The use of STMP as crosslinking agent also played an important role in the application of this composite in bone tissue engineering, considering the bonds formed would improve phosphate groups availability to the microenvironment, this way assisting with mineralization and turning the matrix more osteoinductive [154]. Saeaeh et al. took a different approach and tested the reinforcement of pullulan hydrogel with multi-walled carbon nanotubes (MWCNT). MWCNT/pullulan hydrogel composites were produced (crosslinking with STMP) and analyzed for their electromechanical properties and deflection responses under applied electric field. The MWCNT/pullulan hydrogel with a content of 0.01% v/v of MWCNT was the one that guaranteed the highest storage modulus sensitivity [(ΔG'/G′0) of 71.2 in the applied electric field intensity of 800 V/mm] and force density of dielectrophoresis [155].

Atila et al. explored the combination of a pullulan hydrogel (inner layer; STMP used as a crosslinking agent) with a bacterial cellulose membrane (outer layer; produced by the bacterial species *Glucanoacetobacter xylinum*) (BC/pullulan composite) as a platform for the release of vitamin C (VtC) and vitamin E (VtE). More specifically, after producing the hydrogel (with incorporated VtC), it was immobilized on BC membrane by crosslinking. Only then, the bilayer composite was loaded with VtE. Macroscopic images revealed that the composite had a semi-transparent appearance (the hydrogel had a transparent appearance and the membrane was yellowish). Data also showed that in the BC/pullulan membranes near 80% of the pores had dimensions between 20 and 100 μm. The difference in porosity observed in the two layers made the BC/pullulan composite permeable to microorganisms on the pullulan side and impenetrable on the BC side. The pullulan hydrogel (pullulan layer) assumed a large part of the thickness (3.36 ± 0.58 mm; approximately 92% of the weight of the bilayer membrane) and absorbed a larger amount of water compared to the pure BC layer. Considering the cumulative weight loss profiles of BC layer, pullulan layer and BC/pullulan structure in simulated wound fluid (SWF), the authors found that there was no major decomposition of the BC layer. In contrast, the pullulan layer lost >90% of its initial weight, which proves its high biodegradability. Mechanical tests on the individual layers were performed to evaluate if both layers had the necessary properties to be applied in wounds. The BC layer showed a resistance of about 22 MPa which confirmed its suitability for enduring the tensile stress associated to a wound surface. Nevertheless, the pullulan layer was shown to have a flexible structure with deformation values greater than 70%, which conferred the hydrogel adaptability to the wound bed under compressive stress without causing any damage [44]. Based on this study and considering the characteristics of the BC/pullulan-VtC/VtE structure, it can be concluded that the addition of a reinforcement (BC layer) to the pullulan hydrogel increased the potential of this structure for uses in wound dressing production. More recently, a magnetic pullulan-based hydrogel was engineered for antibiotic removal from wastewater. Here, polydopamine was used as a bridge between the magnetic Fe_3O_4 nanoparticles and the pullulan matrix, by increasing the stability of the hydrogel via hydrogen bond, hydrophobic and electrostatic interactions [12]. This study unequivocally demonstrated the high versatility of pullulan hydrogels and their importance not only for human-directed uses but also for biomedical-related secondary purposes, including environmental contamination by everyday use of pharmaceutical therapies.

6. Pullulan hydrogels as drug delivery systems

Drug delivery systems are developed with the purpose of improving and directing the therapeutic benefits of the drug, by protecting the payload, and regulating its release [25,58,125,156,157]. Hydrogels are regarded as attractive for drug delivery, considering their swelling behavior can be tuned, can reach high water contents, are easy handled and produced, are biodegradable and have enhanced porosity [25,158]. Furthermore, most hydrogels are highly biocompatible due to their high water content and physicochemical similarities with the native extracellular matrix [159]. In terms of porosity, the presence of pores in the hydrogel matrix facilitates the loading and release rate of large quantities of drugs [160]. In a porous structure, penetration and adhesion of cellular elements such as human dermal fibroblasts is facilitated (average diameter of 10–15 μm) throughout the entire hydrogel [133]. However, knowing that many drugs (49%) and therapeutic compounds (90%) have low solubility in water can condition the dispersion of these drugs in hydrogels (given its hydrophilic nature) and, consequently, influence their degradation profile. Regardless, there are ways to overcome this problematics, including raising the payload concentration or incorporating trigger-based nanocapsules containing the desired

therapeutic formulation [50].

Depending on the application (disease to be treated), hydrogel drug delivery systems can be applied via enteral/oral administration, locally implanted (parenterally) or topically positioned [161]. Drug release from hydrogels can occur through specific mechanisms: diffusion, degradation and swelling [138,162]. Diffusion is considered the most used mechanism to describe the release of drugs in hydrogels [160]. Here, the drug may be encapsulated and surrounded by a polymeric hydrogel (reservoir drug delivery systems) or may be dispersed in the polymeric matrix [162,163]. In both cases, drugs are incorporated non-covalently into the hydrogel matrix [164]. It is described that reservoir systems have constant release rates (time-independent), while matrix systems have release rates that tend to decrease over time [165]. Drug movement within a hydrogel may delay diffusion, which can also be condition by the interactions established with the system components. In general, when a drug is released from the interior of the hydrogel into the surrounding medium, there is a mass transfer from a highly concentrated region to an area of low drug content [137]. Both drug movement and release can be triggered by physical and chemical stimuli, such as pH, time, light, temperature and magnetic field [52,137]. The use of these variables allows the release of the drug to be stimulated at a given time, inducing a controlled distribution at a desired therapeutic concentration [52]. Additionally, knowing the potential chemical bonds generated between the drug and the polymeric chains can also assist in controlling drug release (Fig. 4) [3,166]. This is because it is possible to pre-engineer polymer chains using a variety of physical and chemical interactions to increase the thickness of the drug bond (e.g., through covalent conjugation) [159]. The hydrogel pore size/drug size ratio also influences drug diffusion. Diffusion can be free (drugs much smaller than the mesh size), slow (drugs of similar size to the pores), or it can occur only when there is hydrogel deformation (e.g., degradation, swelling or mechanical forces; drugs larger than the hydrogel pores). If a rapid initial burst release occurs, it is often necessary to modify the hydrogel structure to prevent drug local toxicity. This rapid release is often associated with small drugs and large meshes that generally cannot restrict the diffusion of small molecules from the hydrogels. In these situations, the effect of polymer-drug interactions can have a significant impact on the release kinetics [142,166–168]. On the other hand, the absorption/swelling properties are extremely important for defining the drug release profile of any molecule from any material. When the hydrogel network structure is opened by swelling, the drug can be released from the surface and internal pores in a controlled manner. This happens because the absorption of solvent molecules by the hydrogel network occurs [142].

Biodegradable hydrogels control drug release through their degradation profile (e.g., by hydrolytic or enzymatic cleavage). During the degradation/erosion process, there is a gradual loss of the structural integrity of the matrix (the polymer disintegrates), which triggers the slow release of the immobilized drug molecules. When the drug is chemically linked to the polymeric structure of the hydrogel, the rate of cleavage of the drug-polymer bond will determine the rate of release. Also, it is not necessary for the hydrogel to be removed after the entire payload has been released and the drug does not need to be soluble in water for its release to occur [164,169].

Drug release kinetics in hydrogels can be adjusted by means of chemical, molecular weight and morphology alterations in the hydrogel, through the size of the drug molecules (e.g., drug hydrodynamic diameter) and their solubility, and via the type of interactions established between hydrogel and drug (Fig. 4). For example, the relationship between the hydrodynamic diameter of the drug and the pore size of the hydrogel network influences the initial release of the drug [170]. Furthermore, factors such as ionic strength, presence/absence of enzymes and agitation (normally increases the diffusive release of drugs), are parameters that can be adjusted to obtain the desired outcomes [161]. To understand the drug release mechanism/profile, authors often times resort to mathematical models, like the Higuchi, Ritger–Peppas, Peppas–Sahlin, Hixson–Crowell and Korsmeyer–Peppas [142,167,168]. The release kinetic curves may present different phases. We can have zero order kinetics (constant amount of drug released over time), first order kinetics (constant percentage of drug load released over time; release rate depends on drug concentration) or a sudden first phase of release followed by a second, more stable release until reaching a plateau. The selection of the mathematical model to be used must be adjusted to the type of release in question. For example, the Higuchi model should not be used when release is due to swelling of the hydrogel. The most used model for drug release data treatment in hydrogels is the Peppas equation [161]. All these mathematical models are beneficial to better characterize and explain drug release mechanisms. Furthermore, they facilitate the development of drug carriers to meet the needs of a given application and allow the elucidate on of the transport processes that control the release kinetics of new formulations [166].

In the last decades, many studies have explored the potential of pullulan-based hydrogels for biomedicine and drug delivery (Table 4). Pullulan and its derivatives can act as efficient carriers in drug delivery systems, reducing the toxicity of drugs and improving their activity and stability [44,153,156]. Pullulan has in its chemical structure, in each repetitive unit, multiple –OH groups available for binding with bioactive agents [73]. Also, pullulan is capable of quenching free radicals, highly useful in cell delivery, for instance in skin wound therapies [13]. Natural

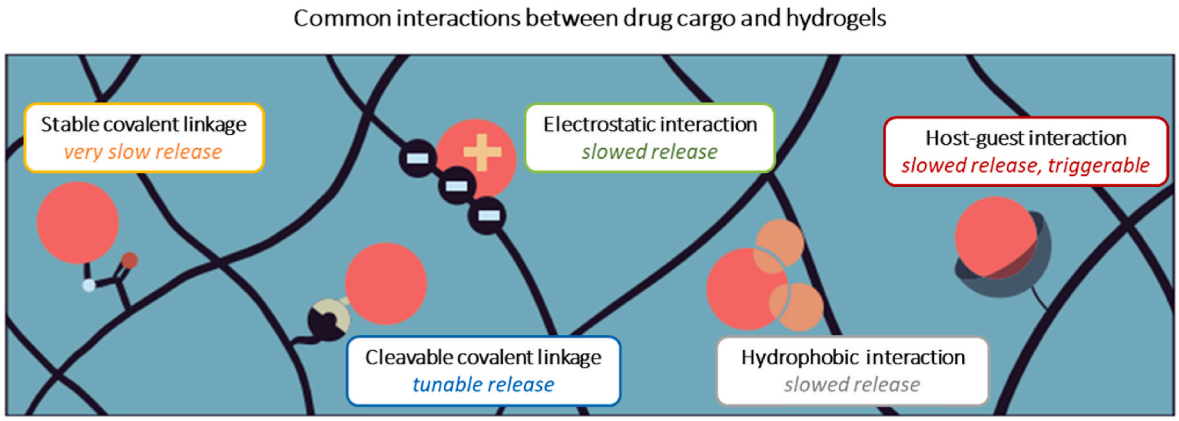

Fig. 4. Drug release is intrinsically related with the bonds established by the drug with the hydrogel. The hydrogel network is represented in blue and the drug in red. Adapted from Ref. [166] with CC BY 4.0 permission. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Pullulan hydrogels produced from different polymeric formulations, encasing bioactive agents for increased biomedical potential.

Composition of the system	Bioactive additives	Reinforcing agent properties	Main observed effects	Potential applications	Ref.
Pullulan/BC composite	Vitamin C (VtC) and E (VtE)	Antioxidant activity; VtC triggers fibroblast proliferation and collagen deposition; VtE is effective against the negative influence of ROS attack on cells.	- VtC (100 μg/mL) was incorporated into the pullulan hydrogel, whereas VtE (100 μg/mL) was incorporated into the composite (hydrogel and membrane); - The individual release and co-release profiles of the vitamins did not differ significantly; - Pullulan hydrogel (thickest layer of the composite) presented a high rate of degradation, which facilitated drug release; - The VtE release profile from pullulan/BC membranes may be associated with the relatively rapid degradation of the pullulan side membranes; - More than one release mechanism may be involved in the release of VtC: diffusion (related to its solubility in water) and fragmentation of the polymeric network; - In the co-release study, the results showed that about 60% of VtC and about 45% of VtE were released after 6 h (burst release phase). By the fourth day more than 80% of each vitamin had left the hydrogel; - In both release profiles (single and co-release), VtC and VtE were fully released from the structure after 14 days; - From the seventh day, the positive effect of the vitamins (loaded in pullulan/BC) on cell proliferation was more evident compared to the control (empty pullulan/BC); - There was a greater impact on cell viability when vitamins were used together.	Wound healing	[44]
Hydrogel pullulan nanoparticles	pBUDLacZ plasmid	Not mentioned.	- Glutaraldehyde was used to crosslink the nanoparticles; - The nanoparticles had a spherical shape with an average size of 45 ± 0.80 nm in diameter; - Pullulan nanoparticles were successfully endocytosed by cells; - At nanoparticle concentrations up to 1000 μg/mL, cells did not exhibit toxicity; - The effective internalization and *in vitro* transfection of these nanoparticles suggests that these pullulan structures are an efficient local delivery system for genetic material.	DNA delivery carrier	[178]
CHPOA/PEGSH	BMP2 and FGF18	Important role in many physiological processes: BMP2 intervenes in the commitment and differentiation of osteoprogenitors (precursors to the more specialized bone cells); FGF18 intervenes in osteogenesis and angiogenesis during skeletal development and wound healing.	- CHPOA/hydrogel was deemed an efficient delivery system capable of inducing bone formation; - The addition of FGF18 to the hydrogel promoted the same outcomes as the control, whereas the addition of BMP2 induced imperfect bone repair; - After 8 weeks, it was verified that there was an effective bone repair caused by CHPOAPEGSH/FGF-18 + BMP-2 hydrogel; - Disintegration of the BMP2-CHPA nanogels from the hydrogel triggered the release of BMP2 molecules; - Based on the gradual degradation of the CHPOA/hydrogel and the associated disintegration of the nanogel particles, the authors expected that the FGF18 and BMP2 proteins to be released at the same rate and that their effects would last for 3 weeks.	Bone tissue engineering	[179]
Pemulen® TR1/Pullulan hydrogel (HGPP)	Clotrimazole (loaded into cationic nanocapsules; CTZ-NC)	Antifungal activity (effective in inhibiting ergosterol, which consequently causes damage to the membrane structure of the fungus).	- The developed Pemulen® TR1/pullulan hydrogel contained cationic nanocapsules loaded with CTZ (HGPP-CTZ-NC); - The addition of pullulan to the hydrogel, in high concentrations, causes an increase in the percentage of CTZ adhered to the mucin gel; - After 8 h, higher drug release was achieved in HGPP3 (3% pullulan)-CTZ (released 70.63 ± 6.2 μg/cm^2) compared to HGPP3-CTZ-NC (drug incorporated in nanocapsules, which released 20.14 ± 2,33 μg/cm^2). A slower/	Treatment of vulvovaginal candidiasis	[168]

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Table 4 (*continued*)

Composition of the system	Bioactive additives	Reinforcing agent properties	Main observed effects	Potential applications	Ref.
			prolonged release of the drug when it was loaded into nanocapsules incorporated into the hydrogel was attained, which suggested that nanoencapsulation made the delivery system more controlled; - HGPP3-CTZ-NC potentiated the adhesive strength (values greater than 50%) in both layers used (mucin gel or animal mucosa).		
CMP-TA/SF hydrogel	Mesenchymal stem cells	Not mentioned.	- Injectable CMP-TA/SF hydrogel was manufactured by enzymatic crosslinking (HRP enzyme); - Cytocompatibility of injectable hydrogels was confirmed by encapsulating rabbit MSCs. After 7 days of culture, near 90% of the cells (encapsulated in hydrogel) were still alive; - Degradation assays with the enzyme protease XIV showed that the degradation rate decreases with increasing CMP-TA percentage.	Musculoskeletal tissue engineering	[92]
Plx-g-CMP hydrogel	AM	Used in the treatment of degenerative disc disease or other related disorders.	- The Plx-g-CMP hydrogel was synthesized at two different concentrations (13% and 22%, w/v) and modified with AM; - The release assays (in phosphate buffer at pH = 7.4) indicated that in the first 6 h about 41% of the drug was released from the hydrogel Plx-g-CMP 13% (w/v), and that in the hydrogel with concentration of 22% (w/v) half of the drug was lost; - After 48 h, the release profiles end up having a parallel release pattern, maintaining a higher drug release in the hydrogel Plx-g-CMP 13% (w/v); - At the end of 168 h (duration of the assays), none of the hydrogels released completely the drug (Plx-g-CMP 13% w/v with about 84% and Plx-g-CMP 22% w/v with near 58%); - The reduction in the rate of diffusion that accompanies the increase in the concentration of Plx-g-CMP confirmed that steric impediments (between the drug and the polymer network) control the rate of drug release; - This hydrogel guaranteed a sustained release of AM.	Injectable hydrogel for drug delivery	[167]
Hydroxyethyl methacrylate-derivative pullulan (pullulan-HEMA) hydrogel	Dexamethasone (drug model)	Not mentioned.	- The pullulan-HEMA hydrogel was produced by crosslinking under visible light irradiation (with carboxylated camphorquinone as photo-initiator, folic acid as co-initiator and diphenyliodonium tetrafluoroborate as accelerator); - Several hydrogels with different degrees of substitution were produced (DS = 0.025, 0.038, 0.065, 0.089); - An increase in the swelling rate of pullulan-HEMA hydrogels was observed with decreasing copolymer DS; - In the first 24 h, there was an initial burst release of drug (10–25% based on DS values of 0.089–0.025), which was associated with rapid and high hydrogel swelling and poor crosslinking density of hydrogels; - The rapid and cumulative total dexamethasone released increased gradually, particularly in the low DS copolymer. After 15 days, the cumulative value of drug release varied between 90% (DS = 0.025) and 40% (DS = 0.089); - Modifying the DS of the copolymer changed the *in vitro* release profile from fast release and high burst to a sustained rate (seen with higher DS values).	Drug carrier and tissue engineering	[138]
Pullulan hydrogel	MSCs	Antioxidant capacity.	- Using the salt-induced phase inversion method (and with STMP present), a pullulan-based hydrogel containing 5 wt% collagen was produced; - To realize the potential of pullulan hydrogels as cell delivery system, mouse MSCs were seeded onto the hydrogels *in vitro*;	Wound healing	[13]

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Table 4 (*continued*)

Composition of the system	Bioactive additives	Reinforcing agent properties	Main observed effects	Potential applications	Ref.
			- MSCs seeded inside the porous hydrogel demonstrated a rounded cell morphology and high viability (95% viable) for a period of up to two weeks, which suggested that the hydrogel is highly biocompatible with MSCs; - A reduction in intracellular generation of ROS was observed in MSCs seeded on the hydrogels; - The ability of pullulan hydrogels to deliver MSCs *in vivo* was tested using a subcutaneous implantation model. Hydrogel application and consequently MSC delivery significantly prolonged cell and engraftment survival for up to 30 days; - Results indicated that pullulan hydrogel can protect seeded MSCs from initial insults (e.g., acute inflammatory responses), which allows for increased cell viability.		
Pullulan grafted poly (acrylic acid-*co*-itaconic acid) (Pu-*g*-p (AA-*co*-IA)) hydrogel	Ampicillin sodium salt (model drug)	Broad-spectrum antibiotics with high solubility in aqueous solutions.	- Pu-g-p (AA-co-IA) hydrogels synthesized via UV crosslink graft copolymerization of acrylic acid (AA) and itaconic acid (IA) monomer in pullulan. BIS was used as a crosslinking agent and APS as an initiator; - Hydrogels with ampicillin sodium salt (0.1 wt %) incorporated (Pu-g-p (AA-co-IA)-D) were developed; - Swelling tests carried out under different pH conditions, to simulate the wound exudate (pH = 5.2), the skin layer (pH = 7.4) and to understand the impact of acidic environments, showed greater degrees of swelling at the two highest pH. Pu-g-p (AA-co-IA)- D hydrogels displayed a greater degree of swelling than the Pu-g-p (AA-co-IA) hydrogels; - The results showed that under conditions that simulate the wound environment (pH 5.2 and temperature 37 °C), after 4 days of incubation, the cumulative release rate of ampicillin sodium salt was 5.36 mg of drug/hydrogel; - In the first 7 h, there was an ampicillin sodium salt explosion release (about 42%). In the following 168 h the release rate was around 67%; - In the Pu-g-p (AA-co-IA)-D hydrogel, drug release occurs essentially by diffusion; - Pu-g-p (AA-co-IA)-D hydrogels showed the highest cell viabilities (mouse fibroblast cell line L929; greater than 120%).	Wound healing	[180]

Abbreviations: AA – acrylic acid; AM – amoxicillin; APS – ammonium persulfate; BC – bacterial cellulose; BIS – N,N′-methylene bisacrylamide; BMP2 – bone morphogenetic protein 2; CHPOA – acryloyl group-bearing cholesterol-modified pullulan; CMP – carboxymethyl pullulan; DS – degree of substitution; FGF18 – factor 18; IA – itaconic acid; MSCs – mesenchymal stem cell; PEGSH – Polyoxy-ethylene; Plx-g-CMP – Poloxamer-graft-carboxymethyl pullulan; SF –silk fibroin; STMP – sodium trimethaphosphate; TA – tyramine.

extracts such as curcumin [171] and *Rhus verniciflua* extract [11], and many cell and protein elements like mesenchymal stem cells [13,92] and BSA [137,153] have been successfully incorporated within pullulan hydrogels. For instance, Cutiongco et al. produced a pullulan/dextran hydrogel composite with chitosan/alginate fibers (produced by interfacial polyelectrolyte complexation; IPC) incorporated (PD-IPC) and studied the bioactivity and release kinetics of BSA and vascular endothelial growth factor (VEGF). Incorporating BSA into IPC fibers alone was conducive to a faster release of the payload than from within the composite. In the first situation, there was an initial high burst release of BSA that stabilized after 1 day, reaching a plateau in just 20 days (with a release greater than 90% after 2 months). Yet, when BSA was incorporated into the composite, an attenuated burst release was observed followed by a constant/sustained release profile over 2 months (total release greater than 90%). In terms of VEGF release, the incorporation of different types of VEGF (VEGF 1 to 3; heparin was added in combination with the growth factors to bind, stabilize and increase VEGF activity) in the PD-IPC composite was compared, showing that VEGF2 and VEGF3 were released similarly, reaching approximately 5% after 8 days. The release of VEGF1 (smaller amount of heparin) was more limited, with only a release of about 3% being observed in 8 days. Data demonstrated that in long term applications, a constant therapeutic delivery could be guaranteed regardless of the typology of VEGF and that release could be sustained for the two loaded biomolecules (VEGF and BSA) [153]. Su et al. developed a pH-responsive drug delivery system (hydrogel based on pullulan and polydopamine fibers; PHG-PDA) loaded with crystal violet (prototype drug; CV) by swelling diffusion, with its release profile being studied in PBS (pH = 7.4 and 5.0). The cumulative release rate of CV from the PHG-PDA_3 (composite with higher amount of PDA fibers dispersion) was higher at lower pH (at pH 7.4 it was of 60.3% and at pH 5.0 it was 87.0%). Under acidic conditions, the hydroxyl and amine phenolic groups in PDA are protonated, which can increase the electrostatic repulsive force between CV (positively charged) and the protonated PDA. In this way, the environmental pH can effectively influence the drug release behavior [125].

Recently, Bercea et al. developed a hybrid hydrogel composed of

polymers (PVA/pullulan) and proteins (BSA or lysozyme)/peptide (reduced glutathione; GSH) loaded with neomycin for prospective wound dressing and/or drug delivery uses. Neomycin is an aminoglycoside antibiotic, soluble in water, with good bactericidal and bacteriostatic action, very effective against skin and mucous infections caused by Gram-negative organisms. Hydrogels with different percentages of BSA (0–90 wt%) were produced. The results demonstrated the stability of the engineered hydrogel, its porosity (average size between 15.7 and 24.5 μm) and interconnected structure. Pore dimensions were not affected by protein content; however, the increase in BSA content altered the hydrogel morphology. Presence of BSA was seen to facilitate the delivery of neomycin, with hydrogels containing 10% and 30% of BSA, as well as the hydrogel with 2% lysozyme (0% BSA) achieving the greatest release kinetics. However, at higher concentrations than 2%, lysozyme caused phase separation of the polymeric matrix. Samples without BSA guaranteed fast drug release during the first 4 h of incubation; however, after that time-period release was slower and lower than on the other hydrogel systems, because of the greater density of molecular interactions formed in the hydrogel that ended up conditioning neomycin release [137].

Natural extracts have been recurrently used for the treatment of various pathologies and injuries [172–174]. The extract of *Rhus verniciflua* (RVE) is described has having beneficial effects on blood homeostasis, aside from presenting good antibacterial activity, anti-inflammatory, anti-allergic, antitumor and neuroprotective effects. A topical film composed of a pullulan hydrogel loaded with RVE (RVE@PH) was proposed by Jeong et al. for the treatment of atopic dermatitis (AD). According to the *in vitro* release tests, about 64.94 ± 2.79% of the total amount of RVE was released from the RVE@PH film within 6 h time. After 12 h, the plateau was reached, with near 86.98 ± 0.53% of RVE being already lost. Drug release was stabilized within 24–48 h. It was seen that the RVE-containing film reduced epidermal thickness and mast cell infiltration, hence confirming the RVE therapeutic efficacy against AD which was boost by the physical actions (e.g., barrier against excessive scratching or anti-evaporation) of the pullulan hydrogel [11]. Curcumin is a natural extract with enhanced antioxidant, antibacterial and anti-inflammatory properties that can be highly beneficial in wound healing processes. However, its low bioavailability and poor water solubility condition its action/application and, therefore, its incorporation into drug delivery structures [171,175,176]. Ahmed et al. developed an injectable hydrogel of hyaluronic acid-pullulan-grafted-pluronic F127 to be used as a sustained and target delivery platform for curcumin. Before producing the hydrogel, the authors incorporated curcumin dissolved in methanol (10 mg/mL) into the polymeric solution. This work aimed at stimulating the regeneration of the skin in diabetic wounds. Biological tests showed that the presence of curcumin in the hydrogel increased cell proliferation, inhibited inflammatory cells presence, and consequently enhanced wound closure. More specifically, a wound recovery rate of 13 days was observed when the wounds were treated with a hydrogel loaded with curcumin, whereas in the control group recovery was only achieved after 35 days. Curcumin release rate was instigated in the first 8 h, releasing 50% of the hydrogel content. Yet, after this point, curcumin liberation was a sustained for up to 24 h. These studies also showed that curcumin release was improved when the amount of hyaluronic acid was superior (from 3.0 to 5.0% w/w; greater hydrophilic substitution enables greater interaction with water molecules). On the other hand, an increase in pullulan concentration from 2.0 to 4.0% w/w caused the opposite effect and decreased the percentage of released curcumin. Furthermore, due to the porous and permeable nature and easy erosion of PF127 in contact with water, increasing its content in the hydrogel (between 20 and 24%) triggered the release of curcumin. Regardless of the initial curcumin release kinetics observed, the authors considered that the hydrogel could sustain curcumin release overtime. In general, by adjusting the composition of the hydrogel and the gelling time, for example, it was possible to control the explosive release of the drug, and thus tune the biological effects of curcumin [171].

Li et al. produced a hydrogel based on pullulan (carboxymethylpullulan; CMP) and cystamine with incorporated gentamicin by chemical crosslinking. In this work, different percentages of crosslinking (30–60%; 1-(3-dimethylaminopropyl)-3-ethyl- carbodiimide hydrochloride as coupling agent) in the hydrogel were tested. In the first 2 h, there was a rapid release of the drug (one third of the incorporated drug), as hydrogel swelling occurred. In the following hours (up to almost 40 h in total), a gradual release was observed. The test lasted 72 h, and at the end, the hydrogel with the highest total release was the one subjected to the lowest degree of crosslinking (90% of the loaded drug released). On the other hand, the hydrogel that experienced the most crosslinking (60% degree of crosslinking), because of restriction in drug movement, was the one that released the least amount of drug (about 60%). Data also indicated that gentamicin sulfate maintained its bioactivity, including exceptional antimicrobial properties, overtime regardless of crosslinking degree [177].

Many therapies have been used to treat surface skin infections. Polyvinylpyrrolidone (povidone)-bound iodine (PI), an antiseptic reagent, considered an active agent against several pathogenic strains (e.g., gram-negative and gram-positive bacteria, fungi, protozoa, and several viruses) has been shown effective against skin infections. Emam et al. developed a Pectin@carboxymethyl pullulan (Pe@CMP) hydrogel modified with PI (PI@Pe@CMP). This hydrogel showed improved solution absorption capacities compared to single polymer gels, with swelling rates of 16.0–18.0% in 24 h. In terms of the PI release profiles, it was verified that the PI release in the CMP hydrogel was fast (complete release after 180 min). On the other hand, the increase in pectin content in the CMP hydrogel controlled drug release. Since the Pe@CMP hydrogel (20% pectin) showed a slower/controlled release of PI (about 57.7% was released in 360 min), not reaching total release under the tested conditions, the authors suggested the Pe@CMP hydrogel (20% pectin) to possess a controlled release profile that allows its application as a controlled release system for the treatment of skin infections (Fig. 5) [142].

7. Final remarks and future perspectives

In recent years, an increased number of studies has been carried out on biomaterials for application in areas such as biomedicine, tissue engineering and drug delivery. Considering the final application and the desired properties, the adequate selection of polymers to produce different structures (e.g., fibers, hydrogels and nanoparticles) is extremely important. Due to their biocompatibility, biodegradability, and low environmental impact, natural polymers are regarded as superior materials for biomedical applications. Explicitly, natural-origin polymeric hydrogels have been highlighted for their unique properties, namely their biodegradability and biocompatibility. In addition, their biological and mechanical characteristics can be easily modified using different techniques or combinations of materials, not only applied in the matrix but as well as reinforcing agents. The pullulan polymer is a powerful candidate to be used in the production of hydrogels for application in biomedicine given its properties of high biocompatibility, non-toxicity, non-mutagenicity, non-immunogenicity and biodegradability. Pullulan hydrogels can be combined with other polymers or with other structures for improving their properties, namely structural stability. Since pullulan hydrogels have an excellent ability to incorporate/encapsulate biomolecules and cells, several combinations have been tested for engineering drug delivery platforms. Most incorporated drugs are capable of maintaining their bioactivity and an easily adjusted release profile when loaded onto pullulan-based hydrogels. Additionally, the hydrogel itself, aside from its versatility, has a beneficial effect on various stages of the skin healing process, which makes it extremely attractive for wound treatments. Despite the beneficial properties of pullulan hydrogels, poor mechanical properties and the associated high cost and availability of the raw polymer limit its use. Although

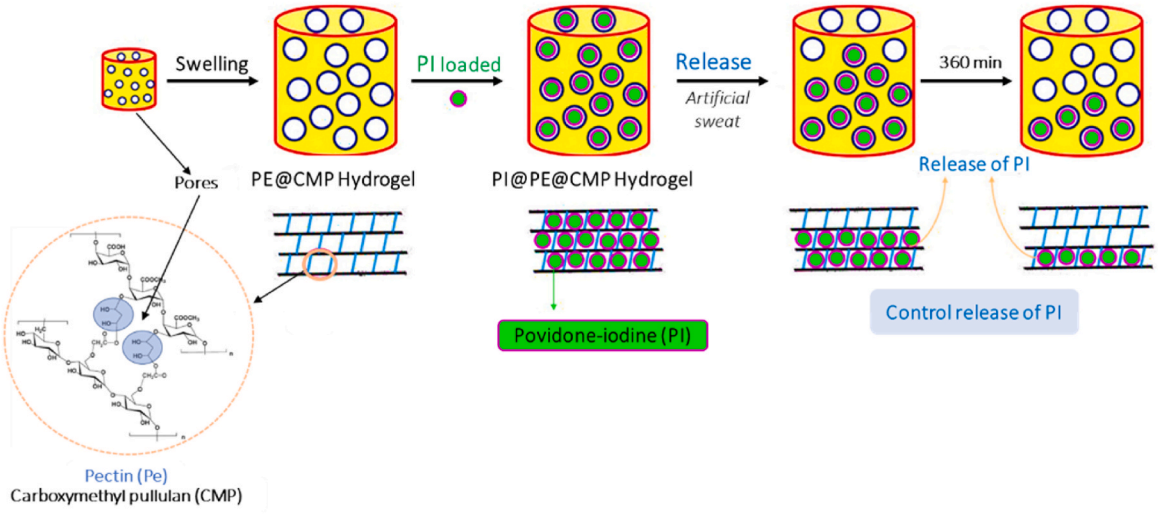

Fig. 5. Schematic representation of the release mechanism of PI from Pe@CMP hydrogel (with 20% pectin). Adapted from Ref. [142] with CC BY 4.0 permission.

investigations in this field have increased, it remains essential to continue exploring new approaches to overcome the limitations of pullulan and expand their application to other fields of biomedicine. Specifically, new formulations that result from the combination of pullulan with other polymers/structures and the production of pullulan derivatives with adequate properties must be studied. Furthermore, to improve therapeutic efficacy and expand the use of pullulan hydrogels in the biomedical field, it is also crucial to investigate new therapeutic and bioactive chemicals for integration into pullulan hydrogels.

Author statement

Conceptualization: M.O.T. and H.P.F.; Funding acquisition – H.P.F.; Supervision; Validation – C.S., J.C.A. and H.P.F.; Roles/Writing, original draft – M.O.T. and E.M.; and Writing, review & editing – C.S., J.C.A. and H.P.F.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

Authors acknowledge the Portuguese Foundation for Science and Technology (FCT), FEDER funds by means of Portugal 2020 Competitive Factors Operational Program (POCI) and the Portuguese Government (OE) for funding the project PEPTEX with reference PTDC/CTMTEX/28074/2017 (POCI-01-0145-FEDER-028074) and 2C2T Strategic project UIDP/00264/2020. M.O.T. and H.P.F. also acknowledge FCT for funding PhD scholarship with reference 2021.06906.BD and auxiliary researcher contract via 2021.02720. CEEIND, respectively.

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