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Review

In situ forming hydrogels based on chitosan for drug delivery and tissue regeneration

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ABSTRACT

In situ forming hydrogels with simple sol–gel transition are more practicable as injectable hydrogels for drug delivery and tissue regeneration. State-of-the-art in situ gelling systems can easily and efficiently be formed by different mechanisms in situ. Chitosan is a kind of natural polysaccharide that is widely exploited for biomedical applications due to its good biocompatibility, low immunogenicity and specific biological activities. Chitosan-based in situ gelling systems have already gained much attention as smart biomaterials in the development of several biomedical applications, such as for drug delivery systems and regeneration medicine. Herein, we review the typical in situ gelling systems based on chitosan and mechanisms involved in hydrogel forming, and report advances of chitosan-based in situ gels for the applications in drug delivery and tissue regeneration. Finally, development prospects of in situ forming hydrogels based on chitosan are also discussed in brief.

1. Introduction

Hydrogels are the polymeric materials with three dimensional networks, which have gained much attention in biomedical fields as carriers for drugs, protein, cells, and others because of their good biocompatibility, solute permeability and tunable release characteristics [1]. The retaining ability of a large amount of water within their structures which results in high water content and soft-surface properties is the character that makes them compromised on the surrounding tissues and leads to a good biocompatibility. Since the development of hydrogels in 1960s, numerous studies on adapting hydrogels as biomaterials have been reported. Especially, the in situ forming hydrogels which usually show sol-to-gel transition at the in-situ site where they are administrated into the body, exhibit promising potentials for clinic applications. It is more practicable to apply in-situ forming hydrogels to tropical drug delivery, injectable implant, tissue engineering scaffold and so on [2–5]. The drug/cell can be mixed with the aqueous sol for convenient administration

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like injection and then a gel depot encapsulating drug/cells is formed in situ. In situ gelling systems could potentially alleviate several drawbacks associated with contemporary regenerative medicine approaches and scaffolds. Primarily, they minimize the invasiveness of the open surgical technique and can conform to complex 3D geometries, which is critical in implant drug delivery system, repair of trauma, and regeneration post-tumor resection. More importantly, this allows for delivery of cells and growth factors locally, which could potentially lead to faster and complete regeneration [6].

Chitosan, the second most abundant natural polysaccharides next to cellulose, has many advantages over other polymers, like nontoxicity, biocompability and biodegradability. Chitosan is a family of cationic polysaccharides with a basic chemical structure of (1,4)-linked 2-amino-2-deoxy-D-glucans, which are produced commercially by the partial deacetylation of chitin obtained from the reprocessing of seafood waste. Members of the chitosan family differ in terms of their molecular weight and degree of deacetylation. Chitosan is biodegradable, as it is broken down in the human system to harmless products (amino sugars) that can be easily absorbed. Nowadays, chitosan and its derivates have been investigated for many diverse medical applications, such as wound dressings, contact lenses, and materials for cell encapsulation, drug delivery and so on [7–9]. Additionally, chitosan has several active functional groups that allow for protein binding and an inherent positive charge that is known to stimulate cell interactions and differentiation.

By far, chitosan-based hydrogels have proven to be very efficient for the delivery of biologically active molecules like insulin, growth factors, and for providing organization of cells and tissues, due to the possibility to create multilayered system [10]. There are also many reports about chitosan-based in situ hydrogels that can be delivered in minimally invasive techniques such as injection, ocular or nasal administration while protecting drugs or cells from the hostile environment. In this paper, several typical in situ gelling systems based on chitosan are reviewed together with their biomedical applications in drug delivery and tissue regeneration.

2. Mechanisms to generate in situ hydrogels

A gel is defined as a three-dimensional network swollen by a solvent. Hydrogels are hydrophilic polymeric networks able to absorb and retain high quantities of water while retaining its shape. Based on how the network is connected, hydrogels can be classified into two categories: the chemical hydrogels where the hydrophilic polymer chains are associated together by covalent bonds, and the physical hydrogels by secondary forces, such as hydrogen bonds, ionic bonds, intermolecular hydrophobic association and so on. Mechanisms involved in in situ gel formation may include the following: gelation in response to temperature or pH change, ionic or covalent cross-linking, solvent exchange or crystallization, or simply thickening upon removal of the injection shear.

Among them, thermoresponsive gelling polymers [11,12] are included with unique characters, which usually exhibit phase change behaviors of sol-to-gel or gel-to-sol transition upon an increase in temperature, and have gained enormous attention to form in situ gels with promising biomedical applications. Commercially available block copolymers of poly(ethylene oxide-b-propylene oxide-b-ethylene oxide) (PEO-PPO-PEO) are the best-known examples of thermally gelling polymers. Aqueous solutions of PEO-PPO-PEO copolymers demonstrate phase transitions from sol to gel (low temperature sol-gel boundary) and gel to sol(high temperature gel-sol boundary) with monotonically increasing temperature when the polymer concentration is above a critical value. Moreover, biodegradable copolymers of polyethylene glycol and poly(lactic/glycolic acid) such as PLGA-PEG-PLGA, PEG-PLGA-PEG, mPEG-b-PCL and so on, were recently developed also with thermal-responsive sol-to-gel transition [13–16]. Aqueous solutions of these copolymers form soft gels at body temperature (37 °C) but flow freely at room temperature. Therefore, subcutaneous injection of the copolymer formulation resulted in an in situ-forming gel that exhibited controlled drug release. Formulations are water based, easy to sterilize by simple filtration of the aqueous solution. Ideally, the duration of the gel should be matched with its function as a temporary tissue scaffold or a drug release depot. By optimizing the composition of copolymers, injectable scaffolds with a broad range of gel degradation time can be designed.

Besides the classic systems mentioned above, enzymatically cross-linked hydrogels [17] have emerged recently as in-situ gelling system with increasing interest, which can be formed by enzyme-catalyzed mild-cross-linking reactions in situ. Hydrogels prepared by using enzyme systems like tyrosinases, transferases and lysyl oxidases show interesting characteristics as dynamic scaffolds and as systems for controlled release. Additionally, unwanted side effects can be avoided because of the substrate specificity of the enzyme. In situ cross-linkable gels are based on aqueous mixtures of gel precursors with bioactive agents that can be administrated via a syringe. Moreover, injectable enzymatically cross-linked hydrogels offer a plausible solution for the generation of functional tissue substitutes of these gels and native tissue, therefore maintaining the cell phenotype, which is highly relevant for tissues like cartilage. Importantly, the poor or limited mechanical properties of some hydrogels can be improved by combining enzyme types such as transglutaminases and horseradish peroxidases systems, after adjusting the material design. For example, transglutaminases are highly interesting as they offer intimate integration between the in situ formed gel and the native host tissue. Engineered peroxidases with higher stability and catalytic efficiency are currently being developed. In the near future, further applications using this enzyme type will be developed and continue to prosper in the tissue engineering field.

3. Typical in situ gelling systems based on chitosan

According to the mechanism of gel formation, chitosan-based in-situ gelling systems can be classified into two categories: in-situ covalent cross-linking system and in-situ phase separation system.

3.1. *In-situ covalent cross-linking system*

In the chemical cross-linking approach, the formation of covalent bonds between polymer chains results in gel matrix of three-dimensional network. There are plentiful of amino groups (-NH_2) and hydroxyl groups (-OH) along the chitosan chain, which can be used as cross-linkable functional groups to react with cross-linking agents for in-situ chemical cross-linking. Genipin is a water-soluble bifunctional cross-linking reagent derived from gardenia fruit extract (geniposide), the most popular non-toxic cross-linker to produce chitosan hydrogels. A series of in situ forming chitosan-based hydrogels have been prepared by chemical cross-linking of chitosan and genipin without/with the cooperation of ionic interaction between chitosan and sodium salts, like sodium orthophosphate hydrate (Na_3PO_4), sodium sulfate (Na_2SO_4), or sodium bicarbonate ($NaHCO_3$), etc. [18–20]. The gelation time, rheological properties and morphology of gelling systems varied with the genipin concentration, the pH condition as well as the different salts. For example, the hydrogel composed of chitosan, genipin and Na_3PO_4 showed short gelation time of 8 min and non-toxicity under physiological conditions [18]. It is noted that the cross-linking reaction mechanisms for chitosan with genipin are different at different pH values [20]. Under acidic and neutral conditions, genipin acts as a similar function as dialdehyde undergoing a Schiff reaction with the amino groups on chitosan and yielding the two newly chemical groups: the monosubstituted amide and the tertiary amine, as shown in Fig. 1. Under basic conditions, genipin formed first homopolymerized ones to be a cross-linker. Though the mixture of chitosan and genipin is almost transparent, they produce blue-colored fluorescent hydrogels after completing sol–gel transition. It is attractive that genipin is a fully biocompatible reagent, capable of meeting the scopes of the current biomedical applications based on chitosan in-situ forming hydrogels. Genipin was also added in some physical in-situ gelling system (like chitosan/GP system) to improve the mechanical properties and chemical stability of hydrogel due to the presence of covalent bonds between the chitosan and genipin [21].

Moreover, in-situ covalent cross-linking hydrogels may also be achieved without the additional cross-linkers by attaching cross-linkable functional groups to chitosan and another polymer. This approach has been explored by many researchers as summarized in Table 1. For example, Schiff's reaction between the amino and aldehyde groups is introduced in different systems to form pH-sensitive in situ forming hydrogel for protein delivery or as wound dressing materials [22–24]. The newly emerged click reactions, like thiol-ene reaction [26,27] and cyclo-addition [28], are also involved to form chitosan-based hydrogels due to their high efficiency in mild reaction conditions. Fabrication of the injectable hydrogels can be modulated from within a minute to hours by controlling the temperature and pHs of the precursor solution. These hydrogels prepared via click reaction exhibit controlled architectures and improved mechanical properties for soft tissue engineering applications.

Some covalent cross-linking approaches mentioned above require several hours for gelation. Such slow processes may impact the functionality of the hydrogel and result in unexpected drug release to surrounding tissues. On the other hand, fast gelation may have difficulty for the mixing and injecting operations. Thus, photo-induced cross-linking has emerged to

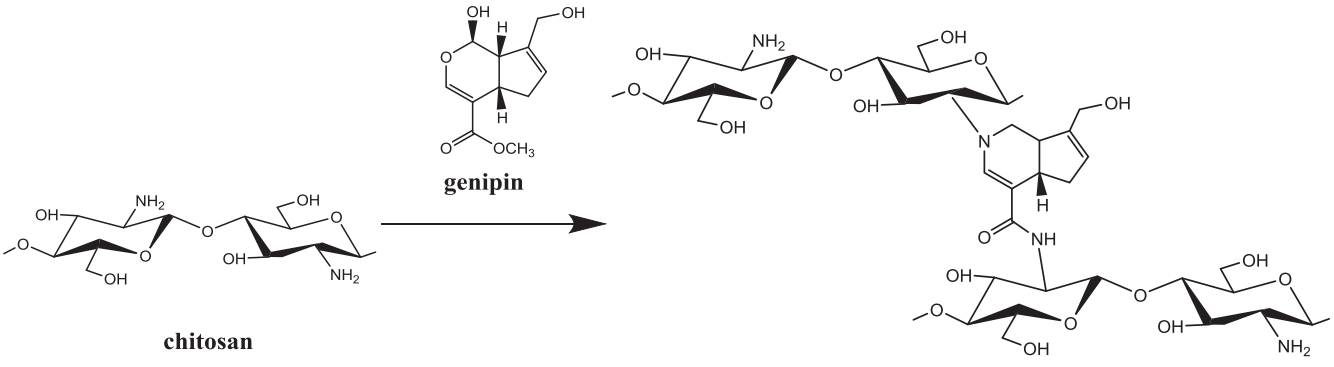

Fig. 1 – Schematic reaction on the cross-linking of two chitosan chains through one molecule of genipin.

Table 1 – Examples of generating chitosan-based in-situ hydrogels through covalent cross-linking of different functional polymers.

Hydrogel precursors		Covalent cross-linking bonds	Ref
Chitosan-based derivate	Second modified polymer		
N,O-carboxymethyl chitosan	Oxidized alginate	Imine bond	[22,23]
Carboxymethyl chitosan	Oxidized carboxymethyl cellulose	Imine bond	[24]
Phenylboronic modified chitosan	Oxidized dextran	Imine bond and phenylboronate ester	[25]
Oxanorbornadiene modified chitosan	11-azido-3,6,9-trioxaundecan-1-amine modified hyaluronan	The triazole ring formed via a metal-free click reaction (cyclo-addition)	[28]
Chitosan-acrylate	PEO-thiol	-S-CH_2- formed via thiol-ene reaction	[26]
Chitosan–thioglycolic acid	Pectin–cysteine	Disulfide linkage	[29]

generate in-situ gelation without this conflict. The starting hydrogel precursors are liquid solutions that can be injected into the local site. Upon exposure to light, the injected precursors polymerize by the initiation of the produced radical species to form cross-linked polymer network in-situ. Yoon Yeo and coworkers [30] developed a semi-interpenetrating network (semi-IPN) by blends of photocross-linkable 4-azidobenzoic acid-modified chitosan (Az-C) and polyethylene glycol (PEG) as an in-situ-forming nerve adhesive. The precursor solutions formed soft gels in <1 min under UV illumination. However, concern may arise regarding the cytocompatibility of UV-initiating systems when applied in tissue engineering for direct cell encapsulation in hydrogels. Min Lee et al. [31] recently developed visible light cross-linkable hydrogel systems using methacrylated glycol chitosan (MeGC) and three blue light initiators: camphorquinone (CQ), fluorescein (FR) and riboflavin (RF). A minimal irradiation time of 120 s was required to produce MeGC gels with CQ or FR, showing no significant effect on the viability of encapsulated chondrocytes. It is advanced to apply photoinitiators absorbing in the visible region over UV light-initiated polymerizations, since exposure to visible light is non-thermogenic and causes less damage to cells. Nevertheless, caution should be exercised because the reactive species in photopolymerization may expose free radicals to the surrounding tissues and affect the incorporated drugs.

3.2. *In situ phase-separation system*

In-situ phase separation is another strategy utilized widely to generate in situ gelling system, which can be induced by changing the solubility of the polymer with respect to changes in temperature, pH or by elimination of solvent. It is a kind of physical cross-linking approach to form chitosan hydrogels in situ by employing secondary bond forces such as hydrogen bonding, electrostatic interaction, or hydrophobic association. As other in-situ gelling systems based on the physical cross-linking, the low mechanical strength of chitosan physical hydrogels and the impact of the drug and environment on their in-situ gelling processes need to be taken into consideration.

3.2.1. *Thermal-sensitive gelation system*

Thermogel-based platforms undergo sol-to-gel transformation upon temperature change. Since thermogels do not require the use of organic solvents, cross-linking agents, or any exterior applied triggers for in-situ gelation, they are especially attractive for delivery of sensitive small molecules and biological molecules. The chitosan-based thermogelling systems with the combinations of chitosan and polyol-phosphates are representative ones since they were developed by Chenite et al. in 2000 using β-glycerophosphate (β-GP) as gelling agent [32]. Chitosan is known to be soluble in acidic solutions, and phase separation happens at pH greater than 6.5. It is interesting that the acidic chitosan solutions were neutralized by the addition of polyol-phosphates, but without phase separation at physiological pH range. These chitosan/polyol-phosphate systems remain in a liquid state at low temperatures and undergo sol–gel transition at body temperature. Such chitosan/polyol-phosphate systems have been extensively studied and reviewed [33,34], opening doors for the development of parenteral drug delivery systems. It was shown that the molecule weight, deacetylation degree, and concentration of chitosan, as well as the kind and concentration of polyol-phosphate had effects on their rheological and physicochemical properties, and the gelation process. Recently, Nicolas Anton et al. [35] elucidated the gelation mechanism of thermal-sensitive chitosan/polyol-phosphate systems particularly by comparing β-GP to glucose-1-phosphate (G1-P) and glucose-6-phosphate (G6-P) and to a polyol-free phosphate salt, Na_2HPO_4 as well. As shown in Fig. 2, polyols create a hydration protective layer around the chitosan chains, largely built through weak intermolecular interactions, like hydrogen bounds. An increase on temperature disrupts this polyol layer and allows the polymers to interact with each other through stronger hydrophobic bonding, thus inducing the gelation. It was disclosed that the size of the polyol part had an impact on the stability of this hydration layer, and thus on the temperature and kinetics of sol–gel transition.

The chitosan/β-GP systems have been proved to be suitable in various pharmaceutical and biomedical applications with promising potentials since GP is a biocompatible component naturally present in the body approved by the U.S. Food and Drug Administration [36,37]. However, the long period of time required for their effective gelation has severely limited their clinical application. Different strategies have been implemented to improve the hydrogel characteristics such as replacement of GP, addition of a second polymer and chemical modification of chitosan, or tailoring the drug release by combination with carrier particles. For example, the chitosan/G1-P system exhibited an enhanced stability compared to the standard chitosan/β-GP system. Chitosan solution with 0.40 mmol/g G1-P is stable for at least 9 months at 2~8 °C versus less than 1 month of the CS/β-GP system [38]. Indeed, hydrogels from chitosan/β-GP may lack appropriate mechanical properties for given applications, especially for cell-carrier systems. To circumvent this problem, several trials have been evaluated by blending the chitosan/β-GP system with another biocompatible compound or polymer, such as g-glycidoxypropyltrimethoxysilane (GPTMS), gelatin, and hydroxyethyl cellulose (HEC) [39–41]. It is noted that commercial-grade HEC contains glyoxal, a dialdehyde, which gives rise to covalent cross-linking of chitosan-GP-HEC hydrogels [41]. In addition, chemical modification of chitosan has also been reported to perform modified-chitosan/β-GP systems as thermogelling solutions. For example, the employment of thiolated chitosan (CS-TGA) exhibited a sol/gel transition within 2 mins at physiological temperature, an improved mechanical property of hydrogel and a decreased bovine serum albumin (BSA) release rate, due to the additional cross-linking by disulfide bonds [42]. Another chitosan-based thermalgelling system has emerged by the incorporation of hydrophobic n-dodecyl groups in chitosan following the mixing with β-GP [43], which induced faster gelation process through formation of smaller but more homogenous hydrophobic micro-domains with the aid of pendant hydrophobic moieties in the lower temperature range.

However, there is still a problem with these thermogelling systems based on chitosan/β-GP system that the excessive use of glycerophosphate salt may practically limit a number of biomedical applications. Alternatively, a series of thermogelling chitosan derivatives have also been developed, like hydroxypropyl chitin (HPCh) [44], glycol chitin [45], poly(ethylene glycol) (PEG) grafted chitosan [46] and poly(ethylene

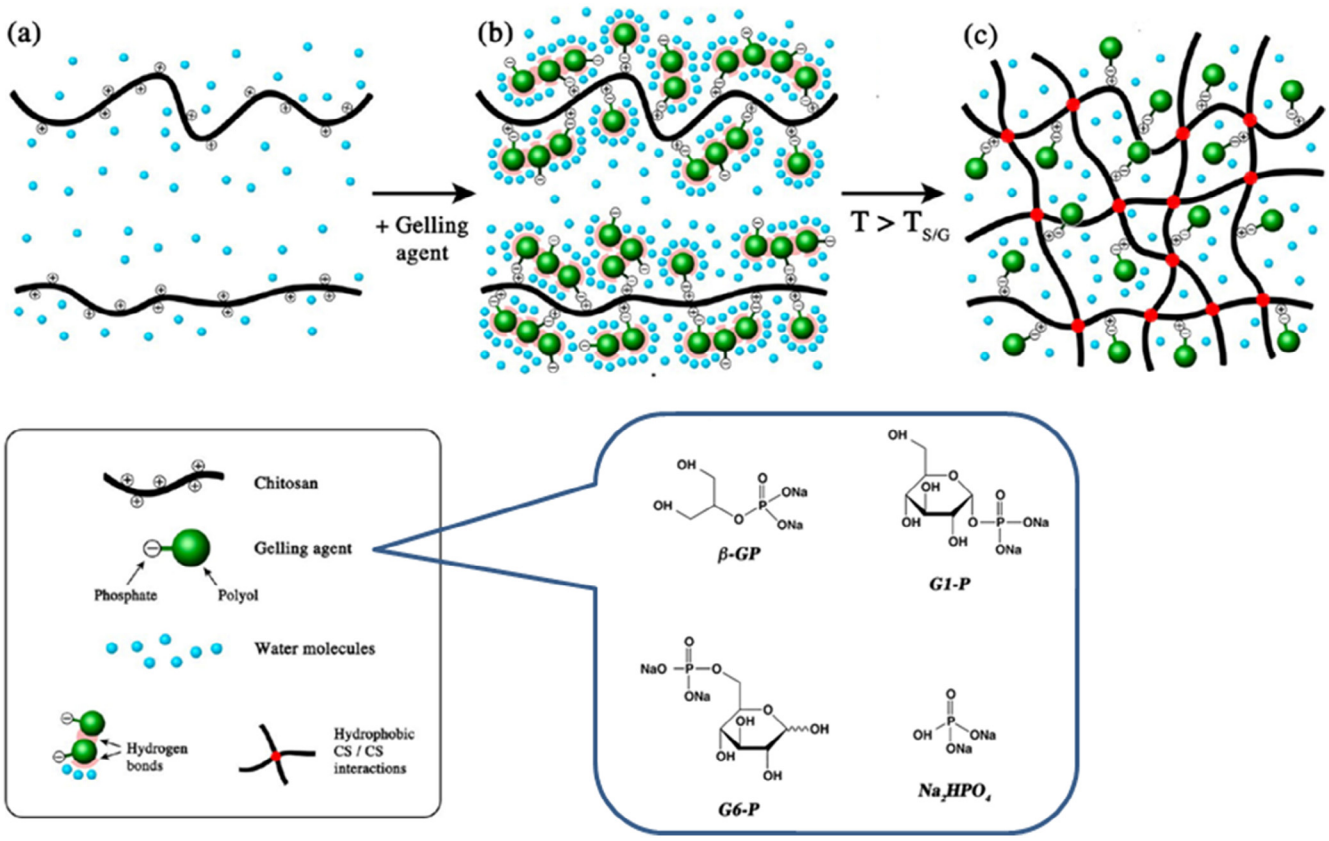

Fig. 2 – Schematic representation of gelation mechanism of thermal-sensitive chitosan/polyol-phosphate systems. Adapted with permission from [35]. Copyright (2013) American Chemical Society.

glycol)-poly(L-alanine-co-L-phenyl alanine) grafted chitosan (CS-g-(PAF-PEG) [47]. These thermogelling chitosan derivatives are water-soluble under physiological conditions after the chemical modification. By varying the polymer concentration, the molecular parameters of grafted branches (groups), such as their composition, molecular weight and/or substituted degree, the sol–gel transition temperature of such thermogelling chitosan derivative could be tuned to match the different application requirements, showing good feasibility as an injectable in situ gelling system. The thermogelation mechanism of CS-g-(PAF-PEG) was investigated on a molecular level by Byeongmoon Jeong et al. [47]. It was suggested that an extensive molecular aggregation might be involved in the sol-to-gel transition, where the CS-g-(PAF-PEG) formed micelles with 10–50 nm in diameter at 10 °C and formed large aggregates ranging from hundreds to thousands of nanometers in size as the temperature increased up to 35 °C.

3.2.2. *pH sensitive gelation system*

Changing pH is a common approach to perform sol-to-gel transition for pH-sensitive, water-soluble polymers. They usually undergo phase transition due to the functional groups on the polymers that either accept or donate protons as a result of pH changes in the environment. Chitosan is a cationic polyelectrolyte, whose solutions would exhibit a liquid–gel transition around pH 6.5, when pH changes from slightly acidic to neutral. Increasing the pH will deionize chitosan, thus generating the three-dimensional chitosan network due to physical junctions of hydrogen bonds. Based on such mechanism, an injectable in situ forming system was developed from acidic chitosan solution with the combination of sodium bicarbonate ($NaHCO_3$) [48]. Similarly, a thermosensitive chitosan gel containing hydroxyapatite (HA) was prepared using Na_2CO_3 as coagulant for bone mesenchymal stem cells (MSCs) loading [49]. It was reported that the chitosan solution remained in a homogeneous sol state in sealed container at low temperature. Then, chitosan gels were formed in situ after subcutaneous injections of either the chitosan/ $NaHCO_3$ sol or the chitosan/ HA/Na_2CO_3 ones in rats. Although their sol-gel transitions appeared to be thermal-sensitive upon the temperature increasing, these systems performed actually the pH-induced gelation process. Both $NaHCO_3$ and Na_2CO_3 are known as a weak base, and the reaction between [HCO_3^-] ([CO_3^{2-}]) and [H^+] leads to the pH increase accompanying with the CO_2 emitting and the hydrogel formation. Such neutralization reaction would reach different balance depending on the temperature and the release of CO_2, which controlled the sol–gel transition. The composition of these in-situ forming systems is simple with no additives of cross-linking agent or organic solvent, giving rise to great potential for their biomedical applications.

3.2.3. *In situ gelation system through electrostatic interaction*

As a cationic polyelectrolyte, the gelation of chitosan can be induced by adding anionic cross-linker like tripolyphosphate (TPP) and alginate, as a result of electrostatic attractions. In this strategy, the cross-linking association occurred usually with

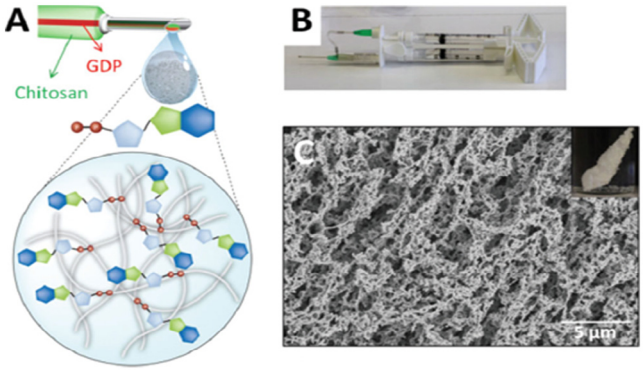

Fig. 3 – (A,B) Schematic representation and image of a double-barrel and double lumen injection system for in-situ gelation. (C) SEM image of the formed chitosan sponge. Adapted with permission from [51]. Copyright (2014) Royal Society of Chemistry.

a fast rate, which has been previously used to fabricate chitosan nanoparticles, microparticles and fibers in many researches. Recently, Maryam Tabrizian and coworkers [50,51] created a rapid in-situ gelation system based on chitosan and guanosine 5'-diphosphate (GDP), where GDP acted as an anionic cross-linker. GDP is a cellular component, which cross-linked chitosan chains very rapidly at the injection location (T_{gel} < 1.6 seconds) due to the electrostatic interaction between the anionic phosphate groups of GDP and the cationic amine groups of chitosan. The fast gelation rate of this system could prevent undesirable flow to surrounding tissues as injectable carriers. But it is necessary to design a specific double-barrel syringe with two independent outlets to mix chitosan and GDP solutions at the tip of the needle during injection as shown in Fig. 3. This avoids clogging of the needle and ensures that the mixture and gelation occurs in situ. The obtained hydrogels showed a porous micro-structure, desirable cytocompatibility and mechanical properties resembling those of human soft tissue.

4. Applications of in-situ forming chitosan hydrogel

4.1. *Drug delivery*

A large amount of pharmaceutical agents have been discovered over the past decades. Many of them possess good therapeutic effects but their bioavailability and pharmacokinetics are poor, showing systemic toxicity after the administration. Therefore, drug delivery system has become an important tool to enhance the therapeutic efficiency using delivery matrices such as nanoparticles, polymeric micelles or hydrogels to deliver drug molecules. In situ gelling systems, especially the biodegradable ones are very attractive for local delivery of drugs, since they can be administrated by injection showing less invasion and less pain as compared to preformed implants and sustained release of drug [52,53]. Injectable chitosan-based in situ gelling systems have been applied as in-situ forming implants and as mucosadhesive carriers in nasal delivery, ocular delivery and others because of their good tissue biocompatibility, sustained-release properties and simple manufacture.

4.1.1. *In situ forming implants*

In the area of parenteral controlled release formulations, in situ forming implants are attractive alternatives to the preformed implants and microparticles, which could be injected as low viscous solutions and transform to a gel or solid depot in the body at the injection site, thus avoiding the use of large needles or microsurgery. The in-situ forming gelling systems consisting of chitosan and β-GP have come into the market as commercially available delivery platforms (like BST-Gel) for in-situ forming implants, which can deliver water-soluble small-molecular drugs, chemotheraputic agents as well as proteins and peptides.

Peng et al. [54] reported the thermosensitive chitosan/GP hydrogels for the sustained delivery of venlafaxine hydrochloride (VH) and the optimization of this formulation. VH is a water-soluble antidepressant of the serotonin-norepinephrine reuptake inhibitor (SNRI) class. Both in vitro drug release and in vivo pharmacokinetic study via subcutaneous administration were investigated, demonstrating that thermosensitive chitosan/GP hydrogels had a better sustained delivery of VH over 24 h compared to VH solution. Dean-Mo Liu et al. [55] described a depot drug delivery system by combining self-assembled nanocapsules of carboxymethyl-hexanoylchitosan (CHC) with glycerophosphate di-sodium salt and glycerol for the highly water-soluble drug Ethosuximide (ESM). In this case, CHC played the role not only to encapsulate the drug, with reduced burst release and release rate as consequences, but also to be the gel network forming constituent. In vivo study through subcutaneously injection using Long Evans Rats as the animal model demonstrated that therapeutic drug levels of ESM remained until day 4, in contrast, for free ESM there was a rapid initial response 1 h after administration.

Additionally, intratumoral injection has served as another way for in-situ forming implants to deliver anti-tumor drugs, which can enhance drug bioavailability to the tumor site and reduce systemic toxicity [2]. The chitosan/GP gelling system has been utilized to deliver several kinds of chemotheraputic agents such as paclitaxel [56], camptothecin [57], Docetaxel [58], melphalon [59], curcumin [19] and so on [60], displaying more efficiency in inhibiting the growth of tumors and less toxic than single intraperitoneal or intravenous injections. Interestingly, the chitosan/GP thermogel was also applied as a vehicle of pingyangmycin (PYM) for the chemoembolization therapy of vascular malformations (VM) in the research by Chen et al. [61]. The injected solution changed into a semisolid embolic agent, which showed the dual occlusion mechanism including occluding blood vessels to interrupt the nutrition supply to VM and sustained release of PYM to keep an effective therapeutic concentration. The rabbit in vivo pharmacokinetics study of PYM loaded thermogels indicated that a small dose of PYM thermogels could make higher localized drug concentrations for a longer time and lower drug concentrations of systemic circulation, compared to PYM injections.

Moreover, a proposal to achieve longer sustained release of drug in chitosan/β-GP hydrogels is to incorporate the drug into liposomes, microspheres or nanoparticles at first and then mix the drug-loaded liposomes with chitosan/β-GP solution. The

release rate of the drug can be tuned by both the properties of liposomes (size and composition) and the properties of chitosan-based thermogels. Topotecan hydrochloride (TPT) has potential for the treatment of ovarian cancer. Scientists have proposed to incorporate TPT liposomes into injectable thermosensitive in situ hydrogel, consisting of chitosan and beta-GP, for sustained release and preservation of active lactone form of TPT [62]. The drug release rate of TPT was found to be slowed down in liposomes-loaded chitosan/GP hydrogel, and the lactone fraction of TPT in the hydrogel matrix remained 40% after 50 h. Therefore, TPT-liposomes-loaded chitosan/GP hydrogel was demonstrated to be a potential formulation for improving the antitumor efficacy of TPT and an important technology platform for intratumoral administration of camptothecin-family drugs. Another injectable hydrogel with an in situ and pH sensitive drug delivery system was developed by mixing conjugated doxorubicin (DOX) conjugated succinated chitosan (S-chi) with oxidized alginate and investigated for cancer treatment in the xenograft breast tumor model [63]. Since DOX was conjugated to S-chi via a pH-sensitive Schiff base, the DOX laden hydrogel exhibited a pH sensitive release of DOX upon the stimulus of an acidic tumor microenvironment and thus significantly inhibited tumor growth.

4.1.2. *Nasal delivery*

Intranasal delivery is one of the most interesting and challenging endeavors in pharmaceutical fields. It could overcome the high first-pass metabolism and drug degradation in the gastrointestinal environment, but show such drawbacks as short residence in the nasal cavity, highly variable efficiency, low permeability, and inconvenient administration. Attempts have been made to overcome these drawbacks and to increase the nasal bioavailability of drugs through use of in situ gelling systems [5]. In the quest for the development of intranasal hydrogels for the drug delivery, the in situ forming chitosan hydrogels have received considerable attention because of the strong mucoadhesivity of chitosan derivates and its effectiveness at opening tight junctions between epithelial cells [64–66]. These in situ gelling systems can carry different drugs through nasal routes for disease treatment, among which the most commonly used are protein drugs like insulin and central nervous system (CNS) acting drugs like doxepin and ropinirole.

An in situ thermogelling hydrogel based on N-trimethyl chitosan chloride, glycerophosphate (GP) and poly(ethylene) glycol (PEG) has been formulated as a carrier matrix for the transmucosal delivery of insulin via the nasal route [67–69]. By the utilization of such in situ thermogelling hydrogel, the formulation can be dropped or sprayed easily into nasal cavity and spread on the nasal mucosa in solution state and then transformed into viscous hydrogel at body temperature. An in vivo investigation in a diabetic-rat model demonstrated a decreased nasal mucociliary clearance rate and a slow release of insulin, resulting in the reduction in blood glucose over ca. 24 hours as compared with the subcutaneous insulin control (maximum reduction of blood glucose of ca. 40% at 1 hour from administration). Thus, such nasal formulation shows the potential as a once-a-day dosage form for the delivery of insulin. There is another thermosensitive in situ gel system based on chitosan and poly vinyl alcohol (PVA) for nasal delivery of insulin [70]. The hydrogel is prepared by mixing chitosan and PVA. The in vitro release of insulin from gel network is observed spectrophotometrically which is good enough to maintain blood glucose level for six hours. Furthermore, the formulation when evaluated for their in vivo hypoglycemic effect, demonstrated its ability to reduce glucose level. The observed in vitro and in vivo results indicate that the proposed thermosensitive in situ gelling system has substantial potential as nasal delivery system for insulin.

As for CNS drug delivery, the chitosan/GP based thermogelling system has been served to delivery doxepin, an agent used in the treatment of depression, to brain through intranasal administration [37]. The prepared systems are evaluated for different parameters like gelling characteristics, rheological characteristics, in vitro drug release, and ex vivo permeation across sheep nasal mucosa. According to the results, such chitosan/GP hydrogels gels have shown good mucoadhesion, enhanced drug permeation, and provided sustained in vitro release of doxepin at 37 °C. The prolonged effect of the formulations endowed the reduced dosing frequency possible and the effective delivery of doxepin to brain via nose. Another mucoadhesive in situ gel formulations consisting of chitosan and hydroxyl propyl ethyl cellulose have been applied to enhance intranasal delivery of ropinirole (a dopamine D2 agonist in treatment of Parkinson's disease) to the brain [71]. The high brain direct drug transport percentage of 90.36% and drug targeting index (DTI) > 1 confirms direct nose to brain transport of the intranasal in situ gel formulation of ropinirole. These chitosan-based in situ gelling systems have been demonstrated to increase the retention of drugs in the nasal cavity, and some of them also show permeation-enhancing capabilities, which can serve as effective systems for delivery of drugs through nose with increased nasal residence time.

4.1.3. *Ocular delivery*

Research on chitosan and its derivatives as promoters of intraocular penetration of topically administered drugs has been reported in recent literature [72]. A major problem in ocular therapeutics with classical formulations is the maintenance of an effective drug concentration at the site of action for a long period of time. Chitosan stands out with its unique advantageous characteristics for different types of formulations like in situ gelling systems, nanoparticles, inserts, etc [73]. Also chitosan seems to be one of the most promising polymeric carriers for both hydrophilic and lipophilic drugs for ocular application. The chitosan-based in situ hydrogels have been adopted in the treatment for age related macular degeneration (AMD), glaucoma as well as some mucosal allergic diseases.

Avastin® tends to be the first choice drug for the treatment of AMD and proliferative diabetic retinopathy in clinic. An in situ injectable hydrogel was developed by simple mixing glycol chitosan and oxidized alginate aqueous solution for potential ocular drug delivery of avastin by Xu et al. [22]. In vitro degradation test and in vitro release study have demonstrated that the release rate of avastin from hydrogels declined accordingly with the increase of oxidized alginate concentration in the hydrogel, and the encapsulated avastin had an initial burst release at early stage (within 4 h) followed by a sustained release manner in a period of 3 days. The obtained injectable polysaccharides cross-linked hydrogel with controllable degradation rate and sustained drug release may be a

versatile carrier for ocular delivery of avastin through intravitreal injection to treat the posterior diseases.

When it comes to glaucoma therapy, timolol maleate was formulated by the gelling agent of combining chitosan (a pH-sensitive polymer) with gellan gum (an ion-activated polymer). Ocular retention was studied by gamma scintigraphy and a significant increase in retention time was observed in the research [74]. An in situ thermogelling system based on chitosan in combination with disodium alpha-d-Glucose 1-phosphate (DGP) has also been developed as ocular drug delivery system for levocetirizine dihydrochloride (LD), which especially does good to cure mucosal allergic diseases. The developed hydrogel presents a characteristic of a rapid release at the initial period followed by a sustained release and remarkably enhances the cornea penetration of LD [75]. The drug-loaded hydrogel has produced more effective anti-allergic conjunctivitis effects compared to LD aqueous solution. These results show that that the chitosan-based in situ gelling system can be a viable alternative to conventional eye drops for the treatment of various ocular diseases.

Overall, the potential of chitosan-based formulations has been confirmed by proving their safety to ocular structures. The above-mentioned results of ocular irritation have demonstrated the excellent ocular tolerance of the chitosan-based in situ hydrogels. Meanwhile, the ocular residence time for the hydrogel is significantly prolonged compared to eye drops.

4.2. *Tissue regeneration*

Tissue regeneration is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore or improve tissue function. In situ-forming hydrogels have emerged in the field of tissue regeneration with potential applications, which can be divided into two main parts: wound dressing materials for wound healing and cell scaffolds in tissue engineering.

Creating new materials that are biodegradable and do not induce scar tissue formation is a challenging and a new area of tissue regeneration [76].

4.2.1. *Wound dressing material for wound healing*

Wound healing is a complex process involving three interrelated dynamic and overlapping phases including inflammation, granulation and remodeling. In this field, numerous materials have been discovered as wound dressing to promote wound healing, such as chitosan, hyaluronic acid, poloxamer and so on [24,77]. We take advantage of chitosan by designing various types of in situ forming hydrogels, since chitosan itself shows antibacterial nature and capability to enhance wound healing and not to induce scar tissue formation.

An in situ gelling system composed of rutin- and tyramine-conjugated chitosan derivatives, horseradish peroxidase (HRP) and hydrogen peroxide was reported as injectable dressing for dermal wound repair [78]. It was found that rutin-conjugated hydrogel exhibited enhancement of wound healing as compared with treatments with PBS, hydrogel without rutin, and a commercialized wound dressing (Duoderm). Moreover, an in situ injectable nano-composite hydrogel composed of N,O-carboxymethyl chitosan and oxidized alginate (CCS-OA) encapsulating nano-curcumin has been successfully developed as a novel wound dressing for the dermal wound repair application. Histological study revealed that the application of nano-curcumin/CCS-OA hydrogel could significantly enhance the re-epithelialization of epidermis and collagen deposition in the wound tissue [79]. All these results suggest that the developed chitosan-based in situ forming hydrogels might have potential application as a promising wound dressing for wound healing.

4.2.2. *Cell scaffold in tissue engineering*

In situ forming hydrogels show interesting characteristics as dynamic scaffolds to provide 3D network for seed cells and to provide the less invasive surgical procedures for tissue engineering application. Scaffolds based on chitosan hydrogels have great potential due to their minimal foreign body reaction, biocompatibility, biodegradability, adhesion to cells and the ability to be molded in various geometries. Especially, the chitosan-based gelling systems have been applied as injectable scaffold materials mostly in the regeneration of cartilage [40,80], bone [26,52,81–84], nerve [51,76] and other soft tissue [85,86].

Hydrogels based on chitosan/GP thermosensitive formulation can be implanted in a minimal invasive manner, representing a great promise as injectable scaffold choice for cartilage and bone tissue engineering. However, their poor mechanical property is a limitation for cartilage lesions repair as well as bone repair. Some modified formulations of chitosan/GP combined with starch were investigated to generate a firm texture gels encapsulating adipose derived stromal (ADSC) cells for cartilage-engineered regeneration [80]. The storage modulus of the hydrogels increased within increasing starch concentration. The encapsulated ADSC remained viable and proliferated within chitosan hydrogels, and maintained the expression of typical chondrogenic markers genes and deposited cartilage ECM molecules in vitro, demonstrating the chondrogenic differentiation of ADSC. In another case, chondrogenic factors or human mesenchymal stem cells (hMSCs) were included in the chitosan-β glycerophosphate-hydroxyethyl cellulose (CH-GP-HEC) systems, and injected into the site of injury to fill the cartilage tissue defects using minimal invasive techniques [40]. During the 28-day investigation, chitosan-based injectable hydrogels provided suitable conditions for chondrogenic differentiation of the encapsulated hMSCs.

Regarding the complex features of bone tissue, the composite materials combining of inorganic ceramic materials with organic hydrogels are highly advantageous for bone tissue regeneration. Dessi et al. [82] prepared thermosensitive chitosan-based hydrogels, cross-linked with β-glycerophosphate and reinforced via physical interactions with β-tricalcium phosphate (β-TCP) as promising candidates for injectable in situ gelling bone analogues. Moreover, in order to enhance osteogenic differentiation of seed cells, an ideal scaffold should also provide controlled release of the active substance. Therefore, a serial of composite materials were reported in the literature in order to enhance antibacterial activity and maximize bone induction, like the chitosan–collagen composite hydrogels [84], the demineralized bone matrix (DBM) powder loaded thermogelling chitosan [83], the zinc doped chitosan/GP hydrogel [81] and the in situ chitosan–PEO hydrogel containing recombinant human bone marrow protein-2 (rhBMP-2) [26].

Moreover, injectable hydrogels offer a more applicable strategy for soft tissue regeneration, by which an easy and homogenous drug or cell distribution could be performed within any defect size or shape. For example, Valmikinathan CM et al. [76] designed the photocross-linkable chitosan hydrogels to encapsulate neural stem cells (NSCs), which facilitated the differentiation of NSCs into tubulin positive neurons and astrocytes and proved to be a suitable scaffold for neural tissue engineering.

5. Conclusion and prospects

In-situ gelling system is a minimally invasive and interesting solution in the development of drug delivery and tissue engineering approaches. Various in-situ forming hydrogels based on chitosan have been designed as injectable hydrogels-based devices for in situ drug or cell release, which rheological or mechanical and functional features could be tailored and enhanced by the composites and methods modification.

In fact, the drug delivery and scaffold could be consistent in the field of tissue engineering. The key point in tissue engineering is to construct a suitable microenvironment which includes mechanical strength, growing factors, and others for seed cells to grow, ensuring their long-term survival and further differentiation in vivo. Three dimensional porous scaffolds with growing factors are commonly used in the field of tissue engineering. In situ forming chitosan hydrogels can be used not only as exogenous implant carrier of seed cells but as the carrier for controlling the release of growing factors as well. However, if we carry the growing factors directly inside the chitosan hydrogels, several drawbacks emerge, like very fast releasing rate. It is always a difficult and hot spot in tissue engineering that the additive cell growing factors play the role in a timely and appropriate way effectively in order to promote cell proliferation and directional differentiation and benefit the reconstruction of damaged tissues. Organic combination of novel growing factors controlling release systems based on chitosan microspheres, chitosan nanoparticles, chitosan nanoparticle gene complexes and chitosan-based in situ gelling systems turns out to be a possible solution to solve the above-mentioned problems. Therefore, the combination of drug delivery system and tissue engineering scaffold based on in situ chitosan hydrogels is expected to enjoy a definite promising prosperity in the medical field.

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