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Review

Glycerophosphate-based chitosan thermosensitive hydrogels and their biomedical applications

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ABSTRACT

Chitosan is non-toxic, biocompatible and biodegradable polysaccharide composed of glucosamine and derived by deacetylation of chitin. Chitosan thermosensitive hydrogel has been developed to form a gel in situ, precluding the need for surgical implantation. In this review, the recent advances in chitosan thermosensitive hydrogels based on different glycerophosphate are summarized. The hydrogel is prepared with chitosan and β-glycerophosphate or αβ-glycerophosphate which is liquid at room temperature and transits into gel as temperature increases. The gelation mechanism may involve multiple interactions between chitosan, glycerophosphate, and water. The solution behavior, rheological and physicochemical properties, and gelation process of the hydrogel are affected not only by the molecule weight, deacetylation degree, and concentration of chitosan, but also by the kind and concentration of glycerophosphate. The properties and the three-dimensional networks of the hydrogel offer them wide applications in biomedical field including local drug delivery and tissue engineering.

Contents

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1. Introduction

Hydrogels are composed of three-dimensional polymer networks that have a high number of hydrophilic groups. The hydrogels can absorb large quantities of water and will neither disintegrate nor dissolve. Fully swollen hydrogels are soft, pliable, and low interfacial tension with water or biological fluids. The high water content of hydrogel renders it compatible with most living tissue and the viscoelastic nature of hydrogel minimizes damage to the surrounding tissue when it is implanted in the host. These characteristics make hydrogel an ideal candidate in biomedical applications such as remedying injuries to living systems (Bhattarai, Gunn, & Zhang, 2010). In addition, hydrogels can serve as a supporting material for cells during tissue regeneration as well as drug delivery system because the hydrogel's physicochemical properties are similar to the native extracellular matrix both compositionally and mechanically (Lee & Mooney, 2001; Tessmar & Gopferich, 2007). The hydrogel will become responsive to environmental stimulation if the polymer network of hydrogel is endowed with functional groups (Buenger, Topuz, & Groll, 2012). The stimulation may be temperature, pH, biomolecules, electric field, magnetic field, light rays and so on. When the environment stimulation is changed, the hydrogels undergo a volume-phase transition due to molecular interactions resulting in abrupt changes in the network such as swelling, collapse or sol-to-gel transition.

Glycerophosphate is an organic compound naturally found in the body which is usually used as a source of phosphate in the treatment of unbalance of phosphate metabolism and its veinal administration has been approved by FDA. β-Glycerophosphate has been shown as an osteogenic supplement when added to cultures of human bone marrow stem cells. αβ-Glycerophosphate is the mixture of α-glycerophosphate and β-glycerophosphate, and α-glycerophosphate has linear chain structure and shows less steric hindrance than β-glycerophosphate. Glycerophosphate also has been used as a catalyst to cause a sol-to-gel transition in chitosan solutions at physiological pH and temperature.

Chitosan, the cationic (1-4)-2-amino-2-deoxy-β-D-glucan, partly acetylated to the typical extent close to 0.25 is industrially produced in medical/pharmaceutical grade from marine chitin (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar et al., 2010; Muzzarelli, 2009; Muzzarelli, 2010; Muzzarelli, 2012; Muzzarelli et al., 2012). The development of chitosan hydrogel has been an area drawing intensive investigation and a large amount of works have been reported on chitosan hydrogel and its potential use in various applications (Lee et al., 2009; Nagahama et al., 2009; Sung et al., 2010; Tang, Du, Hu, Shi, & Kennedy, 2007; Zhou et al., 2012).

Chitosan hydrogels have been prepared with a variety of different geometries and formulations including liquid gels, beads, films, tablets, capsules, microspheres, microparticles, sponges, and textile fibers (Denkbas, 2006; Hamidi, Azadi, & Rafiei, 2008; Khan, Tare, Oreffo, & Bradley, 2009; Ladet, David, & Domard, 2008). In each preparation, the polymer binding is accomplished either by non-covalent physical association, such as secondary forces (hydrogen, ionic, or hydrophobic bonding) and physical entanglements, or by covalent cross-linked chemical association (Hoffman, 2002). Physical associated networks can often be obtained by simply mixing the components which make up the gel under the appropriate conditions. Furthermore, the gelation, requiring no toxic covalent linker molecules, is always safe for clinical applications.

This review focuses on the hydrogel based on different glycerophosphate including the preparation and applications, which is a temperature-sensitive physical associated network. There are several excellent reviews of hydrogel or chitosan-based hydrogel (Bhattarai et al., 2010; Buenger et al., 2012; Hoffman, 2002; Lee & Mooney, 2001) and there is a review about thermosensitive chitosan/glycerophosphate-based hydrogel and its derivatives (Supper et al., 2014), but an in-depth review on chitosan/glycerophosphate hydrogel based on the different glycerophosphate cannot be found ever. In this review, we will summarize the various categories of chitosan/glycerophosphate hydrogels prepared with chitosan and β-glycerophosphate or αβ-glycerophosphate, describe the preparation methods, introduce the properties and gelation mechanism, and present recent advances in biomedical applications.

2. Chitosan/β-glycerophosphate hydrogels

2.1. Development process and gelation mechanism

Chitosan dissolves in acidic environments via protonation of its amine groups. Once dissolved, chitosan remains in solution up to pH value in the vicinity of 6.2. Neutralization of chitosan aqueous solutions to pH value exceeding 6.2 systematically leads to the formation of a hydrated gel-like precipitate. Effort on chitosan neutral solution is first proposed by Chenite et al. (2000). They find that chitosan solutions remain liquid below room temperature, even with pH values within a physiologically acceptable neutral range from 6.8 to 7.2, in the presence of β-glycerophosphate salt. The system becomes thermally sensitive, which is liquid at room temperature and solidifies into gel as temperature increasing to body temperature (Fig. 1) (Ruel-Gariépy et al., 2004). β-glycerophosphate plays three essential roles in the system: (1) to increase the pH into the physiological range of 7.0–7.4; (2) to prevent immediate precipitation or gelation; and (3) to allow for controlled gel formation when an increase in temperature is imposed. The sol-to-gel transition temperature is pH-sensitive and gelling time is shown to be temperature-dependent.

The molecular mechanism of gelation may involve multiple interactions between chitosan, β-glycerophosphate, and water (Crompton et al., 2005; Ruel-Gariépy, Chaput, Guirguis, & Leroux, 2000). The effective interactions responsible for the sol/gel transition included: (1) the increase of chitosan interchain hydrogen bonding as a consequence of the reduction of electrostatic repulsion due to the basic action of the salt, (2) the chitosan-glycerol-phosphate electrostatic attractions via the ammonium and the phosphate groups, respectively, and (3) the chitosan–chitosan hydrophobic interactions which should be enhanced by the structuring action of glycerol on water.

The solution behavior of chitosan/β-glycerophosphate hydrogel is affected by the concentration of chitosan and β-glycerophosphate (Cho, Heuzey, Bégin, & Carreau, 2006a). The pH of chitosan solution is slightly increased with increasing of β-glycerophosphate concentration due to the neutralizing effect of the phosphate groups. The pH increase with polymer concentration is due to the consumption of H^+ ions in solution by the protonation of the free amine groups. The relationship between gelation temperature and concentration of chitosan (C_C) and β-glycerophosphate (C_{GP}) is shown in Fig. 2. The region below the surface is the sol state, while that above is a gel. T_{gel} is gradually decreased with increasing of C_{GP} and C_C. However, a synergetic effect at high C_{GP} and C_C results in a sudden drop of the gelation temperature and a phase transition which is on the edge between concentration-induced and heat-induced gelation.

The rheological and physicochemical properties of the chitosan/β-glycerophosphate system in terms of temperature are investigated sequentially (Chenite, Buschmann, Wang, Chaput, & Kandani, 2001; Cho, André Bégin, & Carreau, 2005). Increasing temperature has no effect on the pH value of the chitosan/β-glycerophosphate system, while conductivity is increased. The results indicate that the decrease of potential ionic interactions

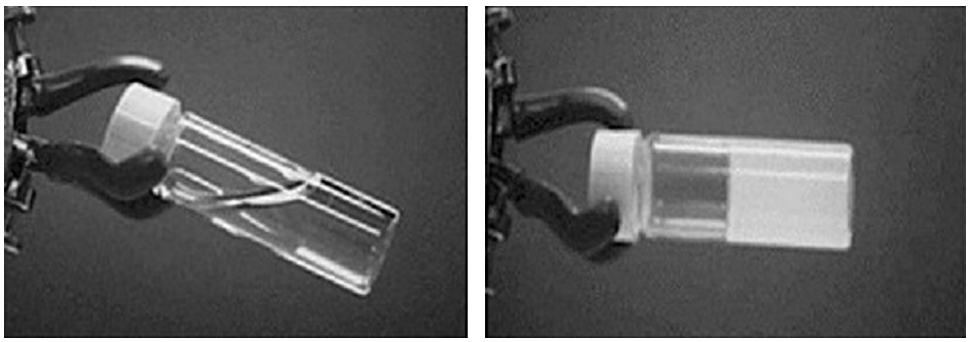

Fig. 1. The CS/GP formulation at room temperature (left) and at 37 °C (right). Figure was reprinted from Ruel-Gariépy et al. (2004).

such as ionic bridging is resulted in by the reduction of the ratio of $-NH_3^+$ in chitosan and $-OPO(O^-)^2$ in β-glycerophosphate at high temperature. On the other hand, the favorable conditions for gel formation resulted in by the increased ionic strength as a function of temperature involved which enhanced screening of electrostatic repulsion forces and hence more polymer–polymer hydrophobic interactions. Since it is generally assumed that hydrogen bonding interactions are not predominant at high temperature and hydrophobic effect is the main driving force for the chitosan/β-glycerophosphate gelation at high temperature. To expand this standpoint, physicochemical and rheological properties of the chitosan/β-glycerophosphate system is investigated in the presence of urea, which is a hydrogen bonding disrupting agent and can also affect hydrophobic interaction (Cho, Heuzey, Bégin, & Carreau, 2006b). Heat-induced gelation of the chitosan system in the presence of urea shows higher gelation temperature in nonisothermal tests and longer gelation time in isothermal conditions. At low temperature, urea strongly affects polymer–polymer interactions by weakening hydrogen bonds. With temperature increasing, it hinders hydrophobic effect since the reduction of ionic strength results in less screening of electrostatic repulsion between protonated glucosamine groups. It is confirmed that hydrophobic effect is the main driving force for the chitosan/β-glycerophosphate heat-induced gelation.

Aliaghaie, Mirzadeh, Dashtimoghadam, and Taranejoo (2012) have investigated the gelation mechanism of injectable thermosensitive hydrogels comprising chitosan and β-glycerophosphate by rheological measurements for a drug delivery. According to the gelation behavior, three regions are defined: (1) a liquid-like behavior at low temperature, (2) a fast gelation process near the gel point, and (3) a slow gelation process at higher temperatures (seen in Fig. 3). The theory initially worked out by Fredrickson and Larson for block copolymers near their order–disorder transition is extended and proposed to precisely determine the gel point in such systems by considering the aggregation of hydrophobic acetylated blocks of chitosan chains and gelation in chitosan/glycerophosphate systems as an order–disorder transition of block copolymers. Upon heating, the water sheaths around chitosan chains are removed and the heat induced transfer of protons from protonated amino groups to glycerophosphate occurs, and consequently the formation of hydrophobic interactions among chitosan chains is facilitated. Then the gelation process beyond the crossover point goes through a nucleation and growth mechanism and the growth of hydrophobic domains could happen through the reaction limited cluster aggregation. In the third region, the observed rheological behavior could be associated with the tendency of system to minimize its interfacial area through the Ostwald ripening process.

In addition, the effect of molecular weight and deacetylation degree of chitosan on gelation process is researched. The results of the temperature sweep measurement (Aliaghaie, Mirzadeh, Dashtimoghadam, & Taranejoo, 2012) have been shown that the G' and G'' crossover point for lower molecular weight chitosan sample have been shifted to 33 °C, which is 2 °C higher than that for high molecular weight chitosan sample. This implies that the hydrophobic interactions in lower molecular weight chitosan sample have been formed at a higher temperature, which might be a result of lower entanglement and shorter hydrophobic domains. Such an observation could be attributed to the lower molecular weight and consequently lower viscosity of the sample, which provides more possibility for aggregation and percolation of hydrophobic domains through reaction limited cluster aggregation theory. It

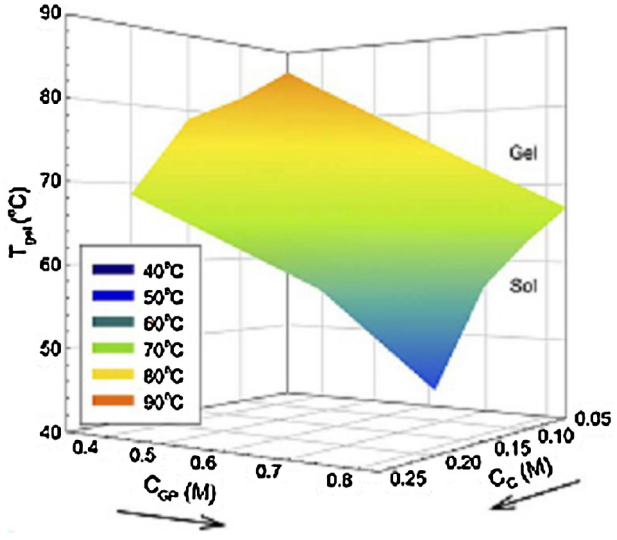

Fig. 2. Phase diagram for T_{gel} under various β-GP (C_{GP}) and chitosan (C_C) concentrations. Figure was reprinted from Cho et al. (2006a).

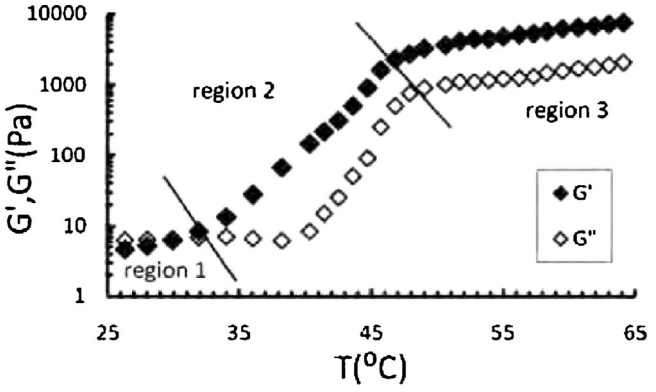

Fig. 3. The evolution of the storage (G') and loss (G'') moduli of the chitosan hydrogel during heating from 25 to 65 °C. Figure was reprinted from Aliaghaie et al. (2012).

Table 1
Parameters, gel temperature and gel time of chitosan/glycerophosphate hydrogels: some examples..

Chitosan			C_{GP} (w/v)		$T_{gel/°C}$	Gel time	Reference
Mw/kD	DD	C (w/v)	β-GP	αβ-GP			
700/200–300	74%/99%	1%(1/1)	23%		31–33	120–280 s	Aliaghaie et al. (2012)
350	91%	1–2%	0–8%		32–42	13–2 min	Chenite et al. (2001)
552–808	70–91%	2%	5.6%		37–66	quickly	Chenite et al. (2000)
850	93%	0.05–0.20 M	0.33–0.83 M		58–79	7–11 s	Cho et al. (2006a,b)
140cps	96.5%	0.1512–0.1728%		2–8%	30.1–45.7	1–3 min	Dang et al. (2011)
140cps	96.5%	0.1512–0.162%		5–8%	21–35	30–90 s	Dang et al. (2012a,b)
463–862	81.8–83.4%	2.05%	9.09%		37	1400–2400 s	Douglas et al. (2013)
455.2	95%	2%	5.6%,7%		33, 37	/	Hastings et al. (2012)
20–525cps	91%	2%	8%		37	4–10 min	Huang et al. (2009)
1080	86%	2%		8.33–9.09%	25–37	20–3 min	Ji et al. (2010)
/	95%	2.5%	6–16%		50–37		Kempe et al. (2008)
310	75%	1.5%	0.045–1.155 M		37	60–330 s	Kim et al. (2010)
200–800cps	75–80%	1.6%	30%		37	/	Kwon et al. (2012)
/	75–85%	0.9%	5.8%		37	6 min	Niranjan et al. (2013)
/	95%	1.4–2.2%w/w		8–16% w/w	37	/	Peng et al. (2013a,b)
421.8/455.2	84%/95%	1.5% w/w	7.27% w/w	7.27% w/w	37	5/140 min	Ruel-Gariépy et al. (2000)
113–900	65–88%	2%	2–8%		24.3–91.7	/	Tsai et al. (2011)
300–400	81.0–94.8%	1.8%	5%		37	2–60 min	Zan et al. (2006)
50	95%	2%	10%		37	6 min	Zhang et al. (2013)
1360	75%	1.7–1.9%		2.5–7.5%	37	10 min	Zhou et al. (2009)
88–1360	56.5–90.3%	1.0–3.0%		5%	37	10 min	Zhou et al. (2008)

β-GP: β-glycerophosphate; αβ-GP: αβ-glycerophosphate; T_{gel}: gel temperature.

could be suggested that the lower molecular weight has facilitated the aggregation and percolation of hydrophobic domains, which is consistent with the lower percolation temperature obtained from temperature sweep measurements. Furthermore, it is understood that the deacetylation of chitin chains to achieve chitosan preferably occurs at the amorphous zones, and hence blocktype distribution of hydrophobic and hydrophilic segments are the result. So deacetylation affects the gelation process by affecting the hydrophobic and hydrophilic properties of chitosan. Chitosan could be considered as a block copolymer of acetylated and deacetylated units and the thermo-gelation of the chitosan/glycerophosphate system happens due to aggregation of hydrophobic acetylated blocks, and could be considered as an order–disorder transition or phase separation. It is the same result as Ruel-Gariépy et al. (2000) that gelation could occur at relatively low temperatures depending on the deacetylation degree of chitosan. The increase in gelation rate observed with chitosans of higher deacetylation degree might be attributed to the increase in cross-link density between the phosphate groups of glycerophosphate and the ammonium groups of chitosan. The parameters, gel temperature and gel time of chitosan/glycerophosphate hydrogels from some references are shown in Table 1.

Tsai, Chang, Yu, Lin, and Tsai (2011) has explored the effect of nanosilver, chitosan characteristics, and solution conditions on gelation temperature of chitosan/β-glycerophosphate thermosensitive hydrogel. The gelation temperature of hydrogel is decreased with the increasing of deacetylation degree of chitosan, concentration of β-glycerophosphate, and pH value (6.5–6.8). It is also decreased with the decreasing of chitosan molecular weight. The present of 12 ppm nanosilver has no effect on gelation temperature. Then, effects of chitosan characteristics on the physicochemical properties are also studied by Chang, Lin, Tsai, and Tsai (2013). It is concluded that the gelation temperatures for the hydrogels are measured in the range of 32–37 °C by manipulating the molecular weight and deacetylation degree of chitosan and the glycerophosphate concentration. The structure of hydrogel with 88% deacetylation degree of chitosan is more porous, uniform, and connective than that of the hydrogel with 80% deacetylation degree of chitosan.

Li, Fan, Ma, et al. (2014) and Li, Fan, Zhu, and Ma (2014) have prepared the chitosan/β-glycerophosphate hydrogel included human-like collagen and studied the gelation mechanism and its properties. The results show that an amide bond (—CONH) is formed between the carbonyl (C═O) of human-like collagen and the amino of chitosan and —NRH^{2+} is formed between the amide bond and β-glycerophosphate. The temperature sensitivity depends on the hydrophobic–hydrophilic interaction of molecular chains, and the pH sensitivity depends on the electrostatic interaction of ionic groups (—NRH^{2+} and —OPO_3^{2-}). In addition, the chitosan/human-like-collagen/β-glycerophosphate hydrogel with crosslinking agent of carbodiimide is prepared and the properties are also examined (Li et al., 2013). The results show that the gelation time, structure, equilibrium swelling and degradation in vitro and in vivo are dependent upon crosslinking and structure of composite hydrogels. The hydrogel shows desirable gelling time, swelling ratio, smooth surface, regular porous networks and biodegradability. Furthermore, Li, Fan, Ma, et al. (2014) and Li, Fan, Zhu, et al. (2014) have prepared chitosan/human-like collagen/hyaluronic acid/β-glycerophosphate hydrogels based on the self-assembly of chitosan/human-like collagen/hyaluronic acid fibers. The results indicate that a new amide bond (—CONH—) and —NRH^{2+} are formed. The gelling time and swelling behaviors are dependent on the intertwining, overlap and adsorption of the polymer chains at various temperatures and pH values.

Furthermore, the biological properties of the chitosan/β-glycerophosphate system are investigated and the effects of different factors are analyzed. The effects of chitosan characteristics on the antibacterial activity, and cytotoxicity of chitosan/β-glycerophosphate/nanosilver hydrogels are studied (Chang et al., 2013). It is concluded that the hydrogel could be prepared with lower molecular weight chitosan and lower concentration of nanosilver in order to reduce the cytotoxicity of nanosilver, while maintaining similar antibacterial activity with the hydrogel prepared with higher concentration nanosilver and higher molecular weight chitosan. Then the effect of the self-assembly of chitosan/human-like collagen/hyaluronic acid fibers on the histocompatibility of the hydrogels is studied (Li, Fan, Ma, et al., 2014; Li, Fan, Zhu, et al., 2014) and the result reveals that the fibers inside the hydrogel pores reduce the quantity of macrophages, decrease the degree of inflammation, and improve the anti-degradation of the modified hydrogels.

2.2. Preparation and properties

A novel injectable in situ gelling thermosensitive chitosan/β-glycerophosphate formulation has been proposed in recent years (Chenite et al., 2000, 2001; Ruel-Gariépy, Hildgen, Gupta, & Leroux, 2002). First, chitosan and β-glycerophosphate solutions are prepared in deionized water. Second, the two solutions are chilled in an ice bath for 15 min. Then the β-glycerophosphate solution is added dropwise to the chitosan solution under stirring and the resulting mixture is stirred for another 10 min. The release of macromolecules from the system could sustain over a period of several hours to a few days while the release of low-molecular-weight hydrophilic compounds is generally completely within 24 h. The gelation rate and gel strength is slightly increased by the adding of liposome to the chitosan/β-glycerophosphate solution, and the liposome-chitosan/β-glycerophosphate system rapidly gels at body temperature (Ruel-Gariépy et al., 2002). The similar preparative method has been used to control the release of different model compounds (Ruel-Gariépy et al., 2000). Ruel-Gariepy et al. (2004) have proposed to use the chitosan thermosensitive hydrogel for the sustained release of paclitaxel at tumor resection sites in order to prevent local tumor recurrence.

The macro- and microstructure of chitosan/β-glycerophosphate systems is exosyndromed by rheology and electron paramagnetic resonance spectroscopy (Kempe et al., 2008). Increasing β-glycerophosphate concentration leads to a lowering of the gelation temperature from about 50 °C to 37 °C, and higher β-glycerophosphate concentrations lead to faster gelation. The results indicate that the insulin is incorporated into the aqueous environment of the gel and released in its native form. The viscosity of the gelled system is much higher compared to the sol systems of the same composition. The pH of chitosan/β-glycerophosphate solution is between 6.6 and 6.8 which do not change during gelation irrespective of the proportion of β-glycerophosphate. The gelation formation process does not change the microacidity inside the sample.

Sharma, Italia, Sonaje, Tikoo, and Ravi Kumar (2007) have developed a chitosan-based in situ gelling system for subcutaneous administration of ellagic acid. The ellagic acid/chitosan/β-glycerophosphate hydrogel system shows an initial burst release in vitro with about 85% drug releasing in 12 h followed by a steady release till 160 h. The data indicate that formulations are effective against cyclosporine induced nephrotoxicity, where the ellagic acid/chitosan/β-glycerophosphate hydrogel shows activity at 10 times lower dose compared to orally given ellagic acid. So the bioavailability of ellagic acid can be improved by subcutaneous formulations administered as simple ellagic acid hydrogel. Kim et al. (2010) have reported a thermosensitive chitosan hydrogel for the delivery of ellagic acid for the treatment of brain cancer. The gelation temperature and time are affected by the final pH of the chitosan/β-glycerophosphate solution. Dialyzed chitosan solution with final pH 6.3 greatly reduces β-glycerophosphate needed for gelation, thereby significantly improves the biocompatibility of gel. The chitosan gels containing 1% (w/v) of ellagic acid significantly reduces viability of U87 cells and C6 cells compared with the chitosan gels at 3 days incubation.

Cui et al. (2011) have fabricated bioabsorbable chitosan/β-glycerophosphate composite membranes through a relatively pH neutral and mild sol-to-gel process for guided bone regeneration. The results show that the chitosan/β-glycerophosphate composite membranes have a porous structure both at the surface and in sub-layers and the incorporation of β-glycerophosphate in the chitosan matrix decreases the initial tensile strength of the membrane. The concentration of β-glycerophosphate is proportional to the pore size and thickness but is inversely proportional to the tensile strength of the chitosan/β-glycerophosphate membrane.

Hydrogel beads are proposed with two sequential gelation steps based on chitosan/β-glycerophosphate system and the model drug/cell (dexamethasone and L929 cells) are immobilized inside (Lima, Correia, Oliveira, & Mano, 2014). Superhydrophobic surfaces are used to produce the spherical hydrogel particles that provided favorable conditions to encapsulate cells or bioactive agents. First, the chitosan acidic solution is neutralized with β-glycerophosphate at room temperature to pH 6.2. Suspended cells (or dexamethasone) in the formulation are dispensed in controlled volumes onto biomimetic polystyrene superhydrophobic surfaces, to form spherical shapes. The addition of sodium tripolyphosphate on the top of each sphere induces an ionic gelation process of the chitosan through electrostatic interactions. At 37 °C, the hydrophobicity of the chitosan/β-glycerophosphate system increases and a second gelation step occurs, which increase the elastic modulus. The softness and flexibility of the system can potentially be utilized to implant cells and therapeutic molecules using less invasive procedures.

Recent studies have been reported that the physical properties including flexibility and mechanical properties of CS/β-GP hydrogel can be improved by blending chitosan with other materials. Ngoenkam, Faikrua, Yasothornsrikul, and Viyoch (2010) have developed a thermosensitive hydrogel which forms gel rapidly by blending chitosan/β-glycerophosphate solution with the pregelatinized starch. The presence of starch in the system could increase the water absorption and average pore size of the hydrogels. The hydrogel could degrade in lysozyme slowly during 56 days of incubation. In vivo studies indicate that the hydrogel shows rapid formation and localization at the injection site (shown as Fig. 4) and has a potential to stabilize chondrocytes which is characterized by the expression of type II collagen mRNA and protein. Sun, Jiang, Wang, and Ding (2012) have reported that hydrophilic polymer poly(vinyl alcohol) is blended into the thermosensitive hydrogel composed of chitosan and glycerophosphate to mitigate the body responses and promote the drug bioavailability. The results show that the presence of poly(vinyl alcohol) improves the surface hydrophilicity of the hydrogel and inhibits the cell attachment on the hydrogel, which alleviates the further cell infiltration and tissue integration in body. In addition, the presence of poly(vinyl alcohol) leads to the more rapid gel formation and more compact network, which resisted the dehydration and survived the hydrogel from cell division. These advantages benefit the controlled release and absorption of cyclosporine A, and contribute to the higher drug bioavailability.

Fatimi et al. (2012) have developed the chitosan/β-glycerophosphate hydrogel which is injectable, radiopaque and contains sodium tetradecyl sulfate, a well-known sclerosing agent, in order to combine blood flow occlusion and endothelium ablation properties. The hydrogel is shown to exhibit rapid gelation and good mechanical properties, as well as sclerosing properties. The preliminary animal study shows that no endoleak is detected in any of the three aneurysms treated with chitosan/sodium tetradecyl sulfate/β-glycerophosphate hydrogel at 3 months. Generally, the prepared hydrogels have great potential as embolizing and sclerosing agents for endovascular aneurysm repair and possibly other endovascular therapies. Then chitosan radiopaque hydrogels are prepared using chitosan, β-glycerophosphate, iopamidol, and different sodium tetradecyl sulfate concentrations to investigate whether embolization with chitosan/β-glycerophosphate hydrogel with or without a sclerosant can induce chemical endothelial ablation and prevent endothelial recanalization in a rabbit model (Chabrot et al., 2012). It is concluded that the viscosity obtained with chitosan, β-glycerophosphate, and 3% sodium tetradecyl sulfate permits better control during injection and longer vascular occlusion. These findings, combined with the intravascular neovascularization observed with the chitosan/β-glycerophosphate

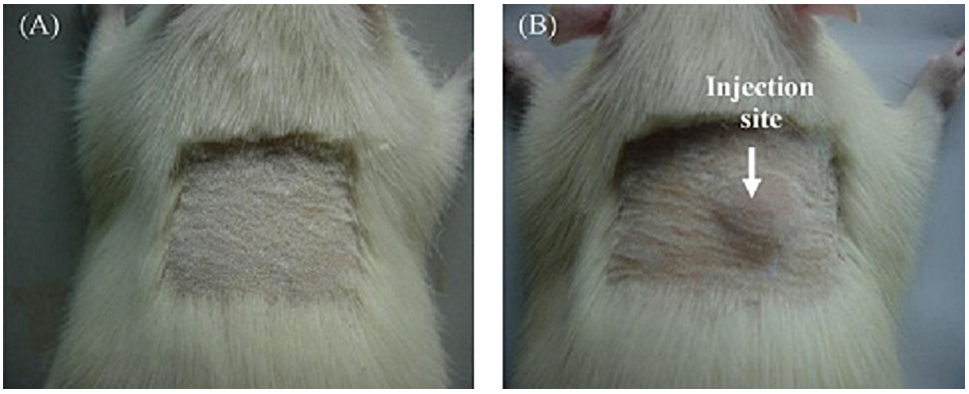

Fig. 4. In vivo formation of gel after subcutaneous injection of the starch CS/β-GP hydrogel on the back of Sprague-Dawley rat: before (A) and after 1 day (B) of administration. Figure was partly reprinted from Ngoenkam et al. (2010).

hydrogel, led to prefer the combination with sodium tetradecyl sulfate.

Then thermosensitive chitosan/β-glycerophosphate hydrogels are prepared by crosslinked with β-glycerophosphate and reinforced via physical interactions with β-tricalcium phosphate (Dessi, Borzacchiello, Mohamed, Abdel-Fattah, & Ambrosio, 2013). The kinetics of sol-to-gel transition and the composite hydrogel properties are investigated by rheological analysis. The hydrogels exhibit a gel-phase transition at body temperature, and a three-dimensional network with typical rheological properties of a strong gel. Mirahmadi, Tafazzoli-Shadpour, Shokrgozar, and Bonakdar (2013) have added degummed chopped silk fibers and electrospun silk fibers to the thermosensitive chitosan/β-glycerophosphate hydrogels to reinforce the hydrogel constructs. The results show that mechanical properties of the hydrogel are significantly enhanced when a hybrid with two layers of electrospun silk fibers is made. The mechanical characteristics of these composites may be further improved by the number of fiber layers in the laminate, combination of both degummed fibers and nanofiber sheets, crosslinking of hydrogels and silk fibers by nontoxic agent during gelation, content and surface treatment of fibers, control of water uptake of the hydrogel and fiber orientation. Coutu, Fatimi, Berrahmoune, Soulez, and Lerouge (2013) have developed a new injectable radiopaque embolizing agent based on chitosan thermogelling properties by associating different commercial contrast agents (Isovue (R), Visipaque (R), and Conray (R)) with chitosan/β-glycerophosphate. Addition of contrast agents does not prevent gelation at body temperature, whereas it significantly increases the viscosity of the solution before gelation, delays gelation, and reduces the gelation rate. In vitro studies have demonstrated rapid release of contrast agents from hydrogels when immersed in a saline solution (>50% within 4 h) which is suitable for embolization.

Giuseppe Molinaro, Jacques, and Albert (2002) have studied biocompatibility of thermosensitive chitosan/β-glycerophosphate hydrogel. The results show that the inflammatory response is dependent on the injection site. Subcutaneous injections in the rat hindpaw yield an acute edematous response, which is substantially more pronounced than that observed following the transdermal injection in the rat dorsal region, due to differences in blood flow, tight geometry of injection space, etc. Chitosan chain with higher deacetylation degree shows mild inflammation and minimal osteogenesis and seems to be desirable for superior biocompatibility. Cheng, Yang, Liu, Gefen, and Lin (2013) have developed ferulic acid-gelatin and chitosan/β-glycerophosphate hydrogel which is biocompatible and can prolong the release period of ferulic acid. From the results of mRNA gene expression, the hydrogel may treat H_2O_2-induced oxidative stress nucleus pulposus cells through down-regulation of matrix metalloproteinase-3 and up-regulation aggrecan and collagen-II. The sulfated-glycosaminoglycan production of 100 μM H_2O_2-induced oxidative stress nucleus pulposus cells can be increased to the normal level in the post-treatment of hydrogel group. Then the chitosan hydrogels containing β-glycerophosphate are functionalized by incorporation of alkaline phosphatase, an enzyme involved in mineralization of bone (Douglas et al., 2013). The results demonstrate that alkaline phosphatase accelerates formation of thermosensitive chitosan/β-glycerophosphate hydrogels and induces their mineralization with calcium phosphate, which paves the way for applications as injectable bone replacement materials.

An injectable thermosensitive double crosslinking hydrogel based on thiolated chitosan and β-glycerophosphate is prepared by combining physical and chemical crosslinking (Chen, Dong, Yang, & Deng, 2012). By the physical interaction of thiolated chitosan and β-glycerophosphate, the hydrogel undergoes a fast gelation at body temperature within 2 min. In addition, the hydrogel contains a low concentration of β-glycerophosphate, which significantly decreases the toxicity of the gel. Owing to the chemical crosslinking of disulfide bonds, the gel is durable and possesses high-mechanical strength without introducing any potential cytotoxicity. The integrity of the hydrogel maintains for more than 30 days both in vitro and in vivo, and the interior morphology reveals that the gel has interconnected porous network structure. In vitro cytotoxicity, hemolysis, and histopathological analysis reveal that the gel is biocompatible. To improve the biocompatibility and application properties of injectable chitosan/β-glycerophosphate hydrogel, an injectable triple crosslinking network hydrogel is prepared by physical interaction, Michael addition and disulfide bond formation based on thiolated chitosan, β-glycerophosphate and poly(ethylene glycol) diacrylate without the addition of cytotoxic crosslinkers and catalysts (Chen, Wang, Deng, Hu, & Dong, 2013). It is found that the triple crosslinking network hydrogel containing different molecular weight of poly(ethylene glycol) diacrylate exhibits controllable gelation times from 1–22 min, which could meet the different demands in clinical application. The presence of poly(ethylene glycol) diacrylate in the triple crosslinking network hydrogel imparts better swelling property, and there is a sustained protein release from the hydrogel without any initial burst.

In order to get a water-soluble in situ gel-forming system, a thiolated chitosan (chitosan-4-thio-butylamidine conjugate) is synthesized and used to replace the unmodified chitosan in the application of the in situ gel-forming system (Liu, Zang, et al., 2014; Liu, Chen, et al., 2014). A thermosensitive hydrogel was prepared based on chitosan-4-thio-butylamidine conjugate, hydroxyapatite, and β-glycerophosphate. The chitosan-4-thio-butylamidine conjugate/hydroxyapatite/β-glycerophosphate hydrogel shows a higher storage modulus (G') and loss modulus (G'') and a decreased

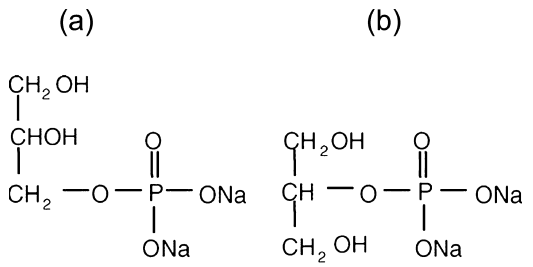

Fig. 5. Molecular structure of (a) α-GP and (b) β-GP.

bovine serum albumin release rate which is maintained the protein release for a longer time compared with the unmodified chitosan/hydroxyapatite/β-glycerophosphate hydrogel, due to the existence of thiol groups and/or disulfide bonds. The chitosan-4-thio-butylamidine conjugate/hydroxyapatite/β-glycerophosphate gel has a porous structure with a uniform distribution of nano-hydroxyapatite, an appropriate degradation rate and low cytotoxicity.

3. Chitosan/αβ-glycerophosphate hydrogels

3.1. Development process and gelatinize mechanism

αβ-glycerophosphate is the mixture of α-glycerophosphate and β-glycerophosphate. Wu, Su, and Ma (2006) have reported a hydrogel composed of quaternized chitosan and β-glycerophosphate or αβ-glycerophosphate. It is observed that quaternized chitosan can form thermosensitive hydrogels not only with β-glycerophosphate, but also with αβ-glycerophosphate which shows similar thermosensitivity. The molecular structures of α-glycerophosphate and β-glycerophosphate are sketched in Fig. 5. α-glycerophosphate has linear chain structure and shows less steric hindrance than β-glycerophosphate that hydrophobic interaction force between chitosan molecules is formed more easily. So αβ-glycerophosphate may have better gelation capacity compared with β-glycerophosphate. With the increase of αβ-glycerophosphate amount, more quaternary amino groups are combined with glycerophosphate by ionic interaction. As a result, electrostatic repulsive force between quaternary amino groups is weakened, and the aggregation of polymer chains forms more easily. Therefore, the gelation time is markedly shortened. The gelation time is also affected by the quaternized chitosan concentration and becomes shorter at higher quaternized chitosan concentration. However, if the concentration is too high, quaternized chitosan is hard to dissolve completely and the solution is too viscous to be injected by syringe. Comparison between chitosan and quaternized chitosan hydrogel is also practiced. Drug is released from chitosan/αβ-glycerophosphate hydrogel with high initial release rate at both acidic and basic circumstances without apparent pH-sensitivity. Release from quaternized chitosan/αβ-glycerophosphate hydrogel shows better pH-sensitivity. The trapped drug is released quickly in acidic condition and quite slowly with low initial release in basic condition. The quaternized chitosan/αβ-glycerophosphate hydrogel is subsequently evaluated for intranasal vaccine delivery with adenovirus based Zaire Ebola virus glycoprotein antigen (Wu, Wu, et al., 2012). Results show that moderate quarternized chitosan/αβ-glycerophosphate hydrogel/antigen formulations induce highest IgG, IgG1, and IgG2a antibody titers in serum, as well as mucosal IgA responses in lung wash, which may be attributed to the prolonged antigen residence time due to the thermal sensitivity of the hydrogel. The same research progress has been practiced on quarternized chitosan-polyethylene glycol/αβ-glycerophosphate hydrogel (Wu, Wei, Wang, Su, & Ma, 2007) and the results show that quarternized chitosan-polyethylene glycol/αβ-glycerophosphate formulation can be used as nasal drug delivery system to improve the absorption of hydrophilic macromolecular drugs.

3.2. Preparation and properties

Zhao, Ji, et al., 2009; Zhou, Chen, et al., 2009have developed a preparation of chitosan/αβ-glycerophosphate thermosensitive hydrogel. In simple terms, chitosan is added into the acetic acid/sodium acetate buffer solution, with stirring until it dissolves thoroughly. Then, the chitosan solution is chilled in ice bath for 20 min. A 50% (w/v) αβ-glycerophosphate aqueous solution is prepared in distilled water and chilled along with the chitosan solution in ice bath. Predetermined value of αβ-glycerophosphate solution is added dropwise to the chitosan solution with stirring, and the final chitosan/αβ-glycerophosphate solution is mixed for another 20 min. The thermosensitive characteristics, appearance, and structure of the hydrogel are all affected by the pH, ionic strength, chitosan/aβ-glycerophosphate ratio and concentration, molecule weight, deacetylation degree of chitosan (Zhou, Chen, Kong, Liu, Cha, & Kennedy, 2008; Zhao, Ji, et al., 2009; Zhou, Chen, et al., 2009). The hydrogel is stable for at least 3 months at 4 °C and the appearance of the hydrogel become more compact and regular with increasing concentration and molecule weight of chitosan. The hydrophilic model drug adriamycin is released 60-70% over 24 h which is slower than that of the hydrophobic model 6-mercaptopurine. Biocompatibility research (Zhou, Zhang, Zhang & Chen, 2011) shows that the injected chitosan/aβ-glycerophosphate hydrogel don't produce any significant changes in the haematology of SD rats such as white blood cell, red blood cell, platelet and the volume of haemoglobin. In addition, the results show that the level of serum alanine aminotransferase, blood ureic nitrogen and creatinine has no obvious changes in SD rats of different groups. The morphology of the implanted gel shows that the delivery system could sustain a relatively long time in vivo and could be degraded gradually with time lasting. The parameters, gel temperature and gel time of chitosan/glycerophosphate hydrogels from some references are shown in Table 1.

Zhao, Ji, et al. (2009) and Zhou, Chen, et al. (2009) have prepared thermosensitive hydrogels of chitosan and αβ-glycerophosphate. The formed hydrogels are dried at room temperature to form films. The hydrogel films show better elasticity and lower tensile strength comparing with pure chitosan films. Scanning electron microscope reveals that the hydrogel films have rough surfaces and porous structure with interconnected pores. The pore diameters depend on the chitosan/aβ-glycerophosphate ratio. The gelation temperature decreases by increasing αβ-glycerophosphate content. Degradation experiments show that the degradation behaviors of these hydrogels via bulk erosion are relied heavily on the chitosan/αβ-glycerophosphate ratio (Dang, Yan, Li, Cheng, Liu, & Chen, 2011).

Dang et al. (2012b) has investigated the influences of different solvents on highly porous chitosan/αβ-glycerophosphate hydrogel by monitoring the characterizations of the hydrogel. The results of rheological analysis show that all the chitosan dissolved in different solvents used in the study could transform into a highly porous three-dimensional hydrogel at a certain temperature when neutralized by αβ-glycerophosphate solution. The SEM photographs show that the aperture size and shape of the pores are related to solvent variety and solvent strength. The cytocompatibility and in vivo injection experiments demonstrates that the hydrogels prepared with acetic acid, hydrochloric acid, or lactic acid as the solution of chitosan have low toxicity and high histocompatibility. The results suggest that the gelation temperatures, pH values, turbidity, complex viscosities, and porous structures of the hydrogels for certain

experimental applications could be tailored by adjusting the solvent variety and solvent strength of the chitosan solution.

Based on the basic quality research of chitosan/αβ-glycerophosphate hydrogel, Dang et al. (2012a) has developed a hydrogel with low gelation temperature by changing the solvent strength of chitosan solution and the chitosan/αβ-glycerophosphate ratio. The gelation time of this hydrogel is 90s at 25 °C and 30s at 37 °C. In vitro study shows that the hydrogel has low protein adsorption and cytotoxicity. The surface and interior morphology of the hydrogel displays a highly porous structure and makes it a potential 3D culture scaffold for cells. Wu, Wei, et al. (2012) have developed a thermal-sensitive hydrogel with quaternized chitosan and αβ-glycerophosphate intranasal vaccine delivery system for H_5N_1 influenza immunization. The solution of quaternized chitosan/glycerophosphate could gelate rapidly at body temperature, and significantly prolong the H_5N_1 split antigen residence time in nasal cavity.

4. Biomedical applications of chitosan/glycerophosphate based hydrogels

Hydrogels make a volume change with water filled in the cavity of networks or drained by the shrinkage of networks accompanying with the variation of the extra condition, such as pH, temperature and ion (Lai, Li, & Luo, 2010; Liu & Lin, 2010). This stimuli-responsive property makes them have many potential applications, such as smart drug delivery systems. Drugs release by diffusion through the porous structure under specific stimulation. The biochemical properties and the three-dimensional networks of chitosan hydrogel offer them wide applications in biomedical field including local drug delivery and tissue engineering.

4.1. Local drug delivery

The oral administration of therapeutics leads to internalization by the body at the mouth, stomach, small intestine, or colon. Using drug delivery systems, clinicians can specifically target these different tissue systems for local drug action within the gastrointestinal tract, or achieve drug delivery to the vasculature through the expansive capillary beds of the small intestine (Bhattarai et al., 2010). Due to the advantages of pH sensitivity and mucoadhesive properties, chitosan/glycerophosphate based hydrogels are effectively used in delivery of proteins/peptides, growth factors, anti-inflammatory drugs, antibiotics, as well as, in gene therapy application.

Peng, Sun, et al. (2013) and Peng, Li, et al. (2013) have prepared chitosan/αβ-glycerophosphate thermosensitive hydrogels for the sustained delivery of venlafaxine hydrochloride and studied the optimization of the formulation. Release mechanism is investigated by applying various mathematical models to the in vitro release profiles. Overall, drug release from the hydrogel shows best fit in first-order model and drug release mechanism is diffusion-controlled release. The results present that higher strength and glycerophosphate concentration result in higher initial release and rate constant, which support the hypothesis that the kinetic gelation mechanism of this system is nucleation and growth. Furthermore, biodegradable poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) nanoparticles containing insulin phospholipid complex are loaded in chitosan/β-glycerophosphate hydrogels for long-term sustained and controlled delivery of insulin (Peng, Sun, et al., 2013 and Peng, Li, et al., 2013). In the in vitro release studies, only 19.11% of total insulin is released from the nanoparticle-loaded hydrogel within 31 days. Most importantly, the hypoglycemic effect of nanoparticle-loaded hydrogel following subcutaneous injection in diabetic rats lasts for >5 days, much longer than the effect caused by free insulin-loaded chitosan/β-glycerophosphate or other long-acting insulin formulations.

The drug release process from hydrogel is affected by composition of chitosan/glycerophosphate solution, pH, temperature as well as the nature of drug. Huang et al. (2009) have developed a thermosensitive hydrogel system for local delivery of ^{188}Re colloid drugs. Solution of chitosan/β-glycerophosphate mixed with ^{188}Re–Tin colloid and free $^{188}ReO_4^-$ is injected through intramuscular to two groups of rats, respectively. Scintigraphic images show that chitosan/β-glycerophosphate hydrogel is capable of immobilizing the ^{188}Re-Tin colloid locally at the injection site with no diffused radioactivity for up to 48 h post injection (Fig. 6). In addition, the chitosan/β-glycerophosphate hydrogel is induced as an implanted carrier to combine the Re-188–Tin colloid and doxorubicin as a treatment strategy (Huang et al., 2014). The results of the biodistribution and scintigraphy studies show the highest hydrogel uptakes in the tumor at different time points, as well as localized radioactivities for a certain time.

Zan et al. (2006) find that the properties of chitosan/β-glycerophosphate hydrogel influence the release profiles of a hydrophilic drug-5-fluorouracil. There is a rapid initial release of the drug because of the hydrophilic environment induced by the large “pores” occurred during the gel-forming process. Once the drug is captured into a scaffold such as poly-3-hydroxybutyrate, the initial release decreases from 85% to 29%, and the release could sustain for about 10 months. Then, sterile chitosan/β-glycerophosphate hydrogels are developed for intraperitoneal delivery of the antineoplastic agent 5-fluorouracil (Depani, Naik, & Nair, 2013). The formulations provide prolonged release of the drug. Tumor volume measurements shows comparable efficacy of 5-fluorouracil administered as gel and commercial injection with a greatly improved safety profile of the former as adjudged from mortality and body weight measurements.

The effectiveness of chitosan as mucoadhesive agent is impaired by its insolubility at pH above 6 (for example at the physiological pH of the buccal cavity and nasal cavity). So chitosan derivatives like methyl pyrrolidinone chitosan and N-trimethyl chitosan are synthesized. Methyl pyrrolidinone chitosan and N-trimethyl chitosan are mixed with β-glycerophosphate and prepared into hydrogel, respectively, and investigated their use in buccal administration of benzydamine hydrochloride in the treatment of oral mucositis (Rossi et al., 2010). Release and wash away measurements prove that the formulation based on N-trimethyl chitosan/β-glycerophosphate hydrogel is able to control the release of benzydamine and to withstand the physiological removal mechanism. Mixing the N-trimethyl chitosan solution with polyethylene glycol and αβ-glycerophosphate, the formation hydrogel is also used for nasal drug delivery (Nazar et al., 2011). The hydrogel exhibits good affinity for mucosal surfaces.

The burst release of loaded drug is a commonly observed problem and drug release of small hydrophilic molecules is often completed in less than 24 h. To solve this problem, the drug is encapsulated into carriers and subsequently embedded in the thermo-gelling matrix (Ruel-Gariépy & Leroux, 2004; Zhang, Jo Lewis, & Chu, 2005). Hsiao et al. (2012) have proposed a chitosan based nanoscale drug-carriers by amphiphilic chitosan, carboxymethyl-hexanoyl chitosan. The carboxymethyl-hexanoyl chitosan demonstrates self-assembly into nanocapsules about 200 nm in size in aqueous environment, and forms thermo-gelling solution when mixed with glycerol and β-glycerophosphate. The hydrogel is demonstrated to be a depot drug delivery system in vivo through the therapeutic effect of ethosuximide, suppressing spike wave discharges in Long Evan rat model.

In order to enhance the targeting of the hydrogel, Zhang et al. (2013) have developed a magnetic thermosensitive hydrogel as intravesical Bacillus Calmette-Guérin delivery system which is

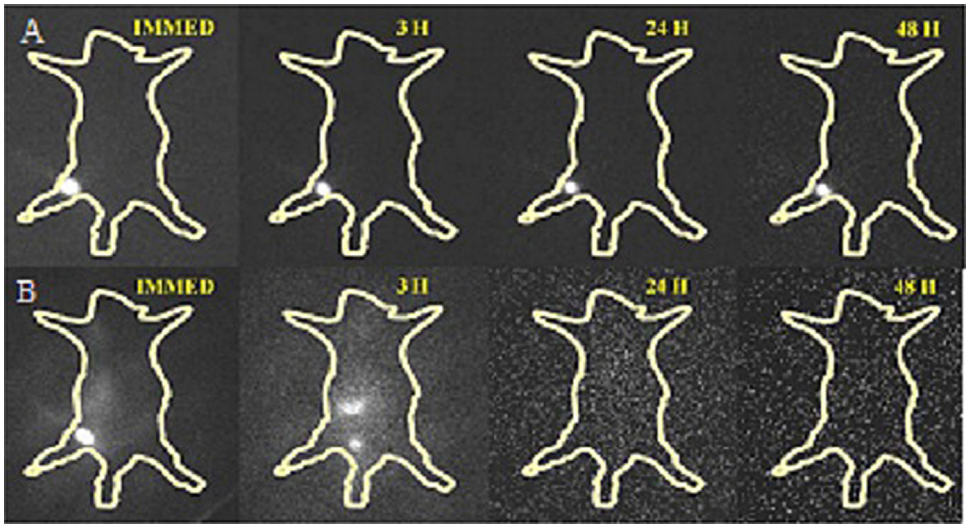

Fig. 6. The scintigraphic images of rats at 0, 3, 24 and 48 h after IM injection at the site of the hind limb: (A) with C/GP/188Re-Tin colloid, (B) with C/GP/188ReO^{4-}. Figure was reprinted from Huang et al. (2009).

formulated with chitosan, β-glycerophosphate and Fe_3O_4 magnetic nanoparticle. The gel permits an intravesical continuous release of Bacillus Calmettee-Guérin over the period of 48 h in the presence of magnetic field. Sustained delivery of Bacillus Calmettee-Guérin markedly increases the antitumor efficacy and induces a high local immunity in bladder.

Alinaghi, Rouini, Daha, and Moghimi (2013) have developed a novel delivery concept based on the integration of liposomes in chitosan/β-glycerophosphate hydrogel for the controlled release of liposomes. The results show that free label leave the peritoneal cavity rapidly in both solution and hydrogel forms, such that the activity is 2.5 and 3.8 (%ID) after 1 h, respectively. The values for liposomes and liposomal hydrogel are 25.8 and 51.2 (%ID) and decrease to 1.9 and 19.2 after 24 h, respectively. Furthermore, it is studied about the influence of lipid composition and surface charge on biodistribution of intact liposomes releasing from hydrogel-embedded vesicles (Alinaghi, Rouini, Johari Daha, & Moghimi, 2014). Interesting results are observed in that the hydrogel could reverse the peritoneal retention of negatively charged liposomes, increasing to eight times its area under the curve (AUC) value, to attain the highest amount among all formulations.

Lajud et al. (2013) have developed an inner ear drug delivery system using chitosan/β-glycerophosphate hydrogel to evaluate the effectiveness of chitosanase as a "switch off" mechanism for the drug delivery system when side effects and potential ototoxicities appear during treatment. The results show that the chitosanase effectively digested the chitosan/β-glycerophosphate hydrogel, quickly releasing gentamicin and Texas Red-labeled gentamicin from the system in vitro. The use of chitosanase to digest the chitosan/β-glycerophosphate hydrogel results in the rerouting of the loaded drug away from the round window niche, effectively down-regulating its delivery to the inner ear. This important modification to the drug delivery system has the ability to deliver therapy to the inner ear until desired effect is achieved and to stop this process when side effects or treatment-related ototoxicities start to occur, providing a novel and salient approach for safe and effective delivery to the inner ear.

4.2. Tissue engineering applications

Tissue engineering is the technology of remodeling of living organisms in vitro and involves architecture of artificial cellular scaffolds, which mimic extracellular matrix. It is a highly interdisciplinary field that combines life science and material science. The materials used in tissue engineering must be biocompatible, non-toxic and have proper mechanical property.

Being an osteogenic medium supplement, β-glycerophosphate is used in bone tissue engineering in form of hydrogel with chitosan. Tissue-engineered cartilage reconstructions are made in vitro by mixing sheep chondrocytes with a chitosan hydrogel, which is prepared by combining chitosan, β-glycerophosphate and hydroxyethyl cellulose (Hao et al., 2010). The results show that the chondrocytes in the reconstructed cartilage could survive and retain their ability to secrete matrix being cultured in vitro. Transplanted in vivo, the reconstructions repair cartilage defects completely within 24 weeks. Naderi-Meshkin et al. (2014) have reported chitosan/β-glycerophosphate/hydroxyethyl cellulose hydrogel included chondrogenic factors or mesenchymal stem cells, and injected into the site of injury to fill the cartilage tissue defects with minimal invasion and pain.

Combining with human mesenchymal stem cells and desferrioxamine, chitosan/β-glycerophosphate gel acts as a multimodal pro-angiogenic therapeutic for the treatment of critical limb ischemia (Hastings, Kelly, Murphy, Barry, O'Brien, & Duffy, 2012). The combination results in a synergistic enhancement in bioactivity, as measured by increased vascular endothelial growth factor expression in gel exposed human umbilical vein endothelial cells. Richardson, Hughes, Hunt, Freemont, and Hoyland (2008) have cultured human mesenchymal stem cells in chitosan/glycerophosphate hydrogel which have been used clinically for the regeneration of the degenerate human intervertebral disc. Gene expression analysis for chondrocytic-cell marker genes demonstrates differentiation of human mesenchymal stem cells to a phenotype which showed similarities to both articular chondrocytes and nucleus pulposus cells. Then the injectable chitosan/β-glycerophosphate hydrogels have been prepared with dexamethasone/recombinant human bone morphogenetic protein loaded hydroxyapatite nanoparticles (Gao, Cai, Kong, Han, & Yao, 2013). The results show that hydroxyapatite nanoparticles could be dispersed homogeneously in the matrix of chitosan/β-glycerophosphate hydrogels and the combination of dexamethasone and recombinant human bone morphogenetic protein could promote the proliferation and osteoblastic differentiation of mesenchymal stem cells.

The chitosan/β-glycerophosphate hydrogel containing collagen is prepared and its biocompatibility and effects on the osteogenic

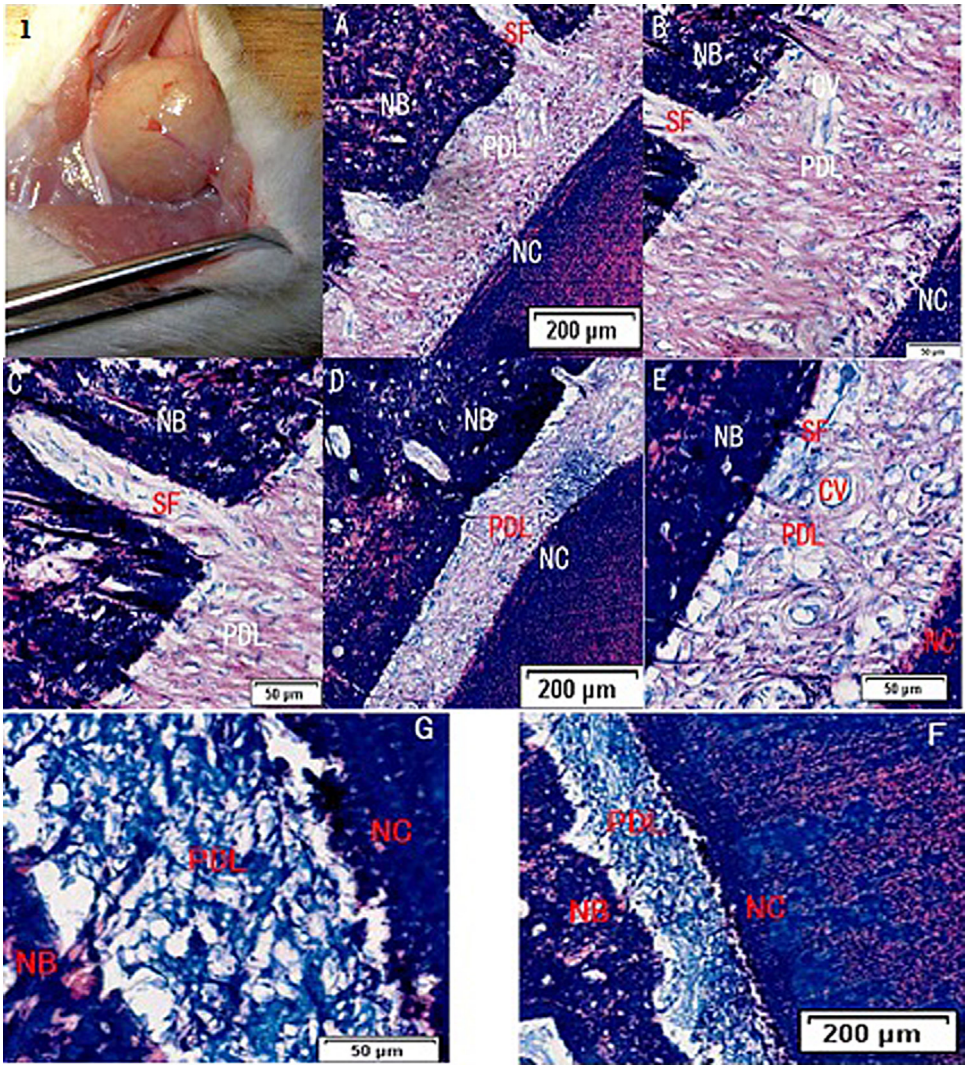

Fig. 7. (1) The CS-HTCC/GP aqueous solution formed a round gel-like plug at the injection site. A–F: Histological examination of periodontal tissue regeneration in different groups over 8 weeks post-surgery. NB, new bone; NC, new cementum; PDL, new periodontal ligaments; SF, Sharpey's fibers; CV, capillary vessel (A–C: CS-HTCC/GP-bFGF group; D, E: CS-HTCC/GP group; F,G: negative control group). Figure was reproduced from Ji et al. (2010).

differentiation of mesenchymal stem cells are evaluated (Ding, Zhang, Yang, & Xu, 2013). The results prove that the hydrogel exhibits good biocompatibility and enhances the in vitro osteogenic differentiation of mesenchymal stem cells. In the experiment of ectopic bone formation, the hydrogel demonstrates its capability of supporting neovascularization and differentiation of mesenchymal stem cells toward osteogenic lineage.

Wang and Stegemann (2010) have encapsulated adult human bone marrow-derived stem cells in hydrogels composed of chitosan, collagen and β-glycerophosphate. The cells exhibit high viability at 1 day after encapsulation, but DNA content dropped by about half over 12 days in pure chitosan materials while it increased twofold in materials containing collagen. The presence of chitosan in materials results in higher expression of osterix and bone sialoprotein genes in medium with and without osteogenic supplements. Chitosan also increases alkaline phosphatase activity and calcium deposition in osteogenic medium. Then Wang and Stegemann (2011) have embedded human bone marrow stem cells in chitosan/collagen materials by initiating gelation using β-glycerophosphate at physiological temperature and pH, examined the use of glyoxal to crosslink these materials, and characterized the resulting changes in matrix and cell properties. The results reveal that glyoxal is cytocompatible at concentrations below 1 mM for periods of exposure up to 15 h, though the degree of cell spreading and proliferation are dependent on matrix composition.

Sun et al. (2011) has added type I collagen to the composite chitosan solution in a ratio of 1/2 to build a physical cross-linked self-forming chitosan-collagen/β-glycerophosphate hydrogel. SEM observations indicate good spreading of bone marrow mesenchymal stem cells in this hydrogel scaffold. Mineral nodules are found in the dog-bone marrow mesenchymal stem cells inoculated hydrogel by SEM after 28 days. After subcutaneous injection into nude mouse dorsum for 4 weeks, partial bone formation is observed in the chitosan-collagen/β-glycerophosphate hydrogel loaded with pre-osteodifferentiated dog-bone marrow mesenchymal stem cells. Mirahmadi et al. (2013) have fabricated chitosan/β-glycerophosphate hydrogel containing two forms of silk fiber used as scaffold for hyaline cartilage regeneration. The results of glycosaminoglycan and collagen type II in cell-seeded scaffolds indicate support of the chondrogenic phenotype for chondrocytes with a significant increase in degummed silk fiber-hydrogel composite for glycosaminoglycan content and in two-layer electrospun fiber-hydrogel composite for collagen type II.

In addition, the thermosensitive and biomimetic gel scaffold is prepared from chitosan, β-glycerophosphate, nano-hydroxyapatite and collagen (Huang, Yu, Feng, Li, Chen, & Luo,

2011). The formed gel acts as a biocompatible substrate for the proliferation of rat bone marrow stem cells in vitro. The injected cells survive in the chitosan/β-glycerophosphate/nano-hydroxyapatite/collagen gel for 28 days in vivo. The gels loaded rat bone marrow stem cells induce less inflammatory reaction in the host tissue than that of the gels without rat bone marrow stem cells.

Then the ability of four gel-forming copolymers to act as in situ dermal fillers in plastic and reconstruction surgery is studied (Li, Ma, Fan, & Zhu, 2012). The four hydrogels are based on chitosan and β-glycerophosphate, included chitosan/β-glycerophosphate, chitosan-human-like-collagen/β-glycerophosphate, chitosan-animal-derived-collagen/β-glycerophosphate and chitosan-gelatin/β-glycerophosphate. The MTT assay shows that the chitosan-human like collagen/β-glycerophosphate hydrogel is less cytotoxic and could promote marrow stromal cell proliferation better than other hydrogels. Furthermore, the chitosan-human-like-collagen/β-glycerophosphate hydrogel rapidly forms a stable gel which maintains its integrity even after 6 weeks in vivo. And abnormalities of cell morphology are observed in chitosan-animal-derived-collagen/β-glycerophosphate and chitosan-gelatin/β-glycerophosphate hydrogels whereas cell morphology in the chitosan-human-like-collagen/β-glycerophosphate hydrogel is regular.

Niranjan et al. (2013) have reported the preparation and characterization of a thermosensitive hydrogel containing zinc, chitosan and β-glycerophosphate for bone tissue engineering. The developed thermosensitive zinc-chitosan/β-glycerophosphate hydrogel exhibits geometrically well-defined pores with crystalline nature and shows swelling ability. The hydrogel enhances antibacterial activity and promoted osteoblast differentiation.

Chitosan/glycerophosphate based hydrogels provide a suitable 3D scaffolding environment for cells cultivation. A polylysine-functionalised thermoresponsive chitosan/β-glycerophosphate hydrogel has been constructed for cell adhesion and neurite outgrowth (Crompton et al., 2007). Chitosan/β-glycerophosphate has good cell adhesion properties and good neuron compatibility at low concentrations. Neuron survival is improved with the covalent attachment of polylysine to chitosan: cell survival doubled although neurite outgrowth did not change. Kwon et al. (2012) use a chitosan/glycerophosphate hydrogel as a 3D substrate for the attachment, proliferation, and differentiation of rat muscle-derived stem cells. In the presence of valproic acid, rat muscle-derived stem cells exhibit higher expression of the neural markers, neuron-specific enolase and β-tubulin III, the oligodendrocyte marker, oligodendrocyte transcription factor 2, and the astrocyte marker, glial fibrillary acidic protein than those in the absence of valproic acid.

Chitosan/glycerophosphate hydrogel's tissue engineering potential is represented in many other respects. Ji et al. (2010) have developed an injectable hydrogel composed of chitosan, quaternized chitosan and αβ-glycerophosphate. The hydrogel is found to have no toxic effects and could promote alkaline phosphatase activity in human periodontal ligament cells in vitro. Being injected into the rumps of SD rats, the chitosan-quaternized chitosan/αβ-glycerophosphate aqueous solution is gelled rapidly due to the increasing temperature of the tissue fluid and formed a gel-like plug at the injection site (Fig. 7(1)) chitosan-quaternized chitosan/αβ-glycerophosphate and that of loading with basic fibroblast growth factor thermosensitive hydrogels are implanted into the periodontal defects of dogs to evaluate the regenerative activity. The histological findings are shown in Fig. 7. There is a significant amount of new bone and cementum, along with periodontal ligaments and corona growth alveolar bone in the chitosan-quaternized chitosan/αβ-glycerophosphate-basic fibroblast growth factor group.

Furthermore, Liu, Zang, et al. (2014) and Liu, Chen, et al. (2014) have synthesized a thermosensitive hydrogel composed of chitosan and αβ-glycerophosphate used as basic substrate for primary culture and subculture of lymphoid cells from shrimp, *Penaeus chinensis*. The peroxidase activity indicates that cells seeded on the hydrogel suffer less stress than cells suspended in control culture flasks. The higher relative viability and alkaline phosphatase activity of primary cultured lymphoid cells indicate that the chitosan/αβ-glycerophosphate hydrogel enhances the growth and proliferation of lymphoid cells.

5. Conclusion

Chitosan is the unique aminopolysaccharide in the nature which is non-toxic, biodegradable, biofunctional, biocompatible and has strong antimicrobial and antifungal activities. Glycerophosphate is an organic compound naturally found in the body which is usually used as a source of phosphate in the treatment of unbalance of phosphate metabolism. In this review, the recent advances in the development of chitosan thermosensitive hydrogels based on different glycerophosphate are summarized including preparation, characteristics, gelation mechanism and biomedical application. The thermosensitive hydrogel has been prepared with chitosan and β-glycerophosphate or αβ-glycerophosphate which is liquid at room temperature and solidifies into gel as temperature increasing to body temperature. The molecular mechanism of gelation may involve multiple interactions between chitosan, glycerophosphate, and water. The theory for block copolymers near their order–disorder transition is extended and proposed to precisely determine the gel point in such systems by considering the aggregation of hydrophobic acetylated blocks of chitosan chains and gelation in chitosan/glycerophosphate systems as an order–disorder transition of block copolymers. The solution behavior, rheological, physicochemical and biological properties, and gelation process of the hydrogel have been studied which is affected not only by the molecule weight, deacetylation degree and concentration of chitosan, but also by the kind and concentration of glycerophosphate. The biochemical properties and the three-dimensional networks of the hydrogel offer them wide applications in biomedical field including local drug delivery and tissue engineering. Furthermore, the hydrogels have been developed based on the integration of nanocapsules, liposomes, magnetic nanoparticle or combining with other materials such as collagen, silk fiber, nano-hydroxyapatite for the controlled release or cell proliferation and differentiation which showed great potential as matrices for drug/cell encapsulation and delivery, or as in situ gel-forming materials for tissue repair.

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References

Aliaghaie, M., Mirzadeh, H., Dashtimoghadam, E., & Taranejoo, S. (2012). Investigation of gelation mechanism of an injectable hydrogel based on chitosan by rheological measurements for a drug delivery application. *Soft Matter*, *8*(27), 7128–7137.

Alinaghi, A., Rouini, M. R., Daha, F. J., & Moghimi, H. R. (2013). Hydrogel-embeded vesicles, as a novel approach for prolonged release and delivery of liposome, in vitro and in vivo. *Journal of Liposome Research*, *23*(3), 235–243.

Alinaghi, A., Rouini, M. R., Johari Daha, F., & Moghimi, H. R. (2014). The influence of lipid composition and surface charge on biodistribution of intact liposomes releasing from hydrogel-embedded vesicles. *International Journal of Pharmaceutics*, *459*(1–2), 30–39.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

Buenger, D., Topuz, F., & Groll, J. (2012). Hydrogels in sensing applications. *Progress in Polymer Science*, *37*(12), 1678–1719.

Chabrot, P., Fatimi, A., Fraine, P. D., Ouchchane, L., Dauplat, M.-M., Rivard, A., et al. (2012). Embolization and endothelial ablation with chitosan and sodium sotradecol sulfate: Preliminary results in an animal model. *Journal of Endovascular Therapy*, *19*(3), 439–449.

Chang, H. W., Lin, Y. S., Tsai, Y. D., & Tsai, M. L. (2013). Effects of chitosan characteristics on the physicochemical properties, antibacterial activity, and cytotoxicity of chitosan/2-glycerophosphate/nanosilver hydrogels. *Journal of Applied Polymer Science*, *127*(1), 169–176.

Chen, C., Dong, A., Yang, J., & Deng, L. (2012). Preparation and properties of an injectable thermo-sensitive double crosslinking hydrogel based on thiolated chitosan/beta-glycerophosphate. *Journal of Materials Science*, *47*(5), 2509–2517.

Chen, C., Wang, L., Deng, L., Hu, R., & Dong, A. (2013). Performance optimization of injectable chitosan hydrogel by combining physical and chemical triple crosslinking structure. *Journal of Biomedical Materials Research Part A*, *101A*(3), 684–693.

Cheng, Y. H., Yang, S. H., Liu, C. C., Gefen, A., & Lin, F. H. (2013). Thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate. *Carbohydrate Polymers*, *92*(2), 1512–1519.

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C. D., et al. (2000). Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*, *21*, 2155–2161.

Chenite, A., Buschmann, M., Wang, D., Chaput, C., & Kandani, N. (2001). Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. *Carbohydrate Polymers*, *46*, 39–47.

Cho, J., Heuzey, M. C., Bégin, A., & Carreau, P. J. (2006). Chitosan and glycerophosphate concentration dependence of solution behaviour and gel point using small amplitude oscillatory rheometry. *Food Hydrocolloids*, *20*(6), 936–945.

Cho, J., Heuzey, M., Bégin, A., & Carreau, P. (2006). Effect of urea on solution behavior and heat-induced gelationof chitosan-β-glycerophosphate. *Carbohydrate Polymers*, *63*(4), 507–518.

Coutu, J. M., Fatimi, A., Berrahmoune, S., Soulez, G., & Lerouge, S. (2013). A new radiopaque embolizing agent for the treatment of endoleaks after endovascular repair: Influence of contrast agent on chitosan thermogel properties. *Journal of Biomedical Materials Research Part B—Applied Biomaterials*, *101B*(1), 153–161.

Crompton, K. E., Goud, J. D., Bellamkonda, R. V., Gengenbach, T. R., Finkelstein, D. I., Horne, M. K., et al. (2007). Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering. *Biomaterials*, *28*(3), 441–449.

Crompton, K. E., Prankerd, R. J., Paganin, D. M., Scott, T. F., Horne, M. K., Finkelstein, D. I., et al. (2005). Morphology and gelation of thermosensitive chitosan hydrogels. *Biophysical Chemistry*, *117*(1), 47–53.

Cui, J., Jiang, B., Liang, J., Sun, C., Lan, J., Sun, X., et al. (2011). Preparation and characterization of chitosan/beta-GP membranes for guided bone regeneration. *Journal of Wuhan University of Technology—Materials Science Edition*, *26*(2), 242–246.

Dang, Q. F., Yan, J. Q., Li, J. J., Cheng, X. J., Liu, C. S., & Chen, X. G. (2011). Controlled gelation temperature, pore diameter and degradation of a highly porous chitosan-based hydrogel. *Carbohydrate Polymers*, *83*(1), 171–178.

Dang, Q. F., Yan, J. Q., Lin, H., Chen, X. G., Liu, C. S., Ji, Q. X., et al. (2012). Design and evaluation of a highly porous thermosensitive hydrogel with low gelation temperature as a 3D culture system for *Penaeus chinensis* lymphoid cells. *Carbohydrate Polymers*, *88*(1), 361–368.

Dang, Q. F., Zou, S. H., Chen, X. G., Liu, C. S., Li, J. J., Zhou, X., et al. (2012). Characterizations of chitosan-based highly porous hydrogel—The effects of the solvent. *Journal of Applied Polymer Science*, *125*, 88–98.

Denkbas, E. B. (2006). Perspectives on: Chitosan Drug Delivery Systems Based on their Geometries. *Journal of Bioactive and Compatible Polymers*, *21*(4), 351–368.

Depani, B. P., Naik, A. A., & Nair, H. A. (2013). Preparation and evaluation of chitosan based thermoreversible gels for intraperitoneal delivery of 5-fluorouracil (5-FU). *Acta Pharmaceutica*, *63*(4), 479–491.

Dessi, M., Borzacchiello, A., Mohamed, T. H. A., Abdel-Fattah, W. I., & Ambrosio, L. (2013). Novel biomimetic thermosensitive beta-tricalcium phosphate/chitosan-based hydrogels for bone tissue engineering. *Journal of Biomedical Materials Research Part A*, *101*(10), 2984–2993.

Ding, K., Zhang, Y. L., Yang, Z., & Xu, J. Z. (2013). A promising injectable scaffold: The biocompatibility and effect on osteogenic differentiation of mesenchymal stem cells. *Biotechnology and Bioprocess Engineering*, *18*(1), 155–163.

Douglas, T. E. L., Skwarczynska, A., Modrzejewska, Z., Balcaen, L., Schaubroeck, D., Lycke, S., et al. (2013). Acceleration of gelation and promotion of mineralization of chitosan hydrogels by alkaline phosphatase. *International Journal of Biological Macromolecules*, *56*, 122–132.

Fatimi, A., Chabrot, P., Berrahmoune, S., Coutu, J. M., Soulez, G., & Lerouge, S. (2012). A new injectable radiopaque chitosan-based sclerosing embolizing hydrogel for endovascular therapies. *Acta Biomaterialia*, *8*(7), 2712–2721.

Gao, C., Cai, Y., Kong, X., Han, G., & Yao, J. (2013). Development and characterization of injectable chitosan-based hydrogels containing dexamethasone/rhBMP-2 loaded hydroxyapatite nanoparticles. *Materials Letters*, *93*, 312–315.

Giuseppe Molinaro, J. C. L., Jacques, D., & Albert, A. (2002). Biocompatibility of thermosensitive chitosan-based hydrogels: An in vivo experimental approach to injectable biomaterials. *Biomaterials*, *23*, 2717–2722.

Hamidi, M., Azadi, A., & Rafiei, P. (2008). Hydrogel nanoparticles in drug delivery. *Advanced Drug Delivery Reviews*, *60*(15), 1638–1649.

Hao, T., Wen, N., Cao, J. K., Wang, H. B., Lu, S. H., Liu, T., et al. (2010). The support of matrix accumulation and the promotion of sheep articular cartilage defects repair in vivo by chitosan hydrogels. *Osteoarthritis and Cartilage*, *18*(2), 257–265.

Hastings, C. L., Kelly, H. M., Murphy, M. J., Barry, F. P., O'Brien, F. J., & Duffy, G. P. (2012). Development of a thermoresponsive chitosan gel combined with human mesenchymal stem cells and desferrioxamine as a multimodal pro-angiogenic therapeutic for the treatment of critical limb ischaemia. *Journal of Controlled Release*, *161*(1), 73–80.

Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, *54*, 3–12.

Hsiao, M. H., Larsson, M., Larsson, A., Evenbratt, H., Chen, Y. Y., & Liu, D. M. (2012). Design and characterization of a novel amphiphilic chitosan nanocapsule-based thermo-gelling biogel with sustained in vivo release of the hydrophilic antiepilepsy drug ethosuximide. *Journal of Controlled Release*, *161*(3), 942–948.

Huang, F. Y. J., Gan, G. Y., Lin, W. Y., Huang, L. K., Luo, T. Y., Hong, J. J., et al. (2014). Investigation of the local delivery of an intelligent chitosan-based Re-188 thermosensitive in situ-forming hydrogel in an orthotopic hepatoma-bearing rat model. *Journal of Radioanalytical and Nuclear Chemistry*, *299*(1), 31–40.

Huang, F. Y., Huang, L. K., Lin, W. Y., Luo, T. Y., Tsai, C. S., & Hsieh, B. T. (2009). Development of a thermosensitive hydrogel system for local delivery of (188)Re colloid drugs. *Applied Radiation and Isotopes*, *67*(7–8), 1405–1411.

Huang, Z., Yu, B., Feng, Q., Li, S., Chen, Y., & Luo, L. (2011). In situ-forming chitosan/nano-hydroxyapatite/collagen gel for the delivery of bone marrow mesenchymal stem cells. *Carbohydrate Polymers*, *85*(1), 261–267.

Cho, J., André Bégin, M. C. H, & Carreau, Pierre J. (2005). Physical gelation of chitosan in the presence of β-glycerophosphate: The effect of temperature. *Biomacromolecules*, *6*, 3267–3275.

Jayakumar, R., Menon, D., Manzoor, K., Nair, S. V., & Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydrate Polymers*, *82*(2), 227–232.

Jayakumar, R., Prabaharan, M., Nair, S. V., & Tamura, H. (2010). Novel chitin and chitosan nanofibers in biomedical applications. *Biotechnology Advances*, *28*(1), 142–150.

Jayakumar, R., Prabaharan, M., Nair, S. V., Tokura, S., Tamura, H., & Selvamurugan, N. (2010). Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. *Progress in Materials Science*, *55*(7), 675–709.

Ji, Q. X., Deng, J., Xing, X. M., Yuan, C. Q., Yu, X. B., Xu, Q. C., et al. (2010). Biocompatibility of a chitosan-based injectable thermosensitive hydrogel and its effects on dog periodontal tissue regeneration. *Carbohydrate Polymers*, *82*(4), 1153–1160.

Kempe, S., Metz, H., Bastrop, M., Hvilsom, A., Contri, R. V., & Mader, K. (2008). Characterization of thermosensitive chitosan-based hydrogels by rheology and electron paramagnetic resonance spectroscopy. *European Journal of Pharmaceutics and Biopharmaceutics*, *68*(1), 26–33.

Khan, F., Tare, R. S., Oreffo, R. O., & Bradley, M. (2009). Versatile biocompatible polymer hydrogels: Scaffolds for cell growth. *Angewandte Chemie (International Edition in English)*, *48*(5), 978–982.

Kim, S., Nishimoto, S. K., Bumgardner, J. D., Haggard, W. O., Gaber, M. W., & Yang, Y. (2010). A chitosan/beta-glycerophosphate thermo-sensitive gel for the delivery of ellagic acid for the treatment of brain cancer. *Biomaterials*, *31*(14), 4157–4166.

Kwon, J. S., Kim, G. H., Kim da, Y., Yoon, S. M., Seo, H. W., Kim, J. H., et al. (2012). Chitosan-based hydrogels to induce neuronal differentiation of rat muscle-derived stem cells. *International Journal of Biological Macromolecules*, *51*(5), 974–979.

Ladet, S., David, L., & Domard, A. (2008). Multi-membrane hydrogels. *Nature*, *452*(7183), 76–79.

Lai, F., Li, H., & Luo, R. (2010). Chemo-electro-mechanical modeling of ionic-strength-sensitive hydrogel: Influence of Young's modulus. *International Journal of Solids and Structures*, *47*(22–23), 3141–3149.

Lajud, S. A., Han, Z., Chi, F.-L., Gu, R., Nagda, D. A., Bezpalko, O., et al. (2013). A regulated delivery system for inner ear drug application. *Journal of Controlled Release*, *166*(3), 268–276.

Lee, K. Y., & Mooney, D. J. (2001). Hydrogels for tissue engineering. *Chemical Reviews*, *101*(7), 1869–1879.

Lee, S. Y., Pereira, B. P., Yusof, N., Selvaratnam, L., Yu, Z., Abbas, A. A., et al. (2009). Unconfined compression properties of a porous poly(vinyl alcohol)-chitosan-based hydrogel after hydration. *Acta Biomaterialia*, *5*(6), 1919–1925.

Li, X., Fan, D.-D., Deng, J.-J., Hui, J.-F., Ma, X.-X., Zhu, C.-H., et al. (2013). Synthesis and characterization of chitosan human like collagen/beta-sodium glycerophosphate-carbodiimide hydrogel. *Asian Journal of Chemistry*, *25*(17), 9613–9616.

Li, X., Fan, D., Ma, X., Zhu, C., Luo, Y., Liu, B., et al. (2014). A Novel injectable pH/temperature sensitive CS-HLC/beta-GP hydrogel: The gelation mechanism and its properties. *Soft Materials*, *12*(1), 1–11.

Li, X., Fan, D., Zhu, C., & Ma, X. (2014). Effects of self-assembled fibers on the synthesis, characteristics and biomedical applications of CCAG hydrogels. *Journal of Materials Chemistry B*, *2*(9), 1234–1249.

Li, X., Ma, X., Fan, D., & Zhu, C. (2012). New suitable for tissue reconstruction injectable chitosan/collagen-based hydrogels. *Soft Matter*, *8*(14), 3781–3790.

Lima, A. C., Correia, C. R., Oliveira, M. B., & Mano, J. F. (2014). Sequential ionic and thermogelation of chitosan spherical hydrogels prepared using superhydrophobic surfaces to immobilize cells and drugs. *Journal of Bioactive and Compatible Polymers*, *29*(1), 50–65.

Liu, T. Y., & Lin, Y. L. (2010). Novel pH-sensitive chitosan-based hydrogel for encapsulating poorly water-soluble drugs. *Acta Biomaterialia*, *6*(4), 1423–1429.

Liu, W. F., Zang, H. D., Zhou, X., Kang, C. Z., Li, Y., Li, J., et al. (2014). The primary culture and subculture of lymphoid cells from shrimp, *Penaeus chinensis* on thermo-sensitive CS/alpha, beta-GP hydrogel. *Aquaculture Research*, *45*(2), 334–340.

Liu, X., Chen, Y., Huang, Q., He, W., Feng, Q., & Yu, B. (2014). A novel thermo-sensitive hydrogel based on thiolated chitosan/hydroxyapatite/beta-glycerophosphate. *Carbohydrate Polymers*, *110*, 62–69.

Mirahmadi, F., Tafazzoli-Shadpour, M., Shokrgozar, M. A., & Bonakdar, S. (2013). Enhanced mechanical properties of thermosensitive chitosan hydrogel by silk fibers for cartilage tissue engineering. *Materials Science and Engineering C—Materials for biological applications*, *33*(8), 4786–4794.

Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*, 167–182.

Muzzarelli, R. A. A. (2010). Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine Drugs*, *8*, 292–312.

Muzzarelli, R. A. A. (2012). Nanochitins and nanochitosans, paving the way to eco-friendly and energy-saving exploitation of marine resources. *Polymer Science: A Comprehensive Reference*, *10*, 153–164.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*(2), 995–1012.

Naderi-Meshkin, H., Andreas, K., Matin, M. M., Sittinger, M., Bidkhori, H. R., Ahmadi-ankia, N., et al. (2014). Chitosan-based injectable hydrogel as a promising in situ forming scaffold for cartilage tissue engineering. *Cell Biology International*, *38*(1), 72–84.

Nagahama, H., Maeda, H., Kashiki, T., Jayakumar, R., Furuike, T., & Tamura, H. (2009). Preparation and characterization of novel chitosan/gelatin membranes using chitosan hydrogel. *Carbohydrate Polymers*, *76*(2), 255–260.

Nazar, H., Fatouros, D. G., van der Merwe, S. M., Bouropoulos, N., Avgouropoulos, G., Tsibouklis, J., et al. (2011). Thermosensitive hydrogels for nasal drug delivery: The formulation and characterisation of systems based on N-trimethyl chitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics*, *77*(2), 225–232.

Ngoenkam, J., Faikrua, A., Yasothornsrikul, S., & Viyoch, J. (2010). Potential of an injectable chitosan/starch/beta-glycerol phosphate hydrogel for sustaining normal chondrocyte function. *International Journal of Pharmaceutics*, *391*(1–2), 115–124.

Niranjan, R., Koushik, C., Saravanan, S., Moorthi, A., Vairamani, M., & Selvamurugan, N. (2013). A novel injectable temperature-sensitive zinc doped chitosan/beta-glycerophosphate hydrogel for bone tissue engineering. *International Journal of Biological Macromolecules*, *54*, 24–29.

Peng, Q., Sun, X., Gong, T., Wu, C. Y., Zhang, T., Tan, J., et al. (2013). Injectable and biodegradable thermosensitive hydrogels loaded with PHBHHx nanoparticles for the sustained and controlled release of insulin. *Acta Biomaterialia*, *9*(2), 5063–5069.

Peng, Y., Li, J., Li, J., Fei, Y., Dong, J., & Pan, W. (2013). Optimization of thermosensitive chitosan hydrogels for the sustained delivery of venlafaxine hydrochloride. *International Journal of Pharmaceutics*, *441*(1–2), 482–490.

Richardson, S. M., Hughes, N., Hunt, J. A., Freemont, A. J., & Hoyland, J. A. (2008). Human mesenchymal stem cell differentiation to NP-like cells in chitosan-glycerophosphate hydrogels. *Biomaterials*, *29*(1), 85–93.

Rossi, S., Marciello, M., Bonferoni, M. C., Ferrari, F., Sandri, G., Dacarro, C., et al. (2010). Thermally sensitive gels based on chitosan derivatives for the treatment of oral mucositis. *European Journal of Pharmaceutics and Biopharmaceutics*, *74*(2), 248–254.

Ruel-Gariépy, E. C. A., Chaput, C., Guirguis, S., & Leroux, J. C. (2000). Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. *International Journal of Pharmaceutics*, *203*, 89–98.

Ruel-Gariépy, E., & Leroux, J. C. (2004). In situ-forming hydrogels—Review of temperature-sensitive systems. *European Journal of Pharmaceutics and Biopharmaceutics*, *58*(2), 409–426.

Ruel-Gariépy, E., Shive, M., Bichara, A., Berrada, M., Le Garrec, D., Chenite, A., et al. (2004). A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*(1), 53–63.

Ruel-Gariépy, E. L. G., Hildgen, P., Gupta, A., & Leroux, J. C. (2002). T hermosensitive chitosan-based hydrogel containing liposomes for the delivery of hydrophilic molecules. *Journal of Controlled Release*, *82*, 373–383.

Sharma, G., Italia, J. L., Sonaje, K., Tikoo, K., & Ravi Kumar, M. N. (2007). Biodegradable in situ gelling system for subcutaneous administration of ellagic acid and ellagic acid loaded nanoparticles: Evaluation of their antioxidant potential against cyclosporine induced nephrotoxicity in rats. *Journal of Controlled Release*, *118*(1), 27–37.

Sun, B., Ma, W., Su, F., Wang, Y., Liu, J., Wang, D., et al. (2011). The osteogenic differentiation of dog bone marrow mesenchymal stem cells in a thermo-sensitive injectable chitosan/collagen/beta-glycerophosphate hydrogel: In vitro and in vivo. *Journal of Materials Science—Materials in Medicine*, *22*(9), 2111–2118.

Sun, J., Jiang, G., Wang, Y., & Ding, F. (2012). Thermosensitive chitosan hydrogel for implantable drug delivery: Blending PVA to mitigate body response and promote bioavailability. *Journal of Applied Polymer Science*, *125*(3), 2092–2101.

Sung, J. H., Hwang, M. R., Kim, J. O., Lee, J. H., Kim, Y. I., Kim, J. H., et al. (2010). Gel characterisation and in vivo evaluation of minocycline-loaded wound dressing with enhanced wound healing using polyvinyl alcohol and chitosan. *International Journal of Pharmaceutics*, *392*(1–2), 232–240.

Supper, S., Anton, N., Seidel, N., Riemenschnitter, M., Curdy, C., & Vandamme, T. (2014). Thermosensitive chitosan/glycerophosphate-based hydrogel and its derivatives in pharmaceutical and biomedical applications. *Expert Opinion on Drug Delivery*, *11*(2), 249–267.

Tang, Y.-F., Du, Y.-M., Hu, X.-W., Shi, X.-W., & Kennedy, J. F. (2007). Rheological characterisation of a novel thermosensitive chitosan/poly(vinyl alcohol) blend hydrogel. *Carbohydrate Polymers*, *67*(4), 491–499.

Tessmar, J. K., & Gopferich, A. M. (2007). Matrices and scaffolds for protein delivery in tissue engineering. *Advanced Drug Delivery Reviews*, *59*(4–5), 274–291.

Tsai, M.-L., Chang, H.-W., Yu, H.-C., Lin, Y.-S., & Tsai, Y.-D. (2011). Effect of chitosan characteristics and solution conditions on gelation temperatures of chitosan/2-glycerophosphate/nanosilver hydrogels. *Carbohydrate Polymers*, *84*(4), 1337–1343.

Wang, L., & Stegemann, J. P. (2010). Thermogelling chitosan and collagen composite hydrogels initiated with beta-glycerophosphate for bone tissue engineering. *Biomaterials*, *31*(14), 3976–3985.

Wang, L., & Stegemann, J. P. (2011). Glyoxal crosslinking of cell-seeded chitosan/collagen hydrogels for bone regeneration. *Acta Biomaterialia*, *7*(6), 2410–2417.

Wu, J., Su, Z. G., & Ma, G. H. (2006). A thermo- and pH-sensitive hydrogel composed of quaternized chitosan/glycerophosphate. *International Journal of Pharmaceutics*, *315*(1–2), 1–11.

Wu, J., Wei, W., Wang, L. Y., Su, Z. G., & Ma, G. H. (2007). A thermosensitive hydrogel based on quaternized chitosan and poly(ethylene glycol) for nasal drug delivery system. *Biomaterials*, *28*(13), 2220–2232.

Wu, Y., Wei, W., Zhou, M., Wang, Y., Wu, J., et al. (2012). Thermal-sensitive hydrogel as adjuvant-free vaccine delivery system for H5N1 intranasal immunization. *Biomaterials*, *33*(7), 2351–2360.

Wu, Y., Wu, S., Hou, L., Wei, W., Zhou, M., Su, Z., et al. (2012). Novel thermal-sensitive hydrogel enhances both humoral and cell-mediated immune responses by intranasal vaccine delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, *81*(3), 486–497.

Zan, J., Zhu, D., Tan, F., Jiang, G., Lin, Y., & Ding, F. (2006). Preparation of thermosensitive chitosan formulations containing 5-fluorouracil/poly-3-hydroxybutyrate microparticles used as injectable drug delivery system. *Chinese Journal of Chemical Engineering*, *14*(2), 235–241.

Zhang, D., Sun, P., Li, P., Xue, A., Zhang, X., Zhang, H., et al. (2013). A magnetic chitosan hydrogel for sustained and prolonged delivery of Bacillus Calmette–Guérin in the treatment of bladder cancer. *Biomaterials*, *34*(38), 10258–10266.

Zhang, X. Z., Jo Lewis, P., & Chu, C. C. (2005). Fabrication and characterization of a smart drug delivery system: Microsphere in hydrogel. *Biomaterials*, *26*(16), 3299–3309.

Zhou, H. Y., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S., & Kennedy, J. F. (2008). Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system. *Carbohydrate Polymers*, *73*(2), 265–273.

Zhao, Q. S., Ji, Q. X., Xing, K., Li, X. Y., Liu, C. S., & Chen, X. G. (2009). Preparation and characteristics of novel porous hydrogel films based on chitosan and glycerophosphate. *Carbohydrate Polymers*, *76*(3), 410–416.

Zhou, H. Y., Chen, X. G., Kong, M., & Liu, C. S. (2009). Preparation of chitosan-based thermosensitive hydrogels for drug delivery. *Journal of Applied Polymer Science*, *112*(3), 1509–1515.

Zhou, H. Y., Zhang, Y. P., Zhang, W. F., & Chen, X. G. (2011). Biocompatibility and characteristics of injectable chitosan-based thermosensitive hydrogel for drug delivery. *Carbohydrate Polymers*, *83*(4), 1643–1651.

Zhou, Y., Zhao, Y., Wang, L., Xu, L., Zhai, M., & Wei, S. (2012). Radiation synthesis and characterization of nanosilver/gelatin/carboxymethyl chitosan hydrogel. *Radiation Physics and Chemistry*, *81*(5), 553–560.