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Review

Injectable chitosan hydrogels for localised cancer therapy

Hang Thu Ta [a], Crispin R. Dass [b,*], Dave E. Dunstan [a]

[a] Department of Chemical and Biomolecular Engineering, The University of Melbourne, Australia, VIC 3010
[b] Department of Orthopaedics, St Vincent's Hospital Melbourne, Australia, VIC 3065

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Abstract

The pace of development of delivery systems that could target drugs to specific body sites and control the release of drugs for prolonged periods of time have been steady though slow. Till a decade ago, mostly microspheres or nanoparticles were widely studied and applied in cancer treatment. However, due to shortcomings of these systems, there has been a surge in interest for in situ hydrogels. This review focuses on the current use of injectable in situ chitosan hydrogels in cancer treatment. Formulation protocols for in situ hydrogel systems, their cytotoxic properties, loading and *in vitro* release of drugs, their effect on cell growth *in vitro* and inhibition of tumor growth *in vivo* using mouse models, and future directions to enhance this technology are discussed.

Keywords: Chitosan; Hydrogel; Controlled release; Cancer; Drug delivery; Therapy

Contents

1. Introduction

Cancer is a disease characterized by an aggressive growth of cells which divide without normal limitations, invade and destroy adjacent tissues, and spread to distant anatomic sites through a process called metastasis, a major cause of death of cancer [1,2]. According to the World Health Organization, cancer is a leading cause of death worldwide. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (or 13%) of all deaths [2]. Cancers may result from the interaction between healthy cells and physical carcinogens such as ultraviolet (UV)

* Corresponding author.
E-mail address: Cris.dass@yahoo.com (C.R. Dass).

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Table 1
Injectable in situ chitosan hydrogels in cancer treatments

Cancer type	Hydrogel system	Method to induce gelation	Therapeutic agent	Route of administration	Effect	Reference
Lung cancer	Azide-chitosan-lactose (Az-CH-LA)	UV irradiation	Paclitaxel	Subcutaneously injected beneath the tumor	●Tumor-growth inhibition ●Tumor-angiogenesis inhibition	[78]
Breast cancer	Chitosan/β-glycerophosphate (C/GP)	Temperature	Paclitaxel	Intratumoral injection	●Tumor-growth inhibition ●Tumor-recurrence prevention	[14]
RIF-1 fibrosarcoma	Chitosan/β-glycerophosphate (C/GP)	Temperature	Camptothecin	Intratumoral injection	●Tumor-growth inhibition	[61]
Cervical cancer	Chitosan/β-glycerophosphate (C/GP)	Temperature	Doxorubicin and Vaccinia virus-based vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1)	Intratumoral injection	●Tumor-growth inhibition	[76]
Mucin-production associated cancers	Chitosan/Glyceryl monooleate (C/GMO)	pH	Paclitaxel	–	–	[77]

and ionizing radiation; chemical carcinogens such as asbestos and tobacco smoke; and biological carcinogens such as infections by virus and contamination of food by mycotoxins. These external agents cause genetic alterations resulting in mutations, and thereby perturbing normal cell growth and repair.

The principal modes of cancer management are surgery, radiotherapy and chemotherapy [2]. Recently, hormonal therapy and immunotherapy are increasingly being used as well, but their applications are limited for a few cancer types such as breast neoplasia [3]. Chemotherapy, the use of cytotoxic drugs to kill cancerous cells, remains the most common approach for cancer treatment. Generally, cytotoxic drugs are highly toxic but poorly specific, and do not differentiate between normal and cancer cells. Therefore, conventional chemotherapy administration or systemic administration has been shown to produce side effects. Most of the drug content is released soon after administration, causing drug levels in the body to rise rapidly, peak and then decline sharply, leading to unacceptable side effects at the peaks and inadequate therapy at the troughs [4]. Due to the short period of actions, repeated injections are often required, which can lead to exacerbation of side effects and inconvenience. In systemic administration, cytotoxic drugs are extensively transported to the whole body, therefore, only a small fraction of the drugs reach the tumor site and other healthy organs or tissues can be affected or damaged by the nonspecific action of the cytotoxic agents [3]. Due to these obstacles, controlled and targeting or localized release technology has been replacing the systemic administration and has shown lots of potential for cancer treatment.

Sustained release injectable formulations are basically designed as microparticulates (microcapsules or microspheres), implants or gel systems [5]. Drugs are commonly loaded into microspheres via a passive absorption method whereby microspheres are added to drug solution. Microspheres swell in solution and the drug molecules enter the gel matrix [6]. However, the efficiency of this loading method for cytotoxic drugs is limited and a high loading capacity is unattainable. An implant requires surgery to insert it near the tumor site, which adds to the costs and the risks of this system. These problems have oriented research towards injectable in situ gelling formulations [7], especially chitosan gelling systems due to their antibacterial, biocompatible, biodegradable [8,9] and mucoadhesive properties [10,11]. Table 1 summarizes the applications of injectable in situ chitosan hydrogels in treatments of various cancers.

2. Method of preparation of chitosan gels

2.1. Crosslinking by ultraviolet irradiation

This method was firstly reported by Ono et al. in 2000 [12]. In this method, lactose moieties were introduced into chitosan to obtain much better water-soluble chitosan at neutral pH, and photoreactive azide groups were added to provide the ability to form a gel through crosslinking azide groups with amino groups. This photocrosslinkable chitosan (Az-CH-LA) was then exposed to ultraviolet light (UV) irradiation to form an insoluble and adhesive hydrogel within 60 s. Az-CH-LA hydrogel has the consistency of transparent and soft rubber (Fig. 1) [13].

Azide and lactose moieties were introduced to chitosan molecules through a two-step condensation reaction [12]. Firstly, the lactose moiety was introduced to chitosan chains using lactobionic acid with the presence of EDC (1-ethyl-3-(3-dimethylaminopropyl carbodiimide). The reaction took place in solution of TEMED (*N*,*N*,*N*',*N*'-tetramethylethylenediamine) containing concentrated HCl. Subsequently, the azide moiety was introduced to the lactose-linked chitosan (CH-LA) using 4-azidobenzoic acid. Again, the reaction was completed in the presence of EDC in TEMED as in the first condensation.

It has been estimated that between 2% and 2.5% of amino groups in the chitosan molecule were replaced by lactobionic acid and by azidobenzoic acid, respectively using this method. CH-LA exhibited a good aqueous solubility at neutral pH and lower. The introduction of azidobenzoic acid by 2.5% into CH-LA showed no additional change in water solubility [12].

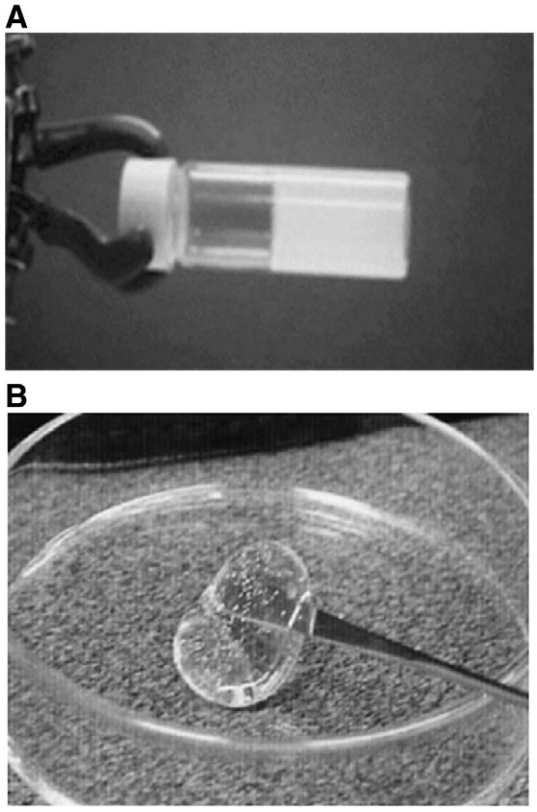

Fig. 1. Chitosan hydrogels. (A) C/GP hydrogel at 37 °C (Source: Ruel-Gariepy et al., Ref. [14]), and (B) Az-CH-LA hydrogel after irradiation (Source: Ishihara et al., Ref. [13]).

Viscous Az-CH-LA aqueous solutions were induced to gel by UV irradiation [12]. It is known that the Azide group ($-N_3$) releases N_2 upon UV irradiation and is converted into highly reactive nitrene groups. The interaction of nitrene groups with amino groups of chitosan leads to the generation of the azo groups ($-N=N-$), causing gelation. Upon irradiation, the formation of insoluble Az-CH-LA hydrogels was completed within 60 s. This time can be shortened with the increase in intensity of UV light.

2.2. Crosslinking by high temperature

This method was invented by Chenite and colleagues, then developed and named as BST-Gel platform technology at Biosyntech Inc. (Laval, QC, Canada) [14]. It is based on the neutralization of a chitosan solution with a polyol counterionic dibase salt such as β-glycerophosphate. Chitosan/glycerophosphate (C/GP) is a thermosensitive solution which is liquid at room temperature and solidifies into a white hydrogel at body temperature (Fig. 1). According to this report, chitosan solution was prepared in 0.1 M acetic acid. Glycerophosphate (GP) solution was prepared and chilled along with the chitosan solution in an ice bath for 15 min. Then, the cold GP solution was added dropwise to the cold chitosan solution with stirring to produce C/GP aqueous formulation with a pH value around neutral. Stability and viscosity of C/GP solutions was dependent on the deacetylation degree of chitosan. Solutions made of less deacetylated chitosan are more stable and their viscosity remains unchanged for a longer time.

The gelation rate was dependent on chitosan deacetylation degree, concentration of β-GP, temperature and pH of final solution as shown by another study [15]. More highly deacetylated chitosan turns to gel quicker when incorporated with GP, which can be explained by the higher cross-linking density between phosphate groups of GP and the ammonium groups of chitosan. The sol-gel transition starts as soon as the polyol salt is added, however, low temperature and low pH slow down the gelation process [7]. The C/GP gel system having pH values between 6.9 and 7.2 is partially thermoreversible upon cooling to 5 °C because of the existence of remaining associations [16]. However, C/GP solutions having lower pH between 6.5 and 6.9 show complete thermoreversibility [17].

It was suggested that in C/GP systems, there are three types of interactions involved in the gelation process [7]. They are (1) electrostatic attraction between the ammonium group of the chitosan and the phosphate group of the glycerophosphate; (2) hydrogen bonding between the chitosan chains as a consequence of reduced electrostatic repulsion after neutralization of the chitosan solution with GP; and (3) chitosan–chitosan hydrophobic interactions. The observation that C/GP solutions gel with increasing temperature implies that some repulsive forces between the chitosan chains are stabilized at low temperature and weakened at high temperature. The polyol part of GP has been considered as the element which prevents or slows down gelation at low temperature. It was hypothesized that polyols reinforced the initial structure of chitosan, thus, more energy was needed to break it [7,15]. Mi et al. reported the occurrence of ionic interactions between positively charged chitosan molecules and negatively charged tri-polyphosphates using FTIR spectra analysis [18,19]. However, Cho et al. reported that hydrophobic interactions and reduced solubility are the main driving force for chitosan gelation at high temperature in the presence of β-GP [16].

Recently, Buschmann et al. have demonstrated that the sol-gel transition occurs via two steps [20]. Firstly, polyelectrolyte chitosan is partially neutralized and brought close to precipitation by the addition of a weak base such as dibasic sodium phosphate or GP. After the neutralization step, the solution is then heated and induces chitosan to release its proton. Phosphate salt acts as a proton sink. If the amount of phosphate is enough to accept these protons, the transfer of protons is sufficient to bring the chitosan to precipitation and induce the sol-gel transition. Therefore, there is no ionic crosslink between the phosphate or polyphosphate and chitosan chain, the gelation is a block precipitation of the chitosan resulting from the homogeneous neutralization of the polyelectrolyte induced by heating. The phosphate salt is then free to diffuse out of the gel.

2.3. Crosslinking by high pH

This method employs the pH-sensitive property of chitosan solutions at low pH. Once injected into the body, these polymer solutions face different environmental pH conditions and form

gels. Ganguly et al. developed a novel mucoadhesive pH-sensitive chitosan/glyceryl monooleate (C/GMO) in situ gel system which consisted of 3% (w/v) chitosan and 3% (w/v) GMO in 0.33 M citric acid [21]. Chitosan is normally insoluble in neutral or alkaline pH. However, in dilute acids ($pH \leq 5.0$), it becomes soluble due to the protonation of free amino groups on the chitosan chains (RNH^{3+}). The solubility of chitosan in acidic medium also depends on its molecular weight [22]. Acidic solutions of chitosan when exposed to alkaline pH or body biological pH lose this charge and form viscous gels [21].

Chitosan and GMO both own mucoadhesive properties which have been applied in drug delivery systems [23–26]. Positive charges on the chitosan backbone may give rise to a strong electrostatic interaction with mucus or a negatively charged mucosal surface [10,27]. The exact mechanism of GMO mucoadhesion is still unknown, but may involve dehydration of mucosa [26]. In the presence of excess water (35% w/w water), GMO can form viscous gels known as a transparent cubic phase constituting a three-dimensional network of curved lipid bilayers. Water content of up to 5% causes GMO forming reverse micelle phase, while water content of up to 20% leads to the formation of GMO lamellar phase [28]. This property of GMO has been used to sustain the delivery of various water-soluble and water-insoluble drugs [29–31]. The mucoadhesive and in situ gel-forming properties of chitosan and GMO can be used in sustaining delivery of both hydrophilic and hydrophobic drugs, and targeting these to cells producing mucin.

To satisfy the requirements of parenteral delivery and proper gelation in reasonable time, chitosan concentration was found to be optimum at 3% (w/v) [21]. The effect of four organic acids including lactic, tartatic, citric and acetic acids on the formation of in situ gel in alkaline pH was investigated. Citric acid exhibited the fastest gel formation and had the best adhesive properties for both glass and cellulose membrane.

In the study of Ganguly and Dash, solutions of 3% (w/v) chitosan in 0.33 M citric acid, pH 3.2, were chosen for sustained release [21]. The incorporation of GMO at 3% (w/v) in chitosan solution retarded the *in vitro* release study of lidocaine hydrochloride from over 80% to 67% within 30 min due to the formation of cubic phases with higher viscosity. The transition of liquid GMO to cubic phase resulted in the penetration of more than 35% (w/w) water into the matrix in less than 30 min. The addition of GMO to chitosan also enhanced its mucoadhesive property by more than three fold.

3. Cytotoxicity of chitosan hydrogels

Chitosan is a natural product which is safe to the human body and contains basic groups. Cells generally have a predominantly negative charge on their surfaces, thus they are known to adhere much more strongly to substrates with basic groups such as chitosan [32]. However, the strongly positive charge causes metamorphosis of cells and inhibits cell growth. Prior studies have reported that epithelial cells grew on a film of chitosan [33], and fibroblast cells could grow on collagen–chitosan blended films [32]. The addition of chitosan to collagen increased cell attachment but decreased cell growth. It has been found to be difficult for cells to adhere to and grow on chitosan hydrogels having high water content.

In vitro cytotoxicity testing of Az-CH-LA gel showed that human skin fibroblasts, coronary smooth muscle cells, and endothelial cells do not adhere and grow on immobilized chitosan gels prepared by UV irradiation [34]. However, they grow normally beside the hydrogels. Az-CH-LA and its gel did not influence both the adhesion and proliferation of these cells. The test confirmed that Az-CH-LA aqueous solutions and their gels are not toxic to the above cells. *In vivo* toxicity testing showed that mice with subcutaneously injected Az-CH-LA gels were alive for at least 1 month. The implanted chitosan hydrogel was partially biodegraded *in vivo* in about 10–14 days when implanted subcutaneously into the mouse back. After 1 month, at the site of administration, no chitosan gel could be visibly detected [12]. In addition, toxicity tests for mutagenicity and cytotoxicity have shown the safety of both Ax-CH-LA and its hydrogel [13]. These results show the safety of Az-CH-LA gels in medical use.

Several C/GP formulations with degree of deacetylation ranging from 40 to 95% were tested *in vitro* with several cell lines and *in vivo* with rats to investigate their cytocompatibility [17]. The absence of any toxic elements was demonstrated by the ability of these C/GP preparations to maintain more than 80% cell viability over several weeks. The safety of these systems was also confirmed by histological analysis. Degree of deacetylation was found to be the key factor governing both the rate of degradation and the inflammatory response. While a lower degree of deacetylation resulted in a short residence time (few days) and inflammatory cell induction, a higher one showed longer residence time (several weeks) and no detectable inflammation. The cytotoxicity of chitosan/GMO systems have not been investigated yet. However, their low pH property imposes a large gap in pH between such delivery solutions and body biological environment, which facilitates gel forming but may lead to significant side effects.

4. Drug agents formulated in chitosan hydrogels for cancer treatment

Paclitaxel is in a class of drugs as taxanesa, originally extracted from the bark of the Pacific yew (*Taxus brevifolia*), known as a potent inhibitor of angiogenesis, cell migration, and collagenase production, and exhibits inhibitory action on tumor cell proliferation [35]. Paclitaxel did not stop cell growth but considerably diminished its rate. It exerts its main anti-tumoral activity by binding to and promoting the assembly of microtubules. This causes the microtubules to become resistant to depolymerization into tubulin. This means that paclitaxel blocks a cell's ability to break down the mitotic spindle during mitosis (cell division). With the spindle still in place the cell cannot divide into daughter cells. Consequently, the cells are arrested at the G2 and M phases of the cell cycle [36–38]. It also has been demonstrated that paclitaxel has significant anti-tumor activity against various solid tumors, including breast and colon cancer, ovarian carcinoma, lung cancer, head and neck carcinoma, malignant melanoma, esophageal adenocarcinoma,

and acute leukemia [39–41]. Paclitaxel also induces apoptosis and could sensitize even multi-drug resistant tumor cells to radiation [42,43].

Traditionally, paclitaxel was administered by intravenous (IV) infusion, and cremophor EL was use as a solvent to enhance paclitaxel solubility [44]. However, this solvent caused severe hypersensitive reactions, cytotoxicity, and was incompatible with polyvinyl chloride (PVC) which was commonly used in IV dosage form. Paclitaxel cannot differentiate between cancer and normal cells, resulting in major toxicity to normal cells. To minimize the cytotoxicity and side effects, localized and targeted delivery of paclitaxel need to be developed. For regional delivery, paclitaxel has been formulated in biodegradable polymeric microspheres [45], hydrogels [14], surgical pastes, and implants [1,14,45].

Camptothecin is an inhibitor of the DNA-replicating enzyme topoisomerase I, leading to the production of a double-strand DNA break during replication and resulting in cell death if the break is not repaired [46]. Camptothecin is believed to break the topoisomerase I-induced single strand in the phosphodiester backbone of DNA, thus preventing replication [47,48]. In preclinical studies, camptothecin was effective against colon [49], lung [50], breast [51], ovarian [49], and melanoma cancers [51]. However, camptothecin was not administered in any clinical trials systemically because of its low solubility in water, unexpected toxicity and low antineoplastic activity [52–54]. In addition, the lactone ring of camptothecin and its analogs is unstable in the presence of human serum albumin which results in the conversion of the active drug to the inactive carboxylate form bound to albumin [55–57].

Alternatively, local delivery of camptothecin has attracted some attention. Camptothecin has been formulated in biodegradable polymeric implant devices, microspheres and hydrogels. Camptothecin was loaded into a controlled-release polymer (ethylene-vinyl acetate co-polymer; EVAc) for brain tumor treatment [58]. It was shown that local controlled delivery by this polymer system significantly extended survival: 59% of the treated animals were long-term survivors (>120 days) compared to 0% of controls. Biodegradable polyanhydride polymer devices [59] and biodegradable poly(lactide-co-glycolide) (PLGA) microspheres [60] were also studied to locally deliver camptothecin and showed promises. However, polymer devices require insertion by surgical intervention and microspheres do not form a continuous film or solid implant and may be poorly retained because of their small size, discontinuous nature and lack of adhesiveness [61]. Recently, camptothecin was formulated in an injectable thermosensitive chitosan/glycerophosphate system for controlled release [61]. This system shows an effective sustained intratumoral delivery of camptothecin.

Doxorubicin or adriamycin is a potent and flourescence-spectrum cytotoxic drug used to treat various human sarcomas and malignancies such as human hepatoma [62], malignant glioma [63,64], fibrosarcoma [65], bone sarcomas, breast and ovarian cancer [66,67], carcinomas of the neck and head [68], lung, prostate, bladder and Ewing's sarcomas [69]. It is one of the most active and widely used anticancer drugs that exerts its cytotoxic activity by inhibiting the synthesis of nucleic acids within cancer cells [70,71]. It is an anthracycline antibiotic produced by the *Streptomyces peucetius varieta caesius*. It blocks DNA and RNA synthesis by inhibiting topoisomerase II [72]. However, nonspecific action of doxorubicin can be harmful to normal cells and causes serious side effects to the patients such as cardiotoxicity and myelosuppression [73,74]. Like many other drugs used to treat cancer, doxorubicin is a potent vesicant that may cause extravasation and necrosis at the injection site or any site that the skin is exposed to [69]. Due to its high toxicity, substantial increase in systemic dose to achieve high concentration of the drug at the target site is not possible and systemic administration of doxorubicin needs to be replaced by local and controlled delivery. Flourescence spectrum of doxorubicin obtained by a 400 nm excitation shows an emission maximum at about 590 nm in water. The excitation spectra of doxorubicin measured at a fixed emission wavelengths of 590 and 550 nm reveal the excitation maxima at about 474 and 496 nm [75]. It is thus easily detected using fluorescence spectroscopy and microscopy.

5. Loading of drug agents

Depending on the properties of chitosan delivery systems, drug agents can be encapsulated into the systems via different approaches (Fig. 2). They can be incorporated in chitosan solution before the addition of gelation-inducing agents [14,61,76] or they can be added into the gelation-inducing agents before mixing with chitosan solution [77]. They can also be added into the gelation mixture solution consisting of both chitosan and gelation-inducing agent [78].

Paclitaxel is a hydrophobic molecule that is poorly soluble in water. Therefore, to incorporate paclitaxel into Az-CH-LA hydrogel, a vehicle must be used. Currently the only available formulation in clinical use is Taxol from Bristol Pharmaceuticals K.K., Tokyo, Japan [78]. This formulation of paclitaxel provides water solubility without detectable precipitation or emulsion formation [40]. 1 ml of Taxol containing paclitaxel (6 mg/ml) in a vehicle composed of Cremophor EL and ethanol at a 50:50 (v/v) ratio was mixed into Az-CH-LA aqueous solution using vortex. The obtained Az-CH-LA solution containing paclitaxel could be converted into an insoluble hydrogel upon UV irradiation. Paclitaxel was retained in this photocrosslinkable chitosan hydrogel and remained biologically active *in vitro* for at least 21 days. It was released by the biodegradation of hydrogel *in vivo* [13,78].

C/GP gels were first formulated with a bone-inducing growth factor preparation [17]. Then, their application of cell transplantation for tissue repair was tested by subcutaneously injecting isolated bovine articular chondrocytes embedded in C/GP gels in rats. In 2004, Ruel-Gariepy reported the application of C/GP hydrogels in cancer treatment by incorporating paclitaxel in these systems [14]. First, a chitosan solution was prepared in deionized water and sterilized in an autoclave (121 °C, 10 min). Autoclaving 2% (w/v) chitosan solutions for as short as 10 min resulted in a 30% decrease in molecular weight, 3–5 fold decrease in dynamic viscosity, and substantial

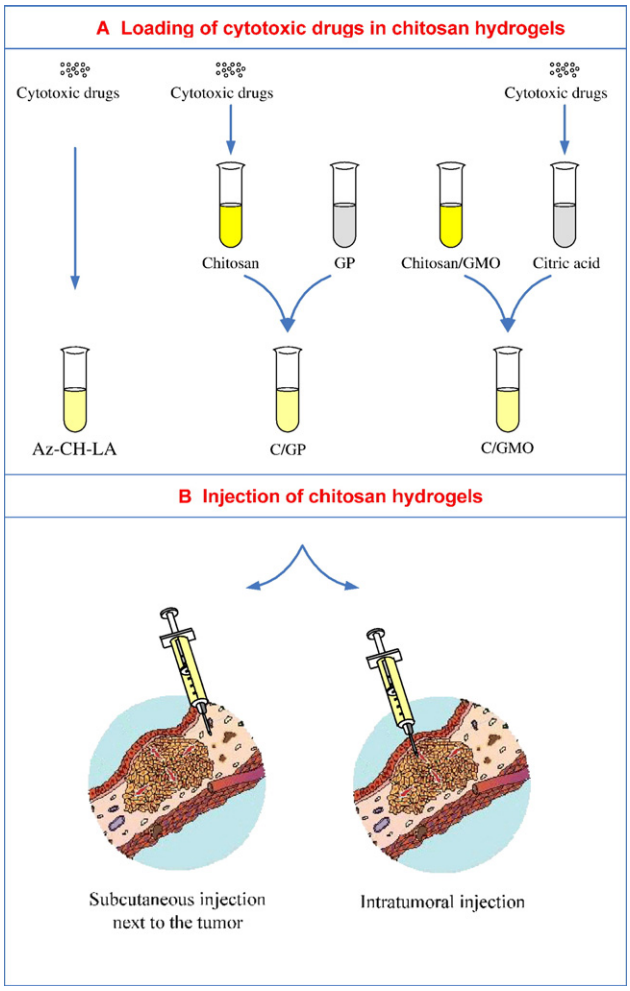

Fig. 2. Injectable in situ forming chitosan delivery systems. (A) Loading of cytotoxic drugs into chitosan hydrogels; and (B) Injection of chitosan hydrogels.

loss of mechanical properties of the resulting gel. However, autoclaving did not modify the deacetylation degree of chitosan and did not impair the ability of the system to form a gel at 37 °C. In addition, autoclaving did not affect the biological performances of C/GP systems *in vivo* [79]. However, the degradation of chitosan following steam sterilization could be reduced by the addition of polyols [80]. Second, chitosan solution was poured directly onto the sterilized palitaxel drug powder and the mixture was stirred for 4 h. Chitosan solution containing paclitaxel and GP solution were chilled in an ice bath for 15 min. β-glycerophosphate was dissolved in distilled water and sterilized by filtration through 0.20 μm filter. GP solution was then added dropwise to the chitosan solution with stirring under aseptic conditions [14].

The preparation of C/GP loaded with camptothecin was similar to that of C/GP loaded with paclitaxel [61]. A 1.7% w/w chitosan solution was autoclaved, and sterilized camptothecin was homogeneously dispersed in chitosan solution at room temperature. Finally, the sterilized GP solution was added to the cooled camptothecin/chitosan mixture under aseptic conditions. In another study [76], the principle steps to prepare C/GP/doxorubicin were the same as the previous report. However, all components were not reported to be autoclaved or sterilized. Medium molecular weight of chitosan (161 kDa) was used and the final concentration of chitosan in the mixture was 2% w/w.

A chitosan/GMO delivery system loading paclitaxel was prepared in 4 steps [77]. GMO was heated in an oven at 45 °C for 30 min then chitosan powder was added to molten GMO solution with stirring. Paclitaxel was dissolved in 0.33 M citric acid followed by sonication for 30 min. The resulting solution was added to chitosan/GMO mixture followed by another sonication step for 45 min. This method of incorporation of paclitaxel in the delivery system provided much better homogeneity of paclitaxel in the formulation compared to the common method in which paclitaxel was added directly to the delivery system containing 3% (w/v) chitosan and 3% (w/v) GMO in 0.33 M citric acid. In this formulation, the solubility of paclitaxel, a highly hydrophobic compound, was also enhanced due to the self-emulsifying property of GMO.

6. *In vitro* release of the drug agents from chitosan hydrogel at 37 °C

The release of paclitaxel from Az-CH-LA hydrogels at room temperature in PBS was observed over 7 days [78]. About 35–40% of both paclitaxel and the vehicle were released within 1 day. The half-releasing time was 45 h. The degree of crosslinking of the polymer influenced the release ability of the hydrogel matrix. Generally, increasing degree of cross-linking enhances the drug loading efficiency and slows the release rate of entrapped drug [34]. As azide moieties introduced into chitosan increased from 2.5% to 5%, the half-releasing time of paclitaxel increased from 45 h to 50 h [78]. Az-CH-LA concentration also affected the release pattern of paclitaxel from the hydrogel matrix. When Az-CH-LA hydrogel was prepared from 20 mg/ml of Az-CH-LA solution, about 30–40% of entrapped paclitaxel was released from the matrix within the first day and the half-releasing time was 45 h. However, when 5 mg/ml of Az-CH-LA was used, more than 80% of paclitaxel was released within 1 day and the half-releasing time was only 7 h.

Washing of paclitaxel-incorporated Az-CH-LA hydrogel resulted in a lowering of an inhibitory effect towards human microvascular endothelial cell (HMVEC), human umbilical vein endothelial cell (HUVEC), and Lewis lung carcinoma cell (3LL) growth [78]. This again confirmed the sustained release of paclitaxel from hydrogel matrix and showed that the release rate was highest (initial burst) in the first day and then gradually decreased in the later 21 days. Addition of chitinase and chitosanase into culture medium led to almost complete recovery of the inhibitory activity of paclitaxel-incorporated Az-CH-LA hydrogels on cell growth. These results strongly suggested that the presence of chitinase and chitosanase caused partial degradation of Az-CH-LA hydrogels, leading to the release of entrapped paclitaxel into culture medium which then inhibited cell growth. This also proved that paclitaxel molecules within the Az-CH-LA hydrogel retained their biological activity during a 21-day period of hydrogel washing [78].

Chitosan/glycerophosphate systems can sustain the release of macromolecules over a period of several hours to a few days [81].

However, more than 80% of the incorporated low-molecular-weight hydrophilic compounds (<1000 g/mol) was released during the first 24 h. To overcome this, low M_w compounds were loaded into liposomes and then the latter were incorporated in the thermosensitive C/GP solutions. The *in vitro* release profiles of paclitaxel from C/GP demonstrated controlled delivery over 1 month. Paclitaxel was released from the gel system to PBS (pH 7.4) containing 0.3% (w/v) SDS [14]. SDS was included in the release medium to increase the solubility of paclitaxel. The initial drug loading was inversely proportional to the release rate. A 64 mg/ml-loaded gel showed the initial burst effect of 7% and the release rate of 2%/day from day 2 to 10, while 6.4 mg/ml-loaded gel produced 16.6% initial burst effect and 4.2%/day release rate. After 1 month, 92% cumulative release was recorded for 6.4 mg/ml-loaded gel compared to only 43% for 64 mg/ml-loaded gel. In PBS containing 0.6% Tween 20, pH 7.4, the C/GP system released 80% of the loaded camptothecin. Approximately 13% was released in the first 72 h after an initial burst of less than 5% in the first day. The release profile of camptothecin was nearly linear under infinite sink conditions.

In vitro release of paclitaxel from the mucoadhesive chitosan/GMO gel delivery systems was performed in Sorensen's phosphate buffer (pH 7.4) [77]. Paclitaxel released from these systems followed a matrix diffusion controlled mechanism. In contrast to the release profile of paclitaxel from C/GP system, loading of paclitaxel in C/GMO systems was directly proportional to its release rate under similar test conditions. The release rate from the gel formulation containing the highest drug load (0.54%, w/w) was found to be 4 times higher than the lowest drug-loaded gel (0.18%, w/w). The addition of surfactant Tween 80 increased the release of paclitaxel from the gel matrix, which might be due to the increased solubility of this hydrophobic drug in Sorensen's phosphate buffer.

7. Effect of drug-incorporated chitosan hydrogels on various cell growths *in vitro*

Paclitaxel released from Az-CH-LA hydrogels was found to inhibit HUVEC, HMVEC and 3LL cells (3LL) but not fibroblast growth [78]. It demonstrated that paclitaxel has the ability to inhibit tumor growth and angiogenesis without damage to surrounding connective tissues *in vivo*. The anti-proliferation activity of paclitaxel depended on its concentration. The use of solvent (Cremophor EL and ethanol) as paclitaxel vehicle did not affect and inhibit the cell proliferation.

Due to their mucoadhesive properties, in situ C/GMO gel delivery systems containing paclitaxel could be targeted to the cancer cells where MUC1 gene is overexpressed as compared with normal cells [77]. MUC1 is a tumor-associated antigen and MUC1 gene encodes a transmembrane mucin glycoprotein that is overexpressed in most of adenocarcinomas [82,83]. It is overexpressed more than 10-fold in 90% of breast carcinoma [82]. This overproduction of mucin in these cancerous cells could be used as a targeting strategy for mucoadhesive drug delivery system for treatment of breast and colon cancer. The bioadhesive properties of these systems can increase the contact time of therapeutic agents at the site of action and thus enhances the efficacy of treatment.

The transport of paclitaxel from C/GMO delivery system to different mucin-producing cell lines (Calu-3 and Caco-2) was investigated in the study of Jauhari et al. [77]. Transport of paclitaxel across the cell monolayers was performed in side by side diffusion chambers from apical to basal (A-B) and basal to apical (B-A) directions. Regardless of the cellular polarity, the transport of free paclitaxel across these cell lines was significantly higher than that of paclitaxel from C/GMO system in both directions. This may be explained by the reduction of free paclitaxel available in the gel delivery system due to the adhesion of the gel to the cellular mucosal secretions. This indicated that C/GMO system can be used to sustain the release of paclitaxel. Transport of paclitaxel from mucoadhesive gels was also shown to be influenced by the mucin-producing capability of cells. In fact, a higher production of mucin in Calu-3 led to a stronger binding with C/GMO gels and provided extra barrier for diffusion of paclitaxel, resulted in the reduced transport of paclitaxel.

8. Effect of drug-incorporated chitosan hydrogels on different cancer treatments *in vivo*

It should be noted that beside its good characteristics such as accelerating wound healing and anti-infection activity, chitosan has also shown a growth-inhibition effect on tumor cells [84] and inhibition of tumor-induced angiogenesis and tumor metastasis [85]. Intratumoral administration of chitosan compounds alone was shown to promote anti-tumoral effects in metastatic breast cancer models [86]. Chitosan was also found to activate macrophages into cytotoxic macrophages and suppressed Meth-A tumor growth in Balb/c mice [87]. Tokoro et al. also showed that two oligosaccharides consisting of the two units which compose chitosan, *N*-acetyl-D-glucosamine and D-glucosamine, were growth inhibitory to Meth-A solid tumors transplanted in mice when administered systemically. Increased sequential production of lymphokines IL-1 and IL-2 was proposed to cause the anti-tumor effect through proliferation of cytolytic T-lymphocytes [88]. Mutara et al. suggested that chitosan directly inhibits tumor cell proliferation by inducing apoptosis [89]. For example, Hasegawa reported in 2001 that chitosan induced apoptosis of bladder tumor cells via caspase-3 activation [90]. In addition, Guminska et al. reported that chitosan inhibited Ehrlich ascites tumor (EAT) cell growth by diminishing glycolysis, that is, aerobic lactate formation, thus decreasing glucose uptake and ATP level in the tumor intact cells. However, chitosan was not inhibitory on glycolytic activity of mouse normal liver and muscle supernatants [91]. *In vivo*, chitosan is phagocytized by macrophages [92] and slowly degraded enzymatically by lysozyme through hydrolysis of acetylated residues [93]. Chitosan is not the only polysaccharide possessing tumor-inhibitory effect. Certain polysaccharides extracted from an edible mushroom were also found to have *in vivo* anti-tumor activity against sarcoma 180 by Chihara et al. [94]. Kodama et al. also reported that polysaccharides extracted from the *Grifola frondaosa* mushroom could activate lymphocyte and macrophage responses and demonstrated *in vivo* anti-tumor effects [95].

Chitosan hydrogel solutions are injected into the body via two main routes of administration including intratumoral administration and subcutaneous administration next to the tumor (Fig. 2). In the study of Obara et al., chitosan hydrogels containing drug were administered intratumorally or locally [78]. Az-CH-LA solution containing paclitaxel was subcutaneously injected beneath the tumor using 18G needle and disposable syringe. Then a tip of optical fiber was inserted into the Az-CH-LA solution through the needle pore and UV-laser irradiation was performed for 30 s to let the Az-CH-LA solution convert to insoluble hydrogel. Both the administration of Az-CH-LA hydrogel and only paclitaxel reduced subcutaneous induced tumor growth of 3LL cells (Lewis cancer cells) within 7 days then they gradually lost their inhibitory activity. Surprisingly, Az-CH-LA hydrogel more strongly inhibited the growth of tumor compared to paclitaxel only, especially in the later days the difference increased.

In the above study, 200 μl of Az-CH-LA solution (20 mg/ml) or Az-CH-LA solution (20 mg/ml) containing paclitaxel (3 mg/ml) or solution of paclitaxel (3 mg/ml) or saline (PBS) solution (control) was subcutaneously injected beneath the tumor. The incorporation of paclitaxel in Az-CH-LA hydrogel resulted in the strongest inhibition on tumor growth compared to only paclitaxel and Az-CH-LA hydrogel administration. Its inhibitory effect lasted for 14 days and subsequently the tumor in almost all the mice grew again. This may be due to the lower release of paclitaxel from hydrogel matrix after time, which is consistent with the results obtained from *in vitro* release study. Applying a new paclitaxel-incorporated Az-CH-LA hydrogel 10 days after the first application resulted in an additional anti-tumor effect. After the first 14 days of administration, approximately 90% of the subcutaneously injected Az-CH-LA hydrogels in mice were biodegraded [78].

The study of Obara et al. also showed that the application of paclitaxel-incorporated Az-CH-LA hydrogel induced significant necrosis on tumor tissue and strongly inhibited angiogenesis in tumors [78]. However, the application of the control and only paclitaxel did not cause any necrotic tissue, and weakly inhibited tumor vascularization. Minor necrotic tumor tissue was induced in tumors treated with the Az-CH-LA hydrogels. Application of hydrogels also showed an intermediate effect on anti-angiogenesis. These results suggested that without a hydrogel carrier, paclitaxel molecules diffused so quickly from the injected site that they were unable to induce any anti-tumor effect.

C/GP solutions containing paclitaxel or camptothecin or doxorubicin were injected intratumorally (IT) using 26G needle inserted in the center of the tumor [14,61,76]. After injection, the needle was held in place for 3–4 s before being withdrawn to prevent the hydrogel from leaking out of the injection site [61].

Local delivery of paclitaxel from the C/GP gel system injected intratumorally in EMT-6 murine mammary tumors (breast cancer) implanted subcutaneously on Balb/c mice showed that one intratumoral injection of the thermosensitive hydrogel containing paclitaxel was as efficious as four intravenous injections of Taxol in inhibiting the growth and recurrence of tumors but in a less toxic manner [14]. The efficacy of the treatment was demonstrated in two separate studies representing two stages of tumor growth. To investigate the ability of palitaxel-C/GP gel on tumor growth inhibition, the treatment was initiated when the tumors reached a volume of 30 mm^3. Treatment group received 1 injection of 10 μl of C/GP solution containing 64 mg/ml of paclitaxel (equivalent to 40 mg/kg) intratumorally. Control groups included saline-injected group (0.2 ml/injection/day, 4 intravenous injections), paclitaxel IV (Taxol)-injected group (10 mg/kg/injection/day, 4 intravenous injections) and C/GP-injected group (10 μl, 1 intratumoral injection). At day 17 of treatment, the saline-treated tumors grew to about 9 times their original size whereas the other groups showed only approximately 5.5 times increase, which represented 38–40% growth inhibition. Tumor growth inhibition in group received 4 Taxol injections and in group received only 1 paclitaxel-C/GP injection was similar.

To investigate the ability of palitaxel-C/GP gel on tumor recurrence prevention, the treatment was initiated on the fourth day of tumor growth when the tumors were very small, which mimicked a population of cancer cells remaining after primary tumor surgical excision [14]. Treatment groups and 3 control groups were treated as in the first study. After 17 days, the saline-treated tumors grew to about 18.5 times their original size while C/GP-treated tumors grew 12 times. Both Taxol-treated and paclitaxel-C/GP-treated group showed around 5.5 times increase in tumor size. All tumors demonstrated some level of necrosis. Tumors from saline-treated group demonstrated the lowest necrotic proportions while those from C/GP groups and Taxol-treated group showed much larger percentages of necrotic regions. During the first 6–7 days of treatment, the mice receiving Taxol displayed weight loss while the weight of mice receiving paclitaxel-C/GP was the same as saline-treated mice. At the end of treatment (17 days), C/GP material was not found in all treated tumors. This may be because of either gel degradation or migration following breakup of the gel over time.

The effectiveness of using the C/GP systems to locally deliver high doses of camptothecin to a RIF-1 fibrosarcoma mouse model was demonstrated in the study of Berrada et al. [61]. In this study, the treatments were begun when the tumors reached a volume of approximately 100 mm^3. The C/GP containing camptothecin was found to be more effective than systemic delivery of camptothecin in delaying tumor growth. Although the camptothecin-treated group received a much higher dose intraperitoneally (60 mg/kg mouse) compared to the C/GP/camptothecin-treated group (24 mg/kg mouse), the latter group had tumors growing 8 times less than the initial group at day 8 of treatment. Tumors injected with blank C/GP showed no inhibition of growth and had the same size as untreated tumors. Tumor from these two groups reached the size of 4× initial tumor volume (end point of treatment) at day 7. Tumors from camptothecin-treated group reached the end point at day 8, while those from C/GP/camptothecin-treated group did not reach the end point until day 25 of treatment. This strongly demonstrated the effectiveness of C/GP on sustained release of camptothecin *in vivo* and thus significantly delayed the growth of tumor. It also indicated that the exposure of tumor cells to drug for a prolonged period of time causes more cell death than the short drug exposure resulting from systemic administration.

Furthermore, mice treated with C/GP/camptothecin showed less weight loss compared to the other groups, which implied less toxicity of local treatment compared to systemic treatment [61].

The effectiveness of C/GP systems were again recently approved in the study of Han et al. [76]. Treatment initiated at day 8 after TC-1 cervical cancer cells were subcutaneously inoculated into C57BL/6 mice. Compared to the PBS-treatment group (positive control group), C/GP hydrogel alone did not cause any inhibition on the growth of tumor. Using the same dose of 6 mg Dox/kg mouse body weight, C/GP/Dox system significantly inhibited tumor growth compared to control groups and other treatments including intravenous injection and intratumoral injection of free Dox. PBS-treated and C/GP alone-treated groups had tumors of 8 times bigger sizes compared to those at the beginning of treatment (day 8). Free Dox-treated groups had tumors of 3–5 times larger while C/GP/Dox-treated groups had tumors of the same size as at day 8. This implied the greater effectiveness of local sustain release of Dox from C/GP hydrogel matrix into the tumor site directly. It was also the result of prolonged exposure of cancer cells to Dox as compared with systemic administration of free Dox. In this study, vaccinia virus-based vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1) was also used as immunotherapeutic agent, which made this study as a pioneer report on the use of a biodegradable hydrogel system as an anticancer drug delivery system for successful chemoimmunotherapy. C/GP/Dox and Vac-Sig/E7/LAMP-1 were injected intratumorally and intravenously, respectively. The combination of these two treatments led to the highest tumor suppression without side effects and remarkably enhanced E7-specific $CD8^+$ T cell immune response. The combined therapy also increased long-term anti-tumor activity and mice survival than monotherapy alone.

In vitro study of paclitaxel delivery from C/GMO systems and its transport across different mucin-producing cell lines demonstrated the strong potential of these in situ gels to be used as controlled and targeted drug delivery systems [77]. When injected close to the site of tumor, the ionic polymer used in the formulation will be deprotonated and will form an instant gel at the site of injection at body pH. This system can provide a sustained release of paclitaxel from the gel at and around the site of cancer, which is impossible to achieve with systemic drug administration. However, an *in vivo* study of C/GMO/paclitaxel in mice has not been reported as yet but is eagerly awaited.

9. Discussion and future directions

In recent years, chitosan has gained lots of interests in biomedical fields and has been formulated in several drug delivery hydrogel systems. Chitosan-based gels have been prepared by chemical or physical crosslinking of the polymer chains. Chemical hydrogels are formed by irreversible covalent links, while physical hydrogels are formed by various reversible links [96]. Dialdehydes such as glyoxal and glutaraldehyde, diethylsquarate (DES) [97], oxalic acid [9], ethylene glycol diglycidyl ether (EGDE) [98] or genipin [99] were used to chemically crosslink chitosan. Among them, dialdehydes are considered to be toxic [100,101], but genipin is an interesting crosslinker as it is a naturally occurring material [99]. β-glycerophosphate [15] and tripolyphosphate [102], alginate [103], pectin [104], α-keratose [105], carboxymethylcellulose [106], or xanthan [107] have been used to physically crosslink chitosan.

Although several chitosan hydrogels have been investigated, only a few of them have in situ gelling properties. Therefore, most chitosan hydrogels were developed under the forms of microspheres or nanoparticles for cancer treatment. Some injectable chitosan hydrogels were invented and presented potential but have not tested *in vivo* or in preclinical trial for cancer application. They are thermosensitive poly(ethylene glycol)-grafted chitosan system [108]; temperature-responsive hydroxybutyl chitosan [109]; poly(vinyl alcohol)/chitosan-blended hydrogels [110]; chitosan/ bifunctional aldehyde hydrogel [111]; and a system composed of *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride/glycerophosphate [112].

Most in situ chitosan hydrogel systems have been tested to deliver cytotoxic drugs *in vivo* for treatment of cancer. However, the effect of toxic agents released from these hydrogel systems on other organs or tissues such as heart and skin have not been studied. Moreover, whether an in situ forming hydrogel can treat metastases has not been investigated yet. It has been widely known that metastasis (the spread of cancer in the body) is the main cause of death for patients with cancers. Malignant cancers can spread preferentially from one organ to another. Cancer cells can break away, leak, or spill from a primary tumor, enter the blood vessels, circulate through the bloodstream, and settle down to grow within normal tissues elsewhere in the body [113]. If the cancer spreads to other tissues and organs, it may decrease the survival of patients. Among various body organs, lung is the most common place for metastasis from tumors in other parts of the body. However, once the cancer is diagnosed early, the whole solid tumor can be fully cured by surgical removal prior to malignant spreading, and then in situ forming hydrogels can contribute their ability to prevent the reoccurrence of cancer cells as what Ruel-Gariepy et al. tried to prove in their work [14]. As described previously, they proposed to use their C/GP thermosensitive hydrogel for the sustained release of paclitaxel at tumor resection sites in order to prevent local tumor recurrence. The introduction of these hydrogels at tumor resection sites can also reduce or stop the travel of remaining cancerous cells to other parts of the body and hence preventing metastasis.

Among in situ forming chitosan hydrogels applied in cancer treatment, C/GP delivery system shows lots of advantages over Az-CH-LA and C/GMO systems. Az-CH-LA system needs the insert of UV-laser optical fiber at the site of injection to induce gelation. And C/GMO system needs to have low pH in order to be gelled when facing body physiological pH. These disadvantages can make Az-CH-LA and C/GMO systems less practical than C/GP system which has physiological pH and owns a very friendly mechanism of gelation based on temperature difference.

The use of these in situ gelling systems should not be limited to cytotoxic drug delivery. They should be investigated for sustained release of biological anticancer agents such as plasmids, short oligonucleotides, hormones, antibodies (and fragments thereof) and peptidic agents as well. The combination of different approaches should be taken into consideration to enhance the efficiency of cancer treatment. It may well be possible to combine

the delivery of two different types of anticancer agents in one gelling system.

With hydrogelling systems that degrade over periods of months to maybe even years, the major advantage would be in greater patient compliance as infrequent injections would be required, with the injections administered subdermally. As lesser amounts of drugs would be required, this would in fact significantly reduce the cost involved with therapy, especially for cancer where treatment usually runs in the tens of thousands of dollars annually. For deeper-seated tumors and those arising in blood, a hydrogel implant may still be worthwhile if small but constant quantities of anticancer drugs are required for efficacy. While touched upon briefly in this discussion paper, such hydrogel systems may also be useful for localised growth of cells, which can serve as bioreactors churning out efficacious quantities of therapeutic proteins. While applications of chitosan hydrogels for cancer therapy only have been covered herein, this same group of technologies may very well be suitable for other pathologies.

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