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Review

Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids

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ABSTRACT

Genipin, a crystalline and well defined chemical compound, is extracted from gardenia fruits according to a modern microbiological process. As a water-soluble bi-functional crosslinking reagent, it reacts promptly with chitosan (and with proteins or amines in general) thus producing blue-coloured fluorescent hydrogels. The reaction mechanism between chitosan and genipin is well understood for a variety of experimental conditions. The resulting crosslinked complexes are not cytotoxic for the animal and human cells so far examined. The safety and the beneficial actions of genipin emerge from a number of research projects in the areas of the therapies of diabetes, periodontitis, cataract, hepatic dysfunction, as well as in wound repair and nerve regeneration. Food sciences, forensic chemistry and cytology have also provided evidence of the safety of its use. The most important applications of genipin in conjunction with chitosan are the preparation of elastic and resistant gels such as the cartilage substitutes, the manufacture of drug carriers for controlled release, the encapsulation of biological products and living cells, and the medication of wounds in animals and humans. Genipin might replace glutaraldehyde with the advantages of stability and biocompatibility of the crosslinked products whose quality assessment and manipulation would be easier.

1. Introduction

Being biocompatible, non-toxic, stable, sterilizable and biodegradable, chitosan has valuable properties as an excipient, but on the other hand it exhibits unique and most valuable properties that enhance its versatility in the biomedical and biotechnological fields, such as immunostimulation, activation of macrophages, mucoadhesion, antimicrobial activity, and well assessed chemistry (Genta et al., 1999; Jiang, Kumbar, Nair, & Laurencin, 2008; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 2009; Muzzarelli & Muzzarelli, 2005, 2008; Senel & McClure, 2004; Yuan, Chestnutt, Haggard, Bumgardner, & Muzzarelli, 2008). Moreover, chitosan can also be prepared in a variety of forms, namely hydrogels and xerogels, powders, beads, films, tablets, capsules, microspheres, microparticles, nanofibrils, textile fibers, and inorganic composites. Chitosan is today a protagonist in advanced fields, for example it is a high performing non-viral vector for DNA and gene delivery (Weecharangsan et al., 2008).

Biodegradable polymers such as chitosan need to be crosslinked in order to modulate their general properties and to last long enough for delivering drugs over a desired period of time. Certain reagents have been used for crosslinking chitosan such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate. However, studies have shown that the synthetic crosslinking reagents are all more or less cytotoxic and may impair the biocompatibility of a chitosan delivery system (Nishi, Nakajima, & Ikada, 1995; Speer, Chvapil, Eskelson, & Ulreich, 1980). Hence, it is desirable to provide a crosslinking reagent for use in biomedical applications that has low cytotoxicity and that forms stable and biocompatible crosslinked products.

2. Isolation of genipin and quality assessment

Genipin (CAS No 6902-77.8) was isolated from *Genipa americana* (Djerassi, Gray, & Kinci, 1960; Djerassi et al., 1961) during an extensive investigation of extracts from Mexican and South American plants, as a "beautifully crystalline substance" with a defined chemical structure (Fig. 1).

The oriental traditional medicine makes wide use of an extract of *Gardenia jasminoides* Ellis, containing the geniposide that is enzymatically hydrolyzed to genipin by intestinal bacteria when administered *per os*, as recent pharmacokinetics studies indicate (Akao, Kobayashi, & Aburada, 1994). Genipin concentration in gardenia fruits is rather low (ca. 0.005–0.01%), whilst geniposide is in the range 3.06–4.12% (Cao, Wang, & Jia, 2001). Because it is recognized that genipin, rather than geniposide, is the main compound that exerts the pharmacological activities of gardenia subsequent to geniposide hydrolysis (Zheng, Dong, & Yu, 2000), there is interest in its isolation and purification for use in therapy and in the manufacture of food commodities and dyes.

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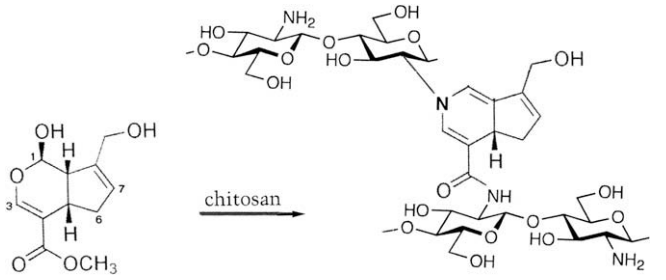

Fig. 1. Genipin reacts with chitosan to yield two main crosslinking reactions. On the right, two chitosan chains (represented by their structural units) are crosslinked by one mole of genipin: the formula shows the two newly formed chemical groups, namely the monosubstituted amide and the tertiary amine. An additional reaction is the homopolymerization of genipin (see text).

Genipin is manufactured today from geniposide, a glucoside, by using β-glucosidase (Fujikawa, Fukui, & Koga, 1987; Fujikawa, Yokota, Koga, & Kumada, 1987; Tsai, Westly, Lee, & Chen, 1994). It should be added that the production protocol and the quality evaluation have been recently re-assessed and established in modern terms (Xu et al., 2008). While it is difficult to extract genipin from gardenia fruits directly by common chemical procedures (Yao, Wu, & Wu, 2004) on the other hand genipin is isolated in large quantity by a microbiological process involving *Penicillium nigricans* that produces β-glucosidase that in turn hydrolyzes the geniposide into the aglycone genipin.

For the preparation of the gardenia extract, the dry fruit of gardenia is finely ground to pass 200-mesh sieve and dried at 60 °C. The gardenia material (100 g) is boiled with 1 l of water twice for 30 min at a time, and the extracts are pooled; the concentration is adjusted to 1% with distilled water. For the TLC analysis of geniposide and genipin, useful to monitor the production process, the fermentation product is deposited on a silica gel TLC plate with mobile phase ethylacetate and petroleum ether mixture (2:1 v/v): the blue spot is visualized by spraying glycine solution (0.44 mmol Gly in 0.1 M phosphate buffer at pH 7.0) and then heating at 70 °C for 30 min. High performance liquid chromatography analysis of fermentation substrate and product is performed on a Reliasil C18 column (5 microm, 25 °C; UV detection at 238 nm; injection volume 10 microl; mobile phase methanol–water 45:55 v/v; flow rate 1 ml/min). The linear regression equations for geniposide and genipin are $Y = 2208.6\ X - 41.419$, and $Y = 2478.4\ X + 14.149$, respectively.

For the production of genipin, macroporous resins D141 and HPD100 (7:3 v/v) soaked in 95% ethanol for 24 h, washed with 95% ethanol and rinsed with distilled water are used to adsorb genipin from the fermented extract and then packed into a column, to be eluted with acetone–ethanol: the purity of genipin at this stage is ca. 30%. Further purification of genipin is carried out in a glass column packed with silica gel in petroleum ether: the crude genipin extract dissolved in petroleum ether is loaded on the silica gel column that is then eluted with ethylacetate–petroleum ether 3:2 mixture. The eluate is then collected, vacuum concentrated and purified again by repeating the procedure till a high purity genipin concentrate is obtained. The latter is distilled, and then crystallized and re-crystallized. The conversion of geniposide into genipin reaches 95% under the optimized fermentation conditions. This procedure has the advantages of speed, efficiency, low environmental impact and low cost. No additional nutrient was needed in the fermentation medium, therefore this cost-effective process may be applied industrially.

Crystalline genipin is identified as follows: UV (CH_3OH) λ_{max} 240 nm. ESI, m/z 226. IR υ_{max}: 1007, 1105, 1686, 2849, 2929, 2935, 3030, 3238, and 3386 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 7.53 (s, H-3), 5.89 (s, H-7), 4.82 (d, J = 8.5 Hz, H-1), 4.35 (d, J = 13.2 Hz, H-10), 4.29 (d, J = 13.2 Hz, H-10), 3.74 (s, —OCH_3), 3.22 (ddd, J = 9.5, 8.5, 8.5 Hz, H-5), 2.89 (ddt, J = 16.8, 8.5, 1.4 Hz, H-6), 2.54 (ddd, J = 8.5, 8.5, 1.5 Hz, H-9), 2.07 (ddt, J = 16.8, 9.5,1.8 Hz, H-6). These data by Xu et al. (2008) are in accordance with those reported previously (Endo & Taguchi, 1973).

3. Biochemical data on genipin safety

Besides the empirical data of the traditional medicine, today some information on the beneficial effects of genipin is available in biochemical terms. Mie, Satoshi, Atsushi, and Kenji (2004) used gardenia for the treatment of liver cirrhosis and their investigation showed that the activated hepatic stellate cells could be suppressed by genipin. Genipin reveals remarkable effects as an anti-inflammatory and anti-angiogenesis agent, and inhibits lipid peroxidation and production of nitric oxide, besides protecting the hippocampal neurons from the toxicity of Alzheimer's amyloid beta protein (Koo, Song, Kim, & Park, 2004). Takeuchi et al. (2005) found that genipin protected mice from the lethal effect of administered GalN-LPS: in fact 8 out of 15 mice survived for at least 24 h, whilst all mice not given genipin died within 12 h. In mice given genipin, serum and liver tumor necrosis factor-alpha levels as well as serum AST and ALT activities were significantly lower; hepatic necrosis and inflammatory cells infiltration were slight. TNF-α, NF-κB activation and TNF-α mRNA expression in the cultured mouse macrophage-like cell line J774.1 were significantly suppressed by genipin administration. Therefore genipin was a remedy for acute hepatic dysfunction thanks to its suppressive effect on TNF-α production.

The extract of *Gardenia jasminoides* Ellis fruits has been used over the years in traditional Chinese medicine to treat symptoms of type 2 diabetes (Danbuo, 1984). Zhang et al. (2006) evaluated gardenia extract and observed that it dose-dependently stimulated insulin secretion by pancreatic islets derived from wild type but not from UCP2-deficient mice. Because UCP2 was required for this effect, it was assumed that gardenia extract contained a UCP2 inhibitor. The uncoupling protein 2 (UCP2) negatively regulates insulin secretion: UCP2 deficiency (by means of gene knockout) improves obesity- and high glucose-induced beta cell dysfunction and consequently improves type 2 diabetes in mice. To identify the active molecule, the gardenia extract was fractionated using silica gel chromatography (1:1 hexane and ethyl acetate as eluent). Fractions were assessed for UCP2-dependent stimulation of insulin secretion. Using H NMR and mass spectroscopic analysis, genipin was identified within the active fraction.

Moreover, Zhang et al. (2006) while investigating if the crosslinking action of genipin is required for inhibition of UCP2, synthesized a derivative of genipin that lacks crosslinking activity. It has been reported that genipin can be dimerized by equimolar amounts of glycine to form the blue pigment, genipocyanin G1, a highly conjugated dimeric adduct (Fujikawa et al., 1987). It has also been reported that cytochrome c can be crosslinked by genipin to form oligomers and that the crosslinking process likely involves formation of complexes in which two primary amino groups belonging to two distinct proteins react with genipin (Fujikawa, Nakamura, & Koga, 1988). As the C10 primary alcohol and the C1 hemiacetal of genipin are likely required for the formation of the oligomeric products (Touyama et al., 1994), it was speculated that genipin derivatives lacking these two active sites may lose crosslinking activity. To confirm this prediction, 1,10-anhydrogenipin was prepared: genipin (5 g, 22.1 mmol) and triphenylphosphine (6.085 g, 23.2 mmol) were dissolved in distilled methylene chloride (110 ml). To the solution at 0 °C diisopropylazodicarboxylate (4.60 ml, 23.2 mmol) was added drop-wise; the resulting mixture

was stirred at 0 °C for 30 min. Concentration in vacuo and purification on silica gel using hexane–ethylacetate (10:1 or 4:1) as eluent yielded 1.9 g of anhydrogenipin as a white solid (41% yield, >99% purity).

A simple color assay was made by treating anhydrogenipin with an equimolar amount of glycine in pH 7 buffer at 80 °C. After 4 h, the anhydrogenipin solution, but not the genipin solution, was still colorless, which indicates that dimerization to form conjugated genipocyanin blue pigments did not occur with anhydrogenipin. To confirm that the latter lacks protein crosslinking activity, the cytochrome c crosslinking assay was performed according to Fujikawa et al. (1988). Thus, 1,10-anhydrogenipin inhibits proton leak, closes K_{ATP} channels and stimulates insulin secretion in UCP2-dependent manner. The lack of crosslinking activity might represent an advantage insofar as it would avoid adverse nonspecific effects due to interaction with other proteins. In isolated kidney mitochondria, genipin specifically inhibits UCP2-mediated proton leak. In pancreatic islets, genipin increases mitochondrial membrane potential, increases ATP levels, closes plasma membrane K_{ATP} channels, and stimulates insulin secretion in UCP2-dependent manner. Importantly, acute inhibition of UCP2 by genipin reverses high glucose- and obesity-induced beta cell dysfunction. These results suggest that the crosslinking activity of genipin is not required for its biological activity as a UCP inhibitor.

Current treatment for diabetes mellitus is based on drugs such as sulfonylureas, thiazolidinediones, or metformin. Of interest, none of these pharmacologic treatments are specifically directed at a pathogenic cause of pancreatic beta cell dysfunction. Also, in many patients, these pharmaceutical agents prove inadequate for maintaining blood glucose at an acceptable level and injection of insulin is unavoidable. Thus, genipin and its chemical variants are today useful research tools for studying biological processes related to insulin secretion and thought to be controlled by UCP2 (Zhang et al., 2006).

A biodegradable composite made of genipin-crosslinked gelatin and tricalcium phosphate ceramic particles was developed as a bone substitute and was evaluated on the basis of the biological response of rabbit calvarial bone White rabbits were used for cranial implantation: bone defects (15 × 15 mm) were filled with the composite, while controls were filled with de-proteinized bovine bone. It was found that the composite genipin–gelatin–ceramic is malleable and easily molded into the bone defect; biocompatible; osteoconductive, with progressive growth of new bone into the defect; and biodegradable, with progressive replacement of the composite by new bone. Radiographic and histological evaluations revealed greater new bone growth into the composite compared to controls (Yao, Liu, Hsu, & Chen, 2005). Therefore, genipin could serve as a safe bone substitute ingredient.

Research on gelatin was a prelude to more extended research on the associations of gelatin and chitosan: the possibility to stabilize gelatin films by crosslinking with genipin was investigated with genipin solutions at different concentrations. Crosslinking of gelatin provokes a significant reduction of the swelling in physiological solution, and enhances the thermal stability of the samples. The data obtained from the gelatin films treated with genipin at concentrations higher than 0.67% are quite similar, and indicative of a good stabilizing effect of genipin. In spite of the small gelatin release (2%) observed after 1 month of storage in buffer solution, the mechanical, thermal and swelling properties of the films were very close to those previously obtained for glutaraldehyde-crosslinked gelatin, and suggested that genipin, by far less cytotoxic, can be considered a valid alternative for crosslinking aminated biomaterials (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002).

Yang et al. (2007) evaluated the therapeutic effects on periodontitis of ketorolac tromethamine gel (KT gel) and the same with genipin (KTG gel). The skin permeation rate of ketorolac from the KT and KTG gels was 5.75 ± 0.53 and 5.82 ± 0.74 μg/cm^2/h, respectively. The tensile strength was larger for the KTG gel than for the KT gel. After 4 weeks, the periodontal pocket depth of the KTG gel group (3.22 ± 0.20 mm) was significantly decreased compared with the control group (4.50 ± 0.25 mm) and the KT group (3.84 ± 0.26 mm). The KTG gel did not induce separation of the stratum corneum and subcutaneous tissue, and the collagen layers of the corium were closer, more fibrous, and showed longer connections than in the other groups. The KTG gel, for which the skin permeation rate of genipin was 10.13 ± 1.47 μg/cm^2/h, was effective against gingivitis in the periodontal pocket thanks to its better anti-inflammatory activity, and to the crosslinkage of the biological tissue by genipin.

Kitano et al. (2006) assayed the epithelial cell line α-TN4 for proliferation, migration, and expression of α-smooth muscle actin (α-SMA, the hallmark of myofibroblast generation). The gene expression of transforming growth factor-β1 and connective tissue growth factor was characterized with real-time reverse transcription-polymerase chain reaction. In addition, p38 mitogen-activated protein kinase (p 38 MAPK) and extracellular signal-regulated kinase (ERK) were evaluated. Cytotoxicity of genipin was tested by using a commercial colorimetric assay kit for nuclear matrix protein 41/7 in culture medium. Genipin suppressed cell proliferation and migration in association with inhibition of p38 MAPK phosphorylation, although ERK signaling was enhanced. Genipin suppressed mRNA expression of transforming growth factor-β1 and connective tissue growth factor. Cytoplasmic fiber formation declined based on less intense α-smooth muscle actin immunocytochemical staining. However, the α-smooth muscle actin expression was actually not altered: this suggests that genipin attenuated formation of α-SMA–containing cytoskeleton. Treatment of the cells with genipin for 48 h did not increase the release of NMP41/7 to the medium, indicating that genipin is not cytotoxic. Because genipin suppressed α-TN4 lens cell fibrogenic behaviors, it may be of therapeutic value in preventing posterior capsule opacification (Kitano et al., 2006).

Lu et al. (2007) evaluated peripheral nerve regeneration using biodegradable genipin-crosslinked gelatin nerve conduits with three different crosslinking degrees, 24%, 36% and 51%. Biocompatibility and biodegradability of the genipin-crosslinked gelatin nerve conduit and its efficiency as a guidance channel were examined based on the repair process of a 10-mm gap in the rat sciatic nerve. They concluded that such conduits with a mean crosslinking degree of 36% can ensure nerve regeneration with a more mature structure, as demonstrated by better developed epineural and perineural organisation and axonal development, as well as better-recovered electrophysiology with a relatively positive sciatic functional index and a shorter latency of the muscle action potential curve. Regenerated nerves in the conduits with mean crosslinking degrees of 24% and 51% were less favourable, due to irritation caused by degradation material and compression by the remaining tube walls, respectively.

The development of stable hydrogels based on blends of agar and k-carrageenan crosslinked with genipin (Meena, Prasad, & Siddhanta, 2008) has generated much interest in further developments of seaweed hydrocolloids that are well established on the food market, in view of increased stability and remarkable swelling capacity imparted by genipin. It became apparent that genipin does not react with said polysaccharides, but with the proteins present at the level of 0.29% and 1.81% in agar and k-carrageenan, respectively, as demonstrated after the thorough removal of the proteins until nitrogen was no longer detected: in such instances the blue color did not develop nor the general characteristics were altered. It is in any case noteworthy that genipin is active on these polysaccharides as a consequence of the usual presence of minor quantities of aminoacids or proteins.

The extreme sensitivity of genipin towards the amino groups at trace levels, together with its fluorogenicity was applied in forensic science to develop latent fingerprints as blue impressions that fluoresce brightly upon illumination at ca 590 nm: Almog, Cohen, Azoury, and Hahn (2004) in fact presented genipin as a new fingermark reagent that forms a visibly blue compound with aminoacids: thus, genipin combines in itself properties simultaneously useful in colorimetry and fluorimetry. Genipin was further studied as a fingerprint reagent, and optimal conditions for fingerprint development have been determined (Levinton-Shamuilov, Cohen, Azoury, Chaikovsky, & Almog, 2005). Latent fingerprints on paper items previously treated with a non-ink running formulation containing 0.17% of the reagent, showed up as both colored and fluorescent images. On brown wrapping paper and on papers with highly luminescent backgrounds, genipin developed more visible and clearer prints than did classical reagents such as ninhydrin. According to the experts in forensic science, an additional important advantage of genipin is that it is a totally harmless and environmentally friendly reagent.

Sung, Huang, Huang, Tsai, and Chiu (1998) and Sung, Huang, Huang, and Tsai (1999) defined the crosslinking protocols for spray-dried chitosan microspheres, and studied their cytotoxicity after intramuscular injection and subsequent inflammatory response. The latter disappeared in 12 weeks for plain chitosan and genipin–chitosan microspheres but persisted in tissues treated with glutaraldehyde–chitosan microspheres. Glutaraldehyde and ethylene glycol diglycidyl ether, which have been used extensively in developing bioprostheses, were more cytotoxic than genipin. *In vitro* results demonstrated that genipin is an effective crosslinking reagent for biological tissue fixation: again, genipin-fixed porcine pericardium implanted sub-cutaneously generated less inflammation as compared to a glutaraldehyde control.

Human fibroblasts cultured in contact with crosslinked chitosan films provided preliminary confirmation of the lower cytotoxicity of genipin compared to glutaraldehyde. The cytotoxicity of genipin studied in vitro with 3T3 fibroblasts and MTT assay resulted ca. 10,000 times lower than that of glutaraldehyde. Also, the colony forming assay showed that the proliferative capacity of the cells after exposure to genipin was approximately 5000 times greater than for cells exposed to glutaraldehyde. It was concluded that genipin may be well suited for clinical usage (Mi, Sung, & Shyu, 2001; Mi, Tan, Liang, Huang, & Sung, 2001; Sung, Chang, Liang, Chang, & Chen, 2000).

4. Reaction mechanism and products of chitosan crosslinking with genipin

The crosslinking reaction mechanisms for chitosan with genipin are different at different pH values. Under acidic and neutral conditions, a nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurs, followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehydo group. In other words, genipin acts as a dialdehyde but its condensation products are much more stable compared to glutaraldehyde (Mi, Shyu, & Peng, 2005; Sung, Huang, Huang, & Tsai, 1999). In the product, short chains of condensed genipin act as crosslinking bridges.

Under basic conditions, the ring-opening reaction of genipin occurs via a nucleophilic attack by hydroxyl ions in aqueous solution to form intermediate aldehyde groups, which subsequently undergo aldol condensation. The terminal aldehyde groups on the polymerized genipin undergoes a Schiff reaction with the amino groups on chitosan to form crosslinked networks. Therefore, the pH condition plays an important role in influencing the crosslinking reactions.

Butler, Ng, and Pudney (2003) found that the fastest reaction is a nucleophilic attack of an amino group of chitosan to carbon 3 of genipin which results in the opening of the dihydropyran ring and the formation of a tertiary amine, i.e. a genipin derivative linked to a glucosamine unit. The subsequent slower reaction is a nucleophilic substitution of the ester group of genipin to form an amide. Simultaneously, polymerization can take place between genipin molecules already linked to amino groups of chitosan which could lead to the crosslinking of amino groups by short genipin copolymers. The dark-blue coloration that appears in the hydrogels exposed to air is associated with the oxygen radical-induced polymerization of genipin as well as its reaction with amino groups.

Poly(vinyl alcohol), poly(vinyl pirrolidone) and poly(ethylene oxide) are synthetic hydrophilic polymers widely used because non-toxic and non-irritating, in contact lenses, vocal chord reconstruction, and artificial cartilage manufacture: chitosan solutions are often blended with these polymers for such technical purposes. In order to understand the effect of a crosslinker such as genipin, it was deemed appropriate to study blends of chitosan and said hydrophilic man-made polymers: for example, chitosan and 80% hydrolyzed poly(vinyl alcohol) were used to prepare semi-interpenetrating networks of varying ratios of the constituents. The hydrogels were crosslinked, and the swelling behavior of the resulting hydrogels was studied in deionized water at 25, 35 and 45 °C and in media of various pH values at 25 °C. The swelling behavior of the gels was found to depend on temperature, pH value, and amount of poly(vinyl alcohol) present in the gel.

The solutions with chitosan: poly(vinyl alcohol) (mass ratio 1 and constant genipin quantity), turned into hydrogels exhibiting a sharp viscosity rise after ca. 500 min at 25 °C, whilst in the absence of poly(vinyl alcohol) the chitosan–genipin hydrogel was formed after ca. 400 min. Thus poly(vinyl alcohol) delayed the crosslinking reaction. PVA-containing hydrogels prepared in Petri dishes were dried at 55 °C to produce 0.2 mm thick blue films (Nand, Rohindra, & Khurma, 2007). Similar semi-interpenetrating polymeric networks were prepared with chitosan and PVP. Genipin was used in all cases at the 0.5 w/v concentration, that is largely lower than the chitosan concentration (Khurma, Rohindra, & Nand, 2005). Basic information such as that reported above was put to use in developing molecularly imprinted materials using xylene isomers as analogues of dioxin and polychlorinated biphenyls suitable for identification or separation purposes (Espinosa-Garcia et al., 2007).

4.1. Chitosan–genipin hydrogels

Mi, Sung, Shyu, Su, and Peng (2003) synthesized novel chitosan gel beads by simultaneous ionic and covalent crosslinking mechanisms involving tripolyphosphate (TPP) and genipin. The UV and IR spectroscopic data indicated that the co-crosslinking depends on pH: the covalent crosslinking dominates at pH values 7.0 and 9.0, whilst ionic crosslinking dominates at pH 1.0, 3.0 and 5.0. There are evident effects on the swelling and the enzymatic degradation of chitosan derivatives.

A novel pH-sensitive hydrogel of N,O-carboxymethyl chitosan and alginate crosslinked by genipin has been reported by Chen et al. (2004). The amount of albumin released from the genipin-crosslinked NOCC/alginate hydrogel at pH 1.2 was relatively low (20%), while at pH 7.4 it increased significantly (80%). Under the same condition, the swelling ratio was higher at high pH than at low pH. The authors suggested that the genipin-crosslinked NOCC/alginate hydrogel may be a suitable polymeric carrier for site-specific proteinic drug delivery to the intestine. Another substituted chitosan, the carboxymethyl-hexanoyl chitosan amphiphatic hydrogel with excellent water-absorption and

water-retention capacity under neutral conditions was crosslinked with genipin and then employed as a carrier for delivering ibuprofen and other amphiphatic agents (Liu, Chen, Lin, & Liu, 2006).

Under other instances genipin was dissolved (0.05–0.20%) into chitosan solutions (1.5%; pH 7.0) containing glycerol phosphate disodium salt to promote gelation. The resulting hydrogels were cured at 37 °C for 12 h before viscosimetric measurements. Genipin at 0.15% reduced the gelation time from 8 to less than 2 minutes as estimated from the crossover of elastic modulus G'' and storage modulus G'. The main conclusion by Moura, Figueiredo, and Gil (2007) was that under physiological conditions the viscoelastic features of a chitosan solution and its gelling ability could be tuned by changing the genipin concentration (very low in all cases); relatively strong elastic gels were obtained. More detailed rheological data for gels obtained at body temperature and physiological pH values have been published by Moura, Figueiredo, and Gil (2008). In view of this behavior the formulations were deemed suitable for encapsulation of cells and bioactive compounds. In any case, also acellular biological tissues isolated from bovine pericardium and treated with genipin were found to be safe and useful for the regeneration of tissues (Liang, Chang, Hsu, Lee, & Sung, 2004).

A 5–20% genipin formulation maintained 95% viability of encapsulated cultured disk cells. The gel did not produce inflammatory reactions when injected sub-cutaneously into C57BL/6 mice and was therefore biocompatible. As a preliminary step toward replicating design and function of the human intervertebral disk, the gel flowed into the clefts without leakage when injected into the degenerated nucleus pulposus of human cadaveric intervertebral disk (Mwale et al., 2005).

The suitability of chitosan-based hydrogels as scaffolds for the encapsulation of intervertebral disc cells and the accumulation of a functional extracellular matrix mimicking that of the nucleus pulposus was further investigated by Roughley et al. (2006). The specific hypothesis under study was that the cationic chitosan would form an ideal environment in which large quantities of newly synthesized anionic proteoglycan could be entrapped. Indeed, all the formulations of cell-seeded chitosan hydrogels, studied in vitro, showed that the majority of proteoglycan produced by encapsulated nucleus pulposus cells was retained within the gel rather than released into the culture medium. The results supported the concept that chitosan may be a suitable scaffold for cell-based supplementation to help restore the function of the nucleus pulposus during the early stages of intervertebral disc degeneration.

As far as the growth of murine chondrocytes is concerned, the chitosan–genipin product permits to reduce to 1/3 the preparation time for a cartilage substitute (Lien, Li, & Huang, 2007). Similar scaffolds were also prepared in the presence of hydroxyapatite for the cultivation of bovine knee chondrocytes: the right choice of pre-freezing temperature or a higher weight percentage of chitin in the chitin–chitosan scaffolds yielded small pore diameters, great porosity, large specific surface area, high Young's modulus and low extensibility (Kuo & Lin, 2006).

For the same purpose, Kuo and Ku (2008) prepared PEO + chitin + chitosan scaffolds. The rationale behind the use of the thermoplastic polyethylene oxide was that it has been widely applied over the past two decades in the pharmaceutical technology for drug delivery systems, wound dressings and generation of cartilage-like tissue; on the other hand, chitin has been extensively used in hemostasis and in wound healing (Muzzarelli, 2009). If chitin is present in a chitosan scaffolds, the tensile strength can be considerably improved. A suitable chitin was prepared as follows: chitin powder (3 g) was rinsed with acetone, suspended in 60 ml of 12 N HCl at 3 °C for 1 h, and filtered: the solid residue was likewise treated again with fresh acid for 1 h. Collected filtrates were mixed, adjusted at pH 7, and centrifuged to obtain a (presumably amorphous) chitin sludge.

Based on the contours of porosity, the percentage of void space in these tertiary scaffolds was higher than 90%; large extensibility of the scaffolds occurred at the following compositions: PEO > 37.5%, chitin < 25%, and chitosan < 62.5%. After cultivation of chondrocytes over 4 weeks, the biodegradation was between 30 and 60%. The formation of neo-cartilage was assessed on the basis of the amounts of chondrocytes, glycosaminoglycans and collagens. The highest chondrogenesis was obtained in the following ranges: PEO 25–40%; chitin 12.5–37.5% and chitosan 30–50% (Kuo & Ku, 2008).

Silva et al. (2008) reacted a mixture of chitosan and silk fibroin with genipin: because genipin preferentially reacts with the aminoacids lysine and arginine of certain proteins, and the fibroin chain contains a very low percentage of those aminoacids (0.6%), the crosslinking sites are low in number and the kinetics are consequently slow for the silk fibroin fraction. On the other hand, chitosan has a high content of amino groups that promptly react with genipin. In fact, the reaction velocity was found to depend on the chitosan/fibroin ratio, based on rheological data: the yellowish formulation with high chitosan content formed a dark-blue gel within 3 h whereas the formulations with low chitosan content required a longer time, up to 24 h at 37 °C. Moreover, the crosslinking degree values of chitosan + fibroin + genipin sponges were 45.2, 29.3 and 23.9 for chitosan contents 80%, 50% and 20%, respectively. Nevertheless, the genipin crosslinking of the chitosan + fibroin blended systems was accompanied by protein conformational changes that promoted the formation of stable and ordered structures. Higher moduli values were found in the sponges prepared with a higher amount of silk fibroin, which suggests that its incorporation into chitosan submitted to genipin crosslinking hardens the sponges notwithstanding the scarce reactivity of fibroin. The infrared spectra seem to indicate that genipin crosslinking promoted the formation of β-sheets in the sponges.

The observed surfaces of the sponges showed distinct morphology with pore sizes ranging from 29 to 167 μm, depending on the composition, and porosity values were all slightly over 80% for the above given chitosan contents. The hydrophilicity of the sponges combined with their porosity sustained uniform cell distribution within the entire sponge volume. Mouse fibroblasts were viable in the presence of the extracts from crosslinked chitosan + fibroin sponges: in fact, the viability close to 100% in all sponges demonstrated their extremely low cytotoxicity. The glucosaminoglycan content increased with the culture time, typical values being ca. 4%, 4% and 8% matrix after 14, 21 and 28 days for samples containing equal quantities of chitosan and fibroin (Silva et al., 2008).

4.2. Chitosan microspheres

Different ways are used to prepare micro/nanoparticles of chitosan for drug delivery such as emulsion crosslinking, coacervation/precipitation, spray-drying etcetera (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). Yuan et al. (2007) mixed chitosan with bovine albumin and genipin to make crosslinked microspheres. The degree of crosslinking of the chitosan microspheres and their swelling ratio increased with increased crosslinking time or genipin concentration. The chitosan microspheres crosslinked with genipin released albumin more slowly than the plain ones. The drug release rates and the swelling ratio of chitosan microspheres were controlled by the degree of crosslinking.

Mi, Sung et al. (2001) and Mi, Tan et al. (2001) prepared chitosan microspheres by a water-in-oil dispersion method, using genipin as a crosslinker. The chitosan microspheres with indomethacin were crosslinked for various time periods and at different pH values. Their results showed that the release of indomethacin from

the chitosan microsphere prepared from a chitosan–indomethacin suspension at high pH exhibited increased dissolution rate compared with that of chitosan microspheres prepared from a chitosan–indomethacin suspension at low pH due to the difference in the ionization of indomethacin. The release of indomethacin from the microsphere was influenced by the degree of crosslinking and by the chitosan/indomethacin ratio. These studies indicate that the crosslinked chitosan microspheres are a good carrier for drug release.

Chitosan–genipin microspheres were obtained by crosslinking in inverse emulsion, and were observed to swell significantly in water at pH values below 6.5 but to a smaller extent at pH values above 6.5. Rate and efficiency of heparin adsorption at pH 7.4 were increased by quaternization of the chitosan–genipin microspheres with the aid of glycidyl trimethylammonium chloride: Whereas at pH 7.4, 40 mg of chitosan–genipin microspheres adsorbed only ca. 20% of heparin from a solution (5 ml) containing 200 μg/ml heparin, the quaternized microspheres could bind 90% of heparin under the same conditions. The 50% decrease in heparin concentration occurred within 5 min. The quaternized microspheres were claimed to be potentially useful for heparin removal in biomedical applications (Kaminski, Zazakowny, Szczubialka, & Nowakowska, 2008), but more in vitro studies and pre-clinical trials are needed to provide evidence of safety, considering the well-known hemostatic activity of chitosan (Muzzarelli, 2009).

Beads containing a chitosan core and a polyelectrolyte complex shell were formed by the drop-wise addition of chitosan to solutions containing genipin together with one of the following: sodium alginate, gellan, pectin, k-carrageenan, or poly(acrylic acid). Hydrogel cores were formed by crosslinking chitosan with genipin. The shell thickness was generally very thin and impermeable to macromolecules, but permeable to low molecular weight organic compounds. The shell was a means to control stability and swelling of the beads (Barck & Butler, 2005).

For example, chitosan–alginate beads with inner crosslinked chitosan core and outer chitosan–alginate complex membrane were prepared by Mi, Sung, and Shyu (2002) and Mi, Tan, Liang, and Sung (2002). These reinforced chitosan–alginate complex beads were prepared by dropping chitosan into a solution containing both alginate and genipin: the gelation of the chitosan droplets depended on chitosan–alginate polyelectrolyte complexation and chitosan crosslinking by genipin. The chitosan–alginate complexation dominates the formation of bead skin layer, whereas the crosslinking of chitosan by genipin dominates the curing of the bead core. The swelling ratio of the crosslinking reinforced chitosan–alginate complex bead decreased as the pH or the concentration of alginate in the gelling solution was decreased. The protonation of the amino group of chitosan or the shield of charge of the ammonium group of chitosan by chloride ions resulted in the decrease of crosslinking density due to the inhibition of nucleophilic attack on the dihydropyran ring of genipin. Contrary to the swelling properties, the rate of indomethacin release out of the chitosan–alginate complex beads increased with the pH decrease, or concentration of alginate in the gelling solution due to the decreased crosslinking density of the beads. The cytotoxicity examination with 3T3 fibroblasts in the vicinity of the chitosan gel after 32 h of culture suggested that the chitosan–alginate beads crosslinked by genipin had good biocompatibility.

According to a different approach, alginate–chitosan microcapsules, composed of an alginate core and a genipin-crosslinked chitosan membrane, proposed for live cell encapsulation and other delivery applications were prepared by Chen, Ouyang, Lawuyi, and Prakash (2006). Results showed that the crosslinking reaction generated the fluorescent chitosan–genipin conjugates. The crosslinked chitosan membrane was clearly visualized by confocal laser scanning microscopy. The shell-like crosslinked chitosan membranes were affected by the reaction temperature but not by reaction time and genipin concentration. Crosslinking at higher temperatures tended to form relatively thinner membranes. The construction of the genipin-crosslinked chitosan membranes could be varied by manipulation of the genipin crosslinking conditions. Their results will be useful in the future exploitation of genipin-crosslinked alginate–chitosan microcapsules for therapy.

4.3. Genipin-modified chitosan gel as a wound dressing

Genipin has been used to crosslink chitosan membranes to control swelling ratio and mechanical properties. It was found that crosslinking of a chitosan membrane using genipin increased its ultimate tensile strength but significantly reduced its strain-at-fracture and swelling ratio. There was no significant difference in antimicrobial activity between the genipin-crosslinked chitosan membrane and its plain counterpart. As already mentioned, the genipin-crosslinked chitosan membrane had significantly less cytotoxicity for human fibroblasts and slower degradation rate compared to the glutaraldehyde-crosslinked membrane (Mi, Sung et al., 2001; Mi, Tan et al., 2001). These results suggested that the genipin-crosslinked chitosan membrane may be a promising carrier for fabricating a wound dressing and an implantable drug-delivery system.

In fact, in vivo experiments were conducted by Liu, Yai, & Fang (2008) to study wounds treated with the composite made of a soybean protein non-woven fabric coated with a genipin-crosslinked chitosan film in a rat model. They presented a bi-layer composite as a wound dressing material: the upper layer of the composite is a non-woven fabric that prevents wound bed dehydration and bacterial infection, while allowing for drainage of wound exudates; the lower layer of the composite is a genipin-crosslinked chitosan film that contacts the wound to provide high absorption capacity for fluids and, most important, to promote ordered tissue reconstruction. Soybean protein fiber was preferred for a number of attractive properties: besides being made of fine monofilaments, it has low density, high strength, biocompatibility with human skin and excellent acid and alkali resistance. It has the tactile feel of natural silk but also the mechanical strength of synthetic fibers. Other general characteristics of the soybean protein non-woven fabric are silk-like luster, warmth retention like that of wool, and moisture absorption/transmission like that of cotton fiber. Genipin–chitosan mixtures with genipin from 0.01 to 0.1%, after stirring for 2 min turn light bluish and become increasingly viscous: they are suitable for immediate casting on glass plates and drying to constant weight at room temperature. After one day, the crosslinked chitosan becomes blue. The dried film is then washed with 1 N NaOH to remove residual acid, and finally rinsed with ultrapure water.

Needling was used to obtain the coalescence of fibers in the non-woven fabric, that was placed on the surface of the genipin–chitosan mixture, air-dried at room temperature for 24 h, frozen at −80 °C for 24 h, and freeze-dried to yield a bi-layer wound dressing material (4 × 4 cm).

The swelling ratios of chitosan crosslinked with 0.01% and 0.015% of genipin were in the range 650–900% after 24 h of soaking. In comparison, the swelling ratios attenuated to 200–300% after 24 h of soaking when the genipin content exceeded 0.025%. Based on the fixation index, the measurements of the swelling ratios, and the thermal properties, the content of genipin required to fully crosslink the chitosan was as little as 0.025%. The water contact angles of the crosslinked chitosan film (>50°) were higher than that of the plain chitosan film (35.5°). The water contact angle increased with increasing genipin content, which indicates that crosslinking depressed the hydrophilicity of the chitosan films.

The in vivo histological assessment revealed that epithelialization and reconstruction of the wound were achieved by dressing the wound with the composite: when a change was necessary, the latter was easily removed from the wound surface without damaging the newly formed tissue. On post-operative day 14, the epithelium was well organized and covered with a horny layer; the dermis had substantial fibroblasts and collagen fibers because the crosslinked chitosan layer had degraded. The newly synthesized collagen in the wound was well organized and all oriented along the same axis parallel to the skin surface, which indicates a perfect regeneration of the damaged tissue (Liu et al., 2008).

In an extension of said work, Liu and Huang (2008) prepared films with a thickness of 1 mm by casting chitosan + genipin + Ag mixtures onto plastic dishes and drying at room temperature for 48 h, and freeze-drying to remove residual solvent. The Ag nanoparticles in water had uniform diameter (ca. 15 nm). While the water contact angle of pure genipin + chitosan (68.4°) was much higher than that reported above for plain chitosan, the Ag nanocomposite films had smaller values, progressively declining with increasing Ag content, down to 48.7° for the genipin + chitosan + Ag film at 200 ppm Ag. The number of fibroblasts attached to the surface of the latter exceeded that of the genipin + chitosan film: the trend was consistent with the recognized fact that surface wettability is a vital parameter that governs cell adhesion. The typical morphology of fibroblasts during prolonged cell culture suggested that the Ag-loaded films had no toxic effect on the attached cells, while the antimicrobial activity of the incorporated Ag was maintained against *Escherichia coli*. In fact, Ag ions kill microorganisms instantly by blocking their respiratory enzyme systems, while having no negative effect on animal and human cells.

By taking advantage from the already mentioned data on gelatin, Chiono et al. (2008) produced genipin-crosslinked chitosan/gelatin blends for biomedical applications: various amounts of genipin were used to crosslink the blends. Composition and crosslinking had effect on the physico-chemical properties of samples such as thermal degradation, surface wettability, dissolution and swelling. Stiffness and crosslinking degree increased with increasing genipin amount. Genipin crosslinking reduced wettability, dissolution and swelling degree of gelatin and blend samples. The blend composition affected mouse fibroblasts adhesion and proliferation, depending on the crosslinker amount.

Crosslinked chitosan–gelatin blends containing 80 wt% gelatin were found to support neuroblastoma cells adhesion and proliferation which made them interesting for nerve regeneration. As an extension of the data by Lu et al. (2007) mentioned above, Yamazaki and Chiba (2008) suggested that genipin induces neurite outgrowth through the NO–cGMP–PKG signaling pathway followed by phosphorylation promoted by extracellular signal-regulated kinase in Neuro2a cells as well as in PC12h cells. Moreover, the genipin-induced phosphorylation by extracellular signal-regulated kinase is mediated by the cGMP-dependent protein kinase (PKG) activation rather than tyrosine kinase-A (TrkA) activation in Neuro2a cells. The findings imply that genipin is a novel neurotrophic compound, which does not require the activation of any neurotrophin receptor, including TrkA.

Gelatin and chitosan crosslinked with several agents among which genipin, were tested in order to deliver timolol maleate, a drug useful in the treatment of glaucoma. The gelatin and chitosan gels obtained with the aid of genipin were found safe and useful for controlled drug release to the eye (Almeida, Fonseca, Batista, Leite, & Gil, 2007).

The results for biopolymers associated to chitosan stimulated further research on the association of generally safe synthetic polymers to chitosan, such as poly(ethylene glycol) (Ferretti, Marra, Kobayashi, Defail, & Chu, 2006; Khurma & Nand, 2008). Novel genipin-crosslinked chitosan-based films, the chitosan and chitosan/poly(ethylene oxide) blend networks, using two types of PEO, one with a molecular weight of 20 kDa and the other of 600 Da, were prepared by Jin, Song, and 'ton (2004) by using the solution casting technique. Compared with the transparent yellow, plain chitosan/PEO blend films, the genipin-crosslinked chitosan-based films had blue color and better mechanical properties: they were more elastic and more stable than plain films and were insoluble in acidic and alkaline solutions.

Jin and Song (2006) also reported that crosslinked chitosan and chitosan-poly(ethylene oxide) (PEO) blend membranes were prepared by using the solution casting technique for *Eleutherococcus senticosus*, a herbal compound similar to Chinese gingseng, and vitamin B-12 release. Genipin was used to form the chitosan and chitosan-PEO blend networks. The drug release rate depended on the pH value and decreased with increasing crosslink density, to which it was very sensitive at low densities. PEO was introduced into the membrane to control the drug release rate thanks to its proportion and molecular weight. In fact there is interest in crosslinking said blends as testified by a number of recent works: for example, in a study by Vondran, Sun, and Schauer (2008) a chitosan-poly(ethylene oxide) copolymer blend was electrospun and crosslinked with glutaraldehyde vapor for various time periods to impart desirable mechanical characteristics to the nanofiber mats prepared for material engineering.

Chao (2008) used glycidoxypropyltrimethoxysilane (crosslinking agent) and NaCl (porogen) to make chitosan–silica porous hybrid membranes, with optimal chitosan/GPTMS weight ratio 1. Macropores (ca. 11–200 microm) in the membrane matrix and micropores (8–10 nm) in the skeleton of the macropores were observed on the membranes. Several aminoacids were individually grafted onto the membrane by genipin for affinity adsorption of tyrosinase from a crude *Agaricus bisporus* extract.

5. Conclusion

Since the time of the introduction of glutaraldehyde as a crosslinking agent promptly reactive towards amines but capable to preserve the activity of biological materials such as immobilized enzymes (Muzzarelli, Barontini, & Rocchetti, 1976) glutaraldehyde became a quite popular reagent widely used today for the preparation of hydrogels containing sensitive compounds. Nevertheless, some drawbacks deriving from glutaraldehyde capacity to transform itself into undesirable reactive species, prompted more research towards safer crossliking agents and procedures, for example phenols, and the tyrosinase-mediated quinone reaction (Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994; Wu, McDermott, Zhu, Ghodssi, & Payne, 2006).

It is understandable that a fully biocompatible reagent such as genipin, capable to meet the scopes of the current regenerative medicine based on chitosan (Muzzarelli, 2009; Muzzarelli et al., 1988; Shi et al., 2006) attracts much interest today as testified by the suddenly increased number of published articles.

References

Agnihotri, S. A., Mallikarjuna, N. N., & Aminabhavi, T. M. (2004). Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of Controlled Release, 100*, 5–28.

Akao, T., Kobayashi, K., & Aburada, M. (1994). Enzymic studies on the animal and intestinal bacterial metabolism of geniposide. *Biological Pharmacology Bulletin, 17*, 1573–1576.

Almeida, J. F., Fonseca, A., Batista, C. M. S. G., Leite, E., & Gil, E. (2007). Immobilization of drugs for glaucoma treatment. *Journal of Material Science. Materials in Medicine, 18*, 2309–2317.

Almog, J., Cohen, Y., Azoury, M., & Hahn, T. R. (2004). Genipin-a novel fingerprint reagent with colorimetric and fluorogenic activity. *Journal of Forensic Science, 49*, 255–257.

Barck, K., & Butler, M. F. (2005). Comparison of morphology and properties of polyelectrolyte complex particles formed from chitosan and polyanionic biopolymers. *Journal of Applied Polymer Science, 98*, 1581–1593.
Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., & Rubini, K. (2002). Stabilization of gelatin films by crosslinking with genipin. *Biomaterials, 23*, 4827–4832.
Butler, M. F., Ng, Y. F., & Pudney, P. D. A. (2003). Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science. Part A: Polymer Chemistry, 41*, 3941–3953.
Cao, J. P., Wang, Y. L., & Jia, Y. J. (2001). Simultaneous determination of geniposide and genipin in Gardenia jasminoides Ellis by high performance liquid chromatography. *Journal of the Dalian Medical University, 23*, 61–62.
Chao, A. C. (2008). Preparation of porous chitosan/GPTMS hybrid membrane and its application in affinity sorption for tyrosinase purification with Agaricus bisporus. *Journal of Membrane Science, 311*, 306–318.
Chen, H. M., Ouyang, W., Lawuyi, B., & Prakash, S. (2006). Genipin crosslinked alginate-chitosan microcapsules: Membrane characterization and optimization of crosslinking reaction. *Biomacromolecules, 7*, 2091–2098.
Chen, S. C., Wu, Y. C., Mi, F. L., Lin, Y. H., Yu, L. C., & Sung, H. W. (2004). A novel pH-sensitive hydrogel composed of N, O-carboxymethyl chitosan and alginate crosslinked by genipin for protein drug delivery. *Journal of Controlled Release, 96*, 285–300.
Chiono, V., Pulieri, E., Vozzi, G., Ciardelli, G., Ahluwalia, A., & Giusti, P. (2008). Genipin-crosslinked chitosan/gelatin blends for biomedical applications. *Journal of Materials Science. Materials in Medicine, 19*, 889–898.
Danbuo, Y. (1984). Zabing Guagyao. In Y. Wang (Ed.) (2nd ed., pp. 414). PR China: People's Health Publishers.
Djerassi, C., Gray, J. D., & Kinci, F. A. (1960). Naturally occurring oxygen heterocycles. IX. Isolation and characterization of genipin. *Journal of Organic Chemistry, 25*, 2174–2177.
Djerassi, C., Nakano, T., James, A. N., Zalkow, L. H., Eisenbraun, E. J., & Shoolery, J. N. (1961). Terpenoids. XLVII. The structure of genipin. *Journal of Organic Chemistry, 26*, 1192–1206.
Endo, T., & Taguchi, H. (1973). The constituents of *Gardenia jasminoides*: Geniposide and genipin-gentiobioside. *Chemical Pharmacology Bulletin, 21*, 2684–2688.
Espinosa-Garcia, B. M., Arguelles-Monal, W. M., Hernandez, J., Felix-Valenzuela, L., Acosta, N., & Goycoolea, F. M. (2007). Molecularly imprinted chitosan-genipin hydrogels with recognition capacity toward o-xylene. *Biomacromolecules, 8*(11), 3355–3364.
Ferretti, M., Marra, K. G., Kobayashi, K., Defail, A. J., & Chu, C. R. (2006). Controlled in vivo degradation of genipin crosslinked polyethylene glycol hydrogels within osteochondral defects. *Tissue Engineering, 12*(9), 2657–2663.
Fujikawa, S., Fukui, Y., & Koga, K. (1987). Structure of genipocyanin G1, a spontaneous reaction product between genipin and glycine. *Tetrahedron Letters, 28*, 4699–4700.
Fujikawa, S., Nakamura, S., & Koga, K. (1988). Genipin, a new type of protein crosslinking reagent from gardenia fruits. *Agricultural and Biological Chemistry, 52*, 869–870.
Fujikawa, S., Yokota, T., Koga, K., & Kumada, J. (1987). The continuous hydrolysis of geniposide to genipin using immobilized b-glucosidase on calcium alginate gel. *Biotechnology Letters, 9*, 697–702.
Genta, I., Perugini, P., Pavanetto, F., Modena, T., Conti, B., & Muzzarelli, R. A. A. (1999). Microparticulate drug delivery systems. In P. Jollès & R. A. A. Muzzarelli (Eds.), *Chitin and chitinases* (pp. 305–313). Basel: Birkhauser Verlag.
Jiang, T., Kumbar, S. G., Nair, L. S., & Laurencin, C. T. (2008). Biologically active chitosan systems for tissue engineering and regenerative medicine. *Current Topics in Medicinal Chemistry, 8*, 354–364.
Jin, J., & Song, M. (2006). Chitosan and chitosan-PEO blend membranes crosslinked by genipin for drug release. *Journal of Applied Polymer Science, 102*(1), 436–444.
Jin, J., Song, M., & Hourston, D. J. (2004). Novel chitosan-based films crosslinked by genipin with improved physical properties. *Biomacromolecules, 5*, 162–168.
Kaminski, K., Zazakowny, K., Szczubialka, K., & Nowakowska, M. (2008). PH-Sensitive genipin-cross-linked chitosan microspheres for heparin removal. *Biomacromolecules, 9*, 3127–3132.
Khurma, J. R., & Nand, A. V. (2008). Temperature and pH sensitive hydrogels composed of chitosan and poly(ethylene glycol). *Polymer Bulletin, 59*(6), 805–812.
Khurma, J. R., Rohindra, D. R., & Nand, A. V. (2005). Swelling and thermal characteristics of genipin crosslinked chitosan and poly(vinyl pyrrolidone) hydrogels. *Polymer Bulletin, 54*(3), 195–204.
Kitano, A., Saika, S., Yamanaka, O., Reinach, P. S., Ikeda, K., Okada, Y., et al. (2006). Genipin suppression of fibrogenic behaviors of the alpha-TN4 lens epithelial cell line. *Journal of Cataract and Refractive Surgery, 32*, 1727–1735.
Koo, H. J., Song, Y. S., Kim, H. J., & Park, E. H. (2004). Anti-inflammatory effects of genipin, an active principle of gardenia. *European Journal of Pharmacology, 495*, 201–208.
Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*, 6017–6084.
Kuo, Y. C., & Ku, I. N. (2008). Cartilage regeneration by novel polyethylene oxide/chitin/chitosan scaffolds. *Biomacromolecules, 9*, 2662–2669.
Kuo, Y. C., & Lin, C. Y. (2006). Effect of genipin-crosslinked chitin-chitosan scaffolds with hydroxyapatite modifications on the cultivation of bovine knee chondrocytes. *Biotechnology and Bioengineering, 95*(1), 132–144.
Levinton-Shamuilov, G., Cohen, Y., Azoury, M., Chaikovsky, A., & Almog, J. (2005). Genipin, a novel fingerprint reagent with colorimetric and fluorogenic activity, part II: Optimization, scope and limitations. *Journal of Forensic Science, 50*(6), 1367–1371.
Liang, H. C., Chang, Y., Hsu, C. K., Lee, M. H., & Sung, H. W. (2004). Effects of crosslinking degree of an acellular biological tissue on its tissue regeneration pattern. *Biomaterials, 25*, 3541–3552.
Lien, S. M., Li, W. T., & Huang, T. J. (2007). Genipin-crosslinked gelatin scaffolds for articular cartilage tissue engineering with a novel crosslinking method. *Material Science and Engineering C*. doi:10.1016/j.msec.2006.12.015.
Liu, B. S., & Huang, T. B. (2008). Nanocomposites of genipin-crosslinked chitosan/silver nanoparticles. Structural reinforcement and antimicrobial properties. *Macromolecular Bioscience, 8*, 932–941.
Liu, B. S., Yai, C. H., & Fang, S. S. (2008). Evaluation of a non-woven fabric coated with a chitosan bi-layer composite for wound dressing. *Macromolecular Bioscience, 8*(5), 432–440.
Liu, T. Y., Chen, S. Y., Lin, Y. L., & Liu, D. M. (2006). Synthesis and characterization of amphiphatic carboxymethyl-hexanoyl chitosan hydrogel: Water-retention ability and drug encapsulation. *Langmuir, 22*(23), 9740–9745.
Lu, M. C., Hsiang, S. W., Lai, T. Y., Yao, C. H., Lin, L. Y., & Chen, Y. S. (2007). Influence of crosslinking degree of a biodegradable genipin-crosslinked gelatin guide on peripheral nerve regeneration. *Journal of Biomaterials Science. Polymer Edition, 18*(7), 843–863.
Meena, R., Prasad, K., & Siddhanta, A. K. (2008). Development of a stable hydrogel network based on agar and k-carrageenan blend crosslinked with genipin. *Food Hydrocolloids*. doi:10.1016/j foodhydr.2008.03.008.
Mi, F. L., Shyu, S. S., & Peng, C. K. (2005). Characterization of ring-opening polymerization of genipin and pH-dependent crosslinking reactions between chitosan and genipin. *Journal of Polymer Science. Part A: Polymer Chemistry, 43*, 1985–2000.
Mi, F. L., Sung, H. W., & Shyu, S. S. (2001). Release of indomethacin from a novel chitosan microsphere prepared by a naturally occurring crosslinker: Examination of crosslinking and polycation-anionic drug interaction. *Journal of Applied Polymer Science, 81*, 1700–1711.
Mi, F. L., Sung, H. W., & Shyu, S. S. (2002). Drug release from chitosan-alginate complex beads reinforced by a naturally occurring crosslinking agent. *Carbohydrate Polymers, 48*(1), 61–72.
Mi, F. L., Sung, H. W., Shyu, S. S., Su, C. C., & Peng, C. K. (2003). Synthesis and characterization of biodegradable TPP/genipin co-crosslinked chitosan gel beads. *Polymer, 44*, 6521–6530.
Mi, F. L., Tan, Y. C., Liang, H. C., Huang, R. N., & Sung, H. W. (2001). In vitro evaluation of a chitosan membrane crosslinked with genipin. *Journal of Biomaterials Science. Polymer Edition, 12*(8), 835–850.
Mi, F. L., Tan, Y. C., Liang, H. F., & Sung, H. W. (2002). In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant. *Biomaterials, 23*, 181–191.
Mie, I., Satoshi, M., Atsushi, M., & Kenji, F. (2004). Japanese herbal medicine inchin-koto as a therapeutic drug for liver fibrosis. *Journal of Hepathology, 41*, 584–591.
Moura, M. J., Figueiredo, M. M., & Gil, M. H. (2007). Rheological study of genipin crosslinked chitosan hydrogels. *Biomacromolecules, 8*(12), 3823–3829.
Moura, M. J., Figueiredo, M. M., & Gil, M. H. (2008). Rheology of chitosan and genipin solutions. *Advanced Materials Forum, IV, 587–588*, 27–31.
Muzzarelli, R. A. A., Barontini, G., & Rocchetti, R. (1976). Immobilization of enzymes on chitosan columns: Alpha-chymotrypsin and acid phosphatase. *Biotechnology and Bioengineering, 18*, 1445–1454.
Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*. doi:10.1016/j.carbpol.2008.11.002.
Muzzarelli, R. A. A., & Muzzarelli, C. (2005). Chitosan chemistry: Relevance to the biomedical sciences. In T. Heinze (Ed.), *Advances in polymer science* (pp. 151–209). Berlin: Springer Verlag. 186.
Muzzarelli, R. A. A., & Muzzarelli, C. (2008). Chitin and chitosan hydrogels. In G. O. Philips & P. A. Williams (Eds.), *Handbook of hydrocolloids* (2nd ed.). Cambridge, UK: Woodhead Publishing Ltd.
Muzzarelli, R. A. A., Baldassarre, V., Conti, F., Gazzanelli, G., Vasi, V., Ferrara, P., et al. (1988). The biological activity of chitosan: An ultrastructural study. *Biomaterials, 9*, 247–252.
Muzzarelli, R. A. A., Ilari, P., Xia, W., Pinotti, M., & Tomasetti, M. (1994). Tyrosinase-mediated quinone tanning of chitinous materials. *Carbohydrate Polymers, 24*, 294–300.
Mwale, F., Iordanova, M., Demers, C. N., Steffen, T., Roughley, P., & Antoniou, J. (2005). Biological evaluation of chitosan salts crosslinked to genipin as a cell scaffold for disk tissue engineering. *Tissue Engineering, 11*(1–2), 130–140.
Nand, A. V., Rohindra, D. R., & Khurma, J. R. (2007). Characterization of genipin crosslinked hydrogels composed of chitosan and partially hydrolyzed poly(vinyl alcohol). *E-Polymers*, 30–38.
Nishi, C., Nakajima, N., & Ikada, Y. (1995). In vivo evaluation of cytotoxicity of diepoxy compounds used for biomaterial modification. *Journal of Biomedical Material Research, 29*, 829–834.
Roughley, P., Hoemann, C., DesRosiers, E., Mwale, F., Antoniou, J., & Alini, M. (2006). The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. *Biomaterials, 27*(3), 388–396.
Senel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Advanced Drug Delivery Reviews, 56*, 1467–1480.
Shi, C. M., Zhu, Y., Ran, X. Z., Wang, M., Su, Y. P., & Cheng, T. M. (2006). Therapeutic potential of chitosan and its derivatives in regenerative medicine. *Journal of Surgical Research, 133*(2), 185–192.

Silva, S. S., Motta, A., Rodrigues, M. T., Pinheiro, A. F. M., Gomes, M. E., Mano, J. F., et al. (2008). Novel genipin-crosslinked chitosan/silk fibroin sponges for cartilage engineering strategies. *Biomacromolecules, 9*, 2764–2774.

Speer, D. P., Chvapil, M., Eskelson, C. D., & Ulreich, J. (1980). Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. *Journal of Biomedical Material Research, 14*, 753–764.

Sung, H. W., Chang, Y., Liang, I. L., Chang, W. H., & Chen, Y. C. (2000). Fixation of biological tissues with a naturally occurring crosslinking agent: Fixation rate and effects of pH, temperature, and initial fixation concentration. *Journal of Biomedical Material Research, 52*(1), 77–87.

Sung, H. W., Huang, R. N., Huang, L. L. H., & Tsai, C. C. (1999). In vitro evaluation of cytotoxicity of a naturally occurring crosslinking reagent for biological tissue fixation. *Journal of Biomaterials Science. Polymer Edition, 10*(1), 63–78.

Sung, H. W., Huang, R. N., Huang, L. L. H., Tsai, C. C., & Chiu, C. T. (1998). Feasibility study of a natural crosslinking reagent for biological tissue fixation. *Journal of Biomedical Material Research, 42*, 560–567.

Takeuchi, S., Goto, T., Mikami, KI., Miura, K., Ohshima, S., Yoneyama, K., et al. (2005). Genipin prevents fulminant hepatic failure resulting in reduction of lethality through the suppression of TNF-alpha production. *Hepatology Research, 33*(4), 298–305.

Touyama, R., Takeda, Y., Inoue, K., Kawamura, I., Yatsuzuka, M., Ikumoto, T., et al. (1994). Studies on the blue pigments produced from genipin and methylamine. I. Structures of the brownish-red pigments, intemediates leading to the blue pigments. *Chemical Pharmaceutical Bulletin (Tokyo), 42*, 668–673.

Tsai, T. H., Westly, J., Lee, T. F., & Chen, C. F. (1994). Identification and determination of geniposide, genipin, gardenoside, and geniposidic acid from herbs by HPLC/photodiode-array detection. *Journal of Liquid Chromatography, 17*, 2199–2205.

Vondran, J. L., Sun, W., & Schauer, C. L. (2008). Crosslinked, electrospun chitosan-poly(ethylene oxide) nanofiber mats. *Journal of Applied Polymer Science, 109*(2), 968–975.

Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Apirakaramwong, A., Rojanarata, T., Ruktanonchai, U., et al. (2008). Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells. *International Journal of Pharmaceutics, 348*(1–2), 161–168.

Wu, L. Q., McDermott, M. K., Zhu, C., Ghodssi, R., & Payne, G. E. (2006). Mimicking biological phenol reaction cascades to confer mechanical function. *Advanced Functional Materials, 16*(15), 1967–1974.

Xu, M., Sun, Q., Su, J., Wang, J., Xu, C., Zhang, T., et al. (2008). Microbial transformation of geniposide of Gardenia jasminoides Ellis into genipin by Penicillium nigricans. *Enzyme and Microbial Technology, 42*(5), 440–444.

Yamazaki, M., & Chiba, K. (2008). Genipin exhibits neurotrophic effects through a common signaling pathway in nitric oxide synthase-expressing cells. *European Journal of Pharmacology, 581*(3), 255–261.

Yang, J. H., Yun, M. Y., Kim, D. K., Kim, M. J., Kim, Y. I., Kim, T. Y., et al. (2007). Preparation and evaluation of ketorolac tromethamine gel containing genipin for periodontal diseases. *Archives of Pharmaceutical Research, 30*(7), 871–875.

Yao, C. H., Liu, B. S., Hsu, S. H., & Chen, Y. S. (2005). Calvarial bone response to a tricalcium phosphate-genipin crosslinked gelatin composite. *Biomaterials, 26*(16), 3065–3074.

Yao, X. S., Wu, L. J., & Wu, J. Z. (2004). *Natural medicinal chemistry* (4th ed.). Beijing, China: People's Medicinal Publishing House. Vol. 220.

Yuan, Y., Chestnutt, B. M., Haggard, W. O., Bumgardner, J. D., & Muzzarelli, R. A. A. (2008). Genipin crosslinked chitosans for drug release. In R. Jayakumar & A. Prabaharan (Eds.), *Chitin and chitosan in biomaterial science*. Trivandrum, India: Research Signpost.

Yuan, Y., Chestnutt, B. M., Utturkar, G., Haggard, W. O., Yang, Y., Ong, J. L., et al. (2007). The effect of crosslinking of chitosan microspheres with genipin on protein release. *Carbohydrate Polymers, 68*, 561–567.

Zhang, C. Y., Parton, L. E., Ye, C. P., Krauss, S., Shen, R., Lin, CT., et al. (2006). Genipin inhibits UCP2-mediated proton leak and acutely reverses obesity- and high glucose-induced beta cell dysfunction in isolated pancreatic islets. *Cell Metabolism, 3*(6), 417–427.

Zheng, H. Z., Dong, Z. H., & Yu, J. (2000). *Modern research and application of Chinese traditional medicine* (Vol. 4). Beijing, China: Academy Press. 3166–72.