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Biological Evaluation of Chitosan Salts Cross-Linked to Genipin as a Cell Scaffold for Disk Tissue Engineering

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ABSTRACT

Degenerative disc disease has been implicated as a major component of spine pathology. However, although biological repair of the degenerate disk would be the ideal treatment, there is no universally accepted scaffold for tissue engineering of the intervertebral disk. To help remedy this, we investigated the gelation kinetics of various concentrations (2.5 to 10%) of two water-soluble chitosan chlorides (low molecular weight Protasan UP CL113 and high molecular weight Protasan UP CL213) and two chitosan glutamates (low molecular weight Protasan UP G113 and high molecular weight Protasan UP G213). Various concentrations (5 to 20%) of genipin, a naturally occurring cross-linking reagent used in herbal medicine and in the fabrication of food dyes, were used to prepare cross-linked chitosan hydrogels. The results show that 2.5% Protasan UP G213 cross-linked to 5% genipin was the best candidate. This formulation gelled fastest at 37°C, and maintained 95% viability of encapsulated cultured disk cells. The gel did not produce an inflammatory reaction when injected subcutaneously into C57BL/6 mice and is therefore biocompatible. Most importantly, when injected into the degenerated nucleus pulposus of human cadaveric intervertebral disk, the gel flowed into the clefts without leakage. This study demonstrates that 2.5% Protasan UP G213 cross-linked to 5% genipin might be a promising scaffold for disk tissue engineering.

INTRODUCTION

Low back pain is a frequent health problem that, by age 70 years, affects about 60% of the population.[1] Although the etiology of low back pain is often unclear, it is believed that intervertebral disk (IVD) degeneration plays a major role.[2] Although the majority of those affected will not require prolonged medical care or absence from work, about one-third will require extensive care involving hospitalization. The present management of disk pathology has been focused on the symptoms associated with disk degeneration, and although surgical procedures produce a good short-term clinical result in the relief of pain, they alter the biomechanics of the spine and can lead to further degeneration of surrounding tissue and disks at adjacent levels.[3,4] In addition, the failure rate for lumbar fusions is 20 to 40% after 5 years.[5]

Disk pathology treatment is now shifting toward disk regeneration, with a view toward replicating the design and function of the human disk.[6,7] Advances in tissue engineering offer the unique opportunity to engineer a replacement nucleus pulposus (NP), using polymer–cell

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constructs and growth factors. In the current studies, we sought to use chitosan salts cross-linked to genipin for NP supplementation via injection, rather than *in vitro* engineering of intact NP for implantation. This is a much simpler and technically more feasible proposition than attempting to engineer an entire disk.

Chitosan, an amino-polysaccharide obtained by the alkaline deacetylation of chitin, derived from crustacean shells, is currently being investigated for many pharmaceutical applications,[8–10] and chitosan/glycerophosphate-thermosensitive solutions, which gel at 37°C, have been described.[11] However, the use of β-glycerophosphate, a nonphysiological substrate for alkaline phosphatase, is known to induce mineralization,[12] which might limit its use in disk engineering. Although cells from the anulus fibrosus (AF) and NP do not initiate calcification of their extracellular matrix, increased endplate vertebral calcification, a hallmark of disk degeneration, is known to limit solute transport.[13] In addition, adult osteoblasts cultured in native collagen gels in the presence of β-glycerophosphate ceased proliferation and underwent mineralization.[14] To avoid the possibility of inducing mineralization, we chose to search for a suitable chitosan formulation for NP supplementation that gels and maintains cell viability in the absence of β-glycerophosphate.

It is also important to prepare a cross-linked hydrogel, because this will decrease biodegradability and allow entrapped cells to synthesize a functional extracellular matrix before the polymer dissolves. It has been discovered that chitosan could be cross-linked with a naturally occurring cross-linking reagent, genipin, which has been used in herbal medicine and in the production of food dyes.[15,16] In the current study, we investigated the feasibility of using two water-soluble chitosan chlorides (low molecular weight Protasan UP CL113 and high molecular weight Protasan UP CL213) and two chitosan glutamates (low molecular weight Protasan UP G113 and high molecular weight Protasan UP G213) cross-linked to genipin as cell scaffolds for NP supplementation of the degenerate IVD. This study demonstrates that 2.5% Protasan UP G213 cross-linked to 5% genipin is a good candidate for this purpose.

MATERIALS AND METHODS

Preparation of genipin cross-linked chitosan

Two ultrapure medical-grade water-soluble chitosan hydrochlorides (Protasan UP CL113 and UP CL213) and two chitosan glutamates (Protasan UP G113 and UP G213) were obtained from FMC BioPolymer (Oslo, Norway). The chitosan hydrochlorides CL113 (M_W 150 kDa) and CL213 (M_W 300 kDa) were 86% deacetylated. The chitosan glutamates G113 (M_W 150 kDa) and G213 (M_W 300 kDa) were 86 and 85% deacetylated, respectively. Protasan ultrapure medical grades have been shown to be nontoxic.[17,18] Various formulations were tested, using different Protasan concentrations (2.5, 5, and 10%, w/v) cross-linked with different genipin concentrations (5, 10, 15, and 20%, w/w Protasan). Routinely, a 2.5% Protasan solution (w/v) was prepared in serum-free medium (Dulbecco's modified Eagle's medium [DMEM] plus streptomycin [100 μg/mL] and penicillin [100 U/mL] [HyClone Logan, UT] plus gentamicin [50 ng/mL], pH 7.4) at room temperature or at 37°C, and cross-linked with genipin (5%, w/w Protasan; Challenge Bioproducts, Taichung, Taiwan). The powders were added progressively to the medium with stirring, and mixing was continued for 1 h, until the chitosan was completely dissolved. Subsequently, genipin powder was added to the chitosan solution. The gels were characterized visually and tested for punchability with a 6-mm Keyes dermal biopsy punch (WorldSource, Niles, IL).

Source of intervertebral disks

Adult bovine tails (2–4 years old) were obtained from Les Abattoirs Collux (Saint-Cyrille-de Wendover, QC, Canada). All IVDs were classified as nondegenerate grade I according to the grading system of Thomson *et al*.[19] Eighty disks from 12 tails were used.

Cell isolation

Cells were isolated immediately after transportation from the abattoir.[20] The IVDs were dissected from their adjacent vertebral bodies and placed in DMEM-high glucose (HyClone), with 20 mM HEPES and 45 mM $NaHCO_3$ (pH 7.4), containing gentamicin (150 ng/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (Fungizone, 0.25 μg/mL) (medium A; Invitrogen, Burlington, ON, Canada). Under aseptic conditions, the IVDs were separated by dissection into regions corresponding to the AF and the NP. The AF and NP were dissected into approximately 2-mm fragments and were washed twice in medium A for 15 min. Cells were enzymatically isolated from the tissue, using a sequential protease type XIV–collagenase protocol.[20] Briefly, 28 g and 10 g of AF and NP tissues, respectively, were incubated at 37°C with stiring (75 rpm) for 1 h in medium A with bacterial protease type XIV (Sigma, Oakville, ON, Canada) at 0.2% (w/v) for the NP and at 0.4% (w/v) for the AF. The tissue was then washed and a second digestion was performed overnight at 37°C in washing medium (medium A without gentamicin and Fungizone) supplemented with 10% fetal bovine serum (FBS) (HyClone) and bacterial collagenase type IA (Sigma) at 0.03% (w/v) for the NP and at 0.06% (w/v) for the AF. The resulting cell suspensions were passed through a 70-μm cell strainer (VWR International, Mis-

sissauga, ON, Canada), washed twice in washing medium containing 10% FBS, and cells were recovered by centrifugation at 400 × *g* for 6 min. Cells were counted with a hemacytometer and the viability was determined with 0.04% trypan blue dye. Approximately 15×10^6 cells were recovered from 28 g of AF per tail and 5×10^6 cells from 10 g of NP per tail.

Entrapment and culture of disk cells in scaffold

Disk cells were entrapped in the chitosan–genipin solution at room temperature. Briefly, 1×10^7 cells were suspended in 100 μL of serum-free DMEM and added to 4 mL of a mixture containing 2.5% Protasan and 5% genipin solution. This solution was mixed to obtain a uniform suspension in a standard-size 35-mm plastic petri dish. After gelling at 37°C, 6-mm-diameter and 2-mm-thick disks were punched from the cell-seeded scaffold and cultured in serum-free medium supplemented with vitamin C (50 μg/mL) and incubated at 37°C with 5% humidified CO_2. Control cells without polymer were cultured in monolayer serum-free DMEM on polylysine-coated coverslips, for 24 h.

Assessment of cell viability

The viability of control cells and of cells entrapped in the scaffold was evaluated 24 h after seeding, using the LIVE/DEAD viability/cytotoxicity assay (kit L-3224; Molecular Probes, Eugene, OR). This is a two-color fluorescence assay that simultaneously determines live (green) and dead (red) cell numbers, using calcein AM and ethidium homodimer, respectively. The cells were exposed to the probes for 10 min. Cells entrapped in scaffolds were visualized by compressing the polymer on a microscope slide, whereas control cells were visualized immediately on the slides. Each image was automatically counted, using ImageJ (Java freeware).

Porosity

Chitosan–genipin gels were examined by scanning electron microscopy (SEM) to study porosity before and after culturing encapsulated cells. The gels were fixed by overnight immersion in 5% glutaraldehyde–0.1 M sodium cacodylate buffer (pH 7.4) at 4°C. After rinsing three times with buffer, the preparations were stepwise dehydrated in graded ethanol (25, 50, 95, and 100%; 60 min each). Control chitosan–genipin gels without cells and chitosan–genipin gels with cultured NP and AF cells were mounted on SEM specimen stubs, and coated with gold–palladium. SEM was performed with a JEOL (Tokyo, Japan) JSM-840A scanning electron microscope at an accelerating voltage of 10 kV. Image acquisition was performed with the EDAX Phoenix microanalysis system (EDAX/AMETEK, Mahwah, NJ).

In vivo *biocompatibility*

A chitosan–genipin aqueous formulation consisting of 0.2 mL of 2.5% Protasan UP G213 freshly mixed with 5% genipin was injected subcutaneously at room temperature into a single site on the back of 3-month-old C57BL/6 mice (100–200 g; Charles River Canada, Saint-Constant, QC, Canada) through a 22-gauge needle. Polymerization occurred *in situ* at body temperature after the injection. Mice were given food and water *ad libitum* and allowed free movement within their cages. The gels were retrieved 1 week, 2 months, and 4 months after implantation, after sacrifice of the mice, fixed in 10% neutral buffered formalin for 24 h, and processed for paraffin embedding and sectioning.[7] Staining was performed with hematoxylin and eosin (to visualize cells in chitosan and surrounding tissue[17]) or Giemsa stain (to detect inflammatory infiltrates[21,22]). All animal experiments were performed according to the guidelines of the Canadian Council on Animal Care.

Injectability in human IVD

Whole human lumbar spine specimens were removed from fresh cadavers, aged 60 to 65 years. Thomson grading was performed as previously described.[23,24] A solution of 2.5% Protasan UP G213 and 5% genipin was used to examine how the solution distributes into the clefts of degenerated disks after injection. Coomassie blue was added to the chitosan–genipin mixture to facilitate visualization of the gel. Each injection was 0.2 mL in volume and was performed through an 18-gauge needle. Gelling was achieved at room temperature within about 30 min.

RESULTS

Effect of molecular weight and salt form of chitosan cross-linked to genipin on gelation time

Figure 1 shows the results of a time course analysis of the gelation of 2.5% Protasan UP G113 cross-linked to 5% genipin. Five distinct gelation phases were identified and used to facilitate a description of results. Separation into the five phases was characterized visually (grades 1–4) and mechanically (grade 5), using a 6-mm Keyes dermal biopsy punch. Phase 1 occurs when genipin is added to the chitosan solution and the color remains yellowish. Phase 2 is characterized by the first appearance of a light blue color, but the solution is still mobile. In phase 3 the solution becomes viscous, but still light blue. Phase 4 is characterized by a dark bluish gel, but from which disks cannot be removed with a tissue punch. This dark bluish color was previously observed with genipin-fixed porcine pericardium.[25] Phase 5 is characterized by a strong dark blue gel from which 6-mm-diameter disks could be punched.

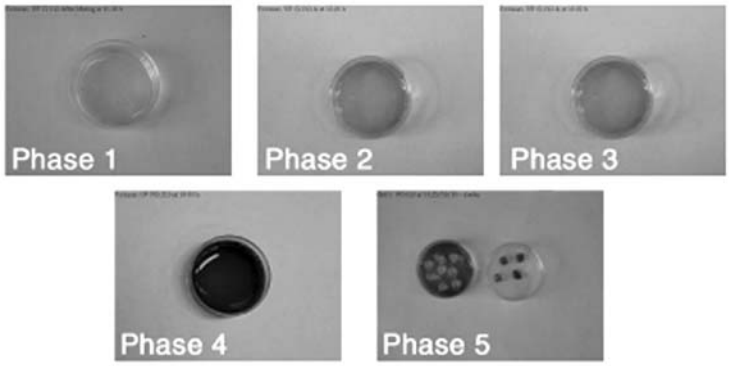


FIG. 1. Five gelling phases of chitosan cross-linked to genipin. Gelling solutions of Protasan UP G113 and genipin were characterized for color and punchability. The gelling phases were (1) liquid and yellow, (2) viscous fluid and light blue, (3) very viscous fluid and darker blue, (4) dark blue and gelled but not removable with a tissue punch, and (5) dark blue firm gel from which disks can be removed with a tissue punch.

Figure 2A shows that in the presence of 5% genipin at room temperature, Protasan UP G213 gelled the quickest whereas Protasan UP CL113 and UP G113 were the slowest at room temperature. At 37°C, Protasan UP G213 still gelled the quickest, but Protasan UP G113 was quicker than UP CL113 (Fig. 2B). All the Protasan preparations gelled quicker at 37°C than at room temperature. These observations indicate that the nature of the glutamate or chloride salts as well as the molecular weight of the polymer contribute to the gelation kinetics. The degree of deacetylation was not a factor in these experiments because all were about 86% deacetylated. The re-

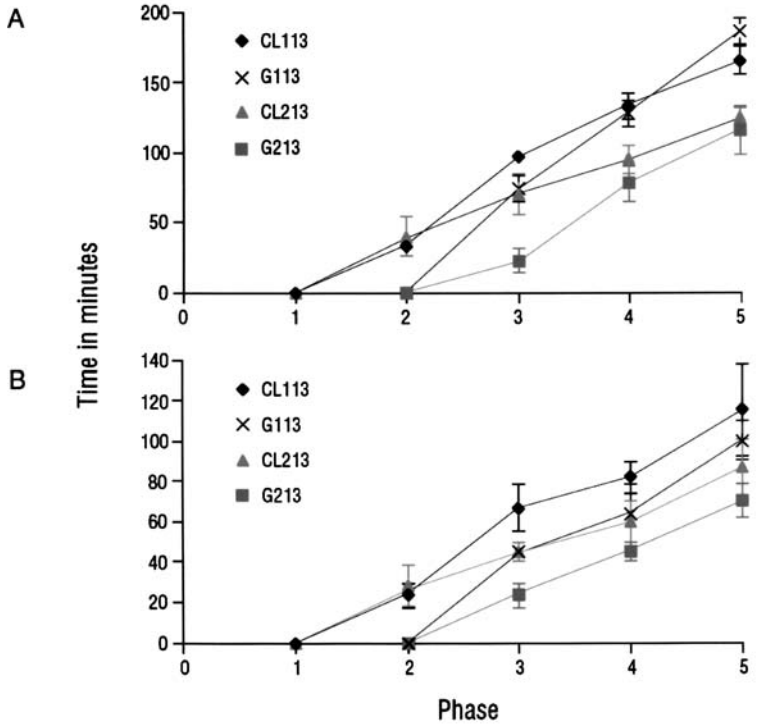


FIG. 2. Temperature dependence of gelation times for genipin cross-linked chitosan salts. The time to reach each gelation phase was assessed for two genipin cross-linked chitosan hydrochlorides (Protasan UP CL113 and UP CL213) and two chitosan glutamates (Protasan UP G113 and UP G213) at room temperature (**A**) and at 37°C (**B**). The gels were prepared by mixing Protasan (2.5%, w/v) in 10 mL of serum-free medium with genipin (5%, w/w Protasan). Note that the chitosan polymer solution solidifies faster when heated to 37°C.

sults were similar for different batches of each Protasan preparation tested.

We next explored whether increasing the Protasan or genipin concentration would decrease the gelation time. Figure 3A shows that increasing the Protasan concentration reduced the gelling time. The gelation time was also reduced by increasing the genipin concentration (Fig. 3B). Addition of 15% genipin was characterized by the rapid appearance of a viscous and light blue solution (phase 2). Increasing the genipin concentration to 20% led to a dark bluish viscous gel that was initially not possible to remove with a tissue punch, but that was rapidly transformed into a firm gel that could be removed with a tissue punch. These results were reproducible with three other Protasan batches.

Assessment of cell viability

Figure 4 presents the results of cell viability examination of NP cells entrapped in different Protasan–genipin gels, measured by LIVE/DEAD assay. Of special interest here was the finding that NP cells entrapped in the glutamate salts of chitosan cross-linked to genipin clearly had better viability than cells entrapped in chloride salts (Fig. 4B). At 24 h postentrapment, cell viability was greater in Protasan UP G113 (82 ± 9%) and Protasan UP G213 (87 ± 7%) than in either Protasan UP CL113 (67 ± 12%) or Protasan UP CL213 (74 ± 4%). Increasing the concentration of Protasan UP G213 to 10%, to decrease the gelling time, substantially decreased cell viability (Fig. 4C). This could be due to the generation of an acidic pH, which has been reported with high chitosan concentrations.[26]

Porosity

Because scaffolds for tissue engineering of IVDs are required to be porous and permit the adherence and proliferation of living cells,[27] we explored the porosity and the behavior of cells in genipin cross-linked to Protasan UP G213 (Fig. 5). Typical SEM examination of the central region of the gel revealed a porous microstructure (Fig. 5A). The pores ranged from 2 μm to greater than 100 μm in diameter. Figure 5B show that cells can adhere to the inner surface of the pores.

In vivo *biocompatibility*

In vivo biocompatibility of 2.5% Protasan cross-linked to 5% genipin was also examined. When the polymer was

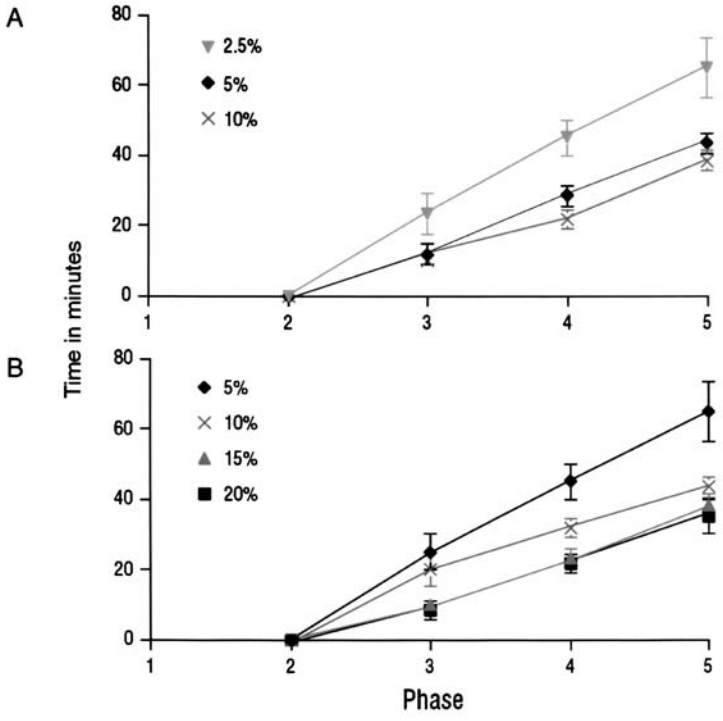
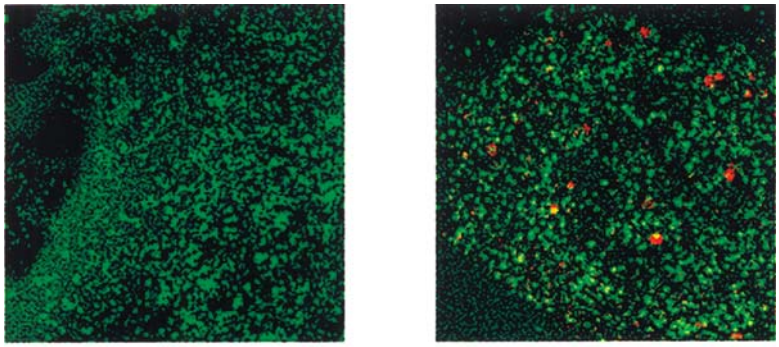


FIG. 3. Concentration dependence of gelation times for chitosan cross-linked solution. The time to reach each gelation phase was assessed for Protasan UP G213. (**A**) Gels with three different concentrations of Protasan (2.5, 5, and 10%, w/v) and the same concentration of genipin (5%, w/w Protasan). (**B**) Gels with four different concentrations of genipin (5, 10, 15, and 20%, w/w Protasan) and the same concentration of Protasan (2.5%, w/v). The gelling was done at 37°C. Note that increasing the Protasan or genipin concentration reduced the gelling time.

A



B

Type Of Protasan®	Protasan® of Low Molecular Weight		Protasan® of High Molecular Weight		Control
	CL 113	G 113	CL 213	G 213	
Cell Viability %	67 ± 12	82 ± 9	74 ± 4	87 ± 7	100 ± 3

C

Protasan® Concentration %	2.5	5	10
Cell Viability %	87 ± 7	60 ± 11	25 ± 10

FIG. 4. Viability of nucleus pulposus cells entrapped in chitosan gels cross-linked to genipin. Viability was measured by LIVE/DEAD viability/cytotoxicity assay. (**A**) Typical confocal images of NP cells cultured in monolayer (control cells) and entrapped in Protasan UP G213. Green fluorescence indicates live cells, red indicates dead cells. (**B**) Effect of chitosan type on cell viability calculated as the percentage of live cells present after 24 h in culture. Note that cells entrapped in the glutamate salts of chitosan cross-linked to genipin had better viability than those entrapped in chloride salts. (**C**) Effect of increasing the chitosan concentration on cell viability, calculated as the percentage of live cells present after 24 h in culture. Note that 2.5% Protasan UP G213 had the best cell viability, and that increasing its concentration reduced cell viability.

injected subcutaneously into C57BL/6 mice, the polysaccharide gelled *in situ* at body temperature. A thin fibrous capsule indicative of minimal foreign body reaction and inflammatory response was observed after 1 week (Fig. 6). The appearance of the fibrous capsule and the shape of the gel were maintained for several months, indicating continuing stability and lack of an immune reaction. The cellular composition of any inflammatory infiltrate present around the fibrous capsule was examined by means of Giemsa stain on histological sections. This stain has been used to detect mast cells, lymphocytes, plasma cells, polymorphonuclear leukocytes, and basophils.[21,22] Some blue staining was detected in the infiltrate (Fig. 6B), suggesting the presence of small amounts of basophil granules, which react with the methylene blue component of Giemsa stain.[22]

Injectability into the human disk

To explore how a chitosan solution distributes into the clefts of a degenerated human disk (Thomson grade 3), it was injected into human cadaveric disks (Fig. 7). The results show that when the solution was injected into the center of the disk, it flowed into the nuclear clefts and then gelled with no detectable leakage through the AF and out of the disk. This is of particular importance be-

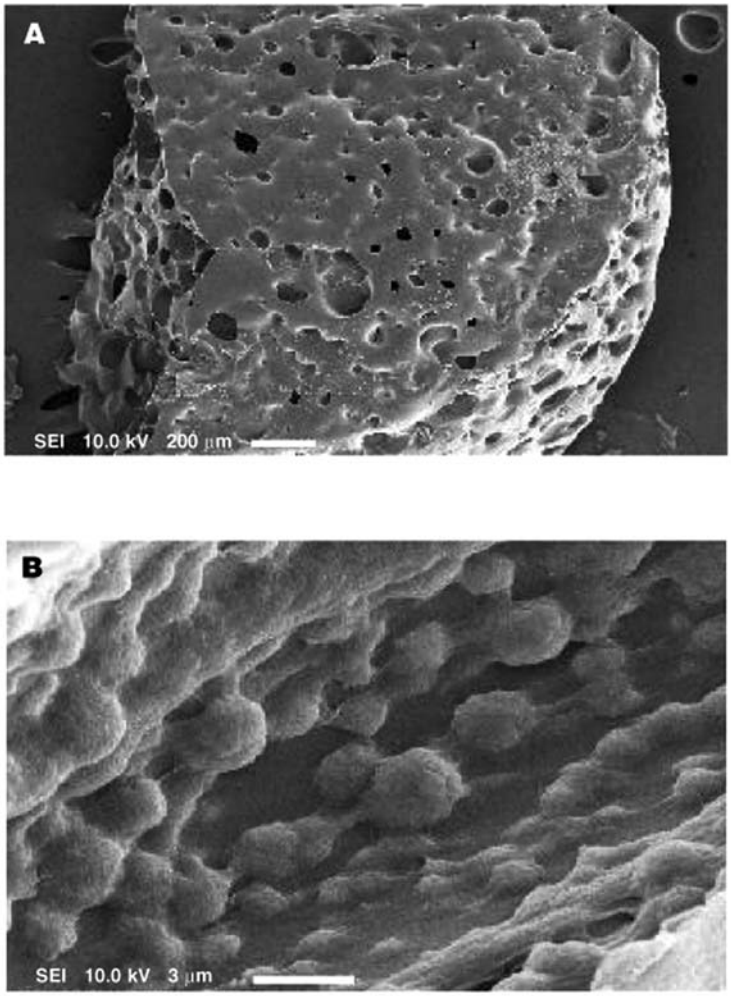


FIG. 5. SEM micrographs of 2.5% (w/v) Protasan UP G213 cross-linked with 5% (w/w) genipin. After gelling, the sample was fractured and processed for SEM imaging. (**A**) Control chitosan without cells. (**B**) Chitosan cultured with NP cells after 24 h. Note the presence of pores ranging from 2 μm to greater than 100 μm in diameter and NP cells adhering to the scaffold.

cause any scaffold designed to supplement the NP must be able to polymerize *in situ* without leakage from the degenerate disk.

DISCUSSION

Chitosan-based gels have long been studied as an injectable material in surgery,[28,29] although no temperature-controlled gel has been developed for encapsulating cells from the IVD while retaining their biological activity at neutral pH. Here we explored two chitosan chlorides, Protasan UP CL113 and UP CL213, and two chitosan glutamates, Protasan UP G113 and UP G213, with the aim of developing a scaffold for culturing disk cells. The availability of Protasan UP grades made it possible to obtain a chitosan that dissolves in a serum-free medium at pH 7.2. Previously, one of the limitations of chitosan was its insolubility in water, with its becoming soluble only when dissolved in acidic solvents.[17,30] An important factor to be considered before an injectable gel can be selected as a candidate for tissue-engineering applications is the gelation kinetics[31]; therefore variation in gelation time between different chitosan salts cross-linked to genipin was investigated. Our data indicate that chitosan salts can be dissolved at neutral pH and that the kinetics of gelation is dependent on the type of Protasan, the temperature, and the polymer and genipin concentrations.

We have shown that chitosan cross-linking to genipin generates a strong, dark blue gel. Previously it was postulated that this change in color might be due to the reaction of genipin with basic amino acid residues such as lysine, hydroxylysine, or arginine.[25,32] We have cross-linked chitosan with genipin in distilled deionized water at 37°C and found that after 1 h only the top part of the

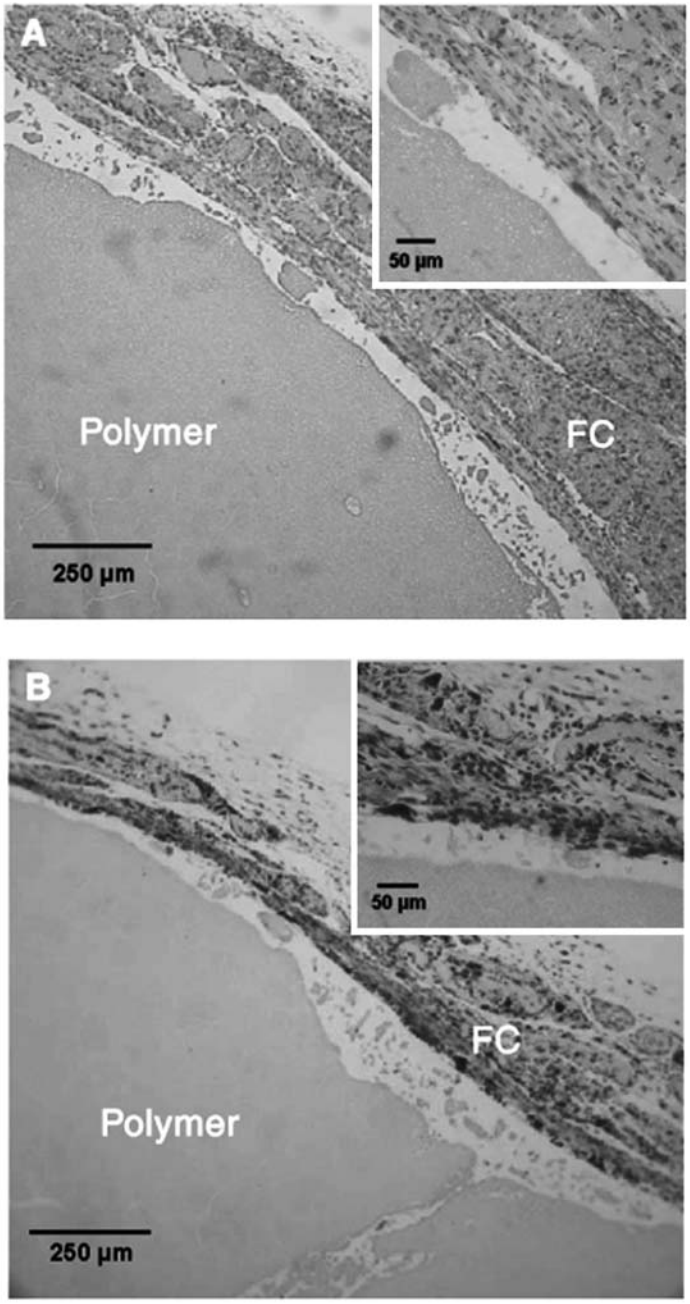


FIG. 6. Histological examination of chitosan injected into C57BL/6 mice. (**A**) Hematoxylin and eosin stain or (**B**) Giemsa stain was used to stain sections for the histologic study. Note the polymer surrounded by a fibrous capsule (FC).

gel (exposed to air) turned bluish (data not shown). On removing the gel and exposing the bottom half of the gel to air, it also turned bluish, suggesting that oxidation is necessary for the change in color to occur. However, although the relationship between cross-linking and color formation is not clear, the blue color is not a problem for disk tissue engineering as its generation does not adversely affect cell viability with the optimal gel formulation.

When NP cells are entrapped in the glutamate salts of chitosan cross-linked to genipin, our analyses revealed better viability than when cells are entrapped in the chloride salts (Fig. 4B). Chitosan glutamate solutions were previously reported to be less harmful to TR146 cells.[33] This could be related to differing effects of increased glutamate or chloride on cell metabolism. On preparing a 2.5% Protasan solution, glutamate concentration is increased by 10.75 mg/mL (about 74 mM) or chloride con-

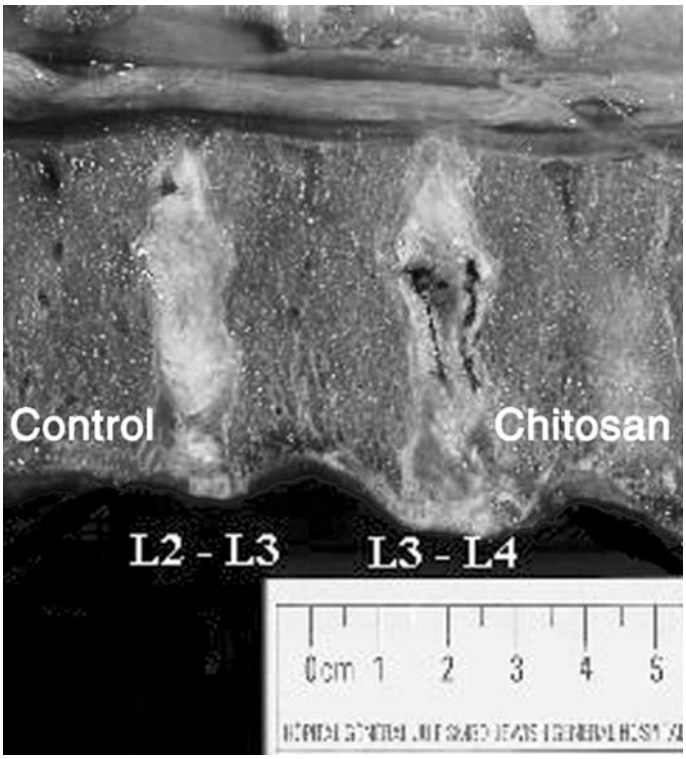


FIG. 7. Injection of chitosan cross-linked to genipin into the degenerated nucleus pulposus of human cadaveric IVD. Note that the solution flowed into the nuclear clefts with no detectable leakage through the anulus fibrosus and out of the intervertebral disk.

centration is increased by 3.5 mg/mL (about 100 mM), depending on the salt type chosen. In addition, the results could be influenced by the lower amount of chitosan present in an equal weight of Protasan glutamate relative to Protasan chloride (1.43 and 2.15%, respectively, in a 2.5% solution of each Protasan salt).

Minimal foreign body reaction and inflammatory response were observed for up to several months when a solution of 2.5% Protasan and 5% genipin was injected into C57BL/6 mice and allowed to gel. Previously, it has been shown that chitosans with a high degree of deacetylation resulted in high residence time and no detectable inflammation.[17] The fact that the gel was not resorbed after several months of implantation is important for tissue engineering by NP supplementation, as this suggests that the gel is stable enough to allow sufficient time for cells to make and accumulate their own extracellular matrix.

This study demonstrates that 2.5% Protasan UP G213 cross-linked to 5% genipin was biocompatible and porous, maintained the viability of entrapped NP cells, and most importantly permitted injection into and distribution within the human degenerated disk without leakage into the AF, suggesting that it might be a promising scaffold for supplementation of degenerated NP if cells can produce an appropriate matrix. In this NP supplementation technique the scaffold is not required to have mechanical strength, as it is not intended to enhance the functional abilities of the disk, but rather to provide a three-dimensional cell support in which such a functional matrix can be generated.

In this study we chose to use chitosan–genipin for NP supplementation for a number of reasons. (1) Chitosan hydrogels are neither cytotoxic[16,34] nor exothermic[11] and have excellent biocompatibility[16,17,34]; (2) chitosan can be maintained in solution below room temperature for encapsulating living cells and therapeutic proteins, but forms a gel at a temperature close to 37°C.[17] Thus, when injected *in vivo,* the liquid formulation turns into a gel implant *in situ*; (3) chondrocytes embedded in chitosan hydrogels proliferate and maintain their phenotype[17]; (4) chitosan can be *in situ* cross-linked with genipin.[15,16] During cross-linking the temperature never exceeds 37°C, making the process compatible with cell viability and preventing protein denaturation in the surrounding extracellular matrix[15,16]; (5) chitosan can be implanted by injection without major surgical disruption of the AF; and (6) most importantly, the chitosan gel permits the accumulation of an appropriate extracellular matrix, and retains more than 80% of the pro-

teoglycan produced by entrapped NP cells.[35] Each of these properties makes cross-linked chitosan an ideal scaffold for NP supplementation.

ACKNOWLEDGMENTS

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