Feasibility of chitosan-alginate (Chi-Alg) hydrogel used as scaffold for neural tissue engineering: a pilot study in vitro

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ABSTRACT
In tissue engineering, scaffolding plays an important role in accommodating and stimulating new tissue growth. Chitosan and alginate are two widely used natural polymers in tissue engineering. Here, we prepared the chitosan-alginate (Chi-Alg) hydrogel from naturally derived chitosan and alginate polymers. The Fourier-transformed infrared spectroscopy and X-ray diffraction results demonstrated that a chitosan-alginate hydrogel was constructed due to the strong ionic interaction between the positively charged amino groups of chitosan and the negatively charged carboxyl groups of alginate. The scanning electron microscopy and contact angle results showed the inner porous structure and highly hydrophilic property of chitosan-alginate hydrogel. As the two most promising cell types in nerve tissue engineering, both olfactory ensheathing cells and neural stem cells proliferated well on the chitosan-alginate hydrogel. All results indicated the good potential application of a chitosan-alginate hydrogel for neural tissue engineering.

KEYWORDS
Chitosan; alginate; hydrogel; cell proliferation; tissue engineering

Introduction
Chitosan [β-(1,4)-2-amino-2-deoxy-D-glucan], a cationic polymer, is the N-deacetylated derivative of chitin, which is abundant in nature. Chitosan has drawn much attention because of its good biocompatibility, biodegradability, low toxicity and ability to be fabricated into various forms in tissue engineering, such as films [1], porous scaffolds [2], hydrogels [3] and tubes [4–6]. Previous studies have shown that chitosan is a potential candidate biomaterial for neural tissue engineering applications [4,6–8]. Alginate, an anionic polymer, is extracted from the cell walls of brown algae and is composed of sequences of α-L-guluronic acid and β-D-mannuronic acid. Alginate is biocompatible, hydrophilic and biodegradable under normal physiological conditions [9] and has been widely used in bone tissue engineering [10] and drug delivery [11]. Water-soluble derivatives of chitosan have been used to blend with alginate to prepare Ca2+-crosslinked hydrogel beads, which generally exhibit pH-sensitive and ionic-sensitive swelling and drug release properties [12].

Most central nervous system (CNS) diseases are often caused by the progressive loss of function and death of neurons. Tissue engineering provides a way of supplementing cells lost in the injury. Among the numerous types of seed cells, olfactory ensheathing cells (OECs) and neural stem cells (NSCs) are the two most promising cell types. OECs are a unique type of glial cells that arise from the olfactory placode and occur both peripherally and centrally along the olfactory nerve [13]. The primary advantage of using OECs may be that they migrate into and through the glial scar and therefore facilitate axonal regrowth through an injury barrier. It has also been shown that OECs transplantation promotes partial functional recovery in rats after spinal cord injury (SCI) [14]. NSCs have the ability to self-renew and the potential of differentiating into any one of the principal CNS cell types (neurons, astrocytes and oligodendrocytes) according to the local environment. These properties make NSCs a good source of cells for cell replacement therapy after CNS injury. However, most transplanted NSCs differentiate into astrocytes, which weakens the effect of implantation of NSCs [15,16]. Our previous work demonstrated that OECs could promote NSCs differentiation into neurons, and co-transplantation of OECs and NSCs might have a synergistic effect on promoting neural regeneration and improving the recovery of locomotion function after SCI [14].

Another important element of tissue engineering is the scaffold, which plays an important role as a seed cell carrier. However, as biomaterials widely used in tissue engineering, there are insufficient data available describing the interaction of a chitosan-alginate hydrogel with nerve cells. In the present study, we fabricated a
chitosan-alginate hydrogel and investigated the cytocompatibility of chitosan-alginate with OECs and NSCs.

Materials and methods

Materials

Chitosan (degree of deacetylation = 93.5%, viscosity-average relative molecular mass $M_n = 1.8 \times 10^6$) was purchased from Qingdao Haisheng (China). Sodium alginate was purchased from Sigma (Saint Louis, MO, USA). Dulbecco’s Modified Eagle Medium and Nutrient Mixture F-12 (1:1) (DMEM/F12), B-27 supplement, and trypsin were purchased from Gibco (Waltham, MA, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Basic fibroblast growth factor (bFGF), rabbit anti-nestin antibody, and rabbit anti-S-100β antibody were purchased from Sigma (Saint Louis, MO, USA). 4’,6-diamidino-2-phenylindole (DAPI) and Cell Counting Kit-8 (CCK-8) were purchased from Dojindo (Kamimashiki District, Kumamoto, Japan). Matrigel® was purchased from Vigorous Biotechnology Beijing Co., Ltd (China). Fibrin glue was purchased from Guangzhou Bioseal Biotechnology Co., Ltd (China). All other reagents were of analytical grade.

Preparation of the chitosan-alginate hydrogel

Chitosan and sodium alginate powders were dissolved in acetic acid (1.2%, v/v) and distilled water, respectively, to obtain separate solutions (2%, w/v). Then, sodium chloride was added into the sodium alginate solution to obtain a 1.2 mol/L concentration of NaCl. The chitosan solution was injected dropwise into the sodium alginate solution under constant stirring to obtain a homogeneous solution (1% chitosan and 1% alginate). The pH of the chitosan-alginate solution was adjusted to pH 7.4. Then, the solution was incubated in a water bath at 60 °C for 1 h and maintained in a refrigerator at 4 °C for 5 h. The product was the chitosan-alginate hydrogel.

Fourier-transformed infrared spectroscopy (FTIR)

A PerkinElmer system 2000 FTIR spectrometer (PerkinElmer, Norwalk, CT) was used for FTIR analysis. The spectra were collected over the range of 4000–400 cm$^{-1}$.

X-ray diffraction (XRD) study

X-ray diffraction patterns of the composite films were measured using an X-ray diffractometer (model D8 Advance; Bruker, Germany) with Ni-filtered Cu radiation generated at 30 kV and 30 mA as the X-ray source. The diffraction patterns were determined over a range of diffraction angles $2\theta = 5°$ to $40°$ at a rate of $1°$ ($2\theta$) per minute and a step of $0.1°$ ($2\theta$).

Scanning electron microscope (SEM) observation

The chitosan-alginate hydrogel was maintained in a freezer at $-20 °C$ for 24 h. Then, the samples were lyophilized in a freeze-dryer. Next, the freeze-dried samples were fixed with 4% paraformaldehyde at room temperature for 30 min followed by washing with 0.1 mol/L phosphate buffered saline (PBS, pH 7.4) three times. Then, the samples were dehydrated through an ascending graded ethanol series (30, 50, 70, 90, 95 and 100%) and allowed to air dry in a fume hood. After being coated with gold on a metal stub in vacuum, the samples were examined with SEM (KYKY-2800; Apparatus Factory, Chinese Academy of Sciences, Beijing, China) with an accelerating voltage of 20 kV.

Contact angle measurement

Wettability was examined by measuring contact angles. The contact angles were measured using a Contact Angle Meter (JY-82, Chengde Test-Machine Factory, China). Redistilled water (10 μL) was gently dropped on the surface of the samples. At least six readings on different parts of the samples were averaged for data collection.

Isolation, purification and culture of OECs and NSCs

NSCs were isolated from the cortices of foetal rats on day 12–14 of embryonic development. In brief, pregnant Sprague-Dawley (SD) rats (purchased from the Animal Center of Haidian, Beijing) were anaesthetized by intraperitoneally injecting 5% chloral hydrate (8 mL/kg body weight). The embryos were isolated from the uterus, and the cortices were dissected under a stereomicroscope in sterile conditions. After removing the meninges and blood vessels under a stereomicroscope, the tissue was cut into 1 mm$^3$ pieces and incubated with 0.25% trypsin at 37 °C for 15 min. Trypsinization was stopped by adding DMEM/F12 with 10% FBS. Then, the cell suspension was centrifuged at 250 g for 5 min, the tissue was resuspended in DMEM/F12 medium with 2% B-27 supplement and 20 ng/mL bFGF. After filtration, cells with a density of 1 × 10$^5$ cells/mL were cultured in a humidified incubator at 37 °C with 5% CO$_2$.

OECs were isolated from the outer two layers of olfactory bulbs. Briefly, adult male SD rats (2 months old; purchased from the Animal Center of Haidian, Beijing) were sacrificed by decapitation, and the olfactory bulbs were placed in ice-cold Hank’s balanced salt solution (HBSS) in sterile conditions. As stated above, after centrifugation,
the cell suspension was centrifuged at 250 g for 5 min and resuspended in DMEM/F12 medium with 10% FBS. The tissue was triturated with a fire polished Pasteur pipette (15–20 times) and filtered through a cell strainer (200 mesh) to obtain a single cell suspension. Cells were then plated in a culture dish at a density of $1 \times 10^5$ /mL and maintained at 37 °C with 5% CO$_2$.

According to the differential rates of attachment of fibroblast cells and OECs, after 12 h of culture, the suspended cells were centrifuged and subsequently expanded in DMEM/F12 medium with 20 μmol/L Forskolin (Sigma, USA) and 20 μg/L bovine pituitary extract (BPE; Invivortoxogen, USA). Then, 12 h later, the supernatant and suspended cells were transferred to another new culture dish coated with poly-L-lysine (Sigma, USA). After a 2-d culture, cells were treated with 1 × 10^{-5} mol/L cytosine-barabinoside hydrochloride (Ara-C; Sigma, USA) for 24 h, washed with PBS and subsequently expanded in DMEM/F12 medium with 20 μmol/L Forskolin and 20 μg/L BPE. The medium was changed every 2 d.

All the animal studies were performed in line with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996 and approved by the Beijing Administration Committee of Experimental Animals.

Immunofluorescence staining

The characterization of OECs and NSCs was performed by using rabbit anti-S-100β antibody (1:200) and rabbit anti-nestin antibody (1:150), respectively. Cells cultured on coverslips were fixed with 4% paraformaldehyde at room temperature for 30 min, washed several times in phosphate buffered saline (PBS, pH 7.4) and rinsed in PBS with 0.1% Triton X100 (PBST) for 30 min. Then, the cells were incubated with a blocking solution including 5% goat serum for 1 h. Coverslips were then incubated at room temperature for 2 h in PBST containing the diluted primary antibodies. After washing, the cells were incubated for another hour in PBST containing the secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or tetraethyl rhodamine isothiocyanate (TRITC). Nuclear staining was performed by incubating the coverslips in a solution PBST with DAPI (1:1000) for 10 min. Finally, after washing several times, the preparations were visualized by using a confocal laser scanning microscope (Olympus FV500, Japan).

Cell proliferation

In order to evaluate the cytocompatibility of the chitosan-alginate hydrogel, three other hydrogels (Matrigel®, Fibrin glue and E-matrix) were selected for comparison. Matrigel® was purchased from Vigorous Biotechnology Beijing Company Limited; Fibrin glue was purchased from Guangzhou Bioseal Biotechnology Company Limited; and E-matrix was prepared according to the method described in [17].

Cell proliferative analysis was performed using 96-well plates that contained the hydrogels. OECs were seeded at a concentration of 1,000 cells/well cultured in DMEM/F12 medium with 10% FBS. NSCs were seeded at a concentration of 5,000 cells/well cultured in DMEM/F12 medium with 2% B27 supplement and 20 ng/mL bFGF. After 1, 3, 5 and 7 d, 10 μL CCK-8 solution was added to each well. After incubation at 37 °C for 4 h, the absorbance of WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] in each well at 450 nm was measured by a Bio-Rad 550 spectrophotometric microplate reader (Tokyo, Japan).

Statistical analysis

Data are presented as the mean values with standard deviation (±SD). Statistical comparisons were performed using analysis of variance (ANOVA), and differences of $p < 0.05$ were considered statistically significant.

Results and discussion

FTIR analysis

The FTIR spectra of alginate, chitosan, and chitosan-alginate complex are shown in Figure 1. The chitosan spectrum (Figure 1(b)) showed a characteristic band of amino group ($-\text{NH}_2$) at 1596 cm$^{-1}$. The intensity of the amide peak for chitosan corresponds to the partial N-
deacetylation of chitin [18]. The 2856 and 2925 cm$^{-1}$ peaks were attributed to −CH$_2$ and −CH$_3$ groups stretching vibration, and the 1424 cm$^{-1}$ peak was attributed to C–H bending vibration [19]. The characteristic peaks of alginate (Figure 1(a)) were observed at 1619 and 1416 cm$^{-1}$, corresponding to two carboxyl groups (−COOH) in the molecular chain. In the chitosan-alginate spectrum (Figure 1(c)), the amide II peak (1579 cm$^{-1}$) was significantly intensified, and the peaks of the amino group (1596 cm$^{-1}$) and carboxyl group (1619 cm$^{-1}$) were absent. These changes demonstrated that the formation of the chitosan-alginate hydrogel was due to the ionic interaction between the negatively charged carboxyl group (−COO$^-$) of alginate and the positively charged amino group (−NH$_3^+$) of chitosan.

**XRD pattern**

Figure 2 shows the XRD patterns of alginate, chitosan and chitosan-alginate complex. The XRD pattern of alginate shown in Figure 2(a) had no crystal peak, indicating that alginate is an amorphous material, whereas the pattern of chitosan (Figure 2(b)) showed the two typical diffraction peaks at $2\theta=11^\circ$ and $20^\circ$. If alginate and chitosan had very low compatibility, each polymer would exhibit its own crystal regions in the blend. However, the XRD pattern of the chitosan-alginate hydrogel had a weak broad profile without an obvious crystal peak. The formation of the chitosan-alginate hydrogel broke the hydrogen bonding between amino groups and hydroxyl groups in chitosan and then resulted in an amorphous structure of the chitosan-alginate complex, which has been similarly discussed before [20]. The decreased crystallinity of the composite hydrogel also implied that the ionic interaction between chitosan and alginate lead to their good compatibility, which was consistent with the result of FTIR.

**SEM observation**

The SEM image (Figure 3) shows the inner structure of the chitosan-alginate hydrogel. The scaffold had a highly porous and interconnected pore structure as a result of phase separation in lyophilization. This porous structure would be beneficial to cell attachment and proliferation as well as tissue growth, and also might provide the passage of nutrients by diffusion and metabolites exchange. The porosities of the chitosan-alginate complex in this study were determined to be 85.6%±4.7%, and the scaffold had a pore size of approximately 70–150 μm, which was favourable for cell attachment and migration [21].

**Wettability**

The contact angle is the angle at which a liquid interface meets the material surface. As is well known, if the material surface is hydrophobic, the contact angle will be larger than 90°. The static water contact angles are shown in Figure 4. Both chitosan and chitosan-alginate had a contact angle smaller than 90°, which indicated their hydrophilicity. With the introduction of alginate, the
contact angle significantly decreased from $71.4^\circ \pm 1.6^\circ$ (chitosan) to $24.4^\circ \pm 1.9^\circ$ (chitosan-alginate hydrogel), demonstrating that the hydrophilicity of the chitosan-alginate hydrogel surface significantly increased and chitosan-alginate was more hydrophilic than pure chitosan. The hydrophilic or hydrophobic property of a biomaterial surface is an important factor affecting cell affinity. In most cases, the capacity for cell attachment and proliferation on a hydrophilic surface is better than that on a hydrophobic surface [22]. To determine whether the chitosan-alginate hydrogel is suitable for nerve cell growth requires further cell proliferation tests.

**Primary culture and characterization of OECs and NSCs**

Figure 5 shows the expression of marker proteins of OECs and NSCs. Immunocytochemical staining of OECs showed positive immunoreactivity to S100\(\beta\) with a spindle morphology as green stain (Figure 5(a)) and NSCs (single cells or neurospheres) showed positive immunoreactivity to nestin, an acknowledged protein marker for NSCs. Primary culture of OECs is often contaminated with fibroblast cells because of the incomplete removal of meninges and blood vessels. If the purification procedure was not carried out, there were many fibroblast cells in the culture dish. With the continuation of the culture, the fibroblasts showed a much faster proliferation than OECs, and eventually led to a useless result in the OECs culture. In our study, we obtained a highly purified culture of OECs (Figure 5(a)) according to the method based on differential rates of attachment of different cells [23].

In vitro, NSCs were small and round with little morphological evidence of differentiation after 1 d incubation. Then, the cells were expanded as floating aggregates called neurospheres during subsequent culture. The neurospheres increased both in size and amount in serum-free medium containing 20 ng/mL bFGF and NSCs could maintain the undifferentiated status for a long time.

**Cell proliferation**

Figure 6 showed the nerve cell proliferation results from day 1 to day 7. We compared the chitosan-alginate
hydrogel with three other commercial gels (Matrigel®, Fibrin glue and E-matrix) in cell proliferation. For OECs (Figure 6(a)), the cells proliferated more on Matrigel® (with 281% increase) and E-matrix (with 234% increase) and less on Fibrin glue (52% increase). On the chitosan-alginate hydrogel, OECs proliferated slightly less (with 149% increase) than on Matrigel® or E-matrix, but more than on Fibrin glue. However, for NSCs, the cell proliferation behaviour on the four different gels showed no significant difference (approximately 230% increase).

In tissue engineering, it is possible to use the hydrogel matrix as a carrier for the direct injection of cells into a host organism without considerable loss of cell viability or function [17]. Moreover, the hydrogel matrix may also serve as a shield to protect the cells from the immune attack of the host organism [17]. As three widely used hydrogels, Matrigel®, Fibrin glue and E-matrix are capable of maintaining cell viability and function during storage when the cells are embedded in the hydrogel matrix, and they have potential applications in various transplantation therapies for the treatment of CNS diseases and other chronic disorders.

Matrigel® is the trade name for a gelatinous protein mixture (laminin, collagen, entactin and other growth factors) secreted by mouse tumour cells. This mixture resembles the complex extracellular environment found in many tissues and is used as a substrate for cell culture (nerve cell, liver cell, vascular endothelial cell and other tissue cells) [24]. Matrigel® is effective for the attachment and differentiation of both normal and transformed anchorage dependent epithelial and other cell types [25–27]. It also supports in vivo peripheral nerve regeneration and has been widely used in the nerve system [28–30].

Fibrin glue is a synthetic substance used to create a fibrin clot. It is made up of fibrinogen and thrombin. Thrombin acts as an enzyme and converts the fibrogen into fibrin in about 10–60 s [31]. Fibrin glue has been used as a scaffold for a long time to culture a variety of cells and tissue analogues [31–34]. As a hydrogel, it has also been widely used in nerve tissue engineering to repair peripheral nervous system (PNS) injuries and CNS injuries [35,36].

E-matrix is a hydrogel matrix for long-term storage and proliferation of cells. E-matrix includes an effective amount of polar amino acids selected among arginine, lysine, histidine, glutamic acid and aspartic acid. E-matrix is able to sustain cells and complex clusters of cells [37]. One advantage of E-matrix is its ability to immobilize water at appropriate storage temperatures and providing binding sites for cells, including nerve cells [38]. The increased level of polar amino-acid groups allows cells stored in E-matrix to be directly injected into a host organism without recognition by the host’s immune system.

In tissue engineering, the scaffold plays a critical role in the successful living tissue construct. Enhancement of cell attachment and proliferation are the main attributes of a beneficial scaffold. Compared with three gels widely used in tissue engineering, including nerve tissue engineering, a chitosan-alginate hydrogel is much cheaper with easy preparation. Moreover, chitosan-alginate hydrogel could well promote the proliferation of nerve cells (OECs and NSCs). According to our previous study [14], with the presence of OECs, NSCs could differentiate into more neurons than NSCs cultured alone in DMEM/F12 with 10% FBS. Moreover, co-transplantation of OECs and NSCs into the injured spinal cord of rats could improve the recovery of locomotion function and reduce spinal cord damage, which indicated that OECs and NSCs were two promising cell types in CNS repair. Chitosan-alginate hydrogel combined with OECs and NSCs as a whole would have a good prospect for application in neural tissue engineering.

Conclusions

In summary, a natural polymer-based complex chitosan-alginate hydrogel was constructed by the strong ionic bonding between the amino groups of chitosan and the carboxyl groups of alginate. The chitosan-alginate hydrogel with a porous structure was highly hydrophilic. The cell proliferation study showed that OECs and NSCs proliferated well on the chitosan-alginate hydrogel. These encouraging results indicated the potential application of a chitosan-alginate hydrogel as an improved alternative for neural tissue engineering.

Disclosure statement

All authors have no conflicts of interest.

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