Cellular Interaction of *In Situ* Chitosan- and Hyaluronic Acid-Based Hydrogel

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Introduction

Development of polysaccharides-based hydrogels to promote tissue regeneration and control the release profile of an encapsulated drug has been extensively investigated by employing different biomedical materials. Among them, hyaluronic acid (HA) and chitosan have attracted great interesting on employment of scaffolds and hydrogel for tissue regeneration and drug carriers because of their interesting properties such as biocompatibility, muco-adhesiveness, biodegradability as well as encapsulation of living cells [1] and drugs. HA- and chitosan-based hydrogel has been employed by numerous groups to various methods and applications such as control release of bioactive molecules [2], interaction of endothelial cells with HA-based scaffold for vascular grafts to generate extra-cellular matrix such as laminin, fibronectin, collagens [3] as well as tissue engineering applications to mimic extra-cellular matrix design [4, 5].

We synthesized and rigorously evaluated the mechanisms of chemistry of novel *in situ* hyaluronic acid- and chitosan-poly(ethylene oxide) (PEO) hydrogels, rheological behaviors, morphologies, FTIR, NMR, in vitro and in vivo tissue regeneration by bone cells and stem cells as well.

Experimental

HA Acrylation. After mixing the APM into the 0.1g HA solution, EDC was slowly added into the mixed solution, thus mixing the APM and EDC at a final molar ratio of 1:4:4 in HA:APM:EDC for 24 hr. After precipitating the products in ethanol, methacrylated HA samples were obtained by lyophilizing.

Chitosan acrylation: 1.5 mL 2-carboxyethyl acrylate was added into the chitosan solution(1.0g) after adding up 2.3 mL EDC into the mixture solution of both chitosan and 2-carboxyethyl acrylate, thus utilizing a final concentration of 1:4:4 (chitosan:EDC:2-carboxyethyl acrylate) in molar ratio. The product was lyophilized overnight.

^{1}H nuclear magnetic resonance (^{1}H-NMR). ^{1}H-NMR spectra were obtained by employing an UI 500 MHz FT-NMR Spectrometer to observe an extent of acrylation of the methacrylated HA. Chemical shift (δ) was measured in ppm by employing deuterium oxide (D_2O) as solvent.

Hydrogel synthesis. HA- and chitosan-PEO hydrogels were synthesized by employing methacrylated HA and PEO 6 thiol arms. First aminopropylmethacrylate-grafted-HA (0.01 g) was dissolved in triethanolamine-buffered solution. A separate 20% (w/v) PEO solution in buffered solution was mixed with the above methacrylated HA solution. HA-PEO hydrogel was spontaneously synthesized without any further treatment via Michael type addition reaction.

Rheological behaviors of hydrogel formation. Analysis of hydrogel formation was performed with rheological behaviors by Rotational Rheometer Gemini. Rheological behaviors were observed over the 1 mL methacrylated HA, chitosan and PEO mixture solution on the sandblast parallel plate under the conditions of frequency sweep at 0.1~10 rad/s, strain at 0.1. Gelation behavior was observed over time by observing the behaviors of the viscous and elastic modulus as well as tan δ.

Swelling behaviors of hydrogels. Hydration of the HA- and chitosan-based hydrogels was quantized by immersing repeatedly its weight changes with a microbalance. Percentages of water absorption of the hydrogel sample were measured over its initial dry weight with a microbalance. As another test, water absorption of the hydrogel was determined by immersing the dry three hydrogels per condition in distilled water at predefined intervals. Percentage of water swelling was calculated.

Mechanical properties. Compression tests were performed three times per condition on the chitosan-EPO and HA-PEO hydrogels to measure its mechanical strength with a MT-LQ material tester. The hydrated HA-PEO hydrogel was inserted into half the mold (d=1 cm x h=3 cm) by pressing down the probe (d = 0.8 cm) until the hydrogel was completely crushed down.

SEM observation of the dehydrated gel network. Dehydrated gel samples were sputter-coated with gold in plasma for 60 sec. Morphological images of the dry hydrogel samples were obtained from 10- to 3,000-times high magnifications to observe the existence of pores and their sizes under vacuum circumstance.

Results and discussion

Graftings of 2-carboxyethyl acrylate to the free amine groups of chitosan and aminopropylmethacrylate to the free acid groups of HA were achieved via EDC chemistry and their chemical graftings were confirmed with analyses of NMR with new acrylate peak appearance and FTIR spectrum with new amide peaks in 1729 cm-1 and 1658 cm^{-1}. Degrees of acrylation to the chitosan and hyaluronic acid were measured as approximately 30% by comparing the hydrogen peaks and the new acrylate hydrogen peaks in NMR. The hydrogel was *in situ* formed by mixing the acrylated chitosan and PEO-thiol solutions over 5 min to 1 day dependent upon choices of degree of hyaluronic acid and chitosan acrylations after mixing the acrylate-derivertized chitosan and hyaluronic acid with PEO-thiols as observed with a rheometer. While viscous modulus increased sharply and constantly over 5,000 sec, the phase angles decreased, indicating that the gel was formed. Formation of a chitosan-PEO hydrogel was further analyzed with viscosity changes with rheology analyses and gel shapes in vials. Chitosan-PEO hydrogelation was further analyzed by swelling the preformed gels in water after dehydration process and then the morphologies of the dehydrated gel demonstrated its cross networks with various pores ranging 10 μm and more.

Cellular interaction of the chitosan-PEO hydrogel was tested with MC3T3 bone cells for 3 day. When we seeded the cells at a density of 200,000 cells/cm^2, cell adhesion was good on the cell-non-adhesive surfaces. *In vivo* bone tissue regeneration was clearly observed on both hyaluronic acid- and chitosan-PEO hydrogels without any cells incorporated in rats for 4 weeks via Masson's trichrome staining. The regenerated tissues include new bones and collagens as stained in red and blue, respectively.

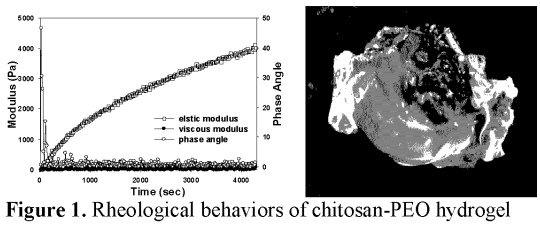

Figure 1. Rheological behaviors of chitosan-PEO hydrogel formation and its formed hydrogel shape.

Conclusions

We synthesized an *in situ* chitosan- and hyaluronic acid-poly(ethylene-oxide) hydrogels via acrylation of chitosan and hyaluronic acid and then its Michael type addition reaction with PEO. The obtained hydrogel demonstrated their gel properties such as water absorption, gel shape, chemical network structures after dehydration and rheological behaviors during gel formation. The hydrogels induced modest induction of cell adhesion on its hydrogel surfaces as well as demonstrated good bone tissue regeneration in rats. The chitosan and hyaluronic acid hydrogels demonstrated cell compatibility, indicating a possibility of application in *in situ* bone tissue regeneration in irregular defects.

References

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