A Heparin/Poly(l-lysine) Nanoparticle-Coated Polymeric Microspheres for Stem Cell Therapy

Kun Na, † Sungwon Kim, †,§ Kyeongsoo Park, †,§ Kwangmeyung Kim, †,§ Dae Gyun Woo, § Ick Chan Kwon, †,§ Hyung-Min Chung, †,* Keun-Hong Park †,*

Division of Biotechnology, The Catholic University of Korea, 43-1 Yokkok2-dong, Wonmi-gu, Bucheon City, 420-743, Republic of Korea, Biomedical Research Center, Korea Institute of Science and Technology, 39-1 Haweolgog-dong, Sungbook-gu, Seoul 136-791, Republic of Korea, Chabiotech Co., Ltd 606-16., Yeoksam 1-dong, Kangnam-gu, Seoul 135-081, Republic of Korea, College of Medicine, Pochon CHA University, CHA Stem Cell Institute 606-16, Yeoksam 1-dong, Kangnam-gu, Seoul 135-081, Republic of Korea

* Co-corresponding authors. Tel: +82-2-3468-3392, Fax: +82-2-3468-3373
E-mail address: phd0410@hanmail.net (Park, K.-H.), hmchung@chacare.com (Chung, H.-M.)
Experimental Methods

**Materials.** Low molecular weight heparin (LMWH, Fraxiparin®), which has an average molecular weight of approximately 4,500 Daltons, was obtained from Sanofi-Synthelabo Co. (Gentilly, France). Poly(l-lysine) (Mw = 3,000 Dalton) was purchased from Sigma-Aldrich. A poly(lactide-co-glycolide) (PLGA) with a molecular weight of 33,000 and a copolymer ratio of lactide to glycolide of 50:50 (R503H, Boehringer Ingelheim, Germany) was used as a wall material for the microspheres. All reagents and organic solvents used were of at least ACS grade.

**Preparation of heparin/poly(l-lysine) nanoparticles.** All materials were obtained from commercial sources, and were used without additional purification. Specific amounts of a concentrated heparin solution (0.5mg/ml) were added to a solution of poly(l-lysine) (0.5mg/ml) in distilled water, yielding complexes with +/- molar charge ratios in a range from 0 to 2.95. The density of charges on heparin was assumed to be 5 mEq/g. DLS analyses were conducted after each incremental addition of heparin. Both stock heparin and polymer solutions were passed through 0.22-μm nylon filters prior to experimentation.

**Preparation of PLGA microspheres.** The PLGA microspheres were prepared using a solvent evaporation in an oil-in-water emulsion. In brief, PLGA (4 g) was dissolved in 30 ml of dichloromethane. Using a glass syringe and needle (needle gauge; 20G), the polymer solution was dropped into 300 ml of aqueous solution containing 2 w/v% of PVA while mixing, using a magnetic stirrer at 600 rpm. The suspension was then gently stirred for 2 to 3 hours at 35°C with a magnetic stirrer at 600 rpm in order to evaporate the dichloromethane, and the microspheres were collected via 2 minutes of centrifugation at 1500 rpm. The collected microspheres were washed four times in
distilled water, and were then lyophilized. The size of the microspheres, as measured by SEM, ranged between 20 ~ 80 μm.

**Immobilization of heparin/poly(l-lysine) nanoparticles onto PLGA microspheres.**

First, the PLGA microspheres were coated with positively-charged PEI. The coating was conducted at the native pH (7.4) of the polymer solution, with no additional salt. Under the same conditions, the polyethyleneimine (PEI) side chain amines (pKₐ≈10) would be extensively protonated. The microspheres (1g) were then soaked for 12 hours in the PEI solution (1mg/ml) with gentle stirring, collected through 2 minutes of centrifugation at 1500 rpm, rinsed 3 times in distilled water, and then dipped into the heparin/poly(l-lysine) nanoparticle solution (1 mg/mL) for 24 hours with gentle stirring. The heparin/poly(l-lysine) nanoparticle-coated PLGA microspheres were then collected via 2 minutes of centrifugation at 1000 rpm and rinsed 3 times in distilled water. Finally, the heparin/poly(l-lysine) nanoparticle-coated PLGA microspheres were washed four times in distilled water and lyophilized.

**Cell seeding and growth on heparin/poly(l-lysine) nanoparticle-coated polymeric microspheres.** Rabbit mesenchymal stem cells (MSC) were isolated from White New Zealand rabbits. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco Co. (Grand Island, NY) and was supplemented with 10% FBS (fetal calf serum), streptomycin at 100 mg/ml, penicillin at 100 IU/ml, and 2 mM L-glutamine. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For the cell seeding and growth test, 10mg of microspheres and 2×10⁵ cells/ml of MSCs were incubated in a Transwell insert in the culture dish with gentle shaking. After 2 hours of incubation, the unattached cells were removed and the Transwell inset was incubated for cell growth. After incubation, the cells were fixed in a 3.7% solution of
formaldehyde in PBS, and were then stained with rhodamine-conjugated phalloidin (Molecular Probes).

**Characterizations.** Dynamic light scattering measurements were conducted using a helium ion laser system (Spectra Physics Laser Model 127-35) operated at 633 nm at 25 °C. The intensity autocorrelation of the sample (1mg/mL in PBS) was then measured at a 90° scattering angle using a Brookhaven BI-9000AT digital autocorrelator. The AFM images were maintained in room temperature air using an Autoprobe CP system (Park Scientific, Inc., Sunnyvale, CA) (Fig. SI1). The tapping mode was conducted with an ultrasharp tip with ca. 3 N/m force constant. Images present the silicon wafer (Fig. SI1A), polyethyleneimine (PEI) coated onto a silicon wafer (Fig. SI1B), and heparin/poly(l-lysine) nanoparticles (NPs) absorbed onto a polyethyleneimine (PEI) modified silicon wafer (Fig. SI1C). Inorganic substrate silicon wafers were utilized as model surfaces for the polyelectrolyte multilayers. These hydrophilic, very smooth substrates were employed in order to obtain data on more ideal polyelectrolyte layer surfaces. As can be clearly observed in the comparisons of images, only a small portion of the coated surface was not covered with NPs. This implies that the polyelectrolyte complex NPs were physically entrapped by the PEI film covering almost the entirety of the silicon wafer surfaces. Particle morphology was observed by field-emission scanning electron microscopy (FE-SEM) (S-5000, Hitachi Ltd., Japan). Confocal microscopy was conducted using a Zeiss 40X/1.20 NA water immersion objective lens. Fluorescence images were acquired using argon (wavelength 488nm) and helium (wavelength 543nm) neon lasers. Simultaneous images corresponding to fluoresceine and rhodamine were acquired using the multitracking function of the microscope.
Differential interference contrast (the Nomarski technique) was employed in the visualization of individual images.

Fig. S11. The several different surfaces of AFM images. (a) Silicon wafer surface, (b) PEI-coated silicon wafer surface, and (c) heparin/poly(l-lysine) nanoparticles attached to the polyethyleneimine surface using a silicone wafer.

**DNA content.** At each time point, the samples and negative controls from each time point were extracted from the sacrificed nude mice, rinsed in 2.5mL PBS, and homogenized with a pellet grinder (Fisher Scientific). The samples were then digested in 500 mL of a proteinase K solution (1 mg/ml proteinase K, 10 mg/ml pepstatin A, and 185 mg/ml iodoacetamide) in PBE buffer (6.055 mg/ml Tris(hydroxymethyl aminomethane), 0.372 mg/ml EDTA, pH 7.6 adjusted by HCl) at 60 °C for 16 h. The specimens subsequently underwent three repetitions of a freeze/thaw/sonication cycle (30 min at 80°C, 30 min at room temperature, 30 min of sonication) to extract the DNA from the cell cytoplasm. DNA and GAG assays were then carried out in triplicate for each experimental and control group at each time point. The number of cells was determined by measuring the double-stranded DNA content using the PicoGreen assay.
(Molecular Probes, Eugene, OR) according to the instructions of the manufacturer. The fluorescence of the negative, cell-free controls was subtracted from the fluorescence values of the experimental groups in order to account for the fluorescence of the material alone.

![DNA content of the embedded MSCs on microspheres for up to 4 weeks.](image)

**Fig. SI2.** DNA content of the embedded MSCs on microspheres for up to 4 weeks.

(a): 0, (b): 1, (c): 2, (d): 3, and (e): 4 weeks.

**RT-PCR.** Total RNA was obtained from freshly cultured chondrocytes with Trizol (GibcoBRL) as described by the manufacturer. Total RNA was used in reverse transcriptase-PCR reactions (Titan RT-PCR system, Roche Molecular Biochemicals) to determine the presence of collagen types II transcripts. The RNA was reverse-
transcribed, and the cDNA was amplified by PCR (Expand High Fidelity, Roche Molecular Biochemicals) with collagen type II collagens. Primer for rabbit collagen type II was designed to amplify a 350 bp fragment from the 3′ end of the collagen type II chain. Total RNA was used in the RT-PCR according to the protocol suggested by the manufacturer under the following conditions: 94°C for 1 min 30 sec, 20 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min plus a 5 sec increase for every additional cycle, and a final extension step at 72°C for 5 min. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide. The size of the products was determined by comparison with the migration of a 1 kb DNA ladder and 100 bp DNA ladder (Gibco-BRL). To confirm the identity of the amplified products corresponding to collagen type II (350 bp) as specific products of amplification, the ethidium bromide-stained bands were excised, and the DNA was extracted and purified using the Qiagen Gel Extraction Kit (Qiagen, Santa Clarita, CA). The sequences were analyzed and compared to known sequences in GenBank.

Fig. SI3. Gene expression profiles of type II collagen as analyzed by RT-PCR.

(a): PLGA microspheres and (b): heparin/poly(l-lysine) NP-coated microspheres.

1: 1 week, 2: 2 week, 3: 3 week, 4: 4 week.
**Histology and Immunohistochemistry.** The injection site was completely excised and processed for classical histology. In brief, samples from each time point were embedded in O.C.T. compound (TISSUE-TEKs 4583, Sakura Finetek USA, Inc) and frozen. The specimens were cut into 10 μm-thick sections at -20°C and stained with hematoxylin and eosin (H&E) for nuclei and cytoplasm. The stained sections of each test sample were examined using light microscopy for cell proliferation, and were photographed with a digital camera. Cryosections (8 μm) of chondrogenic cultures were mounted on adhesion microscope slides (Marienfeld). Cell-hydrogel constructs were placed in 4% paraformaldehyde or 2.5% glutaraldehyde solution for 30-40 min, and the constructs were then fixed on slides. Embedded sections were stained with Safranin-O staining for histological evaluation. Sections were also immunolabeled for the presence of type II collagens, with visualization via horseradish-conjugated secondary antibody (Fig. SI3). Images were taken from stained sections, randomized, and graded by three readers for cell arrangement, proteoglycan, and collagen staining (blind study), as well as collagen-specific staining from immunolabeled sections.

![Image](a) ![Image](b)

**Fig. SI4.** Collagen type II staining of MSCs embedded in heparin/poly(l-lysine) NP-coated PLGA microspheres.
(a): PLGA microspheres and (b): heparin/poly(l-lysine) NP-coated microspheres.