Video Article

Polyelectrolyte Complex for Heparin Binding Domain Osteogenic Growth Factor Delivery

Raymond Wing Moon Lam[1], Sunny Akogwu Abbah[2], Wang Ming[1], Mathanapriya Naidu[1], Felly Ng[1], Hu Tao[1], James Goh Cho Hong[3,4], Kang Ting[5], Wong Hee Kit[1,4]

[1]Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore

[2]Centre for Research in Medical Devices (CÚRAM), National University of Ireland Galway

[3]Department of Bioengineering, Faculty of Engineering, National University of Singapore

[4]Tissue Engineering Program, National University of Singapore

[5]Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, University of California, Los Angeles

Correspondence to: Raymond Wing Moon Lam at doslwmr@nus.edu.sg

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Abstract

During reconstructive bone surgeries, supraphysiological amounts of growth factors are empirically loaded onto scaffolds to promote successful bone fusion. Large doses of highly potent biological agents are required due to growth factor instability as a result of rapid enzymatic degradation as well as carrier inefficiencies in localizing sufficient amounts of growth factor at implant sites. Hence, strategies that prolong the stability of growth factors such as BMP-2/NELL-1, and control their release could actually lower their efficacious dose and thus reduce the need for larger doses during future bone regeneration surgeries. This in turn will reduce side effects and growth factor costs. Self-assembled PECs have been fabricated to provide better control of BMP-2/NELL-1 delivery via heparin binding and further potentiate growth factor bioactivity by enhancing *in vivo* stability. Here we illustrate the simplicity of PEC fabrication which aids in the delivery of a variety of growth factors during reconstructive bone surgeries.

Video Link

The video component of this article can be found at https://www.jove.com/video/54202/

Introduction

The incidence of pseudoarthrosis has been reported to be as high as 10 to 45% in degenerative spinal fusion and revision spinal surgeries[1]. To reduce the rate of pseudarthrosis during spine fusion and other reconstructive bone surgeries, osteogenic growth factors such as BMP-2, Nell-1[1] and platelet derived growth factor (PDGF) have been introduced to promote de novo osteogenesis. Among these, BMP-2 is a popular choice for spinal fusion[2]. Although the potency of BMP-2 in inducing and facilitating new bone formation has been well established[3]; clinically significant complications such as heterotopic bone formation, seroma and hematoma formation, inflammatory response, radiculitis, vertebral body osteolysis, and retrograde ejaculation continue to be issues of concern due to the supraphysiological amounts used[4,5].

Therefore, lowering the dose of BMP-2 remains a relevant strategy in attempts to minimize side effects. Besides, efficient carrier systems are required to suppress the initial burst release of BMP-2 observed in contemporary collagen sponge carrier systems and further enhance prolonged and localized delivery of this potent cytokine. The layer-by-layer self-assembly of alternating cationic and anionic polyelectrolytes can be employed as a tunable method to build up polyelectrolyte complexes on the surface of scaffold matrices or implantable materials[6]. In this respect, heparin (known for having the highest negative charge density of all biological agents) has been recognized to avidly bind with a variety of growth factors via electrostatic and heparin binding domains. Indeed, heparin has been shown to prolong the half-life and thus potentiate the bioactivity of several growth factors.

Based on this, our group adapted a layer-by-layer self-assembly protocol to fabricate a heparin-based polyelectrolyte complex (PEC) that loads and preserves the bioactivities of osteogenic growth factors during immobilization[7,8]. The alginate microbead core was fabricated by crosslinking α-L-guluronate (G) residues of alginate with divalent cation calcium or strontium ions. The alginate core is a biodegradable scaffold matrix; which after implantation, it is resorbed in the fusion bed providing room for bony ingrowth. Poly-L-lysine (PLL) or protamine is used as the cationic layer to interlace with both the scaffold matrix (in this case, the alginate microbead carrier core) and the negatively charged heparin; while the anionic heparin layer functions to stabilize and localize loaded growth factors. The triple layer PEC has been shown to increase growth factor loading capacity in a porcine model[9]. Recently, PEC carriers have been shown to successfully reduce the effective dose of BMP-2 by at least 20-fold in rat[10] and porcine models of spinal fusion[8].

Here, we report the methods of fabricating PECs for enhanced growth factor delivery in spinal fusion and the other reconstructive bone surgeries using BMP-2 as a model osteogenic growth factor.

Protocol

1. Alginate Solution Preparation

1. Dissolve 200 mg of sodium alginate (non-irradiated) or 400 mg of 8 MRad irradiated sodium alginate in 10 ml double distilled water and shake for 1 hr for non-radiated alginate and 15 min for irradiated alginate. Store the alginate solution at 4 °C overnight. Filter the alginate solution with a sterile 0.2 µm syringe filter before alginate microbead fabrication.

2. Alginate Microbead Fabrication

1. Disinfect the electrostatic bead generator and syringe pump with 70% ethanol and place them in a Class II Biological Safety Cabinet (**Figure 1**).
2. Place a glass basin with a magnetic stir bar inside the bead generator.
3. Set the arm electrode of the bead generator 9 cm above the basin.
4. Connect the electrode cable of the bead generator to the knurled screw 2 of the arm electrode, and pour 80 ml of $SrCl_2$ solution into the basin.
5. Load 5 ml of 0.2 µm filtered alginate solution into the syringe and rubber tube. After connecting the rubber tube to the arm electrode, switch on the syringe pump at 5 ml/hr for 2 min to expel the air inside the tubing and deliver the alginate solution to the tip of the nozzle. Turn off the syringe pump.
6. Next, switch on the encapsulator and then, the syringe pump to commence microbead generation. Set the alginate flow rate at 5 ml/hr and the voltage at 5.8 kV on the encapsulator. Discard the microbeads generated during the first two minutes (or the initial 0.5 ml alginate solution pumped out of the syringe), as these microbeads tend to be irregularly sized and shaped.
7. Collect subsequent microbeads in 0.2 M strontium chloride solution. Turn off both the syringe pump and the encapsulator (in that order) after pumping the pre-planned volume of alginate solution. Repeat this for subsequent batches of microbead fabrication. Upon completion, turn off the syringe pump first, followed by the encapsulator.
8. Store the microbeads in 20 ml of 0.2 M strontium chloride solution at 4 °C overnight to complete cross linking and stabilize the gel.

3. Size Measurement of Alginate Microbeads

1. Collect 0.5 ml of alginate microbeads with a plastic pipette and place it on a glass slide. View the microbeads under an optical microscope at 10X magnification. Take ten images of the microbeads with a microscope CCD camera. Save the images with scale bar (500 µm) in TIFF format at resolution 2,048 x 1,536.
2. Using the length tools in ImageJ, measure the size of the microbeads and scale bar (**Figure 2**). Convert the microbeads length from pixel to micrometer.
    1. Click on the line tools and draw a line across the middle of the alginate bead.
    2. Click "Analyze" on the menu bar and select "Measure". A pop-up window will appear.
    3. Repeat steps 3.2.1-3.2.2 to measure all alginate beads within the image. Measure the scale bar on the image.
    4. Convert the diameter of the alginate bead to the actual length by using the formula:length of alginate bead/length of scale bar x 500 µm. For example, 1.420 (diameter measured by ImageJ) / 2.657 (scale bar length measured by ImageJ) * 500 µm = 267 µm.
3. Consider the average size of 100 microbeads (mean ± standard deviation) as the representative size of each batch of microbeads.

4. Sterilization

1. Collect the microbeads using a 100 µm nylon strainer and wash the beads with double distilled water.
2. Using a spatula, transfer all the microbeads made from 0.1 ml alginate solution into a 2 ml microcentrifuge tube and cover with gauze to prevent drying.
3. Finally, sterilize the microbeads by autoclaving using liquid mode (115 °C, 15 min) or in accordance with manufacturer's specifications. Add 1.5 L of distilled water to the chamber to prevent beads from drying.

5. Protamine and Heparin Coating

1. Inside the BSL-2 hood, incubate the sterile microbeads with 1 ml of 2 mg/ml protamine solution (sterilized using a 0.2 µm syringe filter) for 1 hr at room temperature.
2. After incubating the microbeads for 1 hr (Step 5.1), collect 150 µl of the protamine solution for the micro bicinchoninic acid (microBCA) test (section 6).
3. Wash the protamine coated microbeads twice with double distilled water. Spin down using a bench top centrifuge at 200 x g for 3 min at room temperature. After centrifugation, aspirate the water using a syringe.
4. Incubate protamine coated microbeads with 1 ml of 0.5 mg/ml heparin solution (sterilized using a 0.2 µm syringe filter) for 30 min to create polyelectrolyte complex (PEC).
5. After incubating the protamine coated microbeads for 30 min (Step 5.4), collect 400 µl of the heparin solution to determine heparin content (section 7).

6. After incubation, wash off the unbound heparin from the PECs by washing twice with double distilled water.

6. Protamine Content

1. Perform the microBCA test according to manufacturer instructions. Briefly, add 150 µl protamine solution (collected before and after incubation with microbeads) into a 96 well plate. Add 150 µl microBCA working solution.
2. Use albumin solution (0, 0.5, 1, 2, 5, 10, 20, 40 and 200 µg/ml) as calibration standards.
3. Incubate the mixture for 60 min at 60 °C. Measure the absorbance with a spectrophotometer at 562 nm.
4. Use the standard curve to determine the protamine concentration of each unknown sample according to manufacturer's instructions.
5. Determine the protamine content of the microbeads by subtracting the total amount of protamine in the coating solution (before incubation with microbeads) from the amount of protamine remaining in the coating solution (after incubation with microbeads).

7. Heparin Content

1. Prepare the 10 ml of working solution by dissolving 4 mg toluidine blue and 20 mg sodium chloride in 0.01 N hydrochloric acid.
2. Add 400 µl of sample (from step 5.5) to the working solution at a ratio of 2:3 and vortex for 30 sec.
3. Add 600 µl of n-hexane (equivalent volume to the working reagent solution) and vortex the mixture to extract the toluidine blue heparin complex.
4. Aspirate 200 µl of the aqueous phase by syringe after phase separation.
5. Measure the amount of un-extracted toluidine blue contained in the aqueous phase using a spectrophotometer at 631 nm.
6. Prepare heparin standard solutions of 0-20 µg/ml.
7. Plot the 631 nm reading of each heparin standard vs. heparin concentration in µg/ml. Use the standard curve to determine the heparin concentration of each sample.

8. Confocal Image of Layer-by-layer Structure

1. Fabricate protamine, heparin and NELL-1/BMP-2 fluorescent analog CF-405 protamine (blue), CF 594 heparin (red) and FITC labeled NELL-1/FITC labeled (green) BMP-2 + heparin + protamine according to manufacturer's technical datasheet.
2. Coat 100 µg microbeads with 300 µl of fluorescent analog (coating method as described in 5.3-5.6) CF-405 protamine (blue) (2 mg/ml, 1 hr incubation), CF 594 heparin (red) (0.5 mg/ml, 30 min) and FITC labeled NELL-1/FITC labeled (green) BMP-2 (1.5 mg/ml, overnight). Wash the microbeads twice with distilled water to eliminate the unbound fluorescent protamine, heparin and NELL-1/BMP-2.
3. Observe the layer-by-layer structure (**Figure 3**) by using a confocal microscope at 10X magnification.[7]

9. BMP-2 and NELL-1 Uptake and Release

1. Load 13.3 µl of 1.5 mg/ml of BMP-2 or NELL-1 solution on 100 µg of PEC. Incubate PEC at 4 °C under 30 rpm shaking for 10 hr.
2. Immerse the microbeads in 1 ml of phosphate buffered saline (PBS) at 37 °C with constant shaking (30 rpm).
3. Collect 1 ml of the supernatant and replace it with 1 ml PBS after 1, 3, 6, 10 and 14 days.
4. Evaluate the uptake and release efficiency of BMP-2 using the ELISA method according to the manufacturer's protocol. Evaluate the uptake and release efficiency of NELL-1 using the carboxybenzoyl quinoline-2-carboxaldehyde (CBQCA) protein assay method according to the manufacturer's protocol.
5. Determine cumulative release at time (t):
   Cumulative release at time (t) = Release at time (t) + Previous release at time (t-1).
6. Plot cumulative release of BMP-2 and NELL-1 against time.

10. *In Vitro* Bioactivity of NELL-1

Note: The bioactivity of NELL-1 released from PEC was assessed by measuring its ability to increase the expression of alkaline phosphatase (ALP) in rabbit bone marrow stem cells (rBMSC).

1. Seed 20,000 rBMSCs per well in a 24-well plate and allow them to grow for one day with 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) + 10% Fetal bovine serum (FBS) at 37 °C and 5% CO_2.
2. After 24 hr, replace the medium with 1 ml of an osteogenic medium (DMEM supplemented with 10% FBS, 2% penicillin streptomycin, 50 µg/ml ascorbic acid, 10 mmol/L beta-glycerophosphate, and 10^{-8} mol/L dexamethasone) for 7 days at 37 °C and 5% CO_2.
3. Place 300 µg PEC-NELL-1 (from step 8.2) and PEC inside cell culture inserts (TC insert) to keep the PECs separate from the cells (this avoids the washout of PEC microbeads during the osteogenic medium change). Place TC insert into the 24 well plate for 14 days.
4. Once every three days, aspirate 1 ml of the osteogenic medium by placing a needle outside the TC insert, and replace with 1 ml of fresh osteogenic medium.
5. After 7 and 14 days of incubation, determine ALP activity with an ALP assay kit in accordance with the kit manufacturer's protocol.
    1. Lyse cells with assay buffer containing 0.1% TritonX-100 at 4 °C for 10 min. Scrape off adhered cells using a cell scraper. Incubate cell suspension at 4 °C under agitation for at least 60 min.
    2. Centrifuge the cell suspension at 2,500 x g at 4 °C for 10 min. Collect the supernatant for the ALP assay.
    3. Add 50 µl of serially diluted alkaline phosphatase standard solution from 200 to 0 ng/ml to the wells of a 96 well plate. The final amounts of alkaline phosphatase standard are 10, 5, 2.5, 1.2, 0.6, 0.3, 0.15, and 0 ng/well.
    4. Add the supernatant from step 10.5.2 (50 µl/well) and dilute with dilution buffer.
    5. Add 50 µl of *p*-nitrophenyl phosphate (pNPP) substrate solution into each well. Mix the reagents by gently shaking the plate for 30 sec. Incubate the mixture for 30 min in the dark. Measure the absorbance at 405 nm by plate reader.

6. Calculate ALP activity using the calibration curve.

6. Determine protein content using the microBCA protein assay kit according to manufacturer's instructions. Normalize the ALP activity by dividing ALP activity by protein content.

11. Cell Viability

1. Incubate 200 mg of PEC-NELL-1 with 1 ml of DMEM +10% FBS at 37 °C for 24 hr for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
2. Seed 2,000 rBMSCs per well (in 100 µl of DMEM with 10% FBS) in a 96 well plate and incubate for 1 day at 37 °C, 5% CO_2.
3. Replace DMEM + 10% FBS with 100 µl of PEC-NELL-1/PEC extract and incubate at 37 °C, 5% CO_2.
4. After 1 day or 3 days of incubation, add 10 µl of 5 mg/ml MTT solution and further incubate at 37 °C, 5% CO_2 for 4 hr in darkness.
5. Add 100 µl DMSO solution to each well to dissolve formazan crystals.
6. Determine the absorbance at 570 nm using a microplate reader.
7. Calculate the relative growth rate:

$$Relative\ growth\ rate\ = \frac{Absorbance\ of\ Extract\ (e.g.\ PEC + NELL - 1, PEC)}{Absorbance\ of\ cells\ exposed\ to\ DMEM + 10\%\ FBS}$$

12. Packaging into Scaffold and BMP-2 & NELL-1 Loading

1. Pack the PECs into the pores of a bioresorbable medical grade Polycaprolactone - tri-calcium phosphate (mPCL-TCP) scaffold using a sterilized spatula inside a BSL-2 chamber.
2. Add 1.5 mg/ml solution of BMP-2 or NELL-1 onto the mPCL-TCP scaffold packed with PEC and incubate overnight at 4 °C.

Representative Results

In our carrier, protamine was chosen as a substitute of poly-L-lysine as it has similar chemical properties and it is FDA approved as an antidote of heparin. Optical microscope results showed that the non-irradiated microbeads were spherical in shape with a diameter of 267 ± 14 µm. (0.35 mm nozzle, flow rate of 5 ml/hr & 5.8 kV). The majority of the irradiated microbeads are of teardrop shape. The diameter measured on the round portion of the irradiated microbeads was 212 ± 30 µm (0.35 mm nozzle, flow rate of 4 ml/hr & 6 kV). (**Figure 4**).

Confocal images of the PEC microbeads reveal layer-by-layer coating of CF-405 labeled protamine (blue), CF594-labeled heparin (red) and FITC-BMP-2/FITC-NELL-1 (green). The results indicate that the PECs can bind with positively charged BMP-2 and negatively charged NELL-1 via the heparin binding domain (**Figure 5**). This suggests that the interaction between the PECs and the osteogenic growth factors is not charge dependent.

To prove that the PECs can uptake and release BMP-2, we used an ELISA assay to determine the amount of BMP-2 remaining after incubation and the amount of BMP-2 in the PBS on Day 1, 3, 6, 10 and 14. However, a similar approach does not work well with the NELL-1 protein, since heparin blocks the antibody binding site, significantly reducing the signal. Therefore, the CBQCA protein assay was used to determine the difference between PEC-NELL-1 and PEC. From the cumulative release curve, PECs not only show a higher NELL-1 uptake efficiency compared to BMP-2 but also release it much slower than BMP-2 (NELL-1: 20% vs. BMP-2: 25%) (**Figure 6**). This suggests that PECs bind more tightly with NELL-1 than BMP-2.

From the MTT assay, PEC-NELL-1 is not cytotoxic (**Figure 7**). The result matches with the Alamar Blue assay result in a previous study[7]. Heparin neutralizes the positive charge of protamine which plays an important role in maintaining the biocompatibility of PEC.

To determine whether NELL-1 release from PEC affects long-term osteogenic differentiation, the expression level of an osteogenic marker, alkaline phosphatase (ALP), was investigated by a colorimetric assay. NELL-1 release from PEC increases the ALP activity of rabbit bone marrow stem cells by 2.2 fold at day 14 compared to the PEC control group (**Figure 8**). BMP-2 shows maximum increase of ALP activity (3.75 fold) on day 7. Both PEC-BMP-2 and PEC-BMP-2 + extra BMP-2 show decrease of activity on day 9.

ALP activity with BMP-2 in the medium without PEC is shown in **Figure 8C**. Although 70% of the growth factor remains on the PEC, the ALP activity of PEC BMP-2 group is equivalent to the free BMP-group. *In vivo*, growth factor must be delivered by the carrier to avoid washout. From our rodent and porcine model, we can lower the dose of BMP-2 by 20-fold and 6-fold, respectively. The reduction of does not only reduce unwanted side effects but also cuts down the cost of growth factor usage.

Figure 1: Electrostatic bead generator and syringe pump set up in BSL-2 biosafety cabinet. (**A**) Nozzle secured on nozzle holder. (**B**) Big basin for strontium chloride solution. (**C**) Electrode cable. (**D**) Hose delivers alginate solution. (**E**) Nozzle holder and arm. (**F**) Electrodes supply the potential difference to regulate the bead size. (**G**) Magnetic stirrer. (**H**) 5 ml syringe. (**I**) Syringe pump to regulate alginate flow. (**J**) Agitator control knob to control stirring speed. (**K**) Voltage control knob to regulate the potential difference between 0-10 kV. Please click here to view a larger version of this figure.

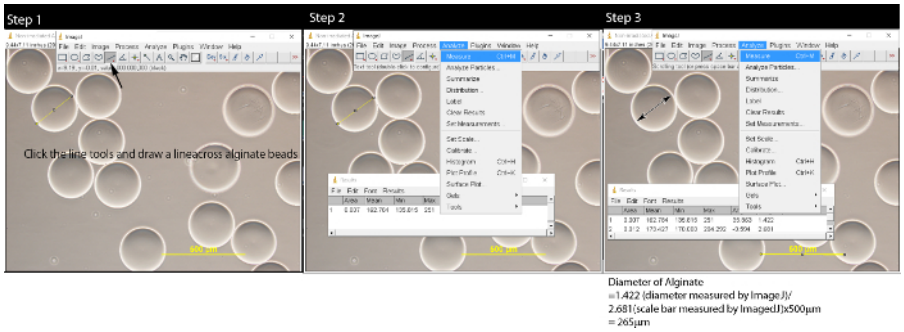

Figure 2: Measuring alginate microbeads with ImageJ software. After opening the image file by clicking File → Open, follow Step 1: click the line tool and draw a line on the alginate microbeads. In Step 2, click Analyze on the menu bar and a pop-up window will appear. Repeat Step 1 and Step 2 until all microbeads are measured. In Step 3, click the line tool, draw a line across the scale bar and measure the length of the scale bar. Convert the microbeads length to µm by using formula: length of alginate/length of Scale bar x 500 µm. Please click here to view a larger version of this figure.

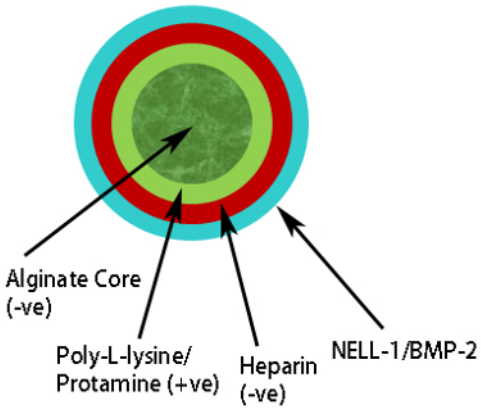

Figure 3: Schematic representation of Polyelectrolyte Complex. Alginate core (dark green) positive charge protamine layer (pale green), negative charge heparin layer (red), osteogenic growth factor, *e.g.*, BMP-2/NELL-1 on outermost layer (pale blue). Please click here to view a larger version of this figure.

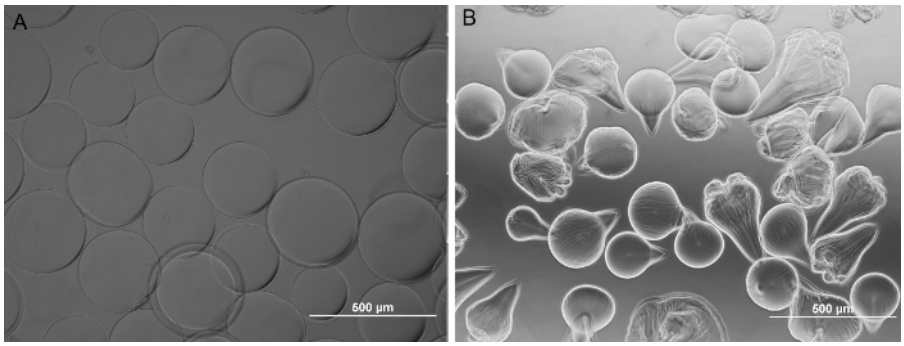

Figure 4: Bright field images of alginate microbeads. (**A**) Non-irradiated. (**B**) 8M Rad irradiated. Non-irradiated alginate microbeads are spherical and the 8M Rad irradiated counterpart is teardrop shaped. Magnification 100X. (Scale bar = 500 µm.) Please click here to view a larger version of this figure.

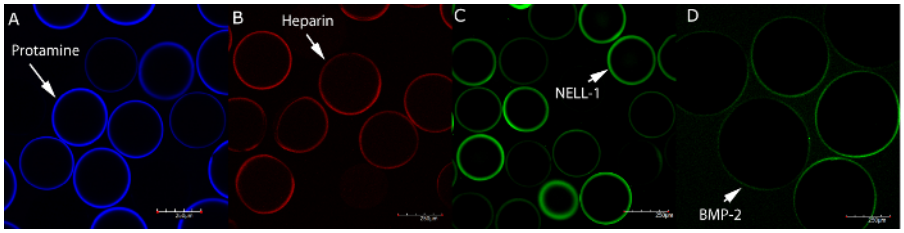

Figure 5: Confocal laser scanning microscopy images of alginate microbeads incubated with fluorescent analogues of protamine, heparin, NELL-1 and BMP-2. (**A**) CF 405 Protamine (blue), (**B**) CF 594 Heparin (red), (**C**) FITC NELL-1 (green), and (**D**) FITC BMP-2 (green). Microbeads remain spherical even after incubation with fluorescent analogues. Scale bar = 250 µm. Please click here to view a larger version of this figure.

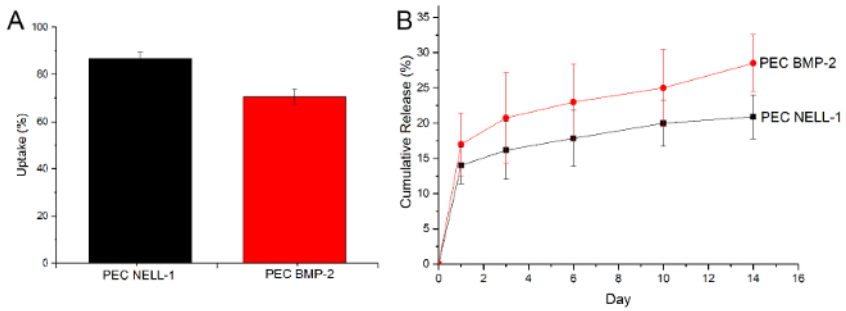

Figure 6: Uptake and release of BMP-2 and NELL-1. (**A**) Uptake of NELL-1 protein (black) BMP-2 (red), (**B**) Cumulative release curve of BMP-2 (red) and NELL-1 (black) from PEC carrier. Results are presented as mean ± standard deviation. Please click here to view a larger version of this figure.

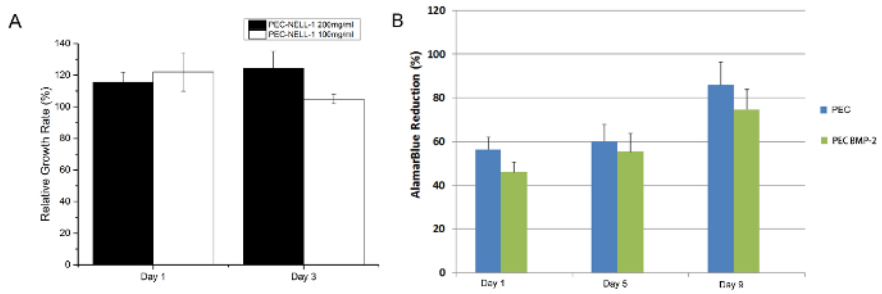

Figure 7: Cytotoxicity Assay. (**A**) MTT assay of rabbit bone marrow stems cell with protamine based PEC NELL-1 200 mg/ml extract (black), PEC-NELL-1 100 mg/ml extract (white) at Day 1 and 3. (**B**) Alamar Blue assay of rabbit bone marrow stems cell with PEC BMP-2 (blue), PEC (green) Experiments were performed in triplicate and results are presented as mean ± standard deviation. Please click here to view a larger version of this figure.

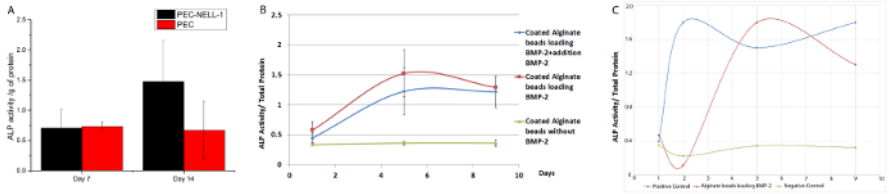

Figure 8: Bioactivity Assay for BMP-2 and NELL-1 release from PEC carrier. (**A**) ALP activity of rabbit bone marrow stem cell incubated with PEC-NELL-1 (black), PEC (red). ALP activity was measured as absorbance at 405 nm. 2.2 fold increase in ALP activity was observed after incubation with PEC-NELL-1 at Day 14. (**B**) ALP activity of rabbit bone marrow stem cell incubated with PEC-BMP-2 (red) PEC-BMP-2 + addition of BMP-2 (green) and PEC (blue). Increase in ALP activity after incubation with PEC-BMP-2 and PEC-BMP-2 + addition of BMP-2 at Day 7, ALP activity drops on Day 9. (**C**) ALP activity of rabbit bone marrow stem cell incubated with PEC-BMP-2 (red) BMP-2 (blue) and Medium (green). Results are presented as mean ± standard deviation. Please click here to view a larger version of this figure.

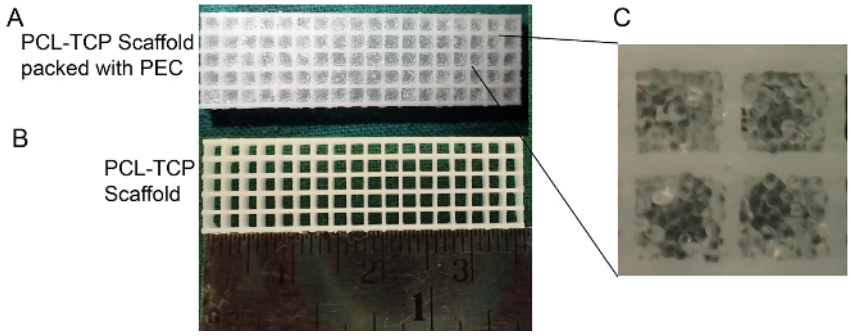

Figure 9: Polycaprolactone Tri-calcium phosphate (PCL-TCP) scaffold packed with PEC carrier. (**A**) Polycaprolactone Tri-calcium phosphate (PCL-TCP) scaffold (pore size 1,300 µm) packed with PEC carrier. (B) PCL-TCP scaffold. (**C**) High magnification: PEC maintains its shape after packing. Please click here to view a larger version of this figure.

Discussion

This protocol presents a method for the preparation of PECs through layer-by-layer self-assembly. The layer-by-layer structure is visualized using fluorescent analogues of protamine, heparin, BMP-2 and NELL-1 and confocal microscopy. Uptake and release tests show that heparin on PEC mediates osteogenic growth factor uptake and release. The uptake efficiency of the PEC method is: NELL-1: 86.7 ± 2.7%, BMP-2: 70.5 ± 3.1%. The PEC carrier has a better modulation of NELL-1 (20%) release compared to a pure surface adsorption carrier such as calcium apatite particles (40-80%)[11].

Besides modulating release, heparin neutralizes the excessive positive charge of polycations such as protamine to avoid unwanted cytotoxicity related issues[12]. The PECs do not show any signs of cytotoxicity, as determined by the MTT assay and the Alamar Blue assay[7]. The ALP assay shows the PEC carrier can maintain the bioactivity of both NELL-1 and BMP-2. Although developed for osteogenic growth factor BMP-2 therapy in spinal fusion surgeries, PEC can also take up other growth factors with heparin binding domains such as NELL-1 and PDGF-BB. Compared to other delivery methods such as encapsulation of growth factors in polyglycolic acid microspheres, PECs do not require organic solvents that tend to inactivate growth factors[13].

A number of factors in the PEC fabrication procedure may affect carrier performance. Firstly, microbead size affects the surface area/volume ratio. Higher osteogenic growth factor loading can be achieved with smaller microbeads. Secondly, the alginate concentration should be sufficient to maintain the stability of the microbead structure. Microbead stability depends on alginate type, chain length (affected by gamma irradiation) and the divalent ion used (barium > strontium > calcium). While 2% alginate solution is sufficient to manufacture PECs with stable microbead structures, 4% alginate is required following 8 MRad gamma irradiation to compensate for the effects of alginate chain shortening during irradiation. Thirdly, the rate of *in vivo* degradation of alginate microbeads is strongly influenced by alginate chain length. Based on our experience from rat and porcine models, PEC fabricated using 8 MRad irradiated alginate shows rapid (28 days) and complete degradation of the alginate core (unpublished data). Degradation of the alginate core provides room essential for bony ingrowth. Fourthly, the overnight incubation of BMP-2 and NELL-1 at 4 °C with constant shaking (*e.g.*, 30 rpm) can improve uptake efficiency. Lastly, the protamine coating thickness is time dependent. Since the extent of the protamine-heparin interaction determines the release of osteogenic growth factors such as BMP-2 or NELL-1, 1 hr of protamine incubation is adopted to improve the stability of the PEC structure.

The use of heparin in this technique is critical in stabilizing the delicate growth factors and thus important for prolonging *in vivo* bioactivity. Given the very limited amount of heparin involved and coupled with the choice of the antidote, *i.e.*, protamine (a highly effective drug in neutralizing the anti-coagulant activities of heparin), prolonged bleeding time in decorticated bone is largely theoretical and practically inconsequential.

Loading the PECs into the PCL-TCP scaffold enhances localization of beads at implant sites. Scaffolds provide necessary mechanical support that is crucial for spine fusion. In the present studies, we used PCL-TCP scaffolds with 1,300 µm pores to facilitate proper packing (**Figure 9**). Although the current illustration shows PEC osteogenic growth factor delivery with the PCL-TCP scaffold, our group has also evaluated carrier performance with a polyetherketoneketone (PEKK) bone chamber in one rabbit study with similar efficacy.

In this study, the lack of comparison with other previously evaluated carriers of rhBMP-2 and NELL-1 could represent a limitation.

In conclusion, the presented procedure provides a useful carrier to control release of osteogenic growth factors with heparin domains such as BMP-2 and NELL-1. The described strategy combines many advantages: it is not restricted to BMP-2 and applicable to other growth factors with heparin binding domains such as NELL-1. Dose reduction on osteogenic growth factor can reduce undesirable side effects such as seroma, heterotrophic bone formation and lower the overall cost of treatment.

Disclosures

We have no conflict of interest.

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References

1. Yuan, W. *et al.* NELL-1 based demineralized bone graft promotes rat spine fusion as compared to commercially available BMP-2 product. *Orthop Sci.* **18**, 646-657 (2013).
2. Anderson, C. L., & Whitaker, M. C. Heterotopic ossification associated with recombinant human bone morphogenetic protein-2 (infuse) in posterolateral lumbar spine fusion: a case report. *Spine.* **37**, E502-506 (2012).
3. Glassman, S. D. *et al.* RhBMP-2 versus iliac crest bone graft for lumbar spine fusion: a randomized, controlled trial in patients over sixty years of age. *Spine.* **33**, 2843-2849 (2008).
4. Tannoury, C. A., & An, H. S. Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery. *Spine J.* **14**, 552-559 (2014).
5. Carragee, E. J., Hurwitz, E. L., & Weiner, B. K. A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. *Spine J.* **11**, 471-491 (2011).
6. Abbah, S. A., Lam, C. X., Hutmacher, D. W., Goh, J. C., & Wong, H. K. Biological performance of a polycaprolactone-based scaffold used as fusion cage device in a large animal model of spinal reconstructive surgery. *Biomaterials.* **30**, 5086-5093 (2009).
7. Abbah, S. A., Liu, J., Lam, R. W., Goh, J. C., & Wong, H. K. In vivo bioactivity of rhBMP-2 delivered with novel polyelectrolyte complexation shells assembled on an alginate microbead core template. J. *Control. Release.* **162**, 364-372 (2012).
8. Wang, M. *et al.* Polyelectrolyte Complex Carrier Enhances Therapeutic Efficiency and Safety Profile of Bone Morphogenetic Protein-2 in Porcine Lumbar Interbody Fusion Model. *Spine* **40**, 964-973 (2015).
9. Abbah, S. A., Lam, W. M., Hu, T., Goh, J., & Wong, H. K. Sequestration of rhBMP-2 into self-assembled polyelectrolyte complexes promotes anatomic localization of new bone in a porcine model of spinal reconstructive surgery. *Tissue Eng. Part A.* **20**, 1679-1688, (2014).
10. Hu, T. *et al.* Novel Protamine-Based Polyelectrolyte Carrier Enhances Low-Dose rhBMP-2 in Posterolateral Spinal Fusion. *Spine* **40**, 613-621 (2015).
11. Hu, J., Hou, Y., Park, H., & Lee, M. Beta-tricalcium phosphate particles as a controlled release carrier of osteogenic proteins for bone tissue engineering. J *Biomed Mater Res A.* **100**, 1680-1686 (2012).
12. Darrabie, M. D., Kendall, W. F., Jr., & Opara, E. C. Characteristics of Poly-L-Ornithine-coated alginate microcapsules. *Biomaterials.* **26**, 6846-6852 (2005).
13. Li, X., Min, S., Zhao, X., Lu, Z., & Jin, A. Optimization of entrapping conditions to improve the release of BMP-2 from PELA carriers by response surface methodology. *Biomed Mater.* **10**, 015002 (2015).

