Chapter 11

Preparation and Evaluation of Natural Scaffold Materials for Kidney Regenerative Applications

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Abstract

Tissue engineering involves the concerted action of biomaterials, cells, and growth factors. Kidney regeneration relies on the same combination of ingredients. Here, we describe an example of gelatin-based biomaterial preparation and its evaluation in the context of kidney biocompatibility and integration. This biomaterial manufacturing technique is simple, cost-effective, highly reproducible and the in vivo evaluation procedure highly informative on the biocompatibility and regenerative potential of the tested construct.

Key words Gelatin, Microspheres, Injectable, Renal

1 Introduction

In the past few decades, tissue engineering emerged as a new field with highly promising therapeutic potential (1). Concepts that appeared unattainable not long ago, such as organ regeneration and de novo organogenesis, have now been successfully translated into concrete clinical cases and tissue engineering and regeneration products are slowly progressing toward commercialization (1). One of the initial accomplishments in the field was bladder engineering, where a biodegradable synthetic scaffold and autologous cells were combined successfully to create a brand-new, functional organ (2). The same design approach widely applies to tubular of hollow organs: a biocompatible, persistent scaffold; cells capable of repopulating the scaffold surface and interacting with their endogenous counterparts; and a proper biological milieu that exposes the "organ to be" to physiologically similar conditions (temperature, pressure, pH, nutrient gradients, etc.) (3). While the number of success stories with hollow organ regeneration increases, the design and successful achievement of functional organs with more complicated architectures (i.e., heart, kidney, liver, etc.) still remains a

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challenge. One crucial parameter of every tissue engineering and regenerative process is the choice of the proper scaffold or biomaterial. Synthetic, semisynthetic, and natural scaffolds are widely available and each class features a comparable array of advantages and disadvantages (4). Synthetics are typically more cost-effective, involve highly controllable manufacturing processes, have longer in vivo persistence which is required for certain applications but often are associated with toxic effects and bio-rejection (5). In contrast, natural scaffolds are characterized by great biological compatibility and controllable and predictable biodegradation rates, but present risks of disease transmission, lot-to-lot variability, and high manufacturing costs. Semisynthetics represent a hybrid of the aforementioned categories, and typically leverage the positive features of their natural and synthetic counterparts (i.e., cross-linked gelatin-based biomaterials—maintain the biocompatibility and biological effects associated with collagens (6–10) and in addition they have improved lot-to-lot consistency, longer in vivo residence, and tailorable stiffness) (4).

For our kidney regeneration-targeted applications, the design of the system was focused on the development and consistent production of biomaterials that (a) would deliver attached cells to the desired sites and create space for regeneration; (b) would be able to persist at the site long enough to allow cells to establish, function, remodel their microenvironment, and secrete their own extracellular matrix (ECM); and (c) would promote integration of the transplanted cells with the surrounding tissue. In other words, process reproducibility, biocompatibility, and biodegradability were key demands. A number of scaffolds were considered: gelatin, hyaluronic acid, alginate, polyglycolic acid (PGA), and poly-lactic-*co*-glycolic acid (PLGA) (11). For this specific application, gelatin-based scaffolds featured most positive parameters, but the long bioresorption rate of commercially available variants appeared to hinder tissue integration and regeneration.

Here, we detail a simple, straightforward method to obtain gelatin-based microspheres with controllable proteolytic susceptibility. By using a carbodiimide widely employed in the manufacturing of collagen-based FDA-approved devices and a physiological chemical cross-linking process, one can manufacture gelatin beads with biodegradation rates spanning across a significant range. In addition, the resulting constructs (cellularized scaffolds) are injectable and easy to evaluate both in vitro and in vivo for their cytocompatibility and biocompatibility.

2 Materials

All solutions should be prepared with deionized water (resistivity of 18 MΩ cm at 25°C). Follow all handling, storage, and disposal recommendation provided by the manufacturer for all chemicals.

2.1 Biomaterial Preparation

1. 10% (w/v) gelatin solution: 10 g of gelatin (low endotoxin, Gelita Inc., Sioux City, IA, USA) into deionized water to a final volume of 100 ml.
2. Heat/stir plate with temperature control.
3. Stir bar.
4. Support stand with rod.
5. Clamp and clamp holder.
6. Deep metal tray.
7. Thin layer chromatography reagent sprayer.
8. Liquid nitrogen.
9. Aluminum foil to line the metal tray.

2.1.1 Microsphere Cross-Linking

1. Cross-linking buffer: 0.1 M MES, 0.9% NaCl pH 4.7. 2-(morpholino) ethanesulfonic acid (MES) buffered saline (Fisher Scientific, Pittsburg, PA, USA).
2. Cross-linking reagent stock solution: 1 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich, St. Louis, MO, USA) in cross-linking buffer. If desired, for differential cross-linking a concentration range of cross-linking reagent solutions (i.e., 10–100 mM) can be obtained from the 1 M EDC stock solution via serial dilutions.
3. Conical tubes (50 ml).
4. Tabletop vortex.

2.1.2 Microsphere Purification

1. Cross-linking buffer.
2. Deionized water.
3. Vacuum flask.
4. Buchner funnel.
5. Filter paper.

2.2 Biomaterial Evaluation

1. Lyophilized cross-linked gelatin microspheres.
2. Phosphate-buffered saline (1× PBS).

2.2.1 Cross-Linking Efficiency Determination

3. Collagenase/dispase digestion mix: 30 U/ml collagenase IV (Worthington Biochemical Corp., Lakewood, NJ, USA), 4 U/ml dispase I (STEMCELL Technologies, Vancouver, BC), 0.5 mM $CaCl_2$ in PBS.
4. Coomassie Plus (Bradford) protein assay solution (Fisher Scientific, Pittsburg, PA, USA).
5. Picrylsulfonic acid (TNBS) 5% w/v in H_2O (Sigma-Aldrich).
6. 1 M NaOH solution
7. pH paper strips (0–14 pH range).
8. Eppendorf tubes (1.5 ml).
9. Multi-well spectroscopy plate (96-well).

2.2.2 Microsphere Sizing

1. Nylon meshes of desired pore size (Small Parts, Inc., Atlanta, GA, USA).
2. 70% v/v ethanol solution.
3. Buchner funnel.
4. Vacuum flask.

2.2.3 Biocompatibility

1. Mammalian primary kidney cells (12, 13).
2. Basal medium: 1:1 Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG; Invitrogen, Carlsbad, CA, USA): Keratinocyte-Serum Free Medium (K-SFM; Invitrogen).
3. Kidney growth medium: Basal medium containing 5% v/v fetal bovine serum (FBS), 5.0 μg/l epidermal growth factor (EGF), 50 mg/l bovine pituitary extract (BPE), 1× insulin-transferrin-sodium selenite medium supplement (ITS; Invitrogen), 1× antibiotic–antimycotic (Invitrogen).
4. LIVE/DEAD mammalian cell viability assay kit (Invitrogen).
5. Injection needle or catheter and 1 ml syringe.

3 Methods

3.1 Gelatin Microspheres Preparation

3.1.1 Bead Production

1. Prepare a concentrated gelatin solution (i.e., 10% w/v) in water and allow the protein to dissolve at 40°C under stirring for 1 h.
2. Set up the necessary equipment for bead spraying (support stand with rod, clamp holder, clamp) (Fig. 1).
3. Rinse tubing and TLC reagent sprayer with hot water for 5–10 min to warm them up. Alternatively, equilibrate in a 50°C oven.
4. Fix the gelatin solution flask onto the stand with a clamp, connect the TLC reagent sprayer to the flask on one end and a forced air source on the other end.
5. Position the TLC reagent sprayer tip perpendicularly to the tray surface at a distance of 25–30 cm (see Note 1).
6. Pour liquid nitrogen into a metal tray lined with aluminum foil to an approximate liquid depth of 1 in.
7. Air spray gelatin solution into liquid nitrogen until the desired amount of gelatin solution is sprayed (see Note 2).
8. Place the tray in a fume hood to evaporate the liquid nitrogen.
9. Collect the beads and lyophilize.

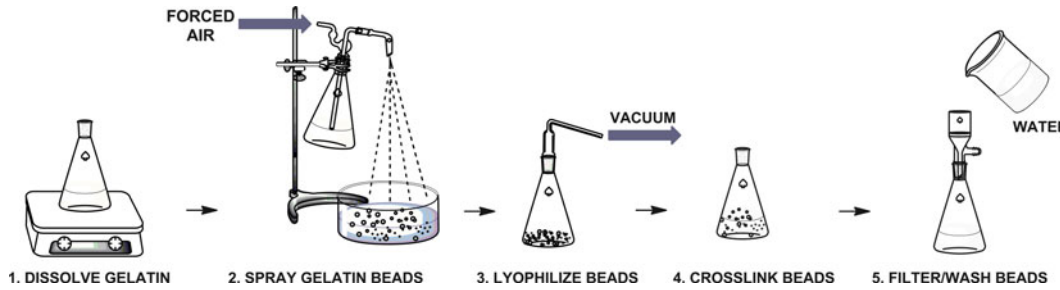

Fig. 1 Schematic illustration of the gelatin microsphere production and cross-linking process

3.1.2 Bead Cross-Linking

Soak 1 g of lyophilized beads in (20 – *X*) ml prechilled cross-linking buffer (where *X* = cross-linking reagent stock solution volume that needs to be added to the beads in order to obtain the final desired cross-linking reagent solution concentration) for at least 1 h at 4°C (see Notes 3 and 4).

1. Briefly vortex samples.
2. Incubate for 24 h at 4°C under static conditions.
3. Add the X ml cross-linking reagent stock solution to the beads, so that the total solution volume is 20 ml.
4. Briefly vortex samples to ensure homogeneity.
5. Incubate for 24 h at 4°C under static conditions.
6. Filter beads and wash extensively with deionized water.
7. Freeze and lyophilize for long-term storage or size separation (Fig. 1).
8. Alternatively, purified beads can be kept in PBS or ethanol solution at 4°C and used within a few days.

3.1.3 Optional: Bead Sizing (see Notes 5 and 6)

1. Transfer beads into 70% ethanol solution.
2. Prepare a filtering flask with a Buchner funnel with the larger pore size mesh (see Note 7).
3. Filter beads through.
4. Remove mesh, resuspend captured beads in ethanol solution, and repeat filtering step two more times (see Note 8).
5. The beads that filtered through the mesh are now all ≤250 μm.
6. Remove the large pore size mesh from the funnel. Thoroughly rinse funnel with hot water, connect to a clean filtering flask, and insert the small pore size mesh (i.e., 100 μm).
7. Filter the ≤250 μm beads through the mesh three times as described above.
8. Collect the beads that get retained on the mesh: these beads will be 100–250 μm in diameter (see Note 9).

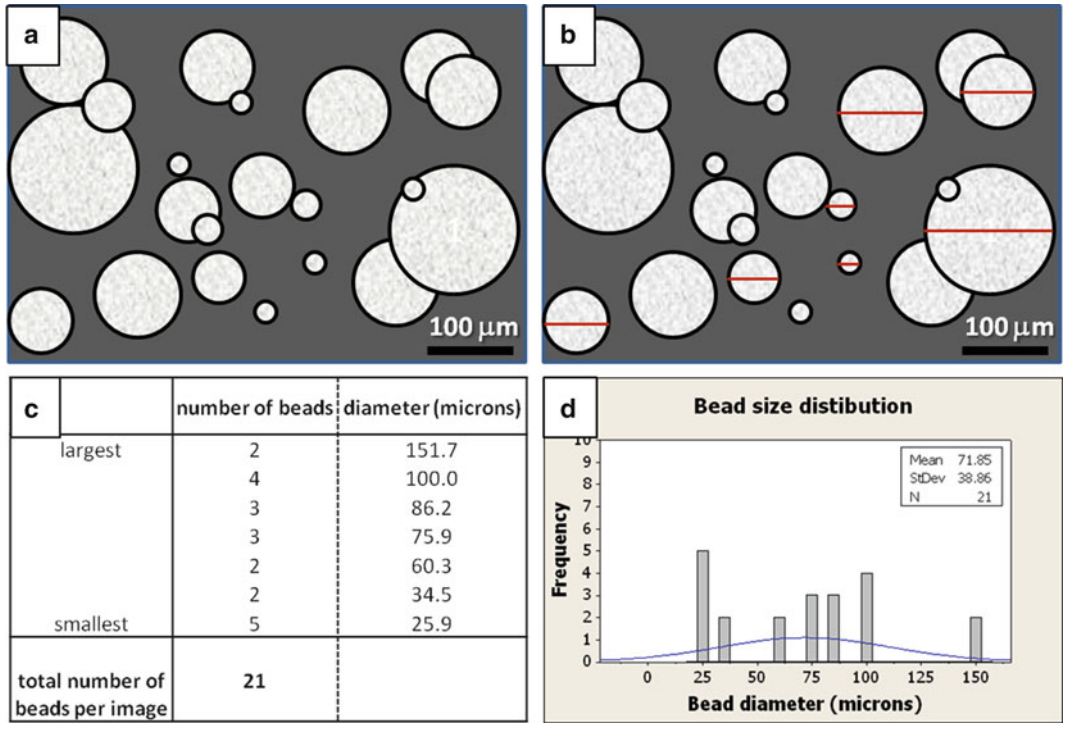

Fig. 2 Methodology for bead size distribution determination. (**a**) Schematic of SEM image of beads; (**b**) illustration of bead diameter measuring procedure; (**c**) example of bead size and diameter compilation; (**d**) determination of bead size distribution

3.2 Gelatin Microsphere Characterization

3.2.1 Morphology and Size Distribution (see Note 10)

1. Cover a scanning electron microscopy (SEM) stub with carbon tape.
2. Apply lyophilized beads onto carbon-taped SEM stubs.
3. Sputter-coat with gold (see Note 11).
4. Image.
5. Verify sizes by measuring the bead diameters from at least ten different images using a software of choice (i.e., ImageJ).
6. Compile bead sizes and numbers, then determine the bead size distribution by using a software of choice (i.e., MiniTab) (Fig. 2).

3.2.2 In Vitro Enzymatic Degradation Assay (see Notes 12 and 13)

7. Weigh out dry cross-linked beads.
8. Suspend in PBS, pH 7.4 to a concentration of ~20 mg/ml.
9. Add 50 μl of collagenase/dispase digestion mix to 0.5 ml bead suspension.
10. Vortex samples.
11. Incubate for 1 h at 37°C on a rocker (use $n=3$ for each cross-linked concentration used) to yield partially digested samples.

12. Subsequently, collect 20 µl supernatant from the partially digested samples and evaluate for soluble protein content via Bradford assay (see Note 14).
13. Further incubate the remaining digestion mix overnight as described above to yield totally digested samples.
14. Determine total protein content by Bradford assay (see Note 15).
15. Determine the percent degradation per hour for each sample by using the formula: ((amount of protein detected in the supernatants of partially digested sample)/(amount of protein in the totally digested samples))×100.

3.2.3 Quantification of Primary Amines (see Note 16): Option 1 (see Note 17)

1. Add 5 µl of 1 M NaOH to each vial of fully digested cross-linked beads solution (see Subheading 3.1, step 2) to increase the pH.
2. Verify by pH paper strip that the final pH value is ~8.5.
3. Add TNBS to each sample to a final concentration of 0.25% w/v.
4. Incubate vials at 37°C for 2 h on a rocker (see Note 18).
5. Determine A_{335} values of the resulting colored reaction product with a plate reader.
6. Normalize values per milligram protein in each sample as determined previously (see step 8 of Subheading 3.2).

3.2.4 Quantification of Primary Amines: Option 2 (see Note 19)

1. Weigh out uncross-linked (see Subheading 3.1, step 1) and cross-linked beads (see Subheading 3.1, step 2).
2. Suspend in PBS, pH 7.4 to a concentration of ~20 mg/ml.
3. Add 50 µl of collagenase/dispase digestion mix to 0.5 ml bead suspension.
4. Vortex samples.
5. Incubate for overnight at 37°C on a rocker.
6. Add 5 µl of 1 M NaOH to each vial of beads solution to increase the pH.
7. Verify by pH paper strip that the final pH value is ~8.5.
8. Add TNBS to each sample to a final concentration of 0.25% w/v.
9. Incubate vials at 37°C for 2 h on a rocker.
10. Determine A_{335} values of the resulting colored reaction product with a plate reader.
11. Determine percent of primary amines in the cross-linked samples according to the formula: (A_{335} for cross-linked sample/A_{335} reference sample)×100.

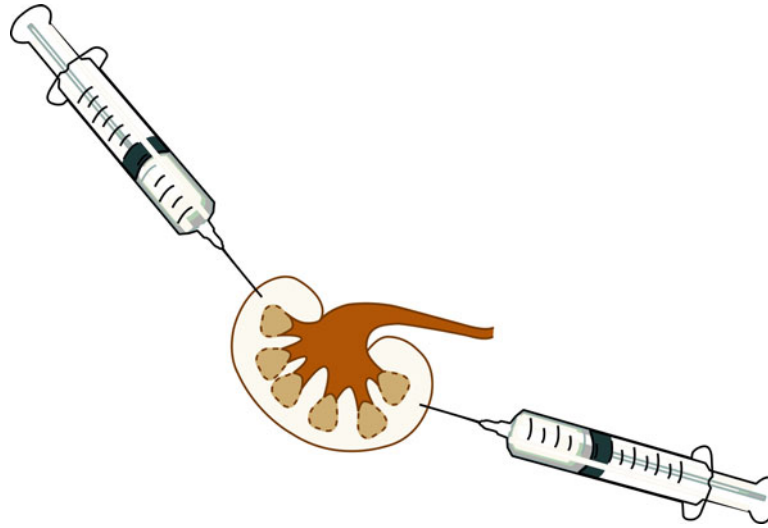

Fig. 3 Illustration of the construct microinjection technique

3.3 Gelatin Microsphere Cytocompatibility Evaluation (see Note 20)

1. Seed kidney cells onto cross-linked beads at a density of 7.15×10^6 cells/100 μl of beads (packed volume).
2. Incubate overnight at 37°C/5% CO_2 with 10 ml basal medium under dynamic conditions (see Note 21).
3. Assess cell viability 24 h later by using a LIVE/DEAD® viability/cytotoxicity kit (see Note 22).
4. Cell laden beads can be easily visualized with bright field and fluorescent microscopes.

3.4 Gelatin Microsphere Biocompatibility

3.4.1 Construct Preparation

1. Sterilize sized beads in 70% ethanol solution for 24 h.
2. Wash three times with sterile PBS.
3. Wash beads one time with sterile basal medium.
4. Seed renal cells at a density of 71.5×10^6 cells/ml packed beads under dynamic conditions (syringe placed on rotating device at 1 rpm, spinner flasks, etc.) in kidney growth medium.

3.4.2 Construct Microinjection (Fig. 3)

1. Expose kidney through midline incision.
2. Inject constructs from each pole into the cortex.
3. Suture incision and allow animal to recover.
4. Sacrifice at required time points and collect kidney for histological evaluation (i.e., hematoxylin–eosin (H&E), trichrome staining, and macrophage phenotyping (M1/M2)) (see Note 23).

4 Notes

1. By decreasing or increasing the distance between the TLC sprayer and the metal tray, the size distribution of the sprayed beads can be varied. Also, various types of TLC reagent sprayers are available, either standalone or bottle-type (Fig. 1)—either one is suitable for this application as long as the nozzle can be positioned perpendicularly to the tray and there is an uninterrupted flow of gelatin solution during spraying.
2. If the sprayer clogs with solidified gelatin, disconnect from air source, rinse rapidly with hot water, verify flow with hot water, reconnect, and continue procedure.
3. This temperature will prevent beads from melting and sticking together.
4. When adding the buffer to the dry beads account for the cross-linking agent solution volume that needs to be added so that the final cross-linking volume is 20 ml (i.e., if the cross-linking stock solution is 1 M EDC and the desired cross-linking concentration is 100 mM, then presoak beads in 18 ml prechilled buffer and subsequently add in 2 ml of the stock cross-linking solution. The final solution volume will be 20 ml of 100 mM EDC per gram of beads).
5. While optional, this bead sizing step narrows the diameter range of the beads and is recommended to facilitate injection and analysis of the in vivo data.
6. Gelatin beads tend to clump together but having them in 70% ethanol solution will diminish clumping.
7. Identify the desired bead size range for a specific application and select nylon meshes accordingly, i.e., 250 μm—for the upper limit and 100 μm for the lower limit. Keep in mind that sizing will decrease the final bead yield (i.e., if 1 g of beads is used for sizing, after this process the bead mass will be less than 1 g, in certain cases significantly less than 1 g depending on the initial size distribution of the beads).
8. The larger size beads that are retained after the filtration steps can be discarded or collected for different applications, as desired.
9. The beads that passed though the mesh pores (≤100 μm in diameter) can be discarded or collected for different applications as desired.
10. The size (before or after sizing) and morphology of the gelatin beads can be visualized in detail by scanning electron microscope (SEM).

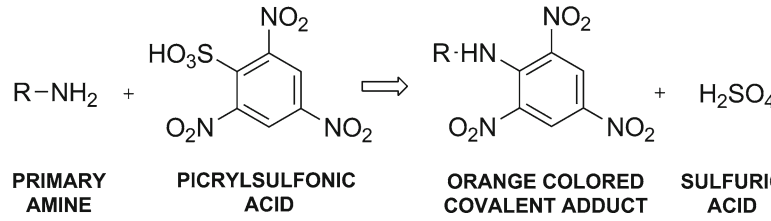

Fig. 4 Reaction scheme illustrating the formation of a covalent adduct between available primary amine functionalities and TNBS

11. Other sputter-coating metals, compatible with SEM, can be used.
12. This assay is highly informative, especially if the production of differentially cross-linked beads was sought (i.e., a range of EDC solution concentrations were used for cross-linking).
13. This assay has a dual function: (a) confirms that cross-linking was successful and (b) differentiates between dissimilar degrees of cross-linking.
14. For gelatin-based biomaterials the Bradford assay needs to be modified, specifically the ratio of the Bradford reagent to protein solution needs to be 1:9 v/v in order to increase sensitivity.
15. The amounts of protein in the samples can be calculated from a gelatin standard curve obtained by plotting the A_{595} values for solutions made from known amounts of gelatin that were fully digested then assayed with Bradford reagent.
16. The EDC cross-linking reaction involves the formation of a covalent bond between adjacent inter- and intramolecular primary amine and carboxyl functionalities. Accordingly, increasing the extent of cross-linking will result in a decrease of free primary amines on the beads. Therefore by determining the number of free primary amines present on the beads, the extent of cross-linking can be determined (Fig. 4).
17. This amine quantification method should be used when analyzing differentially cross-linked beads.
18. Solutions will turn yellow with color intensities inversely proportional to the degree of cross-linking.
19. This amine quantification method should be used to generally detect cross-linking and the percentage of free primary amines in the cross-linked sample compared to the reference (uncross-linked) sample.
20. Because of the three-dimensional nature of the microspheres cell seeding onto the beads should be carried out under dynamic conditions.

21. For incubation under dynamic conditions a syringe placed on rotating device at 1 rpm or a spinner flask can be used.
22. LIVE/DEAD mammalian cell viability kits comprise of a dual calcein AM/ethidium homodimer mix that stains live cells green (calcein AM gets cleaved by the esterases of live cells to yield fluorescent calcein) and dead cells red (ethidium homodimer diffuses through the membranes of dead cells and upon binding to nucleic acids fluoresces red).
23. The histological analysis of the injection sites will provide information on the biocompatibility of the construct and its resorption/tissue integration rate. The H&E staining makes the gelatin beads easily detectable by staining them purple. Additional macrophage phenotyping helps estimate the inflammatory (M1 phenotype) or remodeling/regeneration (M2) phenotypes that are induced by the construct.

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