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Cross-linked collagen gels using gold nanoparticles

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Abstract

Crosslinking collagen results in the formation of a collagen matrix that is one of the most widely used biomaterials for applications in drug delivery, tissue engineering, molecular scaffolds, and materials for wound dressing. The properties of the collagen matrix depend on the crosslinking strategy employed. Here we describe the preparation and characterization of a non-fibrillar collagen matrix stabilized by crosslinking with tiopronin-protected, 3–4 nm polyvalent gold nanoparticles.

Keywords

Collagen; non-fibrillar collagen; gold nanoparticles; polyvalent crosslinking agents; collagen matrix

1. Introduction

Collagen is one of the most common biomaterials used for biomedical applications (1,2). The medicinal applications of collagen originate from its ease of extraction, purification, and processing (3). Decomposition of the matrix and its porosity strongly depend on how the matrix is prepared (4). Crosslinking is the most widely used stabilization method to improve gel longevity and allows the decomposition time to be varied between 24 hours to 6 months (5). The majority of crosslinking agents are linear and their use results in a similar morphology of the gels regardless of the crosslinking agent. Gold nanoparticles are polyvalent crosslinking agents and are able to form, on average, 8 bonds per crystal within the matrix (6); the other polyvalent crosslinking agents used are dendrimers (7). Modifying collagen matrices with polyvalent crosslinkers result in biocompatible matrices with improved thermal and enzymatic stability (6–8).

Additionally, the method of collagen preparation before crosslinking is performed and before gel formation will influence the morphology of the crosslinked collagen matrix. To achieve a homogeneous matrix containing small pores suitable for drug delivery applications and a small size dispersion of the pores, the entire fibrillar component of the collagen has to be removed and only molecular collagen can be used for matrix preparation (9).

The synthesis of a collagen matrix crosslinked with gold nanoparticles has three parts: (1) preparation of a molecular collagen solution via extraction and purification of collagen type I from rat tail tendons, (2) synthesis of tiopronin protected gold nanoparticles (2–3 nm), and (3) crosslinking collagen type I with gold nanoparticles via formation of covalent bonds between the carboxyl group of tiopronin and primary amine groups from collagen lysine residues. The general scheme of the process is outlined in Scheme 1.

2. Materials

All solutions should be prepared with ultrapure water, referred to here as D.I. water (prepared by feeding deionized water into a Milli-Q system to attain a resistivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Follow all waste disposal regulations when disposing of waste materials.

2.1. Monomeric collagen type I

1. 3 rat tails
2. Blade
3. Refrigerated centrifuge
4. 0.01 M HCl (500 mL)
5. 3% (v/v) Acetic acid (1 L)
6. 3.5 kDa cut-off dialysis bags or tubing
7. Millipore filtration system (glass vacuum filter holders) with 20, 5, and 0.65 µm nitrocellulose filter disks
8. Solid NaCl (50 g)
9. Phosphate buffer: 0.02 M phosphate buffer, pH 7.8, prepared by mixing 1.020 g of anhydrous potassium phosphate monobasic and 24.545 g of sodium phosphate dibasic heptahydrate, make up to 5 L with water, store at 4 °C.
10. 5–10 50 mL Falcon tubes
11. 1 L beaker
12. Acetone (1 L)
13. Dry ice (200 g)
14. Lyophilizer
15. Parafilm
16. Mini Protean System for protein gels
17. SDS PAGE precast gel (4–20% polyacrylamide)
18. Low range SDS PAGE MW standard stained with Coomassie R-250

2.2. Synthesis of tiopronin-gold nanoparticles

1. Tetrachloroauric acid (500 mg)
2. N-(2-mercaptopropionyl) glycine (1 g)
3. Fresh NaBH₄ (1 g)
4. Methanol (100 mL)
5. Acetic acid (100 mL)
6. 50 mL of 6:1 (v/v) methanol/acetic acid mixture
7. Concentrated HCl
8. Stir plate
9. 100 mL round bottom flask
10. rotary evaporator
11. 3.5 kDa cut-off dialysis bags or tubing
12. TEM
13. Formvar coated copper TEM grids

2.3. Cross-linking collagen type I

1. 3 mL glass vials
2. 1.5 mL microcentrifuge tubes
3. Fresh EDC (1-(3-dimethyl-aminopropyl)-3-ethyl carbodiimide) (1 g)

2.4. TNBS assay to quantify cross-linking

1. 0.1 M sodium bicarbonate pH 8.5 (10 mL)
2. 0.1% TNBS: 5 mL 0.1% w/v 2,4,6-trinitrobenzene sulfonic acid in 0.1 M sodium bicarbonate pH 8.5, 10 mL.
3. 12 M HCl (10 mL)
4. Plastic cuvette
5. Spectrophotometer

2.5. TEM of cross-linked collagen matrix Araldite 502 resin (20 g) (Ted Pella)

1. 70% ethanol (50 mL)
2. 95% ethanol (50 mL)
3. 100% ethanol (50 mL)
4. Pasteur pipettes
5. DDSA Dodecenyl succinic anhydride (DDSA) (20 g)

6. 2,4,6-tris(dimethylaminomethyl)phenol (DPM-30) (1 g)
7. Propylene oxide (50 mL)
8. Ultratome
9. TEM
10. Formvar-coated copper TEM grids

3. Methods

3.1. Monomeric collagen type I

3.1.1 Extraction

1. Extract tendon fibers from a rat tail with a blade (*see Note 1*).
2. Dissolve 1 g of rat tail tendon in 100 mL of 0.01 M HCl and stir for 4 hours at room temperature.

3.1.2 Purification

1. Centrifuge the solubilized tendon for 30 min at 10,000 rpm ($14,000 \times g$) and 4°C . Discard the pellet and filter the supernatant sequentially through 20, 5, and $0.65 \mu\text{m}$ filters using the Millipore system (*see Note 2*).
2. Obtain a salt concentration of 0.7 M by adding the appropriate amount of NaCl to the filtrate and stir for 1 hour.
3. Centrifuge the mixture for 1 hour at 10,000 rpm ($14,000 \times g$) and 4°C . Collect the pellet.
4. Dissolve the pellet in 100 mL of 0.01 M HCl overnight.
5. Dialyze the sample against phosphate buffer for at least 8 hours at room temperature.
6. Perform another dialysis against fresh phosphate buffer (1L) for at least 4 hours at 4°C .
7. Centrifuge the dialysate for 1 hour at 10,000 rpm ($14,000 \times g$) at 4°C . Perform dialysis on the pellet against 3% acetic acid overnight.
8. Perform another dialysis on the pellet against D.I. water for 24 to 48 hours, changing the water every 8 hours, at 4°C .
9. The collagen solution may be stored at 4°C .

3.1.3 Lyophilization

1. Pour approximately 5 mL of collagen solution into 50 mL Falcon tubes. Prepare at most 5 tubes for each run.
2. Pour acetone onto dry ice in a 1 L beaker and place the tubes in there for about 20–30 minutes to completely freeze the collagen solutions.

3. Remove tubes and dry thoroughly. Unscrew the caps and replace with parafilm. Punch tiny holes into the parafilm to allow for suction and to prevent sample loss during lyophilization.
4. Place the tubes in the lyophilizer jar and leave in the lyophilizer for 2 nights.
5. Store lyophilized collagen in the freezer (-20°C).
6. To confirm purity, dissolve collagen in 0.01 M HCl (overnight) and perform standard SDS-PAGE analysis (Figure 1).

3.2. Synthesis of tiopronin-gold nanoparticles (Au/Tp; size 2.4 nm)

This protocols follows a synthesis originally published by Brust et al. (10). The ratio of tetrachloroauric acid to N-(2-mercaptopropionyl) glycine determines the size of the nanoparticles (*see* Note 3). The protocol below yields 2.4 nm nanoparticles. Perform all steps at room temperature.

1. Co-dissolve tetrachloroauric acid (0.31g, 0.80 mmol) and N-(2-mercaptopropionyl) glycine (0.39 g, 2.4 mmol) in 35 mL of 6:1 methanol:acetic acid.
2. Dissolve NaBH_4 (0.6 g, 16 mmol) in 15 mL of water.
3. Add NaBH_4 dropwise to tetrachloroauric acid with rapid stirring and leave stirring for 15 min (*see* Note 4).
4. Stir for an additional 30 min and remove the solvent under vacuum (with rotary evaporator) without exceeding 40°C (*see* Note 5).
5. Purify the black, crude tiopronin-protected gold nanoparticles by dialysis. Dissolve 150 mg of crude product in 75 mL of water. Adjust the pH of the solution to 1.0 by adding drop-wise concentrated HCl. Load this solution to 8 inch segments of dialysis tubing (3.5 kDa cut off) and place in a 10 L beaker of water with slow and continuous stirring for 72 h, changing the water every 8 h.
6. Collect the black tiopronin-protected gold nanoparticles in a round bottom flask and remove the solvent under vacuum with rotary evaporator without exceeding 40°C . Place the damp nanoparticles under vacuum for 24 h to completely remove any remaining water.
7. The size dispersion of the nanoparticles can be confirmed under TEM (Figure 2). (See section 3.5 for a detailed TEM protocol.)

3.3. Cross-linking collagen type I

This protocol describes preparation of a molecular collagen matrix (0.4 % wt/vol) crosslinked with gold nanoparticles protected by tiopronin. The crosslinking is achieved by amide bond formation between collagen primary amines (lysine; ~3% amino acid content) and the carboxyl group of tiopronin (Scheme 1). This protocol yields 1.5 mL of matrix for 3:1 collagen to nanoparticle weight ratio (*see* Note 6).

1. Make a 1% collagen stock solution in water by weighing out 0.2000 g of freeze-dried collagen and tearing it up into small pieces and then adding to 20 mL of water in a 50 mL Falcon tube. Vortex the solution and then let it dissolve overnight in the refrigerator at 4°C.
2. Prepare a 30 mg/mL nanoparticle (Au/Tp) stock solution by weighing out 0.0600 g of nanoparticles and adding to 2 mL of water in a small vial. Vortex or sonicate until completely dissolved.
3. Pipette 40 µL of Au/Tp stock and 860 µL of water (for 3:1 collagen:Au/Tp ratio; for other ratios *see* Note 6) into a 1.5 mL microcentrifuge tube.
4. Add 30 mg of EDC to each sample (*see* Note 7).
5. Add 600 µL of the collagen stock solution to each sample (*see* Note 8).
6. Vortex the mixture in the tube to mix thoroughly (*see* Note 9).
7. Leave it overnight to fully crosslink and deactivate the EDC (*see* Note 10).

3.4 TNBS assay to quantify cross-linking

The degree of crosslinking can be measured as the loss of free ε-amino groups of lysine upon the addition of Au/Tp nanoparticles. The number of free amino groups can be determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS), as originally described by Cheung et al. and Sheu et al. (11,12)

10. Place 100 µL of collagen treated with different amounts of Au/Tp nanoparticles in a microcentrifuge tube.
11. Add 400 µL of 0.1 M sodium bicarbonate solution and 250 µL of 0.1% TNBS.
12. Mixed well and place in a water bath at 40 °C for 2 h.
13. Add 300 µL of 12 M HCl and raise the temperature to 60 °C. Solubilization of the collagen will be complete after 60 min.
14. Transfer the samples to the cuvette, place it in the spectrophotometer and read absorbance at 425 nm (*see* Note 11).
15. Calculate the degree of cross-linking as a percent loss of free ε-amino groups: $(1 - (Abs_{Au}/Abs_{EDC}))100\%$, where Abs_{Au} represents the Absorbance of the Au/Tp crosslinked collagen and Abs_{EDC} represents the intensity of the collagen treated with only EDC.

3.5 TEM of cross-linked collagen matrix

16. Take the gel sample and lyophilize it.
17. Use a standard protocol for TEM tissue imaging with araldite resin. Dehydrate the sample by progressively increasing the concentration of ethanol (70, 95, 100%), using a Pasteur pipette to add and remove the ethanol solutions to and from the sample. Keep the sample for 20 min in each solution.

18. Thoroughly mix 7.8 g of DDSA with 10.0 g Araldite 502 and 0.27 g DPM-30 and wait for at least 20 minutes.
19. Place the sample in propylene oxide for 1 hour.
20. Place the sample in the mold to be infused with the resin. Add a 1:1 mixture of the resin solution and propylene oxide to fully immerse the sample.
21. Wait until the sample sinks and place it at 60°C for 24–48 h.
22. Cool the sample to room temperature and cut it with Ultratome to 60–80 nm sections before imaging under TEM (Figure 3) (*see* Note 12).

4. Notes

1. Cutting along the entire length gives the best results. One tail should provide 300 to 500 mg of crude material. If performed correctly this protocol results in 30% yield of pure monomeric collagen. The lower yields can occur if the subsequent filtration step is not performed correctly.
2. This step may require several Millipore disk filters due to clogging. Do not skip the larger pore filters, otherwise this step will take many hours and several filter disks to complete. Also make sure the vacuum is at least 100 torr to ensure speedy filtration.
3. The 3:1 ratio of tiopronin to tetrachloroauric acid (2.4 to 0.80 mmol) results in 2.4 nm nanoparticles; the ratio 1:12 (0.1 to 1.2 mmol) results in 3.8 nm nanoparticles.
4. The rapid stirring ensures a small size dispersion in the final solution. The temperature and the pH of the reaction mixture will increase and the color of the solution will turn to black. After 15 min of stirring to complete the reaction, the temperature will return to room temperature.
5. When removing the solvent do not exceed 40°C. Higher temperature can result in partial removal of the protective monolayer of tiopronin and coalescence of gold nanoparticles. If this happens the flask will be covered with a gold mirror.
6. The ratio of collagen to nanoparticle can be adjusted by varying the dilution factor of nanoparticle solution while keeping the final collagen concentration 0.4%. See table below:

Ratio (wt) Coll : Au/Tp	30 mg/mL stock Au/Tp (μL)	water (μL)	EDC (mg)	1% stock collagen (μL)
3:1	40	860	30	600
2:1	60	840	30	600
1:1	120	780	30	600
1:2	240	660	30	600
1:3	360	540	30	600

7. 30 mg of EDC in 1.5 mL corresponds to 1.5×10^{-4} M, giving 10 times excess with respect to carboxyl groups to be activated when the highest concentration of nanoparticles is used for collagen cross-linking.
8. The stock solution is very viscous and hard to pipette accurately, so it is more consistent to use the tube's 1.5mL mark as reference to reach volume.
9. Large purple precipitates will appear. The sample can be left in the tube or placed in the dish that will be used for further tests.
10. The gels have a consistency of semi-set gelatin, VERY fragile and easily broken. If one needs to prepare a matrix that is similar to hydrogels, increase the concentration of the collagen solution to 1.5–2.5% (wt/vol).
11. The reference cuvette should contain the same amount of Au/Tp nanoparticles to avoid scattering losses.
12. This protocol (collagen:Au/Tp = 3:1) results in gel a sample that exhibits the nanoparticles' organization within the gel in island-like structures. With a higher content of gold nanoparticles one should expect chain-like features in the TEM. When a higher concentration of collagen is present in a collagen gel (above 0.5%) the TEM samples result in "shadow areas", where the collagen matrix is present, where nanoparticles are localized.

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References

- (1). Nimni ME, Cheung D, Strates B et al. (1987) Chemically modified collagen: a natural biomaterial for tissue replacement. *J Biomed Mater Res* 21: 741–771 [PubMed: 3036880]
- (2). Ruszczak Z (2003) Effect of collagen matrices on dermal wound healing. *Adv Drug Delivery Rev* 55:1595–1611
- (3). Ruszczak Z, Friess W (2003) Collagen as a carrier for on-site delivery of antibacterial drugs. *Adv Drug Delivery Rev* 55: 1679–1698
- (4). Rault I, Frei V, Herbage D et al. (1996) Evaluation of different chemical methods for crosslinking collagen gel, films and sponges. *J Mater Sci: Mater Med* 7: 215–221
- (5). Barbucci R (ed) (2002) *Integrated Biomaterials Science*. Kulwer Academic/Plenum, New York
- (6). Castaneda L, Valle J, Yang N et al. (2008) Crosslinking Collagen with gold nanocrystals. *Biomacromolecules* 9: 3383–3388 [PubMed: 18959440]
- (7). Duan X, Sheardown H (2005) Crosslinking of collagen with dendrimers. *J Biomed Mater Res, Part A* 75: 510–518
- (8). Castaneda L, Pluskat S, Ky D et al. (2007) Novel Collagen Based Material for Local Drug Delivery Systems In: Naik RR, Perry CC, Shiba K, Ulijn R (eds) *The Nature of Design—Utilizing Biology's Portfolio*, Mater Res. Soc. Symp. Proc. 1008E, Warrendale, PA, p. T09–08.
- (9). Schuetz T, Richmond N, Harmon ME et al. (2013) The microstructure of collagen type I gel cross-linked with gold nanoparticles. *Colloids and Surfaces B: Biointerfaces* 101: 118–125 [PubMed: 22796781]
- (10). Brust M, Walker M, Bethell D et al. (1994) Synthesis of thiol-derivatised gold nanoparticles in a two-phase liquid–liquid system. *J Chem Soc, Chem Commun* 801–802

- (11). Cheung DT, Perelman N, Ko EC et al. (1985) .Mechanism of crosslinking of proteins by glutaraldehyde III: Reaction with collagen in tissues. *Connect Tissue Res* 13: 109–115 [PubMed: 3157539]
- (12). Sheu MT, Huang JC, Yeh GC et al. (2001) Characterization of collagen gel solutions and collagen matrices for cell culture. *Biomaterials* 22: 1713–1719 [PubMed: 11396874]

Figure 1.
SDS PAGE of pure collagen extract. The $\alpha 1$ band is half the size of the $\alpha 2$ band, reflecting a 1:2 stoichiometry of the collagen triple helix.

Figure 2.
TEM image of tiopronin-protected gold nanoparticles.

Figure 3. TEM images of lyophilized collagen gels preserved in Araldite resin. (A) Collagen: Au/Tp = 3:1 and (B) Collagen: Au/Tp = 1:3

Scheme 1.

Synthesis of Au nanoparticles, and cross-linking of a collagen gel. Reproduced from Castaneda *et al.* 2008 with permission from American Chemical Society.