Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications

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Published online 18 January 2007; doi:10.1038/nprot.2006.430

Collagen is a widely investigated extracellular matrix material with extensive potentials in the field of tissue engineering. This protocol describes a method to prepare reconstituted collagen that can be ready-to-use, storable and suitable for further *in vitro* and *in vivo* investigations. Type I collagen was extracted from rat tail tendons and processed in acetic acid solution to obtain sterile soluble collagen. At first, crude collagen was dissolved in acetic acid, then frozen at −20 °C and lyophilized to obtain a sponge, which could be stored at −80 °C. Lyophilized collagen was then dispersed in acetic acid to obtain a sterile solution of collagen at targeted concentrations. The whole low-cost process from the extraction to the final sterile solution takes around 2–3 weeks. The collagen solution, once neutralized, has the potential to be used to produce gels or scaffolds, to deposit thin films on supports and to develop drug delivery systems.

INTRODUCTION

Collagen is the major component of the extracellular matrix and largely present in the mammalian body. As collagen can be extracted in large quantity, it has been widely studied as a natural material in the field of tissue engineering[1], wound dressing[2] and drug delivery[3]. It is a structural protein and its main function is to provide mechanical integrity to different tissues and organs such as tendon, bone, etc. In blood vessels, the structure of which is very peculiar and complex from a mechanical point of view, collagen plays a fundamental constitutive role. Therefore, collagen is largely investigated as a possible candidate for the design of scaffold able to mimic the native structure of vessels. Different techniques have been proposed to obtain a solution of collagen, typically in acidic environment[4–6] or in neutral salt solutions[7,8]. However, the method described here was developed with the aim to obtain a ready-to-use, easily storable collagen, which can then be successfully used in any application requiring type I collagen. In this laboratory, it was used as scaffold for vascular tissue engineering. The main advantage of this protocol is its division into two steps; at the end of the first step, it is possible to obtain a long-term storable collagen sponge, ready to be transformed to a sterile solution when needed. Besides, it is a cost-effective process and each step of the process is well monitored, which makes it easier to minimize errors. Another advantage is the possibility of producing in amounts as required, avoiding wastage of the product owing to expiration of the sterility, for example. Briefly, it consists of isolating tendons from rat tails, extracting collagen from tendons and dissolving the collagen fibers in acetic acid. This gel-like substance is then mixed in a house-ware blender, frozen and lyophilized. At this stage, the resulting product is a dry collagen sponge that may be preserved at −80 °C for 12–18 months. For further use, the lyophilized sponge is then mixed in a blender with acetic acid. After centrifugation and degassing, the solution is sterilized by a dialysis technique[9]. Biological performances of scaffolds derived from processed collagen, following this method have been investigated and showed promising results[10]. This method involves lyophilization for improved long-term storage and reproducibility of subsequent collagen solutions. This method of extraction of collagen can probably be extended to other sources of tendons, for example, bovine, porcine or avian, for a better yield. However, it is necessary to set up an automated extraction method, given the dimensions of the other animals. We have specialized in extraction from rat tail tendons. When compared with other methods[6,11], it takes longer time to prepare, but reproducibility of the concentration of the collagen solution can be guaranteed. The other methods usually require measuring the concentration of collagen each time the solution is prepared and eventually diluting for certain applications. Moreover, in the latter case, the solution may be over-diluted for specific applications, thus implying the difficulty to re-concentrate it, which might be time consuming.

Rat tail tendon collagen has been extensively used to study mesenchymal-derived cells in 3-D scaffolds such as gel. Esdale and Bard[12] were pioneers reporting a method to produce a cell-embedded collagen gel. Moreover, fibroblast-populated collagen gels exhibit gel contraction probably through remodeling of collagen fibrils by cell traction[13]. These primary investigations led to further developments toward the first tissue engineered substitutes, such as a blood vessel[14]. Since then, type I collagen gel has been a major reconstituted extracellular matrix to grow cells in 3-D culture systems and to investigate cell differentiation and maturation mimicking *in vivo* environment.

MATERIALS

REAGENTS

- Tails from rat stored at −80 °C. Mature rats (8–9 weeks old) would be preferable ! CAUTION Animal experiments must comply with national regulations.
- Surgical clamps
- Forceps and scissors
- Surgical mask (optional)
- Clean bench (about 1 m wide and 2 m long)
- Ultrapure water
- Phosphate-buffered saline (PBS; Sigma, cat. no. P3813)
- Pure acetone (99%; Laboratoire Mat Inc., cat. no. AP0102)
- Isopropyl alcohol (HPLC grade, 99.9%; Fisher Scientific, cat. no. AC610080040)
- 0.02 N acetic acid (glacial acetic acid, HPLC grade, 99%; Fisher Scientific, cat. no. FL070494)
- 0.5 liter of ice flakes derived from ultrapure water
- Chloroform solution (99%; Laboratoire Mat Inc., cat. no. CR 0179)
- pH paper

EQUIPMENT

- House-ware blender with pulsation
- Five rectangular containers for freezing at −20 °C (30 cm × 40 cm, depth around 20 cm)
- Freeze-drier with temperature regulator (Labconco)
- Magnetic stirrer and magnetic spin bars × microbalance × balance (2 kg capacity)
- Plastic bags, such as Ziploc for conserving the sponge
- Ultracentrifuge Sorvall, RC 5B plus, ultraspeed centrifuge (DuPont)
- Rotor Sorvall SLA-1000 (DuPont)
- Four bottles suitable for ultracentrifuge (250 ml, PPCO; Nalgene, cat. no. BT 3141-0250)
- Vacuum pump with temperature regulator for degassing
- Autoclave
- SpectraPor dialysis bag (MWCO:6-8000; Spectrum Laboratories, recorder no. 132660)
- Spectra/Por Closures (sealing width 75 mm; Spectrum Laboratories, recorder no. 132738)
- Refrigerator and freezer
- Laboratory glassware washer
- Three plastic beakers (4 liters each, PP; Nalgene, cat. no.1201-4000)
- Two 1-liter glass beakers, cylinders (from 1 liter and above)
- Flat glass containers (4)
- Four glass bottles (1 liter each; Gibco Laboratories)
- Four plastic bottles (1 gallon each; HDPE, Nalgene)
- Four glass bottles (100 ml each, Gibco Laboratories)
- Biohazard bags for waste products

PROCEDURE

1| Remove the rat tails from the −80 °C freezer a day before the extraction and keep at 4 °C for 24 h to soften for future extraction. Always store in a biohazard bag.

2| Process a maximum of 30 rat tails at a time.

▲ CRITICAL STEP Process a maximum of 30 rat tails at a time, mainly because the volumes of all solutions have been calculated for 30 rat tails. In addition, processing the rat tails requires a certain amount of time and if you process a higher number of tails, there might be deterioration in those remaining at room temperature for too long. Moreover, unexpected effects (mainly on the homogeneity) of the final gels could be observed.

3| Prepare a clean bench on which to work and lay a sterile cloth over it.

4| Prepare a beaker with 1 liter PBS.

5| Prepare a bowl with ultrapure water for rinsing the rat tails.

6| Rinse the rat tails by soaking in water and hand-dry to remove excess water before isolation of tendons.

7| Hold the tail at about 5 mm from its thinner extremity with the help of clean surgical clamps. If you are right-handed, use the left hand to hold and with the other hand twist the tail around until its skin breaks apart. At this point, pull off the skin; bundles of white fibers will be exposed and separated. These are the collagen fibers of the rat tail tendon (see **Fig. 1**).

8| Cut the white bundle of fibers near the thinner extremity and collect in the beaker containing PBS.

▲ CRITICAL STEP Do not throw away the tail after the first bundles have been extracted; you can continue the extraction from the same tail.

9| Repeat Steps 6–8 recursively until the remaining part of the tail is too short or too thick to be manipulated.

10| Repeat Steps 6–9 for all the rat tails.

11| Prepare a beaker with 1 liter of acetone.

12| Prepare a beaker with 1 liter of 70% (vol/vol) isopropanol.

13| Prepare 4 liters of 0.02 N acetic acid.

14| Prepare a large 4-liter plastic beaker with a large spin bar.

15| Once all the fibers from all the rat tails are collected in PBS, transfer them to the beaker with acetone for 5 min.

16| Transfer the fibers to 70% isopropanol for another 5 min.

17| Then collect the fibers in the large plastic beaker and add 500 ml to 1 liter of acetic acid.

18| Keep this beaker well stirred on a magnetic stirrer at 4 °C for at least 48 h.

19| At this point, you will observe a viscous solution.

20| Blend this viscous solution in a house-ware blender along with ice flakes and using the pulse mode to avoid overheating of the collagen (this can lead to the denaturation of collagen), until a homogeneous solution is obtained. There is no need for any particular blade for the blender. The blender should be cleaned in a laboratory glassware washer. Its container should be immediately washed in running water to avoid protein adsorption on its surface. The ice flakes should be prepared from ultrapure water, to avoid any contaminants.
▲ **CRITICAL STEP** It is mandatory to minimize all the possible forms of contamination, as this can compromise the whole process. Avoid using dirty surfaces and dirty instruments. Cleanliness is the key to success.

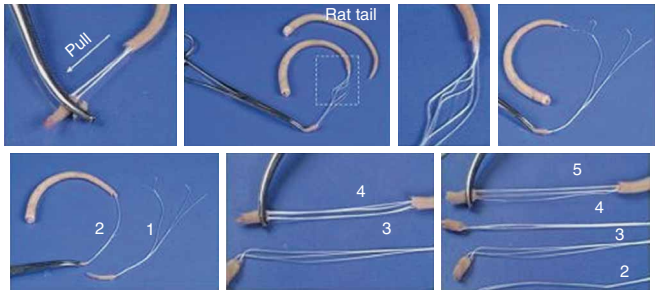

Figure 1 | Sequential images of the procedure to remove the skin and retrieve collagen fiber from a rat tail tendon. The skin of the rat tail is ripped off several times (five in the example given) with a clamp and collagen fibers (white filaments) are exposed as described in Step 7 of the PROCEDURE.

21| Pour the solution in a plastic or metallic container (any shape) to form a liquid film about 5 cm in thickness.

22| Freeze the containers at −20 °C until the solution is completely frozen.
■ **PAUSE POINT** The containers can be kept at −20 °C until the solution is completely frozen. Usually, after 3 days, the solution is well frozen and can be lyophilized.

23| Release the frozen collagen block from the container to facilitate the next step.

24| Cut small blocks of frozen collagen and put in flat glass containers (e.g., diameter 50 cm and height 10 cm). Put the containers in the freeze-drier and lyophilize for 24–48 h or more till all the water has totally disappeared from the collagen.
▲ **CRITICAL STEP** Verify that the temperature is around −20 °C. Make sure the temperature remains constant throughout the whole process of freeze-drying.
▲ **CRITICAL STEP** Use flat or short containers to lyophilize more rapidly. If the collagen blocks are higher than 5 cm, it might even take a week to lyophilize. In this case, it is essential to stop the freeze-drier after 48–72 h. De-ice its ice trap, restart the vacuum pump and then continue the lyophilization process till all the water has disappeared.
■ **PAUSE POINT** The lyophilized collagen, which is in the form of a sponge, can be stored in sealable plastic bags (Ziploc) at −80 °C for 12–18 months.

25| Prepare 8 liters of 0.02 N acetic acid and store at 4 °C for at least 24 h before starting the process.

26| Prepare 4 liters of 0.02 N acetic acid. Sterilize in an autoclave and store at 4 °C for at least 24 h before starting the process.

27| Sterilize a 4-liter beaker with a spin bar at the bottom, cover with aluminum foil and store at 4 °C for at least 24 h before starting the process.

28| Store 4 liters of ultrapure water at 4 °C for at least 24 h before starting the process.

29| Keep the plastic Nalgene bottles for centrifugation at 4 °C for at least 24 h before starting the process.
▲ **CRITICAL STEP** Check the maximum acceleration that these bottles can withstand before plastic deformation or cracking can occur.

30| Keep the container of the blender (house-ware blender) at 4 °C for at least 24 h before starting the process.

31| Keep the appropriate head of the ultracentrifuge at 4 °C for at least 24 h before starting the process.

32| Start cooling the ultracentrifuge to 4 °C at least 2 h before centrifugation.

33| Start cooling the vacuum pump to de-aerate (degassing) the collagen solution at least an hour prior degassing.

34| Verify that all the materials are cold before starting the dissolving process.

35| Weigh the sponge to have the desired final concentration of the collagen solution: to have a final concentration of around 4 g l^{-1}, you should weigh 4 g of collagen sponge and dissolve in 1 liter of 0.02 N acetic acid. Note that the final concentration of the solution will not be exactly 4 g l^{-1}, but a little less owing to the elimination of some impurities during centrifugation. In this case, measuring the concentration the first time could help estimate the standard error in the final concentration of the

solution. Although the concentration of collagen can be measured in different ways, for example, with the BRADFORD assay, Bio-Rad protein assay or the biuretic protein method, the optimal way is to freeze-dry the collagen solution and then calculate the final concentration as a ratio of the weight of the dried powder and the initial volume.

36| Place the sponge in the blender adding an appropriate amount of 0.02 N cold acetic acid.

37| Use pulsatile blending only to avoid overheating of the solution (this can lead to the denaturation of collagen).

38| You will obtain a viscous solution.

39| Pour an equal amount of this solution in plastic Nalgene containers for ultracentrifugation and fill the bottles to the recommended level (for 250 ml bottles, the recommended level is usually between 200 and 250 ml).

40| Weigh two bottles at a time on an appropriate balance and be sure to have the same amount of solution in each.

41| Make sure that the threading of the bottles is not forced and that the bottles are closed properly (note that these bottles may be deformed after several cycles of centrifugation).
▲ **CRITICAL STEP** If the threading is forced the bottles might not be closed as required and collagen might flow out of the container during centrifugation.

42| Place the bottles in the appropriate holes of the ultracentrifuge head in a balanced manner.

43| Read the instructions of the ultracentrifuge.

44| Centrifuge at 4 °C at 15,000 r.p.m. for 45 min. 15,000 r.p.m. in the Rotor Sorvall SLA-1000 is equivalent to 30,000*g* at the bottom of the tube.
▲ **CRITICAL STEP** Check the maximum acceleration that the bottles can tolerate.

45| After centrifugation, collect the supernatant in a beaker and keep the beaker on ice. Normally you should observe deposition of impurities at the bottom of each bottle; typically debris is made of other types of collagen. Be careful not to add this to the supernatant you collect.

46| Pour small quantities (around 100 ml) of this solution into a 1-liter Erlenmeyer flask with side access for a vacuum pump.

47| De-aerate for about 15 min. This step is fundamental, especially if you are planning to use the collagen solution to make thin films.

48| Store the degassed collagen solution in a clean beaker on ice.

49| Repeat Steps 46–48 till you have used the whole solution.

50| Now it is time to sterilize the solution. Prepare a 1-liter beaker with ultrapure water and a 4-liter beaker 0.02 N with cold acetic acid (non-sterile) with a spin bar in it.

51| Cut a 12-cm strip of SPECTRAPOR dialysis bags.

52| Wash the outside of the strip in ultrapure water twice to rinse away any impurities present on the bags.

53| Make two solid knots at one end of the strip and rinse the inside of the strip twice with ultrapure water.

54| Pour the collagen solution stored on ice (Step 50) to 80% of the length of the strip and fix it with an appropriate clamp.

55| Place the filled bag in the 4-liter acetic acid beaker.

56| Repeat Steps 53–57 until you have used the whole solution.

57| Place the 4-liter beaker, now containing all the dialysis bags filled with collagen, on a magnetic stirrer and keep stirring gently for 1 h in a cold room at 4 °C.

58| In the meantime, prepare a solution of 1% chloroform in 4 liters of cold ultrapure water (40 ml chloroform in 4 liters of ultrapure water) in a 4-liter beaker with a spin bar at the bottom.
! CAUTION Concentrated chloroform can lead to nausea and dizziness while breathing. It is a strong anesthetic; so be careful not to breathe over the solution and protect yourself; manipulate with a surgical mask and handle with gloves in a fume hood.

59| After 1 h, transfer the dialysis bags from the acetic acid beaker to the chloroform beaker with the help of surgical clamps.

60| Place the 4-liter beaker, containing of chloroform and the dialysis bags, on a magnetic stirrer and keep stirring for 1 h in a cold room at 4 °C.

61| In the meantime, open the sterile 4-liter beaker (see Step 27) in a sterile safety cabinet and pour cold and sterile acetic acid (see Step 26) into it while keeping the aluminum foil sterile.

62| After Step 60 is completed, transfer the beaker to the safety cabinet and shift the dialysis bags to the 4-liter sterile beaker containing sterile acetic acid and cover with the sterile aluminum foil.

63| Discard the chloroform solution in an appropriate chemical waste container (chlorinated waste products).
! CAUTION Be careful not to breathe the fumes coming out of the container. Wear a mask and use gloves while discarding. Use a funnel in order to avoid leaking of this toxic material.

64| Place the 4-liter beaker, now containing sterile acetic acid and the dialysis bags, on a magnetic stirrer and keep stirring for 2 days in a cold room at 4 °C.

65| Change the acetic acid every 2 days as per Steps 61, 62 and 64. Sterilize a 4-liter beaker as in Step 27 with a spin bar at the bottom and an aluminum foil and also sterilize 4 liters of acetic acid. You can prepare these during the break day between one change and another. Store the sterile beaker and the sterile acetic acid in a cold room.
▲ CRITICAL STEP It is important to keep everything cold to avoid degradation of collagen.

66| In the meantime, sterilize 100-ml glass bottles and 1-liter beakers in which you will store the sterile collagen solution. Also sterilize surgical clamps and scissors.

67| After 1 week of sterilization in acetic acid, open a sterile 1-liter beaker and the surgical instruments in the safety cabinet.

68| Move the 4-liter beaker containing the dialysis bags to the safety cabinet.

69| Take out one dialysis bag at a time and move the bag to the 1-liter sterile beaker with the help of a surgical clamp. Hold the bag at the extremity with the closure clamp and cut open the knot. The sterile solution of collagen will flow into the beaker.

70| Repeat Step 69 for all the bags.

71| With the use of a 25-ml pipette and a pipette pump, prepare aliquots in sterile 100-ml bottles for storage (it is advisable to prepare 100 ml aliquots and not larger aliquots, to avoid wastage in case of contamination). Always store the bottles at 4 °C .

72| Check whether the resulting product is effectively collagen and also check its purity if needed, for example with amino-acid analyses[15]. Collagen can be further characterized by sodium dodecyl sulfate–PAGE electrophoresis after pepsin digestion to look for the α, β and γ bands of type I collagen and compared to a known specimen. Transmission electron microscopy and atomic force microscopy can be used to observe the characteristic periodicity of collagen fibrils and their proportion compared to denatured collagen that loses its periodicity.
▲ CRITICAL STEP It is advisable to store these bottles of collagen for no more than 6–8 months.

73| To neutralize the collagen solution, prepare 0.1 N solution of NaOH in water. Filter this solution to obtain a sterile solution and add small quantities (25 µl) to the sterile collagen solution in a safety cabinet, by means of sterilized microtips, and bring the pH to around 6.5–7. This pH along with the temperature of 37 °C leads to jellification of collagen and the self-assembly of collagen fibrils. To test the pH, take a small aliquot of the solution with a sterile microtip and check the pH with pH papers. In this process, it is fundamental to keep everything sterile.

74| Test the sterility of the solution by storing three Petri dishes containing neutralized collagen solution in a cell culture incubator for 1 week. Gels are incubated in the presence of sterile culture medium. As observed under a microscope, no bacterial growth (cloudy medium) or fungal formation (mold and spreading/branching particles) indicates that the sterilization process was effective.
▲ CRITICAL STEP In case the collagen gel is not sterile, it might lead to contamination of other experiments in the same incubator. Plan the collagen preparation process, for example, by using a separate incubator for sterility testing only.

● TIMING
Preparation of the setup: 20–30 min
Steps 6–10: 5 h considering only one person's work and extraction of around 30 rat tails
Steps 11–18: 30 min

Step 18: 48 h
Step 21: 15 min
Step 22: 3 days
Step 24: 2 days or more (at this stage, the resulting sponge can be preserved for 12–18 months at −80 °C)
Steps 34–49: 2 h
Steps 50–51: 0.5 h
Steps 57 and 60: 2 h
Steps 53, 64, 65: 2 days, to be repeated three times in 1 week, so a total of 6 days
Steps 67–70: 1 h

? TROUBLESHOOTING

This procedure is straightforward and unlikely to cause problems to the user. However, it is necessary to pay attention to the CRITICAL STEPS and verify the sterility of the product.

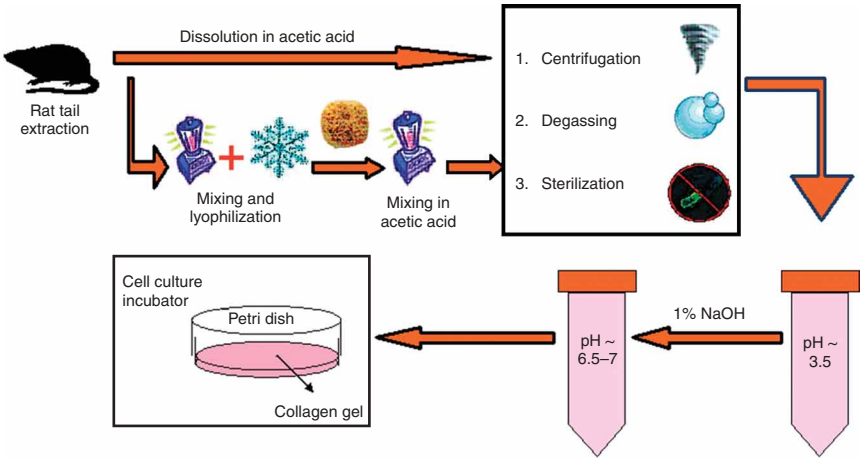

Figure 2 | Schematic representation of the process from extraction to cell culture and through sterilization.

ANTICIPATED RESULTS

Collagen solution in acetic acid at the targeted concentrations will be produced by the protocol described above. A schematic overview of the whole process is presented in **Figure 2**. The appearance of the final sterile solution stored properly in cold room at 4 °C gives the idea of a viscous and grayish solution. The resulting solution can be used for different applications—from tissue engineering in many areas such as skin, cartilage, bone, etc., to drug delivery or substrate for cell growth[16].

ACKNOWLEDGMENTS This work was partially supported by the Natural Science and Engineering Research Council of Canada, the Canadian Institute for Health Research and the Quebec City University Hospital Research Center.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com

1. Berglund, J.D., Mohseni, M.M., Nerem, R.M. & Sambanis, A. A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials* **24**, 1241–1254 (2003).
2. Purna, S.K. & Babu, M. Collagen based dressings––a review. *Burns* **26**, 54–62 (2000).
3. Wallace, D.G. & Rosenblatt, J. Collagen gel systems for sustained delivery and tissue engineering. *Adv. Drug Deliv. Rev.* **55**, 1631–1649 (2003).
4. Sionkowska, A. & Kaminska, A. Thermal helix-coil transition in UV irradiated collagen from rat tail tendon. *Int. J. Biol. Macromol.* **24**, 337–340 (1999).
5. Steven, F.S. & Tristram, G.R. The presence of non-protein nitrogen in acetic acid-soluble calf-skin collagen. *Biochem. J.* **83**, 240–244 (1962).
6. Silver, F.H. & Trelstad, R.L. Type I collagen in solution. Structure and properties of fibril fragments. *J. Biol. Chem.* **255**, 9427–9433 (1980).
7. Wood, G.C. The heterogeneity of collagen solutions and its effect on fibril formation. *Biochem. J.* **84**, 429–435 (1962).
8. Gross, J., Highberger, J.H. & Schmitt, F.O. Extraction of collagen from connective tissue by neutral salt solutions. *Proc. Natl. Acad. Sci. USA* **41**, 1–7 (1955).
9. Habermehl, J. *et al.* Preparation of a ready-to-use, stockable and reconstituted collagen. *Macromol. Biosci.* **5**, 821–828 (2005).
10. Boccafoschi, F., Habermehl, J., Vesentini, S. & Mantovani, D. Biological performances of collagen-based scaffolds for vascular tissue engineering. *Biomaterials* **26**, 7410–7417 (2005).
11. Sionkowska, A. Flash photolysis and pulse radiolysis studies on collagen type I in acetic acid solution. *J. Photochem. Photobiol. B* **84**, 38–45 (2006).
12. Elsdale, T. & Bard, J. Collagen substrata for studies on cell behavior. *J. Cell. Biol.* **54**, 626–637 (1972).
13. Bell, E., Ivarsson, B. & Merrill, C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc. Natl. Acad. Sci. USA* **76**, 1274–1278 (1979).
14. Weinberg, C.B. & Bell, E. A blood vessel model constructed from collagen and cultured vascular cells. *Science* **231**, 397–400 (1986).
15. Deyl, Z., Miksik, I. & Eckhardt, A. Preparative procedures and purity assessment of collagen proteins. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **790**, 245–275 (2003).
16. Lee, C.H., Singla, A. & Lee, Y. Biomedical applications of collagen. *Int. J. Pharm.* **221**, 1–22 (2001).