J Mater Sci: Mater Med (2009) 20:2413–2418
DOI 10.1007/s10856-009-3819-9

Heat denatured/aggregated albumin-based biomaterial: effects of preparation parameters on biodegradability and mechanical properties

Ramin Rohanizadeh · Nima Kokabi

Received: 22 September 2008 / Accepted: 1 July 2009 / Published online: 15 July 2009

Abstract Albumin-based biomaterials prepared using heat-aggregation or cross-linking agents have been used in various biomedical applications such as solder materials for laser-assisted tissue welding, anti-bacterial coatings and drug carriers. In this study, solid albumin-based materials were prepared via heat aggregation of albumin solution. The study aimed to determine the influences of the preparation parameters such as albumin concentration in solution, solution pH and temperature, on the mechanical properties as well as the biodegradation rate of heat-aggregated albumin-based materials. The results demonstrated that the materials prepared from the albumin solution with the pH of 8.5 had the highest mechanical strength. Augmenting the albumin concentration in solution led to an increase in mechanical strength, and the materials prepared from the solution with isoelectric albumin pH (pH 4.8) possessed the lowest biodegradation rate and those prepared at pH 12 showed the highest biodegradation rate.

1 Introduction

Biomaterials prepared from different animal or plant matrix proteins (e.g. collagen, gelatin, elastin, fibrin, hyaluronic acid, chitosan/chitin and alginate) have been widely studied and used in various therapeutic or surgical applications including tissue engineering, drug delivery, anti-post surgical adhesion barriers, wound dressings, and haemostatic control materials [1–3]. Such biomaterials are generally fabricated in various forms and shapes such as sponge, mesh, foam, fibre, film, coating, or as a composite with another type of material. Most protein-derived biomaterials exhibit superior properties over synthetic materials due to their excellent biocompatibility, and high affinity to and compatibility with other matrix proteins and growth factors [1–3]. Such biomaterials are naturally biodegradable, facilitating their eventual replacement with regenerated native tissues. However, the lack of mechanical strength, high biodegradation rate, and risk of adverse reaction of immune system after implantation may limit certain clinical applications of protein-derived biomaterials. To fabricate a proteinaceous solid structure, the protein chains must be cross-linked either by direct reaction between functional groups of protein molecules (self cross-linking) [4], or by chemically cross-linking agents such as aldehydes (e.g. glutaraldehyde, formaldehyde) [5, 6]. To obtain a proteinaceous solid structure, self cross-linking between protein chains offers advantages over using toxic chemical cross-linking agents. Although the percentages of agents added to cross-link the protein chains are generally low and they are trapped between the molecules, some free agents or those leached out over time from the protein structure can interfere with surrounding living tissues, impairing tissue function or regeneration during the healing process.

Heating can result in self cross-linking between protein chains, forming a solid proteinaceous network without the use of any cross-linking agents. Using this technique albumin-based biomaterials in the form of coatings, strips, or tubes have been successfully developed and fabricated [7–10]. Albumin is a water-soluble protein synthesised in the liver, and is the protein with the highest concentration

R. Rohanizadeh (✉)
Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia
e-mail: raminr@pharm.usyd.edu.au

N. Kokabi
Faculty of Medicine, University of Sydney, Sydney, NSW 2006, Australia

in blood plasma (55–60%). Unlike other protein-based biomaterials (e.g. collagen, elastin, hyaluronic acid, etc.) that are largely extracted from different animal tissues through complicated extraction and purification process, albumin can be extracted from human blood. This offers the advantage of minimising the risk of inter-species host responses. Furthermore, human blood is a widely available source of protein, substantially decreasing the fabrication costs of biomaterials that are albumin-based.

Albumin-based biomaterials in different forms and shapes have been studied and applied in several biomedical applications, such as solder materials for laser-assisted tissue welding [7, 8], coating materials for preventing bacterial adhesion [11, 12], and drug carrier materials—particularly for anti-cancer agents [13, 14]. However, the physico-chemical and mechanical properties of albumin-based biomaterials, which have a crucial impact on their function and performance, have as yet received little attention. This study aimed to determine the effects of fabricating parameters such as albumin concentration, pH, and denaturation/aggregation temperature on mechanical properties and biodegradability of proteinaceous solid albumin-based material. The materials were fabricated from albumin solution using heat-denaturation/aggregation process.

2 Materials and methods

2.1 Materials preparation

Albumin-based materials were prepared by heating 50 ml albumin solution (ZLB, Behring) poured into a 15 cm diameter cylindrical container. The container was sealed with aluminium foil, placed in a water bath, and then the ensemble was placed in a pre-heated oven for 60 min. Three fabrication parameters were analysed: albumin concentration in solution, pH of solution and temperature varied in 20 experimental groups. The parameters that were used to prepare the samples in different groups as well as those of other groups which were not prepared due to various difficulties are listed in Table 1. pH of 4.8 is the isoelectric point of albumin, and pH of 7.1 is the pH of purchased albumin solution (adjusted by the manufacturer). The materials were prepared using solutions with 25, 35 and 45% albumin concentrations. At the end of the heat-denaturing/aggregating process, the solid albumin-based materials were removed from the container and cut to 12 strips of 5 mm width, 6 mm thickness and 60 mm length. To keep the water content of the samples constant, the albumin strips were kept in a humidor at 20°C and relative humidity (RH) of 90% for 24 h prior to the tensile test. The tensile tests were carried out the following day of the sample preparations.

Table 1 The parameters used to prepare the albumin-based materials in each experimental group

Group	Conc. (%)	pH	Temp. (°C)	Group	Conc. (%)	pH	Temp. (°C)
1	25	4.8	120	11[a]	25	4.8	140
2	25	7.1	120	12	25	7.1	140
3	25	8.5	120	13	25	8.5	140
4	25	10	120	14	25	10	140
5	25	12	120	15	25	12	140
6	35	4.8	120	16[b]	45	4.8	120
7	35	7.1	120	17	45	7.1	120
8	35	8.5	120	18	45	8.5	120
9	35	10	120	19[b]	45	10	120
10	35	12	120	20[b]	45	12	120

[a] The material was not prepared: torn or broken when were removed from container-because of very low flexibility

[b] The material was not prepared: impossible to adjust the pH—albumin denaturation/aggregation occurred during the pH adjustment

2.2 Tensile test

The strips of materials were placed between two grips of tensile tester (Instron, 5655). The test was performed using a 100 N loading cell with 5 mm/min loading rate. The maximum load at failure, maximum extension before failure and elastic modulus (the ratio between tensile load and material's elongation in the linear region of the stress–strain curve) were measured using the software on Instron machine. At least 10 strips were tested in each group. The statistical differences between groups were determined using analysis of variance (ANOVA) followed by post hoc multiple comparison test (Tukey test). A *P*-value of less than 0.05 was considered significant.

2.3 Biodegradation assay

Once the mechanical tests were carried out, the materials were cut into small cubes (about $2 \times 2 \times 2$ mm^3) and totally dried overnight in an oven at 37°C. The cubes of materials were placed individually in 1.5 ml eppendorf tube and 0.05% of trypsin solution (in Phosphate Buffer Solution, pH 7) was added to each tube. The material weight-to-solution volume ratio was adjusted at 0.08 (e.g. 40 mg/500 µl). The tubes were closed, sealed with paraffin film, vortexed and incubated in oven at 37°C for 1, 2, 3 week incubation time. The samples were vortexed every 3 days until the end of the incubation period. In order to determine the effect of trypsin on protein measurement, the tubes containing only 500 µl of 0.05% trypsin solution (without albumin-based material) were also incubated under the same condition.

2.4 Measuring biodegradation rate using protein assay

At the end of each time point, the samples were vortexed and centrifuged (5 min, 6000 rpm). The solution was carefully transferred from each tube to another tube without disturbing the precipitated materials. The samples were stored in a freezer (−20°C) until usage. The percentage of the protein in the solution was measured using BioRad Dc Protein Assay kit with a plate reader at 750 nm absorption wavelength. The concentration of protein in the solution was calculated by comparing it to that of the standard protein concentration curve. Statistical differences between groups were determined using analysis of variance (ANOVA) followed by post hoc multiple comparison test (Tukey test). A *P*-value of less than 0.05 was considered significant.

3 Results

3.1 Appearance

By varying the pH of albumin solution from 4.8 to 12, the colour of materials changed from white opaque to cream and then to translucent yellow.

3.2 Mechanical properties of heat-denatured/aggregated albumin-based materials

Figure 1 shows the mechanical properties of solid albumin-based materials prepared at 120°C. The maximum tensile load at failure was measured for different groups and presented as a function of albumin concentration and the pH of albumin solution. For all tested albumin concentration (25, 35 and 45%), materials that were prepared from albumin solution with the pH of 8.5 demonstrated the highest tensile strength compared to those prepared using other pHs. The tensile strength was substantially lower for the materials prepared from the solution with the pH of 12. Increasing the albumin concentration from 25% to 45% led to a more than two-fold increase in the tensile strength of the materials (Fig. 1). For the materials prepared from 45% albumin solution, there was no significant difference in tensile strength between pH 7.1 and 8.5. It should be noted that due to the agglomeration of solution during the pH adjustment, the samples corresponding to the pHs above 8.5 and below 7.1 were not prepared for the 45% albumin concentration.

Figure 2 shows the elastic modulus of albumin-based materials increased constantly as a function of albumin concentration in solution, thus the materials became stiffer when they were prepared using a higher concentration of albumin in solution. The effect of pH on elastic modulus was in opposite direction than that of albumin concentration. As Fig. 2 shows, at higher pHs (pH 10 or 12) the materials were more flexible than those prepared at the pH of 7.1 and 8.5. However, the elastic modulus slightly decreased (not significant) by decreasing the pH from 7.1 to 4.8.

An increase in denaturation temperature from 120°C to 140°C decreased the tensile strength of materials only when they were prepared from the solutions with the pH of 10 and 12 (Fig. 3). Increasing the temperature did not demonstrate any significant changes in the tensile strength for the samples prepared from the solution with the pH of 7.1 or 8.5 (Fig. 3). Compared to the samples denatured at 120°C, the elastic modulus of those prepared at 140°C were slightly higher (except for the pH of 12). However, these differences were not significant (Fig. 4).

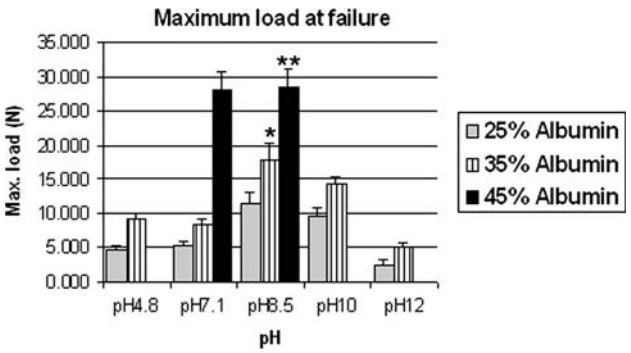

Fig. 1 Maximum tensile load at failure of different albumin-based materials as a function of the albumin concentration in solution and solution pH. The materials were prepared at 120 °C. * Significantly ($P < 0.05$) higher than that of the other pHs for the same albumin concentration (35% albumin). ** Significantly ($P < 0.05$) higher than that of the other concentrations for the same pH (pH 8.5)

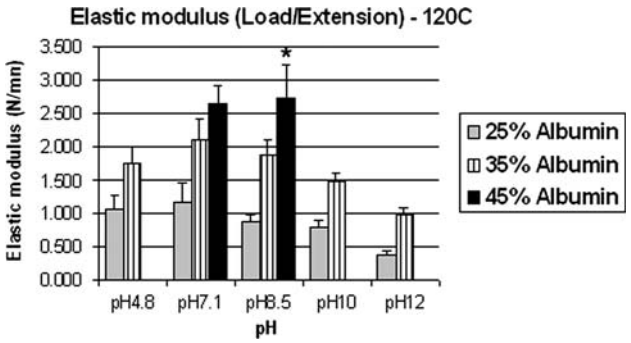

Fig. 2 Elastic modulus of different albumin-based materials as a function of the albumin concentration in solution and solution pH. The materials were prepared at 120 °C. * Significantly ($P < 0.05$) higher than that of the other concentrations for the same pH (pH 8.5)

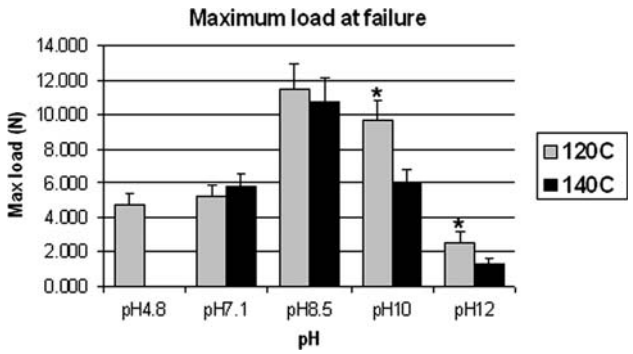

Fig. 3 Maximum tensile load at failure of different albumin-based materials as a function of the denaturation temperature (120 and 140 °C) and solution pH. The concentration of albumin in solution was at 25%. * Significantly ($P < 0.05$) higher than that at 140 °C

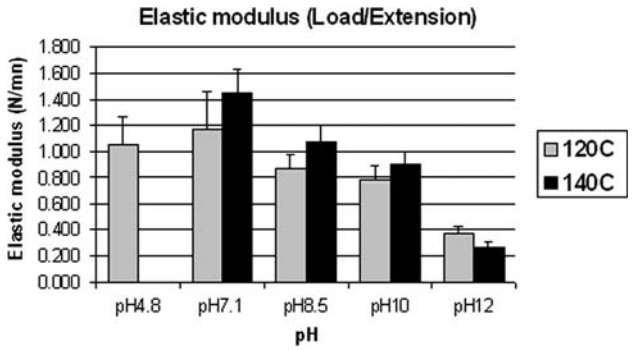

Fig. 4 Elastic modulus of different albumin-based materials as a function of the preparation temperature (120 and 140 °C) and solution pH. The concentration of albumin in solution was at 25%

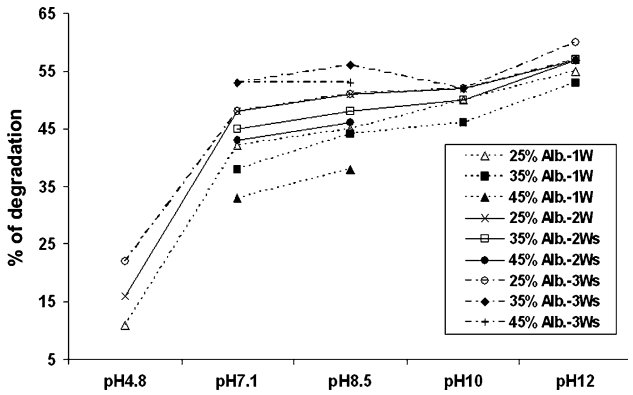

Fig. 5 Percentage of degradation of different albumin-based materials after 1, 2 and 3-weeks incubation in an enzymatic solution. The materials were prepared from 25, 35 and 45% albumin solution with different pHs and aggregated at 120 °C

3.3 In vitro degradation rate of heat-denatured/aggregated albumin-based materials

Figure 5 shows the degradation rate of different albumin-based materials after 1, 2 and 3-week incubation periods in 0.05% trypsin solution. For each time point the degradation rate increased as a function of increasing in the pH of albumin solution (Fig. 5). The degradation rate almost reached a plateau from pH 7.1 to pH 10 and was changed substantially by increasing the pH to above 12, or by decreasing the solution pH to below 4.8 (Fig. 5). For all of the incubation time points (1, 2 and 3 weeks), the degradation rate of samples prepared from the solution with the pH of 4.8 were lower than those prepared using higher pHs (Fig. 5). The amount of proteins detected in solution (representing the percentage of degraded materials) did not increase significantly by increasing the incubation period, especially between 2 and 3 week incubation times (Fig. 5).

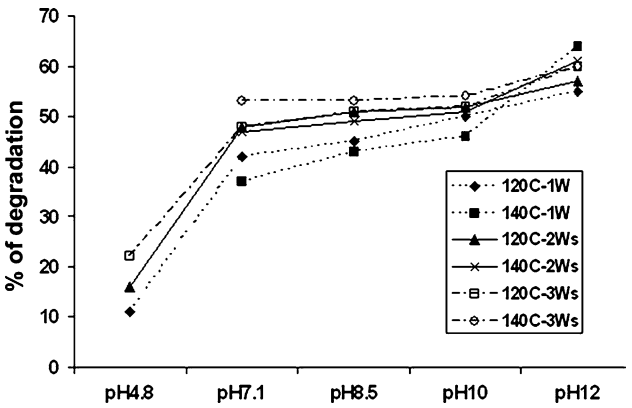

Fig. 6 Percentage of degradation of different albumin-based materials after 1, 2 and 3-weeks incubation in an enzymatic solution. The materials were prepared from 25, using solutions with different pHs and aggregated at 120 and 140 °C

The results did not demonstrate any significant differences in the degradation rates of the samples prepared at 120°C compared with those prepared at 140°C (Fig. 6).

4 Discussion

In the present study, albumin-based solid materials were fabricated by denaturing and aggregating albumin chains in aqueous solution using heat. Heating albumin solution above 65°C results in protein denaturation—that is irreversible three dimensional structural changes of molecules from their native forms [15]. The structural changes in the heat-denatured albumin molecules include the formation of beta-sheets as well as the transformation of some alpha-helices to random coils [15, 16]. These structural changes vary as a function of albumin concentration in solution, pH, temperature and ionic strength [15]. If the concentration of denatured molecules in solution reaches a certain threshold, further heating can lead to an aggregation of the adjacent denatured albumin molecules, forming a three-dimensional solid network enclosing water (a gel structure) [10]. Protein "denaturation" does not always result in protein "aggregation". In a solution with low protein concentration,

although heating can cause denaturation of proteinaceous molecule, cross-linking (aggregation) between the protein chains does not occur due to large spaces between the molecules and their neighbours. In general, the protein chains are heat-aggregated via the formation of hydrogen bonds, disulfide bonds, hydrophobic association, or a combination of these [15]. When an albumin solution is heated, aggregation between the denatured albumin molecules starts through the hydrogen bonding between the beta-sheet orientation of protein chains. Increasing the temperature causes further distortion of beta sheets, unfolding the sheets and thereby exposing the cysteine group (Cys-34) [15]. The exposed Cys-34 groups form disulphide bonds between the adjacent albumin molecules, resulting in a greater rate of aggregation, and therefore a stronger solid albumin-based material. Both the mechanical properties and the degradation rate of the solid protein-based materials are governed by the percentage and the strength of the afore-mentioned hydrogen and disulphide bonds. In turn, the exposure of these bonds depends on preparation parameters, namely: (i) denaturing temperature, (ii) denaturing time, (iii) solution pH, (iv) protein concentration and (v) presence of salts [17–19]. Greater aggregation may be achieved either by increasing protein concentration or by adjusting the pH closer to the isoelectric point. At the isoelectric point, the surface net charge of protein molecules is at its lowest level, minimising the repulsing forces between two adjacent molecules and decreasing the spaces between the protein chains.

The results of the present study showed when the albumin-based materials were prepared from albumin solution at isoelectric pH of albumin (pH 4.8), the materials demonstrated the lowest biodegradation rate compared to those prepared from solutions with the higher pHs. The shorter distance between the albumin molecules at isoelectric pH can result in a greater aggregation between the neighbouring molecules, leading to a lower degradability of the material. Using FTIR analysis Park et al. [10] reported that the albumin solution that denatured at the pH value near isoelectric point (pH 4.8) was more cross-linked (aggregated) than the other samples prepared at other pHs. In our study, the biodegradability of materials varied based on the pH of the solution in which they were prepared. As mentioned above isoelectric pH resulted in the lowest biodegradation, between pH 7 and 10 the biodegradation reached a plateau, and then biodegradation increased substantially by increasing the pH solution from 10 to 12. The materials prepared at pH 12 also demonstrated the lowest mechanical strength. These results suggest that at a pH above 10 the inter-molecular spaces were at their greatest value, allowing the maximum amount of water to be placed in the spaces between the albumin molecules. Due to the largest inter-molecular distances of materials prepared at pH 12, the mechanical properties was the lowest and the biodegradation was the highest in these materials in comparison with those prepared at lower pHs.

As expected, increasing the albumin concentration from 25% to 35% and then 45% decreased the biodegradability of materials. A greater number of molecules per volume (higher albumin concentration) will increase the possibility and the frequency of cross-linking between the albumin molecules, forming a stronger aggregation between them, which will result in lower biodegradability. Accordingly the tensile strength and the stiffness of materials were also increased by augmenting the albumin concentration in the solution.

Unexpectedly, denaturation of albumin solution at the isoelectric pH (pH 4.8) did not lead to a stronger material. The materials were substantially stronger, and achieved the best mechanical properties, when the solution pH increased to 8.5. As mentioned, in solution at isoelectric point the spaces between neighbouring albumin molecules are at their minimum. Very short intra-molecular spaces can cause a rapid aggregation between molecules once heat is applied, causing formation of a non-uniform 3-dimensional structure. The albumin molecules may then be heavily and rapidly aggregated in some areas, and poorly or non-aggregated in other areas. A highly-localised aggregation prevents the formation of a large and homogenous network structure made of the cross-linked albumin chains. In a non-homogenous solid structure, the frequency of structural defaults and inconsistencies are high, resulting in a weaker mechanical strength. The study demonstrated that the materials prepared from a solution with a pH of 8.5 had the highest tensile strength compared to those materials prepared at other pHs. The tensile strength of materials became lower at the pHs 10 and 12, which is probably due to larger intra-molecular spaces at these pHs (particularly at pH 12), decreasing the possibility and concurrency of cross-linking between the neighbouring albumin molecules.

The study of biodegradation revealed that there was no significant increase in the amount of degraded materials between the second and third weeks of incubation in enzymatic solution. Given the trypsin solution was not refreshed during the incubation period, after the first week of incubation, the concentration of soluble proteins detached from the material may reach a saturation threshold, and as a consequence lower degradation or no degradation occurred in the second and third weeks of incubation. Secondly, albumin-based materials may be composed of microscopic areas with different protein conformation and aggregation rates. In the first week of the biodegradation test, the regions with a higher degradation rate may be completely degraded while the remaining materials remain intact and do not degrade in the second or third weeks of incubation.

Antibodies circulating around a protein-based biomaterial implanted in body recognise the three dimensional structure of the native form of protein and, therefore, in general only bind to proteins in a highly specific interaction. Slight changes of the native protein three-dimensional structure have a crucial role in antibodies' recognition/binding to protein-based materials, and will dictate the host's reaction to the materials. Our previous study evaluating in vivo interactions between heat-denatured albumin biomaterials and tissue in a jugular vein ovine model, did not demonstrate any noticeable foreign body response at 1 and 6 weeks post-implantation period [20]. Fabricating solid albumin-based biomaterials by heat-denaturing and aggregating process greatly alters the three dimensional structure of albumin. The altered structure prevents the recognition of the denatured proteinous structure by antibodies, resulting in the biomaterials not inducing the recruitment of marcrophages and/or foreign body giant cells at the tissue/material interface. The animal studies on heat-denatured/aggregated albumin based biomaterials showed that these types of biomaterials are totally biocompatible and non-toxic [7, 8, 20]. It should be noted that the biological activities/tissue responses of the prepared materials in this study are directly affected by the degree and the forms of the cross-linking between and within albumin chains. Further studies to evaluate the correlation between preparation parameters of albumin-based biomaterials and the biological activities of these materials, will help us to have a better understanding of the biomedical applications of such biomaterials.

In conclusion, the study demonstrated that albumin-based materials prepared from the albumin solution with 45% albumin concentration and a pH of 8.5 possessed the highest mechanical strength. The materials prepared from the solution with isoelectric pH (4.8) had the lowest biodegradation in an enzymatic solution. The biodegradation rate of materials was increased by increasing the pH of the solutions from which they had been prepared.

Acknowledgements The study was fully supported by Avastra Ltd (Brisbane, Australia).

References

1. Lanza RP, Langer R, Vacanti J. Principles of tissue engineering. San Diego: Academic Press; 2000.
2. Ratner RD, Hoffman AS, Schoen FJ, Lemons JE. Biomaterials science. San Diego: Academic Press; 1996.
3. Ratner RD, Bryant SJ. Biomaterials: where we have been and where we are going. Annu Rev Biomed Eng. 2004;6:41–75. doi:10.1146/annurev.bioeng.6.040803.140027.
4. Clarke RC, Courts A, editors. The chemical reactivity of gelatin. London: Academic press; 1977.
5. Yapel A. Albumin medicament carrier system, (US, 1979).
6. Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev. 2003;55:1613–29. doi:10.1016/j.addr.2003.08.010.
7. Maitz PK, Trickett RI, Dekker P, Tos P, Dawes JM, Piper JA, et al. Sutureless microvascular anastomoses by a biodegradable laser-activated solid protein solder. Plast Reconstr Surg. 1999; 104:1726–31. doi:10.1097/00006534-199911000-00017.
8. Trickett RI, Wang D, Maitz PK, Lanzetta M, Owen ER. Laser welding of vas deferens in rodents: initial experience with fluid solders. Microsurgery. 1998;18:414–8. doi:10.1002/(SICI)1098-2752(1998)18:7<414::AID-MICR5>3.0.CO;2-W.
9. Bass LS, Treat MR. Laser tissue welding: a comprehensive review of current and future clinical applications review. Lasers Surg Med. 1995;17:315–49. doi:10.1002/lsm.1900170402.
10. Park HY, Song IH, Kim JH, Kim WS. Preparation of thermally denatured albumin geland its pH-sensitive swelling. Int J Pharm. 1998;175:231–6. doi:10.1016/S0378-5173(98)00289-0.
11. An YH, Stuart GW, McDowell SJ, McDaniel SE, Kang Q, Friedman RJ. Prevention of bacterial adherence to implant surfaces with a cross-linked albumin coating in vitro. J Orthop Res. 1996;14:846–9. doi:10.1002/jor.1100140526.
12. Kinnari TJ, Rihkanen H, Laine T, Salonen EM, Jero J. Albumin-coated tympanostomy tubes: prospective, double-blind clinical study. Laryngoscope. 2004;114:2038–43. doi:10.1097/01.mlg.0000147944.20676.17.
13. Yu Z, Garcia AS, Johnston KP, Williams RO. Spray freezing into liquid nitrogen for highly stable protein nanostructured microparticles. Eur J Pharm Biopharm. 2004;58:529–37. doi:10.1016/j.ejpb.2004.04.018.
14. Santhi K, Dhanaraj SA, Joseph V, Ponnusankar S, Sureh B. A study on the preparation and anti-tumor efficacy of bovine serum albumin nanospheres containing 5-fluorouracil. Drug Dev Ind Pharm. 2002;28:1171–9. doi:10.1081/DDC-120014584.
15. Wetzel R, Becker M, Behlke J, Billwitz H, Bohm S, Ebert B, et al. Temperature behaviour of human serum albumin. Eur J Biochem. 1980;104:469–78. doi:10.1111/j.1432-1033.1980.tb04449.x.
16. Clark AH, Saunderson DHP, Suggett A. Infrared and laser-Raman spectroscopic studies of thermally-induced globular protein gels. Int J Pept Protein Res. 1981;17:353–64.
17. Nakamura R, Sugiyama H, Sato Y. Factors contributing to heat-induced aggregation of ovalbumin. Agric Biol Chem. 1978;42: 819–24.
18. Woodward SA, Cotterill OJ. Texture and microstructure of heat-formed egg white gels. J Food Sci. 1986;51:333–9. doi:10.1111/j.1365-2621.1986.tb11123.x.
19. Shimada K, Matsushita S. Thermal coagulation of bovine serum albumin. Agric Biol Chem. 1981;45:1945–52.
20. Wright B, Vicaretti M, Schwaiger N, Wu J, Trickett R, Morrissey L, et al. Laser-assisted end-to-end BioWeld anastomosis in an ovine model. Lasers Surg Med. 2007;39:667–73. doi:10.1002/lsm.20541.