Research Article

Gelatin nanoparticle production: an in-process study using size exclusion chromatography

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Summary. *Gelatin is a good candidate for nanoparticle preparation. An in-process study of an earlier method was performed by characterizing the gelatin species present in solution at various production stages. Gelatin nanoparticles were prepared as described by Farrugia and Groves (1999). Samples from various production stages were analysed by size-exclusion HPLC. Addition of ethanol to the initial gelatin solution removed all but the low molecular weight species. Ultrafiltration was effective at separating and purifying nanoparticles. The glutaraldehyde-metabisulfite addition byproduct present in the first ultrafiltrate was practically absent in the third ultrafiltrate and in the filtered nanoparticle and control preparations. Also, ultrafiltration of gelatin solutions removed species of low to intermediate molecular weights, leaving higher molecular weight species in the retentate. The residual soluble species following desolvation were not present in the final nanoparticle dispersion. Both the nanoparticle filtrates and the ultrafiltrate washings exhibited insignificant concentrations of eluted gelatin species. The chromatogram of a water control taken through the production process was superimposable on that of the filtered nanoparticle dispersion, indicating that soluble gelatin species present post-desolvation were absent following cross-linking and neutralisation. A possible explanation is that the glutaraldehyde crosslinked the residual soluble gelatin onto the surface of existing nanoparticles.*

Keywords: *Gelatin nanoparticles, size-exclusion chromatography, purification*

Introduction

Over the past three decades, considerable research interest has arisen worldwide in the development of new colloidal drug delivery systems. The ideal colloidal delivery system would transport the associated drug to its desired site of action and then release it at an optimum rate. The carrier itself should be non-toxic and able to be degraded in vivo so that it does not accumulate indefinitely in the tissues. The colloidal preparation also needs to be pharmaceutically acceptable with regards to stability and ease of administration. Nanoparticulate colloidal drug carriers can be used to improve the therapeutic index of both established and new drugs by modifying their distribution, and thus increasing their efficacy and reducing their toxicity. This can be achieved because the drug distribution then follows that of the carrier, rather than depending on the physicochemical properties of the drug itself (Barratt, 2000). Gelatin, a natural macromolecule, is widely used in biotechnological and biomedical applications. Thus, it is a good contender for the preparation of nanoparticles for the purpose of controlled release applications of drugs (Akin and Hasirci, 1995), and methods for reproducibly preparing nanoparticles from gelatin have been described (Marty et al., 1978; Yoshioka et al., 1981; El-Samaligy and Rohdewald, 1983; Kreuter, 1983; Farrugia and Groves, 1999). The objective of this study was to carry out in-process development of the method developed by Farrugia and Groves (1999) by characterizing the molecular weights of the species present in solution at various stages of the production using size exclusion HPLC.

Materials and Methodology

Materials: All chemicals were of analytical reagent grade quality. Gelatin, bovine skin, lime-cured (Type B), with a bloom strength of 225, glutaraldehyde 25% w/w and sodium metabisulfite were purchased from Sigma-Aldrich Company. Absolute ethanol, sodium dihydrogenphosphate and disodium hydrogenphosphate were purchased from BDH Chemicals, sodium hydroxide was purchased from Merck Ltd., and sodium chloride was purchased from Timstar Laboratory Suppliers.

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Methodology:

(a) Size-exclusion chromatography: The HPLC system (Shimadzu SCL-10Avp) consisted of a pump system and a tuneable absorbance detector set at 205 nm. The mobile phase consisted of 0.05 mol dm^{-3} sodium dihydrogenphosphate, 0.05 mol dm^{-3} disodium hydrogenphosphate and 0.15 mol dm^{-3} sodium chloride. The mobile phase was maintained at 29ºC. Samples for analyses were filtered through 0.2-µm Whatman Anotop 10 membrane filters and analysed on an Ultrahydrogel Linear 7.8 x 300 mm size exclusion column (Waters Corporation), with an Ultrahydrogel Guard Column, at a flow rate of 0.3 mL/min.

(b) Production and analysis of the gelatin nanoparticles: Gelatin nanoparticles were prepared as described in Farrugia and Groves (1999). Briefly, a 1% w/w B225 gelatin solution was prepared by heating with moderate stirring to 40ºC for 20 minutes. The solution was adjusted to pH 7.0 with dilute sodium hydroxide and incubated at 37ºC for 1.5 hours. A hydroalcoholic solution, similarly incubated, was then added to the gelatin solution, so that the final mixture contained 0.2% w/w gelatin and 70% w/w ethanol. The colloidal dispersion was incubated at 37ºC for a further 20 minutes and then diluted 1:30 by weight, with stirring, in a hydroalcoholic solution of similar composition and at the same temperature, but containing 1% w/w glutaraldehyde. The particles were allowed to react for 2 hours and excess glutaraldehyde neutralised by addition of 3% w/v sodium metabisulfite with stirring for 10 minutes. Separation and purification of the particles was performed by ultrafiltration on an Amicon XM300 membrane (MWCO 300 kDa [Millipore Corporation]), using distilled water as the washing agent. Samples from various stages throughout the nanoparticle production process were filtered through 0.2-µm filters and analysed by size-exclusion chromatography as described above. Similar analyses were also carried out for a control water sample taken through the entire nanoparticle production process, and a B225 gelatin solution taken through the nanoparticle production process without the presence of alcohol as a desolvating agent. A dispersion of B225 gelatin nanoparticles was also incubated at 37ºC for 24 hours and subjected to HPLC analysis.

Results

Addition of the non-solvent ethanol to the initial gelatin solution resulted in removal of all but the low molecular weight species, the original gelatin solution having a characteristic broad peak extending from approximately 22 to 36 minutes of elution time (Figure 1). The filtered desolvated solution contained a much lower concentration of gelatin species with retention times between 27 to 36 minutes. (Figure 1). However, these residual soluble species were not present in the final nanoparticle dispersion, as filtrates of the nanoparticle dispersion did not exhibit any significant concentrations of eluted species (Figure 1), while the ultrafiltrate washings only contained species with retention times greater than approximately 34 minutes. (Figure 2).

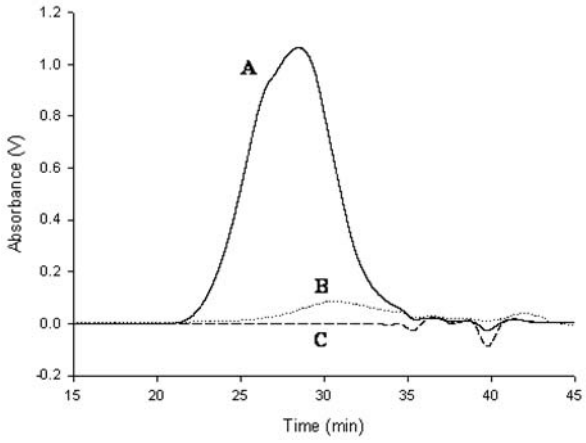

Figure 1: HPLC size-exclusion chromatograms for (A) dilute gelatin B225 solution, (B) residual gelatin species following desolvation, and (C) residual gelatin species in the final nanoparticle dispersion.

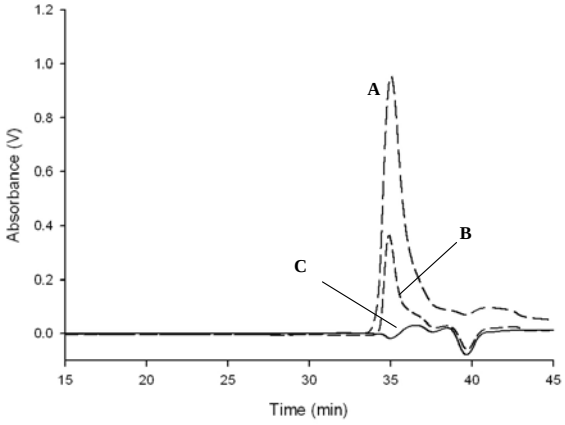

Figure 2: HPLC size-exclusion chromatograms for (A) first ultrafiltrate washing, (B) third ultrafiltrate washing, and (C) residual gelatin species in the final nanoparticle dispersion.

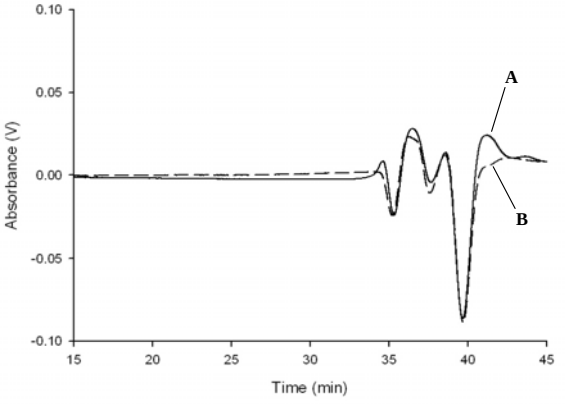

Figure 3: HPLC size-exclusion chromatograms for (A) residual gelatin species in a nanoparticle dispersion after purification and, (B) residual species in a blank control following purification.

The chromatogram of a water control taken through the nanoparticle production process was practically superimposable on that of the filtered nanoparticle dispersion (Figure 3), as was the chromatogram of a dispersion produced by taking a B225 gelatin solution through the entire nanoparticle production process without the presence of the desolvating agent alcohol (Figure 4). The HPLC chromatograms of a filtered nanoparticle dispersion immediately after production and of a filtered nanoparticle dispersion post-incubation for 24 hours at 37ºC were also superimposable (Figure 5).

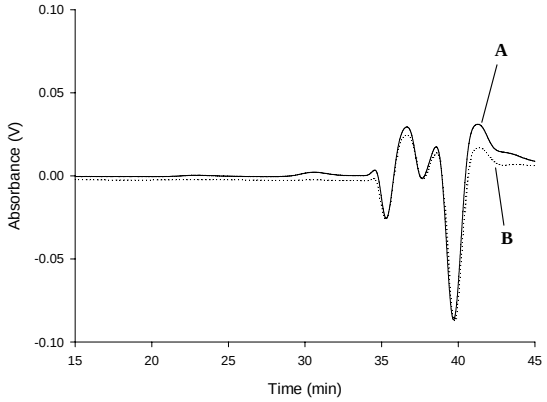

Figure 4: HPLC size-exclusion chromatograms for (A) residual gelatin species in a gelatin solution post-production without desolvation and, (B) residual gelatin species in a nanoparticle dispersion.

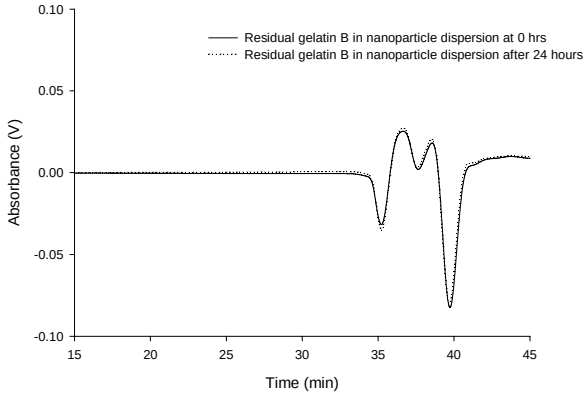

Figure 5: HPLC size-exclusion chromatograms for residual gelatin species in a nanoparticle dispersion at 0 hours incubation and at 24 hours incubation at 37°C.

When the HPLC chromatograms pre- and post-ultrafiltration were compared, the first ultrafiltrate of both the nanoparticle and control preparations exhibited a sharp absorption peak at high retention times (Figure 6). This peak was practically absent in the third ultrafiltrate (Figure 6) and also in the filtered nanoparticle preparation (Figures 1 and 2). Similar ultrafiltrate chromatograms were also obtained when a water control was ultrafiltered following the crosslinking process (Figure 5). Moreover, ultrafiltration of dilute gelatin solutions was shown to be effective at removing gelatin species of low to intermediate molecular weights, with medium to high molecular weight species being detected in the retentate (Figure 6).

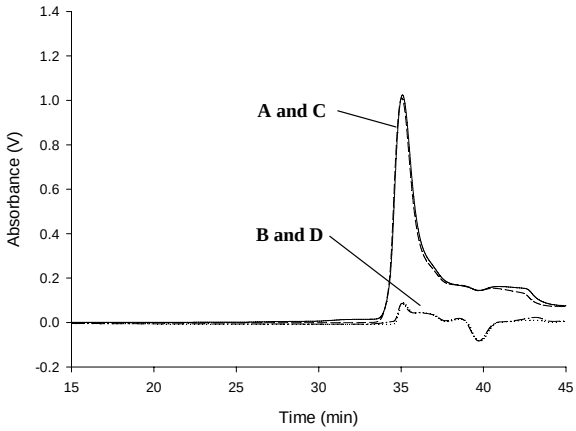

Figure 6: HPLC size-exclusion chromatograms for eluted species in first (A) and third (B) ultrafiltrates from gelatin nanoparticle preparation, and first (C) and third (D) ultrafiltrates from a water control.

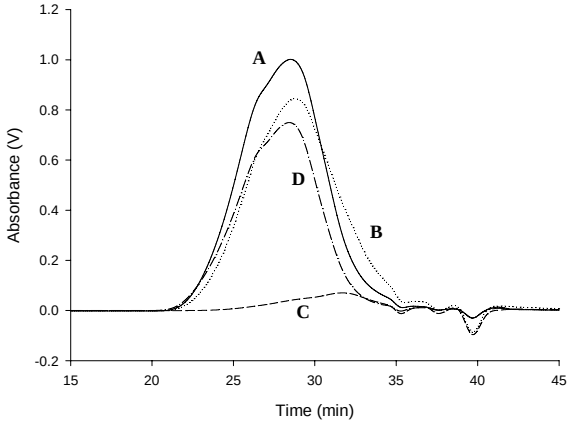

Figure 7: HPLC size-exclusion chromatograms for eluted gelatin species in (A) dilute native gelatin B225 solution, and first (B) and third (C) ultrafiltrates, and retentate (D) of an ultrafiltered dilute gelatin solution.

Discussion

The HPLC chromatograms obtained for native gelatin solutions are consistent with those observed in earlier studies (Farrugia and Groves, 1999, 2000). The addition of ethanol to these solutions, producing a colloidal gelatin dispersion, resulted in removal of all but the low molecular weight species, as indicated by the absence, in the colloid filtrate, of species with retention times between 22 to 27 minutes, these species having been present in the original gelatin solution (Figure 1). These results are consistent with those observed in earlier studies.

Ultrafiltration appeared to be an effective method for separation and purification of the nanoparticles. The ultrafiltrate chromatograms exhibited an absorption peak sharper in intensity and eluting at higher retention times than the low molecular weight species present in either

the native gelatin solution or the desolvated gelatin dispersion (Figures 1 and 2). The absence of these species eluting at 34 minutes or greater in the desolvated gelatin preparation prior to crosslinking (Figure 1), and the fact that these species were also present in a water control subjected to the crosslinking process (Figure 6) indicates that this peak is probably due to the glutaraldehyde-metabisulfite addition product formed during the neutralisation process. Moreover, the intensity of this peak decreased in successive ultrafiltrate washings, decreasing to less than 10% in peak height by the third ultrafiltrate (Figure 6). The absence of this peak in the nanoparticle dispersion retained in the ultrafiltration apparatus (Figures 1 and 2) is indicative of the effectiveness of the technique at removing this by-product of the nanoparticle production method.

The effectiveness of the ultrafiltration process at removing gelatin species should theoretically not have been of any direct concern, since nanoparticle dispersions did not appear to contain any significant amount of residual gelatin species, and dispersed gelatin nanoparticles incubated in aqueous media did not appear to undergo any hydrolysis to release soluble gelatin that could be detected by HPLC (Figure 5). Nevertheless, ultrafiltration of dilute gelatin solutions was shown to be effective at removing gelatin species of low to intermediate molecular weights, with medium to high molecular weight species being detected in the retentate (Figure 7).

It was furthermore observed that the residual low molecular weight species present in solution following desolvation of the gelatin preparation, with retention times between 27 and 34 minutes, were not present in the final nanoparticle preparation, as filtrates of the final nanoparticle dispersion did not exhibit any significant concentrations of eluted species (Figures 1 and 2), while the ultrafiltrate washings only contained species with retention times greater than approximately 34 minutes (Figures 2 and 6). Moreover, the similarity between the chromatograms of the filtered nanoparticle dispersion and that of a water control taken through the nanoparticle production process (Figure 3) indicates that the solution gelatin species present post-desolvation were effectively absent following cross-linking and neutralization. An explanation for this observation is that the glutaraldehyde crosslinked both the desolvated and the soluble gelatin, a hypothesis supported by the fact that crosslinking of an undesolvated gelatin solution also did not have any residual detectable gelatin species (Figure 4). The residual gelatin species following desolvation thus appear to be crosslinked onto the surface of previously existing nanoparticles (nanoencapsulation), possibly establishing a gelatin 'brush border' and accounting for the dispersion stability of the nanoparticles (Mifsud, 2003).

Conclusion

We conclude that, during nanoparticle production, the ultrafiltration process is effective both at removing the addition reaction impurities and low molecular weight gelatin species. However, the latter do not appear to be present in the nanoparticle dispersion prior to purification since the crosslinking process not only crosslinks the colloidal gelatin particles but also removes residual soluble gelatin fractions from solution, probably by crosslinking to the surface of the existing nanoparticles.

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