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Comparison of the Molecular Weight Distribution of Gelatin Fractions by Size-exclusion Chromatography and Light Scattering

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Abstract

Commercial gelatin is a heterogeneous proteinaceous product with a broad range of molecular weights. The use of gelatin to prepare insoluble nanoparticles depends largely on the presence of high molecular weight fractions that can be separated by size-exclusion chromatography. The purpose of this study was to determine the molecular fractions of gelatins from three commercial types (B225, B60 and A60), measure their molecular weight and compare these with data obtained from an absolute light scattering method.

The mean molecular weight of the gelatins decreased in the order B225 > B60 > A60. All samples were polydisperse, with fractions varying from < 50 kDa to > 2 MDa. Each sample was divided into eight fractions based on the molecular weight distribution and using a paired *t*-test the two methods were shown to be in substantial agreement.

The light scattering method would appear to provide an absolute quality control procedure for commercial gelatin, depending on its application and requirements.

Gelatin has a wide variety of applications pharmaceutically including the preparation of gelatin capsules, coatings and the preparation of drug delivery systems. Gelatin is prepared by the hydrolytic degradation of collagen. Collagen consists of three collagen peptide chains (α-chains) wound around each other in a superhelix and stabilized primarily by non-covalent and both inter- and intra-molecular covalent cross-links (Alberts et al 1994). During the commercial production of gelatin from animal collagen, most of these linkages are hydrolytically cleaved to yield a heterogeneous proteinaceous product made up of components with a broad range of molecular weights (Courts 1954; Flory & Weaver 1960; Steven & Tristam 1962). Previous studies have identified various sized fractions in aqueous solutions of gelatin, namely sub-α (50–80 kDa), α (80–125 kDa), β (125–230 kDa), γ (230–340 kDa) and δ (approx. 1·4 MDa) (Veis et al 1962; Lorry & Vedrines 1985). We have found it convenient to separate out the larger fraction as the ε (340–700 kDa), ζ (700–1000 kDa), δ (1000–1800 kDa) and microgel (> 1·8 NDa) sub-fractions (Farrugia & Groves 1999). We found that the formation of nanoparticles ($\simeq$ 200 nm diam.) in aqueous ethanol was strongly influenced by the relative proportion of fractions larger than ε; the higher the content of these fractions the more readily these nanoparticles formed. Size-exclusion chromatography is a relative technique for molecular weight determination, while a laser-light scattering procedure (Wyatt Technology, Santa Barbara, CA) is both rapid and absolute. We compared the two methods using different gelatin samples.

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Materials and Methods

Materials

Lime-cured gelatin from bovine skin (Type B) of bloom strengths 225 and 60 and acid-cured gelatin from porcine skin (Type A) of bloom strength 60 were purchased from Sigma Chemical Co., St Louis, MO. Sodium chloride, sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrous were of analytical grade and were obtained from Fisher Scientific, Itasca, IL. Gel Filtration Standards (Bio-Rad Laboratories, Hercules, CA), containing thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin

(44 kDa), equine myoglobulin (17 kDa) and vitamin B-12 (1·35 kDa) were used to calibrate the column for the size-exclusion chromatography system. HPLC-grade water (Fisher Scientific, Itasca, IL) was used for preparation of mobile phase and samples in the light scattering experiments.

Size-exclusion chromatography

The chromatographic system used consisted of a Micromeritics Model 728 Autosampler (Alcott Chromatography Inc., Norcross, GA), equipped with 0·5-mL glass sample vials; a Waters Model 501 high-performance liquid chromatography (HPLC) pump (Waters Corp., Milford, MA); a mixed-bed Ultrahydrogel Linear 300×7·8 mm column coupled with an Ultrahydrogel Guard Column (Waters Corp., Milford, MA); a Waters Model 486 Tunable Absorbance Detector, set at 205 nm; a computer system running Millennium 2010 Chromatography Manager software. The mobile phase was phosphate-buffered saline (0·15 M NaCl, 0·1 M phosphate, pH 6·8) filtered through a 0·1-μm polyvinylidene membrane filter (Millipore Corporation, Bedford, MA). The flow rate of the mobile phase was 0·3 mL min^{-1}. After equilibration, a 10-μL sample of Gel Filtration Standard was injected onto the system. A 0·2% (w/v) solution of each of the three gelatins was prepared in filtered water by heating to 40°C with stirring for 20 min, followed by cooling to room temperature for 90 min. A 10-μL sample of the solution was injected onto the system and data collection and analysis was performed using the Millennium software.

Static light scattering

The system used consisted of a Waters Model 600 multisolvent delivery system (Waters Corp., Milford, MA) equipped with a high-sensitivity noise pulse dampener (Waters Corp., Milford, MA), connected to a tank of helium (99·9% purity) (AGA Gas Inc., Cleveland, OH); a Bio-Sil SEC-400 300×7·8 mm column (Bio-Rad Laboratories, Hercules, CA); a mini-Dawn static light scattering detector (Wyatt Technology, Santa Barbara, CA), previously calibrated (calibration constant $8{\cdot}681\times10^{-6}$) with toluene, a highly scattering solvent of known Raleigh ratio; a Waters 410 differential refractometer (Waters Corp., Milford, MA), operated at 35°C, a sensitivity setting of 64 and a scale factor of 20, having a calibration constant of $7{\cdot}84\times10^{-5}$ V/refractive index unit; a computer system running Wyatt Astra software, which controlled data acquisition and analysis.

The mobile phase was phosphate-buffered saline (0·15 M NaCl, 0·1 M phosphate, pH 6·8), filtered through a 0·1-μm polyvinylidene membrane filter (Millipore Corporation, Bedford, MA). The solution was degassed with helium (10 mL min^{-1}) before and throughout the experiment. The flow rate of the mobile phase was 0·5 mL min^{-1}. A 0·2% (w/v) solution of each of the three gelatins was prepared in filtered, degassed water by heating to 40°C with stirring for 20 min, followed by cooling to room temperature for 90 min. A 60-μL sample of the solution was injected onto the system and data collection and analysis performed using Astra software.

Results and Discussion

The calibration plot for the standards passed through the Ultrahydrogel Linear size-exclusion column gave the regression equation:

$$\log \mathrm{MW} = -0{\cdot}29t + 13{\cdot}08 \quad (r = 0{\cdot}984, P > 0{\cdot}99) \tag{1}$$

where MW is the molecular weight (Da) and t is the retention time (min). This equation was used to analyse the gelatin chromatograms and calculate the area under the curve (AUC) for the eight arbitrarily defined classes, thereby enabling the relative proportions of each to be determined (Tables 1–3).

Techniques for measuring molecular weight have been classified as relative (e.g. size-exclusion chromatography) and absolute (requiring no calibration). An example of an absolute method is the laser light scattering procedure incorporated in the Wyatt mini-Dawn instrument that has proved to be exceptionally valuable for molecular weight measurements. However, a validation procedure is still required and the chromatography system, calibrated with suitable standards, is suitable for this purpose.

Table 1. Comparison of the relative abundance of the various molecular weight class fractions in a dilute solution of B225 gelatin determined by light scattering and size-exclusion chromatography.

Molecular weight		Composition (%)	
Class	Range (kDa)	Light scattering	Chromatography
LMW + sub-α	< 80	20·1 ± 0·2	19·6 ± 1·0
α	80–125	13·6 ± 0·2	8·1 ± 0·4
β	125–225	17·6 ± 0·3	14·1 ± 0·5
γ	225–340	8·2 ± 0·2	10·5 ± 0·3
ε	340–700	14·7 ± 0·2	16·2 ± 0·3
ζ	700–1000	5·3 ± 0·1	7·3 ± 0·1
δ	1000–1800	8·8 ± 0·3	10·6 ± 0·3
Microgel	> 1800	11·6 ± 0·2	13·4 ± 1·7

n = 3. MW = low molecular weight.

Table 2. Comparison of the relative abundance of the various molecular weight class fractions in a dilute solution of B60 gelatin determined by light scattering and size-exclusion chromatography.

Molecular weight		Composition (%)	
Class	Range (kDa)	Light scattering	Chromatography
LMW + sub-α	< 80	35·5 ± 0·5	35·7 ± 1·6
α	80–125	15·0 ± 0·3	8·5 ± 0·3
β	125–225	15·8 ± 0·3	12·4 ± 0·5
γ	225–340	7·3 ± 0·6	8·7 ± 0·4
ε	340–700	10·7 ± 0·5	13·2 ± 0·7
ζ	700–1000	4·4 ± 0·2	5·3 ± 0·3
δ	1000–1800	5·5 ± 0·3	7·2 ± 0·3
Microgel	> 1800	5·8 ± 0·6	9·0 ± 1·1

n = 3. LMW = low molecular weight.

Table 3. Comparison of the relative abundance of the various molecular weight class fractions in a dilute solution of A60 gelatin determined by light scattering and size-exclusion chromatography.

Molecular Weight		Composition (%)	
Class	Range (kDa)	Light scattering	Chromatography
LMW + sub-α	< 80	47·8 ± 1·4	50·5 ± 2·3
α	80–125	11·1 ± 2·2	9·1 ± 1·4
β	125–225	14·7 ± 0·4	11·0 ± 0·6
γ	225–340	7·4 ± 0·2	6·7 ± 0·3
ε	340–700	9·4 ± 0·2	10·0 ± 0·4
ζ	700–1000	3·7 ± 0·2	3·9 ± 0·2
δ	1000–1800	3·1 ± 0·2	4·9 ± 0·3
Microgel	> 1800	2·8 ± 0·4	3·9 ± 0·6

n = 3. LMW = low molecular weight.

The absolute measurement was carried out by passing the column output sequentially through both a light scattering detector and a differential refractometer acting as a mass detector. This gave two chromatograms, which differed in shape because the light scattering was sensitive to the relatively few molecules of higher molecular weight present (Styring & Hamielec 1989). As might be anticipated, the samples with broad size ranges had high polydispersity indices. Nevertheless, the three gelatin samples could be ordered as B225 > B60 > A60, with weight average molecular weights falling between the number average and the Z-average molar masses in each case (Table 4). The relative proportions of the various fractions were also determined from the light scattering data (Tables 1–3).

Table 5. Statistical analysis by paired *t*-test to assess the correlation between the percentage compositions of the various molecular weight fractions in gelatin B225, B60 and A60 samples measured by light scattering and by size-exclusion chromatography.

Statistical parameter	Gelatin		
	B225	B60	A60
Paired *t*-test			
t	0·0120	< 0·0001	< 0·0001
df	7	7	7
P	> 99%	> 99%	> 99%
Effectiveness of pairing			
Correlation coefficient (r)	0·808	0·947	0·992
P	> 95%	> 99%	> 99%

There was no significant difference between means ($P < 0·05$; paired *t*-test). Pairing was significantly effective ($P < 0·05$) in all cases.

The purpose of this work was to determine the relative abundance of each molecular weight fraction and assist in determining the optimal conditions for the precipitation of insoluble nanoparticles from aqueous ethanol systems. The two sizing techniques were in excellent agreement (Table 5), bearing in mind the arbitrary nature of the fractionation process and the fact that they operate on entirely different experimental principles.

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Table 4. Mean molecular weight data generated by static light scattering for different solutions of gelatin.

Parameter	Gelatin		
	B225	B60	A60
Number-average molecular weight (kDa)	131·6 ± 4·8	98·4 ± 2·3	72·1 ± 5·9
Weight-average molecular weight (kDa)	1120 ± 22	548 ± 31	306 ± 14·8
Z-average molecular weight (kDa)	12 300 ± 101	4576 ± 523	3123 ± 573
Z-average radius (nm)	61·9 ± 1·0	41·6 ± 2·7	42·1 ± 2·6
Polydispersity index	8·52 ± 0·29	5·6 ± 0·3	4·3 ± 0·5

Polydispersity index = weight-average molecular weight/number-average molecular weight.

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