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Characterization of polyamino acids by use of GPC–viscometry technology*

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Abstract: The properties of polybenzyl glutamate, a well known polyamino acid vary considerably with molecular weight in both solid state and in solution. Therefore, accurate determinations of the molecular weights of these polyamino acids is essential. The dual viscometer/refractometer when used as detector system for size exclusion chromatography provides a way of determining accurate molecular weights. An indirect method of determining the molecular weight distribution (*MWD*) and the radius of gyration distribution (*RgD*) of polybenzyl glutamate is described. The *MWD* is calculated from the measured value of intrinsic viscosity (*IV*) and the known *IV*-to-*MW* relationship, at every SEC retention volume slice. Such a technique of determining *MWD* requires no calibration and is more precisely measurable than conventional SEC methods.

Keywords: *Polybenzyl glutamate (PBG); size exclusion chromatography (SEC); intrinsic viscosity* (IV); *molecular weight distribution* (MWD); *radius of gyration distribution* (RgD).

Introduction

Polyamino acids are synthetic polymers that are important because of the close relationship they bear to proteins [1–3]. These synthetic polyamino acids act as protein models and lend themselves to a better understanding of their macromolecular stereochemistry [4]. The polyamino acids are also used as enzyme inhibitors [3], in microencapsulation [5], and drug delivery devices [6, 7].

The polyamino acids can be grouped into neutral polyamino acids such as poly-L-proline and poly-DL-alanine, basic such as polylysine and polyarginine, and acidic such as polyaspartic acid and polyglutamic acid. The basic and acidic polyamino acids are highly electrically charged in solution. Many of these properties of polyamino acids in both solid state and in solution vary considerably with molecular weight. Therefore, accurate determinations of molecular weights of the polyamino acids used in chemical and physical experiments is essential.

The understanding of the macromolecular properties of polyamino acids requires a reliable method for estimating the molecular weight (*MW*) and the distribution of molecular weight (*MWD*) in a suitable solvent that minimizes solute–solute (aggregation), solute–solvent, and solute–column packing material interactions. The use of size exclusion chromatography (SEC) with a combined differential viscometer–differential refractometer for characterizing *MWD*, intrinsic viscosity, Mark–Houwink constants and branching information is a widely accepted technique for both organic and aqueous polymers [8–10]. The method is usually based on the concept of universal calibration [11]. Such calibration techniques are very sensitive to SEC flow rate changes, column deteriorations, instrumental band broadening and sample overloading. Available literature data generally have supported the universal calibration concept. But, there are still significant deviations where calibration curve deviation do occur due to the column packing [10]. It has been also shown that in the presence of sorbent interaction between the oligomers and the sorbent matrix, the oligomer does not conform to universal calibration [12]. As a result, it is not possible to determine the accurate *MWD*.

In this paper, an indirect method of determining *MWD* of polybenzyl glutamate by using an on-line differential viscometer detector [13–16] is described. The *MWD* was calculated from the measured value of intrinsic viscosity

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(*IV*) and the known *IV*-to-*MW* relationship, at every SEC retention volume slice. This technique of determining *MWD* required no calibration and was more precise than conventional SEC methods.

Experimental

Chemicals and reagents

Polyamino acid such as poly-γ-benzyl glutamate (PBG) and lithium bromide (LiBr) was purchased (Sigma Chemical Company, St Louis, MO, USA) and was used without further purification. *N,N*-Dimethylacetamide (DMAC) was from Aldrich Chemical (Milwaukee, WI, USA). The mobile phase consisted of DMAC–LiBr (99.9:0.1, v/w) and was filtered through a Teflon filter (Type FH, 0.5 μm, Millipore, Bedford, MA, USA). The mobile phase was vacuum-degassed before use.

Sample preparation

Poly-γ-benzyl glutamate (PBG) solution was prepared in the mobile phase. Concentrations ranged from 0.90 to 0.333 mg ml^{-1}. The PBG was first dissolved in DMAC–LiBr and allowed to stand for 2 h before injection.

Chromatographic system and conditions

The HPLC–DV system was modular and consisted of an autosampler Model 728 (Micromeritics, Norcross, GA, USA), remote switching valve, Model EQ60 (Valco, Houston, TX, USA) fitted with a 100 μl loop, HPLC pump, Model 222B (Scientific System, State College, PA, USA) and differential viscometer–differential refractometer detector, Model 200 (Viscotek, Porter, TX, USA). Separation was performed on two American Polymer Laboratories (Mentor, OH, USA) mixed bed columns (10 μm, 300 × 7.5 mm i.d.) connected in series after the injector. Run time was 30 min.

The flow rate was maintained at 1 ml min^{-1}. The PBG solutions were processed by triplicate injections on the differential viscometer (range adjusted at 1) and measurement of both the inlet and differential pressures.

A plumbing schematic of the DV detector [15, 16] is shown in Fig. 1. The pneumatic pulse dampener shown was a capped Teflon tube (304 mm × 2.5 mm i.d.). This was found to be adequate for the present experiment. Briefly, the PBG solution from the column flows continuously through the balanced bridge network, which consisted of four capillaries (R1–R4). The reservoir (Res 1), located out of the

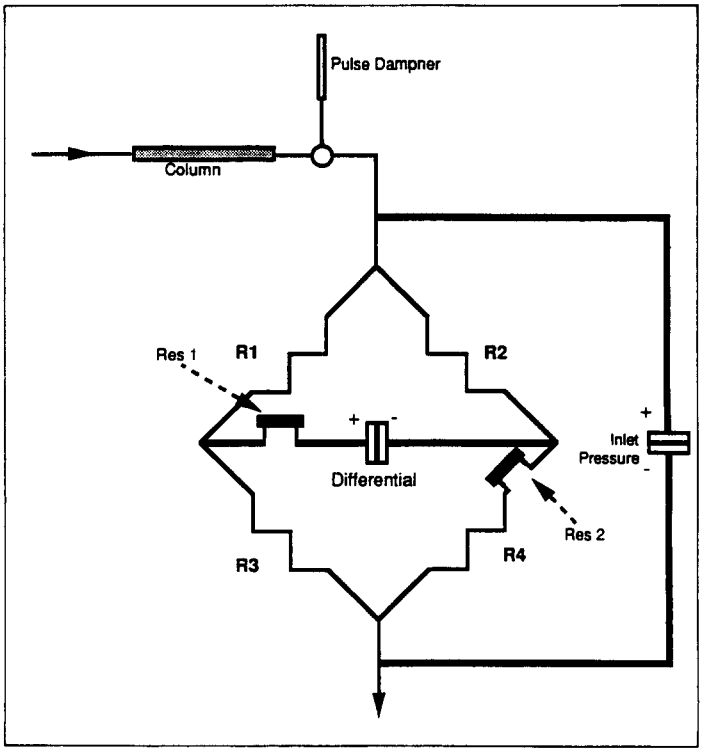

Figure 1
Simplified schematic of differential viscometer.

flowstream acted to compensate volume so that any flow rate fluctuations caused equal pressure changes on each side of the 'differential' pressure transducer. The other reservoir (Res 2) held up the PBG solution and prevented it from entering capillary R4. For any time slice in the chromatogram, PBG solution was in capillaries R1, R2, and R3 but only mobile phase was in capillary R4. Res 1 and Res 2 are large (30 ml) reservoirs so that the elution buffer was not entirely displaced until after the elution peak is completely eluted. Two pressure measurements were normally made by use of transducers. The differential pressure, ΔP, was due to the difference in the viscosity of the PBG solution in capillary R3 and viscosity of mobile phase in capillary R4. Viscosities of PBG solutions in R1 and R2 cancel each other. Another transducer measures P_i, the inlet pressure. The differential viscometer detector nulls the solvent pressure as desired yielding a differential signal (ΔP) proportional to the specific viscosity of the PBG solution. The differential viscometer, being highly sensitive, can measure the specific viscosity near zero concentration, thereby obtaining the intrinsic viscosity from a single measurement. The following expression relates the specific viscosity (η_{sp}) to these two pressure terms [14].

$$\eta_{sp} = 4\Delta P/(P_i - 2\Delta P). \quad (1)$$

The intrinsic viscosity [η], is defined as:

$$[\eta] = (\eta_{sp}/C),\ c \rightarrow 0, \quad (2)$$

where C is the concentration of the PBG solution in mg ml^{-1}. The solutions required were dilute enough that a single-point determination of intrinsic viscosity can be made, without the need to extrapolate from the viscosities of several relatively high sample concentrations.

Calculation

Intrinsic viscosity (IV) was calculated [13–15] from data collected using the above equations on an IBM compatible personal computer system with UNICAL software, Version 4.0 (Viscotek, Porter, TX, USA).

Molecular weight of the unknown PBG was calculated by use of the intrinsic viscosity–molecular weight relationship (Fig. 2). The molecular weight distribution and the radius of gyration distribution was calculated from the intrinsic viscosity distribution curve generated by the UNICAL software from the dual DV–DRI chromatogram of the unknown PBG.

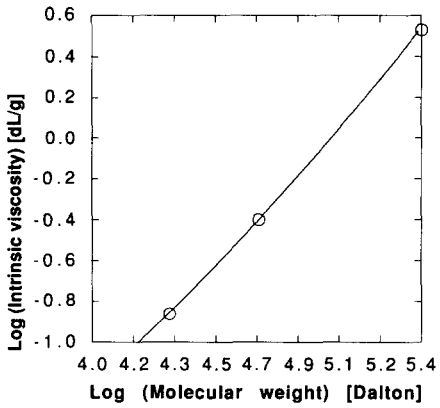

Figure 2
Relationship between intrinsic viscosity and molecular weight of known poly-γ-benzyl glutamates dissolved in DMAC–LiBr (99.9:0.1, v/w).

Results and Discussion

Poly-γ-benzyl glutamate (PBG) tends to aggregate in many solvent systems such as benzene, dioxane and chloroform. Therefore, the elucidation of their conformation in solution was made by making physicochemical measurements in solvents such as DMAC–LiBr (99.9:0.1, v/w) in which molecular solutions are easily obtained [2]. The intrinsic viscosity determination was made on PBG by integrating area under the chromatographic (DV) peaks (Fig. 3). The intrinsic viscosities of PBG (Table 1) increased with increasing molecular weight. The precision of the DV technique for determining intrinsic viscosity was excellent. It had a standard deviation of less than 0.002 for triplicate injections. The true IV of PBG was plotted against the molecular weights (MW) reported by the supplier (Fig. 2). This familiar relationship (Fig. 2) was first expressed by Mark [17] and Houwink [18]. In this relationship:

$$[\eta] = KM^a \quad (3)$$

[η] is the intrinsic viscosity, K and a are Mark–Houwink constants for a particular solute–solvent system and temperature [19]. It has been predicted by universal calibration first suggested by Benoit *et al.* [11] that all molecules having the same [η]M value should have

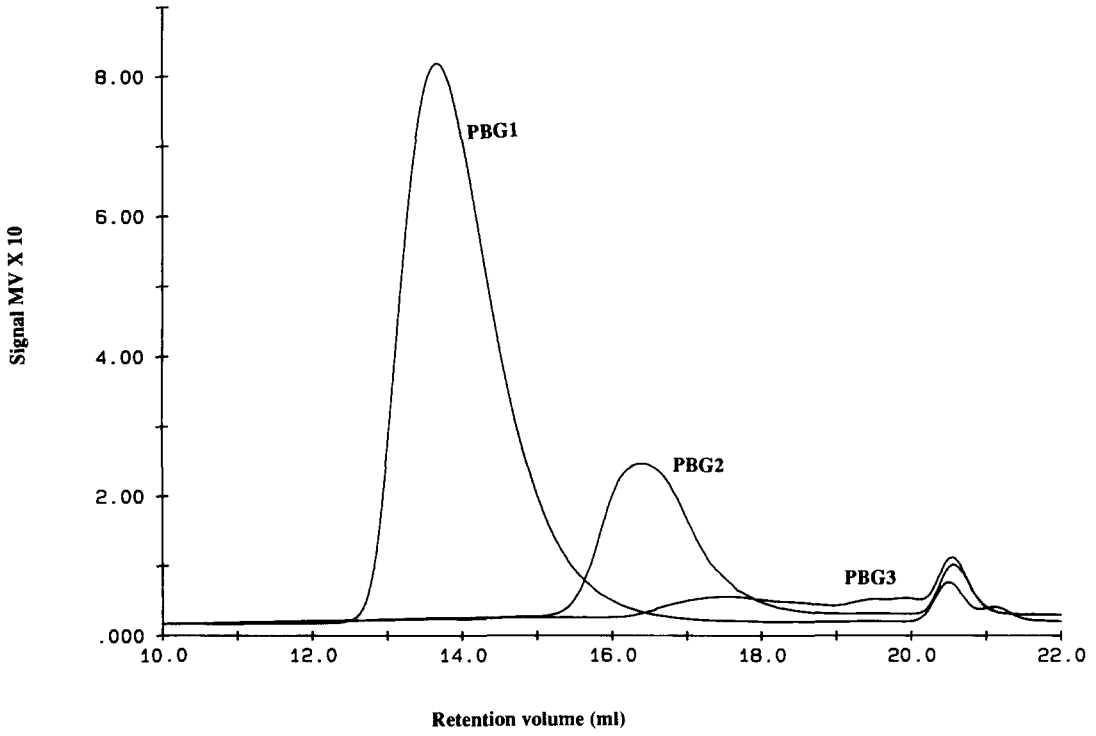

Figure 3
Overlay of differential viscometer chromatograms of poly-γ-benzyl glutamate standards in DMAC–LiBr. Raw chromatograms are not normalized. PBG1, $MW = 2.48 \times 10^5$, conc. = 0.333 mg ml^{-1}; PBG2, $MW = 5.10 \times 10^5$, conc. = 0.75 mg ml^{-1} and PBG3, $MW = 0.21 \times 10^5$, conc. = 0.900 mg ml^{-1}.

Table 1
Intrinsic viscosities and molecular weights* (*MW*) of poly-γ-benzyl glutamate standards in DMAC–LiBr (99.0:0.1, v/w)

Name of polyamino acid	Reported *MW* ($\times 10^3$)	Concentration (mg ml^{-1})	$[\eta]$ (dl g^{-1})
Poly-γ-benzyl glutamate	21	0.90	0.138
	51	0.75	0.398
	248	0.33	3.440

*Molecular weight reported by Sigma Chem. Co. (St Louis, MO, USA).

the same value of V_h, the hydrodynamic volume. Also, if V_h is the parameter that uniquely determines the elution volume, V_e, these molecules should have the same elution volume.

SEC calibration techniques are very sensitive to flow rate changes, column deteriorations, instrumental band broadening and sample overloading. Sources of error encountered in universal calibration procedure have been discussed and illustrated [8, 19]. Therefore, attempts were made to calculate *MWD* indirectly using a technique which did not depend on any SEC retention calibration. The technique was insensitive to SEC experimental conditions like flow-rate changes, column deteriorations, instrumental band broadening, etc. Briefly, 100 μl of the unknown PBG (0.455 mg ml^{-1}) was injected onto the columns. The *IV*-to-*MW* relationship, at every SEC retention volume slice was calculated from viscosity chromatogram from the differential viscometer and concentration chromatogram from the differential refractometer (Fig. 4). This then was used to plot the intrinsic viscosity distribution plot (Fig. 5) by using the UNICAL software. From this plot, the *MWD* and the *RgD* was calculated (Table 2). The calculated *MW* for the unknown is very close to the value reported by vendor (Sigma). The

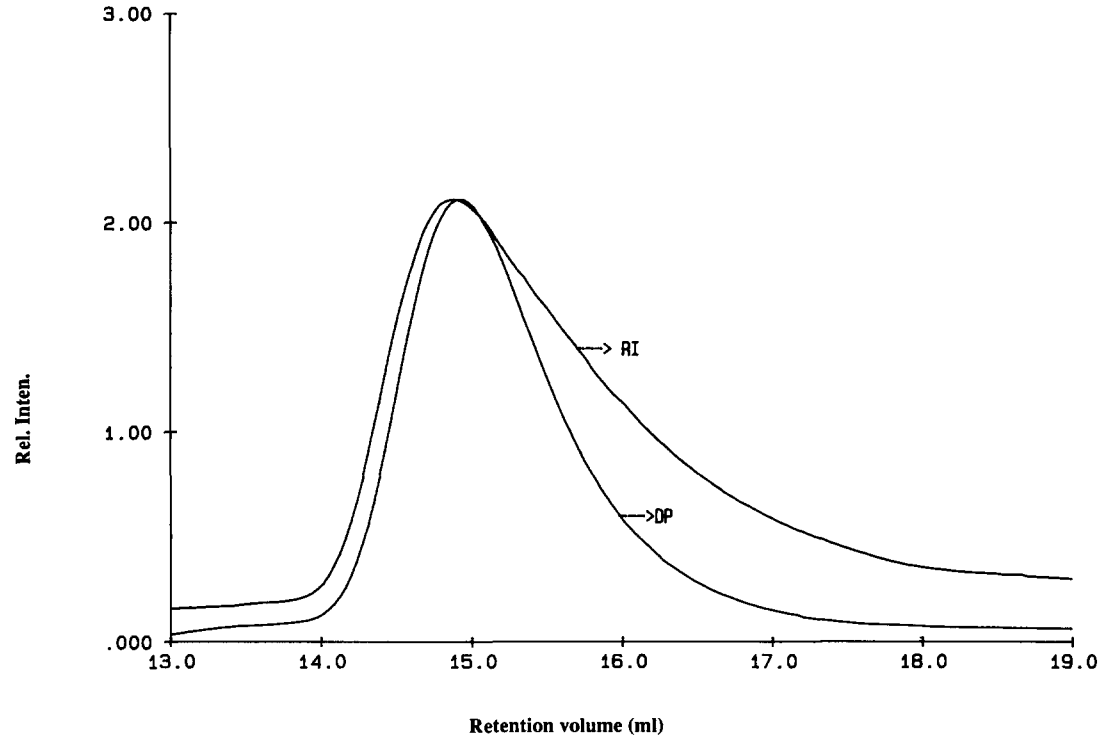

Figure 4
Dual chromatogram showing the elution of unknown poly-γ-benzyl glutamate in DMAC–LiBr from SEC–DV system. A 100 μl sample (0.455 mg ml^{-1}) was injected. RI, differential refractometer and DP, differential pressure.

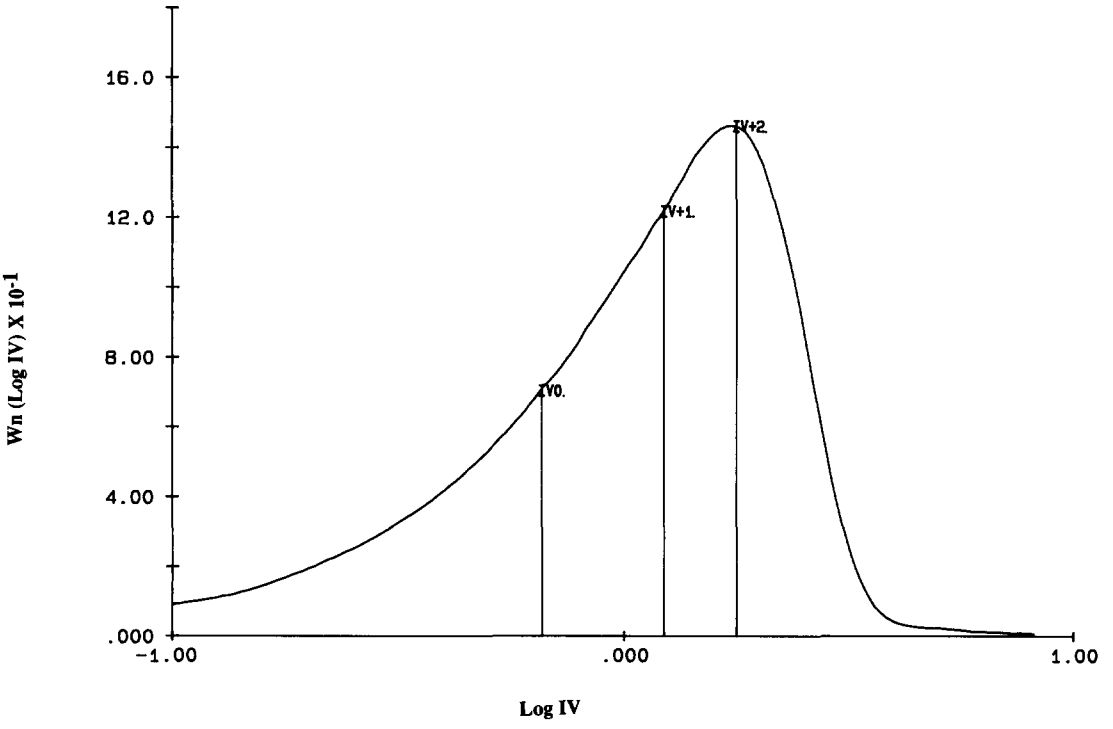

Figure 5
Intrinsic viscosity distribution plot of unknown sample of poly-γ-benzyl glutamate.

Table 2
Summary of SEC–DV results on unknown poly-γ-benzyl glutamate in DMAC–LiBr (99.9:0.1, v/w)

Reported *MW** = 120,000				
Calculated†	*Mw*	= 114,270	*Rgw*	= 18.76 nm
	Mn	= 79,052	*Rgn*	= 15.43 nm
	Mz	= 141,909	*Rgz*	= 21.37 nm

*Molecular weight reported by Sigma Chem. Co. (St Louis, MO, USA).

†The average Mark–Houwink exponent a = 1.26, indicating that the sample is a rigid rod.

high precision of the method offers a good possibility of making SEC-viscometry a tool for determining PGB and other polyamino acid *MWD* and *RgD*.

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