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ORIGINAL CONTRIBUTION

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Behavior of γ-ray-irradiated pullulan in aqueous solutions of cationic (cetyltrimethylammonium hydroxide) and anionic (sodium dodecyl sulfate) surfactants

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Abstract γ-ray-irradiated pullulan macromolecules acquire properties of an anionic polyelectrolyte and, upon aggregation with the oppositely charged surfactant cetyltrimethylammonium hydroxide, are found to precipitate according to their molecular weight. This provides a convenient means for obtaining polymer fractions with a narrower molecular-weight distribution than those of the original samples. The method can be employed to obtain fractions of radiation-modified pullulan required in the production of a blood-plasma substitute. Anionic properties of γ-ray-irradiated pullulan also manifest themselves in interactions with sodium dodecyl sulfate (SDS) in aqueous solution, which result in a significant change in the viscous behavior of the polysaccharide. Upon an increase in the concentration of γ-ray-irradiated pullulan in an SDS solution, the reduced viscosity of the polymer first increases and, upon reaching a certain concentration, C^*, decreases. The C^* values were found to be dependent on the molecular weight of the polymer. The phenomena observed are discussed in terms of the general theory of polymer solutions within which C^* is treated as a critical concentration at which interpenetration of polymer molecules becomes important. Unperturbed dimensions of γ-ray-irradiated pullulan macromolecules were estimated on the basis of experimental viscosimetric data.

Keywords γ-ray-irradiated pullulan · Fractionation · Unperturbed dimensions · Sodium dodecyl sulfate · Cetyltrimethyl ammonium hydroxide

Introduction

Molecular characteristics of biodegradable polysaccharide pullulan have been extensively investigated in recent years for theoretical reasons and because of its industrial applications [1–5]. A number of essential products were developed via chemical modification of this polysaccharide [6, 7]. Most pullulan derivatives possess improved physical and chemical properties and exhibit high biocompatibility, which is important from the viewpoint of their possible biomedical applications [7–10]. In our previous work [11], we reported the hydrodynamic properties of γ-ray-irradiated pullulan used in production of a blood-plasma substitute [9, 10].

The ability of surfactants to conjugate electrostatically with ionic polysaccharide derivatives and to change properties of a polymer solution is well known [12]. In the manufacturing and processing of pullulan, surfactants are used to purify pullulan from biosynthesis by-products (acidic polysaccharides) [13, 14]. The procedure comprises treatment of cultural media by the cationic surfactant cetyltrimethylammonium hydroxide (CTA) and removal of the precipitate [14]. The precipitation of acidic polysaccharides occurs owing to

complexation with CTA by means of ion–ion interactions between charges of the hydrophilic moiety of the surfactant molecule and oppositely charged groups of the macromolecule [12]. Native pullulan, being a neutral polysaccharide, does not interact with CTA and, therefore, remains dissolved.

In contrast with native pullulan, radiation-modified pullulan acquires the properties of an anionic polyelectrolyte, and the concentration of the newly formed functional groups along the polysaccharide chain increases with decreasing molecular weight of fractions of a γ-ray-irradiated sample [15].

In this context, the objective of the present work was to evaluate the possibility of fractionation of γ-ray-irradiated pullulan with the use of CTA and to examine the effect of anionic surfactants on the viscous behavior of γ-ray-irradiated pullulan in aqueous solution.

Experimental

Materials

A sample of pullulan with a molecular weight (M) of approximately 1×10^3 kDa was synthesized microbiologically with the use of *Auerobasidium pullulans* strain [10] and was subsequently purified from proteins and the acidic polysaccharide as described elsewhere [10]. Aqueous solutions of native pullulan (6 g/dl) were exposed to ^{60}Co γ-rays in air (50–70 kGy). The samples of γ-ray-irradiated pullulan thus obtained were lyophilized and used in the experiments.

Sodium dodecyl sulfate (SDS), CTA, and all mobile-phase ingredients purchased from Merck were of analytical reagent grade. Phenolphthalein purchased from Sigma was of ACS reagent grade.

Water for mobile-phase preparation was purified using a Milli-Q system (Millipore).

CTA-mediated fractionation

Two samples of γ-ray-irradiated pullulan with weight-average molecular weights (M_W) of 55 and 35 kDa and coefficients of polydispersity (M_W/M_N) of 2.10 and 1.95, respectively, denoted as IP55 and IP35, were dissolved in water to a final concentration of 1.5 g/dl. Fractionation was performed by adding an aqueous solution of CTA (0.36 g/dl) at 25 °C to the solutions of IP55 and IP35 until the polymer–surfactant systems turned opalescent. These solutions were centrifuged to separate the fractions. The precipitates were sedimented at 15,000*g* using a Suprafuge 22 (Heraeus Sepatech) thermostabilized at 25 °C by using an integrated temperature controller. The supernatants were collected and mixed with CTA for further fractionation. The precipitates were dissolved in 0.1 M HCl. The molecular-weight distributions (MWD) of the fractions collected were analyzed by means of high-performance size-exclusion chromatography (SEC).

SEC procedures were carried out using a system consisting of a Varian 9012 pump and a Waters 410 differential refractometer. Shodex KB 806M and Shodex KB 803M (300×8-mm inner diameter) columns (Waters) operated in series were used for separation. The mobile phase consisting of a 0.05 M aqueous solution of NaH_2PO_4 containing 0.05% sodium azide as a bacterial growth inhibitor was pumped at a flow rate of 0.6 ml/min. All other SEC conditions, including universal calibration for measurements of MWD of fractions, were as described earlier [15].

Viscosimetric measurements

Monodisperse samples of γ-ray-irradiated pullulan were obtained from IP55 by preparative gel filtration on a column (120×5-cm inner diameter) packed with Sephadex G-200 gel [11, 15]. Their M_W ranged from 24 to 180 kDa, and the polydispersity coefficients were 1.15–1.20. Quantification of the carboxylic groups in the structure of the modified fractions was carried out by titration with 0.01 M NaOH using phenolphthalein as an indicator. Prior to titration, the solutions of the fractions were passed through a column filled with Dowex-50 cation-exchange resin in the H form. The average number of carboxylic groups per macromolecule of γ-ray-irradiated pullulan was estimated to be 6–8 for fractions with $M_W > 30$ kDa and 11–13 for $M_W < 30$ kDa.

Weighted amounts of lyophilized samples of γ-ray-irradiated pullulan were dissolved in an aqueous solutions of SDS with a surfactant concentration of 5 g/dl, and in pure water. The solutions obtained were filtered and incubated at 25 °C for 6–8 h and after that were transferred into a viscosimeter.

In order to prevent polymer adsorption on the capillary wall [16] and to achieve accurate measurements within the full concentration range, we used an Ubbelohde-type viscosimeter with a capillary diameter of 0.34 mm having a flow time, t_0, of 380 s for pure water at 25 °C. For 5 g/dl aqueous SDS solution, t_0 was 417 s. The values of the flow times for solutions of γ-ray-irradiated pullulan in pure water and in aqueous SDS solutions averaged over five independent measurements were used to calculate the reduced viscosity, η_{sp}/C, where C is the polymer concentration.

Results and discussion

Efficiency of CTA-mediated fractionation

The first fractions obtained from the solutions of IP55 and IP35 with a polymer concentration of 1.5 g/dl upon addition of CTA are high-molecular-weight components of the original samples, as is evident from the corresponding chromatograms in Fig. 1 a and b. The peaks of the subsequently segregated fractions are shifted toward higher elution volumes. The weight-averaged molecular weights and the polydispersity coefficients of the fractions obtained upon addition of fixed amounts of CTA are given in Table 1. As can be seen from Table 1, fractions segregated from IP55 and IP35 at a certain concentration of CTA (identical for both polymer samples) have different M_W values, which is ascribed to a difference in the molecular-weight characteristics of the original polydisperse γ-ray-irradiated pullulan samples. Precipitation of a polymer from a polymer–surfactant system occurs at the surfactant concentration required to neutralize the charges of the macromolecule [12]. In the case of γ-ray-irradiated pullulan, interactions with CTA are due to the presence of carboxylic groups in the modified polysaccharide structure [15]. The high-molecular-weight fractions in the γ-ray-irradiated pullulan samples possess only weak polyelectrolyte properties [15] and in presence of CTA precipitate first, whereas fractions of lower M_W have a higher degree of modification and, consequently, require a higher amount of CTA bound to precipitate.

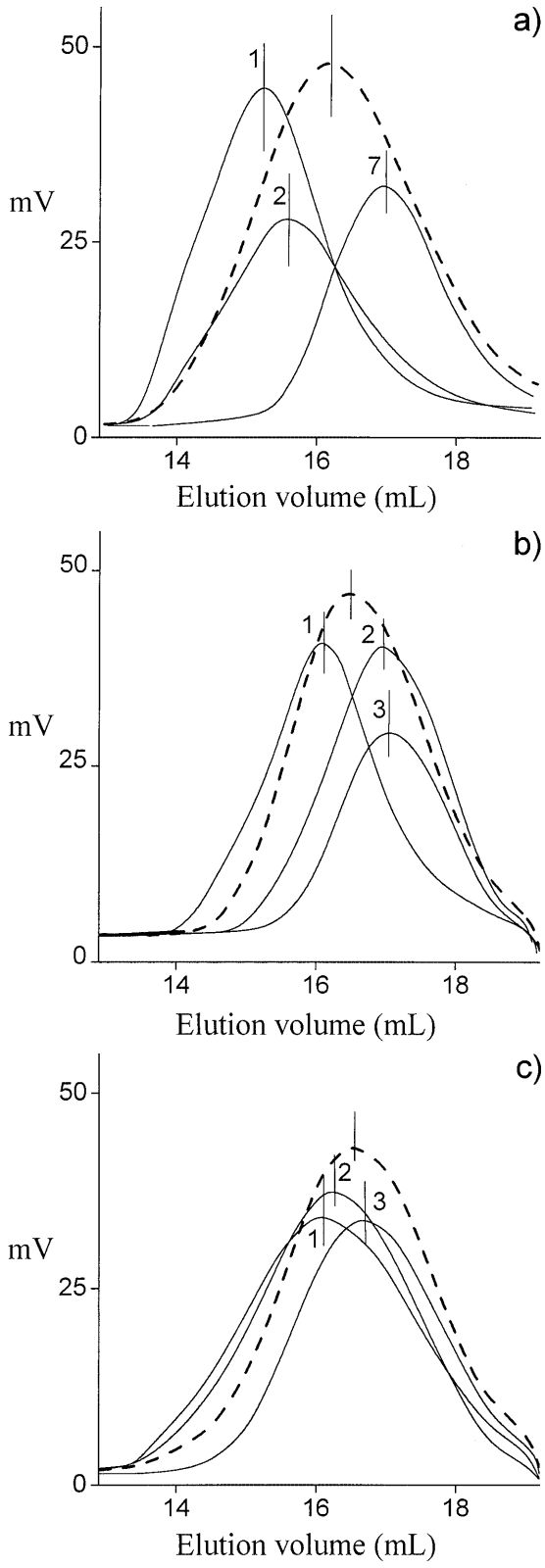

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Fig. 1 Size-exclusion chromatography chromatograms for certain fractions precipitated upon addition of cetyltrimethylammonium hydroxide (*CTA*) to 1.5 g/dl solutions of **a** IP55, **b** IP35, and **c** 5.0 wt% solution of IP35. The positions of the peaks are indicated by *vertical lines*; fraction numbers are shown at the corresponding elution curves. Chromatograms of the original samples are presented by *dashed curves*

The fractions precipitate in the form of polymer–surfactant complexes, and the amount of CTA bound is expected to be equal to the number of carboxylic groups in the segregated γ-ray-irradiated pullulan sample. Taking into account the M_W values (Table 1) and the weight contributions of individual fractions to the MWD of original (nonfractioned) samples, the molar concentrations were calculated for each of the fractions collected. The data obtained were compared with the corresponding molar concentrations of CTA. The average number of carboxylic groups per macromolecule of γ-ray-irradiated pullulan for both IP35 and IP55 was found to be 8–10 for fractions with $M_W > 30$ kDa and 15–17 for $M_W < 30$ kDa, which is consistent with the results obtained by titration (see Experimental) and is comparable with the corresponding data for γ-ray-irradiated dextran of the same molecular-weight range [17].

As a result of the one-stage fractionation, the polydispersity coefficients of the fractions were found to be lower than the original ones ($M_W/M_N \approx 2.0$) and assumed values in the range 1.2–1.6 (Table 1). The methods of SEC and high-osmotic-pressure chromatography [18] are known to be more efficient in obtaining samples with low M_W/M_N values; however, fractionation using CTA has proved to be still efficient in resolving the main component of relatively monodisperse fractions with $M_W/M_N = 1.2$ obtained by means of SEC from the low-molecular-weight tail (Fig. 2). Thus, the method proposed can be of use in producing a clinically active fraction of radiation-modified pullulan free from low-molecular-weight fragments.

The samples obtained upon addition of CTA to the solutions of γ-ray-irradiated pullulan with a polymer concentration of 5.0 g/dl had MWD similar to those of the original samples (Fig. 1c), i.e., in contrast to 1.5 g/dl solutions, no fractionation was achieved in this case. This can be attributed to the simultaneous sedimentation of the chains because of their overlapping. The effect of interpenetration of coils also appears to be pronounced in the viscous behavior of γ-ray-irradiated pullulan in concentrated SDS solutions and is discussed in the following section.

Viscous behavior of γ-ray-irradiated pullulan in concentrated SDS solutions

The typical dependences of the reduced viscosity of γ-ray-irradiated pullulan [15] in aqueous solutions of SDS, $(\eta_{sp}/C)^{SDS}$, on the polymer concentration, C, are

Table 1 Values of M_W and M_W/M_N for fractions obtained from γ-ray-irradiated pullulan samples IP55 and IP35 upon fractionation with the use of cetyltrimethylammonium hydroxide (*CTA*)

Fraction number	Concentration of CTA ($10^6 \times$ M)	IP55		IP35	
		M_W (kDa)	M_W/M_N	M_W (kDa)	M_W/M_N
1	0.36	107.0	1.36	77.0	1.54
2	0.74	73.0	1.30	63.0	1.43
3	0.99	53.7	1.42	58.0	1.27
4	1.37	45.0	1.41	54.0	1.31
5	1.76	34.0	1.28	47.6	1.32
6	2.19	26.7	1.32	39.4	1.26
7	3.29	14.6	1.40	27.0	1.32
8	4.12	12.3	1.25	19.0	1.28

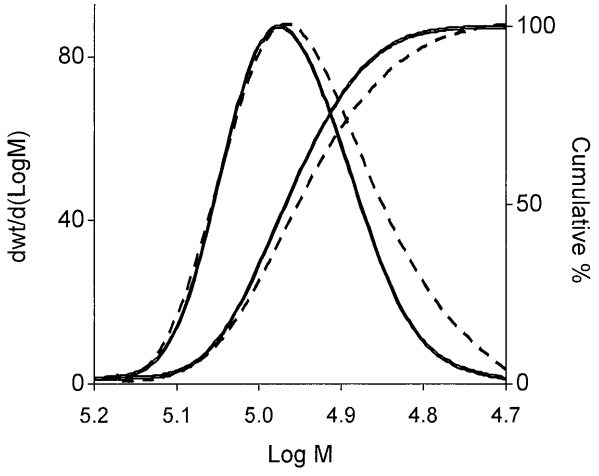

Fig. 2 Molecular-weight distributions of a γ-ray-irradiated pullulan fraction with $M_W = 48$ kDa and $M_W/M_N = 1.2$ before (- - -) and after (——) precipitation with CTA

shown in Fig. 3a. In contrast to the linear dependences of (η_{sp}/C) versus C in pure water, these dependences (Fig. 3b) show pronounced nonlinearity. At the surfactant concentration corresponding to the critical micelle concentration (cmc), a decrease in the surfactant solution viscosity is known to take place [19]. This was indeed found (Fig. 4). The presence of electrolytes in surfactant solutions usually leads to an increase in the cmc value [19] and, therefore, changes in the viscous properties of the surfactant–water system at the cmc can serve as a possible explanation of the character of the $(\eta_{sp}/C)^{SDS}$ versus C dependences in Fig. 3a. However, the maximum value of the cmc (around 2 g/dl) for SDS in the aqueous solution of the γ-ray-irradiated pullulan sample ($M_W = 24$ kDa) having the highest degree of modification among the fractions investigated was obtained at a polymer concentration of 5 g/dl. In experiments investigating of the viscous behavior of γ-ray-irradiated pullulan, a more concentrated SDS solution (5 g/dl) was used and, therefore, the observed character of the $(\eta_{sp}/C)^{SDS}$ versus C dependences for γ-ray-irradiated pullulan (Fig. 3a) cannot be explained as being induced by desegregating action of the polyelectrolyte under investigation.

The reduced viscosity for polymer is a measure of the hydrodynamic volume of macromolecules; therefore, the primary increase in $(\eta_{sp}/C)^{SDS}$ as a function of C is explained by progressive occupation of the volume by isolated macromolecules. The further decrease of $(\eta_{sp}/C)^{SDS}$ and the hydrodynamic volume at higher concentrations can be attributed to the cooperative effect of coils overlapping and chain condensation into dense and compact particles, which result from mutual repulsion between γ-ray-irradiated pullulan and SDS.

A concentration immediately preceding the onset of the interpenetration of coils corresponds to the polymer concentration C^* at which macromolecules uniformly fill the solution volume [20–23]; at this point the dependence of $(\eta_{sp}/C)^{SDS}$ on C shows a downward turn (Fig. 3a). The theoretically predicted value of this concentration, C^*_{th}, for a polymer of molecular weight M lies within the region determined by the inequality [20]

$$3M/4\pi N_A R_g^3 \leq C^*_{th} \leq M/N_A R_g^3 \ , \qquad (1)$$

where R_g is the radius of gyration, and N_A is Avogadro's number.

Using the dependence of the ratio of the reduced viscosities of the polymer in an SDS solution, $(\eta_{sp}/C)^{SDS}$, and in pure water on the polymer concentration, the values of C^* were obtained for each of the fractions investigated. It was found that C^* increases with decreasing M_W of γ-ray-irradiated pullulan.

Taking into account the following relationship between the hydrodynamic radius, R_η, and M_W established for γ-ray-irradiated pullulan in our previous work [11],

$$R_\eta = (0.15 \pm 0.03) \times M_W^{0.52 \pm 0.02} \ ,$$

the values $R_g = R_\eta \xi$, where ξ is a coefficient equal to 0.875 for random coils [24], were calculated. The variation limits for C^*_{th} calculated using Eq. (1) and C^* values obtained from the viscosimetric data are shown in Fig. 5. As can be seen, the experimental points lie within the established range of C^* and are closer to the lower line which

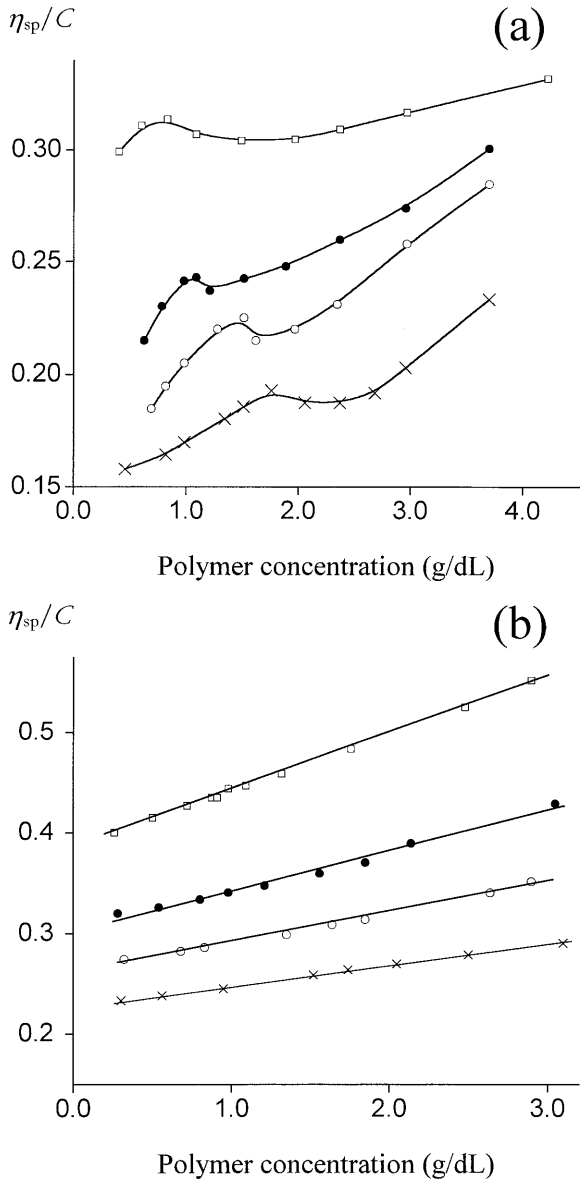

Fig. 3 Relationship between reduced viscosity, $(\eta_{sp})/C)^{SDS}$, at 25 °C and concentration of γ-ray-irradiated pullulan fractions with M_W of 180 kDa (*squares*), 140 kDa (*filled circles*), 90 kDa (*open circles*), and 50 kDa (*crosses*) in **a** 5g/dl SDS solution and **b** pure water

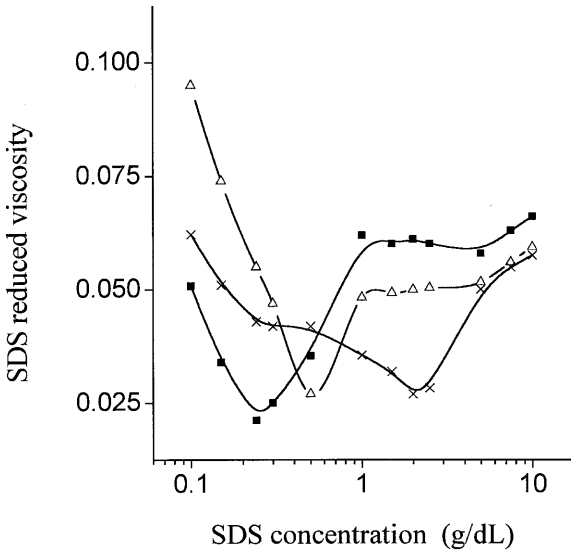

Fig. 4 Relationship between reduced viscosity of sodium dodecyl sulfate (SDS) at 25 °C and surfactant concentration in water (*squares*) and aqueous solutions of γ-ray-irradiated pullulan with polymer concentrations of 1 g/dL (*triangles*) and 5 g/dL (*crosses*)

determines the limit of C^* values for macromolecules in the conformation of random coils [20].

The hydrodynamic size of coils in solution and the concentration at which they start to overlap, as follows from Eq. (1), should depend on thermodynamic properties of the solvent. In a series of works [21–23], Dondos and coworkers have shown that the dependence of C^* on the polymer molecular weight obeys a scaling law with an exponent equal to the exponent α from the Mark–Houwink equation. From the slope of the best linear fit approximating experimental points in Fig. 5 we obtained the value of the exponent $\alpha \approx 0.5$ for the relationship $C^* \sim M_W^{-\alpha}$. This is indicative of the fact that in the SDS solution the attractive interactions between macromolecular segments of irradiated pullulan completely compensate for the chain expansion effect taking place owing to polymer–solvent interactions. Therefore, we believe that, on average, γ-ray-irradiated pullulan molecules in this solution behave as dense compact coils. This collapsed state is favored by the strong repulsion between the negatively charged outer surface of the polar micelle and negatively charged functional groups of γ-ray-irradiated pullulan. The reduced viscosity $(\eta_{sp}/C)^{SDS}$ in SDS solution is substantially lower compared to that measured in pure water, and the radius of gyration in SDS solution corresponds to the unperturbed radius (R_{g0}) of modified pullulan macromolecules. The latter relates [24] to the effective radius of gyration, R_g, as

$$R_{g0} = R_g/\alpha_R \quad , \tag{2}$$

where α_R is the expansion factor.

In calculating C^*_{th} with the use of Eq. (1) the difference between R_{g0} and R_g was neglected and, therefore, C^*_{th} is smaller than C^*, and

$$C^*/C^*_{th} = \alpha_R^3 \quad . \tag{3}$$

The calculated α_R values are plotted in Fig. 6. In order to compare α_R values obtained for γ-ray-irradiated pullulan with known data for similar polysaccharide derivatives, α_R was calculated from the viscosity expansion factor α_η established earlier [26] for γ-ray-irradiated

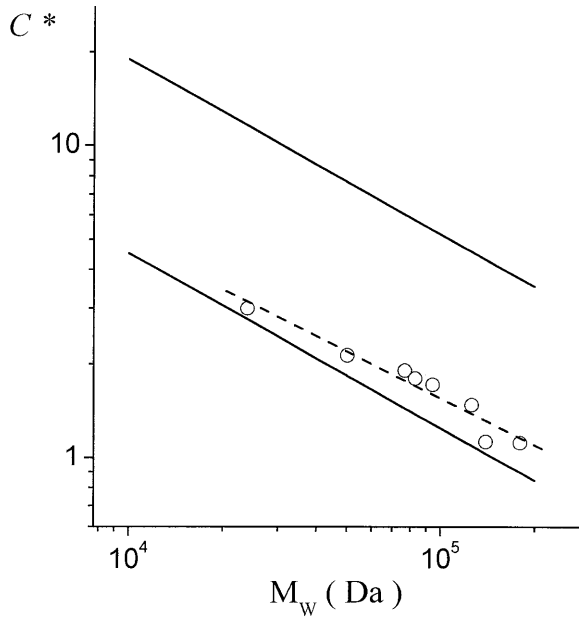

Fig. 5 Dependence of the C^* values calculated using Eq. (1) (*solid lines*) and obtained from viscosimetric data (*points*) on M_W of γ-ray-irradiated pullulan. The *dotted line* represents the best fit approximating experimental points

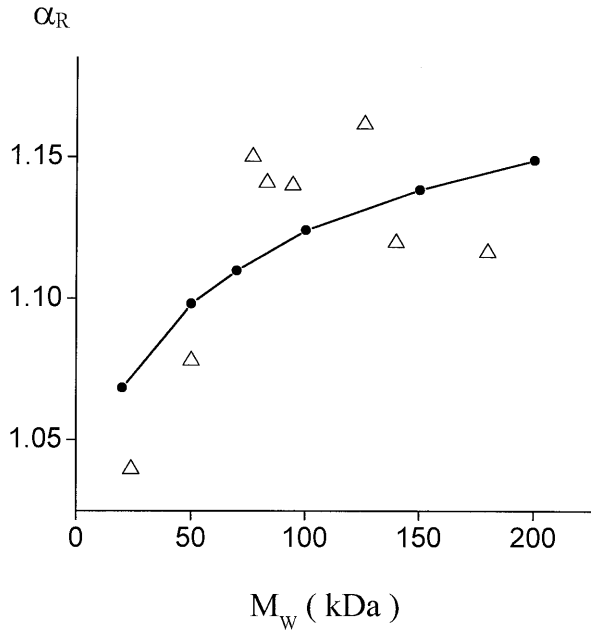

Fig. 6 Expansion factor, α_R, as a function of M_W for γ-ray-irradiated dextran (*circles*) and γ-ray-irradiated pullulan (*triangles*)

dextran according to the relationship $\alpha_\eta^3 = \alpha_R^{2.43}$ [25] (Fig. 6). Interestingly, the α_R values for γ-ray-irradiated pullulan are in good agreement, to within error in determination, with those for γ-ray-irradiated dextran within the range of molecular weights investigated. This allows a characteristic ratio of R_{g0}^2/M to be obtained by using Eq. (2) with the established α_R values. For γ-ray-irradiated pullulan, calculations yielded the average R_{g0}^2/M value of 5.59×10^{-17} cm^2 Da^{-1}, which is intermediate between the values known for γ-ray-irradiated dextran (5.34×10^{-17} cm^2 Da^{-1}) [26] and native pullulan (6.7×10^{-17} cm^2 Da^{-1}) [1]. Summarizing the results on R_{g0}^2/M and α_R for γ-ray-irradiated pullulan as compared with those for reference polysaccharides, one can conclude that the chain dimensions of γ-ray-irradiated pullulan in concentrated SDS solutions are rather close to the unperturbed ones.

Conclusions

In the present work, interactions between polar polysaccharide derivatives and oppositely charged surfactants were successfully applied to separation of γ-ray-irradiated pullulan into a series of fractions with narrow MWD. The method proposed can be employed in the production of a clinically active fraction of radiation-modified pullulan which can further be used as a base of blood-plasma substitute solutions. In the present study, we also demonstrated the influence of SDS on the viscous behavior of γ-ray-irradiated pullulan in aqueous solutions. The reduced viscosity of the radiation-modified polysaccharide in concentrated solutions of the surfactant is sensitive to whether macromolecules are isolated or overlapped. The viscosimetric data obtained allowed the unperturbed dimensions of γ-ray-irradiated pullulan molecules to be estimated.

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