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Binding of poly(amido amine) dendrimer to sodium hyaluronate in aqueous NaCl solution

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Abstract

Binding of poly(amido amine) dendrimer to sodium hyaluronate (NaHA) in aqueous 0.25 M NaCl solution has been investigated by static light scattering. It was observed that the apparent weight-average molecular weight and the radius of gyration increase with the ratio of NH_2 terminal groups in the dendrimer to the carboxylate groups in the NaHA, $[NH_2]/[COO^-]$. Up to $[NH_2]/[COO^-] = 31$, the observed variation of molecular weight was reproduced by the "average binding" model, where an average number of dendrimers binds to each NaHA chain. Based on the "critical binding" model, the maximum number, n_{max}, of dendrimers which can bind to a NaHA chain was calculated to be $n_{max} = 300$ for a solution of $[NH_2]/[COO^-] = 56$. The obtained value corresponds to the binding of one dendrimer per 1.5 repeating units on a NaHA chain. It is suggested from the observed radius of gyration that, while the dendrimer–NaHA complexes of $[NH_2]/[COO^-]$ up to 5 maintain a wormlike character similar to NaHA without bound dendrimers, those of $[NH_2]/[COO^-]$ above 10 behave like rigid rods. It is concluded that the hydrogen-bonding interaction, besides the electrostatic interaction, should play an important role in the formation of the NaHA–dendrimer complexes.

Keywords: Poly(amido amine) dendrimer; Sodium hyaluronate; Static light scattering; Weight-average molecular weight; Radius of gyration; Binding model; Critical binding

1. Introduction

Hyaluronan (hyaluronic acid, HA) is a water-soluble polysaccharide of the glucosaminoglycan family, which is composed of alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine. This polyanion is a biopolymer existing in extracellular matrixes such as tissues and also one of the components of body fluids in vertebrates, acting as lubricant and shock absorber [1]. From its pseudoplastic behavior, non-Newtonian viscosity, and elastic property, aqueous solutions of HA are used for rheological surgery in the biomedical field [2,3]. Synovial fluid includes various solutes, proteins, and salts, besides HA. It has been reported that protein plays an important role in the rheological properties of HA in the flow behavior of synovial fluid [4]. The relatively fast diffusion of coexisting molecules is another important target, since drug release by HA degradation can be used in pharmaceutical research.

The interaction of HA with small molecules was mainly investigated for complexes of sodium hyaluronate (NaHA) to salts of ionic surfactants with opposite charge [5–10]. The binding is cooperative and the aggregates consist of clusters of surfactants bound on an HA chain. It is noted that for those systems the coexistence of free monomers and micelles must be considered in solutions above the critical micelle concentration.

The complex formation of linear polymers with globular proteins, as well as the binding with colloidal particles, is considered from the viewpoint of the molecular design of enzyme reactions, protein separations, and gene delivery processes in the biochemical background. Gene delivery on complexes of DNA is very important in mammalian organisms, and strong complementary activation was investigated for complexes of DNA with polylysine, poly(ethyleneimine), and poly(amido amine) (PAMAM) dendrimers [11]. Among these polymers, since dendrimers

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are a kind of highly branched polymers with spherical structure, the dendrimers should be expected to behave like histone, which is known as a gene transport globular protein. Thus, dendrimers were used for the in vitro investigation of DNA transfer in living cells [12–16]. The electrostatic interaction between the polynucleotide and the PAMAM dendrimer is essential for the effective DNA transfer process [16]. The concentration dependence of the interaction between polynucleotide and dendrimers is just the opposite for small (second generation) and large (sixth generation) dendrimers [15].

Apart from the subject of genes, the binding of dendrimers to polyelectrolytes has been investigated [17–19]. The formation of complexes between polycations and carboxylated dendrimers occurs most readily for the 7.5th generation with high charge density [17] and at a critical pH [18]. The interaction of poly(propyleneimine) dendrimer with linear polyanion is compared with that with DNA [19].

In the present paper, we choose NaHA as a polyanion and a PAMAM dendrimer as a target molecule. The binding is investigated by static light scattering, and the molecular weight and the radius of gyration of the complex are evaluated, followed by the determination of the number of dendrimers bound to NaHA and the evaluation of the complex structure. The dependence of the complex formation on the mixing ratios is discussed.

2. Experimental section

Fourth generation PAMAM dendrimers with NH_2 terminal (molecular weight 14,210; 64 terminal groups) were synthesized as previously reported [20,21]. Microbial NaHA (repeating unit weight 401.3) was obtained from Contipro (Ústín. Orlicí, Czech Republic). Chemical structures of PAMAM dendrimer and NaHA are indicated in Fig. 1. An aqueous 0.25 M NaCl solution, an aqueous 0.25 M NaCl solution of dendrimer (10 wt%), and an aqueous 0.25 M NaCl solution of NaHA (0.2 wt%) were mixed in adequate amounts. The mixed solutions have different mixing ratios of dendrimer and NaHA, whereas the concentrations of NaHA (0.1 wt%) and NaCl (0.25 M) in the solutions are always constant. The number ratios of the NH_2 terminal groups in the dendrimer to the carboxylate groups in NaHA, $[NH_2]/[COO^-]$, were changed from 0 to 56, as listed in Table 1, where molar ratios of dendrimer and NaHA, [dendr]/[NaHA], are included. The solutions prepared were used for the light-scattering experiment within 24–48 h.

Static light-scattering measurements at 30–150° scattering angles (10° interval) were carried out at 25 °C with Otsuka Electronics DLS-700 and DLS-7000 spectrophotometers at a wavelength of 488 nm from an Ar ion laser. The solutions were filtrated through a 0.8-μm Millipore filter. The specific refractive index increment was measured by an Otsuka Electronics RM-102 refractometer with an iodine lamp at 488 nm. The value of 0.126 $cm^3\,g^{-1}$ obtained was

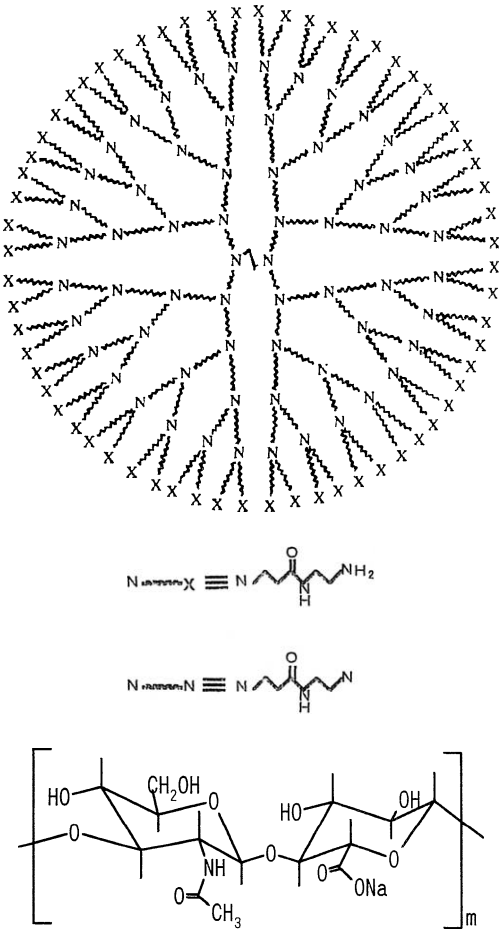

Fig. 1. Chemical structures of a fourth generation amino-terminated poly(amido amine) dendrimer (upper) and a repeating unit of sodium hyaluronate (lower).

used for the calculation of the light-scattering intensity. The specific refractive index increment was found to be independent of composition within experimental error. The apparent weight-average molecular weight $M_{w,\mathrm{app}}$ and radius of gyration R_G were calculated from the equations

$$Kc/\Delta R_\theta = (1/M_w)(1 + Rg^2Q^2/3) + 2B_2c$$

(at low scattering angle),

$$1/M_{w,\mathrm{app}} = 1/M_w + 2B_2c$$

(at extrapolation to zero scattering angle),

$$K = 4\pi^2 n_0^2(\partial n/\partial c)^2/N_A\lambda^4,$$

$$Q = 4\pi n_0 \sin(\theta/2)/\lambda, \quad (1)$$

where K is the optical constant, c is a total solute concentration, ΔR_θ is the reduced scattering intensity at scattering angle θ, M_w is the weight-average molecular weight, Q is the scattering angle magnitude, B_2 is the second virial coefficient, n_0 is the refractive index of the solvent, $(\partial n/\partial c)$ is the specific refractive index increment of the solution, N_A is the Avogadro number, and λ is the wavelength of light in vacuo. The more detailed procedure of the measurements

Table 1
M_w, R_G, and a values for mixed solutions of PAMAM dendrimers and NaHA in 0.25 M NaCl

$[NH_2]/[COO^-]$	[dendr]/[NaHA]	$M_{w,app}$ (10^6)	R_G (nm)	$M_{w,nb}$ (10^6)	$M_{w,ab}$ (10^6)	$M_{w,dp}$ ($f_{dp}=0.1$) (10^6)	$M_{w,dp}$ ($f_{dp}=0.2$) (10^6)	a (nm)
0	0	0.184	45.8					18.5
0.05	0.38	0.252	48.5	0.179	0.189	0.208	0.228	21
0.1	0.66	0.257	52.4	0.162	0.194	0.214	0.234	25
0.5	3.3	0.255	49.0	0.136	0.235	0.258	0.288	22
1	6.6	0.262	49.0	0.115	0.286	0.315	0.355	22
5	38	0.263	34.7	0.053	0.692	0.761	0.891	14
10	66	1.99	119	0.038	1.20	1.32	1.57	
18	158	1.98	89.7	0.027	2.02	2.22	2.64	
31	237	3.34	104	0.023	3.30	3.63	4.33	
56	423	2.77	73.5	0.019	5.83	6.46	7.73	

and analyses is described in the literature [22]. Small-angle neutron scattering (SANS) measurements were performed on a SANS-U spectrometer installed at the Japan Atomic Energy Research Institute research reactor JRR-3M. For SANS measurements, D_2O was used instead of H_2O.

3. Results and discussion

The apparent weight-average molecular weight $M_{w,app}$ and the radius of gyration R_G for mixed solutions of PAMAM dendrimer and NaHA in 0.25 M NaCl were evaluated from the static light-scattering measurements. The numerical values are listed in Table 1 and plotted as a function of $[NH_2]/[COO^-]$ in Fig. 2. The values of $M_{w,app}$ and R_G at $[NH_2]/[COO^-]$ above 10 are remarkably higher than those of NaHA without dendrimer, indicating the complex formation. Under the condition of dilute solute concentration and high salt concentration, the second term in Eq. (1) should be less than the first term. Then M_{app} can be approximated to be M_w.

Considering the coexistence of PAMAM dendrimer and NaHA, one of the possibilities is a mixture of independent molecules. In this "nonbinding" model, dendrimers do not interact with NaHA chains, as illustrated in Fig. 3a. Thus the weight-average molecular weight $M_{w,nb}$ of the mixture is described by

$$M_{w,nb} = \frac{N_{HA}M_{HA}^2 + N_{den}M_{den}^2}{N_{HA}M_{HA} + N_{den}M_{den}}, \tag{2}$$

where N_i and M_i are number of molecules and molecular weight, respectively, of molecule i. The subscripts $i = HA$ and den denote NaHA and dendrimer, respectively.

If the average number n ($= N_{den}/N_{HA}$) of PAMAM dendrimers per NaHA binds on each NaHA chain (see Fig. 3b) and forms complexes with it, n is equal to the value of [dendr]/[NaHA] given in Table 1. Thus, the weight-average molecular weight $M_{w,ab}$ for the "average binding" model can be written as

$$M_{w,ab} = \frac{N_{HA}(M_{HA} + nM_{den})^2}{N_{HA}(M_{HA} + nM_{den})} = M_{HA} + nM_{den}. \tag{3}$$

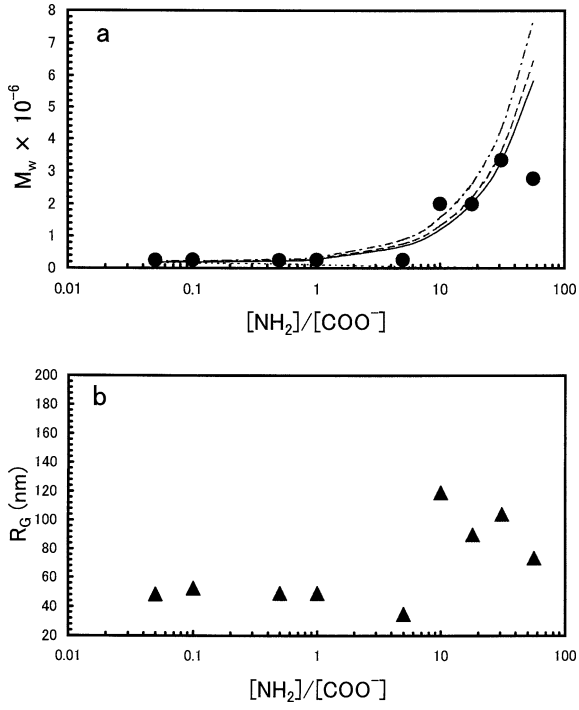

Fig. 2. M_w and R_G for mixed solutions of PAMAM dendrimers and NaHA in 0.25 M NaCl as a function of $[NH_2]/[COO^-]$. (a) Observed and calculated M_w. (●) $M_{w,app}$; (···) $M_{w,nb}$; (—) $M_{w,ab}$; (- -) $M_{w,dp}$ ($f_{dp}=0.1$); (·-·-) $M_{w,dp}$ ($f_{dp}=0.2$). (b) Observed R_G.

When a fraction f_{dp} of NaHA chains bound n dendrimer molecules forms dimers conjugated through dendrimers as seen in Fig. 3c, the weight-average molecular weight $M_{w,dp}$ for the "dimeric polymer" model is

$$M_{w,dp} = \frac{N_{HA}(1-f_{dp})(M_{HA}+nM_{den})^2 + 2N_{HA}f_{dp}(M_{HA}+nM_{den})^2}{N_{HA}(1-f_{dp})(M_{HA}+nM_{den}) + N_{HA}f_{dp}(M_{HA}+nM_{den})}$$
$$= (1+f_{dp})(M_{HA}+nM_{den}). \tag{4}$$

Molecular weights calculated according to Eqs. (2)–(4) are compared with the observed values in Table 1 and Fig. 2. The values calculated by Eq. (2) decrease with increasing dendrimer content, in contrast to the observed ones. On the other hand, the values given by Eq. (3) increase with dendrimer content and produce similar profile to the observed

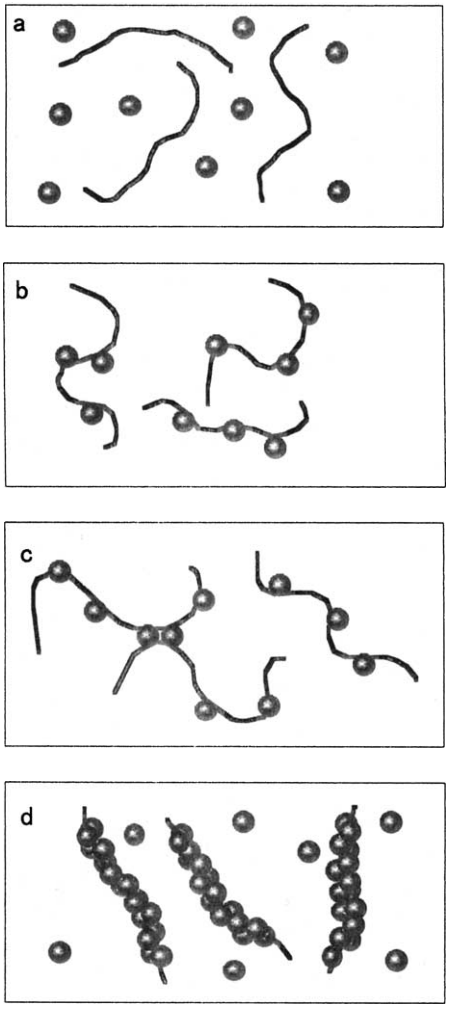

Fig. 3. Models of dendrimer binding to NaHA chains: (a) a nonbinding model; (b) an average binding model; (c) a dimeric polymer model; (d) a critical binding model.

ones. The calculations based on Eq. (4), since $M_{w,\mathrm{dp}}$ is $1 + f_{\mathrm{dp}}$ times larger than $M_{w,\mathrm{ab}}$, show that the coexistence of dimers of dendrimer-bound NaHA raises weight-average molecular weights. The contamination or fraction f_{dp} of dimeric aggregates is less than 20%, as it can be deduced from Fig. 2. The no or slightaggregation behavior originates in the repulsion of amino terminals, which are located in the exterior of the complexes. It has been reported that when protein binds to a polyanion chain in an aqueous solution, the stoichiometric neutralization occurs [23]. Neutral complexes of proteins and polyelectrolytes are subsequently associated into neutral aggregates. It seems that this is not the case for the dendrimer–NaHA complexes, because the complexes carry excess charges on dendrimers.

While the observed weight-average molecular weights are consistent with Eq. (3) or (4), at least, up to $[NH_2]/[COO^-] = 31$, it is noticed that the observed value at $[NH_2]/[COO^-] = 56$ is almost half of the $M_{w,\mathrm{ab}}$ value. This implies that excess dendrimers coexist at the free state. When $n_{\max}$ dendrimers can bind on a NaHA chain, the weight-average molecular weight $M_{w,\mathrm{cb}}$ for the "critical binding" model (see Fig. 3d) can be calculated from

$$M_{w,\mathrm{cb}} = \frac{N_{\mathrm{HA}}(M_{\mathrm{HA}}+n_{\max}M_{\mathrm{den}})^2+(N_{\mathrm{den}}-n_{\max}N_{\mathrm{HA}})M_{\mathrm{den}}^2}{N_{\mathrm{HA}}(M_{\mathrm{HA}}+n_{\max}M_{\mathrm{den}})+(N_{\mathrm{den}}-n_{\max}N_{\mathrm{HA}})M_{\mathrm{den}}}. \quad (5)$$

The $n_{\max}$ value calculated for a solution of $[NH_2]/[COO^-] = 56$ is 300. This value implies the binding of one dendrimer per 1.5 repeating units of a NaHA chain, since the average degree of polymerization of NaHA is 459.

Considering the repeating unit of NaHA as a cylinder of diameter 0.6 nm and length 0.85 nm on the basis of the calculated size, the contour length of NaHA is 390 nm. It is known that NaHA takes on a semiflexible character in an aqueous 0.25 M NaCl solution [24–26]. Thus, the persistence length a of wormlike NaHA chains can be calculated from the observed R_G values and the contour length L of NaHA. The persistence lengths evaluated using the equation [27,28]

$$R_G = a\big[(2a^2/L^2)\{(L/a) - 1 + \exp(-L/a)\} - 1 + (L/3a)\big]^{1/2} \quad (6)$$

are listed in Table 1. Values of 18.5 nm for NaHA and 14–25 nm for dendrimer-bound NaHA at $[NH_2]/[COO^-] = 0.05$–5 are obtained. These results show that the binding of dendrimer scarcely influences the persistence length of dendrimer-bound NaHA at $[NH_2]/[COO^-]$ up to 5. The persistence length of NaHA in aqueous salt solutions is reported to be 4.5 to 9.0 nm [24–26]. These values are close to or slightly smaller than values found in the present system, but it has to be considered that chain length and the salt concentration should influence the persistence length of NaHA. It is reported that NaHA is more extended in an aqueous solution without salt than in solutions with salt because of the electrostatic repulsion [29].

When dendrimer molecules abundantly bind on NaHA, NaHA chains should become elongated and dendrimer–NaHA complexes take a rod shape, because the cross-sectional diameter of the complex becomes greater. The diameter of the spherical shape of the fourth generation PAMAM dendrimer can be 5.2 nm, since the estimate from the CPK model is 3.4–7.0 nm. Thus the rod diameter $2r$ of a dendrimer–NaHA complex can be assumed to be 11 nm. From this, a radius of gyration of 110 nm is evaluated from the equation

$$R_G = (L^2/12 + r^2/2)^{1/2}. \quad (7)$$

The obtained value is close to the observed values for solutions of $[NH_2]/[COO^-] = 10$–30, suggesting the adequacy of the rod-shape assumption for the dendrimer-rich complexes. The decrease of the radius of gyration for a solution of $[NH_2]/[COO^-] = 56$ is due to the coexistence of free dendrimers. The structural change of NaHA before and after binding of PAMAM dendrimers for $[NH_2]/[COO^-] \geqslant 10$ is illustrated in Fig. 4.

At the critical binding, the volume of a dendrimer-bound NaHA chain, which is a cylinder 11 nm in diameter and 390

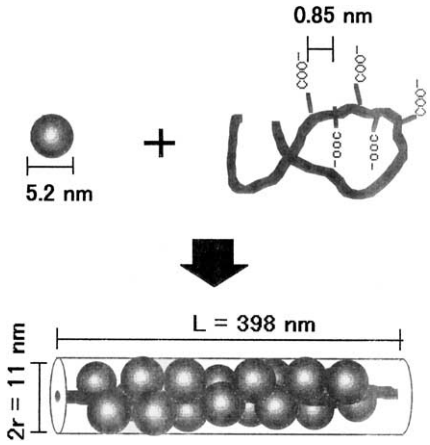

Fig. 4. Structural change of NaHA before and after binding of PAMAM dendrimers at $[NH_2]/[COO^-] \geqslant 10$.

nm in length, is 1.5 times larger than the total volume of a NaHA chain and 300 dendrimer molecules with a diameter of 5.2 nm. Therefore, it is possible that 300 dendrimers can bind on a single NaHA chain, although the dendrimer structure should be distorted changing from a sphere to a corn grain shape. This is possible because the soft structural character of dendrimers allows them to be changeable. The complex structure is morphologically similar to that observed for the tobacco mosaic virus. A maximum of 1.5 protonated amino terminals in a PAMAM dendrimer are able to interact electrostatically with the carboxylate anions of D-glucuronate on NaHA. Is the electrostatic interaction enough to capture many dendrimers on the NaHA chain against the steric hindrance and the repulsion force between dendrimers on same NaHA chain? Hydrogen bonding between amide and amino groups in the dendrimers and the hydroxyl groups of NaHA should also be dominant and important to form the NaHA-dendrimer complex. Complexation was also reported for a human serum albumin–nonionic poly(ethylene glycol) system [30]. In this case, the hydrogen bonding plays an important role in the complexation. The mechanism for the formation of NaHA–dendrimer complex can also be compared with that reported for polyanion–dendrimer and DNA–dendrimer complexes [16,19]. Ionic groups of the polyanion can form ion pairs with all outer primary amino groups and inner tertiary amino groups in protonated dendrimers, while the charged DNA binds only to the charged terminal groups of the dendrimer [19]. The wrapping of dendrimer by DNA occurs for dendrimers of higher generations, although it does not happen for lower generations [16].

Figure 5 shows the curves of the SANS intensity vs scattering vector magnitude Q for mixed solutions of PAMAM dendrimers and NaHA at $[NH_2]/[COO^-] = 0$, 1, and 56 in 0.25 M NaCl. As a comparison, a result for an aqueous 0.25 M NaCl solution of PAMAM dendrimers (1 wt%) is included. At low $[NH_2]/[COO^-]$ values such as unity, SANS intensity is very weak, and this behavior is similar to that of an aqueous solution of NaHA without dendrimer, indicating a small contribution of dendrimers bound to NaHA. This situation is consistent with that observed by light scattering, as described above. However, at high $[NH_2]/[COO^-]$ values such as 56, the intensity increases at $Q < 0.01$ Å^{-1}. A similar increase was also observed for an aqueous NaCl solution of dendrimers, indicating the strong contribution of dendrimers bound to NaHA on SANS. The SANS results then support the difference between complexes at $[NH_2]/[COO^-] = 1$ and 56, which was determined by the light-scattering analysis described above.

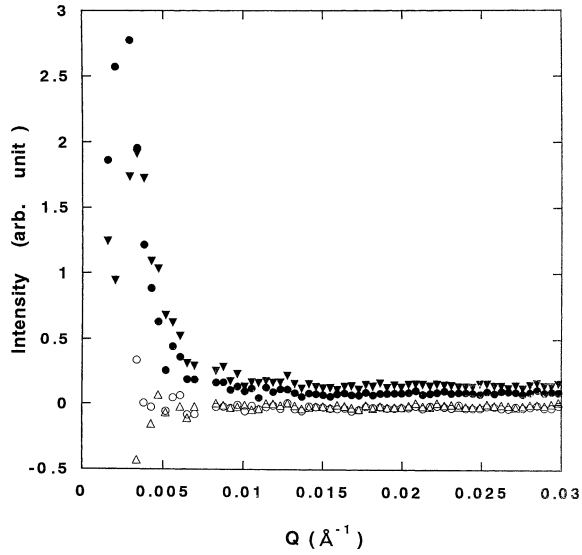

Fig. 5. SANS intensity vs the scattering vector magnitude Q for mixed solutions of PAMAM dendrimers and NaHA in 0.25 M NaCl. $[NH_2]/[COO^-]$: (○) 0; (△) 1; (▼) 56. (●) An aqueous 0.25 M NaCl solution of PAMAM dendrimer (1 wt%).

4. Conclusions

An investigation was carried out for the binding between two kinds of polymers that have different structures and characters, that is, a linear, wormlike NaHA and a highly branched spherical PAMAM dendrimer. When the dendrimers bind to the NaHA chain, the wormlike character of NaHA is scarcely changed at low mixing $[NH_2]/[COO^-]$ ratios. However, the structure of NaHA changes to a rodlike one at high $[NH_2]/[COO^-]$ values, where the terminal numbers of dendrimers exceed at least five times the repeating unit numbers of a NaHA chain. A maximum value of one dendrimer per 1.5 repeating units of the NaHA chain has been evaluated. The electrostatic interaction is essential but not sufficient to connect such large numbers of dendrimers with NaHA, because the number of amino terminal groups is 42 times larger than the number of carboxylates. In addition to the electrostatic interaction, the hydrogen bonding between hydroxyl groups in NaHA and the amide and amino groups in the dendrimer must play an important role in the formation of the complex to overcome the repulsive interaction between the dendrimers.

Acknowledgments

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