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Analysis of Dimer Impurity in Polyamidoamine Dendrimer Solutions by Small-angle Neutron Scattering

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Abstract Dimer impurity in the solution of a generation five (G5) polyamidoamine (PAMAM) dendrimer has been investigated by small-angle neutron scattering (SANS). The existence of dimer impurity in dendrimer solution was evidenced by indirect Fourier transform (IFT) analysis of the SANS data, in which the maximum dimension of particles in solution was found to be about twice the diameter of G5 dendrimer. We then developed an analytical model which accounts for the scattering contribution from both dendrimer monomer and dimer. The experimental data were well fitted by using the established model. The results showed that the amount of dimer impurities is significant for the measured three batches of G5 PAMAM dendrimers.

Keywords PAMAM dendrimer; Dimer impurity; Small-angle neutron scattering; Scattering model

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INTRODUCTION

Dendrimers are known as a family of synthetic macromolecules with well-defined tree-like, hyperbranched structure, and molecular size.^[1] Among the different types of dendrimers, starburst polyamidoamines (PAMAM) are one of the most characterized and investigated molecules.^[2–5] They can be synthesized in a well-controlled manner, generation by generation, which results in high monodispersity, high density of surface functionalities, and internal space available for supramolecular inclusion.^[6] Due to these unique structural and physicochemical properties, they have received intense attention as candidates for a variety of biomedical applications such as drug and gene delivery in the past decades.^[7–10] Although PAMAM dendrimers are highly monodisperse (PDI as low as 1.0007),^[11] which is one of the most important features over traditional polymers, these synthetic materials are not as perfect as biomacromolecules such as proteins and nucleic acids. Structural defects on PAMAM dendrimers were found during the very first synthesis by Tomalia *et al.* in 1980s.^[12] The incomplete Michael addition reaction, intramolecular cyclizations, fragmentation due to retro-Michael reactions, and solvolysis of terminal

functionality were concluded to cause structural defects during dendrimer synthesis. Dendrimer bridging *via* intermolecular transamidation reactions has also been observed during dendrimer generation growth, which leads to the formation of dendrimer dimers. This dendrimer bridging reaction depends very much on synthesis conditions, namely the amount of sequencing reagent and the reaction temperature, and therefore needs to be precisely controlled. Up-to-date, the defective structure of PAMAM dendrimers, including missing arms, trailing dendrimer generations, and dimer impurities, have been carefully investigated by several groups using mass spectrometry (MS), high-performance liquid chromatography (HPLC) and so on.^[13–16] However, given the low polydispersity index of dendrimer samples, the defects were usually thought to be only a small fraction and did not catch much research attention. More recently, Mullen *et al.* carefully characterized different batches of commercial products of a generation 5 (G5) PAMAM dendrimer by HPLC and gel permeation chromatography (GPC),^[17] in which they confirmed the existence of trailing dendrimer generations and dimer impurities. In a separate study, the same research group employed reverse phase HPLC to isolate generation defects in the samples and successfully produced G5 dendrimer with high purities.^[18] The dimer impurities were found to vary with different commercial batches, from 8% to 15%, which are actually non-negligible for biomedical applications. Therefore, it is necessary for researchers to be aware of the effect of dimer impurity in

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commercial dendrimer products or to carry out post-market purification before using these products.

During the past two decades, the molecular structure of dendrimers has been a focus of research interests due to not only its importance in the application aspects but also the unique dendritic structural properties different from those of typical polymers and colloids.^[19] Extensive characterization of PAMAM dendrimers has been carried out by different kinds of experimental techniques, such as small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), laser light scattering, NMR, MS, and X-ray diffraction.^[20] In particular, SANS and SAXS have been widely used due to the fact that they enable the researchers to directly investigate the solution structure of macromolecules.^[21] Structural aspects including the radius of gyration,^[22,23] the spatial distribution of terminated groups,^[24,25] the size and shape of different generation dendrimers,^[26–29] the intramolecular space for supramolecular inclusion,^[30] and the intermolecular interactions at varying solvent conditions^[31] have been addressed. However, the structural defects on dendrimers have not yet been investigated in these SAXS and SANS studies.

Unlike HPLC, which separates and determines the dendrimer impurities by using the size-dependent mobility of different components, SANS directly detects the spatial structure of macromolecules in solution. SANS is sensitive to structural impurities of dendrimers in solution, especially the large size species such as dimers and other oligomers. We therefore performed SANS studies on G5 PAMAM dendrimers in diluted aqueous solutions to investigate the dimer impurities in different batches of dendrimer products. Indirect Fourier transform (IFT) analysis of the SANS data showed that the maximum dimension is about twice the diameter of a single dendrimer (monomer), indicating the existence of dendrimer dimer. Then, we developed an analytical model for the form factor of two spatially correlated spherical particles in SANS analysis. Based on this model and the so-called “fuzzy-sphere with internal blob model” developed by Rathgeber *et al.*,^[26] we successfully fitted the SANS data for PAMAM dendrimers, and estimated the amount of dimer impurities based on the fitting results.

EXPERIMENTAL

Materials

G4 and G5 ethylenediamine (EDA)-cored, amine-terminated PAMAM dendrimers (G4, No. 0713-09-E4.0-LD; G5 Batch-1, No. 1213-08-E5.0-LD; G5 Batch2, No. 0815-07-E5.0-LD-W; G5 Batch3, No. 0113-07-E5.0-LD) were purchased from Dendritech, Inc. (Midland, MI). The dendrimer samples were distilled or free-dried to remove the solvents, and then dissolved in deuterium oxide (D₂O) to obtain aqueous solutions at a concentration of 5 mg/mL. The solution samples were freshly prepared one night before the SANS measurement. The samples were measured by SANS without further purification. D₂O was purchased from Sigma-Aldrich (St. Louis, MO).

SANS

SANS measurements were performed on the V4 SANS ins-

trument at BERII research reactor HZB.^[32,33] In order to cover a wide wave vector q range from 0.16 nm⁻¹ to 5.0 nm⁻¹, two configurations of the instrument have been used, with the wavelengths of incident neutron beam selected at 0.45 and 0.6 nm, and the sample-to-detector distances set at 1 and 4 m, respectively. The samples were contained in 1 mm path length quartz cells. All the experiments were carried out at room temperature. The SANS data were reduced by using the BerSANS software.^[34] The measured intensity was corrected for scattering contribution from the empty cells and D₂O and placed in an absolute scale using water scattering as a reference standard.

Theory of SANS Data Analysis

When analyzing the SANS data, both model-independent and model-dependent methods can be employed. The indirect Fourier transform (IFT) method, developed by Glatter *et al.*, can be used to transfer the scattering data into real space function,^[35] namely pair distance distribution function. It has been shown that IFT is very suitable for determining the maximum dimension of particles investigated. On the other hand, modeling is useful for providing researchers with quantitative structural information in real space on their particular systems.

IFT analysis

The intensity $I(q)$ measured in a SANS experiment is given by the Fourier transform of pair distance distribution function $P(r)$ of the particle:^[35]

$$I(q) = 4\pi \int_0^\infty P(r) \frac{\sin(qr)}{qr} dr \quad (1)$$

$P(r)$ function gives direct information about the structure in real space. The function is a measure of the probability of all distances between a pair of points within the particles weighted by excess scattering length density at the points. From the function, the maximum dimension ($D_{\max}$) of the particle can be directly determined where $P(r)$ goes to zero. Noticing the superposition property of Fourier transform (FT), if there are both dendrimer monomer and dimer impurity in the investigated system, the deduced $P(r)$ will be simply the sum of those of the monomer and the dimer. Therefore, the existence of dimer will result in a larger (doubled) value of $D_{\max}$. IFT method has been well developed and can be applied to calculate the $P(r)$ function. In particular, we used the program GNOM by Svergun *et al.* for the calculation of $P(r)$ from SANS data.^[36]

SANS modeling

For data modeling, the theoretical result of a given model can be calculated so as to get fitted with the experimental data using the non-linear least square method. For the investigated system, the SANS intensity distribution $I(q)$ can be given by the following analytic expression:^[37]

$$I(q) = n_p P(q) S(q) + I_{\text{INC}} \quad (2)$$

where n_p is the number density of molecules, $P(q)$ is the form factor (also known as the intra-molecular structure factor), $S(q)$ is the inter-molecular structure factor, and I_{INC} is the incoherent scattering background. The contribution of the inter-dendrimer correlation to $I(q)$ is found to be negligible in our diluted samples.

Therefore, the scattering intensity of our dendrimer system including both monomer and dimer can be expressed as:

$$I(q) = n_m[P_m(q) + \gamma P_d(q)] + I_{\text{INC}} \quad (3)$$

where n_m is the number density to monomer, γ is the molar ratio of dimer to monomer, and $P_m(q)$ and $P_d(q)$ are the form factors of monomer and dimer, respectively. Based on the model fitting results of γ , molar fraction of the dimer impurity can be determined.

Form factor of two spatially correlated spherical particles

The form factor of the randomly oriented particles in solutions can be described as:

$$P(q) = \left\langle |F(\vec{q})|^2 \right\rangle = \left\langle \left| \int_V (\rho - \rho_s) \exp(i\vec{q} \cdot \vec{r}) d\vec{r} \right|^2 \right\rangle \quad (4)$$

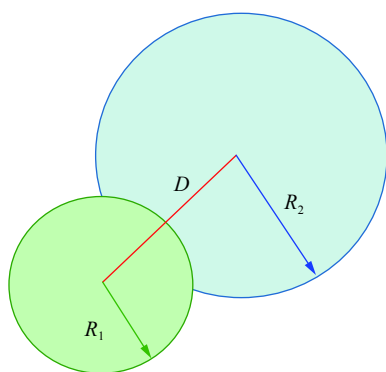
where ρ is the coherent scattering length density of the particle at position r , ρ_s is the scattering length density of the solvent, and V is the volume of the particle. Considering the system of two correlated spherical symmetric particles separated by an arbitrary center-to-center distance D (as shown in Scheme 1), the form factor then can be expressed as:

$$\begin{aligned} P(q) &= \left\langle |F(\vec{q})|^2 \right\rangle = \left\langle \left| \int_{V_1} (\rho_1 - \rho_s) \exp(i\vec{q} \cdot \vec{r}_1) d\vec{r}_1 \right. \right. \\ &\quad \left. \left. + \int_{V_2} (\rho_2 - \rho_s) \exp(i\vec{q} \cdot (\vec{D} + \vec{r}_2)) d\vec{r}_2 \right|^2 \right\rangle \quad (5) \\ &= |F_1|^2 + |F_2|^2 + 2F_1 F_2 \frac{\sin(qD)}{qD} \end{aligned}$$

where F_j is the scattering amplitude of the j^{th} particle and $F_j = \int_{V_j} (\rho_j - \rho_s) \exp(i\vec{q} \cdot \vec{r}_j) d\vec{r}_j$. In fact, it can be found that

Eq. (5) is similar to the widely known Debye equation which is developed for group of atoms in molecules.^[38] Particularly, for two identical spherical particles, we have $F_1 = F_2 = F$, and therefore the form factor can be written as:

$$P(q) = (2 + \frac{2\sin(qD)}{qD})|F|^2 \quad (6)$$



Scheme 1 Two spatially correlated spherical particles

Form factor of dendrimer monomer

An analytic model for PAMAM dendrimer monomer has

been developed by Rathgeber *et al.*,^[26] known as the fuzzy sphere with internal blob model, and can be expressed as:

$$\begin{aligned} P_m(q) &= P_{\text{shape}}(q, R, \sigma) + a_b P_{\text{blob}}(q, R, \sigma) \\ &= [v(\rho - \rho_s)]^2 \left\{ \frac{3}{(qR)^3} [\sin(qR) - qR \cos(qR)] \exp\left(\frac{q^2 \sigma^2}{4}\right) \right\}^2 \\ &\quad + a_b P_{\text{blob}}(q, R, \sigma) \quad (7) \end{aligned}$$

In this expression, the first term gives the contribution of the whole shape, a homogeneous hard sphere with radius R and fuzzy edge σ . v is the volume of the dendrimer molecule, ρ is the coherent scattering length densities of the dendrimer, and ρ_s is the scattering length density of the solvent. The second term $P_{\text{blob}}(q)$ gives the contribution from the intramolecular density fluctuations on length scales smaller than the blob radius. The amplitude a_b is given by the ratio of the scatter number in one blob N_b to the total dendrimer segment number N_t , $a_b = N_b/N_t$.

Form factor of PAMAM dimer

Next, particularly for dimer impurities, we have $F_1 = F_2$, so the form factor can be written as

$$P_d(q) = (2 + \frac{2\sin(qD)}{qD})P_m(q) \quad (8)$$

where $P_m(q)$ represents the form factor of a dendrimer monomer, and $D = 2R$ is the center-to-center distance of the monomers in one dimer. Therefore, the overall scattering is given by:

$$\begin{aligned} P_d(q) &= \left(2 + \frac{\sin(2qR)}{qR}\right) [v(\rho - \rho_s)]^2 \left\{ \frac{3}{(qR)^3} [\sin(qR) - \right. \\ &\quad \left. qR \cos(qR)] \exp\left(\frac{q^2 \sigma^2}{4}\right) \right\}^2 + a_b P_{\text{blob}}(q, R, \sigma) \quad (9) \end{aligned}$$

RESULTS AND DISCUSSION

In Fig. 1, the SANS intensities for solutions of three different batches of G5 PAMAM dendrimer in D₂O, here termed as Batch1, Batch2, and Batch3, are shown as a function of scattering vector amplitude q . The dendrimer concentration for all the samples was kept at a constant of 5 mg·mL⁻¹. It is clearly shown that the scattering is very different for the three samples, especially in the low q ranges. Because the dendrimer solution is dilute, the intermolecular structural factor has essentially a constant unit value for the measured q range. The difference in scattering curve therefore firmly shows some structural difference at the molecular level in these samples.

We then carried out the IFT analysis on these data. The best fitting results to the experimental data are also shown in Fig. 1. The resulting $P(r)$ functions are shown in Fig. 2. IFT method is sensitive in determination of the maximum dimension (D_{max}) of the scattering system. For the measured samples, the values of D_{max} are around 11 nm. As what is known from previous experimental results by TEM or SAXS/SANS, the diameter of a single G5 PAMAM dendrimer molecule is around 6 nm.^[26] Therefore, the larger D_{max} must be due to dendrimer impurities possessing a larger size, such as dimer or other oligomers. If our measured intensity is the sum of

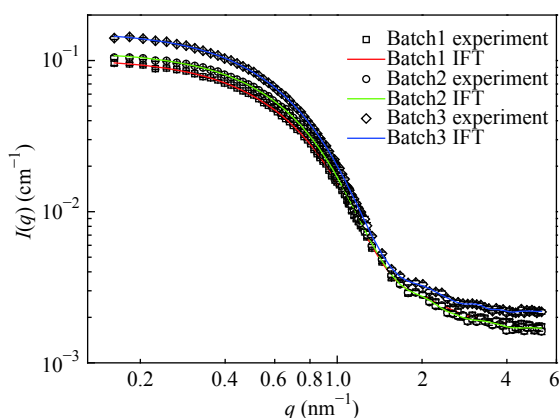


Fig. 1 SANS data from three different batches of G5 PAMAM dendrimer in D₂O at a concentration of 5 mg·mL^{−1} and the corresponding IFT analysis results

scattering from both monomer and dimer, the $P(r)$ function which is FT of the scattering $I(q)$ will be the sum of those from monomer and dimer. This is because of the superposition property of FT. It can be found that the $P(r)$ function decreases to smaller values at $r > 6$ nm, which is not likely the case for a spherical-like dendrimer molecule but can be well explained by the existence of dimer impurity in the samples. The resulting $P(r)$ function for the three batches at $r > 6$ nm also shows relatively small values. This means that although dimer impurity is found in the samples, monomers are still

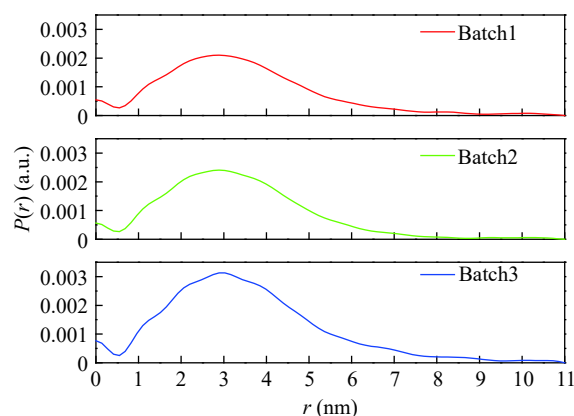


Fig. 2 The pair distance distribution function $P(r)$ determined by IFT analysis of the SANS data from three batches of G5 PAMAM dendrimer in D₂O aqueous solution

the dominate components. It should be mentioned that the non-zero value for $P(r)$ at $r = 0$ is mainly due to the incoherent scattering background and density fluctuations at the atomic scale, which provides non-useful structural information at the interested length scale.

We then fitted the data with the developed model including contributions from dimer impurities, and the results are shown in Fig. 3. It can be found that the experimental data are well fitted by the model. Based on the fitting results, the scattering contributions from dendrimer monomers and di-

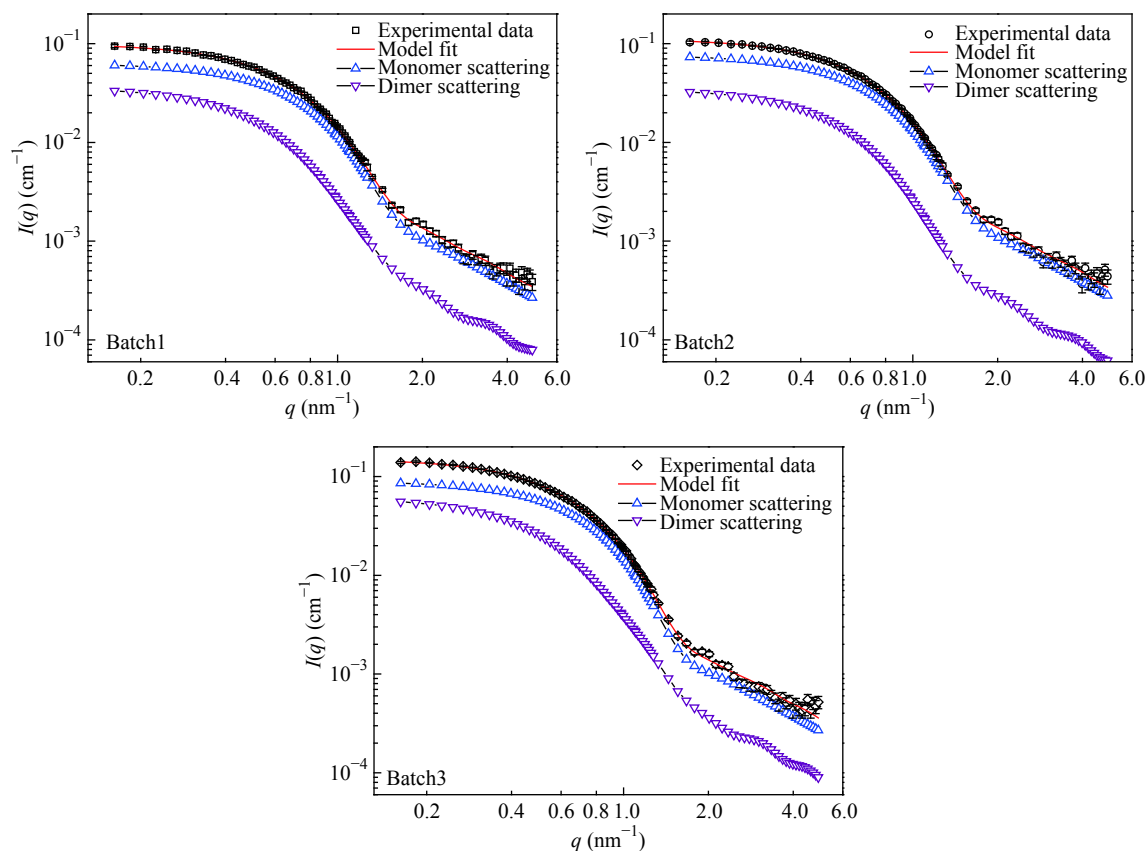


Fig. 3 Model fitting results of SANS data for the three different batches of G5 PAMAM dendrimer products. The incoherent background scattering was subtracted according to the model fitting results. The scattering contributions from both dendrimer monomer and dimer are also shown separately based on the fitting results.

Table 1 Structure parameters of different batches of G5 PAMAM dendrimer

Sample	Sphere radius R (nm)	Fuzzy edge σ (nm)	Blob scattering a_b	Dimer molar fraction
Batch1	2.01 ± 0.14	1.38 ± 0.08	0.097 ± 0.004	0.12 ± 0.03
Batch2	1.84 ± 0.22	1.52 ± 0.10	0.084 ± 0.003	0.10 ± 0.04
Batch3	2.27 ± 0.05	1.26 ± 0.04	0.069 ± 0.003	0.14 ± 0.02
Batch1 + 5%G4	1.82 ± 0.17	1.47 ± 0.07	0.110 ± 0.006	0.14 ± 0.06

mers can be separated. For all the three batches, the contribution from dimer is smaller than that from dendrimer monomer, and is mainly to the scattering at low q . The structural parameters according to the model fitting results are shown in Table 1. It can be found that the molar fractions of dimer impurity in Batch1 and Batch2 are around 10%–12%, and a slightly higher value of 14% is found for Batch3. The intramolecular structure for Batch3 also shows a little difference from Batch1 and Batch2, for which a slightly larger sphere radius R and smaller fuzzy edge thickness σ can be found. These model fitting results are also in agreement with the previous IFT analysis. Taking Batch1 for example, the radius R of the monomer is (2.0 ± 0.2) nm and the fuzzy edge σ equals (1.4 ± 0.1) nm; $D_{\max}$ can also be calculated as (10.8 ± 1.0) nm, while in the IFT analysis $D_{\max}$ was found to be around 11 nm.

We also tried to fit the SANS data without including the dimer contribution, but could not get a good fitting result. This is due to the relatively large amount of dimer impurity in the dendrimer samples which are prepared directly from commercial products without any purification. The amount of dimer impurity is significant and thus detectable by SANS analysis. Therefore, our results indicate the importance of sample purification when characterizing PAMAM dendrimers.

There should also be fragment defects in the measured samples, which are not included in our model fitting analysis. However, because these fragment defects should only account for a relatively small part, as shown by previous HPLC analysis,^[17] and because the scattering is proportional to the square of particle size, their scattering contribution can be essentially ignored. This is proved by comparing the scattering from G5 PAMAM dendrimer (Batch1) and the same dendrimer product (Batch1) with the addition of 5% G4 PAMAM dendrimer, which shows little difference in scattering as shown in Fig. 4. The changes in structural parameters according to model fitting results are also within error for the Batch1 sample and Batch1 + 5%G4 sample, as shown in Table 1. This means the low generation impurities merely change the analysis results. On the other hand, the existence of trimer or tetramer in the product may also be possible. However, the amount should be much less than that of dimer. Moreover, trimer and tetramer should also introduce a larger $D_{\max}$ in the system than that of dimer, because the maximum dimension of a trimer or tetramer should be ranging from two to three or four times of a monomer depending on the internal structure of the oligomer. This is not found in the IFT analysis of SANS data, which means the amounts of trimer or tetramer impurities in the dendrimer solution are negligible.

The existence of large amount of dimer impurity and its

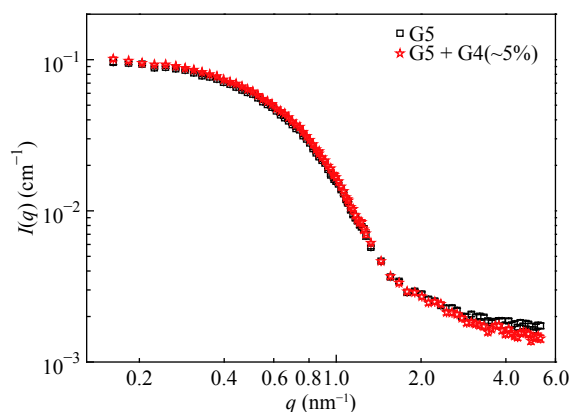


Fig. 4 SANS data of G5 PAMAM dendrimer from Batch1 and the same product added with 5% G4 in mass fraction. Little difference between the two scattering curves can be found.

variation on different batches of dendrimer products can be understood by recalling the PAMAM dendrimer synthesis process. As pointed out by Tomalia *et al.*, dendrimer bridging *via* intermolecular transamidation reactions can significantly influence the monodispersity of final products.^[12] And this bridging phenomenon depends very much on the reaction conditions, including the amount of sequencing reagent and the reaction temperature. The formation of dimer impurity should be due to the dendrimer bridging process. Because bridging amidation and dendrimer amidation are kinetically similar,^[12] it is almost impossible to avoid the formation of dimer impurities during the synthesis. A large number of dimer impurities are likely formed which may vary on different synthesis conditions, or in other words, different batches of dendrimer products.

The structural heterogeneity may have a big effect on the performance of materials concerning their applications where the particle size plays an important role. The large number of dimer impurities in PAMAM dendrimers found in this study are in consistent with previous HPLC results by Mullen *et al.*^[17] Here, we found the molar fractions of dimer impurity for the three measured batches of G5 PAMAM samples are as high as $(12 \pm 3)\%$, $(10 \pm 4)\%$, and $(14 \pm 2)\%$, respectively. Therefore, it is suggested that special care needs to be taken for dendrimer applications wherever particle size and uniformity are important.

CONCLUSIONS

We show that the SANS technique is well-suited for the investigation of dimer impurity in PAMAM dendrimers. Model-independent IFT analysis of SANS data clearly shows the evidence of dimer existence in three independent batches of G5 PAMAM products. We developed an analytical model

for the form factor of two-spatially-correlated-spherical-particles system and then a specific model for our dendrimer system including dimer contribution. Using this model, we successfully fitted the experimental data. The relative amount of dimer impurity was deduced and found to be significant, around 12% in molar fraction, based on the model fitting results. The molar fraction of dimer impurity varies a little among different batches of dendrimer products. The large amount of dimer formation and its variation on different batches of products can be explained by the unavoidable intermolecular transamidation reactions during dendrimer synthesis, as pointed out by Tomalia *et al.*^[12] The large amount of dimer structural defects may play roles in the biomedical applications of dendrimers, wherever particle size and uniformity are important. Therefore, special care should be taken for the related researches. In addition, we would also like to point out that the developed two-spatially-correlated-spherical-particles model is not only restricted to the dendrimer dimer investigations but also generally applicable for other related systems.

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