Interactions of Poly(amidoamine) Dendrimers with Human Serum Albumin: Binding Constants and Mechanisms

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ABSTRACT The interactions of nanomaterials with plasma proteins have a significant impact on their in vivo transport and fate in biological fluids. This article discusses the binding of human serum albumin (HSA) to poly(amidoamine) [PAMAM] dendrimers. We use protein-coated silica particles to measure the HSA binding constants \( K_d \) of a homologous series of 19 PAMAM dendrimers in aqueous solutions at physiological pH (7.4) as a function of dendrimer generation, terminal group, and core chemistry. To gain insight into the mechanisms of HSA binding to PAMAM dendrimers, we combined \(^1\)H NMR, saturation transfer difference (STD) NMR, and NMR diffusion ordered spectroscopy (DOSY) of dendrimer-HSA complexes with atomistic molecular dynamics (MD) simulations of dendrimer conformation in aqueous solutions. The binding measurements show that the HSA binding constants \( K_d \) of PAMAM dendrimers depend on dendrimer size and terminal group chemistry. The NMR \(^1\)H and DOSY experiments indicate that the interactions between HSA and PAMAM dendrimers are relatively weak. The \(^1\)H NMR STD experiments and MD simulations suggest that the inner shell protons of the dendrimers groups interact more strongly with HSA proteins. These interactions, which are consistently observed for different dendrimer generations (G0-NH2 vs G4-NH2) and terminal groups (G4-NH2 vs G4-0H with amidoethanol groups), suggest that PAMAM dendrimers adopt backfolded configurations as they form weak complexes with HSA proteins in aqueous solutions at physiological pH (7.4).

KEYWORDS: dendrimers • proteins • human serum albumin • nanobiotechnology • nantoxicology • NMR epitope mapping and atomistic molecular dynamics simulations

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of proteins with NPs depend on their surface curvature. Very small NPs (i.e., with highly curve surfaces) have been shown to suppress protein adsorption in some cases. Thus, key unanswered questions are whether the observations and hypotheses of Dawson and coworkers on the interactions of proteins with relatively large and hard NPs are applicable to smaller and softer organic nanostructures with size comparable to those of proteins.

Dendrimers are ideal model systems for probing the interactions of soft organic nanomaterials (NMs) with proteins in biological fluids. Dendrimers are highly branched 3-D globular and monodisperse nanostructures with controlled composition and architecture consisting of three components: a core, interior branch cells, and terminal branch cells. Poly(amidoamine) (PAMAM) dendrimers were the first class of dendritic macromolecules to be commercialized. They have been referred to as artificial proteins based on their similarity in size (e.g., 2 – 13 nm in diameter), shape (e.g., globular), electrophoretic mobility and hydrodynamic behavior. The potential use of PAMAM dendrimers in biomedical applications such as gene therapy, drug delivery, and magnetic resonance imaging (MRI) make them good model systems for probing protein binding to NMs in biological fluids. Human serum albumin (HSA) is the most abundant protein (40 mg/mL) in the human blood circulatory system. HSA binds and transports a broad range of compounds including metabolites, drugs, xenobiotic compounds, and nanomaterials. In this article, we combined experimental characterization and atomistic molecular dynamics (MD) simulations to probe the interactions of HSA with PAMAM dendrimers. We utilized protein-coated silica particles to measure the HSA binding constants ($K_b$) of a homologous series of 19 PAMAM dendrimers (Figure 1) in aqueous solutions at physiological pH (7.4) as a function of dendrimer generation, terminal group, and core chemistry. To gain insight into the mechanisms of HSA binding to PAMAM dendrimers, we combined $^1$H NMR, saturation transfer difference (STD) NMR and NMR diffusion ordered spectroscopy (DOSY) investigations of complexes of dendrimer + HSA with atomistic molecular dynamics (MD) simulations of dendrimer conformation in aqueous solutions. The binding measurements show that the HSA binding constants ($K_b$) of PAMAM dendrimers depend on dendrimer size and terminal group chemistry. The $^1$H NMR and DOSY experiments indicate that the interactions between HSA and PAMAM dendrimers are relatively weak. The NMR STD experiments and MD simulations suggest that the inner shell protons of the dendrimers and their neighboring amide groups interact more strongly with HSA proteins. These stronger interactions, which are consistently observed for different dendrimer generations (G0-NH$_2$ vs G4-NH$_2$) and terminal groups (G4-NH$_2$ vs G4-OH), suggest that PAMAM dendrimers adopt backfolded conformations as they form weak complexes with HSA proteins in aqueous solutions at physiological pH (7.4).

RESULTS AND DISCUSSION

Equilibrium dialysis, ultrafiltration, and ultracentrifugation are commonly used techniques to measure the binding of organic solutes to biological macromolecules in aqueous solutions. The use of dialysis to measure the binding constants of dendritic macromolecules to proteins has yielded mixed results. Purohit et al. employed dialysis (with a regenerated cellulose membrane of molecular weight cut off of 10 and 25 kDa) to measure the binding of a dendron (with 32 terminal amine groups and molecular weight 1.5 – 4.7 kDa) to HSA. They found that more than 50% of the dendrons (on a mass basis) were adsorbed onto the 10 kDa membrane. Our initial attempts to use dialysis to measure the binding constants of PAMAM dendrimers to HSA (using 10 – 30 kDa RC membrane) were not successful due to dendrimer adsorption to the RC membranes and limited diffusivity of PAMAM dendrimer through RC membrane (data not shown). Note that Shcharbin et al. investigated the interactions between HSA and a G4-NH$_2$ PAMAM dendrimer [with ethylene diamine (EDA) core] using a suite of analytical techniques including zeta-potential measurements, capillary electrophoresis, isothermal titration calorimetry, circular dichroism (CD), and fluorescence spectroscopy. They reported that the binding affinity of PAMAM dendrimers to HSA depends on dendrimer generation and terminal group chemistry. The estimated binding constant ($K_b$) of a G4-NH$_2$ PAMAM using the different techniques varied by 2 orders of magnitude ranging from $10^3$ to $10^9$ M$^{-1}$. Froehlich et al. combined FT-IR, UV-visible, CD, and fluorescence spectroscopy to probe the binding of HSA to PAMAM dendrimers with ethylene diamine core and terminal amine groups (G4-NH$_2$) and terminal polyethylene glycol (PEG) groups [G3-PEG and G4-PEG] in aqueous solutions at physiological pH (7.4). They reported dendrimer–HSA binding constants ($K_b$) of 1.3 ± 0.19 × 10$^5$ M$^{-1}$ for G3-PEG, 2.2 ± 0.4 × 10$^5$ M$^{-1}$ for G4-PEG and 2.6 ± 0.5 × 10$^5$ M$^{-1}$ for G4-NH$_2$. Although these previous investigations have provided valuable information and insight into the interactions of PAMAM dendrimers with HSA, no consistent quantitative binding data were derived from these studies. Thus, one of the key objectives of this research was to measure the binding constants ($K_b$) of PAMAM dendrimers to HSA in aqueous solutions at physiological pH (7.4).

Binding Constant Measurements. In this study, we employed protein-coated TRANSIL beads from Sovicell as alternative to dialysis to measure the binding constants ($K_b$) of PAMAM dendrimers (Figure 1) to HSA.
Solid-supported biomacromolecules are increasingly being utilized as (i) separation media for affinity chromatography and (ii) high-throughput bioassay systems. The TRANSIL assay system has emerged as a versatile platform of solid-supported biomacromolecules for probing the interactions of solutes (e.g., drugs, metabolites, and xenobiotic compounds) with lipid bilayers, plasma proteins, and membrane-bound proteins. TRANSIL albumin kits consist of HSA proteins that are immobilized onto porous silica particles with average surface areas of $\sim 10 \text{ m}^2/ \text{g}$. A typical SEM image of an HSA-coated silica particle is shown in Figure S1 of the Supporting Information (SI). In this case, a polymer cushion (with optimal chemistry, spacer length and coupling functionality) is first covalently attached to the surfaces of the silica particles before immobilization of the HSA proteins. The main purposes of this polymer cushion are to (i) shield the HSA proteins from the support, (ii) maintain the conformation integrity of the proteins, (iii) preserve the accessibility of the HSA binding sites, and (iv) eliminate nonspecific binding of solutes to the surfaces of the TRANSIL silica particles. Note that the selection of the TRANSIL-HSA binding assay was motivated by several considerations. First, TRANSIL beads have high HSA contents. Second, the polymer shields on the surfaces of the TRANSIL silica particles ensure that there is no significant difference between the conformations of free and immobilized HSA proteins. Third, TRANSIL beads can be easily separated from aqueous solutions by centrifugation. Finally, we would like to point out that the TRANSIL albumin-binding assay was validated by Schumacher et al. They showed that the bound fractions ($f_b$) of various small drugs molecules to HSA that were measured using HSA-coated TRANSIL beads agree very well with those determined using dialysis.

Table 1 lists the measured HSA binding constants of $G_x$-NH$_2$ PAMAM dendrimers with EDA core. We tested eight different dendrimer generations ($G_0$, $G_1$, $G_2$, $G_3$, $G_4$, $G_5$, $G_6$, and $G_8$). In these experiments, we varied the molar ratio of protein to dendrimer NH$_2$ groups from 0.01 to 0.1 by increasing the concentration of HSA (0.5 to 7.5 $\mu$M). By keeping the concentration of NH$_2$ groups constant at 64 $\mu$M for all $G_x$-NH$_2$ PAMAM dendrimers, we were able to decouple the effects of size and terminal group concentration on their HSA binding constants. Figure 2 illustrates the effect of generation and size (as measured by the hydrodynamic radius) on the HSA binding constants ($K_b$) of PAMAM dendrimers. The corresponding fractional binding (FB) curve is plotted in Figure S2 of the SI. In all cases, each reported
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The $K_b$ value is the average of four measurements with different molar ratios of HSA to dendrimer NH$_2$ groups. Similarly, each reported error is the standard deviation of the average of the four measured $K_b$ values. Figure 2 shows that the HSA binding constants of the G$_x$-NH$_2$ PAMAM dendrimers (with EDA core) gradually increase with generation (from 1.67 ± 0.19 × 10$^{-6}$ for G0-NH$_2$ to 5.42 ± 1.09 × 10$^{-6}$ for G6-NH$_2$) followed by a slight decrease (3.30 ± 0.98 × 10$^{-6}$) for the G8-NH$_2$ dendrimer. Interestingly, the G6-NH$_2$ PAMAM dendrimer has the largest $K_b$ value (Figure 2). We believe this might be the result of a size-based selective binding mechanism similar to that reported by Chiba et al.$^{24}$ They used a fluorescence-based competitive displacement assay to investigate the binding of Gx.5 PAMAM dendrimers [with terminal COONa groups] to proteins including cytochrome-c and chymotrypsin. Chiba et al.$^{24}$ found that a dendrimer has a higher binding affinity toward a protein with comparable molecular surface area. For example, the G2.5 PAMAM dendrimer (with a molecular surface area of 1200 Å$^2$) has the highest binding affinity to cytochrome-c, which has a molecular surface area of 1100 Å$^2$. Similarly, the G3.5 PAMAM dendrimer (with a molecular surface area of 2250 Å$^2$) has the highest binding affinity for chymotrypsin, which has a molecular surface area of 2400 Å$^2$. As discussed by He et al.$^{15}$ HSA folds into a heart-shaped 3-D structure that can be approximated by an equilateral triangular prism with sides of ~8 nm and height of ~3 nm. The similarity between the sizes of the sides of the HSA model triangular prism and the hydrodynamic diameter of the G6-NH$_2$ PAMAM dendrimer (7.62 nm) [Table 1] is consistent with the size selective binding mechanism observed by Chiba et al.$^{24}$ Note that this size-selective binding mechanism is also consistent with the nanoperiodic patterns of dendrimers (i.e., quantized size effect) discussed by Tomalia.$^{25}$

**Figure 2.** Effects of dendrimer generation and hydrodynamic radius on the binding constant ($K_b$) of PAMAM dendrimers with EDA core and terminal NH$_2$ groups at room temperature and pH 7.4. In all cases, the concentration of dendrimer NH$_2$ groups was kept constant at 64 μM.

**Figure 3.** Effects of dendrimer terminal group on the HSA binding ($K_b$) of G4-X and G3.5-COONa PAMAM dendrimers at room temperature and pH 7.4. All the dendrimers have an ethylenediamine (EDA) core except the G4 dendrimer with amidoethylethanolamine terminal groups. This dendrimer has a diaminobutane (DAB) core. In all cases, the concentration of dendrimer terminal groups (X and COONa) was kept constant at 64 μM.

**Table 2.** Binding Constant $K_b$ and Other Physical Parameters of PAMAM Dendrimers with Different Terminal Groups

<table>
<thead>
<tr>
<th>generation</th>
<th>terminal group</th>
<th>$N_{terminal}$</th>
<th>$M_{theo}$ (Da)</th>
<th>$K_b$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>amine$^c$</td>
<td>64</td>
<td>14215</td>
<td>1.67 ± 0.19 × 10$^5$</td>
</tr>
<tr>
<td>4</td>
<td>amidoethylethanolamine$^d$</td>
<td>64</td>
<td>34492</td>
<td>1.47 ± 0.12 × 10$^5$</td>
</tr>
<tr>
<td>4</td>
<td>succinamic acid$^c$</td>
<td>64</td>
<td>20619</td>
<td>2.52 ± 0.75 × 10$^6$</td>
</tr>
<tr>
<td>3.5</td>
<td>sodium carboxylate$^c$</td>
<td>64</td>
<td>26252</td>
<td>4.62 ± 1.21 × 10$^6$</td>
</tr>
<tr>
<td>4</td>
<td>pyrrolidinone$^c$</td>
<td>64</td>
<td>22285</td>
<td>2.46 ± 1.5 × 10$^5$</td>
</tr>
<tr>
<td>4</td>
<td>Tris$^c$</td>
<td>64</td>
<td>18121</td>
<td>2.95 ± 0.77 × 10$^5$</td>
</tr>
<tr>
<td>4</td>
<td>amidoethanol$^c$</td>
<td>64</td>
<td>14277</td>
<td>1.29 ± 0.93 × 10$^5$</td>
</tr>
<tr>
<td>4</td>
<td>Polyethylene glycol (PEG)$^c$</td>
<td>64</td>
<td>49414</td>
<td>1.77 ± 0.28 × 10$^6$</td>
</tr>
</tbody>
</table>

$^a$ $N_{terminal}$: number of terminal groups. $^b$ $M_{theo}$: theoretical molar mass. $^c$ Ethylenediamine core. $^d$ Diaminobutane core.
polyethylene glycol (PEG). Except for the PAMAM dendrimer with sodium carboxylate terminal groups (G3.5), all dendrimers were fourth generation (G4) and thus have similar size and same number of tertiary amine and amide groups. Here again, we kept the concentration of dendrimer terminal groups constant at 64 μM for all the G4-X and G3.5 PAMAM dendrimers. This enabled us to decouple the effects of terminal group chemistry and concentration on the HSA binding constants of PAMAM dendrimers. Figure 3 and Table 2 show that the lowest K_b values are observed for the G4 PAMAM dendrimers with neutral terminal groups. The lower HSA binding constants of the G4-X PAMAM dendrimers with neutral (OH, PEG, and Pyro) terminal groups are consistent with a binding mechanism involving weak interactions (e.g., hydrogen bonding) between dendrimer terminal groups and the protein amino acid residues. Enhanced hydrogen bonding could be the reason why the HSA binding constant of the G4 Tris terminal groups (G4-Tris) is larger than that of the G4-OH dendrimer. Note that even though both dendrimers have the same total concentration of OH groups, the G4-Tris PAMAM dendrimer has 64 x 3 = 192 terminal OH groups that provide more sites for hydrogen bonding with the HSA protein.

Figure 3 and Table 2 also show that the HSA binding constants (K_b) of the PAMAM dendrimers with anionic and cationic terminal groups are significantly larger than those of the dendrimers with neutral terminal groups. These larger K_b values are consistent with a binding mechanism involving both electrostatic and hydrophobic interactions between the dendrimers and HSA amino acid residues. For the Gx-NH2 PAMAM dendrimers, our postulated mechanisms of binding with HSA are consistent with the electron paramagnetic resonance (EPR) studies by Ottaviani et al. They employed continuous wave (CW) and electron-spin echo (ESE) EPR to probe the interactions of G2-NH2 with HSA are consistent with the electron paramagnetic (EPR) studies by Ottaviani et al.26 They employed continuous wave (CW) and electron-spin echo (ESE) EPR to probe the interactions of G2-NH2 and G6-NH2 PAMAM dendrimers (labeled with nitroxides) with selected amino acids (Gly, Glu, Arg, and Leu) and proteins (chymotrypsin and HSA) in aqueous solutions (D_2O). By varying the pH and extent of protonation of the dendrimers, Ottaviani et al.26 showed that the binding of the Gx-NH2 PAMAM dendrimers to the amino acids, chymotrypsin and HSA is “mainly promoted when both electrostatic and hydrophobic interactions take place”.

Not surprisingly, the K_b value of the G4-EtNH PAMAM dendrimer (1.47 ± 0.12 x 10^6 M^-1) is comparable to that of the G4-NH2 PAMAM dendrimer (1.67 ± 0.19 x 10^6 M^-1). This dendrimer has 64 secondary amine (NH2) and 48 tertiary amine groups and the positively charged amino-acid residues of the Gx-NH2 PAMAM dendrimer are unprotonated and negatively charged (COO^-). As discussed by Diallo et al.,28 the pK_a of the internal tertiary amine groups (pK_a(ter)) and the pK_a of the pK_a of the terminal carboxylic group of the G3.5 PAMAM dendrimer are unprotonated and negatively charged (COO^-), whereas its internal tertiary amine groups are unprotonated and neutral in aqueous solutions at physiological pH (7.4). This suggests that the G3.5 PAMAM binds to HSA primarily through electrostatic interactions between its COO^- groups and the positively charged amino-acid residues of the carboxylic acid binding sites of the protein.27

Finally, we would like to mention that the magnitude of the HSA binding constant (4.62 ± 1.21 x 10^6 M^-1)
for the G3.5 PAMAM further supports the absence of specific interactions between dendrimers and the silica particles of the HSA-coated Transil beads. At physiological pH (7.4), silica particles are negatively charged. If there were specific interactions between dendrimers and bare TRANSIL silica particles, one would expect the positively charged dendrimer (G4-NH2 PAMAM) to exhibit a larger HSA binding constant than the negatively charged dendrimer (G3.5-COONa PAMAM) with comparable size and same number of terminal groups. However, we find that the $K_b$ value of the G3.5 PAMAM (4.62 ± 1.21 × 10^6 M$^{-1}$) is approximately 2 times larger than that of the G4-NH2 dendrimer (1.67 ± 0.15 × 10^6 M$^{-1}$). Figure 4 and Table 3 highlight the effects of core chemistry on the HSA binding constants of G4-NH2 PAMAM dendrimers. Four different dendrimer cores were evaluated: ethylene diamine (EDA), diaminobutane (DAB), diaminohexane (DAH), diaminododecane (DA), and cystamine (Cys). The corresponding fractional binding curve is plotted in Figure S4 of the SI. We found no significant variation of dendrimer HSA binding constant with core chemistry.

**NMR Investigations.** Although binding constants can provide valuable information on the relative strengths of dendrimer–HSA complexes, no reliable information about molecular interactions can be derived from binding constant measurements alone. We used NMR spectroscopy to probe the interactions of PAMAM dendrimers with HSA in D$_2$O at physiological pD of 7.4. Recall that albumin-coated TRANSIL beads contain polymer cushions that shield the immobilized HSA proteins from the silica particles while maintaining their conformational integrity. Thus, we expect no significant difference between the interactions of PAMAM dendrimers with free and immobilized HSA proteins in aqueous solutions. Three model dendrimers were evaluated during the NMR experiments: G0-NH$_2$, G4-NH$_2$, and G4-OH (amidoethanol) PAMAM dendrimers with EDA core. For the G4 PAMAM dendrimers, 5 mg of dendrimer was mixed with 20 mg of protein to achieve an HSA–dendrimer molar ratio of 1.0. We initially tested an equimolar mixture of G0-NH2 and HSA. However, the signals from the dendrimer could not be distinguished from the protein background signals due to the very low amount of G0-NH$_2$ PAMAM (0.16 mg) needed in this case to achieve a molar ratio of dendrimer–HSA of 1.0. We subsequently mixed 5 mg of G0-NH$_2$ PAMAM with 20 mg of HSA. In this case, the dendrimer–HSA molar ratio was equal to 32.0. Figure 5 compares the $^1$H NMR spectra of the PAMAM dendrimers (A) and their mixtures with HSA (B). We observe little changes in the chemical shifts or line shapes of the $^1$H NMR spectra of the G0-NH$_2$ dendrimer following the addition of HSA. This indicates that the local chemical environments of the dendrimer protons are relatively unaffected by the addition of HSA thereby suggesting that the interactions between HSA and the G0-NH$_2$ PAMAM are relatively weak and/or in fast exchange. Owing to molar excess of the G0-NH$_2$ dendrimer, it is possible that the perturbations in chemical shifts induced by HSA–dendrimer interactions are masked by the free dendrimers. However, the DOSY experiments (Figure 6) clearly suggest that the interactions between HSA and the G0-NH$_2$ PAMAM are weak and/or highly dynamic in solution. Note also that the weak interactions between the G0-NH$_2$ PAMAM and HSA are consistent with the results of the EPR studies carried by Ottaviani et al. They observed that the EPR spectra of

![Figure 5.](image-url)
HSA complexes with the G2-NH2 PAMAM are “quite similar” to those of the dendrimer in D2O. For the G4-NH2 PAMAM dendrimer, we observed significant chemical shifts to lower fields for all resonances of the following addition of HSA (Figure 5). This suggests that all the dendrimer protons are exposed to different chemical environments, an indication of interactions between HSA and the G4-NH2 PAMAM. These interactions are also consistent with the noticeable broadening of the peaks of the dendrimer protons in the presence of HSA (Figure 5) as most clearly seen for protons 4i, 1o, and 1i (see Figure S6 of the SI for the labeling of the protons). We also observed small chemical shifts in all the resonances of the G4-OH dendrimer following addition of HSA (Figure 5). This suggests the interactions of HSA with the G4-OH dendrimer are weaker than those with the G4-NH2 dendrimer. These results are consistent with the binding constant measurements, which show that the HSA binding constant of the G4-NH2 dendrimer \(1.67 \pm 0.19 \times 10^6 \text{ M}^{-1}\) is approximately 2 orders of magnitude larger than that of the G4-OH dendrimer \(1.29 \pm 0.93 \times 10^4 \text{ M}^{-1}\).

We carried out diffusion ordered spectroscopy (DOSY) NMR experiments\(^{31}\) to measure the diffusion constants of the PAMAM dendrimers, HSA, and their complexes. Figure 6 shows the 2-D \(^1\text{H}\) spectra and projected diffusion profiles of the HSA (A), G0-NH2 dendrimer (B), and HSA-dendrimer complex (C). The different regions of the NMR spectra of Figure 6 are well resolved and separated; thus highlighting a significant difference between the diffusion coefficients of the G0-NH2 PAMAM and HSA. Note the small decrease in the diffusion coefficient of the G0-NH2 dendrimer in the presence of HSA (Figure 6). The small decrease of the diffusion coefficient of the G0-NH2 PAMAM also suggests that its interactions with HSA are weak, dynamic,\(^{30}\) and fast during the time scale \((\sim 200 \text{ ms})\) of NMR DOSY experiments. We found that the DOSY diffusion profiles of the G4-NH2 dendrimers are similar to that of HSA (data not shown). Because of this, we were unable to extract reliable diffusion coefficients for the dendrimer-HSA complexes due to the overlap between the diffusion peaks of the dendrimer and protein macromolecules.

We employed saturation transfer difference (STD) to probe the mechanisms and strengths of the interactions between PAMAM dendrimers and HSA. STD is commonly referred to as NMR epitope mapping.\(^{32–35}\) A typical STD NMR experiment begins with the selective saturation of the host, HSA in this case. Saturation is then transferred to the guest molecules (i.e., dendrimers in this case) that are interacting with the HSA macromolecules. During an STD experiment, the dendrimer protons closest to an HSA protein receive the greatest amount of saturation while those furthest from the protein receive the least. Note that only the interacting dendrimers are detected during an STD experiment while all other signals (e.g., water or non-interacting dendrimers) cancel and are not observed (see the section Experimental and Computational Methods). Thus, the quantitative information about the strengths of the underlying molecular interactions between dendrimer and HSA macromolecules become encoded into the integrals of the dendrimer signals in a \(^1\text{H}\) NMR STD spectrum. Figure 7 compares the epitope maps of the interactions of HSA with the G0-NH2, G4-NH2, and G4-OH PAMAM dendrimers. In an NMR epitope map, the percentage value for each type of proton represents an interaction strength that has been normalized by that of the strongest interacting proton, which is assigned a value of 100%. Note also that the % value for each proton is an “indirect” measure of the relative interaction strength of the functional groups in close proximity to the proton. In the case of the G0-NH2 dendrimer (Figure 7A), we find that proton 2 adjacent to the CO of the amide group interacts more strongly with HSA. Surprisingly, the protons adjacent to the dendrimer NH2 groups (i.e., those protons attached to position 4) show the weakest interactions.

Figure 7B,C show the epitope maps of the interactions between HSA and the G4-NH2 and G4-OH PAMAM...
dendrimers. Figure S5 of the SI displays a 2-D structure of the G4-NH$_2$ PAMAM dendrimer with CH$_2$ groups labeled as 1, 2, 3, and 4. In Figure S5 of the SI, the terminal groups of the dendrimer outside the dashed circle are assigned to the outer shell, while the other groups within the dashed circle are assigned to the inner shells. Because of spectral overlaps, we could not generate the epitope maps of each specific dendrimer shell. Note that the protons at position 2 adjacent to the CO of the amide groups of the G4-NH$_2$ and G4-OH dendrimers also display the strongest interactions (Figures 7B and 7C). Figure 7C also shows the protons adjacent to the OH groups (protons 3 and 4) of the G4-OH PAMAM display weaker interactions. These weaker interactions are consistent with the lower HSA binding constant of this dendrimer and strongly suggest that the OH groups do not drive the interactions with the protein in the case of the G4-OH dendrimer. For all the G4-X dendrimers, we find a significant enhancement of signals of the protons from the inner shells compared to those from the dendrimer outer shells. While these observations cannot be completely quantified to produce separate epitope maps for each specific dendrimer inner and outer shells, Figure S6 of the SI clearly shows that the inner protons receive more saturation from the protein (i.e., stronger interactions) and thus are more enhanced in the difference spectrum than the corresponding outer shell protons.

**Figure 7.** Epitope maps for the three dendrimers created from STD NMR (A–C). The percentage values for each epitope map are all relative to the strongest binding proton in each dendrimer, which is expressed as 100%. Red indicates the strongest interacting proton, pink the second, green the third, and blue the weakest. Only one branch of the dendrimers has been labeled for clarity but readers should understand that the interactions apply equally to all branches. Note only interactions from nonexchangeable protons can be measured in $^1$H NMR STD experiments.

**Atomistic Molecular Dynamic Simulations.** The overall results of the NMR epitope mapping experiments suggest that protons from the inner shells of the G0-NH$_2$, G4-NH$_2$, and G4-OH PAMAM dendrimers interact more strongly with HSA proteins. To get a molecular level insight into dendrimer–HSA interactions, we carried out atomistic molecular dynamics (MD) simulations to estimate the “contact” areas between the interacting protons of the dendrimer and HSA. We selected the G4-NH$_2$ PAMAM dendrimer as a model system and performed MD simulations to predict the structure and conformation of this dendrimer in water at physiological pH. Figure 8 displays the equilibrated structure of the G4-NH$_2$ PAMAM dendrimer. The atom coloring schemes used in Figure 8 are the same as those in the epitope maps (Figure 7) obtained from the NMR experiments. We employed the solvent accessible surface areas (SASA) of the equilibrated macromolecule (Figure 8) to estimate the relevant “contact” areas between the G4 PAMAM and HSA. The partial solvent accessible surface areas (PSASA) were calculated by decomposing the total SASA of the dendrimer into components for each relevant chemical group. Several probe radii ($p = 1, 4, 3, 6, 12, 24,$ and $48 \text{ Å}$) were employed in these calculations to mimic interacting molecules ranging from water to large protein macromolecules such as HSA. The van der Waals (vdW) radius of HSA (12 Å) was estimated using the radial
distribution function of a HSA macromolecule in the protein crystal structure (see Figure S7 of the SI). Figure 9 shows the calculated PSASA values using \( p = 12 \) Å. Consistent with the proton labeling scheme used in Figure 7B, we denote the nonexchangeable protons of the G4-NH\(_2\) PAMAM as H1, H2, H3, and H4, respectively, when they are connected to the CH\(_2\) next to a primary amine, the CH\(_2\) next to an amide CO, the CH\(_2\) next to an amide NH, and the CH\(_2\) next to a tertiary amine. Figure 9 shows the normalized PSASA of the protons of the CH\(_2\) groups of the G4-NH\(_2\) PAMAM relative to H2, which has been assigned a value of 100\% as in the NMR STD spectrum (Figure 7B). The relative strength of interactions between the G4-NH\(_2\) PAMAM and HSA estimated from the NMR epitope mapping experiments (Figure 7B) is shown for comparison. Table S1 of the SI lists the calculated PSASA. When the probe radius is equal to the van der Waals (vdW) radius of HSA (12 Å), we find that the calculated PSASA for the G4-NH\(_2\) PAMAM are equal to 58.4\% for H1, 100.0\% for H2 (reference), 94.3\% for H3, and 77.3\% for H4. These values correlate well with the estimated interaction strengths from the NMR epitope mapping experiments (Figure 4B): 82.4\% for H1, 100.0\% for H2 (reference), 92.3\% for H3, and 74.4\% for H4 (Figure 4B). Thus, the PSASA analysis predicts the correct trend of the interaction strengths found in the NMR experiments except for those of H1 and H4. We attribute this discrepancy mainly due to the spatial distribution of chemical groups of the dendrimer as discussed below.

Recall that NMR epitope mapping experiments can only “measure” interaction strengths averaged over all chemical groups of the entire guest. To obtain molecular level information about the groups of the G4-NH\(_2\) PAMAM that interact with HSA, we computed the PSASA for the inner shell and outer shell protons of the dendrimer. In this case, the outer shell protons refer to protons that are connected to the dendrimer terminal NH\(_2\) groups; whereas the inner shell protons refer to all protons from the remainder of the G4-NH\(_2\) PAMAM excluding its EDA core (see Figure S5 of the SI). Figure 9 shows the calculated PSASA of the inner shell and outer shell protons (H1—H4) of the G4-NH\(_2\) PAMAM. We find that the PSASA values of the outer shell

Figure 8. Atomic structure of a G4-NH\(_2\) PAMAM dendrimer in aqueous solutions at neutral pH (snapshot taken from MD trajectory). The atom coloring schemes are the same as those in the epitope maps (Figure 7B) obtained from the NMR experiments: H1 (red balls) and H2 (green balls) represent the inner shell protons, whereas H3 (pink balls) and H4 (blue balls) represent the outer shell protons. The remainder of the dendrimer atoms are represented as ball-and-sticks.

Figure 9. 3-D Partial solvent accessible surface area (PSASA) of the protons of CH\(_2\) groups of G4-NH\(_2\) PAMAM dendrimer relative to H2 (defined as 100) calculated in the MD simulations. The relative strength of interactions between G4-NH\(_2\) PAMAM and HSA calculated from the NMR epitope mapping experiments is shown for comparison. A probe radius \( p = 12 \) Å, corresponding to the vdW radius of a HSA molecule, is used. H1—H4 represent the protons of the CH\(_2\) groups defined in the same way as those in the epitope maps (Figure 7B) in the NMR experiments. The hydrogen atom type goes outward topologically in the order of H1, H2, H3, and H4.
protons of the dendrimer increase as H1 < H2 < H3 < H4, which follows the trend expected from an ideal (topological) branched macromolecule. Note that the PSASA values of the inner shell protons of the G4-NH2 PAMAM reach a maximum value for H2. Similarly, the sum of the PSASA values for all inner and outer shell protons of the G4-NH2 PAMAM reaches a maximum value for H2. This suggests that the predicted PSASA trend from the MD simulations agree with the trend of the NMR interaction strengths only if the contributions of the inner shell protons are included, thus indicating the importance of HSA interactions with dendrimer inner shell protons. This result is consistent with the STD NMR spectra (Figure 8A), which show significant enhancement of the signals of the inner shell protons of the G4-NH2 PAMAM following the addition of HSA.

Although the magnitudes of the contact areas can provide insightful information, we expect the spatial distributions of the groups of G4-NH2 PAMAM to also affect their interactions with HSA. To test this hypothesis, we computed the partial radial mass density distributions of the protons of the CH2 groups of G4-NH2 PAMAM, using its center of mass as reference. Figure 10 shows the calculated partial radial density distributions averaged over 200 ps of the MD simulation trajectories. We found that H1 and H2 are distributed more outward than H3 and H4, whereas H3 exhibits a maximum density at a radial distance r = 16.5 Å, which is lower than those of H1, H2, and H4 at r = 19.5 Å. The maximum density for H4 (4.8 kg/m²) is much smaller than that of H1 (6.0 kg/m²) and H2 (6.1 kg/m²). For an ideal (topological) G4-NH2 PAMAM, we expect the protons to distribute more outwardly in the order H1 < H2 < H3 < H4. The abnormal proton distributions found from the MD simulations are consistent with the backfolding of the terminal amine groups of the G4-NH2 PAMAM as discussed by Liu et al.26 Our calculated radial distributions (Figure 10) indicate that the H1 and H2 protons and their neighboring amide CO groups are more exposed and/or closer to the solvent or protein molecules than the H3 and H4 protons and their neighboring amide NH or primary amine groups. This binding scenario is consistent with the trend found in the NMR epitope mapping experiments. The overall results of the binding measurements, NMR epitope mapping experiments and atomistic simulations suggest PAMAM dendrimers form weak complexes with HSA in aqueous solutions at physiological pH 7.4. These results are consistent with the EPR studies of the interactions of PAMAM dendrimers with amino acids and HSA in aqueous solutions published by Ottaviani et al.26

**Biological Implications.** As discussed in the introductory section, the binding of plasma proteins to nanoparticles (NPs) can significantly alter their in vivo transport in biological fluids. The formations of protein coatings on the surfaces of NPs have a significant impact on their fate in biological fluids as the proteins undergo conformational changes and/or dynamic exchanges with other proteins.8,9 Because the magnitude of the hydrodynamic diameter of HSA (8 nm) at physiological pH (7.4) is comparable to those of Gx-NH2 PAMAM dendrimers (2–10 nm) [Table 1], there are significant differences between the structures and dynamics of dendrimer–HSA complexes and HSA-coated NPs. For example, Lindman et al.39 found that HSA forms dense coronas consisting of 53 protein macromolecules on the surfaces of copolymeric (N-iso-propylacrylamide/N-tert-butylacrylamide) NPs (120 nm in diameter) in aqueous solutions at physiological pH (7.5). Conversely, Shcharbin et al.19 have shown that HSA proteins form 1:5 complexes with G4-NH2 PAMAM dendrimers. Several recent studies have established that dendrimer–protein complexes have a significant impact on a number of important biological processes.24,40,41 For example, PAMAM dendrimers can serve as inhibitors to protein–protein binding and aggregation.24,38 Protein aggregates such as amyloid fibril assemblies play a critical role in neurodegenerative diseases including Alzheimer’s and prion (mad cow) diseases.40 Note that Klajnert et al.41 have found that G5-NH2 PAMAM dendrimers bound to HSA proteins are “significantly less harmful to red blood cells” than the bare G5 dendrimers. Conversely, Shcharbin et al.42 have reported that PAMAM dendrimers can inhibit or enhance the activity of acetylcholinesterase (AChE) depending on their concentration and terminal group chemistry. AChE is a membrane bound enzyme that plays a key role in neurotransmission and signal transduction.42 Collectively taken together, these data and the overall results of our investigations of dendrimer interactions with HSA suggest that the hypothesis Dawson and co-workers12 might not be applicable to dendrimers with size comparable to those of plasma
proteins. In this case, “what the cell sees” might not be a NP core surrounded by a corona of long-lived plasma-proteins as suggested by Dawson and co-workers. In the case of PAMAM dendrimers, we hypothesize that a cell will “see” and interact with weakly bound and dynamics protein-dendrimer complexes. We expect to learn more about the structures and functions of dendrimer-protein complexes as advances are made in the science and application of dendrimer nanotechnology to nanomedicine and nanobiotechnology.

CONCLUSIONS

This article describes an integrated experimental and computational modeling study of the interactions of poly(amidoamine) with human serum albumin (HSA) in aqueous solutions at physiological pH (7.4). We used protein-coated silica particles to measure the HSA binding constants \( K_b \) of 19 PAMAM dendrimers as a function of dendrimer generation, terminal group, and core chemistry. To gain insight into the mechanisms of HSA binding to PAMAM dendrimers, we combined \(^1\text{H} \) NMR, saturation transfer difference (STD) NMR and NMR diffusion ordered spectroscopy (DOSY) of dendrimer-HSA complexes with atomistic molecular dynamics (MD) simulations of dendrimer conformation in aqueous solutions. We found that the HSA binding constants \( K_b \) of PAMAM dendrimers depend on size and terminal group chemistry. The \( K_b \) values suggest several mechanisms of interactions between PAMAM dendrimers and HSA proteins including (i) electrostatic interactions between charged dendrimer terminal groups and protein residues, (ii) hydrogen bonding between dendrimer internal groups (e.g., amide moiety where the carbonyl O act as donor and the amide H as acceptor), and protein amino acid residues, (iii) hydrophobic interactions between the nonpolar dendrimer and HSA groups, and (iv) specific interactions between dendrimer carboxylic groups and protein aliphatic acid binding sites. The NMR \(^1\text{H} \) and DOSY experiments showed that the interactions between HSA and PAMAM dendrimers are relatively weak. The NMR STD experiments and MD simulations indicate that the inner shell protons of the dendrimers and their neighboring amide groups interact more strongly with HSA proteins. These stronger interactions, which are consistently observed for different dendrimer generations (G0-NH\(_2\) vs G4-NH\(_2\)) and terminal groups (G4-NH\(_2\) vs G4-OH), suggest that PAMAM dendrimers adopt backfolded conformations as they form weak complexes with HSA proteins in aqueous solutions at physiological pH (7.4). Finally, we would like to point out that in the case of dendrimers, “what the cell sees” might not be a NP core surrounded by a corona of long-lived plasma-proteins as suggested by Dawson and co-workers. For the PAMAM dendrimers evaluated in this study, we hypothesize that a cell will "see" and interact with weakly bound and dynamics protein-dendrimer complexes.

**EXPERIMENTAL AND COMPUTATIONAL METHODS**

**Materials.** PAMAM dendrimers of different generations, core and terminal groups (Figure 1) were purchased [as methanol solutions or solids] from Dendritech, Sigma-Aldrich, and Dendritic Nanotechnologies. Essentially fatty acid free human serum albumin (HSA) was purchased from Sigma-Aldrich (USA). TRANSIL beads coated with HSA were purchased as suspensions (201 mM in 0.15 M solution of phosphate buffered saline (PBS)) from Sovicell (Leipzig, Germany). All materials were used as received.

**Binding Constant Measurement Methods.** The binding constants \( K_b \) of PAMAM dendrimers were determined by mixing suspensions of HSA-coated TRANSIL beads with dendrimers in 1.5 mL Eppendorf vials at room temperature. All dendrimer solutions and TRANSIL suspensions were prepared in 0.015 M PBS solution (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM Na\(_2\)HPO\(_4\), 0.147 mM KH\(_2\)PO\(_4\)). In a typical binding assay, aliquots of dendrimers (dry solid or methanol-free solution) were mixed with PBS, HCl, or NaOH to prepare stock solutions with pH 7.4. Then, 60 mL of dendrimer stock solutions and differing volumes of TRANSIL bead suspensions were added to each vial. This was followed by the addition of PBS (pH 7.4) to prepare 600 mL of dendrimer solution + TRANSIL bead suspensions. In all experiments, the total concentration of dendrimer in each vial \([\text{dent}]_{\text{total}}\) was kept constant at 64 \( \mu \text{M} \) of equivalent terminal groups. For example, this corresponds to a concentration of 1.0 \( \mu \text{M} \) for all G4 PAMAM dendrimers with 64 terminal groups. For each dendrimer, we varied the protein concentration in each vial (from 0.5 to 7.5 \( \mu \text{M} \)) to prepare four suspensions with different molar ratios of HSA:dendrimer equivalent terminal groups. The vials containing the samples (dendrimer and HSA in PBS), reference solutions (dendrimer in PBS), controls (HSA in PBS), and buffer solutions were subsequently placed on a LabQuake shaker (Barnstead Thermolyne) and slowly rotated for 60 min. After equilibration, the vials (with TRANSIL bead suspensions) were centrifuged at 5000 G for 10 min followed by 10000 G for 15 min. Aliquots of sample (supernatant), reference, and buffer solutions were analyzed using a UV spectrometer (model T60 from PG Instruments). The measured absorbance (wavelength of 203 nm) of each sample supernatant was corrected (by subtracting the absorbance of the corresponding control) and used to determine the equilibrium concentration of dendrimer in the aqueous phase \([\text{dent}]_{\text{free}}\). Similarly, the measured absorbance of each reference solution was corrected (by subtracting the absorbance of the buffer solutions) and used to determine \([\text{protein}]_{\text{free}}\). The concentration of dendrimer bound \([\text{dent}]_{\text{bound}}\) to the HSA \([\text{dent}]_{\text{bound}} = [\text{dent}]_{\text{total}} - [\text{dent}]_{\text{free}}\) was determined by mass balance. The HSA–dendrimer dissociation constant \( K_d \) was estimated using eq 1:

\[
K_d = \frac{[\text{dent}]_{\text{bound}}}{[\text{protein}]_{\text{bound}} \cdot [\text{dent}]_{\text{free}} / [\text{protein}]_{\text{free}}} [1]
\]
where $f_{cor}$ is a correction factor estimated using eq 2.22

$$f_{cor} = \frac{V_{head}}{V_{total}} = \frac{V_{total} - V_{head}}{V_{total}}$$ (2)

In eqs 1 and 2, $V_{total}$ is the total volume of the suspension and $V_{head}$ is the volume of the Transil beads. Equation 1 is derived from the well-known Scatchard-equation for a system with one binding-site. A detailed description of this derivation is given elsewhere.21,23,43 In all cases, each reported $K_d$ value is the average of four measurements with different molar ratios of HSA to dendrimer $N_H$ groups. The overall binding constant ($K_d = 1/K_b$) is taken as the inverse of the dissociation constant ($K_b$). Each reported error is the standard deviation of the average of four measured $K_d$ values.

**Electrostatic Microscopy.** Aliquots of Transil HSA suspensions were deposited on a coverslip glass (10 mm diameter) and dried under vacuum overnight. After sputter coating with gold for 90 s, the Transil beads were imaged by scanning electron microscopy (SEM) at 20 kV using a FEI SIRION-SEM instrument.

**NMR Spectroscopy.** Three PAMAM dendrimers were evaluated in the NMR studies: $G_0$-NH$_2$, $G_4$-NH$_2$, and $G_4$-OH. Human Serum Albumin (20 mg) was mixed with 5 mg of dendrimer and dissolved in D$_2$O. For studies where HSA and the dendrimers were dissolved in D$_2$O. For studies where HSA and the dendrimers were studied in isolation, identical concentrations were prepared. All samples were adjusted using minimal quantities of NaOD/DCl such that a meter reading of 7.0 was obtained on an Acura pH meter (Fisher Scientific) that was fitted with a glass electrode. All NMR pH probe (Wilmad). Note that this corresponds to a pD of 7.4 after correction.30 In the case of the $G_0$ dendrimers, the molar ratio of dendrimer is $\sim$1:1. An equimolar mixture of the $G_0$-HAS was initially tested; but the signals from the dendrimer could not be distinguished from the protein background because of the very low quantities of $G_0$-NH$_2$ dendrimer required. As such 5 mg of $G_0$-NH$_2$ dendrimer were used in the experiments reported. Note that in this case the $G_0$-NH$_2$ dendrimer is present in molar excess. All NMR spectra were acquired using a Bruker Avance 500 MHz spectrometer equipped with a HBB-13C Triple Resonance Bandwidth Inverse (TBI) probe fitted with an actively shielded 2 gradient. Until stated otherwise water suppression was carried out using presaturation utilizing relaxation gradients and echoes (PURGE).45 All assignments were made using a combination of 2D heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), correlation spectroscopy (COSY), and phase-modulated CLEAN chemical exchange spectroscopy CLEANEX-PM (data not shown).45

Diffusion ordered spectroscopy (DOSY) experiments were performed with a bipolar pulse longitudinal encode–decode sequence.26 256 Scans (256) were collected at a temperature of 298 K, using a diffusion time of 200 ms, and 16384 time domain points. A 2.5 ms sine-shaped encoding/decoding gradient pulse was ramped from 0.98 to 49 gauss/cm in 16 linear increments. Solvent suppression in DOSY was achieved with a presaturation of the water resonance using a 60 W amplifier attenuated at 60 db. Spectra were apodized through multiplication with an exponential decay corresponding to 1 Hz line broadening in F2 dimension in F1. Saturation transfer difference (STD) experiments were carried out using the approach described by Mayer et al.22 Incorporating a Carr– Purcell– Meiboom– Gill filter of 50 ms to attenuate the protein signals and presaturation for solvent suppression. Residual protein signals were subtracted using a double difference approach.23 Selective saturation of the protein was achieved by a train of 50 ms Gaussian shaped pulses, truncated at 1%, and separated by a 100 $\mu$s delay; 40 selective pulses were applied, leading to a total length of the saturation train of 2.004 s. The on-resonance irradiation of the HSA was performed at a chemical shift of 0.82 ppm and off-resonance irradiation at 114 ppm, where HSA signals were not present. Selective irradiation was carried out using a very carefully calibrated effective field of 81 Hz. The spectra were subtracted internally via phase cycling after every scan using different memory buffers for on- and off-resonance irradiation; 12 288 scans were accumulated for each STD experiment. Reference spectra were recorded using the identical sequence with the exception that no irradiation power was applied and that the phase cycle was changed such that each of the 256 scans were additive. All experiments were performed with 32 768 time domain points, 256 dummy scans, and additional recycle delay of 100 ms (in addition to the saturation time). Spectra were apodized through multiplication with an exponential decay corresponding to 1 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. Spectral subtractions to produce the double difference spectra were performed in the interactive mode of Topspin 2.1 (Bruker BioSpin Ltd.).

**Computational Methods.** Acid–base titration experiments have shown that the terminal NH$_2$ groups of Gx-NH$_2$ PAMAM are fully protonated at physiological pH (7.4); whereas their tertiary amines remain neutral.16 Therefore, we built a $G_4$ PAMAM model with all primary amines protonated at neutral pH. The PAMAM dendrimer was solvated with explicit water molecules (∼42000) and Cl$^-$ counter-ions (64) in a cubic periodic box with ∼11 nm side length. The system was minimized and then heated to 300 K over 10 ps. We ran MD simulations in NPT ensemble at 300 K and 1 atm for 2 ns, followed by NVT MD at 300 K for 1 ns. In all these simulations, we used Dreiding III force field that was developed recently for accurate description of hydrogen bonding interaction in dendrimers.46 The further details of computational model and simulations are described in ref 36.

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**Supporting Information Available.** Additional figures and data as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

*Note Added after ASAP Publication:* After this paper was published online April 1, 2011, a correction was made to the bottom panel of Figure 2, and the y-axes of Figures 2–4 were amended. The revised version was published April 12, 2011.

**REFERENCES AND NOTES**


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