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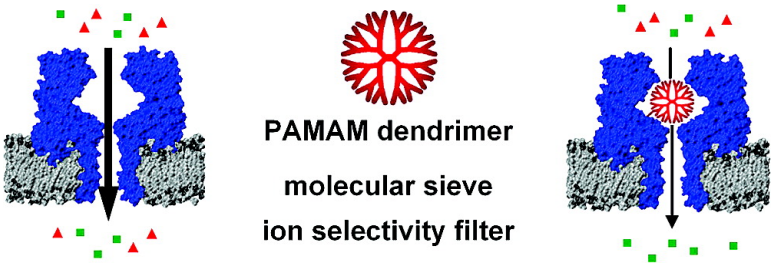

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Nanoscale Protein Pores Modified with PAMAM Dendrimers

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Abstract: We describe nanoscale protein pores modified with a single hyperbranched dendrimer molecule inside the channel lumen. Sulfhydryl-reactive polyamido amine (PAMAM) dendrimers of generations 2, 3 and 5 were synthesized, chemically characterized, and reacted with engineered cysteine residues in the transmembrane pore α-hemolysin. Successful coupling was monitored using an electrophoretic mobility shift assay. The results indicate that G2 and G3 but not G5 dendrimers permeated through the 2.9 nm cis entrance to couple inside the pore. The defined molecular weight cutoff for the passage of hyperbranched PAMAM polymers is in contrast to the less restricted accessibility of flexible linear poly(ethylene glycol) polymers of comparable hydrodynamic volume. Their higher compactness makes sulfhydryl-reactive PAMAM dendrimers promising research reagents to probe the structure of porous membrane proteins with wide internal diameters. The conductance properties of PAMAM-modified proteins pores were characterized with single-channel current recordings. A G3 dendrimer molecule in the channel lumen reduced the ionic current by 45%, indicating that the hyperbranched and positively charged polymer blocked the passage of ions through the pore. In line with expectations, a smaller and less dense G2 dendrimer led to a less pronounced current reduction of 25%. Comparisons to recordings of PEG-modified pores revealed striking dissimilarities, suggesting that differences in the structural dynamics of flexible linear polymers vs compact dendrimers can be observed at the single-molecule level. Current recordings also revealed that dendrimers functioned as ion-selectivity filters and molecular sieves for the controlled passage of molecules. The alteration of pore properties with charged and hyperbranched dendrimers is a new approach and might be extended to inorganic nanopores with applications in sensing and separation technology.

Introduction

Dendrimers are a rapidly expanding class of highly branched globular nanoscale polymers.[1,2] Their synthesis proceeds via either the divergent or the convergent route[3] and offers control over molecular mass, size, shape, degree of branching, and type and number of terminal functional groups. Reflecting their special characteristics, many potential applications have been developed in materials science and nanotechnology for separation technology, surface coatings, and catalysis[4] and in biological sciences for drug and gene delivery, vaccines, and bioimaging.[5] Polyamido-amine (PAMAM) Starburst dendrimers are an important subclass of dendrimers (Figure 1A). They were historically the first dendrimers to be synthesized using the divergent strategy.[1,6] Their synthesis starts with a Michael addition of methyl acrylate to the ethylene diamine core followed by amidation of the tetraester with ethylene diamine, yielding a generation 0 dendrimer. Subsequent Michael addition/amidation cycles provide dendrimers of increasing generation with the number of surface groups doubling each generation. The mass of the polymer also approximately doubles with each extension step until crowding between the terminal surface groups blocks further growth,[7] leading to increasingly rigid compact polymers.[8] The compact structure of PAMAM polymers has been characterized with chemical[9,10] and physicochemical techniques.[11,12]

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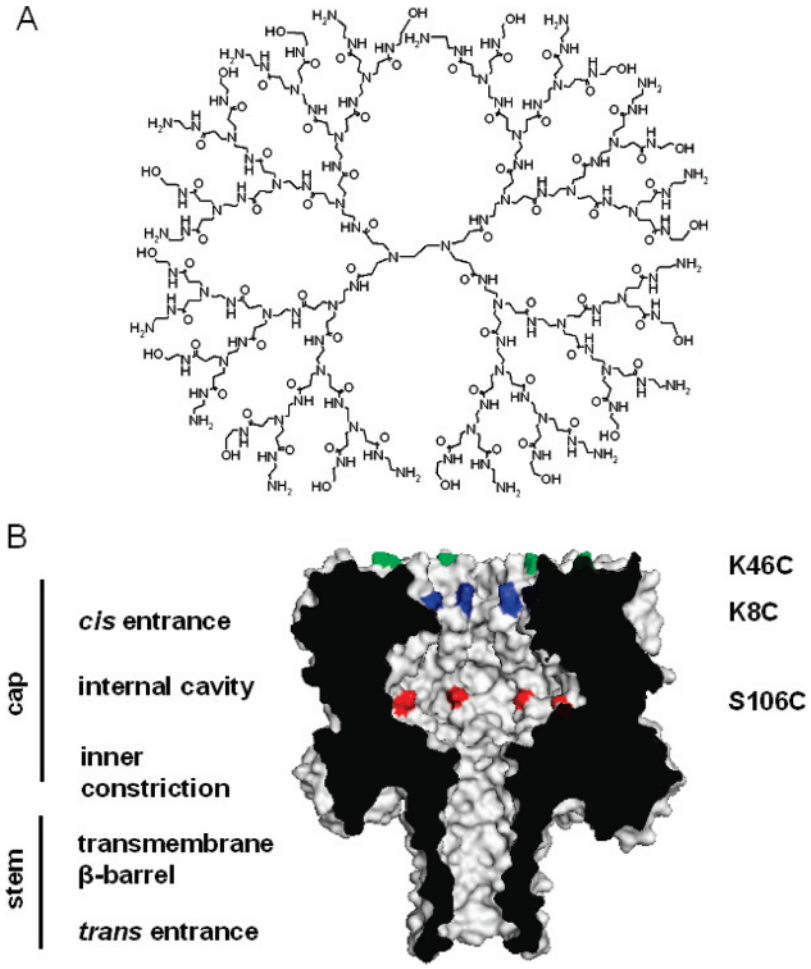

Figure 1. (A) Structure of a polyamido amine dendrimer of generation 3 with 50% amine and 50% hydroxyl terminal groups. (B) Cross-sectional view of the heptameric αHL pore with cysteine substitutions K46C, K8C, and S106C. The model was generated using crystallographic data[13] and PyMol. The internal diameters of the channel are as follows: 2.9 nm, cis entrance; 4.1 nm, internal cavity; 1.3 nm, inner constriction; 2 nm, trans entrance of the β-barrel.

The α-hemolysin (αHL) polypeptide is a bacterial toxin which self-assembles to form a heptameric protein pore. The X-ray structure of the αHL pore resembles a mushroom with a wide cap and a narrow stem which spans the lipid bilayer (Figure 1B).[13] The external dimensions of the heptameric αHL pore are 10 × 10 nm, while the central channel is 2.9 nm in diameter at the cis entrance and widens to 4.1 nm in the internal cavity (Figure 1B). In the transmembrane region, the channel narrows to 1.3 nm at the inner constriction and broadens to 2 nm at the trans entrance of the β-barrel. The defined structure of αHL has facilitated extensive engineering studies and led to the development of tools for the targeted permeabilization of cells[14] as well as new biosensor elements which permit the stochastic sensing of molecules.[15] In stochastic sensing, individual molecules are detected by their ability to modulate ionic current flowing through a single pore. This approach has been used for analytes such as toxic metal ions,[16] drugs,[17] enantiomers,[18] TNT,[19] and nucleotides.[20] Stochastic sensing is also an attractive label-free strategy to study single-molecule kinetics of chemical transformations such as the cis−trans isomerization of azobenzene[21] or the multistep formation or breaking of covalent bonds.[22]

An essential component in these sensors has been the covalent attachment of small molecules and linear polymers within the pore. For example, tethering of DNA oligonucleotides to engineered pores enabled the sequence-specific detection of individual free DNA strands.[23] Organic polymers such as polyethylene glycol (PEG) were also tethered to pores via engineered cysteines. Single-channel current recordings of these pores demonstrated that a single PEG chain modulated the ionic current passing through the pore.[24] On the basis of the

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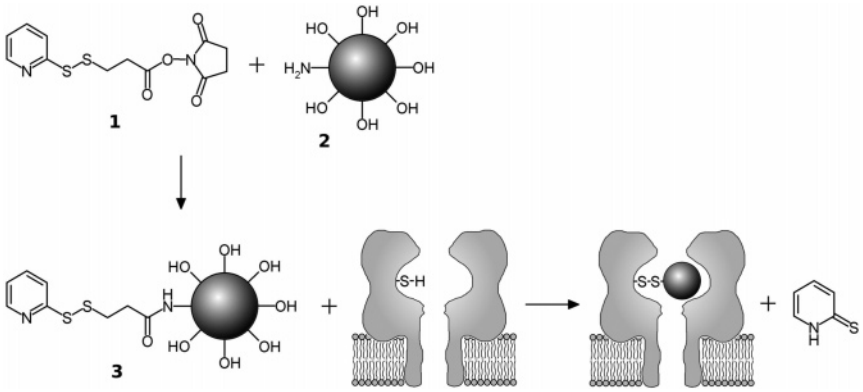
Scheme 1. Preparation of Sulfhydryl-Reactive Pyridyldithiopropanoyl PAMAM Dendrimers, 3, from Heterobifunctional Cross-Linker Succinimidyl-3-(2-pyridyldithio)-propanoate (SPDP), 1, and PAMAM, 2, Followed by Coupling To Engineered Cysteines of the Heptameric Protein Pore αHL[a]



[a] For visual clarity, only one of the seven thiol groups of the protein pore is shown. Drawing not to scale.

characteristic current modulations, differences in the conformational dynamics of individual linear polymers of different chain length could be observed.[24] PEG-modified pores also led to the development of biosensor elements capable of detecting protein analytes at the single-molecule level.[25] Given the past work on flexible, "soft" linear polymers, we were interested in placing a "hard" polymer such as a dendrimer into the pore lumen and investigating this approach for its potential in single-molecule studies and development of designed biomaterials.

In this report, we describe the preparation and characterization of sulfhydryl-reactive PAMAM dendrimers and their coupling to cysteine residues of αHL to form an engineered protein pore. PAMAM dendrimers of generations 2, 3, and 5 (G2, G3, and G5) with a mixed surface of terminal hydroxyl/amine groups were modified with the heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)-propanoate) (SPDP) (Scheme 1). The resulting pyridyldithiopropanoyl (PDP) PAMAM reagents were characterized using RP-HPLC, MALDI-MS, SDS gel electrophoresis, and an electrophoretic mobility shift assay based on the formation of a thiol-specific linkage to a cysteine-bearing polypeptide. Dendrimers were coupled to cysteine residues outside and inside the lumen of the heptameric nanoscale αHL pore. The size-dependent coupling of PAMAM into the protein pore was studied because the permeation properties of some dendrimers into narrow nanoscale openings were not known in advance. In this respect, the αHL pore of known X-ray structure served as a reference and calibration standard. The permeation of the compact PAMAM dendrimers was compared to the permeation of a more flexible linear polyethylene glycol derivative. Current recordings of αHL pores carrying a single PAMAM molecule either inside or outside the channel were performed to show that the presence of the dendrimer altered the conductance of the channel in a manner depending on the size and position of the dendrimer. Recordings also demonstrated that PAMAM dendrimers functioned as an ion-selectivity filter and a molecular sieve for the passage of biopolymers. Comparisons of PAMAM protein pores to recordings of PEG-modified pores revealed striking differences, hence demonstrating how the dissimilar structural and dynamic properties of hard and soft polymers affect ion permeation at the single-molecule level.

This study is of interest to the areas of protein research, materials engineering, and single-molecule science. In protein research, sulfhydryl-reactive PAMAM dendrimers can be used as a new type of reagent to study the molecular structure of membrane proteins. Currently used reagents for the substituted-cysteine accessibility method (SCAM)[26] are too small or too flexible[27] for membrane proteins with pore diameters of more than 2 nm. Sulfhydryl-reactive derivatives of rigid PAMAM dendrimers with diameters up to 10 nm overcome these limitations. In materials science, the engineering of the permeation properties of pores or porous structures is an intensive area of research with applications, for example, in warfare sensing and the membrane-based separation of organic molecules and biomolecules.[28] Placing charged and dense dendrimer polymers inside pores represents a new strategy for altering pore properties that can potentially be applied to inorganic structures with the purpose of controlling the passage of molecules based on their charge and/or size. In single-molecule research, current recordings of pores with single dendrimer molecules can reveal dissimilarities to linear polymers, implying that differences in the structural dynamics of compact dendrimers vs flexible linear polymers can be observed at the single-molecule level.

Results

Selection of PAMAM Dendrimers. Dendrimers of different generations were selected to construct αHL protein pores carrying PAMAM outside and inside the channel lumen. The choice of dendrimers was guided by molecular models of αHL and the known dimensions of PAMAM dendrimers. For the preparation of pores with a PAMAM outside the lumen, we selected G5 dendrimer with a hydrodynamic diameter of 6.2 nm and a hard-sphere diameter of 4.2 nm, obtained from the

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solvent exclusion volume.[11,29] The difference between the hydrodynamic and hard-sphere diameter suggests that the dendrimer molecule is less compact than, e.g., a native globular protein of comparable mass.[11,30] On the basis of these considerations, G5 was predicted to stay outside the pore because it is too big to pass the 2.9 nm wide cis entrance of αHL. For the generation of αHL protein pores carrying a dendrimer inside the lumen, we chose PAMAM dendrimers of generations 3 and 2. The hydrodynamic and hard-sphere diameter of G3 is 4.1 and 2.9 nm, respectively,[11,29] while the hydrodynamic diameter of G2 is 2.9 nm (hard-sphere diameter not available). G3 may or may not couple inside the pore lumen depending on whether the permeation across the 2.9 nm wide cis entrance is governed by the hard-sphere diameter (2.9 nm) or the hydrodynamic diameter (4.1 nm). In any case, the smaller G2 with a hydrodynamic diameter of 2.9 nm was predicted to permeate into the pore.

Sulfhydryl-reactive polyamidoamine dendrimers were generated using G5 PAMAM with a mixed surface of terminal −OH and $-NH_2$ groups at an average ratio of 90:10, G3 PAMAM with a mixed surface of 50:50 hydroxyl/amino groups, and G2 PAMAM with terminal NH_2 groups. A mixed surface with hydroxyl groups avoids formation of multimeric aggregates which can be found in purely amino-terminated but not in hydroxyl-functionalized G5 PAMAM dendrimers.[11] The relative percentages of amine groups of the G3 and G5 PAMAM dendrimers were initially chosen to yield approximately the same number of sulfhydryl-reactive groups for each generation of dendrimer (13 for G5 and 16 for G3 and G2).

Synthesis and Chemical Analysis of Sulfhydryl-Reactive Dendrimers. To introduce sulfhydryl-reactive groups, G5, G3, and G2 PAMAM dendrimers were reacted with the heterobifunctional cross-linker SPDP (Scheme 1). The *N*-succinimidyl-activated ester of SPDP couples to the terminal primary amines to yield amide-linked 2-pyridyldithiopropanoyl (PDP) groups. After reaction with SPDP, PAMAM-PDP was extracted with dichloromethane and precipitated in acetone to remove unreacted cross-linker and the hydrolysis product 3-(2-pyridyldithio)-propanoic acid, as confirmed by TLC (see Experimental Procedures, Supporting Information, S4).

RP-HPLC analysis was used to determine the extent to which SPDP had reacted with dendrimers. The samples were compared to unmodified PAMAM that had been subjected to an identical purification procedure. All samples were initially run on a linear gradient from 90:10 H_2O(0.1 wt % TFA)/acetonitrile to 50:50 H_2O(0.1 wt % TFA)/acetonitrile over 30 min. During this gradient, G3-PAMAM was eluted after 9 min compared to the modified G3-PAMAM-PDP that was eluted after 15 min (Figure 2A and B). The increased retention time is in line with addition of hydrophobic PDP groups. The slight broadening of the peaks and appearance of shoulder peaks present for both PAMAM and PAMAM-PDP might reflect structural defects that occurred during synthesis of the dendrimer, for example, by incomplete alkylation of the primary amines or intramolecular cyclization.[10] Unmodified dendrimer starting material was absent from the traces of the pyridyldisulfide-modified dendrimers, indicating

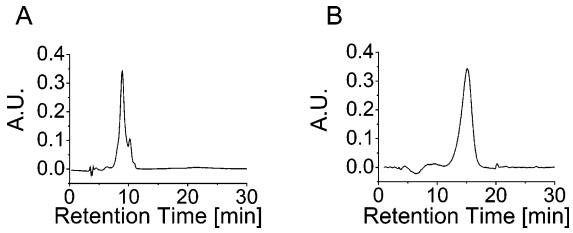

Figure 2. RP-HPLC analysis of the derivatization of PAMAM dendrimer with the heterobifunctional cross-linker SPDP. G3-PAMAM before (A) and after (B) reaction with SPDP.

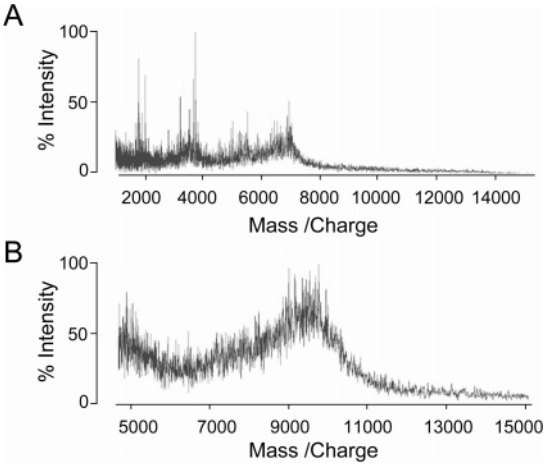

Figure 3. MALDI-TOF analysis of SPDP-modified PAMAM dendrimers to determine the average number of coupled pyridyl disulfide groups. G3-PAMAM before (A) and after (B) reaction with SPDP.

that the reaction had gone to completion. Similar findings on the complete conversion of the starting material were also obtained for G2 and G5 (Supporting Information, S8).

The number of PDP groups coupled to PAMAM dendrimers was determined by MALDI-TOF. Analysis of the unmodified G3 dendrimer gave a broad M^+ peak at 6.9 kD (Figure 3A), which closely corresponds to the theoretical molecular mass of the dendrimer (6909 D).[10] Coupling of the G3 terminal amine groups with SPDP resulted in a shift in the major peak to 9.6 kD (Figure 3B). Each pyridyldithiopropanoyl group has a molecular mass of 199.3 D. Therefore, these data indicate an average of 13 ± 2 pyridyldisulfide groups per dendrimer molecule ($n = 3$; n, number of independent experiments). This is slightly lower than would be expected for complete modification of the 16 amine groups. The same type of MALDI analysis yielded approximately 5.2 ± 1.0 for the G2 and 14 ± 2 PDP groups for the G5 PAMAM dendrimer (Table 1 and Supporting Information, S8 and S9). The number of PDP groups was also determined via photometric analysis, which involved treatment of samples with excess reducing agent dithiothreitol (DTT) to cleave the disulfide bond of PDP, and detection of the cleavage product pyridyl-2-thione at 343 nm. The results of the photometric analysis yielded 5.0 ± 1.5, 11 ± 2, and 13 ± 2 PDP groups for G2-PDP, G3-PDP, and G5-PDP, respectively, which is very similar to the numbers obtained from MALDI-MS (Table 1).

The yield of PAMAM-PDP dendrimers preparations was estimated using sodium dodecylsulfate gel electrophoresis (SDS-PAGE) and Coomassie staining (Figure 4A). The gel band intensities of PDP-modified dendrimers were compared with the intensities of PAMAM dendrimers of a known amount.

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Table 1. Chemical Characteristics and Dimensions of G2-PDP, G3-PDP, and G5-PDP

	number of PDP groups in PAMAM-PDP[b]			diameter [nm][c]			
	number of terminal amine groups[a]	MALDI-MS analysis	photometric analysis	hydrodynamic	hard-shell	permeation through 2.9 nm entrance[d]	modification of a highly accessible cysteine residue[d]
G5-PDP	13	14 ± 3	13 ± 2	6.2	4.2	−	+
G3-PDP	16	13 ± 2	11 ± 2	4.2	2.6	+	+
G2-PDP	16	5.2 ± 1.0	5.0 ± 1.5	2.9	na	+	na

[a] Obtained from ^{1}H NMR data. [b] Average of three independent experiments on the photometric detection of pyridyl-2-thione at 343 nm released by treatment of pydridyldithiopropanoyl-PAMAM with the reducing agent DTT. [c] Derived from sedimentation analysis.[11,30] [d] Permeation characteristics through a 2.9 nm pore represent the modification results with αHL heptamer S106C$_7$ and K8C$_7$ (see Supporting Information, S11). For the modification with very accessible residues, heptamer K46C$_7$ was used. Three independent experiments were performed for each modification reaction and gave the same results.

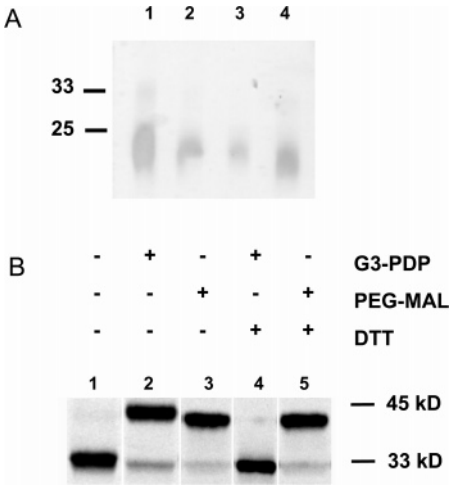

Figure 4. (A) Quantifying the yield of a G3-PAMAM-PDP preparation using SDS gel electrophoresis and Coomassie staining. Solutions of unmodified dendrimers of known concentration and SPDP-modified dendrimers of unknown concentrations were analyzed. The resulting electropherograms were subjected to densitometric analysis to determine the unknown concentrations: lane 1, 67 μM G3-PAMAM; lane 2, 33 μM G3-PAMAM; lane 3, 17 μM G3-PAMAM; lane 4, G3-PAMAM-PDP, concentration determined to be 50 μM. (B) Sulfhydryl-reactive G3-PDP couples specifically to monomeric αHL cysteine mutant K46C and causes a gel shift in SDS-PAGE autoradiographs: lane 1, K46C; lane 2, K46C treated with G3-PDP; lane 3, K46C treated with PEG-MAL 5 kD; lane 4, K46C with G3-PDP and excess reducing agent DTT; lane 5, K46C with PEG-MAL 5 kD and excess DTT.

Densitometric analysis indicated yields of approximately 15% for G2-PDP, 80% for G3-PDP, and 50% for G5-PDP relative to the amount used as starting material (Table 1 & Supporting Information, S10).

PAMAM-PDP Forms a Disulfide Bond with a Cysteine Residue of a Polypeptide Chain. To test their coupling efficiencies, PAMAM-PDP dendrimers were reacted with a water-soluble polypeptide carrying a single engineered cysteine in an accessible position. The [^{35}S]methionine-labeled αHL polypeptide of mutant K46C was generated by coupled in vitro transcription/translation using a cell-free extract. The radiolabeled protein was combined with a 10-fold molar excess of G3-PDP over thiol groups in the reaction mixture and incubated for 20 min at room temperature. The mixture was analyzed by SDS-PAGE and autoradiography. Unmodified αHL polypeptide migrated at a relative molecular mass of 30 kD (Figure 4B, lane 1), while the major protein band after G3-PDP modification was up-shifted to 40 kD (Figure 4B, lane 2). No up-shifted band was observed when the reaction mixture was treated with excess reducing agent DTT (Figure 4B, lane 4), indicating that G3-PDP had coupled specifically to αHL through a disulfide bridge. By quantifying the intensity of the up-shifted αHL-G3 band relative to the unmodified αHL protein band (Figure 4B, lane 2), we deduced that 90% of the cysteine mutant was modified. The same extent of modification was found for G2-PDP and G5-PDP (data not shown). These results confirm that the preparations of SPDP-derivatized PAMAM dendrimers were highly reactive and did not contain contaminating SPDP or the hydrolysis product 3-(2-pyridyldithio)-propanoic acid which might block coupling to cysteines. A similar high extent of modification of 95% was obtained using the sulfhydryl-active reagent mono-methyl polyethylene glycol maleimide 5 kD (PEG-MAL 5 kD) (Figure 4B, lane 3). In line with formation of a thioether bridge between PEG and αHL, treatment with excess DTT did not cleave the conjugate (Figure 4B, lane 5).

A Dendrimer Molecule Can Be Coupled to a Protein Pore Outside the Channel Entrance. Dendrimers G5-PDP and G3-PDP were reacted with the cysteine mutant K46C in an assembled αHL homoheptamer. The seven cysteine residues at the cap of the pore form a ring surrounding the cis entrance (Figure 1B). On the basis of the exposed position of the cysteine residues, both G3-PDP and G5-PDP were expected to react with heptamer. To test the reactivity, radiolabeled homoheptamer K46C$_7$ was generated, treated with G5-PDP, G3-PDP, or, for comparison purposes, PEG-MAL 5 kD, and analyzed via SDS-PAGE and autoradiography to detect the appearance of up-shifted bands. αHL heptamers are not denatured in SDS-PAGE and therefore migrated as defined bands as seen for unmodified K46C$_7$ (Figure 5A and B, lane 1). Upon reaction with G5-PDP, an additional major up-shifted band appeared (Figure 5A, lane 2). This band represents the specific covalent coupling product of G5-PDP and the αHL cysteine mutant as no up-shifted band was observed with unmodified G5 (data not shown). Coupling of G5-PDP to K46C$_7$ produced one but not a second up-shifted band, strongly indicating that coupling of a second G5 dendrimer was disfavored by either steric clash or charge repulsion with the first tethered dendrimer. The first dendrimer could also have coupled to a second or third cysteine residue, thereby preventing reaction with a second molecule. Indeed, some residual cysteine residues of G5-PDP-treated heptamers could still couple to flexible and neutral PEG-MAL 5 kD to produce additional up-shifted bands (Figure 5A, lane 3). A higher extent of modification was obtained when K46C$_7$ was treated with PEG-MAL 5 kD alone (Figure 5A, lane 4). The highly up-shifted and partly unresolved bands represent pore species with 5, 6, or 7 tethered PEG chains as observed in another study.[24] It is worth mentioning that coupling of seven PEG chains with a total mass of up to 7 × 5 kD = 35 kD led to a dramatic gel upshift of the αHL band (Figure 5A, lane 4) while addition of one G5 dendrimer molecule with a comparable mass of 29 kD produced a lesser gel shift (Figure 5A, lane 2) even though addition of

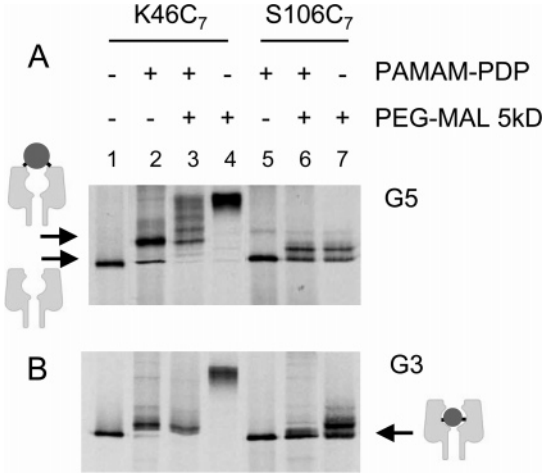

Figure 5. Coupling of a PAMAM-PDP dendrimer inside and outside the lumen of a protein channel. Homoheptameric αHL cysteine mutants were reacted with G5-PDP, G3-PDP, and PEG-MAL and analyzed via gel electrophoresis and autoradiography. $K46C_7$ (lanes 1−4) and $S106C_7$ (lanes 5−7) were treated with PAMAM-PDP (lanes 2 and 5), PAMAM-PDP followed by PEG-MAL (lanes 3 and 6), or PEG-MAL (lanes 4 and 7). The modification reactions were performed with G5-PDP (A) or G3-PDP (B).

the positively charged and unfolded PAMAM polymer would have been expected to slow down the electrophoretic migration of the protein. This difference between PEG and PAMAM likely reflects the different ways in which flexible vs compact polymers interact with the polyacrylamide gel meshwork. Most probably, PEG chains became entangled within the holes of the meshwork, resulting in a retarded migration of the protein band, while PAMAM is too compact to get intertwined.

Reaction of $K46C_7$ heptamers with the smaller G3-PDP resulted in two up-shifted and closely migrating bands (Figure 5B, lane 2 and Supporting Information, S11 for a magnified image of the double band), which likely represent heptamers with one and two G3 dendrimers. In line with the lower molecular mass of G3 (M_r 6.9 kD), both G3-heptamer conjugates migrated lower than the conjugate with G5-PDP (M_r 28.9 kD) (Figure 5A, lane 2). Interestingly, reaction of G3-modified $K46C_7$ heptamers with PEG-MAL 5 kD did not produce additional up-shifted bands (Figure 5B, lane 3), which were however observed in the case of G5-modified heptamers (Figure 5A, lane 3). This indicates that two G3-PDP dendrimers reacted with most of the cysteines, thus blocking further coupling to the flexible PEG-MAL polymer.

A Single PAMAM Dendrimer Can Be Attached Inside the Lumen of a Protein Pore. After establishing that G5-PDP and G3-PDP coupled to very accessible residue K46C, we examined position S106C which is located inside the internal cavity of the αHL pore (Figure 1B). First, we tested the reactivity of the engineered cysteine residue with PEG-MAL 5 kD. Gel electrophoretic analysis of radiolabeled $S106C_7$ heptamers revealed one up-shifted band (Figure 5A, lane 7), confirming successful modification with the polymer. A single and not multiple up-shifted band was obtained because the narrow pore accommodates only one PEG 5kD molecule as found in theoretical[31] and other experimental studies.[24,25] Next, we tested the reaction of $S106C_7$ with the dendrimeric reagents. On the basis of the known hydrodynamic and hard-sphere diameters of the PAMAM dendrimers, we did not expect G5-PDP to permeate into the lumen. By contrast, G3-PDP may couple to the cysteine residues in the internal cavity provided its permeation across the 2.9 nm wide cis entrance is governed by the hydrodynamic diameter (2.9 nm) rather than the hard-sphere diameter (4.1 nm). Gel electrophoretic analysis revealed that reaction with neither G3-PDP nor G5-PDP led to a major up-shifted band (Figure 5A and 5B, lane 5). The lack of up-shifted bands upon PAMAM-PDP addition suggests that $S106C_7$ did not react with the dendrimers. Another possible explanation is that coupling occurred but failed to produce an appreciable gel electrophoretic shift as the tethered dendrimer would reside inside the lumen of the pore. To discriminate between these two possibilities, we treated the $S106C_7$ heptamer with G5-PDP followed by PEG-MAL. Reaction with the flexible PEG chain is known to yield an up-shifted band and can therefore be used to probe whether S106C is accessible or the internal cavity is blocked by a dendrimer molecule. Gel electrophoretic analysis revealed that dual treatment by G5-PDP and then PEG-MAL 5 kD led to an up-shifted band (Figure 5A, lane 6), implying that G5-PDP did not permeate into the internal cavity. By contrast, reaction with G3-PDP and then PEG-MAL 5 kD did not yield an up-shifted band (Figure 5B, lane 6), indicating that G3 permeated into the pore and coupled to S106C, thereby blocking subsequent modification with PEG-MAL. A similar finding on the differential accessibility of G5-PDP, G3-PDP, and PEG-MAL 5 kD was obtained by testing the reactivity of $K8C_7$, which is positioned at the 2.9 nm wide cis entrance (Figure 1B and Supporting Information, S11). These results demonstrate that G3 but not G5 permeated through the cis entrance into the internal cavity. The finding that G5 did not enter the pore is remarkable considering that PEG-MAL 10 kD whose hydrodynamic diameter of 6.2 nm is identical to G5-PAMAM permeated into the pore to couple to residue S106C (Supporting Information, S12). This highlights the different degrees of structural flexibility of dendrimers vs flexible PEG polymers.

Single Dendrimer Alters the Current Flow Through a Protein Pore. The presence of a PAMAM molecule inside the lumen of the heptamer was expected to alter the passage of ions through the channel. We used single-channel current recordings to investigate to what extent the ion conductance was influenced. The recordings were performed with heptamers $S106C_7$, $S106C_7$-G3-PAMAM, $S106C_7$-G2-PAMAM, and $K46C_7$-G5-PAMAM, which had been eluted from preparative PAGE gels. At a potential of +100 mV (chamber on the cis side of the protein grounded), current traces of unmodified $S106C_7$ exhibited a unitary conductance of 925 ± 73 pS ($n = 7$) and an rms noise of 1.82 ± 0.17 normalized to the noise at 0 mV (Figure 6A). By contrast, traces of $S106C_7$ with a single G3-PAMAM inside the lumen had a lower current (Figure 6B) with an average conductance of 512 ± 63 pS and a normalized rms noise of 2.82 ± 0.33 ($n = 8$). The current blockade of 45 ± 6% relative to the open channel indicates that the presence of the dense dendrimer inside the pore lumen largely blocked the permeation of ions by either steric or electrostatic effects or a combination of both. Placing a smaller and less dense G2 dendrimer led to a less pronounced current reduction of 25 ± 5% (Figure 6C) (conductance of 686 ± 114 pS, normalized rms noise of 4.35 ± 0.48, $n = 5$).

The altered channel properties are specific for the presence of dendrimer because addition of excess reducing agent DTT

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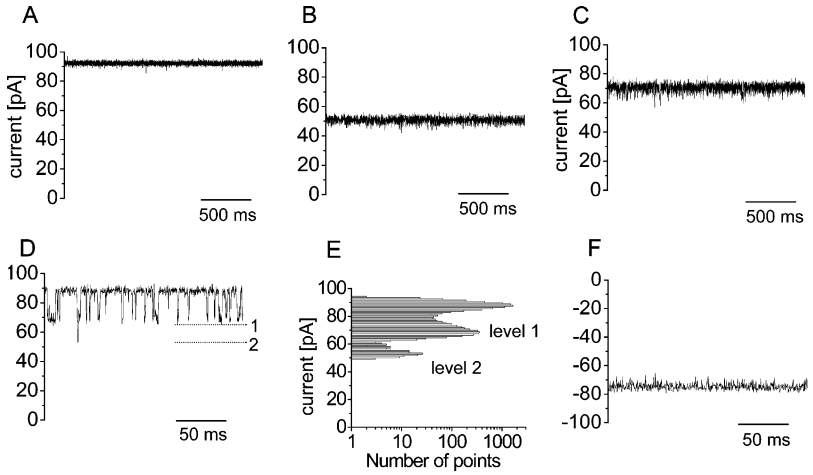

Figure 6. Representative single-channel current traces of (A) $S106C_7$, (B) $S106C_7$-G3-PAMAM, (C) $S106C_7$-G2-PAMAM, and (D and F) $K46C_7$-G5-PAMAM. (E) All-points histogram of $K46C_7$-G5-PAMAM (D) with a duration of 2 s. The recordings were performed in 1 M KCl, 20 mM Tris·HCl pH 7.5 at a transmembrane potential of +100 mV (A−D) or −100 mV (F) with the chamber on the cis side of the protein pore grounded. The currents were filtered at 1 kHz and sampled at 5 kHz.

Table 2. Permeability Ratios for αHL Heptamers Modified with PAMAM Dendrimers

				properties of PAMAM inside protein pore	
heptamer	V_r [mV][a]	P_{Cl^-}/P_{K^+}[a]	P_{K^+}/P_{Cl^-}[a]	hydrodynamic diameter	no. of free amino groups on surface[b]
$S106C_7$	−4.5[c]	1.47 ± 0.06	0.68 ± 0.03	na	na
	5.4[d]	1.59 ± 0.06	0.62 ± 0.2	na	na
$S106C_7$-G2	−9.9[c]	2.41 ± 0.14	0.41 ± 0.02	2.9	11
	11[d]	2.69 ± 0.25	0.37 ± 0.03		
$S106C_7$-G3	−4.9[c]	1.52 ± 0.06	0.65 ± 0.03	4.2	3
	6.6[d]	1.77 ± 0.05	0.56 ± 0.02		

[a] Average of at least three independent experiments ± standard deviation. [b] Determined by subtracting the number of amide-coupled PDP groups from the total number of primary amino groups prior to modification with SPDP. [c] 300 mM KCl (cis), 100 mM (trans). [d] 100 mM KCl (cis), 300 mM (trans).

cleaved the disulfide bonds between the cysteine and PAMAM upon which the conductance state rose to the open state value (data not shown). After examining the conductance changes caused by dendrimers inside the pore lumen, we investigated the effect of G5-PAMAM tethered to K46C just outside the lumen. A typical trace of $K46C_7$-G5-PAMAM is displayed in Figure 6D. The trace with a conductance of 880 pS was decorated with fast downward current fluctuations. An all-points-current histogram generated from a trace of $K46C_7$-G5-PAMAM with a duration of 2 s displays the current levels for the fluctuations (Figure 6E). The peak at 88 pA corresponds to the open channel, while the peak at 68 pA and a minor peak at 53 pA reflect blockade level 1 and blockade level 2, respectively. Blockades to level 2 were not investigated further due to their low frequency. The dominating current blockades from the open channel to level 1 were characterized by an amplitude of 210 ± 23 pS and an average duration of 2.2 ± 0.4 ms. The events occurred at a high frequency of 150 ± 30 s^{-1} ($n = 3$, total of 2000 events). The current fluctuations of $K46C_7$-G5-PAMAM only occurred at a potential of +100 mV but not at −100 mV (Figure 6F), suggesting that the events were dependent on movement of the charged PAMAM relative to the polarity of the potential.

PAMAM Inside the Pore Lumen Acts as an Ion Selectivity Filter and Molecular Sieve. To test the utility of coupling a dendrimer inside a pore, we examined how PAMAM altered the movement of ions through the modified αHL pores. The permeability ratio P_{Cl^-}/P_{K^+} was determined for heptamers $S106C_7$, $S106C_7$-G2-PAMAM, and $S106C_7$-G3-PAMAM. $I-V$ curves were constructed for currents recorded with both cis/trans and trans/cis KCl gradients (Supporting Information, S13), and charge selectivities were calculated from the reversal potential, V_r (Table 2). For $S106C_7$, this analysis yielded a permeability ratio of $P_{Cl^-}/P_{K^+} = 1.47 \pm 0.06$ ($n = 3$), implying that αHL is a weakly anion-selective channel in agreement with previous studies.[32] For $S106C_7$-G2-PAMAM, the permeability ratio was found to be 2.41 ± 0.14 (Table 2), suggesting that PAMAM made the pore more anion selective. This preference for anions is most likely due to the 11 additional protonated positively ionized primary amino groups on the surface of the dendrimer inside the channel lumen. By comparison, $S106C_7$ pores modified with G3-PAMAM displaying only three terminal primary amino groups had a P_{Cl^-}/P_{K^+} ratio of 1.52 ± 0.06 (Table 2), which is very similar to the value for the unmodified pore. The G3 dendrimer had on average only three primary amino groups because the other amines were converted into amide bonds in the course of the attachment to the PDP moiety.

The permeability ratio for $S106C_7$-G2-PAMAM of 2.41 might be further enhanced by increasing the number of positive charges in the dendrimer. It can be expected that PAMAM dendrimers with more surface primary amines will lead to a bigger preference for anions. Quaternary amines[33] could also be used to generate permanent positive charges. In addition, the preferred passage of anions over cations might also be enhanced by changing the size of the dendrimer. Currently, pore-lodged G2 PAMAM with a diameter of 2.9 nm is 1.2 nm smaller than the

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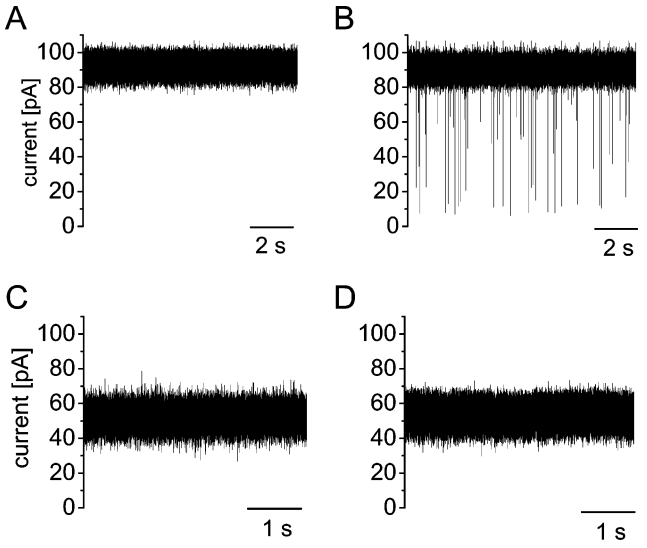

Figure 7. PAMAM acts as a molecular sieve for the passage of RNA polymers through the αHL pore. Single-channel current traces of (A) $S106C_7$, (B) $S106C_7$ with 1 μM RNA oligonucleotide C_{30} at the cis side, (C) $S106C_7$-G3-PAMAM, and (D) $S106C_7$-G3-PAMAM with 1 μM RNA oligonucleotide C_{30} at the cis side with a potential of +100 mV. The traces were filtered at 10 kHz and sampled at 50 kHz.

4.1 nm wide internal cavity. The resulting gap between the dendrimer surface and the channel wall is probably big enough to accommodate the major flow of ions around rather than through the dendrimer. Minimizing the gap is expected to force ions to pass closer to or through the dendrimer and hence increase the effect of positive dendrimer charges on ion flow.

After establishing that PAMAM dendrimers can alter the flow of small ions through the pore, we examined to what extent the dendrimer would affect the permeation of larger molecules. In particular, we tested whether PAMAM would function as a molecular sieve for translocation of oligoribonucleotides. The experiments were conducted with heptamers $S106C_7$ and $S106_7$-G3-PAMAM and oligoribonucleotide C_{30}. When 1 μM C_{30} was added to the cis side of $S106C_7$ with a potential of +100 mV at the trans side, frequent short current deflections appeared (Figure 7B) which were, however, absent in recordings without RNA (Figure 7A). The high-amplitude blockades had an average current amplitude of 87 ± 4% of the open-channel conductance equivalent to a residual conductance level of 120 ± 30 pS and a dwell time of 133 ± 34 μs and occurred at a frequency of 6.5 ± 1.2 s^{-1} ($n = 3$), which are similar to the findings from other nanopore recordings of oligoC-RNA.[34] The high-amplitude events represent the translocation of nucleic acids strands from the cis to the trans side of the pore which temporarily blocks movement of ions through the narrow inner constriction.[34] By contrast, current traces of heptamer $S106C_7$-G3-PAMAM before (Figure 7C) and after addition (Figure 7D) of 1 μM C_{30} did not show any apparent differences. Analysis of extended C_{30} traces revealed that the frequency of occurrence of high-amplitude events for C_{30} traces was 0.005 s^{-1}, which is close to the background noise of the recordings without RNA. The absence of high-amplitude events suggests that the dendrimer blocked the passage of the nucleic acids strands. It is very unlikely that translocation of RNA molecules occurred while their current signatures were masked by the lower conductance level of $S106C_7$-G3-PAMAM pores. If they had occurred, RNA translocation events with a residual conductance of 120 pS should have given rise to highly distinctive current deflections clearly visible in traces of $S106C_7$-G3-PAMAM with a mean conductance of 512 ± 63 pS (Figure 7D). Our data illustrate that a PAMAM dendrimer acts as a size-dependent molecular sieve for the passage of matter through the pore. G3 PAMAM reduced the flux of potassium (M_w 39 D) and chloride ions (M_w 35.5 D) by 45% compared to unmodified pores, while the translocation of RNA polymers (M_w 8614 D) was reduced by more than 3 orders of magnitude. We note that the observed molecular weight cut off is specific for the PAMAM dendrimer of generation 3 and a potential of +100 mV.

Interpretation of Conductance Properties of PAMAM-Modified Pores and Comparison to Pores Modified with PEG. Current traces of dendrimer-modified pores provided insight into the biophysical nature of engineered constructs. For example, recordings from αHL pores with internal G2 and G3 dendrimers established a direct and positive dependence between the size of the polymer inside the pore lumen and the extent of the current blockade. In addition, comparison of G2 and G3 traces with those of G5 revealed information about the conformational freedom of the dendrimer located inside and outside the narrow channel lumen, respectively. In these pores there were no large current steps, indicating that large conformational rearrangements of the dendrimer did not occur most likely due to the steric restraints in the narrow lumen. However, the normalized rms noise of G2 (4.35 ± 0.48) and G3 (2.82 ± 0.33) traces was higher than that of unmodified pores (1.82 ± 0.17), suggesting some minor movement of the dendrimer relative to the pore and/or small conformational changes within the dendrimer structure. Placing G5 outside the pore removed some of the steric constraints, and in line with our simple model, larger current fluctuations occurred in the traces indicating a movement of the dendrimer. The fluctuations oscillated at a rate of 150/s between the conductance level for the open channel and a 22% blockade level. These fluctuations can be interpreted as a rattling movement of the dendrimer between a state very close to the pore entrance and a state further away. The molecular forces for the fast dynamic changes could be the voltage-driven movement of the positively charged dendrimer outside the pore (positive potential at trans side) and the entropy-driven contraction of the stretched dendrimer branches bringing the polymer back to the pore entrance. When the potential was reversed to −100 mV at the trans side, no large current fluctuations were observed. This is in line with the model on the voltage-triggered movement of the dendrimer. Accordingly, the positive PAMAM ball would be expected to move toward the negative pole at the trans side and remain at the pore entrance without subsequent movement out of the channel. The voltage-dependent dynamic behavior of the tethered PAMAM dendrimer indicates that it could function as a voltage-sensing molecular-ball valve.

Current traces also highlighted the different structural dynamics of compact PAMAM as opposed to flexible PEG polymers at the single-molecule level. Pores with a single G2 or G3 PAMAM inside the lumen had a constant current implying that conformational changes of the dendrimer were too small to alter the conductance. In contrast, pores modified with a single PEG chain (5 kD) of comparable hydrodynamic volume to G3

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PAMAM exhibited short large amplitude events suggesting significant changes in the polymer structure. The recordings, which were performed in another study,[24] displayed events characterized by a current blockade of 51 ± 3%, an average duration of 14 ± 2 ms, and a frequency of occurrence of 0.2 ± 0.02 s^{-1}. The short events originated from the threading of the free end of the polymer chain into a narrow constriction of the pore through the β-barrel toward the trans side of the pore (Figure 1B).[24,25,31] Our single-molecule data therefore offer a glimpse of the different structural dynamics of individual "hard" positively charged PAMAM dendrimers as compared to "soft" neutral PEG chains.

Discussion

This report describes the generation of sulfhydryl-reactive PAMAM dendrimers and their coupling to cysteine mutants of a protein pore of known structure. The extent of reaction of the dendrimers was found to depend on the PAMAM generation and position of the engineered residues in the protein pore. G5 with a hydrodynamic diameter of 6.2 nm did not enter the cis entrance, while G3 with 4.2 nm coupled inside the pore. This clear cutoff in the permeation properties is in contrast to the greater accessibility of flexible PEG chains. For example, PEG-MAL 10 and 5 kD with comparable hydrodynamic diameters of 6.2 and 4.4 nm[35] coupled inside the pore lumen. The results of the present study are in line with another report on the use of PEG. Using the same αHL pore as a model system it was found that linear PEG polymers 1−3 kD (hydrodynamic diameters of 2.1−3.0 nm) could pass through the 1.3 nm wide inner constriction of αHL.[27] Only PEG 5 kD with a diameter of 4.4 nm translocated poorly. Hence, a PEG chain was only restricted in its permeation when the hydrodynamic parameter was at least 3.5 times higher than the pore constriction. By contrast, this value is 2 for G5-PAMAM and expected to be even lower for higher generation dendrimers with more rigid structure.

The sulfhydryl-reactive dendrimers presented in this study are a new type of research reagent for examination of the molecular structure of proteins. The reagents can be used in the substituted-cysteine accessibility method (SCAM),[26] which infers the surface accessibility of residues by determining how fast sulfhydryl-reactive reagents couple to single-cysteine mutants of a protein. SCAM has been widely exploited to probe the structure of membrane proteins and ion channels in combination with patch clamp or lipid bilayer recordings,[26,36] and gel electrophoretic mobility shift assays.[24,27,37,38] Despite the wide range of sulfhydryl-active organic reagents or polymeric reagents, most are too small or too flexible[27] for membrane proteins with pore diameters of more than 2−3 nm because they can either access all pore-lining residues or their coupling to cysteine residues does not give rise to an appreciable current block or gel shift, making it difficult to detect successful modification. Sulfhydryl-reactive PAMAM dendrimers with diameters of several nanometers can overcome these constraints as shown in this study. In calibration experiments with the structurally defined membrane pore αHL, dendrimers exhibited a sharp size-dependent permeation cutoff and readily identified a 2.9 nm wide pore entrance. PAMAM-PDP dendrimers with diameters from 2 to 10 nm are well suited to probe various nanometer-sized pore-forming proteins with important biomedical roles such as bacterial pore-forming toxins,[39] pores of the complement system, or dilating purinergic receptors.[40] In addition, the approach can also be applied to explore the molecular structure of interesting biological nanomaterials such as porous S-layer proteins.[38,41]

The study presents a new way to alter the properties of proteins via targeted chemical modification with hyperbranched dendrimers. Coupling of nonbranched linear organic polymers or biopolymers has been used in the past to modify or expand the natural characteristics of proteins such as in pharmacology to increase the half-life of therapeutic proteins via PEGylation,[42] in molecular biology to introduce sequence specificity into nucleases via attachment of a DNA oligonucleotides,[43] or in microarray technology to achieve targeted immobilization of PNA-modified proteins onto DNA-microarrays.[44] While dendrimers have been attached to proteins to produce new types of immunoreagents with enhanced binding capacity,[45] their use to alter the conductance properties of pores is new. Our results show that a dendrimer molecule filled at least the lumen of the engineered pore and thus regulated the ion flux.

The hyperbranched dendrimers were demonstrated to function as ion-selectivity filters for the passage of small ions through the pore. As expected, the positively charged primary amines on the surface of the PAMAM dendrimer led to a preferred permeation of anions over cations. The advantage of PAMAM over other ion-selective elements such as cyclodextrins[32] is that the dendrimer filters are available in different sizes up to 10 nm. This offers the possibility to use dendrimers for inorganic pores with wide lumen. PAMAM was also demonstrated to function as a molecular sieve to control the passage of molecules through the pore based on their molecular weight. Our results show that the RNA oligonucleotides were blocked while smaller ions were only minimally affected. It is expected that the molecular weight cut off of the molecular sieves can be tuned by increasing or decreasing the size of the dendrimer or changing its chemical composition. While movement of RNA through G3 PAMAM-filled pores is blocked, use of smaller dendrimers—in combination with higher potentials—could possibly enable passage of nucleic acids at reduced translocation speeds with applications in DNA sensing.[46]

Our approach to alter pore permeation properties by placing a spherical dendrimer inside the lumen is new. Use of hyperbranched dendrimers has specific advantages over other spheri-

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cal materials such as quantum dots of similar size or functionality. First, it would be difficult to couple a solid quantum dot into a pore lumen of similar diameter because the hard, highly symmetrical sphere would likely clash with the corrugated protein surface of the less symmetrical, imperfectly circular pore opening. By contrast, compact dendrimers have some residual degree of structural flexibility which can help overcome small steric permeation barriers. Indeed, G3 PAMAM with a diameter of 2.9 nm successfully passed the 2.9 nm wide opening of the αHL pore. Furthermore, the hyperbranched character of the dendrimer is important in controlling and tuning the flow of matter through the engineered pore, while solid impermeable spheres would likely lead to much more drastic and less tunable changes in the permeation properties. In summary, placing dendrimers into a pore lumen is a unique approach to introduce ion-selectivity filters or molecular sieves. The approach is not only restricted to protein pores but might be applied to engineer the permeation properties of inorganic or metallic porous structures for the separation of biopolymers or linear polymers for purification or sensing purposes.

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Supporting Information Available: Experimental procedures, HPLC and MALDI-MS analysis of SPDP-modified dendrimers of generations 2 and 5, quantification of the yield of the PAMAM-PDP preparations, coupling of G3-PAMAM-PDP and G5-PAMAM-PDP to homoheptamer $K8C_7$, coupling of MAL-PEG 10 kD to homoheptamer $S106C_7$, and $I-V$ curves for $S106C_7$, $S106C_7$-G2-PAMAM, and $S106C_7$-G3-PAMAM. This material is available free of charge via the Internet at http://pubs.acs.org.

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