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Precisely Defined Protein–Polymer Conjugates: Construction of Synthetic DNA Binding Domains on Proteins by Using Multivalent Dendrons

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ABSTRACT Nature has evolved proteins and enzymes to carry out a wide range of sophisticated tasks. Proteins modified with functional polymers possess many desirable physical and chemical properties and have applications in nanobiotechnology. Here we describe multivalent Newkome-type polyamine dendrons that function as synthetic DNA binding domains, which can be conjugated with proteins. These polyamine dendrons employ naturally occurring spermine surface groups to bind DNA with high affinity and are attached onto protein surfaces in a site-specific manner to yield well-defined one-to-one protein–polymer conjugates, where the number of dendrons and their attachment site on the protein surface are precisely known. This precise structure is achieved by using *N*-maleimido-core dendrons that selectively react *via* 1,4-conjugate addition with a single free thiol group on the protein surface— either Cys-34 of bovine serum albumin (BSA) or a genetically engineered cysteine mutant of Class II hydrophobin (HFBI). This reaction can be conducted in mild aqueous solutions (pH 7.2–7.4) and at ambient temperature, resulting in BSA– and HFBI– dendron conjugates. The protein– dendron conjugates constitute a specific biosynthetic diblock copolymer and bind DNA with high affinity, as shown by ethidium bromide displacement assay. Importantly, even the low-molecular-weight first-generation polyamine dendron (1 kDa) can bind a large BSA protein (66.4 kDa) to DNA with relatively good affinity. Preliminary gene transfection, cytotoxicity, and self-assembly studies establish the relevance of this methodology for *in vitro* applications, such as gene therapy and surface patterning. These results encourage further developments in protein– dendron block copolymer-like conjugates and will allow the advance of functional biomimetic nanoscale materials.

KEYWORDS: dendrimers and dendrons · DNA · multivalency · protein functionalization · self-assembly

The combination of biological macromolecules with synthetic polymers to achieve well-defined bioconjugates is of particular interest for applications in nanobiotechnology.[1] Nature offers a vast repository of proteins with unique functional properties, which have been extensively exploited in biotechnology and medicine.[2] Such properties are, however, only as good as conferred by nature, and efforts to improve or to alter the biological properties of proteins have been made by covalently attaching a polymer chain onto the protein surface. Examples of such modifications have yielded high-affinity binding to biomolecules,[3] tissue or intracellular targeting by multivalent binding to cell-surface receptors,[4] prolonged circulation lifetime,[5] thermal switching of enzyme activity,[6] and size-dependent binding.[7] In particular, protein modification with poly(ethylene glycol) (PEG) has gathered wide interest in therapeutic applications.[8] PEGylation masks the protein's surface, thereby making it unrecognizable to the plasma components and increasing the molecular size of the conjugate, thus improving systemic distribution, circulation lifetime, and resistance against enzymatic degradation.

There has been an increasing interest in using protein–polymer conjugates as nonviral vectors in gene therapy. Nonviral vectors rely on their ability to bind and to condense genetic material into such a form that it can navigate through various extra- and intracellular barriers to the cell nucleus, where genes can be expressed. A range of synthetic materials, such as cationic liposomes,[9] polymers, and dendrimers,[10] have been utilized for this task. However, they tend to be relatively inefficient in transfection and often lack cell-specific targeting. To achieve sufficient transfection efficiencies and cell-specific targeting, protein–polymer conjugates containing a cationic polymer such as polyethyleneimine (PEI) or poly(L-lysine) and an immunoglobulin have been developed.[11] These conjugates rely on the ability of cationic polymers to bind and to compact DNA, with the antibody being selected to facilitate receptor-mediated gene delivery into various cell types.[12]

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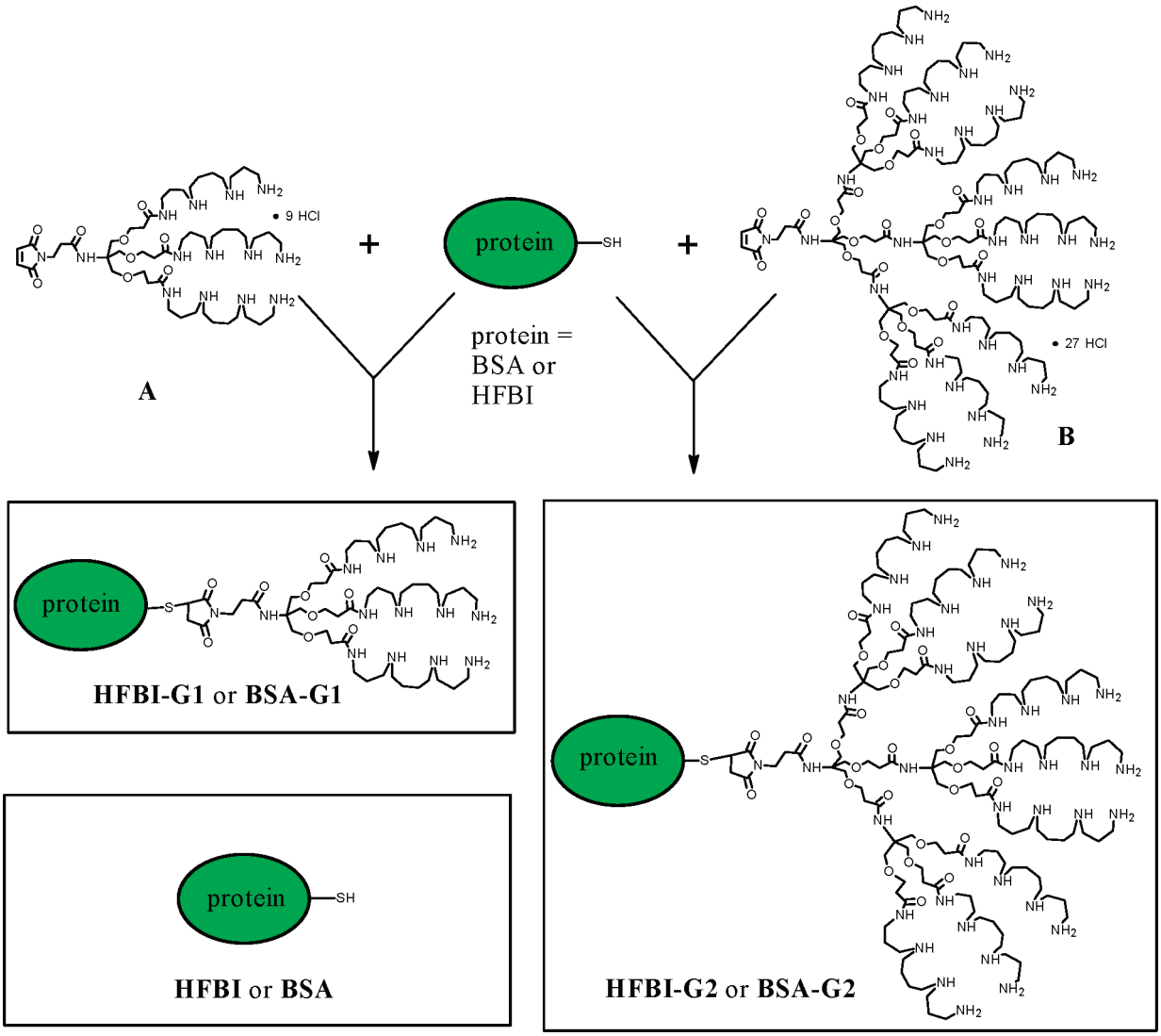
Various methods have been used to prepare well-defined protein–polymer conjugates, and these have been thoroughly reviewed in recent literature.[13] Two different routes are commonly utilized: either grafting a protein-reactive polymer onto reactive groups on protein surface ("*grafting to*")[14] or synthesizing polymers directly from initiation sites attached on the protein ("*grafting from*").[15] Heme proteins modified with tailored cofactor have also been reported.[16] Most studies concentrate on linear polymers, but a drawback to their use is that they induce a degree of heterogeneity in the form of both the polydispersity of the attached polymer and often the protein attachment sites, resulting in varying biochemical properties.[8b,17] However, branched macromolecules, dendrimers and dendrons, are not afflicted by polydispersity problems—indeed, such molecules can be considered to constitute a unique nanoscale construction kit, with each generation of growth modifying the size of the building block.[18] In particular, dendrons exhibit a well-defined, discrete branching structure and focal point, which can be precisely controlled. In addition, dendrons can display a high density of functional surface groups that can offer multiple simultaneous interactions. This leads to enhanced binding—the multivalent effect,[19] which is correlated with the dendritic generation and constitutes an additional advantage of dendritic polymers over their linear analogues. Dendrons also have an "umbrella-like" structure, which is interesting as it has been shown that the properties of proteins modified by branched PEG can be different from those of proteins modified with a linear chain.[20] These properties make dendrimers of great interest for protein conjugation. However, only relatively few studies on protein–dendron conjugates exist. A number of studies have conjugated polyamidoamine-type spherical dendrimers to antibodies or proteins.[21] In other studies, dendritic bisphosphonic acid has been shown to act as a bone-targeting unit for proteins[22] and modification of the heme propionate groups of myoglobin with branched anionic moieties.[23] Alternative reports have looked to the development of dendritic proteins with applications in immunochemistry[24] or neutron capture therapy.[25] Meijer and co-workers have employed native chemical ligation as a method to prepare dendrimers with exactly one copy of recombinant protein.[26] In a key study,[27] Davis and co-workers generated synthetic glycoproteins by conjugation of a dendritic saccharide to an inserted cysteine residue of a target protein, demonstrating that the resulting conjugate inhibited bacterial aggregation. This type of strategy had previously been hinted at by Janda and co-workers for antibody modification.[28] Nishimura and co-workers modified the N-terminus of insulin with a dendritic sialic acid, with the conjugate causing prolonged blood-glucose-lowering activity compared with native insulin.[29]

In our research, we have focused on optimizing DNA binding and have developed biomimetic DNA binding dendrons. We have previously reported a series of dendrons utilizing the Newkome-type dendritic scaffold which display multiple spermine units on the dendritic surface.[30] Spermine, a naturally occurring linear polyamine, is often utilized by nature itself to achieve DNA binding.[31] These dendrons are capable of high-affinity multivalent DNA binding using their surface groups in a generation-dependent manner and showed modest gene delivery profiles.[32] We have subsequently used protein conjugation to achieve well-defined functional biohybrid materials, in which the DNA binding properties of the dendrons were transferable to larger nanoscale conjugates, *i.e.*, to small proteins (class II hydrophobin, HFBI).[3] Such compounds are of potential interest for protecting DNA and other gene medicines (like siRNA) and for delivering genetic information into cells. HFBI is potentially useful because its natural function as an adhesion protein and its high surface activity may endow it with useful properties.[33] In order to explore the full potential of this strategy, we have now chosen a much larger model protein (bovine serum albumin, BSA) and prepared a series of BSA–dendron conjugates with different generations of dendrons. A comparison of these conjugates to our previously reported HFBI–dendron conjugates is presented. We demonstrate the generality of our approach to prepare precisely defined protein–polymer complexes with synthetic DNA binding domains and report on their DNA binding ability, surface self-assembly. and biocompatibility. Importantly, we identify HFBI protein modified with the second-generation dendron (**HFBI-G2**) as a self-assembling protein–dendron conjugate capable of enhancing gene transfection. It is interesting to note that, although cationic amphiphiles have been extensively exploited as nonviral vectors,[9,34] to our knowledge, this is the first report utilizing a cationic protein amphiphile in an analogous manner. The results presented in this paper give new insight into structure–activity relationships in functional protein–dendron conjugates and DNA binding protein–polymer conjugates in general. Understanding structure–activity relationships in the area of gene transfection is a goal of considerable importance in the field of gene therapy.[35]

RESULTS AND DISCUSSION

Preparation of Protein–Dendron Conjugates. We chose to prepare maleimide-functionalized dendrons that react with free cysteines on protein surfaces. The *N*-maleimido group is well known to react very selectively with free cysteine sulfhydryl groups *via* 1,4-conjugate addition. Ideal proteins for precise conjugation should contain only one reactive sulfhydryl group, although methods to modify native disulfide-bridged cysteines using a thiol-specific, cross-functionalized



Scheme 1. Preparation of protein–dendron conjugates BSA-G1, BSA-G2, HFBI-G1, and HFBI-G2. Reaction conditions: H_2O, pH 7.

monosulfone have also been studied recently.[38] Free cysteines that are reactive because they do not take part in disulfide bridge formation are rare in proteins: for example, globular BSA contains naturally only one reactive cysteine (Cys-34). However, approximately 50% of these cysteine residues are oxidized and therefore unavailable for conjugation.[39] Nonetheless, BSA is readily available commercially and exhibits other potential advantages, such as long circulation time and low toxicity. Indeed, a commercial drug formulation, Abraxane, incorporates albumin to improve the solubility and reduce the toxicity of paclitaxel.[40] Due to these advantages, BSA was chosen as our large (66.4 kDa) model protein. If the protein that is studied does not contain a single free cysteine, it can be added to the amino acid sequence using site-directed mutagenesis methods. To demonstrate this, we used our previously reported cysteine- and dendron-modified HFBI as another model.[3] HFBI is a small (8.7 kDa), surface-active protein[41] from *Trichoderma reesei*, known to form various structures through spontaneous self-assembly.[33,37] Drawing on these attributes, we reasoned that amphiphilic protein–dendron conjugates could enhance gene transfection in an analogous manner to cationic lipids.

Polyamine dendrons with spermine surface groups and an *N*-maleimido core (Scheme 1, compounds **A** and **B**) were synthesized using a divergent methodology, as reported previously.[3] Protein–dendron conjugates were assembled by Michael addition reactions between the free cysteine of the protein and the *N*-maleimido group of the dendron (Scheme 1). This reaction is highly selective for sulfhydryl groups and can be carried out in mild aqueous solution at neutral pH (see Scheme 1). The high hydrophilicity of the dendrons allows easy purification of the target conjugates (Scheme 1, compounds **BSA-G1**, **BSA-G2**, **HFBI-G1**, and **HFBI-G2**) by semi-preparative reversed-phase high-performace liquid chromatography (HPLC). Peaks were fractionated, pooled, and finally lyophilized to yield the products as white solids. Yields for the BSA–dendron conjugates are close to 50%, whereas the

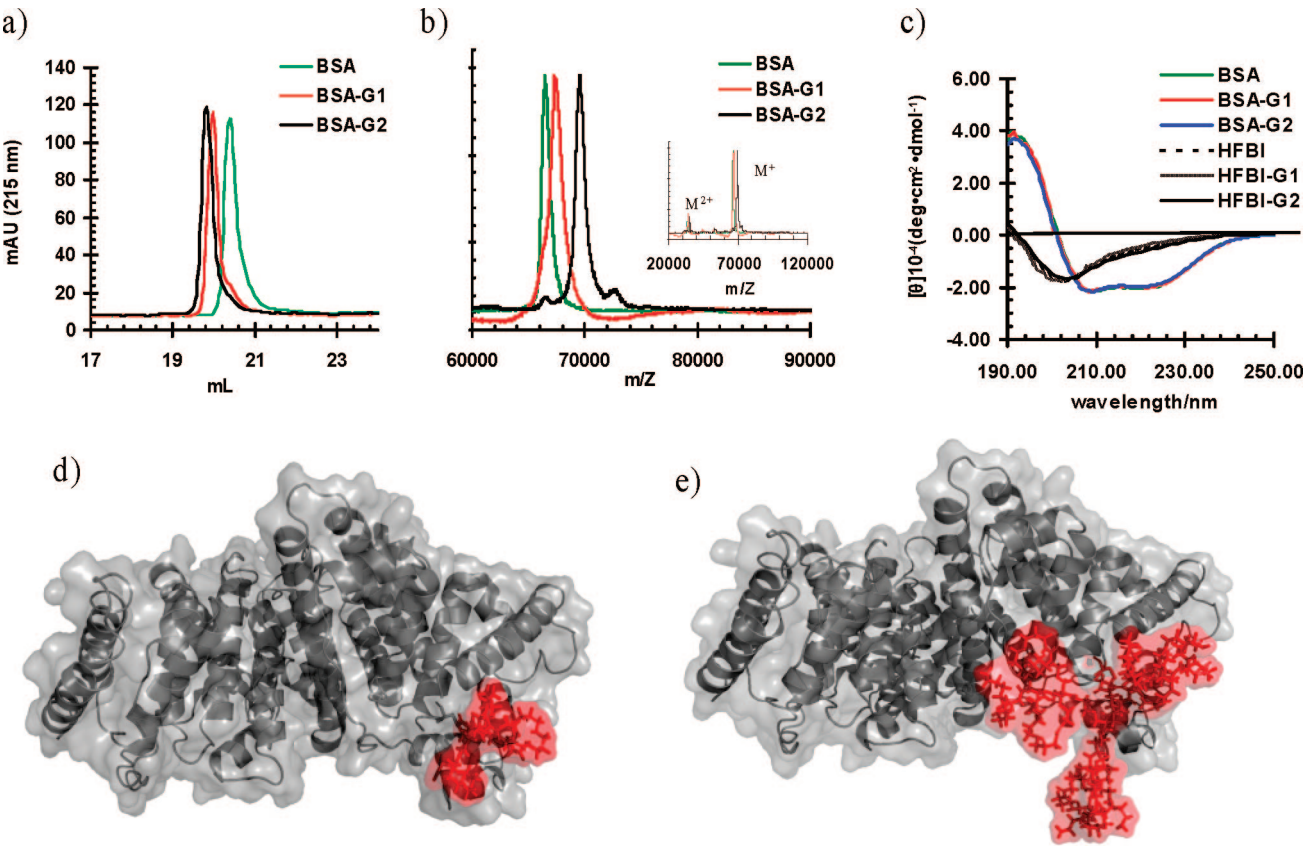


Figure 1. Analytical data for protein–dendron conjugates. (a) Analytical HPLC chromatogram of the purified BSA, BSA-G1, and BSA-G2, showing decreasing retention volume with increasing dendritic generation; see Table 1 for values. (b) MALDI-TOF spectra of purified BSA, BSA-G1, and BSA-G2, showing increasing mass with increasing dendritic generation; see Table 1 for values. (c) CD spectra for all studied proteins and their dendron conjugates, confirming that the protein structure is not detectably changed by the attached dendron. Schematic computer-generated models of (d) BSA-G1 and (e) BSA-G2. Cys-34 and the attached dendron are shown in red.

yields for the HFBI–dendron conjugates are much higher, approximately 80%. These results are consistent with the reports that, in approximately 50% of the BSA molecules, but in nearly 100% of the HFBI molecules, the cysteine residue on the surface of the protein is available for conjugation.

Characterization of Protein–Dendron Conjugates. Analytical data for BSA and BSA–dendron conjugates are presented in Figure 1 and Table 1, where values are also compared to those previously reported for HFBI and its dendron conjugates. High purity after pooling was confirmed by analytical HPLC, which shows a single symmetric peak for all compounds (Figure 1a). For both proteins, smaller retention volume was observed with dendron attachment and increasing dendritic generation, as would be expected because of the high hydrophilicity of the dendrons. Overall, the larger retention volume for the HFBI conjugates is consistent with their higher hydrophobicity when compared with BSA. Analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry shows clear signals with good accordance to the calculated mass of the conjugates, confirming the covalent structure of the target molecules (Table 1, Figure 1b). CD measurements were used to confirm that the protein structure is not detectably changed by the attached dendron (Figure 1c). CD spectra for **BSA** and its conjugates are consistent with the high content of helical secondary structure, while the spectra for **HFBI** and its conjugates indicate rich random coil and β-sheet content, consistent with the protein native structures. Details of the analytical data are presented in Table 1.

TABLE 1. Analytical Data for Protein–Dendron Conjugates

compound	calcd mass (g/mol)	MALDI-TOF (*m/z*)	retention volume (mL) (semi-preparative HPLC)	conjugation yield (%)[a]	retention volume (mL) (analytical HPLC)
BSA	66 430.3[b]	66 444.3	76.96		20.33
BSA-G1	67 471.7	67 404.1	75.70	53	20.00
BSA-G2	69 535.6	69 552.5	72.71	48	19.82
HFBI	8 676.7	8 676.5	107.45		23.86
HFBI-G1	9 718.1	9 722.4	98.49	79	22.87
HFBI-G2	11 782.0	11 782.8	91.97	83	21.13

[a]Approximated from peak heights (semi-preparative HPLC). [b]Reference 42.

Results obtained from the preparation and analysis of the protein–dendron conjugates demonstrate that the maleimide route is an effective and straightforward method to prepare extremely well-defined protein–dendron conjugates. Conjugates can be purified in one

step to yield pure products without free starting materials. Products formed have virtually no polydispersity, and proteins retain their native structure even after the modification. We then performed experiments to demonstrate the DNA binding functionality of the dendrons as well as the self-assembly and surface-activity properties of the protein, leading to bioactive hybrid materials.

DNA Binding Properties of Protein–Dendron Conjugates. The DNA binding properties of compounds were evaluated by using an ethidium bromide (EthBr) displacement assay.[43] This assay measures the fluorescence derived from the DNA-bound EthBr, which is intercalated into the DNA duplex. When EthBr is displaced from the DNA duplex by an effective DNA binding agent, its fluorescence is quenched. It is worth noting that, although this is a powerful comparative method, the resulting data reflect a competition assay rather than an absolute binding strength—it is nonetheless an excellent method for comparing a series of related ligands. Such a study leads to profiles that define CE_{50} values (Table 2), which represent the "charge excess"[44] required to achieve 50% reduction in the relative fluorescence intensity.

In the EthBr displacement assay, neither unmodified protein, **BSA** or **HFBI**, shows affinity toward DNA. This was expected, as neither of the proteins contains any DNA binding motifs or significant surface positive charge and should therefore exhibit only weak interactions with polyanionic DNA. No quenching of the fluorescence was observed, even with high protein concentrations, and thus no CE_{50} values were obtained (Figure 2a,d, Table 2). However, the dendron-conjugated proteins showed significantly enhanced DNA binding and

TABLE 2. Results for Spermine BSA, BSA-G1, BSA-G2, HFBI, HFBI-G1, HFBI-G2, G1, and G2 from an Ethidium Bromide Displacement Assay[a]

compound	nominal charge	calcd FW (g/mol)	CE_{50}	
			9.4 mM NaCl	150 mM NaCl
spermine	+4	362.5	6	>400
BSA	(+9)[b]	66 430.3[e]	>400	>400
BSA-G1	+9	67 471.7	3.5	6.3
BSA-G2	+27	69 535.6	1.0	0.6
HFBI[c]	(+4)[d]	8 676.7	>200	>200
HFBI-G1[c]	+9	9 718.1	0.6	0.9
HFBI-G2[c]	+27	11 782.0	0.6	0.5
G1[c]	+9	1 024.4	0.7	2.7
G2[c]	+27	3 088.30	0.8	0.8

[a]Conditions: buffered water pH 7.2 (2 mM HEPES, 0.05 mm EDTA). DNA (1 μM) and ethidium bromide (1.26 μM) concentrations were kept constant. Total added polyamine solution did not exceed 5% of the total volume; therefore, corrections were not made for sample dilution. Results are averages of three titrations. [b]BSA has an overall negative surface charge ($p < 6$) at neutral pH; however, nine positive charges were assumed for comparison. [c]Values for HFBI conjugates[3] and **G1**, **G2**[43] have been reported previously by us. [d]According to protein amino acid sequence and the number of protonatable side chains, four positive charges were assumed. [e]Reference 42.

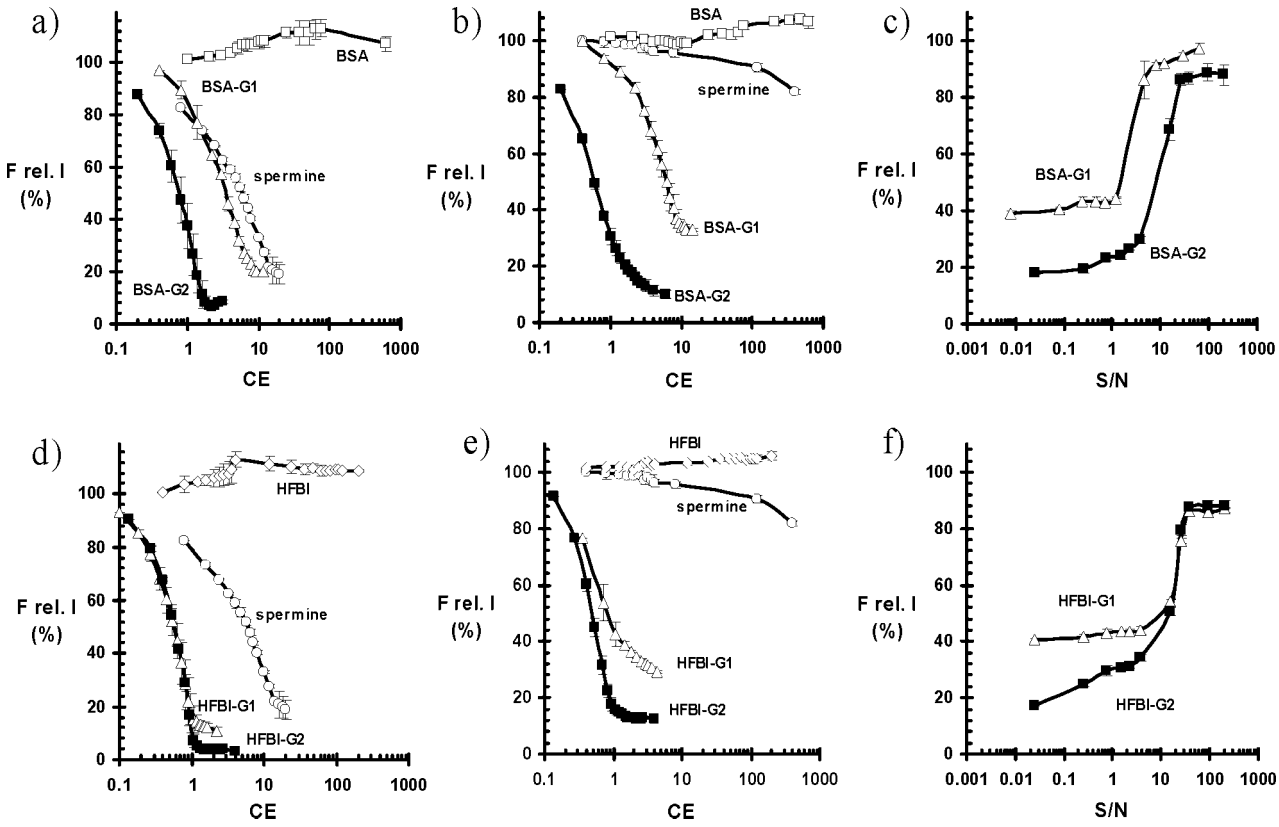


Figure 2. Ethidium bromide displacement assay curves for spermine, BSA, BSA-G1, BSA-G2, HFBI, HFBI-G1, or HFBI-G2 in a solution of 1 μM DNA and 1.26 μM ethidium bromide in buffered water (pH 7.2). EthBr fluorescence quenching in the presence of (a,d) 9.4 mM and (b,e) 150 mM NaCl. DNA–polycation complex relaxation with chondroitin sulfate in the presence of (c,f) 150 mM NaCl. Results are the average of triplicates; error bars represent standard deviation.

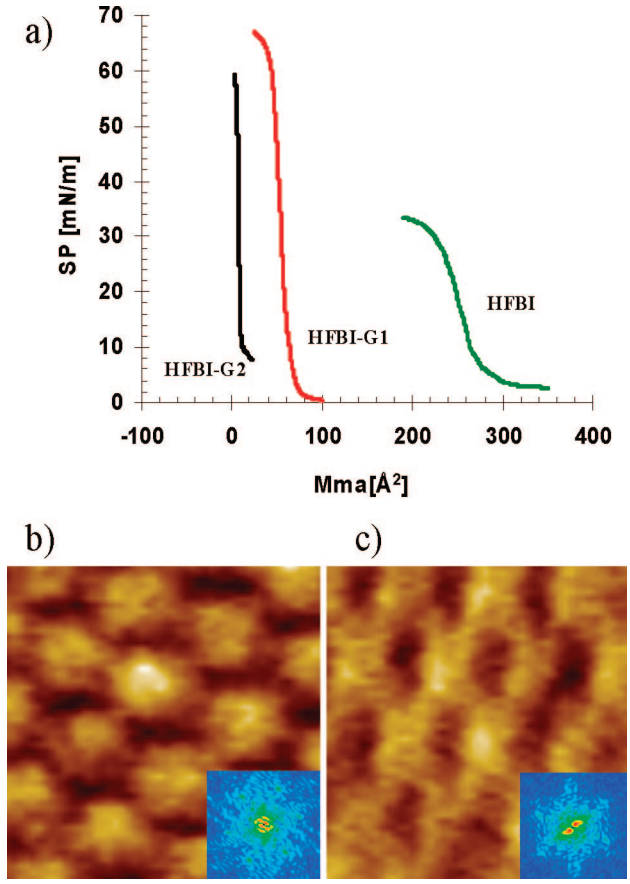


Figure 3. (a) Surface pressure–mean molecular area isotherms obtained by compressing HFBI-G1 or HFBI-G2 Langmuir films. The HFBI isotherm is plotted as a reference. (b,c) Correlation-averaged AFM topography image of HFBI-G1 (b) and phase image of HFBI-G2 (c). Langmuir film showing a regular ordered, nearly hexagonal patterns of objects. Image size is 19 nm × 19 nm. Insets: fast Fourier transforms of single crystalline areas of HFBI-G1 or HFBI-G2 film (see Supporting Information).

displaced ethidium bromide effectively owing to the strong electrostatic interactions between the protonated dendron amines and the polyanionic DNA. The DNA binding affinity of **HFBI** and its dendron conjugates has also been discussed previously.[3] Under low-salt conditions (9.4 mM NaCl), **HFBI-G1** and **HFBI-G2** bind DNA very strongly and with similar affinity (CE_{50} = 0.6, Figure 2d, Table 2). **BSA-G2** also binds strongly, but with slightly lower affinity (CE_{50} = 1.0, Figure 2a, Table 2). **BSA-G1** exhibits lower binding affinity (CE_{50} = 3.5, Figure 2a, Table 2) when compared to other protein–dendron conjugates. The lower binding affinity for this dendron-conjugated BSA might be expected because the rather small dendron (*ca.* 1 kDa) must adhere a much larger BSA protein (*ca.* 66 kDa) to DNA. At physiological salt concentration (150 mM), spermine itself loses its DNA binding ability ($CE_{50} > 400$ Figure 2b,e); however, the multivalent dendron conjugates are less adversely affected by the increase of competitive Na^+ ions. **BSA-G1** and **HFBI-G1** both exhibit weaker binding at high salt concentration (CE_{50} = 6.3 and 0.9, respectively; Figure 2b,e, Table 2). The binding affinity of **BSA-G1** is affected the most. In contrast, **BSA-G2** and **HFBI-G2** are little affected by the increase in salt concentration, and they bind DNA with extremely strong affinities (CE_{50} = 0.6 and 0.5, respectively; Figure 2b,e, Table 2) as a consequence of their multivalent nature. Gel electrophoresis was used to confirm the affinities of **BSA**, **BSA-G1**, and **BSA-G2** for DNA (see Supporting Information).

Interestingly, after the DNA binding assay, the fluorescence of displaced EthBr can increase if reintercalation into the DNA double helix becomes possible. This enables the strength of the resulting DNA–dendron complexes to be studied by using chondroitin sulfate B (csB), which is a sulfated polyanionic glycosaminoglycan known to effectively relax weak DNA–cation complexes.[45] These results are presented as a function of sulfonic acid /protonatable dendron amine (S/N) ratio.[46] We used two different salt concentrations (9.4 and 150 mM NaCl) at a physiologically relevant pH value of 7.2, and spermine as a reference compound. Evaluation of the strength of the DNA complexes using csB as relaxing agent shows that **BSA-G2** packs DNA into a strong complex that can be fully opened only with a large excess of csB (S/N ratio >25, Figure 2c). Complexes formed with **BSA-G1** are significantly weaker and can be opened at a relatively low S/N ratio (S/N ratio *ca.* 5, Figure 2c). Smaller HFBI–dendron conjugates are able to pack DNA even more strongly than BSA conjugates. Both **HFBI-G1** and **HFBI-G2** pack DNA into complexes with similar high strengths, only being fully relaxed at S/N ratios of approximately >30 (Figure 2f).

In summary, therefore, the binding values measured for the protein–dendron conjugates are in general agreement with those measured for the dendrons alone.[30] However, results from the EthBr displacement assay and gel electrophoresis show that **BSA-G1** binds DNA with noticeably lower affinity than **G1** alone. Indeed, at low salt conditions, **BSA-G1** behaves only a little better than unmodified spermine—this is presumably a consequence of the entropic cost of binding the high-molecular-mass BSA protein to the DNA. However, at high salt concentrations, **BSA-G1** is significantly better than simple spermine, clearly demonstrating the benefits of the multivalent dendron in competing for the DNA under these more competitive conditions. The binding affinity of **BSA-G1** shows that a rather small (*ca.* 1 kDa) **G1**–dendron can convey reasonable binding affinity even to a much larger protein (*ca.* 66 kDa). This kind of behavior much resembles the binding of natural proteins that rely on DNA binding domains.[47] Dendrons attached to the protein surface can therefore be described as synthetic DNA binding domains.

Self-Assembly at the Air–Water Interface. One of the remarkable properties of hydrophobins is their ability to form self-assembled films on hydrophobic surfaces or at the air–water interface.[37] These features have been

demonstrated previously for various different types of hydrophobins, and the **HFBI-G2**–dendron conjugate was also found to adsorb effectively on hydrophobic surfaces.[3] We wanted to further study the amphiphilicity of the HFBI–dendron conjugates, as considering the possible applications of these conjugates in gene therapy, the hydrophobic part of the carrier is known to strongly influence the DNA transport into cells.[48] We now demonstrate that the surface-activity of the HFBI–dendron conjugates is dependent on the attached dendron and that the conjugates can self-assemble at the air–water interface into a similar hexagonal array as HFBI alone.

The surface-activities of **HFBI-G1** and **HFBI-G2** were studied by compressing Langmuir films. The isotherm for unmodified HFBI is plotted as a reference (Figure 3a). Isotherms for **HFBI-G1** and **HFBI-G2** were recorded on a water subphase (50 mM NaCl, pH 7.5). The unmodified HFBI isotherm shows a steep liquid-condensed behavior at mean molecular area (Mma) *ca.* 250 Å^2 and a collapse at *ca.* 35 mN/m. **HFBI-G1** shows a rapid rise of the surface pressure at Mma *ca.* 60 Å^2 and a collapse point at 60 mN/m, whereas the measured Mma value of **HFBI-G2** is shifted even lower to *ca.* 10 Å^2, with a collapse point at 56 mN/m. Pleasingly, these results show a dendritic effect on the film formation, where increasing the dendritic generation on the protein surface makes it more soluble in the subphase, therefore shifting the Mma to lower Å^2 values because an increasing amount of the material is lost into the subphase.

We also wanted to observe whether the protein–dendron conjugates show self-assembled packing in films similar to that of pure HFBI. The films were prepared using a Langmuir trough compression and then deposited onto a graphite substrate, after which they were dried and imaged with atomic force microscopy (AFM). The results obtained by AFM indicate that dendron-modified hydrophobins can form stable films at the air–water interface and that the films have a regular, nearly hexagonal-like structure with the dimension of a few nanometers (Figure 3b,c). The same kind of structure was observed for both **HFBI-G1** and **HFBI-G2** films (see Supporting Information). Analyzing the structured parts of the surfaces using fast Fourier transform yielded 2D crystal unit cells of $a = 5.9$ nm, $b = 5.4$ nm, $\gamma = 119°$ for **HFBI-G1** and $a = 5.3$ nm, $b = 4.9$ nm, $\gamma = 115°$ for **HFBI-G2**, indicating nearly hexagonal packing in both cases (Figure 3b,c, inset).

Cytotoxicity and Gene Transfection. Finally, we investigated the gene transfection efficiency and cytotoxicity of our protein–dendron conjugates. Our initial *in vitro* results show that the protein–dendron conjugates are biocompatible and can enhance gene transfection. Proteins have diverse effects on cellular metabolism; however, HFBI and BSA in particular are well known for their safety. Polycationic compounds, on the other hand, are known to damage cell membranes as a result of

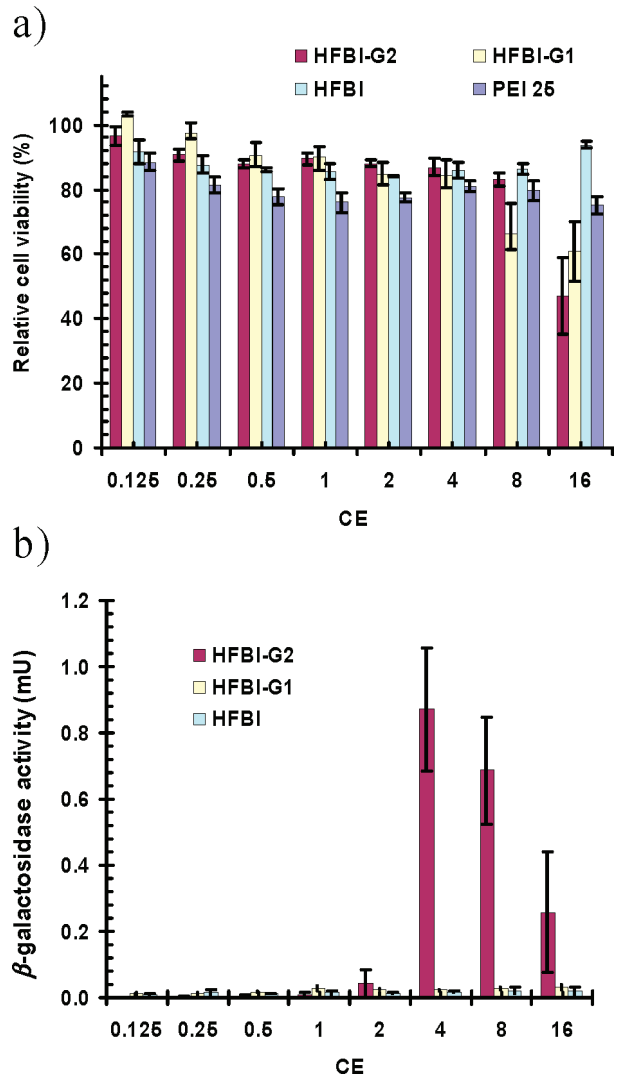


Figure 4. (a) Cytotoxicity of HFBI, HFBI-G1, HFBI-G2, and PEI 25k in kidney fibroblast cells (CV1-P) reported as relative cell viability (%). (b) Transfection efficiency of HFBI, HFBI-G1, or HFBI-G2 in CV1-P cells, given as milliunits of β-galactosidase activity. Results are the average of triplicates; error bars represent standard deviation.

their electrostatic interactions with the plasma membrane.[49] The cytotoxicity of the protein–dendron conjugates was assessed using the MTT assay. None of the studied compounds showed any toxicity at CE ratios 0.125–4. However, **HFBI-G1** and **HFBI-G2** were observed to be slightly cytotoxic at high CE ratios, where the relative cell viability decreased markedly (Figure 4a). **HFBI** did not reduce cell viability at any CE ratio. At CE 16, **HFBI-G1** decreased the relative cell viability to *ca.* 62% and **HFBI-G2** to *ca.* 50% (Figure 4a). **BSA** and its dendron conjugates did not indicate any toxicity (data not shown).

We investigated gene delivery into kidney fibroblasts cells (CV1-P) with varying CE ratios of protein–dendron conjugates. Gene transfection efficiency was measured as β-galactosidase expression. PEI 25k was used as a commercial standard (positive control), and plain pDNA without any complexing agents was used

as a negative control. We observed clearly enhanced transfection only for **HFBI-G2** with high CE ratio (≥4), while all the other protein–dendron conjugates were unable to mediate efficient transfection. Optimal transfection efficiency was achieved at CE 4, and notably, no cytotoxicity was observed at this CE ratio. Interestingly, we have previously observed that the dendrons are relatively ineffective transfection agents in their own right,[32] and the current results therefore show that the protein can play an active role in transfection—enhancing it. Furthermore, the results presented here show that both the dendron and the protein affect transfection efficiency, as BSA and its dendron conjugates did not induce measurable β-galactosidase activity (data not shown), even though both **HFBI-G2** and **BSA-G2** bind DNA in a similar manner. It is possible that the transfection enhancing ability of HFBI is due to its unusual amphiphilic structure, which may lead to a favorable interaction with membrane structures. Interactions between cell membranes and hydrophobins have not been studied previously, although their structure suggests that an interaction could occur. However, higher surface-activity does not alone increase transfection, because **HFBI-G1** is not efficient even though it is more surface-active than **HFBI-G2**. We have therefore demonstrated that HFBI, modified with a synthetic DNA binding domain, functions as a cationic surfactant capable of delivering DNA across a biological membrane. It must be noted that the overall transfection efficiency of **HFBI-G2** is fairly low when compared to that of PEI 25k, which induced over 20-fold higher β-galactosidase activity. However, this is significantly better than the previously reported behavior of simple **G2** dendron as a transfection agent.[32] Dendron **G2** was several orders of magnitude less efficient as a transfection agent than PEI and only demonstrated significant transfection in the presence of chloroquine, added to enhance endosomal escape. These results therefore indicate that the new protein–dendron conjugates can enhance the extent of gene transfection and are not markedly cytotoxic at low CE ratios.

CONCLUSIONS

In conclusion, we have demonstrated the successful synthesis and characterization of precisely defined protein–polymer conjugates, consisting of either HFBI or BSA protein, combined with a first- or second-generation polyamine dendron. Both **HFBI-G2** and **BSA-G2** showed extremely strong DNA binding affinity, irrespective of salt concentration, and packed DNA into strong complexes that could only be opened using a large excess of csB. Surprisingly, even though the first-generation dendron has a relatively low molecular weight (1 kDa) when compared to the much larger BSA protein (66.4 kDa), the conjugate still has a reasonable binding affinity. This approach of attaching low-molecular-weight dendrons onto a protein surfaces mimics natural proteins with DNA binding domains and can therefore be described as a useful approach to tethering synthetic DNA binding domains onto protein surfaces. Furthermore, this approach utilizes free thiol groups and can therefore be applied to virtually any protein with a free cysteine residue. Thus, our method differs in its generality from an approach such as cofactor reconstruction, which is applicable for only a limited range of proteins. If a free sulfhydryl group is not available, it can be added *via* site-directed mutagenesis methods. The functionality of the protein part can be retained, as we have demonstrated that dendron conjugation does not affect protein structure according to our data. The protein part of the conjugate can, for example, promote adhesion onto a hydrophobic surface or self-assembly at an air–water interface. AFM imaging revealed that **HFBI-G1** and **HFBI-G2** can self-assemble into hexagonally ordered arrays onto hydrophobic surfaces, a result which could have relevance for functional surface patterning. MTT and gene transfection results indicate that these protein–dendron conjugates are biocompatible and promote gene transfection *in vitro*. Interestingly, the protein functionality of HFBI endowed the conjugate with enhanced transfection efficiency when combined with the DNA binding function of dendrons. It is further possible to expand the scope of this approach outside applications in gene therapy by choosing different proteins and dendron functionalities, thus allowing novel approaches to achieve bioactive protein–dendron conjugates with wide-ranging applications in bionanotechnology.

EXPERIMENTAL METHODS

All reagents and starting materials were commercially available and used as supplied without further purification. 3MPA-G1-spermine · 9HCl (**A**), 3MPA-G2-spermine · 27HCl (**B**), **HFBI-G1**, and **HFBI-G2** were synthesized as reported previously (subject to minor modifications), and all data were in full agreement with those previously published.[3]

Experimental Procedures for the Synthesis of Protein Conjugates. A typical procedure for conjugation of **BSA** and 3MPA-G2-spermine (**B**) is described. Conjugation of **B** with **BSA** was carried out in buffered water. **B** (2.5 mg, 0.81 nmol) was dissolved in water (100 μL) and mixed with buffered water (400 μL of 0.2 M sodium phosphate pH 7, 30 μL of 0.5 M EDTA pH 7.52). The mixture was then mixed with **BSA** solution (16 mg, 0.24 nmol in 400 μL of water) and left standing at room temperature for 16 h with occasional mixing.

Purification and Analysis of Protein–Dendron Conjugates. The protein and protein–dendron conjugates were purified using a preparative reversed-phase chromatography system coupled to a UV–vis detector probing at 215, 230, and 280 nm (RP-HPLC, Äkta explorer), using a Vydac C4 (1 cm × 20 cm) column and a gradient elution from 0.1% trifluoroacetic acid to 100% acetonitrile (ACN) containing 0.1% trifluoroacetic acid. Peak fractions were pooled and lyophilized. Analytical HPLC samples were analyzed with

the same system using a 4.6 mm × 5 cm Vydac C4 column. Blank water was subtracted from each sample. The protein identity was confirmed using MALDI-TOF mass spectroscopy. MALDI mass spectrometric data were obtained at the protein chemistry core facility at the University of Helsinki using a Biflex (Bruker Daltonic) instrument. 3,5-Dimethoxy-4-hydroxycinnamic acid was used as a matrix with 1:1 0.1% TFA–H_2O/ACN. The CD spectra were recorded at room temperature in the far-ultraviolet region (190–250 nm) using a JASCO J-720 spectropolarimeter by accumulation of 30 spectra. A quartz cuvette and buffered water (2 mM HEPES, 10 μM EDTA, 9.4 mM NaCl) were used for all measurements. All **HFBI**, **HFBI-G1**, and **HFBI-G2** samples were kept briefly in an ultrasonic water bath prior to measurement.

Computer-Generated Pictures in Figure 1d,e. BSA structure was based on a model (SWISS-MODEL repository, P02769). The dendron structure was generated by ChemDraw. Final images were produced and rendered with pyMol (DeLano Scientific LLC, Palo Alto, CA).

Ethidium Bromide Displacement Assay. A Varian Cary Eclipse fluorescence spectrophotometer was used to record the data. Excitation of the sample was done in a 3 mL quartz cuvette using 546 nm excitation light and measuring emission at 595 nm. The buffer designated 0.01 SHE was of ionic strength 0.01 and contained 2 mM HEPES, 10 μM EDTA, and 9.4 mM NaCl. The pH was adjusted to 7.2 with NaOH. Biological SHE contained 2 mM HEPES, 10 μM EDTA, and 150 mM NaCl. The pH was adjusted to 7.2 with NaOH. Ethidium bromide (1.26 μM) was dissolved in the buffer. After mixing, the fluorescence was measured. Type III DNA from salmon testes (1 mM nucleotide concentration in 0.01 SHE) was added to provide a concentration of 1 μM, and the fluorescence was increased to measurement maxima.

Ethidium Bromide Displacement (Figure 2a,b,d,e). The test agent (spermine, **HFBI**, **HFBI-G1**, **HFBI-G2**,**BSA**, **BSA-G1**, or **BSA-G2**) in aqueous solution was added in small portions to reduce the fluorescence of the DNA–ethidium complex to 50% (or plateau).

Complex Relaxation by Chondroitin Sulfate B (Figure 2c,f). DNA was first completely complexed with the test agent (see above; spermine CE = 10, **BSA-G1** CE = 10, **BSA-G2** CE = 2, **HFBI-G1** CE = 2, **HFBI-G2** CE = 2), resulting in EthBr displacement and fluorescence decrease. Chondroitin sulfate B was then added in small portions to relax the formed complexes, allowing re-intercalation of EthBr and an increase in fluorescence.

Each fluorescence measurement was repeated two times, and each titration series was repeated three times. Finally, the results were averaged. For the DNA, a molecular weight of 330 g mol^{-1} and one negative charge per nucleotide were assumed. For csB, a molecular weight of 444 g mol^{-1} and one negative charge per repeat unit were assumed.

Preparation Langmuir Films on Air–Water Interface. The Langmuir films were prepared using a KSV Minimicro trough (KSV Instruments, Finland) instrument. The subphase contained 1 mM Tris-HCl and 50 mM NaCl at pH 7.5. Samples were diluted into the subphase buffer to a concentration of 50 μM (native HFBI[36]), 100 μM (**HFBI-G1**), or 400 μM (**HFBI-G2**), of which 100 μL was spread on the subphase. The surface was allowed to stabilize for 30 min, after which the compression of the monolayer (255 mm^2/min) was initiated. Langmuir–Schaefer (LS) films for AFM imaging were obtained by compressing the monolayer to the deposition pressure of 20 mN/m. The monolayer formed at the air–water interface was then transferred to a 10 mm × 10 mm square piece of freshly cleaved highly oriented pyrolytic graphite (HOPG, ZYA quality) (NT-MDT) by bringing the substrate horizontally into contact with the monolayer at the air–water interface.

Atomic Force Microscopy. The protein–dendrimer LS films were imaged with a NanoScope IIIa Multimode atomic force microscope with an "E"-scanner (Digital Imaging/Veeco) as described earlier.[37] The images were acquired in air using tapping mode and NSC15/AIBS probes (MikroMasch) with a typical constant cantilever force of 40 N/m, a resonant frequency of 325 kHz, and a tip radius of curvature of <10 nm.

Cytotoxicity Determination. Cytotoxicity was characterized using a MTT assay. Monkey kidney fibroblast (CV1-P) cells were plated in 96-well plates at a density of 15 000 cells/well in 100 μL of growth medium consisting of Gibco-DMEM with 10% fetal bovine serum and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL). Cells were incubated for 24 h at 37 °C and 5% CO_2, after which time the growth medium was replaced with fresh serum-free medium. DNA–polyamine complexes were prepared in 96-well plates by adding a solution of polyamine to an equal volume of plasmid DNA (pDNA) solution. Complex (50 μL) containing 0.2 μg of DNA and varying proportions of carrier (CE = 0.125−16) were added to the serum-free medium. The cells were incubated for 6 h and washed with 150 μL of 1× phosphate-buffered saline (PBS), after which the medium was replaced with complete growth medium for 18 h. The growth medium was replaced with serum-free medium (100 μL). Next, 20 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10 mL, 5 mg/mL) was added, and cells were incubated for 2 h. Finally, 100 μL of sodium dodecyl sulfide (SDS)/dimethylformamide (DMF) buffer (200 mg/mL SDS, 50% DMF, pH 4.7) was added, and the mixture was incubated for 20 h. Absorbance was measured at 620 nm with a Victor^2 1420 multilabel counter (Wallac, Turku, Finland). Survival percentage was calculated by comparison to blank cells (100% survival).

Transfection Protocol. Gene transfection efficiency was measured as β-galactosidase enzyme activity. CV1-P cells were plated in 96-well plates at a density of 15 000 cells/well in 100 μL of growth medium consisting of Gibco-DMEM with 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Cells were incubated for 24 h at 37 °C, after which time the growth medium was replaced with fresh serum-free medium. DNA–polyamine complexes were prepared in 96-well plates by adding a solution of polyamine to an equal volume of pDNA (pCMVβ plasmid, Clontech Laboratories) solution. Complex (50 μL) containing 0.2 μg of DNA and varying proportions of carrier (CE = 0.125−16) were added to the serum-free medium. Cells were incubated for 6 h and washed with 150 μL of 1× PBS, after which the medium was replaced with complete growth medium for 42 h. Cells were lysed with 2% Triton-X100, and β-galactosidase activity was detected spectrophotometrically (Victor^2 1420 multilabel counter) using *o*-nitrophenol galactoside (ONPG, Sigma) as the substrate. Purified β-galactosidase enzyme was used as the reference standard.

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Supporting Information Available: Gel retardation assay (Figure S1), AFM images of **HFBI-G1** and **HFBI-G2** Langmuir films (Figure S2), and additional images of protein–polymer conjugates (Figure S3). This material is available free of charge *via* the Internet at http://pubs.acs.org.

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