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Immunoreactivity changes of human serum albumin and alpha-1-microglobulin induced by their interaction with dendrimers

Tatyana Serchenya[a], Dzmitry Shcharbin[b,*], Iryna Shyrochyna[a], Oleg Sviridov[a], Maria Terekhova[b], Volha Dzmitruk[b], Viktar Abashkin[b], Evgeny Apartsin[c], Serge Mignani[d,e], Jean-Pierre Majoral[f,g], Maksim Ionov[h], Maria Bryszewska[h,*]

[a] Institute of Bioorganic Chemistry of NASB, Minsk, Belarus
[b] Institute of Biophysics and Cell Engineering of NASB, Minsk, Belarus
[c] Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia
[d] Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologique, Université Paris Descartes, PRES Sorbonne Paris Cité, CNRS UMR 860, Paris, France
[e] Centro de Química da Madeira, MMRG, Universidade da Madeira, Funchal, Portugal
[f] Laboratoire de Chimie de Coordination du CNRS, Toulouse, France
[g] Université de Toulouse, UPS, INPT, Toulouse, France
[h] Department of General Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

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ABSTRACT

Dendrimers are hyperbranched polymers for delivery of therapeutic genetic material to cancer cells. The fine tuning chemical modifications of dendrimers allow for the modification of the composition. The architecture and the properties of dendrimers are key factors to improve their *in vitro* and *in vivo* properties such as biocompatibility with cells and tissues and their pharmacokinetic/pharmacodynamic behavior. The side effects of dendrimers on structure and function of proteins is an important question that must be addressed. We herein describe the effect of newly synthesized piperidine-based cationic phosphorous dendrimers of 2 generations and commercial cationic, neutral and anionic poly(amidoamine) (PAMAM) dendrimers of 4th generation on immunochemical properties of 2 serum proteins: human serum albumin (HSA) and alpha-1-microglobulin (A1M). Both can bind and transfer ligands in blood, including hormones, fatty acids, toxins and drugs, and have immunoreactivity properties. Comparing the effects of piperidinium-terminated phosphorus and cationic, neutral and anionic PAMAM dendrimers on HSA and A1M, we conclude that, in the case of equimolar complexes, these dendrimers had no significant effect on immunoreactivity of proteins. In contrast, the formation of complexes in which a protein is fully bound to dendrimers leads to partial (1.2–2.3 times) reduction in protein immunoreactivity. The most important fact is that dendrimer-induced change in immunoreactivity of proteins is not complete, even if the protein is entirely bound by dendrimers. This means that the application of dendrimers *in vivo* will not totally hamper the immunoreactivity of these proteins and antibodies.

1. Introduction

Dendrimers are hyperbranched polymers that could be used for delivery of therapeutic genetic material to cancer cells [1–4]. Their well-defined structure with multivalent positive surface groups allows siRNAs to bind to them, thereby forming stable complexes called dendriplexes. Dendrimers protect siRNAs against enzymatic degradation, enable cellular uptake and release of siRNAs inside cells [2,3]. The toxicity of dendrimers *in vitro* and *in vivo* mainly depends on the nature of their terminal groups (positive, neutral or negative). Understanding the pharmacokinetic behavior and biocompatibility of dendrimers, which depends on their structure, composition and size, is essential if reproducible and desirable results in medical applications are to be achieved [5,6]. In addition, the immune activities of dendrimers *in vitro* and *in vivo* are currently poorly understood. They can induce pro-inflammatory cytokines, hypersensitivity as well as activation of toll-like receptors, and finally immunostimulation can occur [7]. In this paper we will be comparing 2 kinds of dendrimers: commercial cationic, and neutral and anionic poly(amidoamine) (PAMAM) dendrimers, which are widely used for many different applications [1,4,8,9]; and newly synthesized cationic piperidine-based phosphorous dendrimers of 2 generations. It is important that both are explored in detail. PAMAM

* Corresponding author.
E-mail addresses: shcharbin@gmail.com (D. Shcharbin), majoral@lcc-toulouse.fr (J.-P. Majoral), maria.bryszewska@biol.uni.lodz.pl (M. Bryszewska).

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dendrimers are based on an ethylenediamine core, their branches being based on methyl acrylate and ethylenediamine [1,4,10,11]. Half generations of PAMAM dendrimers have surface carboxyls, whereas complete generations have surface amino groups. Degradable PAMAM dendrimers have also been synthesized [1,4,10–12]. Synthesis of multifunctional conjugates on the basis of PAMAM dendrimers produces safe and biocompatible nano-carriers with the potential of having prolonged circulation half-life, bioresponsiveness and target-specific degradability [8,9,13,14]. Generation 4 (G4) of cationic PAMAM dendrimers proved to be the most significant carrier for DNA and RNA [8,9,13,14]. Previously we synthesized new cationic piperidine-based phosphorous dendrimers [15], which had excellent binding ability and could transfect siRNAs, providing 80–100% siRNA uptake by HeLa cells in serum-containing medium, whereas the widespread transfection agent, Lipofectamine, showed only ˜40% uptake, with cationic PAMAM dendrimers of 3–4 generations giving only ˜60–80% uptake (data not presented) [15]. Thus, the newly synthesized cationic AE2G3 and AE2G4 phosphorous dendrimers can be applied in RNAi-based tools for treatment of cancer and as efficient vehicles for gene-based drugs in transfection studies. Serum albumins are the main transport proteins in plasma (50–60% of total plasma proteins), giving an FW of 66 kDa [16–18]. Albumins can bind anionic and cationic ligands with high affinity due to the presence of charged groups and hydrophobic pockets in their structure, and due to their negative surface charge [16–20]. Human alpha-1-microglobulin (A1M) is a glycoprotein of ˜30 kDa present in blood plasma and some tissues of the human body [17,21]. It belongs to the lipocalin family. Family of these proteins is able to bind and transport low molecular weight hydrophobic compounds [17,22]. The 3-dimensional structure of the proteins is conserved and resembles a cylinder composed of 8 antiparallel β-strands with a binding site inside a hydrophobic cavity, the so-called "lipocalin pocket". It can interact with retinol or steroid hormones [17,23–25]. Serum A1M circulates in a monomeric form, and as a component of covalent macromolecular complexes with IgA (50% of A1M), albumin (7%) and prothrombin (1%) [26]. The function of these protein conjugates remains unclear. However, A1M immunomodulatory properties *in vitro* have been described. A1M is a sensitive biomarker in the clinical diagnosis of early stage renal tubular damage [27,28].

Here we describe the effect of newly synthesized cationic piperidine-based phosphorous dendrimers of 2 generations (G3 and G4) and commercial cationic, neutral and anionic PAMAM G4 dendrimers on immunoreactivity of these important proteins.

2. Materials and methods

Commercial dendrimers PAMAM-NH3, PAMAM-OH, PAMAM-COOH of 4th generation were purchased from Sigma-Aldrich Chemical Company (USA). Generation 4 (G4) contains 64 surface cationic (NH4 + -), neutral (-OH) or anionic (-COO–) end groups, Mw: ˜ 14kDA (NH4 + -), 12kDA (-OH) and 14kDA (-COO–).

The protonated amino-terminated dendrimers (which were characterized in details in ref. [15]) (Fig. 1) were obtained by firstly grafting of 1-(2-aminoethyl)-piperidine onto the periphery of precursor P(S)Cl2-terminated phosphorous dendrimers of third (G3, AE2G3 H +) and fourth (G4, AE2G4 H +) generation in the presence of N,N-diisopropylethylamine, and then protonation reaction with HCl. The dendrimeric precursors were obtained by growing from a cyclotriphosphazene core as described previously by several of us in [29].

Human serum albumin (HSA) from RSP Center of Transfusiology and Medical Biotechnology was purified by dialysis [30]. Human A1M was purified by immunoaffinity chromatograhy [30].

2.1. Zeta potential

Zeta potential experiments involved phase analysis light-scattering with a Malvern Instruments Zeta-Sizer Nano S90 (Malvern, UK) at 37 °C. The electrophoretic mobility of the scattering samples was determined from the average of 6 cycles of an applied electric field in an electrophoretic disposable plastic cell. The zeta potential of complexes was determined from the electrophoretic mobility by Smoluchowski approximation.

2.2. Enzyme-linked immunosorbent assay (ELISA) for HSA

The materials and reagents used were: 96-well microplates (Greiner Bio-one, Germany), HSA, polyclonal antibodies (Abs) against human serum albumin [30,31], horseradish peroxidase (Sigma-Aldrich, USA). Other reagents were obtained from Sigma-Aldrich (USA). Conjugates of horseradish peroxidase with HSA and the antibodies were synthesized using periodate oxidation of oligosaccharide chains of the enzyme. HSA samples were prepared in 50 mmol/l sodium phosphate buffer (pH 7.4) at a protein concentration of 1 µmol/l. Dendrimers were added to protein in molar ratios of 1:1 and 1:10. Ligand-protein systems were kept at 20–25 °C for 2 h prior to measurement of their immunochemical parameters in several ELISA systems. In the first construction of ELISAs, samples of HSA with dendrimers and without ligands (control sample) were diluted 50-fold using a buffer for immobilization (50 mmol/L sodium carbonate, pH 9.6). Microplates were coated by adding 100 µL/well diluted samples in duplicate or tetraplicate (control sample) and incubated for 18 h at 4–8 °C. The plate was emptied and washed 3 times with 200 µL/well wash buffer (20 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 0.05% Tween 20). Conjugate of Abs against albumin with peroxidase (100 µL at a titer of 1:70 000) was added to coated wells and the plate was incubated at 37 °C for 1 h. After incubation, unreacted components were removed and the plate was washed as above. 100 µL chromogenic substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H2O2 was added to each well and the plate was incubated for 15 min at 20–25 °C. The enzymatic reaction was stopped by addition of 5% H2SO4 (100 µL/well). Absorbance was measured at 450 nm (OD450) with an Infinity M 200 spectrophotometer (Tecan, Austria). In the second series of experiments, HSA was immobilized on microplate wells by physical adsorption for

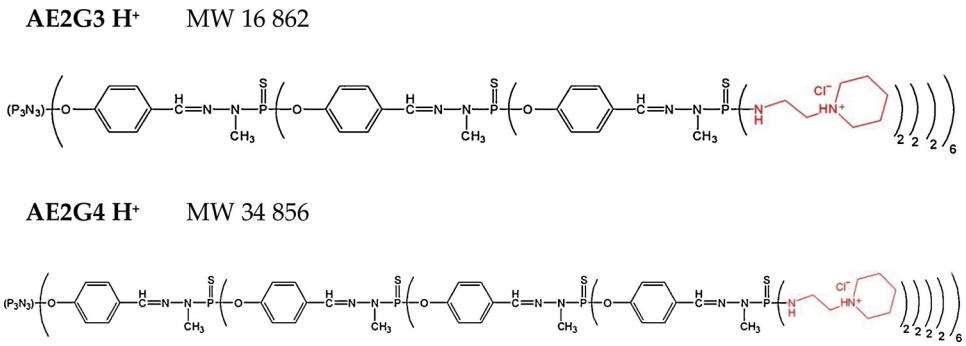

Fig. 1. Structures of the G3 (AE2G3 H +) and G4 (AE2G4 H +) G4 cationic phosphorous dendrimers.

18 h at 4–8 °C, after which the adsorbed protein was incubated with dendrimers or the buffer (control sample) for 1 or 4 h. The plate was washed and ELISA tests were carried out using Abs against albumin conjugated with horseradish peroxidase as described above. In the third series of experiments using the construction of the indirect competitive ELISA, HSA was immobilized on microplate wells by adsorption for 18 h at 4–8 °C. Samples of HSA with dendrimers and without ligands (control samples) were diluted 5-fold using a buffer for assay (50 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl, 0.05% Tween 20, and 0.5 g/L bovine serum albumin). Then 20 µL diluted samples of HSA with dendrimers or HSA calibration samples in the range of 0, 0.5–300 µg/L and 100 µl conjugate Abs with peroxidase at a titer of 1:70 000 were added to the coated wells. The plate was incubated at 37 °C for 1 h before the contents of the wells were removed and the plate was washed 3 times with 200 µL/well wash buffer, as described above. 100 µL chromogenic substrate solution containing TMB and H2O2 were added to each well and the plate incubated for 15 min at 20–25 °C. The intensity of coloration was measured at 450 nm, as above. In the fourth series of experiments using a direct competitive ELISA, polyclonal Abs against human albumin were immunochemically immobilized on microplates through binding to adsorbed secondary (anti-rabbit) antibodies, and 20 µL 50-fold diluted test or control samples of HSA, or HSA calibration samples in the concentration range of 0, 0.1–30 µg/L and 100 µL of HSA-peroxidase conjugate (a titer of 1:50 000), were added. Plates were incubated for 1 h at 37 °C, the unreacted components were removed, and the plates washed. 100 µL chromogenic substrate solution was added to the wells, and the plates were incubated for 15 min at 20–25 °C before absorbance was measured. HSA concentration in ELISAs was determined from a calibration curve plotted on semi-logarithmic coordinates, depending on optical density in the wells and HSA concentration in the calibration samples. Immunoreactivity of HSA was calculated as the ratio of protein concentration of the test to that of the control samples.

2.3. Enzyme-linked immunosorbent assay (ELISA) for A1M

The following materials and reagents were used: 96-well microplates (Greiner bio-one, Germany), A1M, monoclonal antibodies (MAbs) against A1M of the clones G6, F9 and H8 [30], horseradish peroxidase (Sigma-Aldrich, USA), goat anti-mouse immunoglobulin (IBOCH NASB, Belarus). The MAbs, all belonging to IgG1 family, are characterized by reacting primarily with linear epitopes of A1M in the case of MAb G6 and H8, and conformational sites in the case of MAb F9. Other reagents were obtained from Sigma-Aldrich (USA). Conjugates of horseradish peroxidase with antibodies were synthesized using periodate oxidation of oligosaccharide chains of the enzyme. A1M samples were prepared in 50 mmol/l sodium phosphate buffer (pH 7.4) at a protein concentration of 10 µmol/l. Dendrimers and hormones – thyroxine (T4) and estradiol (E2) - were added to protein each in molar ratios of 1:1 and 1:10. Ligand-protein systems prior to measurement were kept at 20–25 °C for 2 h before their immunochemical parameters were measured in 2 constructions of the ELISA systems. In the first series of experiments, samples of A1M with dendrimers and without ligands (control sample) were diluted 100-fold using a buffer for immobilization (50 mmol/L sodium carbonate, pH 9.6). Microplates were coated by adding 100 µL/well diluted samples in duplicate or tetraplicate (control sample) and incubated for 18 h at 4–8 °C. The plate was emptied and washed 3 times with 200 µL/well wash buffer (20 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 0.05% Tween 20). MAbs G6 or F9 (100 µL at 0.25 mg/L) were added to coated wells and the plate incubated at 37 °C for 1 h. After incubation, unreacted components were removed and the plate washed as above. The conjugate of secondary (anti-mouse) antibodies with horseradish peroxidase (100 µL, at a titer of 1:10 000) was added to the wells, and the plate was incubated at 37 °C for 1 h, after which the contents of the wells were removed and the plate was washed. 100 µL chromogenic substrate solution containing TMB and H2O2 was added to each well and the plate was incubated for 15 min at 20–25 °C. The enzymatic reaction was stopped by the addition of 5% H2SO4 (100 µL/well). Absorbance was measured at 450 nm with an Infinity M 200 spectrophotometer (Tecan, Austria). In the second series of experiments, A1M was immobilized on microplate wells by physical adsorption for 18 h at 4–8 °C, after which the adsorbed protein was incubated with dendrimers or the buffer (control sample) for 1 or 4 h. The plate was washed and ELISA tests were carried out using MAb G6, F9 or H8 and the secondary antibodies conjugated with horseradish peroxidase, as described above.

2.4. Statistical analysis

Data are expressed as mean ± S.D. of 6 independent experiments. Significance was assessed using the one-way analysis of variance with the post-hoc *t*-test or Newman-Keuls multiple comparisons test.

3. Results

Zeta-potential findings on the interaction between proteins and dendrimers showed that the anionic PAMAM G4 dendrimers did not form complexes with A1M. In contrast, the data indicate the changes of zeta-potential of these proteins after additng cationic and neutral dendrimers. We had earlier obtained similar results on the interaction between cationic, neutral and anionic PAMAM G4 dendrimers with bovine and human serum albumin [20,32–35]. From Fig. 2, it is clear that the zeta-potential of both proteins changed after adding AE2G3 and AE2G4 dendrimers, reaching a plateau at 10:1 M ratio of dendrimer:protein. This means that, at these molar ratios, proteins are fully

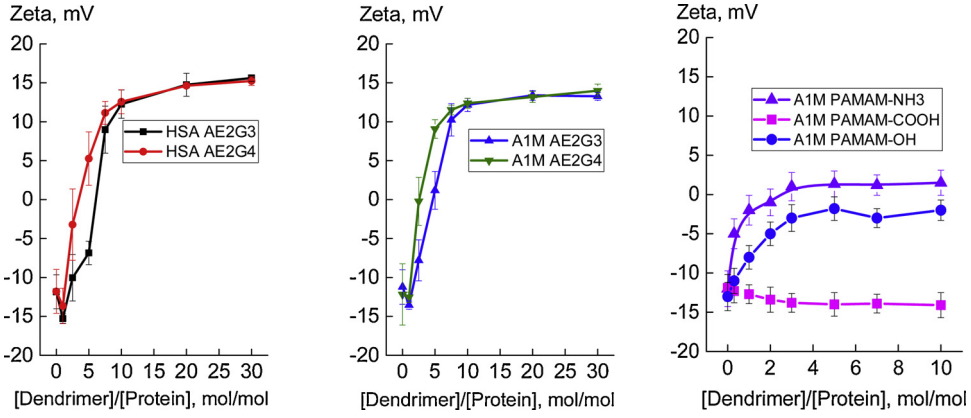

Fig. 2. Zeta-potentials of HSA and A1M upon addition of cationic AE2G3, AE2G4 and cationic, neutral and anionic PAMAM G4 dendrimers.

Table 1
Immunoreactivity of HSA (in %) after complexation with piperidine-based phosphorous dendrimers by ELISA.

Ligand	HSA-ligand, mol/mol	Solid-Phase ELISA (immobilization of complexes)	Solid-Phase ELISA (adsorbed HSA)	Indirect ELISA	Direct ELISA
AE2G3+	1:1	90.5 ± 2.5	99.0 ± 2.5	77.1 ± 2.3	82.5 ± 1.8
	1:10	70.3 ± 2.4	95.7 ± 2.4	52.4 ± 2.0	59.6 ± 2.5
AE2G4+	1:1	80.9 ± 2.4	102.0 ± 2.5	78.2 ± 2.1	81.3 ± 2.4
	1:10	52.5 ± 2.3	101.0 ± 2.8	44.8 ± 2.2	44.3 ± 1.9

bound by dendrimers. In contrast, A1M could bind ˜5 molecules of cationic and neutral PAMAM G4 dendrimers, and was unable to bind anionic PAMAM G4 dendrimer. These results are in close agreement with our previous data on the interaction between HSA and cationic, neutral and anionic PAMAM G4 dendrimers [20,32–35]. HSA could also bind 5–6 molecules of cationic and neutral PAMAM G4 dendrimers, whereas its interaction with anionic PAMAM G4 dendrimer was very weak. To check the effect of dendrimers on immunoreactivity of proteins, based on results of zeta-potential we chose 2 uniform molar ratios - 1:1 and 1:10. At 1:1 the protein-dendrimer complex is negatively charged as its charge is determined mainly by protein net charge. At 1:10 (protein to dendrimer) the protein is fully surrounded by dendrimers and it surface potential is determined by positively charged dendrimers. The same ratios were also chosen for neutral and anionic dendrimers.

Results on immunoreactivity of HSA in presence of piperidine-based phosphorous dendrimers are shown in Table 1.

The interaction of albumin with modified phosphoric dendrimers was studied in 2 solid-phase ELISA formats and 2 formats of competitive ELISA of direct and indirect variants. The samples of albumin with dendrimers were incubated for 2 h at 25 °C and examined in ELISA systems. Subsequently, immunochemical properties were evaluated by polyclonal antibodies (see Materials and Methods) interaction with albumin immobilized on a solid phase or present in solution. In solid-phase, ELISA albumin was immobilized on microplate wells. In the first series of experiments, the protein was immobilized after interaction with dendrimers, and in the second series the dendrimers were interacted with the protein absorbed on the solid phase. To evaluate the immunoreactivity of the solid-phase protein, a conjugate of anti-albumin antibody with peroxidase was used. In the direct competitive ELISA, antibodies to albumin were immobilized in the wells of the microplate, and a competitive interaction of human albumin in the test and calibration samples and the albumin-peroxidase conjugate for binding to antibodies was measured during the assay. In the indirect competitive immunoassay, human albumin was immobilized on the wells of the microplate and, in the assay, a competitive distribution of antibodies to albumin conjugated with peroxidase, between albumin in the test and calibration samples and solid phase albumin occurs. The experimental data (Table 1) clearly show that inhibition of albumin immunoreactivity occurs when interacting with the modified phosphoric dendrimers being used. The outcome depends on the molar ratio of dendrimer-protein complexes. In composition of equimolar complexes, inhibition of albumin immunoreactivity is low, and the efficiency of process is from 77 to 100%. In contrast, a 10-fold excess of dendrimers during the interaction in solution significantly (two fold) reduces the albumin immunoreactivity in the case of free albumin in solution. Dendrimer AE2G4 has a stronger inhibitory effect on the immunochemical properties of albumin compared to AE2G3. However, in the interaction of dendrimers with immobilized albumin, no significant changes in the immunoreactivity of this protein occurred.

Results on immunoreactivity of A1M in presence of piperidine-based phosphorous dendrimers, thyroxine and estradiol are shown in Table 2.

The influence of the modified phosphoric dendrimers and known ligands for this protein (thyroxine and estradiol) on the immunochemical properties of A1M were studied by ELISA using monoclonal antibodies [30]. To estimate the immunoreactivity of A1 M, 3 clones of MAbs – G6, F9 and H8 – were used; they belong to IgG1 family, and are primarily directed toward linear (MAbs G6 and H8) and conformational (MAb F9) epitopes of the protein. Two formats of enzyme immunoassay systems using different MAbs against A1M were tested. In the ELISAs, A1M was immobilized on the surface of a solid phase. In the first series of experiments, A1M was immobilized after interacting with dendrimers, T4 or E2. In the second series of experiments, dendrimers and the hormones interacted with the protein adsorbed onto microplate wells. Immunoreactivity of A1M was determined by interaction with MAbs. MAbs G6, F9 and H8 bound to A1M were detected using a conjugate of anti-mouse immunoglobulin antibodies with peroxidase. As a control, plates treated with only T4 or E2 dendrimers did not react with MAbs. From the experimental data (Table 2), either dendrimers or natural ligands (T4 and E2) had no significant effect on immunoreactivity of adsorbed A1M on a solid phase. Dendrimers, after complexing with the protein, partially prevent the recognition of A1M by antibodies, and A1M immunoreactivities at its equimolar complexes were in the range of 93.3–94% or 83.4–85.6%, as determined by the ELISAs with MAb G6 or MAb F9, respectively. As in the case of albumin, a 10-fold excess of dendrimers during the interaction in solution significantly reduced (48–57%) A1M immunoreactivity (immobilized complexes). Also, dendrimer AE2G4 was a stronger inhibitor on A1M compared to AE2G3. In contrast to dendrimers, the interaction of A1M with natural ligands T4 and E2 does not occur on the surface of the protein globule, but mainly in the hydrophobic cavity of the ligand-binding site of this lipocalin.

We also studied the effect of piperidine-based phosphorous dendrimers on antibodies, *i.e.* antigen-binding activity of the antibodies (Table 3). The experiment was conducted in a single construction: antibodies and dendrimer complexes interacted, then they were immobilized on a solid phase, and immunoassay performed with the use of a conjugate of alpha-1-microglobulin peroxidase MAb G6 and F9 and of albumin conjugate peroxidase for polyclonal antibodies against human serum albumin.

Piperidine-based phosphorous dendrimers, after complexing with the antibody, partially prevented the recognition of A1M and HSA by them in molar ratios 1:10, while A1M immunoreactivities at its equimolar complexes were in the range of 99.5 − 89.4%, as determined by the ELISAs with MAb G6 or MAb F9, respectively, and HSA immunoreactivity – 96.7 − 89.2%, as determined by the ELISA with ABs. As in the case of albumin and A1M, a 10-fold excess of dendrimers during the interaction in solution significantly reduced (60.4 − 50.2%) antigen-binding activity of monoclonal and polyclonal antibodies immunoreactivity.

The influence of the PAMAM G4 dendrimers on the immunochemical properties of A1M were examined by ELISA using monoclonal antibodies [30]. To estimate the immunoreactivity of A1M, 3 clones of MAbs – G6, F9 and H8 – were used; they belong to the IgG1 family, and are primarily directed toward linear (MAbs G6 and H8) and conformational (MAb F9) epitopes of the protein. Two formats of the enzyme immunoassay systems using different MAbs against A1M were tested. In the ELISAs, A1M was immobilized on the surface of a solid phase. In the first series of experiments, A1M was immobilized after interacting with PAMAM dendrimers. In the second series of experiments, PAMAM dendrimers interacted with the protein adsorbed onto

Table 2
Immunoreactivity of A1M (in %) after complexation with piperidine-based phosphorous dendrimers, thyroxine (T4) and estradiol (E2) by ELISA.

Ligand	A1M-ligand, mol/mol	Solid-Phase ELISA (immobilization of complexes)		Solid-Phase ELISA (adsorbed A1M)		
		MAb G6	MAb F9	MAb G6	MAb F9	MAb H8
AE2G3+	1:1	94.0 ± 2.1	93.3 ± 1.9	98.9 ± 2.3	100 ± 2.3	100 ± 2.7
	1:10	49.8 ± 1.7	57.7 ± 2.5	93.7 ± 2.2	94.3 ± 2.5	98.8 ± 2.6
AE2G4+	1:1	83.4 ± 2.5	85.6 ± 2.3	97.7 ± 2.4	98.9 ± 2.1	98.5 ± 2.5
	1:10	35.5 ± 2.1	48.3 ± 1.7	96.3 ± 2.4	97.2 ± 2.4	97.3 ± 2.7
T4	1:1	95.9 ± 2.4	92.3 ± 2.1	95.3 ± 2.0	98.4 ± 2.4	96.6 ± 2.1
	1:10	90.1 ± 2.7	83.6 ± 2.2	92.3 ± 1.9	92.6 ± 1.7	94.9 ± 2.3
E2	1:1	95.4 ± 2.0	91.2 ± 2.7	94.5 ± 2.1	94.8 ± 2.0	100 ± 2.4
	1:10	93.3 ± 2.3	85.5 ± 2.1	93.9 ± 2.5	91.2 ± 2.0	97.9 ± 2.1

Table 3
Antigen-binding activity of monoclonal and polyclonal antibodies (in %) after complexation with piperidine-based phosphorous dendrimers by solid-phase ELISA.

Ligand	Abs-ligand, mol/mol	Solid-Phase ELISA (immobilization of complexes Abs-ligand)		
		MAb G6 against A1M	MAb F9 against A1M	Abs against HSA
AE2G3+	1:1	99.5 ± 1.1	97.2 ± 2.1	96.7 ± 1.3
	1:10	80.0 ± 2.0	76.4 ± 1.9	60.4 ± 1.8
AE2G4+	1:1	89.4 ± 2.0	94.4 ± 2.5	89.2 ± 2.1
	1:10	77.0 ± 1.8	53.4 ± 2.3	50.2 ± 2.2

Table 5
Immunoreactivity of HSA (in %) after complexation with PAMAM G4 dendrimers by ELISA.

Ligand	HSA-ligand, mol/mol	Solid-Phase ELISA (immobilization of complexes) Polyclonal Abs (PolyAbs)	"Sandwich"-ELISA (2 stages)	
			MAT H-A1/ PolyAbs	MAT H-C15/ PolyAbs
PAMAM -NH3+	1:1	91.5 ± 2.0	94.5 ± 2.1	98.0 ± 1.8
	1:10	80.1 ± 2.5	89.4 ± 1.8	94.5 ± 1.9
PAMAM -COO-	1:1	99.1 ± 1.5	98.2 ± 2.4	97.2 ± 2.1
	1:10	95.4 ± 2.2	97.1 ± 2.2	96.0 ± 1.9
PAMAM -OH	1:1	90.5 ± 2.0	88.4 ± 2.0	90.2 ± 2.2
	1:10	70.5 ± 2.4	86.2 ± 1.8	88.1 ± 1.8

microplate wells. Immunoreactivity of A1M was determined by interaction with MAbs. MAbs G6, F9 and H8 bound to A1M were detected using a conjugate of anti-mouse immunoglobulin antibodies with peroxidase. All the PAMAM G4 dendrimers in equimolar concentrations had no significant effect on immunoreactivity of adsorbed A1M on a solid phase (Table 4). After complexing with the protein, PAMAM dendrimers partially prevented the recognition of A1M by antibodies, and A1M immunoreactivities at its equimolar complexes were in the range of 90–96% (cationic), 100% (anionic) or 88–91% (neutral), as determined by the ELISAs with MAb G6 or MAb F9, respectively. A 10-fold excess of cationic and neutral dendrimers significantly reduced (60–70%) A1M immunoreactivity (immobilized complexes), whereas anionic ones had no effect.

The effects of PAMAM G4 dendrimers on the immunochemical properties of HSA were also studied by ELISA using monoclonal (H-A1, H-C15) and polyclonal (polyAbs) antibodies [30], with 2 formats of enzyme immunoassay systems being used. In the first series, HSA was immobilized after interacting with PAMAM dendrimers. In the second series, PAMAM dendrimers interacted with HSA adsorbed onto microplate wells. All PAMAM G4 dendrimers in equimolar concentrations had no significant effect on immunoreactivity of adsorbed HSA on a solid phase (Table 5). After complexing with HSA, cationic and neutral PAMAM G4 dendrimers partially prevented the recognition of HSA by antibodies (for cationic ˜92%, for neutral ˜91%). A 10-fold excess of cationic and neutral PAMAM G4 dendrimers in interaction in the solution partially reduced (from 100% to 70–80%) HSA immunoreactivity (immobilized complexes), whereas anionic ones had no effect. This effect was similar to the that of PAMAM dendrimers on A1M.

4. Discussion

Interaction between dendrimers and antibodies, as well as the impact of dendrimers on immunoreactivity of proteins and antibodies, have been intensively explored ([36–39] and see below). It can be divided in 2 main directions, the first being where dendrimers are used to improve the action of antibodies [36,37] or multivalent antibody-antigen interactions [40,41], to present new kinds of adjuvants [42–44], to improve visualization of proteins [45,46], to construct dendrimer-conjugated peptide vaccines [47,48] and dendrimer-antibody complex systems for siRNA or drug delivery [49–51]. In second direction, immunoreactivity of proteins as well as the interaction of dendrimers with antibodies are examined as a side effect of possible application of dendrimers [52–56]. In particular, Wangler et al. [53] showed that in antibody-dendrimer conjugates, the number, but not the size of the dendrimers, determines the immunoreactivity of these complexes.

Our main concern has been to estimate the effect of newly synthesized perspective piperidine-based phosphorous dendrimers on the

Table 4
Immunoreactivity of A1M (in %) after complexation with PAMAM G4 dendrimers by ELISA.

Ligand	A1M- ligand, mol/mol	Solid-Phase ELISA (immobilization of complexes)		"Sandwich"-ELISA (2 stages)	
		MAT G6	MAT F9	MAT G6/H8	MAT F9/ H8
PAMAM -NH3+	1:1	80.9 ± 2.5	82.2 ± 2.0	90.3 ± 2.0	96.2 ± 2.7
	1:10	64.2 ± 2.1	70.7 ± 2.7	74.1 ± 2.5	82.5 ± 1.9
PAMAM -COO-	1:1	99.2 ± 1.9	99.7 ± 2.5	99.5 ± 2.3	99.6 ± 2.1
	1:10	99.4 ± 2.2	100.2 ± 2.0	99.1 ± 2.5	99.4 ± 2.1
PAMAM -OH	1:1	83.1 ± 2.2	84.3 ± 2.0	88.1 ± 1.9	91.3 ± 2.2
	1:10	60.8 ± 2.1	61.3 ± 2.5	86.2 ± 2.1	75.4 ± 2.7

immunoreactivity of HSA and A1M. Another aim was to study the effect on immunoreactivity of proteins of commertial cationic, neutral and anionic PAMAM G4 dendrimers to analyze the impact of surface-charge of a dendrimer in such interactions. Initially by using zeta-potential, we estimated the number of dendrimers that can be bound by both proteins. HSA and A1M bound up to 10 molecules of cationic AE2G3 and AE2G4 dendrimers, and up to 4–6 molecules of PAMAM-NH3 and PAMAM-OH G4 dendrimers (we had previously reported on the interaction between HSA and all PAMAM G4 dendrimers [20,32–35]). The binding of cationic dendrimers to proteins can be explained by electrostatic forces acting between positively charged dendrimers and negatively charged regions of proteins [20,32–35]. It was also noteworthy that the effect of cationic dendrimers on proteins was generation-dependent: AE2G4 interacted on protein globules more strongly than AE2G3 because of an increased number of cationic charges. The effect of neutral PAMAM G4 dendrimers on the structure and activity of proteins can be explained by (1) interaction between internal NH3+-groups of PAMAM-OH G4 dendrimers and anionic regions of proteins (internal NH3+-groups of dendrimers can be exposed in solution because of their flexible structure), (2) non-electrostatic interactions between neutral dendrimers and proteins [1,4,34,57–60]. In contrast, electrostatic interactions between anionic dendrimers and anionic regions of proteins can prevent their interaction [1,4,34,57–60]. In the second stage, we analyzed the effect of newly synthesized AE2G3 and AE2G4 dendrimers on the immunoreactivity of proteins and antibodies. In our work we used various ELISAs to study the interaction of A1M and albumin with dendrimers in order to convincingly show that the effect on immunoreactivity is not related to the choice of ELISA test. Also, variations of tests allowed to divide the direct effect of dendrimers on proteins from their direct effect on antibodies. Three cases can be identified: (1) in the case of equimolar complexes, inhibition of immunoreactivity of proteins or antibodies is low (a decrease in efficiency from 100% to 70%); (2) in contrast, a 10-fold excess of dendrimers during the interaction in solution significantly reduces (a decrease in efficiency from 100% to 44%) the immunoreactivity of free proteins or monoclonal and polyclonal antibodies in solution. In this case, dendrimer AE2G4 hads a stronger inhibitory effect compared to AE2G3; (3) however, in the interaction of dendrimers with immobilized proteins, no significant changes in the immunoreactivity of proteins occurred even with a 10-fold excess of dendrimers. This might be because proteins can bind dendrimers at special non-specific sites [32]. When proteins are free and native in solution, dendrimers can contact with these sites to reduce their immunoreactivity. When proteins have been immobilized, most of these sites become inaccessible to dendrimers [35]. In our third step, we studied the effect of commercial widely used cationic, neutral and anionic PAMAM dendrimers of 4th generation on the immunoreactivity of proteins. Three cases can be identified: (1) the effect of cationic PAMAM-NH3 G4 dendrimers was similar to that of cationic AE2G3/AE2G4 denrimers; with equimolar complexes, inhibition of immunoreactivity of proteins was low. With a 10-fold excess of dendrimers, this inhibition increased for only the free proteins in solution (the inhibition remained low for immobilized proteins). (2) The effect of neutral PAMAM-OH G4 dendrimers was similar to those of cationic dendrimers. As mentioned in detail above, neutral PAMAM dendrimers can interact with proteins [1,4,34,57–60]. (3) Anionic PAMAM-COOH G4 dendrimers had no effect on immunoreactivity of proteins even as a 10-fold excess, which might be due to their very weak interaction with proteins (see above). In addition, we can notice that effect of dendrimers on HSA and A1M immunoreactivity is also connected with two effects: the direct effect of these dendrimers on proteins, and the effect of dendrimers on antibodies. However, the adsorption of HSA and A1M on solid phase leads to disappearance of effect of dendrimers on these proteins.

5. Conclusions and perspective

Comparing effects of piperidine-based phosphorus dendrimers as well as cationic, neutral and anionic PAMAM dendrimers on HSA, A1M, and antibodies we conclude that, in the case of equimolar complexes, the dendrimers had no effect on all proteins. The possible reason is that the dendrimers do not occupy these macromolecules and the sites for MAbs/Abs binding are free. In contrast, the formation of complexes in which a protein is fully surrounded by dendrimers leads to partial reduction (1.2–2.3 fold) in protein/antibody immunochemical properties. The most important outcome is that dendrimer-induced reduction in immunoreactivity of proteins is only partial, even if protein is fully bound by the dendrimers. This means that the application of dendrimers *in vivo* do not entirely hamper the immunochemical properties of these proteins.

Conflict of interest

The authors declare no conflict of interest.

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