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Effect of poly(amidoamine) dendrimers on the structure and activity of immune molecules

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ABSTRACT

Background: Poly(amidoamine) (PAMAM) dendrimers are widely used biomedical polymers, which are extensively applied in drug delivery, gene delivery, contrast agent, etc. In these biomedical applications, the bio-safety of the PAMAM dendrimers is a critical issue, which affects not only their toxicity to the host but also the expected in vivo biofunctions of the materials. To clarify the bio-safety of PAMAM dendrimers, the effects of generation 5 PAMAM dendrimers with amine, hydroxyl or carboxyl groups on immune molecules were explored in this work.

Methods: Specifically, the effect of the PAMAM dendrimers on the secondary structure and conformation of immune molecule γ-globulin was studied by using ultraviolet-visible, fluorescence, and circular dichroism spectroscopies. The effect of the PAMAM dendrimers on complement activation was determined by enzyme-linked immunosorbent assay. Further, the effect of the PAMAM dendrimers on antigen–antibody reaction was studied by using human red blood cell agglutination assay.

Results: The results showed that, the PAMAM dendrimers could affect the secondary structure and conformation of γ-globulin, and inhibited complement activation. Generation 5 PAMAM dendrimer with carboxyl group at 10 mg/mL impaired red blood cell (RBC) antigen–antibody reaction.

Conclusions: From these results, the effects of the PAMAM dendrimers on immune molecules depend on their bulk structure and surface groups.

General significance: This work provides important information for the immunocompatibility evaluation, preclinical design, and clinical applications of PAMAM dendrimers.

1. Introduction

Poly(amidoamine) (PAMAM) dendrimers are a type of highly branched polymers that have unique physical and chemical properties, such as three-dimensional globular architecture, and functional groups on their surface. Therefore, PAMAM dendrimers have extensive biomedical applications in drug delivery [1], gene delivery [2], imaging [3], etc. In these biomedical applications, the bio-safety of PAMAM dendrimers is a critical issue, which mainly includes hemocompatibility, tissue-compatibility, and immunocompatibility. Bio-safety of the biomedical materials affects not only their toxicity to the host but also their pre-designed in vivo biofunctions. Based on this perspective, the biocompatibility of PAMAM dendrimers should be comprehensively evaluated. To clarify the bio-safety of PAMAM dendrimers, many researchers have investigated their biological effects on blood tissue, various tissue-cultured cells, etc. For example, some researchers reported the effects of PAMAM dendrimers on the structure and function of key blood components, including aggregation, morphological alteration and hemolysis of human red blood cells (RBCs), structural and conformational change and polymerization function of fibrinogen, and blood coagulation [4–6]. Some researchers found that PAMAM dendrimers promote acute lung injury by inducing autophagic cell death [7].

For any biomedical materials, their effect on host immune system (i.e., immunocompatibility) is also a key aspect of their bio-safety evaluation. Compared to hemocompatibility evaluation, immunocompatibility evaluation of biomedical materials is much less clarified, probably because of the complexity of immune system and the difficulty in immunity-related experimental studies. As for PAMAM dendrimers, some related studies include the mechanism of cell death induced by PAMAM dendrimers in RAW 264.7 murine macrophage-like cells [8], biological responses of J774A.1 murine macrophage-like cells induced by PAMAM dendrimers such as intracellular reactive oxygen species, cytokine production and cytotoxicity [9].

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To explore the potential impact of PAMAM dendrimers on the immune system, this work focuses on the effects of PAMAM dendrimers on immune molecules. Besides, it is reported that the surface group of PAMAM dendrimers plays a key role in their biocompatibility [7, 10–12]. Therefore, PAMAM dendrimers with amine, hydroxyl or carboxyl groups (their chemical structures illustrated in Fig. 1) were used to compare the influence of different surface groups. Specifically, the effect of PAMAM dendrimers on the secondary structure and conformation of immune molecule γ-globulin was studied by using UV–vis, fluorescence, and circular dichroism (CD) spectroscopies. The effect of PAMAM dendrimers on complement activation was determined by enzyme-linked immunosorbent assay (ELISA). Further, the effect of PAMAM dendrimers on antigen–antibody reaction was studied by using human RBC agglutination assay. This work provides important information for the bio-safety evaluation, preclinical design, and clinical application of PAMAM dendrimers.

2. Materials and methods

2.1. Materials

PAMAM dendrimers (with an ethylenediamine core) with amine (–NH_2, generation 5), hydroxyl (–OH, generation 5) or carboxyl (–COOH, generation 4.5) groups were purchased from Weihai CY Dendrimer Technology Co., Ltd (Weihai, China), as abbreviated G5 PAMAM–NH_2 (catalog number CYD-150A), G5 PAMAM–OH (catalog number CYD-150H), or G4.5 PAMAM–COOH (catalog number CYD-145C), respectively. The sizes of the PAMAM dendrimers were measured with a Zetasizer Nano-ZS zeta potential analyzer (Malvern Instruments), as shown in the supplementary information. γ-Globulin was purchased from Sigma-Aldrich (catalog number G4386-1G, St. Louis, MO, USA). Commercial C3a ELISA kit was purchased from eBioscience (catalog number 83919001, San Diego, USA). Blood grouping reagents of monoclonal antibodies anti-A and anti-B were purchased from Hemo-Pharmaceutical & Biological Reagent Co., Ltd (catalog number 5012012, Shanghai, China). Fresh whole blood was donated by healthy consenting volunteers and was collected in sodium citrate vacuum tubes. The whole blood was centrifuged at 1000 ×*g* for 5 min, and the supernatant plasma was collected. The remaining RBC pellet was washed with phosphate buffered saline (PBS, pH 7.4). The PAMAM dendrimers and γ-globulin were dissolved in PBS before use.

2.2. Effect of PAMAM dendrimers on the structure and conformation of γ-globulin

UV–vis absorption spectra of γ-globulin (0.1 mg/mL) containing different concentrations of PAMAM dendrimers were recorded with a UV-2550 spectrometer (Shimadzu Corporation, Kyoto, Japan). The spectra were recorded from 200 to 400 nm at room temperature in quartz cuvettes of 1 cm optical path length.

Fluorescence emission spectra of γ-globulin (0.1 mg/mL) containing different concentrations of PAMAM dendrimers were recorded with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The spectra were recorded at λ_{exc} 280 and λ_{em} from 290 to 450 nm at 25 °C. All fluorescent spectra were recorded with 1 × 1 cm path length quartz cells, excitation and emission slit widths of 5 nm, and scan rate of 1200 nm/min.

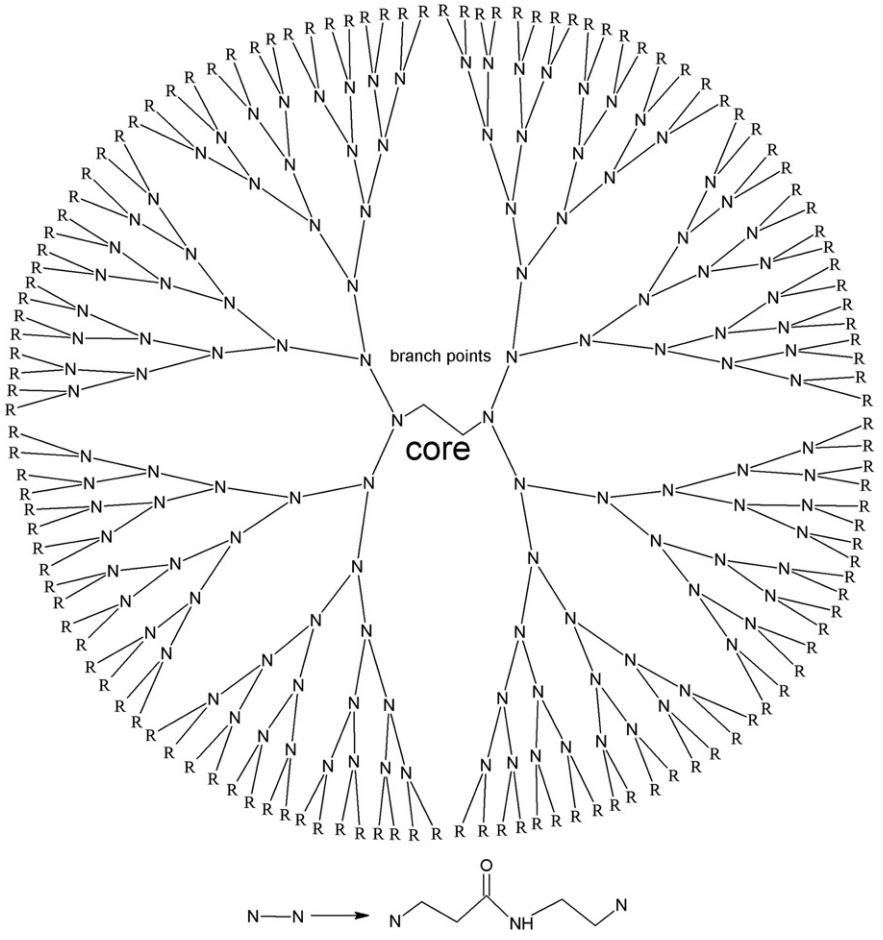

Fig. 1. Chemical structure of the PAMAM molecule (G5 PAMAM–NH_2 as R═NH_2, G4.5 PAMAM–COOH as R═COOH, and G5 PAMAM–OH as R═OH).

CD spectra of γ-globulin (0.1 mg/mL) containing different concentrations of PAMAM dendrimers were recorded in a nitrogen atmosphere with an Applied Photophysics™ Chirascan CD spectrometer (Applied Photophysics Ltd, Leatherhead, UK). The CD spectra were collected at 25 °C from 200 to 250 nm at a scan speed of 60 nm/min by using quartz cells of 1 cm path length and PBS as a running buffer.

2.3. Effect of PAMAM dendrimers on complement activation

Complement activation in the presence of PAMAM dendrimers was evaluated by measuring the content of C3a fragment released during complement activation. Briefly, fresh human plasma (180 μL) was incubated at 37 °C for 60 min with 20 μL of PAMAM solution in PBS. The content of C3a fragment from complement activation was determined with the commercial C3a ELISA kit.

2.4. Effect of PAMAM dendrimers on RBC agglutination

Washed RBC pellet (25 μL) was added to 225 μL of PAMAM dendrimer solutions in PBS. The RBC suspension was mixed with anti-A or anti-B solution at a volume ratio of 1:1. After 2 min, the agglutination phenomena were recorded with a digital camera (Canon, Japan).

2.5. Statistical analysis

Data were represented as the mean ± SD (standard deviation) and analyzed by one-way ANOVA. Statistical analysis was performed using OriginPro 8.0 software (OriginLab Northampton, MA, USA). Difference is considered significant when $p < 0.05$.

3. Results and discussion

3.1. Effect of PAMAM dendrimers on the structure and conformation of γ-globulin

Biomacromolecule proteins play key roles in various metabolism processes such as transporting, signal transduction, catalysis, and recognition. The "smart" bio-functions of proteins depend on their special structure and conformation, which are sensitive to their surrounding environment. Besides, some drugs or administered biomedical materials can also interact with proteins and affect their structure, conformation and biofunction. For example, PAMAM dendrimers have been shown to prevent fibril assembly and provide potential therapeutic strategy for Creutzfeldt–Jakob disease, which is characterized by the deposition of amyloid fibrils—aggregates of normally soluble prion protein [13]. The inhibition of fibril formation could be mediated via interference with beta-sheet assembly of the prion protein [14]. Subtle alteration of protein structure and conformation can be monitored by many techniques, among which UV–vis, fluorescence and CD spectroscopies are widely employed. In this work, the three techniques were used to detect the effect of PAMAM dendrimers on γ-globulins, a family of immunoglobulins with high abundance in plasma. Up to now, many researchers studied the effect of PAMAM dendrimers on proteins such as serum albumins [15,16] and fibrinogen [4], and found that these proteins could undergo structural and conformational alterations in the presence of PAMAM dendrimers [17]. However, few reported the effect of PAMAM dendrimers on immunoglobulins.

UV–vis spectroscopy can be used to monitor the secondary structure change of proteins. Presented in Fig. 2 are the UV–vis adsorption spectra of γ-globulin in the presence of PAMAM dendrimer with amine, hydroxyl or carboxyl groups. The absorption peak at 200–240 nm is induced by the n–π* electronic transition of peptide bonds. Regardless of the different surface groups, the peaks of γ-globulin at 200–240 nm uniformly reduced in intensity and shifted to longer wavelengths with increasing concentration of the PAMAM dendrimers. In addition, the dendrimers also affected the intensity of the peaks of γ-globulin at 280 nm. Specifically, G4.5 PAMAM–COOH from 0.125 to 1 mg/mL enhanced the peak intensity while the other two dendrimers irregularly affected the peak intensity. These suggest that the PAMAM dendrimers

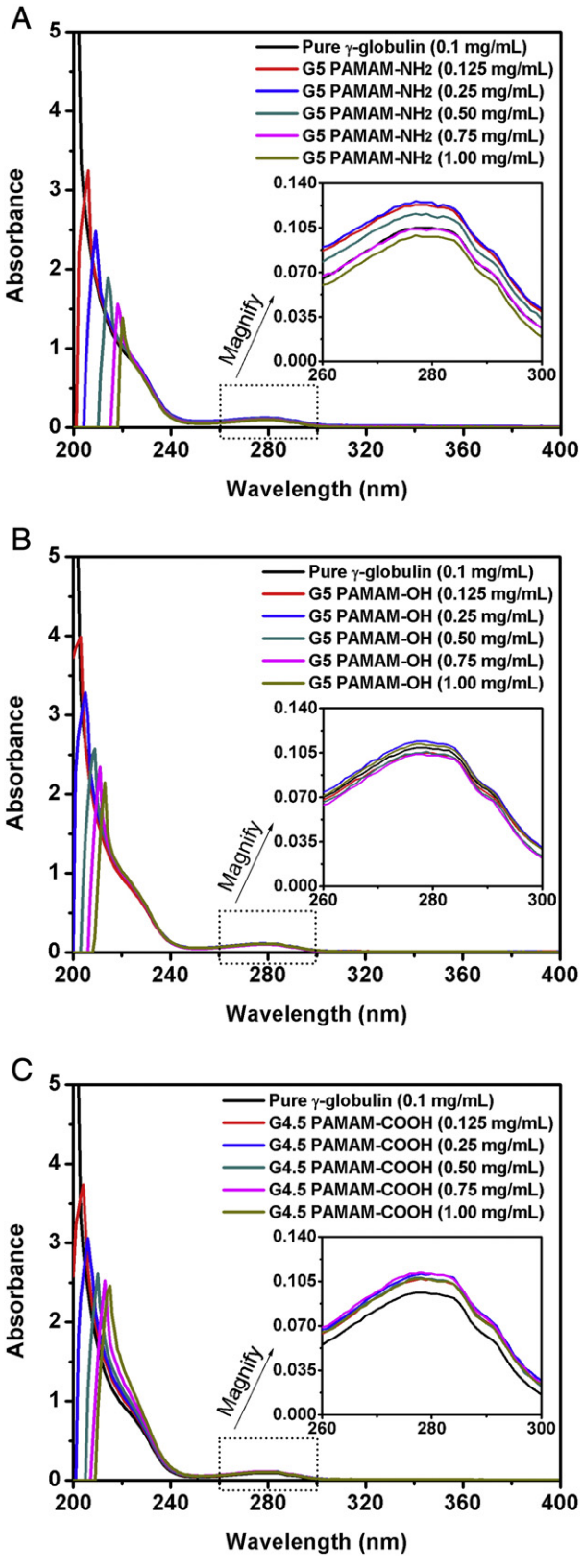

Fig. 2. UV spectra of γ-globulin (0.1 mg/mL) in the presence of the PAMAM dendrimers: A, different concentrations of G5 PAMAM–NH_2; B, different concentrations of G4.5 PAMAM–COOH; C, different concentrations of G5 PAMAM–OH.

all interacted with γ-globulin and affected its secondary structure. Moreover, the interaction was not simply electrostatic force since the PAMAM dendrimers with positive, neutral, or negative charge all caused the spectral change. The interaction could be driven by other forces such as H-bonding and hydrophobic interaction.

Fluorescence spectroscopy can be used to monitor the microenvironmental change surrounding some intrinsically fluorescent amino acids such as tryptophan, phenylalanine and tyrosine. The fluorescence emission of proteins excited at 280 nm is usually dominated by tryptophan residues, which are mainly located in hydrophobic core and are highly sensitive to their local polarity. Each γ-globulin molecule contains 30 tryptophan residues, 20 on the H chain, and 10 on the L chain, respectively [18]. When proteins undergo conformational alteration, tryptophan residues would contact the surrounding polar solvent, usually causing significantly reduced fluorescence emission intensity. Therefore, the fluorescence spectroscopy can be used to detect conformational alteration of γ-globulin molecule. Presented in Fig. 3 are the fluorescence emission spectra of γ-globulin in the presence of the PAMAM dendrimers with amine, hydroxyl or carboxyl groups. The peaks at 335 nm, deriving from the fluorescence emission of tryptophan residues of γ-globulin excited at 280 nm, did not shift with increasing concentration of the PAMAM dendrimers. The intensity of the fluorescence peaks decreased along with increasing G5 PAMAM–NH_2 concentration (Fig. 3A), but increased slightly with increasing G5 PAMAM–OH concentration (Fig. 3B) and increased significantly with increasing G4.5 PAMAM–COOH concentration (Fig. 3C). This indicates that the exposure to the dendrimers altered the microenvironment around the tryptophan residues of γ-globulin. Specifically, G5 PAMAM–NH_2 attenuated but G5 PAMAM–OH and G4.5 PAMAM–COOH enhanced the nonpolarity of the microenvironment around the tryptophan residues. The difference could be due to different interaction forces between the dendrimers and γ-globulin. Positively charged G5 PAMAM–NH_2 should interact with negatively charged γ-globulin mainly through electrostatic attraction, which could be strong enough to expose the tryptophan residues to a polar solvent environment. Neutral G5 PAMAM–OH and negatively charged G4.5 PAMAM–COOH could not interact with γ-globulin through electrostatic attraction but could by hydrophobic interaction, H-bonding, and so on, which could however help expose the tryptophan residues to a non-polar microenvironment.

CD spectroscopy is a powerful analytical tool used to study the interaction of proteins with other molecules and to determine the conformational change of the proteins in solution or adsorbed onto other molecules [19]. Presented in Fig. 4 are the CD spectra of γ-globulin in the presence of the PAMAM dendrimers with amine, hydroxyl or carboxyl groups. The typical CD spectrum of pure, native γ-globulin had a peak at 217 nm, indicating the presence of β-sheet secondary structure [20]. It is known that γ-globulin is mainly composed of β-sheets, and therefore the denaturation degree of γ-globulin can be determined from the ellipticity at 217 nm [21]. It is obvious that the peaks at 217 nm of γ-globulin gradually disappeared along with increasing concentration of the PAMAM dendrimers. This indicates that γ-globulin underwent conformational change (i.e., the reduction of β-sheet) in the presence of the PAMAM dendrimers and that the alteration of the ellipticity at 217 nm depended on the dendrimer concentration. It is well known that β-sheet is maintained by H-bonding, and hence the dendrimer–γ-globulin interaction forces as mentioned above could be stronger than the H-bonding of β-sheet, leading to the damage of β-sheet structure of γ-globulin.

All the spectra results demonstrate that the PAMAM dendrimers could alter the secondary structure and conformation of γ-globulin, regardless of their different surface groups and surface charges. It indicates that all the dendrimers had an interaction with γ-globulin, the driving force of which could be electrostatic attraction/repulsion, hydrophobic interaction, H-bonding, van der Waals force, etc. Moreover, the dendrimer–γ-globulin interaction depended on the properties of the bulk structure and surface groups of the dendrimers.

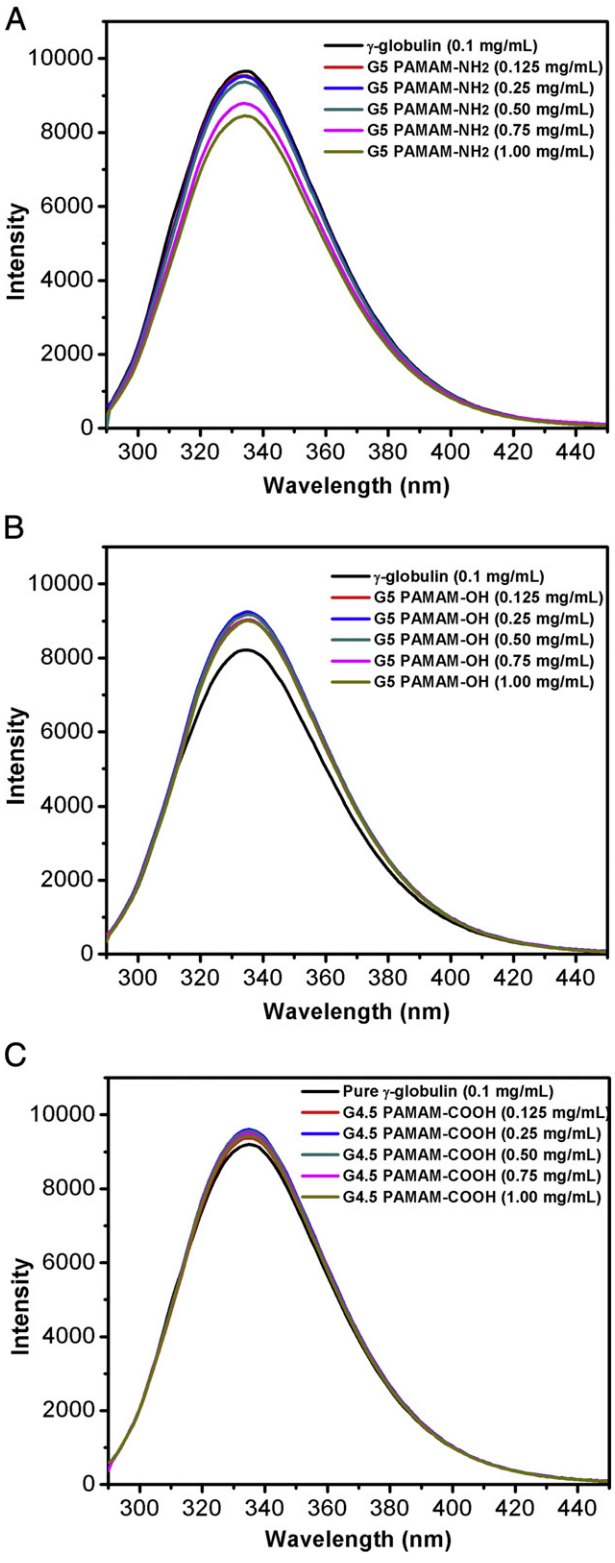

Fig. 3. Fluorescence spectra of γ-globulin (0.1 mg/mL) in the presence of the PAMAM dendrimers: A, different concentrations of G5 PAMAM–NH_2; B, different concentrations of G4.5 PAMAM–COOH; C, different concentrations of G5 PAMAM–OH.

3.2. Effect of PAMAM dendrimers on complement activation

The complement system in humans is an immune molecule family consisting of about 35–40 proteins mainly existing in blood plasma and on cell surface [17]. The complement system helps or "complements"

the ability of the innate and adaptive immune defense systems and serves to recognize and clear invading microorganisms/pathogens/xenobiotics as well as foreign biomaterials coming in contact with blood [22]. For the development of biomedical materials potentially used in vivo, complement activation degree is an important parameter for their biosafety evaluation. Some biomaterials have been found to cause complement activation to different extents [23], and many researchers explored the relationship between the structure of biomaterials and their complement activation degree in order to improve their hemocompatibility and immunocompatibility [24]. Most of these studies focused on biomaterial membrane or nanoparticles which have enough surface area to touch with complement proteins, and by contrast less reports on biopolymers existing in mono-molecular state.

In this work, complement activation was indicated by measuring the production of C3a in fresh plasma in the presence of the PAMAM dendrimers with amine, hydroxyl or carboxyl groups, as shown in Fig. 5. Compared with PBS (negative control), the PAMAM dendrimers from 0.001 to 0.1 mg/mL all inhibited C3a production. For G5 PAMAM–NH_2, G4.5 PAMAM–COOH, and G5 PAMAM–OH, their concentration increase from 0.001 to 0.1 mg/mL did not make a significant difference in C3a production. At 0.001 or 0.01 mg/mL, there was no significant difference in C3a production among the three PAMAM dendrimers. At 0.1 mg/mL, C3a production was significantly lower in the presence of G5 PAMAM–OH than in the presence of G5 PAMAM–NH_2, while the difference between G5 PAMAM–NH_2 and G4.5 PAMAM–COOH and that between G4.5 PAMAM–COOH and G5 PAMAM–OH were not significant in activating C3a production.

The complement system in humans can be activated via three different pathways: classical, lectin, and alternative pathways. In this work, the PAMAM dendrimers could activate the complement system only through the alternative pathway, since the preconditions on which the dendrimers could trigger the complement system by classical and lectin pathways were not available in this study. In the alternative pathway, C3 (one of the complement components) in plasma is constantly cleaved to two fragments C3a and C3b in normal physiological process. In vivo, C3a exists in plasma in a free state within a certain concentration range. Normally, C3b rapidly degrades. However, C3b will bind B factor to form a C3 convertase in the presence of invaders. The formed C3 convertase can in turn cleave C3 to C3a and C3b in a higher degree, which subsequently causes amplified complement activation cascade reactions. According to this mechanism, the reduction of C3a production in the presence of the PAMAM dendrimers could be due to the interaction of these relating proteins/peptides with the dendrimers. The interaction could impair the bio-functions and activities of the proteins/peptides by disturbing their structures and conformations as shown by the above UV–vis, fluorescence, and CD spectroscopies.

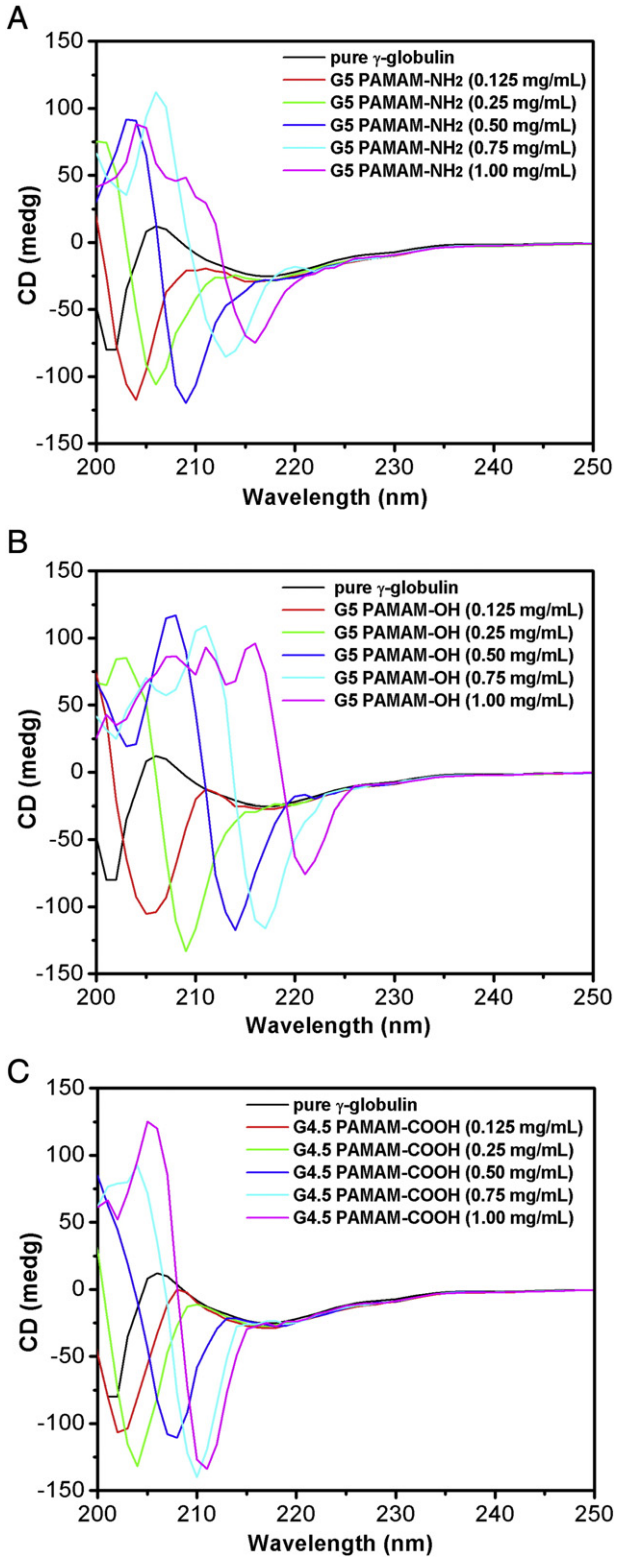

Fig. 4. CD spectra of γ-globulin (0.1 mg/mL) in the presence of the PAMAM dendrimers: A, different concentrations of G5 PAMAM–NH_2; B, different concentrations of G4.5 PAMAM–COOH; C, different concentrations of G5 PAMAM–OH.

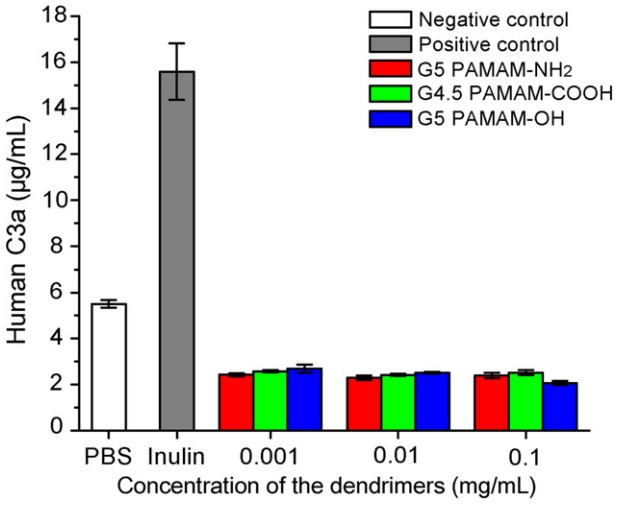

Fig. 5. Complement activation in the presence of the PAMAM dendrimers, PBS (negative control), or inulin (a potent complement activator as positive control).

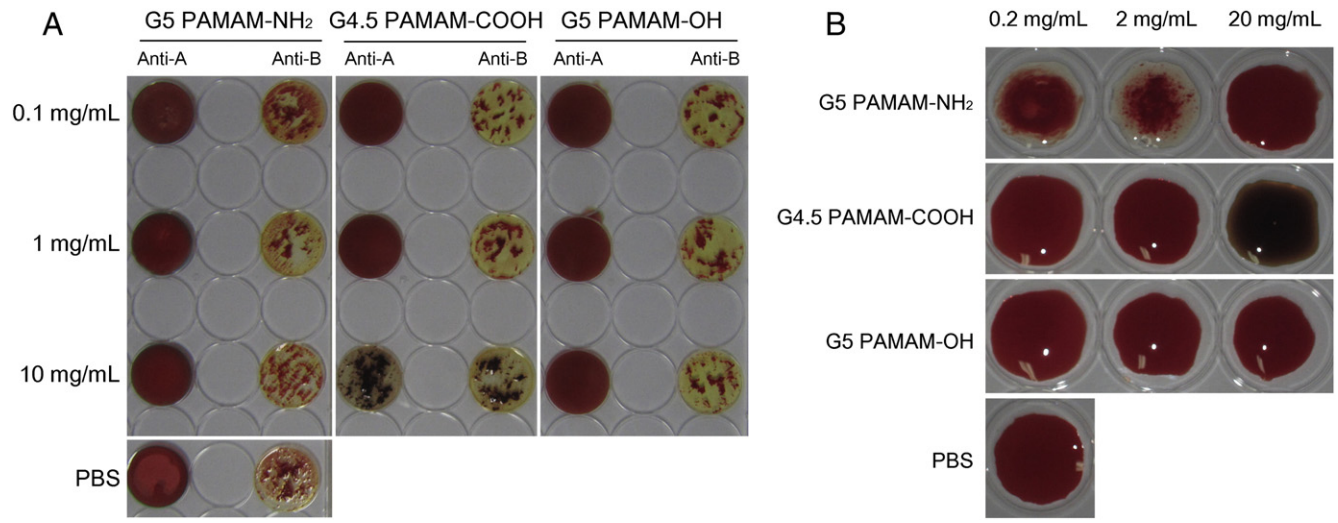

Fig. 6. RBC agglutination in the presence of the PAMAM dendrimers.

3.3. Effect of PAMAM dendrimers on RBC agglutination

Antigen–antibody specific binding reaction is a key step in adaptive immune response. In this work, RBC agglutination in the presence of the PAMAM dendrimers was used to study if the dendrimers could interfere with the RBC antigen–antibody reaction. The agglutination of RBCs was detected by using blood grouping reagents of monoclonal antibodies anti-A and anti-B. The antigen A on RBC surface can bind its specific antibody anti-A, and the antigen B on RBC surface can bind its specific antibody anti-B, both of which result in RBC agglutination. RBC agglutination reaction is used to identify ABO blood group in clinical applications such as transfusion and organ transplantation.

Presented in Fig. 6 are the RBC agglutination phenomena in the presence of different concentrations of the PAMAM dendrimers. From Fig. 6A, 10 mg/mL of G4.5 PAMAM–COOH interfered with the RBC–anti-A reaction. Except it, 0.1 and 1 mg/mL of G4.5 PAMAM–COOH and 0.1–10 mg/mL of G5 PAMAM–NH_2 and –OH had no visible impact on the RBC agglutination, suggesting that the dendrimers did not interfere with the antigen–antibody reactions. It further implies that the dendrimers did not impair the conformation of key domains of the antigens and antibodies, which are responsible for the antigen–antibody specific binding.

To clarify the effect of 10 mg/mL of G4.5 PAMAM–COOH on the RBC agglutination, RBC pellet was added to different concentrations of the dendrimers, as shown in Fig. 6B. G5 PAMAM–NH_2 at 0.2 and 2 mg/mL caused RBC aggregation, but did not cause RBC aggregation at 20 mg/mL, which is in well agreement with a previous report [4]. RBCs in 0.1 or 1 mg/mL of G4.5 PAMAM–COOH or in 0.1–10 mg/mL of G5 PAMAM–OH did not show any visible difference compared with the PBS control. RBCs in 20 mg/mL of G4.5 PAMAM–COOH became dark but did not aggregate, indicating that RBC agglutination in 10 mg/mL of G4.5 PAMAM–COOH (Fig. 6A) was not caused by the dendrimer itself. The possible reason could be due to the interaction of RBC surface, G4.5 PAMAM–COOH and anti-A.

4. Conclusion

In this work, it is shown that the PAMAM dendrimers could affect the secondary structure and conformation of γ-globulin, and inhibit complement activation, regardless of their surface group and charge. G4.5 PAMAM–COOH at 10 mg/mL impaired RBC antigen–antibody reaction. These results indicate that all the dendrimers had an interaction with γ-globulin, the driving force of which could be electrostatic attraction/repulsion, hydrophobic interaction, H-bonding, van der Waals force, etc. Moreover, the dendrimer–γ-globulin interaction depended on the properties of the bulk structure and surface groups of the dendrimers. Generally, positively charged G5 PAMAM–NH_2 could interact with negatively charged γ-globulin mainly through electrostatic attraction. Neutral G5 PAMAM–OH could interact with γ-globulin by hydrophobic interaction, H-bonding, van der Waals force, and so on. Negatively charged G4.5 PAMAM–COOH might generally have electrostatic repulsion with γ-globulin but still could interact with it by hydrophobic interaction, H-bonding, van der Waals force, and so on.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.11.016.

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