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FLUORESCENCE STUDY ON THE INTERACTION OF PAMAM G3.5 DENDRIMER, COPPER IONS, AND DENDRIMER-COPPER COMPLEX WITH HUMAN SERUM ALBUMIN (HSA)

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Dendrimers are new, highly branched polymers. Their specific structure gives them an ability to interact with many different molecules, also with metal ions. The interactions between dendrimer, copper ions and dendrimer-copper complex with human serum albumin were investigated using fluorescence and dialysis methods. The results demonstrate that relatively high number of copper ions which can be bound per single molecule of G3.5 PAMAM dendrimer compare with some well known metal chelators (EDTA, BAL) allow to conclude that this generation can be an efficient chelator for Cu^{2+} ions in water solution and play a role in environment protection to remove these metal from aqueous reservoirs. On the other hand G3.5 dendrimers rather cannot protect the human serum albumin before a destructive influence of copper on the protein. What is more the dendrimer – copper complex generally stronger influence on the structure of human serum albumin compared to the alone copper ions.

INTRODUCTION

Copper ions (Cu^{2+}) are very important for correct functions of living organisms. They are necessary as a cofactor for many important enzymes, like cytochrome c oxidase, tyrosinase, lysil oxydase, dopamine beta hydroxylase, Cu-Zn superoxide dismutase and others (Turnlund, 1999; Uauy *et al.,* 1998). Despite their crucial roles in the proper functioning of enzymes, copper can be also toxic to organisms. Concentrations of copper higher than physiological amounts could be a reason for different disorders. Humans can uptake larger amounts with Cu-contaminated food and water. It may cause development of gastrointestinal symptoms (Knobeloch, Schubert & Hayes 1998; Spitalny *et al.,* 1984). Copper ions are also responsible for development of such pathological states as liver cirrhosis, damage of renal tubules, episodes of hemolysis, brain damage and others. Cu-poisoning can results in coma, vascular collapse, hepatic necrosis and death (Winge & Mehra, 1990). Intoxication by copper ions can be also observed in patients after dialysis via Cu tubing (Klein, Metz Jr. & Price, 1972). A high concentrations of copper could be also responsible for development of Wilson's disease. It should be noted that in patients with Wilson's disease, lipid peroxidation in liver mitochondria and reduced level of antioxidant vitamin E in liver and blood were observed (Myers *et al.,*1993). Copper is also responsible for etiology of some neurodegenerative diseases like Alzheimer (an increase in Cu, Fe and Zn ions level, an increase in amyloid β proteins level in the brain tissue, degeneration of neurons), Parkinson and amyotrophic lateral sclerosis (Cookson & Shaw, 1999; Strausak *et al.,* 2001; Bains & Shaw, 1997; Sayre *et al.,* 2000; Smith *et al.,* 1997). Despite many different ways which explain the toxicity of copper ions, probably the main mechanism is the generation of reactive oxygen species (ROS). Copper can exist in two ionic forms: cupric and cuprous, and both of these types can play a crucial role in ROS production. Presence of superoxide or some natural reducing agents like glutathione (GSH) or vitamin C leading to the reduction of Cu^{2+} (cupric form) to Cu^{+} (cuprous form). Cu^{+} can be used to catalyze Haber – Weiss reaction. This way enables a formation of hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 (hydrogen peroxide) (Bremner, 1998; Kadiiska *et al.,* 1993). It is well known that $^{\cdot}OH$ is one of the most reactive oxygen species in biological systems and possesses an ability to interact with almost all biomolecules (Buettner, 1993).

Dendrimers are nanoscale, hyper – branched and globular polymers. Practically, all types of these molecules have the same plan of architecture. Dendrimers are builds from a central core (e.g.

ammonia, ethylenediamine, single atom) and many branches (builds from repeating fragments) connected with the core. At the end of the branches units called ''dendrons'' are present different terminal groups (Caminade, Laurent & Majoral, 2005). Many biological, chemical and physical properties could enable a potentially use of these polymers, for example as drug carriers (Kukowska-Latallo *et al.,* 2005; Lai *et al.,* 2007), gene carriers (Kukowska-Latallo *et al.,* 1996), contrast agents for magnetic resonance (Bryant Jr. *et al.,* 1999), agents against amyloid fibril formation (Klajnert *et al.,* 2006) and detoxication agents (Shcharbin & Bryszewska, 2006). Terminal groups are able to react with and attach many different biological or chemical molecules (also different metal ions). These specific and very interesting properties could be used to use the dendrimers as chelators for different metal ions. Polyamidoamine (PAMAM) dendrimer generation 3.5 has 64 (-COONa) end groups. Molecular weight of these polymers is approximately 12419 Da and the diameter is approximately 4 nm (Klajnert *et al.,* 2003). Weaker toxicity of the lower generations of dendrimers compared to the higher generations is the main reason why this group of dendrimers is investigate.

This paper describe the investigation of interactions between PAMAM G3.5 dendrimer, copper ions and dendrimer-copper complex with human serum albumin (HSA).

MATERIALS

PAMAM G3.5 dendrimers (in methanol), essentially-fatty-acid-free HSA and benzoylated dialysis tubing were purchased from Sigma-Aldrich (St. Louis, MO, USA). $CuSO_4$ x $5H_2O$ was obtained from POCh (Gliwice, Poland). Other chemical compounds were on analytical grade. Double-distilled water was used to prepare all solutions. Methanol from the PAMAM G3.5 dendrimer solution was evaporated and the thin film of dendrimer was dissolved in 10 mM PBS (phosphate buffer saline). PBS used for the experiments had been filtrated before its application.

METHODS

Equilibrium dialysis against water and PBS (before each experiment the pH of the saline buffer solution was verified) was performed using a MicroEquilibrium Dialyzer (Harvard Apparatus, USA) with 1.2 kDa ultra-thin dialysis membranes. Cu^{2+} ions was added to the first chamber and a mixture of Cu^{2+} and PAMAM 3.5 dendrimer to the second, and the solutions were dialyzed overnight at room temperature. Different concentrations of Cu^{2+} ions were used against a constant concentration of PAMAM 3.5 dendrimer. The concentrations of Cu^{2+} ions in the two chambers were equal before the dialysis. The concentrations after the dialysis were determined in both chambers by atomic absorption spectroscopy using a SpectrAA-300 apparatus (Varian, USA). Fluorescence and anisotropy data were collected using a Perkin-Elmer LS-50B spectrofluorimeter (Perkin-Elmer, UK) in 37^{o}C. The excitation wavelength was set at 280 nm (slit 11.0 nm) and the emission wavelength at 350 nm (slit 11.5 nm) for fluorescence measurements. For anisotropy the excitation wavelength was set at 280 nm (slit 8.0 nm), the emission wavelength at 350 nm (slit 8.5 nm) and value of GF factor was 1.06. The fresh stock solution of human serum albumin (100 μM) was prepared before each experiment in 10 mM PBS, pH=7.4. The final concentration of HSA was 5 μM in PBS. The concentrations of PAMAM G3.5 dendrimer, Cu^{2+} ions and dendrimer-copper complex (molar ratio 1:1) added to the albumin solution ranged from 5 to 100 μM. The Stern-Volmer Eq. (1) was used to analyze fluorescence quenching descending from tryptophan (Trp) residues in albumin.

$$\frac{F_0}{F} = 1 + K_{SV} \cdot [Q] \qquad (1)$$

where: F_0 - the fluorescence intensities in the absence of quencher; F - the fluorescence intensities in the presence of quencher; K_{SV} - the Stern–Volmer dynamic quenching constant; [Q] - the concentration of the quencher.

The equation assumes a linear plot of F_0/F versus [Q] and the slope equals K_{SV}. The Stern–Volmer constants informed about the accessibility of the chromophore to the quencher (Klajnert & Bryszewska, 2002; Suresh Kumar *et al.,* 2006).

All data are expressed as mean ± S.E.M. of at least 5 independent experiments. The Shapiro-Wilk test was used to assessed normal distributions. Statistical significance was assessed using single T-test. Statistics were analyzed using Statistica 8 StatSoft software.

RESULTS AND DISCUSSION

In the first part of the experiments the interaction between dendrimer and copper was investigated using equilibrium dialysis and atomic absorption spectroscopy (AAS). The aim of this investigation was to assess the number of copper ions which can be bound per one molecule of the dendrimer. The experiments were conducted in water and PBS environment. The concentration of PAMAM G3.5 dendrimer was 33 μM. The concentration range of $CuSO_4$ x $5H_2O$ was from 50

to 1500 μM in each chamber of dialyzers. After dialysis the concentrations of copper ions were estimated using AAS. In the chamber containing dendrimer solution the results show Cu_{bound} + Cu_{free} and in the chamber containing only copper salt solution the Cu_{free} was detected.

Scatchard-Klotz plots (Fig. 1, Fig. 2) were used to estimate the number of Cu ions „n", which can be bound by a single molecule of the dendrimer and to estimate the binding constant K_b (Scatchard, 1949). If the Scatchard-Klotz plot is a straight linear curve and is a mathematical function "y = ax + b" then: b = 1/n; a = 1/($K_b \cdot$ n)

The number of copper ions that can be bound by a single dendrimer molecule in water solution is n ≈ 17 ± 2 and the $K_b = 1.60 \pm 0.58 \times 10^5$. In the phosphate buffer saline solution (PBS), the number of copper ions bound with dendrimer is n ≈ 26 ± 9 and the $K_b = 5.81 \pm 2.52 \times 10^3$.

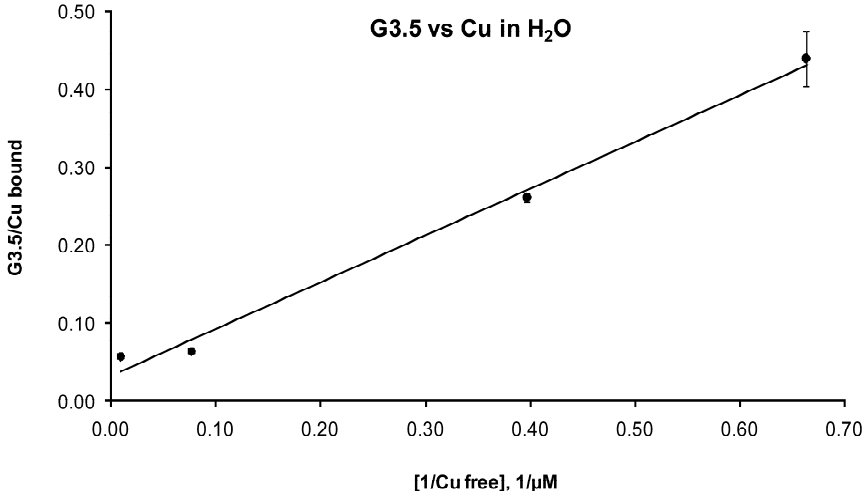

Fig. 1. The dependence of the concentration of bound Cu ions on the concentration of free Cu^{2+} ions in a solution after equilibrium dialysis (Scatchard-Klotz plot). The concentration of PAMAM G3.5 dendrimer is 33 μM, water, 25°C.

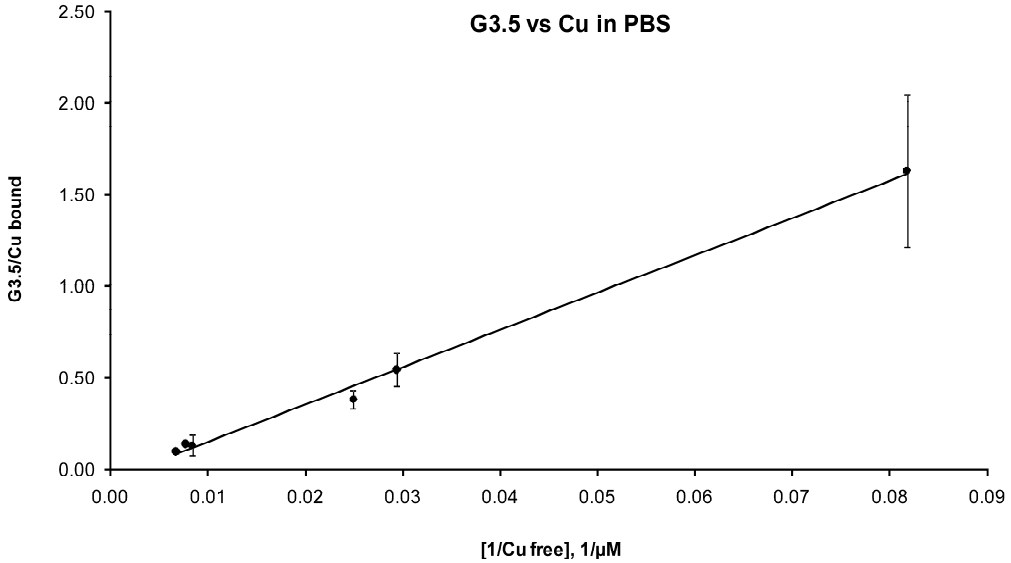

Fig. 2. The dependence of the concentration of bound Cu ions on the concentration of free Cu^{2+} ions in a solution after equilibrium dialysis (Scatchard-Klotz plot). The concentration of PAMAM G3.5 dendrimer is 33 μM, 10 mM PBS, pH = 7.4, 25°C.

The above results show that in the PBS environment, dendrimer G3.5 possesses a bit stronger ability to interact and bind copper ions. Both in water and PBS, the number of bound Cu^{2+} ions is lower compared to a

theoretical number of the binding centers (n = 182) estimated using Tomalia-Mansfield-Rakesh equation (Tomalia *et al.*, 2002). The average number of copper ions that can be bound per single molecule of G3.5 PAMAM dendrimer in these two environments is n ≈ 22. The main reason for these differences is the fact that mathematical calculations do not take into consideration some chemical and physicochemical factors like: a chemical structure of a dendrimer, ions forces and the reaction environment. In the structure of the dendrimer 64 terminated groups are observed. The copper ions have a charge 2+, thus one ion of Cu^{2+} can be potentially bound by two terminated groups, therefore single molecule of dendrimer should bind 32 copper ions. Some ions can be also bound by interior, tertiary amine dendrimer groups which are neutral in high pH. The next reason is that G3.5 dendrimer does not have carboxyl (-COOH) but sodium carboxyl (-COONa) end groups. The presence of sodium ions blocks carboxyl groups and impedes reactions between copper ions and dendrimers.

It is unclear why in PBS G3.5 dendrimers binds a bit better copper ions compare to water. It could be feasible that in PBS some amounts of copper ions create unsolvable copper salt with phosphate compounds of PBS. This could violate the equilibrium state during all process of dialysis and leads to occur some deviations.

On the other hand it is also possible that some molecules of dendrimers create aggregates built-up from several dendrimer particles. Then some terminal groups can be blocked and lose the ability to bind copper ions.

Interactions between dendrimer, copper ions and dendrimer-copper complex with human serum albumin were studied using fluorescence methods. The fluorescence signal comes from tryptophan (Trp 214) amino acids residues. Human protein have only one Trp, thus the signal is lower compared to bovine serum albumin (BSA). A decrease of fluorescence intensity gives information about the interaction with HSA molecules and conformational changes of protein. The results shows that dendrimer G3.5 causes the least fluorescence quenching among all ligands. The highest fluorescence quenching is observed for PAMAM G3.5-Cu^{2+} complex. Figure 3 shows that the highest Stern-Volmer constant (15.9 ± 0.3 mM^{-1}) is observed for the dendrimer-copper complex.

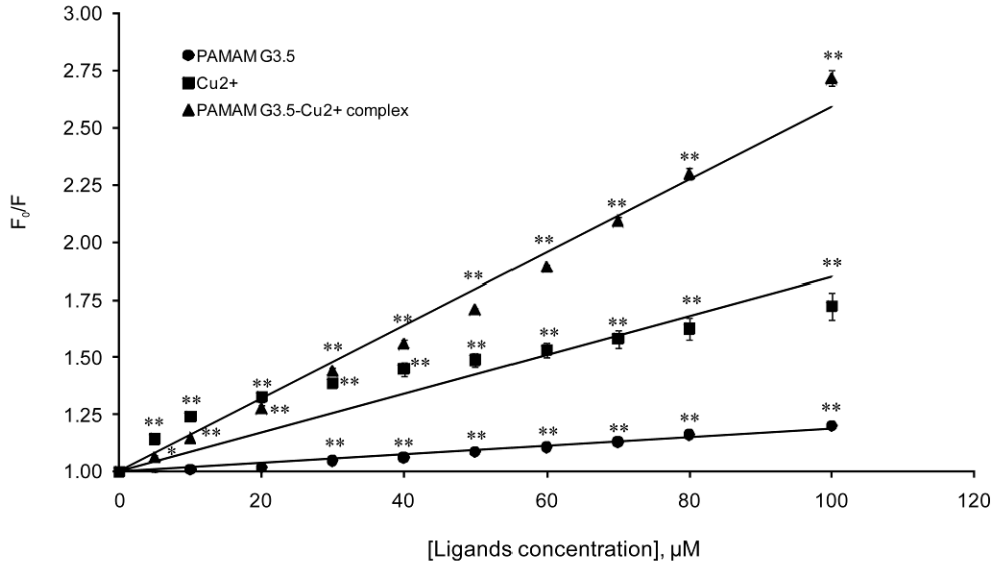

Fig. 3. Stern-Volmer plots of HSA fluorescence quenching by G3.5 PAMAM dendrimer, Cu^{2+} and G3.5-Cu^{2+} complex. [HSA] = 5 μM. $\lambda_{ex.}$=280 nm, $\lambda_{em.}$=350 nm, 10 mM PBS, pH = 7.4. 37ºC. (*p<0.001, **p<0.0001).

The Stern-Volmer constants for the dendrimer and copper are 1.9 ± 0.1 mM^{-1} and 8.5 ± 0.6 mM^{-1} respectively. According to Cheger (Cheger, 1996) and Peters (Peters Jr., 1996) human serum albumin has three domains. The first two domains possess negative charge (-9 domain I and -8 domain II). Only domain III has a positively charge +2, thus positive copper ions can interact with the first two domains and the anionic G3.5 PAMAM dendrimer can interact with domain III of HSA. This is the main reason why copper ions affect more powerfully the changes of the structure of protein compared to the dendrimer. Besides, the copper ions are

very small and can penetrate the inner structure of albumin leading to changes in microenvironment of Trp residues. The result of these changes can be a decrease in fluorescence. The dendrimer – copper complex generally decreases the fluorescence to the highest degree. Probably, a very high concentration of sodium ions in the environment of PBS solution disorganizes electrostatic interactions between the dendrimer and copper ions. It could be possible that the dendrimer – copper complex disintegrates at a high concentration of sodium ions. Fluorescence quenching occur by two mechanisms: dynamic and static quenching.

The mode of fluorescence quenching can be determined using the Eq. (2) (Lakowicz & Weber, 1973):

$$k_q = K_{SV} / \tau_0 \quad (2)$$

where: k_q is the rate constant of quenching; τ_0 is the life time of biomolecule without fluorescence quencher (for biomolecules the typical value is 10^{-8} s); K_{SV} is the Stern-Volmer constant.

The calculated values of k_q for G3.5 PAMAM dendrimer, copper and dendrimer-copper complex are: 1.9 x 10^{11} L/mol^{-1} s^{-1}, 8.5 x 10^{11} L/mol^{-1} s^{-1}, 1.59 x 10^{12} L/mol^{-1} s^{-1}, respectively. Because k_q values are greater than maximum scatter collision quenching constant (2 x 10^{10} L/mol^{-1} s^{-1}) (Lakowicz & Weber, 1973; Yu, 2008), the quenching mode is rather static than dynamic.

Despite the most changes of fluorescence intensity caused by the dendrimer – copper complex, the highest change in fluorescence anisotropy is caused by copper ions (Fig.4).

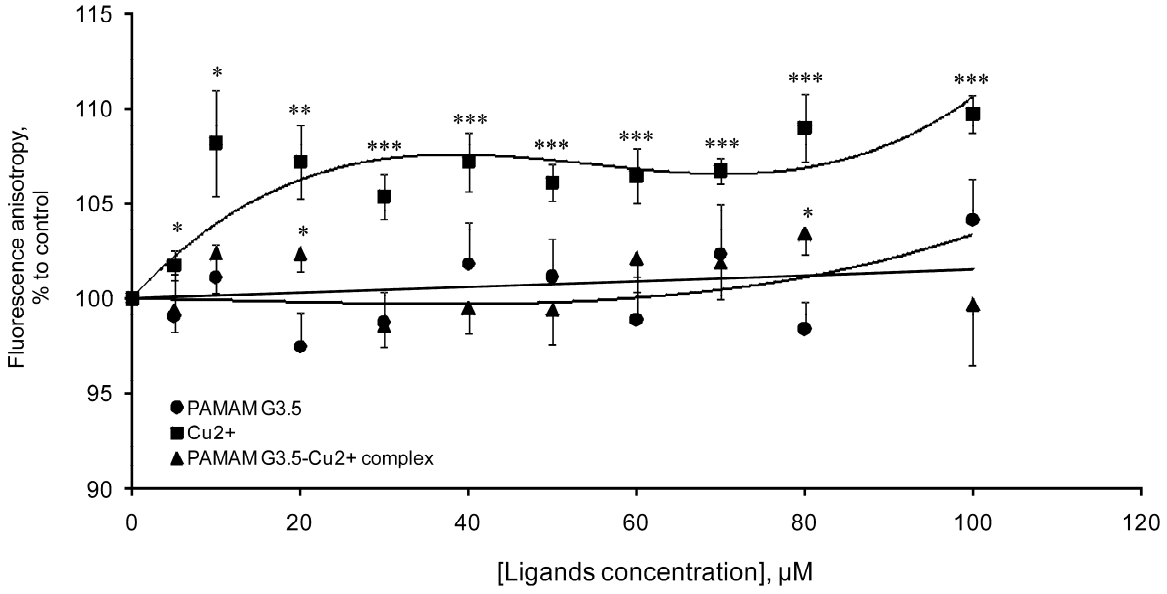

Fig. 4. The dependence of HSA fluorescence anisotropy on the concentration of PAMAM G3.5 dendrimer, Cu^{2+} and G3.5-Cu^{2+} complex. [HSA] = 5 μM. $\lambda_{ex.}$=280 nm, $\lambda_{em.}$=350 nm, 10 mM PBS, pH = 7.4, 37ºC. (*p<0.05; **p<0.005; ***p<0.001).

Binding the copper ions causes a decrease in protein dynamics, which is the reason for an increase in fluorescence anisotropy. PAMAM G3.5 dendrimer practically did not change the anisotropy of HSA. Because the dendrimer – copper complex has similar anisotropy values compared to the dendrimer, probably PAMAM G3.5 protects albumin from a negative influence of Cu^{2+} ions. It is necessary to emphasize that the dendrimer protects only protein dynamics but not protein structure.

CONCLUSIONS

The above results show that PAMAM G3.5 dendrimer possesses an ability to interact with copper ions. Comparison of the water and PBS environments show that in PBS PAMAM G3.5 dendrimer can bind a bit more copper ions compare to water. Obtained results have very important biological consequences. The studies shows that the G3.5 PAMAM dendrimers could be a chelators for copper ions in water environments. What is more single molecule of G3.5

can bind higher number of copper ions compare to other metal chelators like EDTA or BAL. These results clearly show that G3.5 PAMAM dendrimer can play an important role in develop some new technology of environmental protection.

On the other hand a very strong interaction between HSA and the dendrimer – copper complex (very high Stern-Volmer constant $K_{sv} = 15.9 \pm 0.3$ mM^{-1}) compared to other ligands show that using this generation of PAMAM dendrimers as a chelators in biological organisms (for example to remove copper ions from blood) is probably not possible. It should be note that in lower concentrations of dendrimer-copper complex (5μM and 10 μM) there is some protect effect in relation to human serum albumin. Probably in these concentrations the protein is isolated before negative influence from copper ions. Unfortunately it could be not enough reason to use these generation of dendrimers as chelators in living organisms. Also protection of the dynamic structure of the protein do not classify this polymer as a chelator. What is more it is very important to evaluate how this generation of dendrimers could influence on copper toxicity in the living cells. If dendrimers have to be applied as drug or gene carriers it is very important to estimate how they could influence on the toxicity level of copper ions in cells. Here many other research are still needed.

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