Interaction between polyamidoamine (PAMAM) dendrimers and bovine insulin

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Abstract

OBJECTIVE: In this study the mechanism of interactions between polyamidoamine (PAMAM) dendrimers and bovine insulin was examined. The insulin is a 51 amino acid peptide-hormone involved in the homeostasis of blood glucose levels. This molecule consists of two chains – A and B – linked by two disulphide bridges. As insulin contains four tyrosine residues it was possible to evaluate dendrimers effect on protein conformation by measuring changes in the fluorescence spectra of insulin after addition of dendrimers or classical quenchers.
METHODS: PAMAM dendrimers are based on ethylenediamine core and branched units which are built from methyl acrylate and ethylenediamine. PAMAM dendrimers with different surface groups (–COOH, $–NH_2$, –OH) were used. Their size is comparable to insulin.
RESULTS: The experiments show that interactions exist between PAMAM dendrimers and insulin. It was found that these interactions depend on a kind of dendrimer surface groups.
CONCLUSIONS: It is very likely that interactions are of electrostatic nature and cause insulin conformational changes.

INTRODUCTION

The bovine insulin is a 51 amino acid peptide-hormone involved in the homeostasis of blood glucose levels. This molecule consists of two chains – A and B – linked by two disulphide bridges. The A-chain it is composed of 21 amino acid residues and the B-chain contains 30 ones. The secondary structure of insulin is reported to be 58% α-helical with a 6% β-sheet region (Bekard & Dunstan 2009, Whittingham *et al.* 2002). Insulin has the ability to undergo conformational changes resulting in formation of fibrillar agregates that can lead to injection-localized amyloidosis in diabetic patients. Numerous diseases are associated with fibril deposits, among them diabetes, Alzheimer disease, prion diseases, Parkinson's and Huntington's diseases. Therefore there exists a serious demand for new therapeutic agents preventing formation of fibrils. Dendrimers, a relatively new class of polymeric materials, are among the most promising as it was found that they were able to destroy already exist-

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ing Aβ fibrils and protect peptides from forming the new ones (Klajnert *et al.* 2007). Also, the fibrillation of α-synuclein, the protein crucial in Parkinson's disease, was inhibited by dendrimers (Milowska *et al.* 2012). In order to study whether dendrimers are also able to block the formation of insulin fibrils and aggregates, it is first necessary to scrutinize their interactions with insulin.

Dendrimers are a specific group of nanoparticles characterized by their tree-like morphology and a globular, three-dimensional nanostructure. The first and the most extensively studied family of dendrimers are PAMAM (polyamidoamine) dendrimers, which are based on an ethylenodiamine core and branched units built from methyl acrylate and ethylenediamine (Klajnert *et al.* 2003, Svenson & Tomalia 2005).

Dendrimers have shown excellent promise in biomedical applications due to their unique physical and chemical properties. They possess a number of reactive end groups on the surface and empty internal cavities. These features make them effective carriers of drugs or other guest molecules to penetrate through the membrane (Dong *et al.* 2010; Klajnert & Bryszewska 2007). Drug molecules can be attached to the terminal groups on the surface or encapsulated in the dendrimer's interior. Both strategies are absolutely promising in targeted antitumor therapy (Klajnert *et al.* 2007). It is believed that cationic dendrimers can be used as non-viral vehicles for gene delivery. Amino groups on the surface of PAMAM dendrimers interact with phosphate groups of nucleic acids. In this way transfection complexes are created (Klajnert & Bryszewska 2001, Ciolkowski *et al.* 2011). Some kinds of dendrimers have been found to be useful as antiviral drugs against for example: human immunodeficiency virus (HIV) or herpes simplex virus (Svenson & Tomalia 2005).

In this work the interactions of bovine insulin with polyamidoamine dendrimers were studied using intrinsic insulin fluorescence quenching. Proteins intrinsic fluorescence comes from phenyloalanine, tyrosine and tryptophan residues. The bovine insulin possesses four tyrosine and two phenyloalanine residues. Tyrosine fluorescence has a much higher quantum yield than fluorescence of phenylalanine. Moreover, fluorescence of one tyrosine residue is blocked by the disulfide bridge in insulin molecule. Thus, it is believed that the recorded fluorescence of insulin comes from three tyrosine residues. We were able to evaluate dendrimers effect on protein conformation by measuring changes in the fluorescence spectra of insulin after addition of dendrimers. For that, the wavelength at the maximum of fluorescence (λ_{max}) and the fluorescence intensity in the maximum were taken into account. We used dendrimers of two generations G3 and G4 with different surface groups (–COOH, $–NH_2$, –OH) which size is similar to insulin. Also the quenching of insulin emission by the classical quenchers: acrylamide, caesium chloride and potassium iodide in the presence or absence of dendrimers was studied.

MATERIALS AND METHODS

Materials

PAMAM dendrimers with different surface groups were used. Their characteristics are given in Table 1. All types of dendrimers, the bovine insulin, acrylamide, ceasium chloride, potassium iodide were obtained from Sigma-Aldrich. All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

Fluorescence spectra of tyrosine residues in bovine insulin

Bovine insulin was dissolved in NaOH (20mM) and diluted in phosphate buffered saline (PBS: 67 mmol/l NaCl, 16.7 mmol/l NaH_2PO_4, 16.7 mmol/l Na_2HPO_4, pH 7) to a concentration of 95 μmol/l. Data were collected for bovine insulin after adding different concentration of dendrimers (0.5–11 μmol/l). Fluorescence spectra were taken with a Perkin-Elmer LS55 spectrofluorometer. Samples were measured at room temperature, in 1-cm path length quartz cuvettes and were continuously stirred. The excitation wavelength of 274 nm was used and the emission spectra were recorded from 280 to 350nm. Next, increasing concentrations of dendrimers were added and fluorescence spectra were recorded.

Fluorescence quenching measurements

Fluorescence quenching studies were carried out with a neutral quencher acrylamide and two ionic quenchers: caesium chloride (CsCl) and potassium iodide (KI). Increasing aliquots of the quencher were added from a stock solutions to 95 μmol/l sample of bovine insulin. The stock for CsCl and KI was 3 mol/l and for acrylamide it was 4 mol/l. A stock solution of KI contained 0.1 mmol/l $Na_2S_2O_3$ to prevent oxidation of I^- to I_3^-. Data were collected for each quencher after adding (0.01 μmol/l) concentration of dendrimers to bovine insulin. The excitation wavelength of 274 nm was used and the emission spectra were recorded from 280 to 350 nm.

RESULTS AND DISCUSSION

Fluorescence spectra of insulin tyrosine residues in presence of dendrimers

Fluorescence spectroscopy is a sensitive method that has been exploited in studying the structural, physicochemical and functional properties of proteins. It is a technique sensing changes in the local environment of the fluorophore. Intrinsic protein fluorescence originates from the aromatic amino acids: tryptophan, tyrosine, and phenylalanine. As insulin contains four tyrosine residues we were able to evaluate dendrimers effect on protein conformation by measuring changes in the fluorescence spectra of insulin after addition of dendrimers (Bekard & Dunstan 2009, Lakowicz 2006).

The quenching of the fluorescence is a powerful and widely used method to study molecular interactions

involving proteins because is highly sensitive, rapid and simple.

The basic information contained in fluorescence measurements relates to the molecular microenvironment of a chromophore (Klajnert *et al.* 2003). Fluorescence quenching seems to be quite easy to interpret when protein contains only one fluorophore. The analysis is more complicated and difficult for molecules with several fluorophores such as bovine insulin. In our study the mechanism of interactions between polyamidoamine (PAMAM) dendrimers and bovine insulin was examined. There were three reasons for choosing this protein: (a) it plays an important physiological role; (b) it is a protein with well-known structure; (c) it possesses four tyrosine residues, which is important for fluorescent measurements.

Bovine insulin fluorescence quenching was carried out for all above-mentioned PAMAM dendrimers. The emission spectra of insulin tyrosine residues in absence and presence of PAMAM dendrimers are given in Figure 1. For clarity only results for PAMAM G4 are presented in this figure. The wavelength of the emission maximum for insulin did not change when increasing amounts of dendrimers were added. This means that the tyrosine residues did not become exposed either to more or less polar environment. However, the fluorescence intensity decreased with the successive addition of each dendrimer. These results indicate that PAMAM dendrimers quench the fluorescence of bovine insulin. The effect was the strongest for G3 and G4 PAMAM dendrimers possessing positively charged amino groups on the surface and the weakest for PAMAM dendrimers G2.5 and G3.5, negatively charged.

All dendrimers decreased the fluorescence intensity of insulin so significantly that their behaviour could be compared to the quenchers and the data analysed by Stern-Volmer equation:

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

where F is the fluorescence intensity in the presence of a quencher, F_0 is its value without a quencher, K_{sv} is so called Stern-Volmer quenching constant and [Q] is the quencher concentration (Klajnert *et al.* 2003). As an example the Stern-Volmer plot for G4 dendrimer is shown in Figure 2 and the Stern-Volmer constants for all dendrimers are given in Table 2. A downward curvature of the Stern-Volmer plots for all dendrimers was observed, therefore two lines for each PAMAM dendrimers were drawn. Also, two K_{sv} were calculated. K_{sv1} it is the constant for first three points on the plot and K_{sv2} applies to other points.

The downward curvature of the Stern–Volmer plots is observed when a system contains either more than one fluorophore or a fluorophore in different environments. Data in the Table 2 show that tyrosine residues in insulin can be divided into two fractions: fluorophores accessible to the quencher and inaccessible to the quencher. Non-linear Stern-Volmer plots can be analyzed via the following double reciprocal plot, introduced by Lehrer (1971):

$$\frac{F_0}{F_0 - F} = \left(\frac{\Sigma f_i \cdot K_{sv,i} \cdot [Q]}{1 + K_{sv,i} \cdot [Q]} \right)^{-1} \quad (2)$$

where $K_{sv,i}$ is the Stern-Volmer constant for the *i*-th fluorescent species accessible to the quencher and f_i is the fraction of the total fluorescence that is accessible to

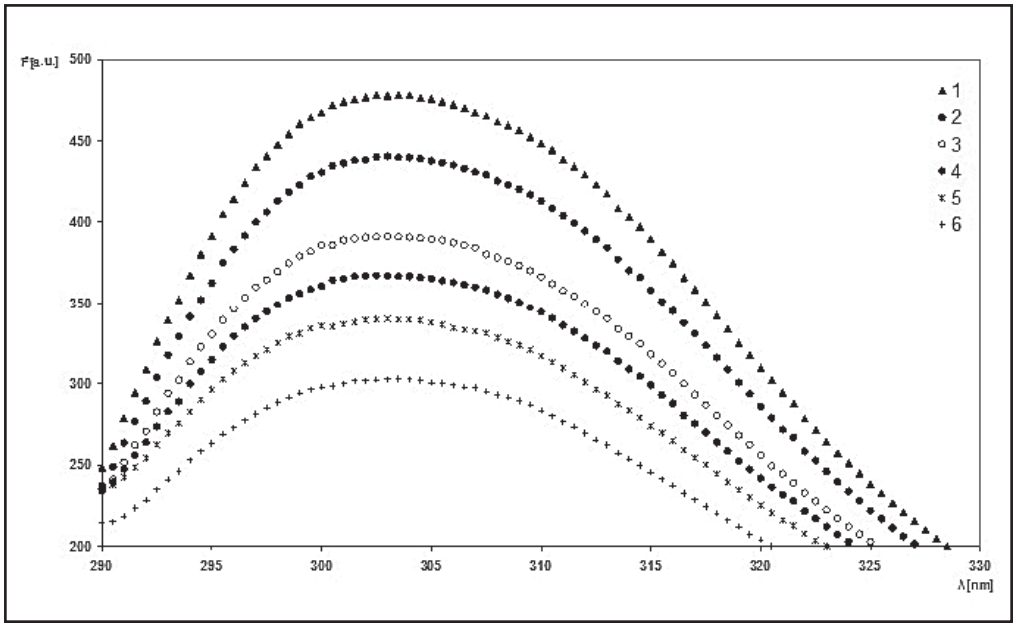

Fig. 1. Bovine insulin fluorescence quenching by G4 PAMAM dendrimer, λ_{exc}=274 nm. 1→ insulin 95μmol/l; 2–6→ after addition of dendrimer at a concentration of (2) 0.1 μmol/l; (3) 0.5 μmol/l; (4) 1 μmol/l; (5) 3 μmol/l; (6) 11 μmol/l.

the quencher (Eftink 1991). If there are only two fractions of fluorescent residues and if one is inaccessible to the quencher (i.e. $K_{sv,2} = 0$), then Eq. (2) becomes:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_1} + \frac{1}{f_1 \cdot K_{sv,i}} \cdot \frac{1}{[Q]} \quad (3)$$

This is a straight-line function. From the intercept of a plot $F_0/(F_0–F)$ vs $1/[Q]$ with Y axis, one can obtain f_1, the fraction of the fluorescence that is accessible to the quencher.

The modified Stern-Volmer plots for all dendrimers are shown in Figure 3 and f_i values are given in Table 3.

F_1 values are the highest for PAMAM G3, G4 and OH-G4 dendrimers indicating that almost 40% of tyrosyl fluorescence is accessible to these quenchers.

It has been demonstrated that all types of PAMAM dendrimers can quench the fluorescence that comes from bovine insulin. The strongest quenchers were dendrimers with amine group: PAMAM G3 and G4. Similar experiments were carried out by scientists studying the interactions between dendrimers and albumin (BSA). The strongest quenching of BSA fluorescence was observed for PAMAM G4 dendrimers (Klajnert *et al.* 2003). It was suggested that fluorescence quenching by these molecules occurs primarily through physical contact between the quencher and the fluorophore (Klajnert *et al.* 2003; Narahari & Swamy 2009).

Quenching data for all dendrimers used in this study were analysed by Stern-Volmer equation. A linearity of Stern-Volmer plot is indicative of a single class of fluorophores, with the same accessiblility to the quencher (Lakowicz 2006). It is believed that if in the solution two classes of fluorophores are present, and one population is not accessible to quencher, the plots are downward curving (Eftink 1991). The Stern-Volmer plots for the quenching of bovine insulin with PAMAM dendrimers (quenchers) deviated from linearity toward the x-axis. The most hyperbolic plots were observed for PAMAM G3 and G4, amino-terminated dendrimers.

Fluorescence quenching measurements

Insulin fluorescence quenching studies, both in the absence and in the presence of dendrimers, have been carried out using three different quenchers: acrylamide, caesium chloride and potassium iodide. Quenching data for all the quenchers used in this study were analysed by Stern-Volmer equation (Eq. (1)). The exemplary plots for PAMAM G3 dendrimer are shown in Figure 4 and the K_{sv} constants for all quenchers and dendrimers are presented in Table 4.

The strongest quenching of bovine insulin fluorescence was observed for acrylamide. Addition of the charged quenchers KI and CsCl led to a much smaller degree of quenching. The upward-curving behaviour of

Tab. 1. Charges, the number of surface groups and end groups of dendrimers.

Dendrimer	Nr. of surface groups	Surface groups	Charge
PAMAM G2.5	32	–COOH	–
PAMAM G3.5	64		
PAMAM G3	32	–NH;	+
PAMAM G4	64		
PAMAM-0H G3	32	–OH	0
PAMAM-OH G4	64		

Tab. 2. Stern Volmer constants for quenching of tyrosine fluorescence of bovine insulin by dendrimers K_{sv}=[l/mmol].

Dendrimer	$K_{sv\ 1}$	$K_{sv\ 2}$
PAMAM G2.5	33.49	12.54
PAMAM G3.5	35.37	9.52
PAMAM G3	194.29	25.99
PAMAM G4	239.95	14.29
PAMAM-0H G3	30.24	12.06
PAMAM-OH G4	46.05	30.97

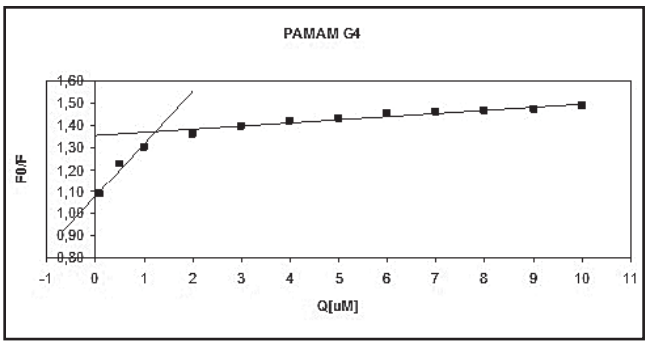

Fig. 2 Stern-Volmer plot for insulin fluorescence quenching by G4 PAMAM dendrimer.

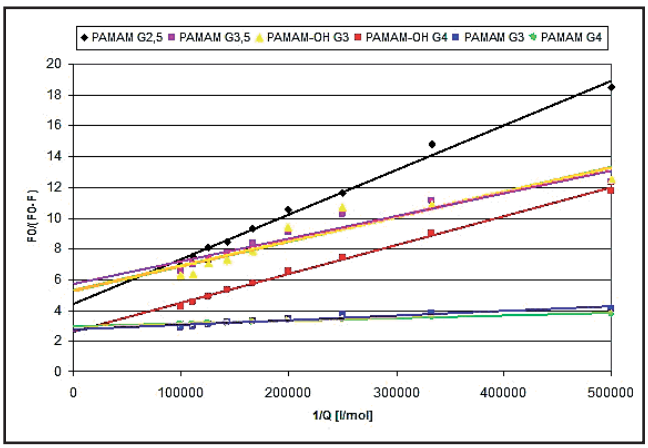

Fig. 3. Modified Stern-Volmer plots.

Stern-Volmer plots for quenching by acrylamide was observed. It indicates the contribution of static quenching for acrylamide. For two other quenchers, plots were linear which suggests the dynamic mechanism of quenching. PAMAM dendrimers did not affect the values of K_{sv} constants in either case.

When small molecules bind independently to a set of sites on macromolecules, the equilibrium between free and bound molecules is given by the equation:

$$\log\left(\frac{F_0}{F_0 - F}\right) = \log K_a + n \cdot \log[Q] \quad (4)$$

where K_a and n are the apparent binding constant and the number of binding sites, respectively (Zhang *et al.* 2008).

The number of quencher molecules (n) bound to bovine insulin and binding constants (K_a) were calculated from equation (Eq. (4)). Linear plots of log $[(F_0–F)/F]$ as a function of log [Q] are shown in Figure 5. Values of n and K_a parameters for all quenchers: acrylamide, KI and CsCl are presented in Table 5. The binding constants for acrylamide and KI were close to one that means the highest affinity between the quencher and insulin.

CONCLUSION

Studies on insulin fluorescence quenching by the classical quenchers in the presence of dendrimers show that dendrimers are not able to block the penetration of these quenchers to the interior of insulin molecule, as Stern-Volmer plots were exactly the same regardless the polymers were present or not in the system. Although the dendrimers themselves quench the insulin fluorescence, the mechanism of their action is different as compared to the typical small molecular quenchers. Dendrimers used in this study have the dimensions comparable to the insulin molecule, therefore they cannot penetrate inside insulin. Their quenching action may be due to the formation of non-fluorescent complexes with tyrosine residues via structural (conformational) modification of the hormone molecule.

Tab. 3. Values of "f" for all dendrimers.

Dendrimer	f [%]
PAMAM G2.5	23
PAMAM G3.5	18
PAMAM G3	36
PAMAM G4	38
PAMAM-0H G3	19
PAMAM-OH G4	38

Tab. 4. Stern-Volmer constants for the quenching of fluorescence of bovine insulin after the addition of a quencher and a dendrimer (0.01 mmol/l). K_{sv}=[l/mol].

	CsCl	KI	Acrylamide
insulin	1.62	3.76	24.71
PAMAM G2.5	1.06	3.53	23.86
PAMAM G3.5	1.22	3.60	23.41
PAMAM G3	1.33	3.42	24.36
PAMAM G4	1.28	3.86	22.57
PAMAM-0H G3	1.40	3.50	24.08
PAMAM-OH G4	1.20	3.73	23.52

Tab. 5. Binding constants and the number of quencher molecules bound to bovine insulin molecule.

Quenchers	Ka [L/mol]	n
Acrylamide	$3.42* 10^2$	1.0517
KI	$29.21 *10^2$	0.9637
CsCl	$109.92*10^2$	0.7540

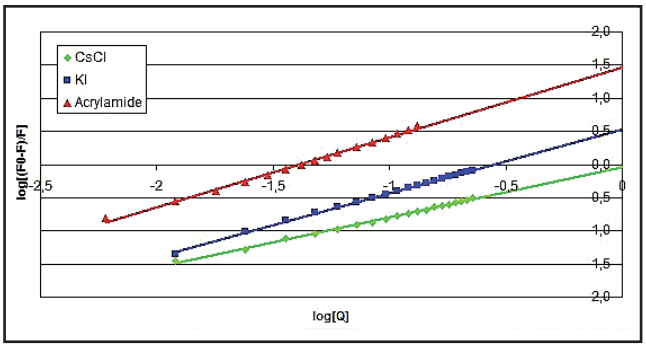

Fig. 4. Stern-Volmer plots for fluorescence intensity of tyrosine residues in bovine insulin, both in the absence (–) and in the presence (+) of PAMAM G3 0.01 μM dendrimer.

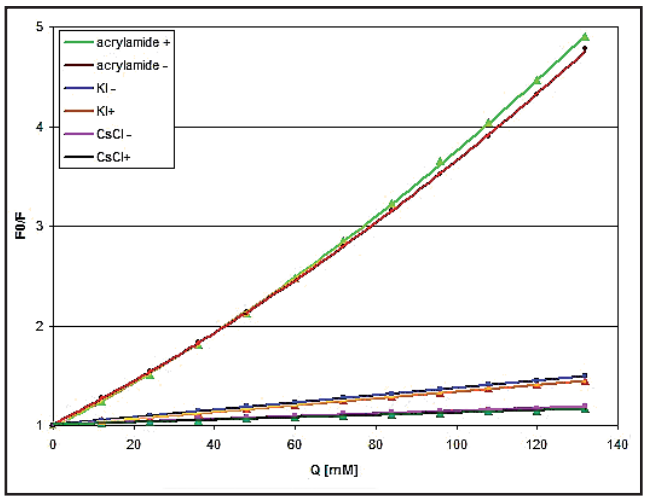

Fig. 5. Plots of log $[(F_0–F)/F]$ versus log [Q].

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