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Interaction of PAMAM dendrimers with bovine insulin depends on nanoparticle end-groups

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ABSTRACT

We have looked at the interactions between polyamidoamine (PAMAM) dendrimers with different terminal groups (–COOH, $–NH_2$, –OH) and bovine insulin. The influence of PAMAM dendrimers on insulin was tested by measuring zeta potential and fluorescence quenching. The secondary structure of insulin in the presence of dendrimers was examined by circular dichroism. The effect of dendrimers on dithiotreitol-induced aggregation of insulin was investigated by spectrophotometry. Dendrimers quenched the fluorescence of insulin, but did not change its secondary structure. Thus dendrimers neither induce hormone aggregation nor inhibit the aggregation process induced by dithiotreitol (DTT), except at 0.01 μmol/l. Dendrimers–insulin interactions are mainly electrostatic.

1. Introduction

Insulin is composed of 2 (A and B) chains containing 51 amino acids, which are connected by 2 disulfide bridges linking and stabilizing the molecule, with the A chain also having one intra-chain disulfide bridge. In solution, insulin mainly consists of the α-helix structure [1–5], and at pH 7 it is negatively charged.

It is also a protein that can form insoluble fibril protein aggregates [6,7]. Upon heating or reduction with dithiol compounds, it loses its function and has the ability to form amyloid-like structures. Dithiotreitol (DTT) is a reducing agent that denatures insulin, which then forms amorphous aggregates and exposes hydrophobic sites on its surface. Insulin interacting with DTT releases the A chain, whereas the B chain aggregates and precipitates [1,2,8]. Aggregation and fibrillation of insulin occurs in insulin-dependent diabetic patients after repeated administration [9], due to these processes being very easily triggered by the conditions of drug administration.

Dendrimers are polymers that have several properties and applications. These well-defined polymers have a characteristic spherical shape and nanometer dimensions. These synthetic molecules can be used as potential drug carriers or delivery systems in targeted medicine [10,11]. They can destroy insoluble protein aggregates or fibrils existing in pathogenesis of many neurodegenerative diseases, as in their influence on Alzheimer's disease deposits of Aβ fibrils and tau protein, fibrillation of α-synuclein in Parkinson's disease, and prion proteins in Creutzfeldt–Jakob disease [12–15].

We have investigated the interaction between bovine insulin molecules and 3 polyamidoamine (PAMAM) dendrimers, which all have an ethylenediamine core, but differ in surface groups (–COOH, $–NH_2$, –OH) and charge at the surface (Table 1). Our main aim has been to see the effect of low concentrations of PAMAM dendrimers on bovine insulin and its aggregation process induced by DTT, measured by spectrophotometeric, fluorimetric, circular dichroism and zeta potential methods.

2. Materials and methods

2.1. Materials

The polyamidoamine (PAMAM) dendrimers (generation 2.5 and 3) and PAMAM–OH dendrimers (generation 3) were purchased from Sigma-Aldrich. Bovine pancreatic insulin and dithiotreitol (DTT) were from Sigma (USA). In all measurements, double-distilled water was used to prepare the solutions. Samples were prepared and measured at 25 °C in 10 mmol/l phosphate buffer at pH 7.

2.2. Effect of dendrimers on zeta potential of insulin

Zeta-potential measurements were carried out using Malvern Instruments Zetasizer 2000 (UK) with capillary plastic cells (DTS1061). Phosphate buffer was filtered through 22 nm filter paper prior to use. The zeta potential was calculated directly from

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Table 1
Dendrimers characteristics.

Dendrimer	Surface groups (type and the number)		Charge on the surface
PAMAM G2.5	**–COOH**	**32**	–
PAMAM G3	**–NH_2**	**32**	+
PAMAM–OH G3	**–OH**	**32**	0

the Helmholtz–Smoluchowski equation using the Malvern software. First, the zeta potential of native insulin dissolved in phosphate buffer was measured. We used increasing concentrations of dendrimers to measure consecutive values of zeta potential. The concentration of bovine insulin was 95 μmol/l and dendrimers were ranged from 1 to 40 μmol/l.

2.3. Effect of dendrimers on insulin fluorescence

Fluorescence spectra of tyrosine residues in bovine insulin were analyzed using a Perkin-Elmer LS55 spectrofluorometer at 25 °C and a 1 cm path-length quartz cuvettes with a volume of 1500 μl in which samples were continuously stirred. An excitation wavelength of 274 nm was used and the emission spectra were recorded at from 280 to 350 nm. Initially, insulin fluorescence spectra were recorded before increasing concentrations of dendrimers were added to for renewed measurements. Spectra were collected for a constant concentration of 95 μmol/l insulin with different concentrations of dendrimers (0.001–0.1 μmol/l). It was checked that dendrimers were not excited at 274-nm wavelength and did not emit fluorescence.

2.4. Effect of dendrimers on insulin circular dichroism spectra

Circular dichroism (CD) spectra of insulin in absence or presence of our 3 dendrimers were analyzed using JASCO-815 CD spectrometry (JASCO, Japan) at 25 °C in 120 μl quartz cuvettes with a path length of 0.5 mm. Each spectrum was an average of 3 scans, with the baseline spectrum subtracted. The insulin concentration in this experiment was 95 μmol/l and measurements were made using all 3 different concentrations of PAMAM dendrimers (0.001, 0.01 and 0.1 μmol/l). We also measured samples containing insulin and DTT (20 mmol/l).

2.5. Dendrimer effect on DTT-induced aggregation of insulin

The turbidimetric method is one of the instrumental methods to monitor protein aggregation by measuring the optical density of the sample based on light scattering in the near UV or visible region. Amino acids like tyrosine and tryptophan absorb virtually no light at 360 nm. Thus, the wavelength selected in this experiment allows observation of the formation of protein aggregates by light scattering or turbidity [1,2].

To investigate the influence of PAMAM dendrimers with different end-groups on the insulin aggregation process, Cary 50Bio UV–visible Spectrophotometer to monitor the process was used. To start the process of insulin aggregation, DTT was added to the samples. We also measured samples containing dendrimers and insulin without DTT as a control. Data were collected for dendrimers at from 0.001 to 0.1 μmol/l. The process was monitored by light absorbance at 360 nm after 100 min in 10 mM phosphate buffer (pH 7.0). Final insulin concentration in the samples was 95 μmol/l. Experiments were repeated 6 times. DTT in the samples was kept at 20 mmol/l.

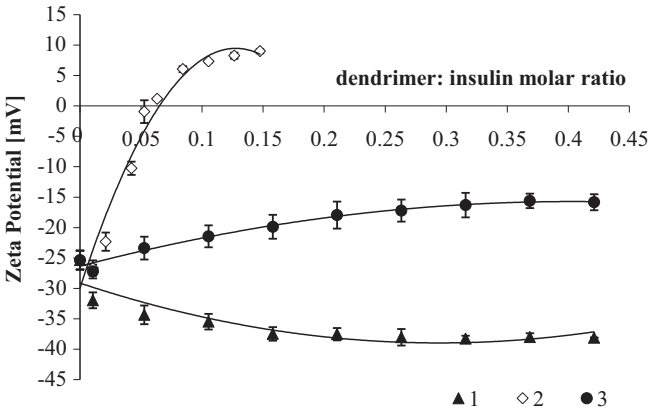

Fig. 1. Changes of zeta potential of insulin with increasing concentrations of: (1) G2.5 PAMAM, (2) G3 PAMAM and (3) G3 PAMAM–OH dendrimers.

3. Results and discussion

3.1. Zeta potential of insulin

The zeta potential is calculated from the electrophoretic mobility of colloidal particles, being the potential that describes the charge present on the particle at the shear plane. The magnitude of the zeta potential can be related to the stability of colloidal dispersions or the ability of the system to maintain the particles in a colloidal dispersion [17]. Changes of zeta potential of insulin under the influence of increasing concentrations of PAMAM dendrimers are plotted in Fig. 1.

The zeta potential of insulin has a mean value of −25.3 mV. After addition of dendrimers with carboxyl functional groups, this value became more negative, reaching −38 mV. In the presence of dendrimer with the hydroxyl group, the potential increased with increasing polymer concentration coming up to −15 mV, but did not reach zero. In increasing concentrations of G3 PAMAM dendrimers, the potential reached +10 mV. The plots show that the potential is dependent on the nature of surface groups on PAMAM dendrimers. Potential measurement not only allows one to determine that of the formed complex, but also the number of dendrimer molecules bound to the insulin molecule. The zeta potential plots vs dendrimer/insulin molar ratio show that 10 molecules of insulin can attach to a single molecule of G3 PAMAM dendrimer, whereas only 5–7 insulin molecules can bind to one PAMAM G2.5 or PAMAM–OH G3 molecule, respectively. This difference can be due to net charge of insulin, being negative the pH 7, so positively charged dendrimer (PAMAM G3) binds due to electrostatic interactions. However, despite the overall negative charge, insulin also has positively charged patches that can be sites for binding of negatively charged molecules.

3.2. Fluorescence spectra of insulin

Protein fluorescence is a very sensitive technique to study the structural, physicochemical and functional properties of proteins. Bovine insulin contains 6 aromatic amino acids: 2 phenylalanine and 4 tyrosine residues, which are responsible for its fluorescent properties. Insulin fluorescence comes mostly from 3 tyrosine residues, because one of residues is blocked by the disulfide bridge in the insulin molecule [18]; fluorescence emitted by phenylalanines has much lower quantum yield than fluorescence of tyrosines. The basic information obtained from fluorescence measurements comes from the immediate environment of the chromophore. Fluorescence quenching data are quite easy to interpret if the protein contains