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Short Communication

Attenuation of the insulin amyloid aggregation in presence of Fe_3O_4-based magnetic fluids *

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Abstract. Presence of protein amyloid deposits is associated with pathogenesis of amyloid-related diseases. Insulin amyloid aggregates have been reported in a patient with diabetes undergoing treatment by injection of insulin. We have investigated the interference of insulin amyloid aggregation with two Fe_3O_4-based magnetic fluids. The magnetic fluids are able to inhibit insulin amyloid fibrillization and promote disassembly of amyloid fibrils. The cytotoxic effect of amyloid fibrils is attenuated in presence of magnetic fluids probably due to reduction of the fibrils. We suggest that present findings propose the potential use of Fe_3O_4-based magnetic fluids as the therapeutic agents targeting insulin-associating amyloidosis.

Key words: Amyloid aggregation — Insulin — Magnetic fluid — Cell viability

Amyloid aggregates of proteins are highly ordered structures whose occurrence is associated with a number of pathologies, such as Alzheimer's and Huntington's diseases, spongiform encephalopathies, type-II diabetes and various systemic amyloidosis. The amyloid deposits localized in various parts of human body are characteristic of the presence of a single predominant protein that is typical for a given disease (Sipe 2005). Up to now, there are more than 25 human proteins related to amyloid diseases. Although the amyloid-related proteins do not reveal any similarities, the amyloid aggregates (oligomers, pores, protofibrils, fibrils) share similar structural and physicochemical properties (Klunk et al. 1989; Sunde and Blake 1997).

Recently, it has been found that ability to form amyloid structures is not restricted to the proteins linked to amyloid diseases, but represents the generic feature of the polypeptide chain (Bucciantini et al. 2002). It allows *in vitro* formation of amyloid aggregates which can be induced by high protein concentration, extreme values of pH (acid, basic), heating, interaction with various surfaces, agitation, etc. (Hamada and Dobson 2002; Dobson 2004).

Insulin amyloid deposits have been found in the sites of subcutaneous insulin application in patients with diabetes long-term treated by insulin. Moreover, insulin amyloid aggregation causes a serious problem in the production, storage and delivery of this important biopharmaceutical compound as well as in application of the insulin pumps. *In vitro* insulin amyloid fibrillization can be achieved at acid pH and presence of strong denaturants (guanidine hydrochloride, urea) or salts (NaCl) (Brange et al. 1997).

No real cure is currently directed toward the amyloid-related diseases and still remains unclear which step in the cascade of amyloid formation is the most toxic. The current models suggest a direct effect of amyloid structures on cell membrane stabilization (pore formation, permeation effect) (Anguiano et al. 2002) or an indirect effect of amyloid assemblies on cellular mechanisms (Conte et al. 2003). The current approaches, however, suggest that reduction of the amyloid aggregation by direct inhibition of amyloid fibrillization or clearance of amyloid aggregates are beneficial (Cohen and Kelly 2003).

The growing attention concerns to the study of the effect of nanoparticles on protein amyloid aggregation due to the

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specific properties as the high ratio surface/volume, size, surface charge and composition. Recent works report that peptide-conjugated fluorescent-maghemite nanoparticles, CdTe quantum dots capped with N-acetyl-L-cysteine, fluorinated nanoparticles and hydrophobic Teflon nanoparticles inhibit Aβ fibrillization (Rocha et al. 2008; Xiao et al. 2010). Recently, we have observed that Fe_3O_4-based magnetic nanoparticles are able to reduce amyloid aggregation of lysozyme or insulin (Bellova et al. 2010; Siposova et al. 2012).

In the present work, we have investigated the ability of two Fe_3O_4-based magnetic fluids (MFs) to interfere with amyloid aggregation of insulin. The anti-amyloid effect was observed for magnetic fluid consisted of Fe_3O_4 magnetite nanoparticles electrostatically stabilized by $HClO_4$ (MF-pH) or sterically stabilized by sodium oleate and coated with bovine serum albumin (BSA) (MF-BSA) to improve their biocompatibility.

The magnetite nanoparticles were prepared by co-precipitation of Fe^{2+} and Fe^{3+} salts by NH_4OH at 60°C. In a typical synthesis to obtain 1 g of Fe_3O_4 precipitate, 0.86 g of $FeCl_2 \cdot 4H_2O$ and 2.35 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 40 ml of deionized water by vigorous stirring, such that the molar ratio of $Fe^{3+}/Fe^{2+} = 2$. As the solution was heated to 60°C, 5.6 ml of 25% NH_4OH was added. The precipitate was isolated from the solution by magnetic decantation with distilled water. Then the freshly prepared magnetic nanoparticles were dispersed in water and stabilized electrostatically with $HClO_4$ (MF-pH) (Massart 1981) or sterically with sodium oleate (MF-BSA). In the case of MF-BSA the BSA was added as a modifying agent during stirring and heating up to 50°C in the w/w ratio BSA:Fe_3O_4 = 0.35. The prepared magnetic fluids were characterized by scanning electron microscopy (SEM) and photon cross correlation spectroscopy (PCCS) to obtain information about morphology, particle size and size distribution. Hydrodynamic diameter of the prepared magnetic fluids, considering the magnetic core and the coating layer of BSA were determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS from Malvern Instrument (Antosova et al. 2010; Gazova et al. 2012; Siposova et al. 2012).

The insulin amyloid aggregates (Iagg) were achieved by incubation of the soluble protein (10 μM) in 100 mM NaCl, pH 1.6 at 65°C and constant stirring (1200 rpm) of the solution for 2 h. Formation of amyloid aggregates was observed by Thioflavin T (ThT) and 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence assays. For both assays the presence of amyloid aggregates is associated with significant increase of fluorescence intensity which is not observed for native protein (data not shown). ThT fluorescence signal increases as a consequence of the interaction between the dye and the assembled β-structured amyloid aggregates. Enhancement of ANS fluorescence responds mainly to the interaction of ANS with hydrophobic protein regions. The presence of amyloid fibrils was confirmed by atomic force microscopy (AFM). AFM images exhibit typical amyloid morphology of insulin fibrils displaying long fibrilar structure and protofibrilar twisting (Fig. 1A).

The abilities of MFs to inhibit amyloid fibrillization of studied proteins as well as to destroy pre-formed amyloid fibrils were determined by ThT and ANS assays as the decrease of the fluorescence intensities indicates reduction of amyloid aggregation. The inhibiting activity of magnetic fluids was investigated by adding of MF to the soluble insulin before the process inducing protein aggregation (described above). Depolymerizing activity of MF was observed after an overnight incubation of insulin fibrils with a magnetic fluid. As a control, the protein was replaced with water to measure the fluorescence of the magnetic fluid. The observed anti-amyloid activities of magnetic fluids were studied at fixed protein concentration (10 μM corresponding to 58 μg/ml) for a range of MF concentrations (concentration of magnetite in MF) from 0.0029 to 1.16 mg/ml and are shown in Fig. 2. The half-maximum inhibition and depolymerization values (IC_{50} and DC_{50}) were calculated from curves fitting the experimental data and are presented in Table 1.

Presented data suggest that both studied magnetic fluids are able to inhibit insulin fibrillization and destroy pre-formed amyloid fibrils. Values of IC_{50} and DC_{50} parameters indicate that MFs interfere with insulin amyloid aggregation already at stoichiometric concentrations. However, the IC_{50} and DC_{50} values detected for MF-pH are in one-order lower than those determined for MF-BSA. It can be assume that modification of the MF with BSA attenuate anti-amyloid activities of magnetic fluid. One of the explanations is that increasing of the nanoparticle size due to the adsorption of BSA affects the interaction of MFs with amyloid aggregation and leads to a decline of MF-BSA activities. The smaller nanoparticles have less steric barriers to interact with bonds involving in the forming or stabilizing the β-sheets of amyloid fibrils. The similar response of nanoparticles was observed for amyloid aggregation of transthyretin and Abeta peptide (Cabaleiro-Lago et al. 2008, Yoo et al. 2011). Interestingly, IC_{50} and DC_{50} values calculated from data obtained by ANS assay are comparable. It is probably due to similar decreasing

Table 1. IC_{50} and DC_{50} values determined for studied magnetic fluids MF-pH and MF-BSA

	IC_{50} (mg/ml)		DC_{50} (mg/ml)	
	ThT assay	ANS assay	ThT assay	ANS assay
MF-pH	0.065	0.101	0.016	0.049
MF-BSA	0.221	0.109	0.233	0.069

IC_{50}, the concentration of half-maximum inhibition; DC_{50}, the concentration of half-maximum depolymerization.

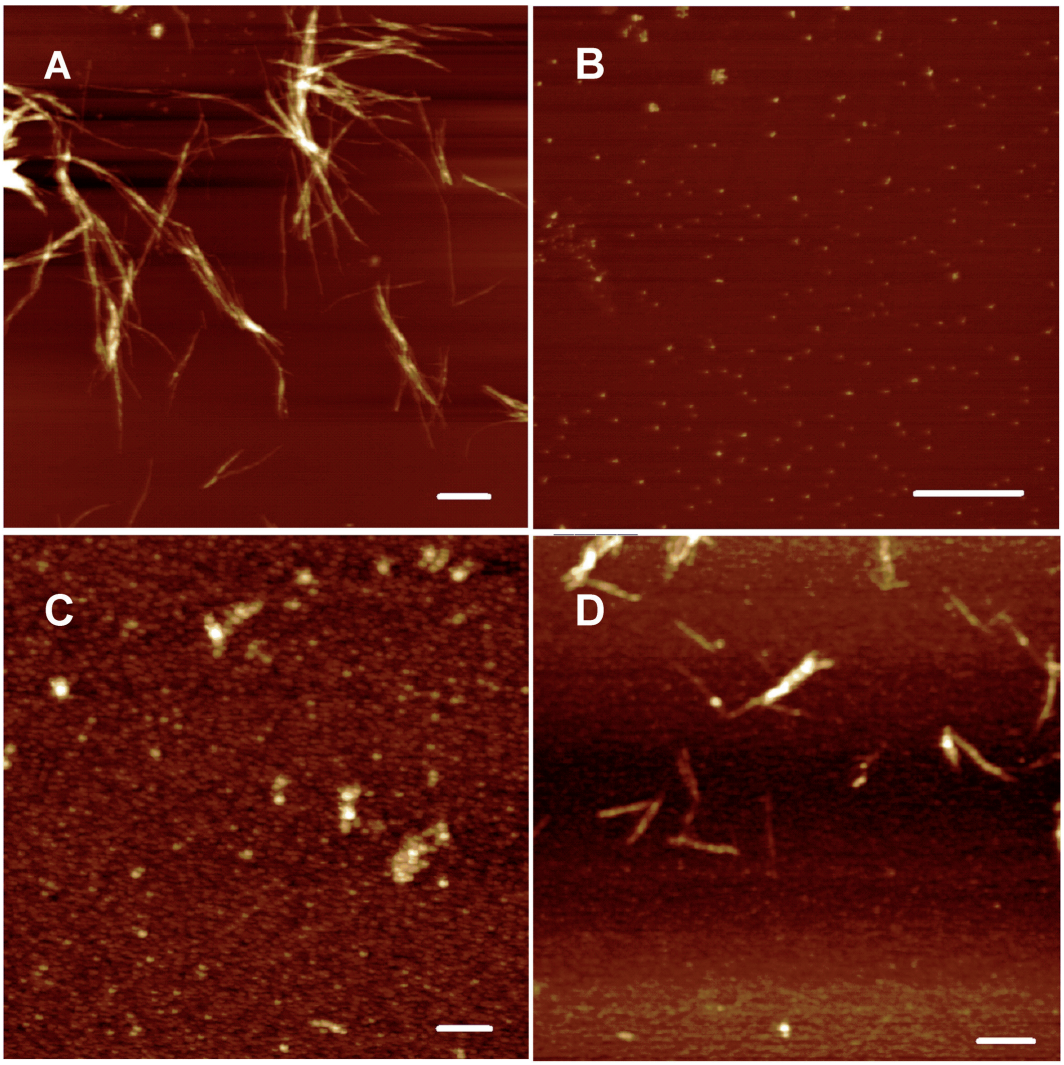

Figure 1. AFM images of insulin amyloid fibrils (**A**), magnetic fluid MF-pH (**B**), inhibition of insulin amyloid fibrillization by MF-pH (ratio insulin/MF = 1:2) (**C**) and depolymerization of insulin amyloid fibrils after incubation with pH-MF (ratio Iagg/MF-pH = 1:2) (**D**). Samples were prepared by drop casting of solution on the surface of freshly cleaved mica and left to adsorb for 2 min, rinsing with UHQ water and dried prior to scan. Solution concentration was always 1 μg/ml. AFM images were taken by a Scanning Probe Microscope (Veeco di Innova, Bruker AXS Inc., Madison, USA) in a tapping mode in ambient conditions, using uncoated silicon cantilevers NCHV (Bruker AFM Probes, Camarillo, USA) with nominal resonance frequency 320 kHz and spring constant 42 N/m. (Scale bar is 1 μm).

of the hydrophobicity resulting from the reduction of the amyloid aggregation with MF.

We were interested if agents used for stabilization ($HClO_4$, sodium oleate) and modification (BSA) of magnetic fluids have any potential to influence the insulin amyloid aggregation. We investigated their effect on amyloid aggregates at the same concentrations occurring for inhibiting or depolymerizing experiments with MFs. The fluorescence intensities observed for amyloid fibrils treated with agents were similar to that detected for amyloid fibrils alone. It suggests that agents had no significant ability to affect protein aggregation. These findings indicate that Fe_3O_4 magnetic core plays a significant role in the anti-amyloid activities of studied MFs.

The reduction of the insulin amyloid aggregation due to interaction with MFs was established also by AFM. Representative AFM images show that incubation of insulin solution with magnetic fluid (Fig. 1B) leads to significant inhibition of fibril formation (Fig. 1C) and destruction of pre-formed fibrillar aggregates (Fig. 1D). The extensive de-

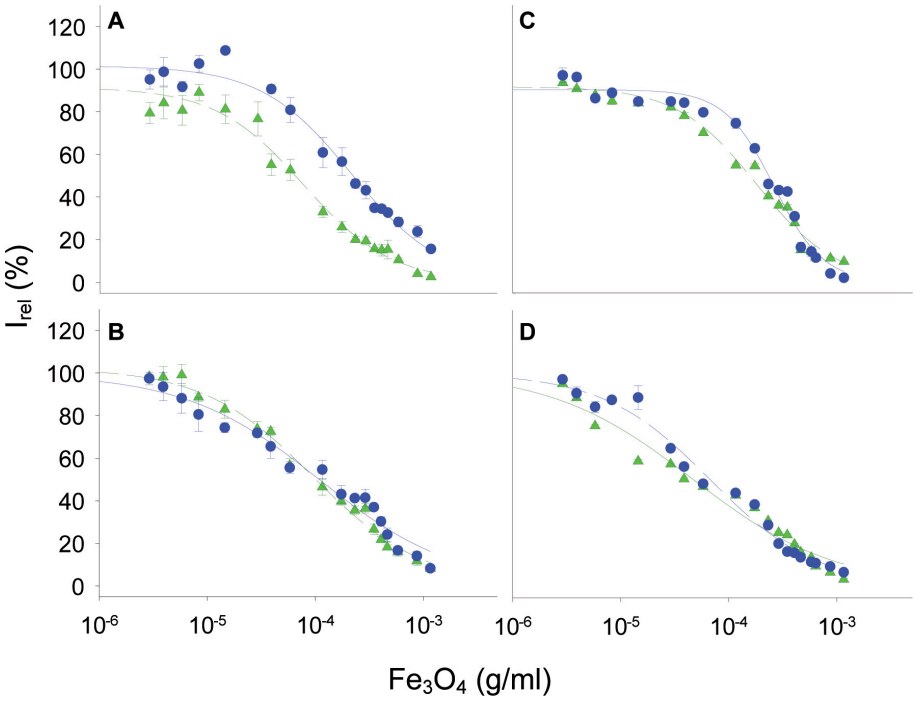

Figure 2. Fluorescence intensities corresponding the ability of MF-pH (triangles) and MF-BSA (circles) to inhibit (**A, C**) and depolymerize insulin amyloid aggregates (**B, D**) detected by Thioflavin T (ThT) and 8-anilinonaphthalene-1-sulfonic acid (ANS) assays. The relative fluorescence intensities (I_{rel} (%)) were normalized to the control (fluorescence intensity of the insulin aggregates in the absence of MF – taken as 100%). ThT or ANS was added to the insulin samples (10 μM) to a final concentration of 20 μM. Measurements were performed in a 96-well plate by a Synergy MX (BioTek) spectrofluorimeter. The excitation was set at 380 (ANS) / 440 (ThT) nm and the emission recorded at 485 nm. The excitation and emission slits were adjusted to 9.0/9.0 nm and the top probe vertical offset was 6 mm. All ThT/ANS fluorescence experiments were performed in triplicate and the final value is the average of measured values. The curves were obtained by fitting of the average values by nonlinear least-squares method with the 3 parameter equation.

crease of the amount of amyloid aggregates and very short parts of fibrils were observed.

The obtained results inspire us to examine the effect of MFs on cells affected by insulin amyloid fibrils inducing significant cytotoxicity. The cytotoxic effect was investigated by MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) in viable cells undergoes reduction by mitochondrial dehydrogenases (the succinate-tetrazolium reductase system) to yield insoluble formazan, which reports on the fraction of metabolically active cells. The fibroblast cells V79 (plated in 24-well plate at a concentration of 5×10^3 cells/well) were incubated with insulin amyloid fibrils for 24 h and MTT reduction was assessed. The experimental data indicate high cytotoxic effect of insulin amyloid fibrils as only about 60% viability of the cells is observed compare to untreated cells (Table 2). Interestingly, the viability was significantly improved when MF was added together with insulin fibrils (ratio Iagg/MF = 10 μg/ml/10 μg/ml). The viability was increased to 80% for MF-pH and 90% for MF-BSA. It has been noted that MF alone caused no significant changes in viability at studied concentration.

Table 2. Viability of the V79 cells in presence of insulin amyloid fibrils (Iagg), insulin fibrils in presence of magnetic fluids (Iagg+MF) and MFs alone determined by MTT assay

	viability (%)
control[a]	93.71
Iagg	63.39
Iagg+MF-pH	79.72
Iagg+MF-BSA	88.25
MF-pH	80.30
MF-BSA	88.62

The viability of treated cells was normalized to the cells incubated alone (taken as 100%). [a] Control experiments were performed by exposing cells to solutions of buffer for the same lengths of time.

The obtained results indicate that MFs are able to alleviate the negative effect of insulin amyloid fibrils on the cells probably by reduction of the fibrils in medium. Presented data also support the importance of the biocompatibility of the magnetic fluid as the modification of

the magnetic fluid by BSA caused higher viability of the cells. The toxicity of MFs can be decreased also by their functionalization with other biocompatible polymers (e.g. dextran, poly(ethylene glycol)) to avoid the massive Fe^{2+} iron mobilization, which can be a source of toxic hydroxyl radicals in organism at some specific conditions (Babincova and Babinec 2005).

It can be concluded that studied Fe_3O_4-based magnetic fluids interfere with amyloid aggregation of insulin. It was found that MFs are able to inhibit insulin amyloid fibrillization and destroy pre-formed insulin fibrils *in vitro*. Results suggest that the extent of anti-amyloid activity of MFs is significantly related to the composition and size of the magnetite core of MFs. The higher efficiency was determined for MF-pH characteristic the smaller size of magnetite nanoparticles. From presented data it is difficult to clarify the exact role of the magnetisms of the Fe_3O_4 core which can be determined only by experiments investigating the direct effect of the magnetic field on the anti-amyloid activity. At least, the magnetic properties of the MF can be utilized for the easy extraction of the insulin amyloid fibrils/MF assemblies from the aqueous phase (possibly also from organism) *via* magnetic field as it was shown by Skaat et al. (2009). Moreover, our results reveal that fibrils prepared from insulin are toxic to the cells. The cytotoxic effect is attenuated in presence of MFs probably by reduction of the insulin fibrils. We suggest that present findings propose the potential use of Fe_3O_4-based magnetic fluids as the therapeutic targeting insulin-associating amyloidosis.

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