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Research articles

Effect of nanoparticles coated with different modifications of dextran on lysozyme amyloid aggregation

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ABSTRACT

More than 50 diseases are associated with a conversion of proteins or peptides from their soluble functional states to highly organized fibrillar aggregates called amyloid fibrils. Due to the specific physico-chemical properties, specifically designed iron oxide nanoparticles may be an effective strategy for inhibiting the amyloid fibrillization of proteins. The inhibitory effect of three types of iron oxide nanoparticles coated with differently modified dextran on lysozyme fibrils formation was studied by Thioflavin T assay and atomic force microscopy. The size and zeta potential of studied nanoparticles were determined by dynamic light scattering. It had been found that nanoparticles are able to inhibit formation of lysozyme amyloid aggregates in concentration-dependent manner. Our results suggest that size of nanoparticles influenced the extent of their inhibitory properties. Moreover, the most effective nanoparticles were not toxic after 48 h incubation with neuroblastoma SH-SY5Y cell line at concentration close to IC_{50} values.

1. Introduction

Proteins are important for functioning of cells and organisms. Therefore, the interaction of nanoparticles with proteins is a critical issue that is attracting increasing attention from researchers. Protein aggregation into characteristic amyloid fibrils is a major cause of various amyloid-related diseases such as Alzheimer's disease, diabetes type II, and different systemic amyloidosis [1]. In these diseases, a conversion of proteins or peptides from their soluble functional states to amyloid oligomers and highly organized fibrillar aggregates occurs. Their following accumulation exerts toxicity by disrupting intracellular transport, overwhelming protein degradation pathways and/or disturbing cell functions.

Amyloid fibrillization follows nucleated growth mechanism, which includes a lag phase followed by an exponential elongation phase [2]. In the lag phase nuclei are formed and further association of monomers or oligomers is the starting point for fibril formation of proteins. The molecular events during amyloid fibrillization remain elusive, but many studies suggest that fibril formation involves a number of intermediate oligomeric and fibrillar states [3]. The *in vitro* fibril formation can be modulated by several factors including solution properties such as ionic strength, pH, temperature, and stirring [4]. Thermal denaturation of proteins leads to the exposure of hydrophobic residues, which increases hydrophobic attraction that overcomes electrostatic repulsion and triggers the aggregation. Raccosta *et al.* have indicated that formation of lysozyme amyloid aggregates occur not only at higher temperatures, where proteins undergo partial unfolding, but also at pH levels far from their isoelectric point where proteins are electrically charged [5].

It has been shown that modified surfaces and interfaces have specific and significant effects in promoting or inhibiting amyloid formation [6]. Nanoparticles (NPs) possess a large surface area and can affect both protein structure and function. It was found that they influence the amyloid fibrillization of proteins very controversially [7]. Changes in protein conformation upon binding with nanoparticles or increased local protein concentration on the nanoparticle surfaces could promote aggregation. On the other hand, trapping of early aggregation intermediates by nanoparticles may inhibit further aggregation [8]. Therefore, specifically designed and engineered nanoparticles may be an

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effective strategy for inhibiting the fibrillization of proteins at its various stages, while keeping in mind safety issues such as biocompatibility and biodegradability [9]. Lee *et al.* have demonstrated that an inhibition by nanoparticles could be done at the nucleation phase leading to increasing the lag phase, at the polymerization phase by its decreasing or via destruction of formed amyloid fibrils. One of the possible explanations is that nanoparticles decrease the rate of fibrillization by altering the amount of free monomeric peptides present in solution [10]. Cabaleiro-Lago and coworkers pointed out that some types of nanoparticles added at the beginning of the kinetic experiment lock the fibrillation process in lag phase. However, these nanoparticles may not show any inhibitory effect when added at the end of the lag phase indicating that once critical nuclei are formed, the elongation process is so favorable that the addition of nanoparticles does not have any effect on monomer/oligomer interactions with nanoparticles [11]. It is known that high protein concentrations lead to the increased formation of amyloid aggregates. But, in the case of nanoparticles, high local protein concentrations at their surface inhibit fibril formation, as the interaction between nanoparticles and proteins leads to blocking of the active sites for fibril formation [12].

Magnetic NPs have been intensively developed, not only for their fundamental scientific interest, but also for many medical applications, such as magnetic resonance imaging, hyperthermia for tumor treatment, cell labeling and sorting, DNA separation and drug delivery [13]. However, so far only a few studies have been related to magnetic NPs and amyloid protein aggregation. Skaat *et al.* demonstrated the significant inhibition of the insulin fibrillation process in the presence of γ-Fe_2O_3/PHFBA nanoparticles [14]. In our previous works, we have demonstrated that magnetic Fe_3O_4 NPs are able to inhibit the formation of lysozyme amyloid aggregates and have the ability to destroy amyloid fibrils by depolymerization of amyloid structures [15–17]. Although nanoparticles have been used to tackle the problem of amyloid protein aggregation, their biological safety is not fully elucidated. One of the main factors in the cytotoxicity of nanoparticles could be the size [18], concentration [19], and surface characteristics [20,21]. However, it has been shown that iron oxide nanoparticles are less toxic when compared with other metal oxide nanoparticles. To further decrease their cytotoxic properties, different types of molecules are used as coating agents. Among them, small proteins, saccharides, amino acids, organic molecules and others are frequently used.

The aim of this study was to investigate the effect of magnetic nanoparticles coated with different types of dextran on the hen egg white lysozyme amyloid fibrillization using spectroscopic and microscopic techniques. It was found that nanoparticles are able to inhibit formation of lysozyme amyloid aggregation. Our results suggest that size of nanoparticles influenced the extent of their inhibitory properties. Moreover, the most effective nanoparticles were not toxic after 48 h incubation with neuroblastoma SH-SY5Y cell line at concentration close to IC_{50} values.

2. Material and methods

2.1. Materials

Hen egg white lysozyme (HEWL, ≥40,000 units/mg protein, Sigma L6876), glycine, sodium chloride, Thioflavin T, Fetal bovine serum (FBS), Dulbecco's modified Eagle Medium (DMEM), penicillin, streptomycin and L-glutamine were obtained from Sigma Aldrich and were of analytical reagent grade. The other chemicals such as WST-1, were purchase from Roche company.

2.2. Preparation of dextran – coated magnetic nanoparticles

The particles were prepared similar to the well-known wet chemical precipitation methods but using another alkaline medium and a slower reaction velocity. In detail, a 1 M $NaHCO_3$ solution was slowly added under permanent stirring to a $FeCl_2/FeCl_3$ solution (total Fe-concentration: 0.625 M; Fe^{2+}/Fe^{3+} ratio = 1/1.3). This procedure was stopped when the pH value reached 8. During this routine a brownish precipitate was formed. This precipitate was heated to 100 °C for 5 min and iron oxides with a spinel structure were formed under the release of CO_2. To remove excess reaction products from the prepared particles they were washed with de-ionized water three times by magnetic separation. For the production of sedimentation stable suspensions of the MNP, the particles were coated with different shells: plain dextran (Dex), carboxymethyldextran (CMD) or diethylaminoethyldextran (DEAE). For this, after washing of the particles the pH value of the dispersed particles was adjusted to pH 2–3 by the addition of diluted HCl. Then the suspension was homogenized by ultrasonic treatment for a few seconds (Sonopuls GM200, Bandelin electronic) and then temperated at 45 °C. An aqueous solution of coating material with a coating/MNP ratio of about 1/3 was added to the suspension and stirred for 60 min at 45 °C. Finally, the coated particles were washed with de-ionized water twice to remove the remaining salts and to adjust the desired particle concentration.

2.3. Amyloid aggregation of HEWL in vitro

Lyophilized lysozyme powder was dissolved in 70 mM glycine, 80 mM NaCl buffer solution (pH 2.7). The protein concentration was determined by UV–Vis spectroscopy using extinction coefficient of $\varepsilon_{280} = 38{,}940\ M^{-1}\ cm^{-1}$. Formation of amyloid aggregates was achieved by exposing the 10 μM protein to 65 °C and continuous shaking (1 200 rpm) for 2 h. The formation of amyloid fibrils was confirmed using Thioflavin T (ThT) fluorescence assay and atomic force microscopy (AFM).

2.4. Thioflavin T fluorescence assay

After binding with amyloid fibrils, Thioflavin T (ThT) fluorescence intensity significantly increasing upon excitation at 440 nm with emission maximum at 485 nm. Thioflavin T was added to the samples containing 10 μM protein solution to reach a final ThT concentration of 20 μM. All measurements were performed in triplicate using Synergy Mx (BioTek) spectrofluorimeter in 96-well plate with excitation wavelength set at 440 nm and emission wavelength in range between 465 nm and 550 nm. The emission and excitation slits were set at 9.0/9.0 nm.

2.5. Atomic force microscopy

Samples of 10 μM protein alone or after treatment with nanoparticles were placed dropwise on the surface of mica. After 10 min adsorption, the samples were washed with ultra-pure water and left to dry on air. Images were obtained using microscope Veeco di Innova in tapping mode with NCHV cantilever. All images are unfiltered and no smoothing was applied.

2.6. Effect of dextran-coated nanoparticles on the kinetics of amyloid aggregation of HEWL

The effect of nanoparticles on formation of lysozyme amyloid aggregates was studied by adding NPs to the native HEWL solution (10 μM) at the concentration ratios 10:1, 1:1 and 1:10. Samples were then exposed to specific conditions for lysozyme amyloid fibrillization (65 °C, 1200 rpm, pH 2.7). ThT fluorescence assay was used to measure the extent of lysozyme amyloid aggregation at different time intervals.

2.7. Half-maximal inhibitory activity – the IC_{50} values

The IC_{50} values were determined as the ability of NPs to inhibit lysozyme amyloid aggregation for NPs concentration ranging from 7.4 to 2940 μg/mL and fixed lysozyme concentration (10 μM = 147 μg/

mL). The activities of NPs on amyloid aggregation were observed by ThT fluorescence assay. In each experiment the final values represent the average of three independently measured values. The IC_{50} values were determined from curves obtained by fitting of the final values with using the equation $y = a/(1 + (x/x_0)^b)$ in the SigmaPlot software, where x_0 corresponds to IC_{50} values.

2.8. Cytotoxicity measurements

The neuroblastoma cell line SH-SY5Y, obtained from German Collection of Microorganisms and Cell Culture (DSZM) company in Germany, was grown in Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin/100 µg/mL streptomycin and 2 mM L-glutamine (Sigma Aldrich). The cells were seeded at the concentration 1×10^6 cells/75 cm^2 for sub-culturing. For the experiments with nanoparticles, 7×10^3 SH-SY5Y cells were seeded in 96-well flat-bottom plate and cultured for 24 h and 48 h with 147 µg/mL solution of nanoparticles (concentration ratio protein: NPs = 1:1). After the corresponding incubation time the viability and proliferation of cell were measured using WST-1 assay. WST-1 assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Briefly, after 24 h and 48 h incubation of the SH-SY5Y cells alone and in the presence of nanoparticles, the solution of WST-1 diluted to 1:10 with medium was added to each well and incubated for 10 min at 37 °C, 5% CO_2 in dark. Then, the formazan absorbance was measured at 480 nm with correction at 600 nm using Synergy Mx spectrophotometer. Control represents non-treated SH-SY5Y cells incubated for 24 and 48 h in medium with glycine buffer (pH 2.7). The formazan absorbance of nanoparticles in medium without cells was used as a negative control. All experiments were performed in triplicate and data represents average with standard deviation.

3. Results and discussion

3.1. Characterization of dextran-coated nanoparticles

We have studied the ability of magnetic nanoparticles coated with differently modified dextran to affect the amyloid aggregation of lysozyme at acidic conditions. For a nanoparticle surface modification the carboxymethyldextran (CMdex NPs), dextran (Dex NPs) and diethylaminoethyldextran (DEAEdex NPs) were used to alter their surface characteristics. The colloidal samples of dextran coated nanoparticles were diluted to 0.01 g/L and size and zeta potential were measured at pH 2.7. The obtained data are presented in Table 1. All samples had positive values of zeta potential equal to 6.7 mV in case of dextran NPs, 21.4 mV was measured for Dex NPs and 42.8 mV for DEAEdex NPs. The hydrodynamic diameter of nanoparticles also depended on the type of dextran used as coating material. The smallest nanoparticles were CMdex NPs with hydrodynamic diameter of 207 nm. Dex NPs were a bit bigger with 246 nm in diameter. The DEAEdex NPs have the largest hydrodynamic diameter ~309 nm.

Table 1
The size, zeta potential and IC_{50} values (concentration of nanoparticles with 50% inhibitory activity on lysozyme fibrillization) of studied nanoparticles.

Sample	Size (nm)	Zeta potential (mV)	IC_{50} (µg/mL)
CMdex NPs	207	21.4	315.2
Dex NPs	246	6.7	364.1
DEAEdex NPs	309	42.8	529.1

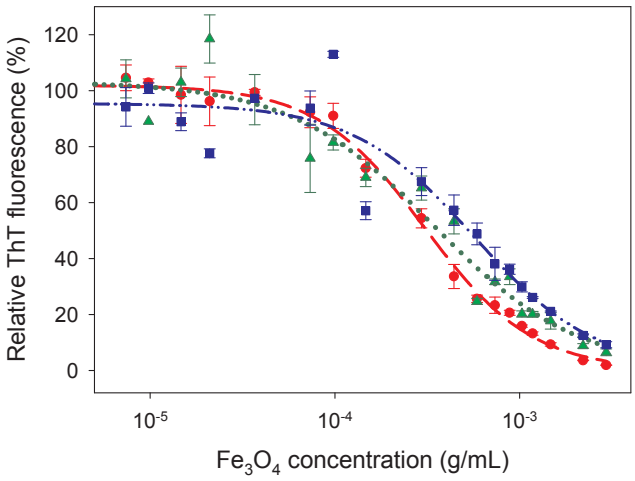

Fig. 1. The inhibition of lysozyme fibrillization induced by increasing concentrations of nanoparticles detected using ThT assay; CMdex NPs (red circles), Dex NPs (green triangles) and DEAEdex NPs (blue squares). The degree of inhibition was plotted as a percentage of the control (lysozyme samples in the absence of nanoparticles). The fits were calculated as three parameter logistic curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Inhibitory effect of nanoparticles towards lysozyme amyloid aggregation

Lysozyme from hen egg white was chosen as a model of amyloidogenic protein to investigate the effect of magnetite nanoparticles coated with different types of dextran on its amyloid aggregation. The lysozyme fibrils formation in the presence of three types of nanoparticles in concentration range from 7.4 to 2940 µg/mL was studied using ThT assay. The observed relative ThT fluorescence intensities normalized to the fluorescence signal of amyloid aggregates alone are shown in Fig. 1. From obtained data it is clear that increasing concentration of nanoparticles led to a decrease in ThT fluorescence intensities implying the significantly lower amount of formed amyloid fibrils after treatment with studied nanoparticles. Data were fitted to calculate IC_{50} values which correspond to a concentration of nanoparticles with 50% inhibitory activity. These data indicate that the IC_{50} values can be correlated to the size of nanoparticles. The biggest DEAEdex NPs (Fig. 1, blue squares) have the smallest tendency to inhibit lysozyme fibrils formation as the IC_{50} value is the highest (529.1 µg/mL). On the other hand the smallest nanoparticles, CMdex NPs (Fig. 1, red circles) showed a stronger inhibiting activity with IC_{50} value equal to 315 µg/mL. The Dex NPs (Fig. 1, green triangles) have IC_{50} values of 364 µg/mL. The determined IC_{50} values are listed in Table 1.

3.3. Study of the kinetics of lysozyme fibrillization in presence of nanoparticles

Thioflavin T fluorescence assay could also be employed to quantify the kinetic process of protein fibrillization. In our study, fibrillization of the HEWL was characterized by a sigmoidal curve which is in consistence with literature (Fig. 2) [22]. Lysozyme fibrils formation (purple curve) represents nucleation-dependent kinetics with characteristic lag phase lasting about 10 min followed by elongation phase (a steep increase in fluorescence intensity due to the elongation of oligomers to protofibrils). The plateau phase with steady-state fluorescence values was reached after 20 min of incubation. To shed more light on inhibition mechanism of nanoparticles, we have studied their effect on kinetics of lysozyme amyloid aggregation at three concentration ratios of protein to NPs = 10:1, 1:1 and 1:10. As shown in Fig. 2, neither type of

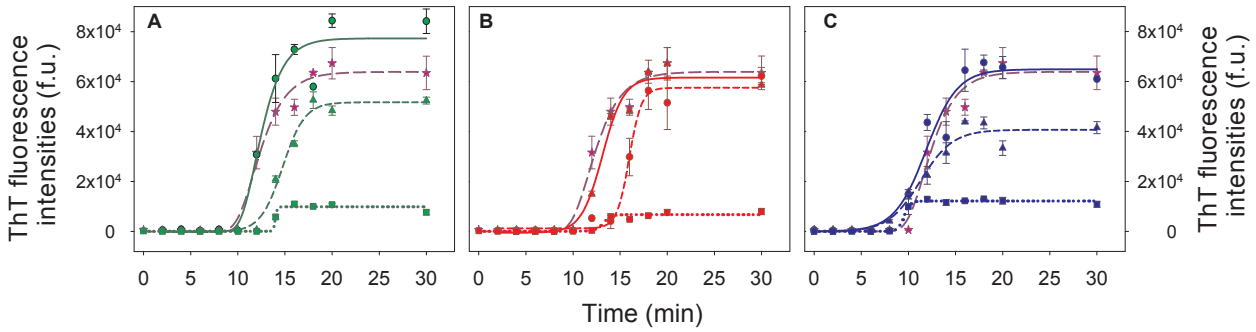

Fig. 2. Kinetics of lysozyme fibrillization (10 μM) alone (pink stars) and in presence of A) Dex (green), B) CMdex (red) and C) DEAEdex (blue) nanoparticles at three protein: NPs ratios – 10:1 (circles), 1:1 (triangles) and 1:10 (squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studied nanoparticles at concentration ratio 10:1 (circles) did affect the formation of lysozyme fibrils as the kinetics profiles are in all three cases similar as the profile of lysozyme fibrillization alone. However, at the ratio 1:1 and higher, Dex NPs and CMdex NPs were able to slow down the rate of fibrillization as they prolonged lag phase. In case of Dex NPs (Fig. 2A) the lag phase was about 12 and 14 min for ratio 1:1 and 1:10 (protein:NPs), respectively. Nanoparticles modified by carboxymethyldextran (CMdex NPs) (Fig. 2B) decreased the lag phase by 4 min in both ratios 1:1 and 1:10. In both cases, increased concentration of nanoparticles led to a significant reduction in steady-state fluorescence intensities suggesting formation of lower amount of fibrillar aggregates. On the other hand, DEAEdex NPs did not influence the formation of nuclei as the length of lag phase was found to be similar to the one observed for lysozyme alone (Fig. 2C). However, these NPs significantly reduced the amount of lysozyme fibrils as the steady-state fluorescence intensities at the concentration ratio of 1:1 (blue triangles, Fig. 2C) are lowest in comparison to the other two types of nanoparticles. These results imply that smaller nanoparticles (CMdex NPs and Dex NPs) slow down the formation of nuclei most likely by altering the amount of free monomeric peptides present in solution after conjugation of monomers on surface of nanoparticles. We assume that at higher concentrations the nanoparticles were able to suppress the association of oligomers and the formation of fibrils. This is consistent with the work done by Cabaleiro – Lago and co-workers [8,11]. The biggest nanoparticles (DEAEdex NPs) were not able to affect formation of nuclei and association of oligomers, suggesting different mechanisms of their inhibition. We assume that DEAE dextran NPs are able to interact with the active sites of prefibrillar aggregates and thus inhibit the formation of mature fibrils.

3.4. AFM study

Atomic force microscopy was used as a direct method for observation of morphology of lysozyme amyloid aggregates. Lysozyme after 2 h incubation at acidic condition, shaking and higher temperature formed amyloid fibrils with typical amyloid morphology (Fig. 3A) – long, unbranched fibrils. After treatment with all studied nanoparticles at concentration ratio protein to NPs = 1:1, the morphology of lysozyme fibrils was changed (Fig. 3B–D). The treatment with CMdex NPs caused the generation of significantly lower amount of lysozyme fibrils and several bigger amorphous aggregates (Fig. 3B). Dextran coated nanoparticles (Dex NPs) also decreased amount of lysozyme fibrils, moreover the huge amount of small globular aggregates can be observed (Fig. 3C). The biggest nanoparticles (DEAEdex NPs) possess smallest inhibitory activity (Fig. 3D). The results from AFM are in agreement with data obtained by the ThT assays.

3.5. Toxicity of dextran nanoparticles

The toxicity of iron oxide nanoparticles coated with different types of dextran towards neuroblastoma cell line SH-SY5Y was monitored after 24 and 48 h incubation using WST-1 assay (Fig. 4). SH-SY5Y cells are routinely used as a model system in the studies of amyloid aggregation; therefore it was employed as a first evaluation step for using these nanoparticles as potential therapeutical agents towards amyloid aggregation of proteins. WST-1 assay provides a simple and accurate method to measure cell proliferation, which is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. In the Fig. 4 is presented the cell viability after 24 h (black bars) and 48 h (red bars) treatment with studied nanoparticles. The obtained data were normalized to the signal of non-treated cells (control, taken as 100%). As one can see, only DEAEdex NPs showed significant decrease in cell viability after 48 h incubation when compared to results for 24 h incubation. This might be connected to the surface charge and size of NPs as DEAEdex NPs are the biggest one. Naqvi *et al.* have shown that iron oxide nanoparticles with size about 30 nm are not toxic to murine macrophage (J774) cells up to the concentration of 200 μg/mL and after 6 h incubation [23].

4. Conclusions

In this study, the ability of magnetic iron oxide nanoparticles coated with differently modified dextran to affect the amyloid aggregation of lysozyme at acidic conditions was determined using several techniques. For a nanoparticle surface modification the carboxymethyldextran (CMdex NPs), dextran (Dex NPs) and diethylaminoethyldextran (DEAEdex NPs) were used to alter their surface characteristics. The size and zeta potential of nanoparticles was measured using DLS method. The nanoparticles are stable colloids and differ in size in order CMdex NPs < dex NPs < DEAEdex NPs. Using Thioflavin T assay and AFM we have shown that studied nanoparticles are able to inhibit formation of lysozyme amyloid fibrils in concentration-dependent manner. Their inhibitory activity was quantified through IC_{50} values. The smallest IC_{50} value and the highest inhibitory effect were observed for the smallest CMdex NPs. On the other hand, the biggest DEAEdex NPs have the highest IC_{50} value. These results implying that size of nanoparticles is an important factor for inhibitory activity of studied nanoparticles. Their cytotoxic properties were also studied using WST-1 assay. Obtained data suggest that dextran coated iron oxide nanoparticles are not toxic at concentration close to IC_{50} values.

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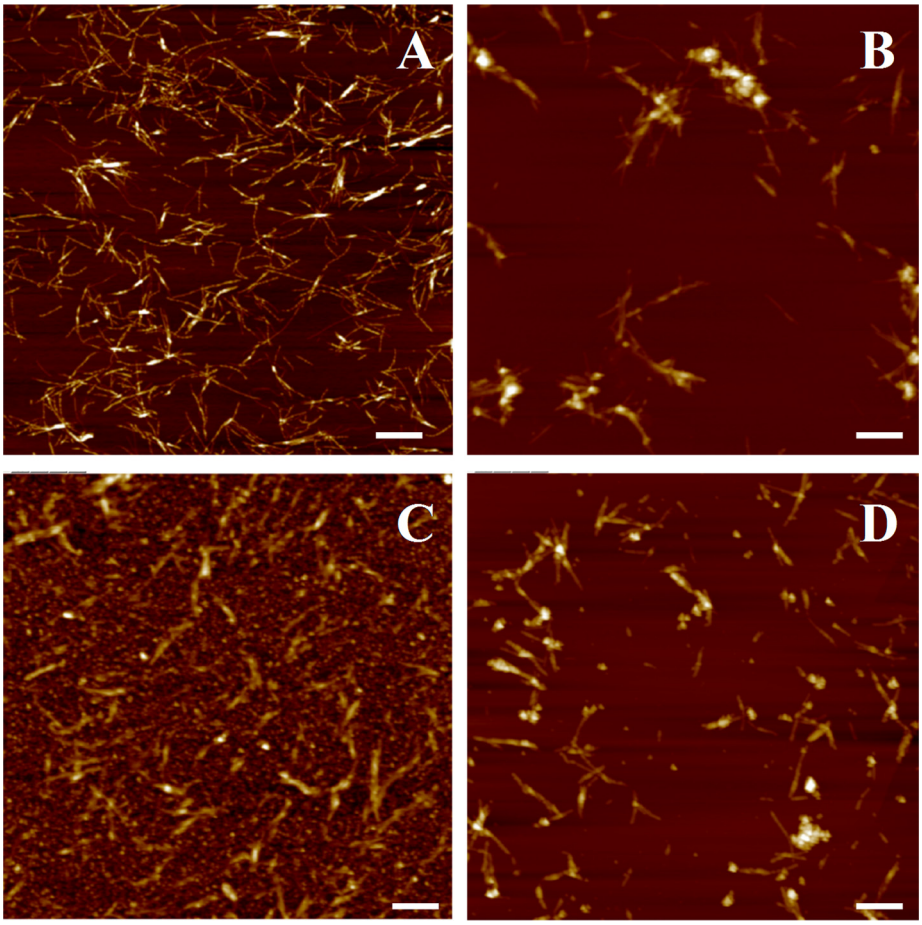

Fig. 3. AFM images of 10 μM lysozyme fibrils formed alone (A) and in the presence of CMdex NPs (B), Dex NPs (C) and DEAEdex NPs (D) at protein: NPs ratio 1:1. Bars represent 1 μm.

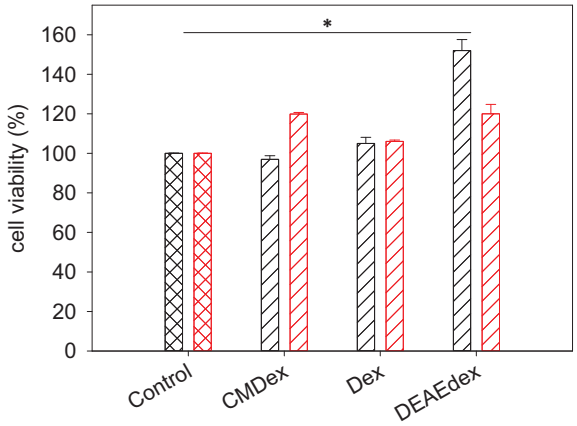

Fig. 4. The SH-SY5Y cell proliferation and survival after 24 h (black bars) and 48 h (red bars) treatment with all three types of nanoparticles determined using WST-1 assay. The concentration of MNPs used in the experiment was 147 μg/mL. Each value represents average of three independent samples with standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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