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Heparin Nanoparticles for β Amyloid Binding and Mitigation of β Amyloid Associated Cytotoxicity

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Abstract

Accumulation of β amyloid ($A\beta$) in the brain is believed to play a key role in the pathology of Alzheimer's disease. Glycosaminoglycans on surface of neuronal cells can serve as nucleation sites to promote plaque formation on cell surface. To mimic this process, magnetic nanoparticles coated with heparin have been synthesized. The heparin nanoparticles were demonstrated to bind with $A\beta$ through a variety of techniques including enzyme-linked immunosorbent assay, gel electrophoresis and thioflavin T assay. The nanoparticle exhibited little toxicity to neuronal cells and at the same time can effectively protect them from $A\beta$ induced cytotoxicity. These results suggest that heparin nanoparticles can be a very useful tool for $A\beta$ studies.

Keywords

amyloid β ; binding study; cytotoxicity; heparin; nanoparticle

1. Introduction

Alzheimer's disease (AD) has become the most common form of dementia, which is affecting about 5.2 million Americans.¹ The number of AD patients is predicted to increase significantly and expected to triple by 2050.² One of the main pathological hallmarks of AD is the senile plaques formed by $A\beta$. $A\beta$ is derived from amyloid precursor protein processing by β - and γ -secretases and proposed to be a causative agent of AD.³ $A\beta$ can aggregate into highly toxic oligomers and deposit as plaques on the cerebral cortex damaging the nervous system.^{4–6} Glycosaminoglycans (GAGs) are believed to play a central role in the amyloidosis pathway with many GAG bearing proteoglycans (PGs) found in both diffuse

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Supporting Information. Materials, methods and additional experimental procedures can be found, in the online version, at #####.

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and neuritic amyloid plaques.^{7–8} GAGs on surface of neuronal cells have been suggested to serve as nucleating sites for A β aggregation, contribute to the formation of neurotoxic A β deposits on cells.^{9–14}

Heparin is a member of the GAG family, which is known to be able to bind with A β .^{12, 15–16} We envision that nanoparticles coated with heparin can be utilized to mimic PG bearing neuronal cells and potentially compete for their interactions with A β . Although heparin nanoparticles have been utilized for cancer targeting, anti-coagulation, tissue engineering and drug delivery applications,^{17–22} their interactions with A β have not been studied before. Herein we report the synthesis of heparin-functionalized magnetic glyconanoparticles. These nanoparticles can bind with A β , induce the formation of fibril, and protect neuronal cells from A β induced cell death.

2. Results and Discussion

2.1. Preparation and Characterization of Hep-SPION

We selected magnetic nanoparticles as the core of our heparin nanoparticles, since magnetic nanoparticles are a powerful platform for biological applications due to their high surface area, biocompatibility and magnetic relaxivity.^{23–25} Two methods were explored to immobilize heparin polysaccharides onto magnetic nanoparticles. In the first approach, we adapted our previous synthesis of colloidal hyaluronan nanoparticles.²⁶ Magnetite nanoparticles were first produced through the thermal decomposition method, resulting in hydrophobic magnetic nanoparticles mainly coated with oleic acid (Scheme 1A). Exchanging the oleic acid ligand with heparin was performed in a water/toluene biphasic system. However, although the resulting nanoparticles could be dispersed in water or phosphate buffered saline (PBS), they quickly precipitated out of aqueous solutions. This was most likely due to the lower efficiency of ligand displacement from the hydrophobic nanoparticles by heparin as compared to hyaluronan, as heparin is more charged and presumably less soluble in the organic solvent for ligand exchange.

An entirely aqueous solution based synthetic route was tested next. The magnetite core was constructed by the co-precipitation method by mixing ferric chloride and ferrous chloride with ammonium hydroxide in water (Scheme 1B).²² The resulting superparamagnetic iron oxide nanoparticles (SPION) were collected with a magnet and resuspended in water. Heparin sodium salt was then added, which could chelate with the SPIONs. Removal of excess heparin by ultrafiltration produced the heparin-coated SPIONs (Hep-SPION), which was a stable colloidal suspension in water with no visible precipitates for weeks at the concentration of 2.5 mg/mL.

Hep-SPION was characterized with a series of techniques including transmission electron microscopy (TEM), dynamic light scattering (DLS), zeta potential and thermogravimetric analysis (TGA). TEM images showed that the magnetite core had an average diameter around 10 nm (Figure 1A), with the hydrodynamic diameters of 68 nm in water and 59 nm in PBS buffer respectively. The successful attachment of heparin was supported by TGA analysis. While the amount of organic compounds only accounted for 3% of the gross weight of SPION by TGA analysis, heating the Hep-SPION to above 700 °C led to 63%

weight loss suggesting that about 60% of the Hep-SPION mass was due to heparin attachment (Figure 1B). Furthermore, the zeta potential of the nanoparticles changed from +12.3 mV (SPION) to -53.3 mV (Hep-SPION), consistent with the high negative charge of heparin on Hep-SPION.

2.2. Assessment of Binding between A β and Hep-SPION by ELISA

With the Hep-SPION in hand, its interaction with A β was investigated first by an enzyme linked immunosorbent assay (ELISA). Naturally isolated A β peptides exist in variable lengths ranging from 36 to 42 amino acid residues. We chose A β 1–42 for our study, as it is the more amyloidogenic A β form.^{27–28} Furthermore, it is prone to aggregation^{6, 29} and is one the major species found in the senile plaques of AD brains.³⁰

For the ELISA, A β 1–42 monomers were dissolved in 10 mM NaOH, neutralized with HCl and were incubated at 37°C for 2 days. The resulting fibrils were added to a 96-well plate, which could adhere to the surface of the wells. Upon removal of the unbound peptide, the bound A β was detected by an anti-A β IgG monoclonal antibody 6E10 (mAb) using a horseradish peroxidase (HRP) conjugated anti-IgG secondary antibody and 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate. The relative amounts of A β bound in each well could be determined from the absorbance at 450 nm.

To test heparin nanoparticle binding, A β fibrils were pre-mixed with varying concentrations of Hep-SPION and added to ELISA plate wells. After incubating overnight and washing off unbound material, the amounts of A β remaining in the well were semi-quantified by ELISA. A concentration dependent decrease in absorbance at 450 nm with increasing amounts of Hep-SPION was observed (Figure 2). This was interpreted that as Hep-SPION bound A β , it should coat the surface of the fibril. As a result, it could shield the A β from adhesion to the plate or sterically block the recognition of A β by the antibody leading to reduced absorbance from the wells. Hep-SPION did not bind to the surface of wells (data not shown), thus precluding the possibility that the decrease in absorbance was due to Hep-SPION passivating the wells. Incubation of A β with uncoated SPION showed no effect on the absorbance, revealing the crucial role of heparin on the surface of nanoparticles (Figure S1). The ability of heparin to inhibit A β adhesion to the plate was also measured, which was similar to that of Hep-SPION.

2.3. Effect of Hep-SPION on A β Aggregation

As Hep-SPION can bind with A β , we analyzed its effects on A β aggregation by native polyacrylamide gel electrophoresis (PAGE). A β monomers (25 μ M) were incubated in the presence or absence of Hep-SPION at 37°C for 2 days followed by analysis via native PAGE. Without any Hep-SPION, A β existed as a mixture of high molecular weight fibrils and a major oligomer species with molecular weight close to that of a trimer (Figure 3A). The formation of the trimer-like oligomer was consistent with the quiescent condition utilized for fibril formation.^{31–32} With increasing concentrations of Hep-SPION (0.0078, 0.0156, 0.0312, 0.125 mg/mL), the relative amounts of the low-molecular-weight A β oligomers decreased (Figure 3A). At the concentration of 0.125 mg/mL Hep-SPION, almost

all A β (>90%) had formed large fibrils appearing at higher molecular weight region on the gel (Figure 3A,B). This suggested that Hep-SPION can reduce the amount of the oligomers.

To further confirm the effect of Hep-SPION on A β aggregation, a thioflavin T (ThT) binding assay was performed. ThT is a cationic benzothiazole dye³³ that displays enhanced fluorescence when interacting with β -sheeted structures (Figure 4, 1st vs 2nd column). While A β monomer can undergo spontaneous aggregation towards fibril (Figure 3a, lane 1), Hep-SPION accelerated β -sheeted fibril formation and gave rise to markedly enhanced ThT fluorescence (Figure 4, 3rd – 5th column). Hep-SPION itself did not result in any fluorescence enhancement of ThT even at the highest nanoparticle concentration tested (Figure 4, 6th column), which excluded the direct effect of Hep-SPION on ThT fluorescence. Uncoated SPION without any heparin did not impact the fluorescence of ThT (Figure S2), which further confirmed the imperative role of heparin.

The disappearance of oligomer upon Hep-SPION incubation (Figure 3) was attributed to the fact that A β peptides exist in a dynamic equilibrium among monomer, oligomers and fibrils.³⁴ Although binding with the monomer and oligomer forms of A β has not been well studied, heparin is known bind to the fibrillar form of A β .^{15, 31} It has been proposed that heparin can function as a structural template and facilitate A β aggregation and the growth to larger sized fibril.¹³ Thus, Hep-SPION can possibly perturb the equilibrium favoring the formation of fibril due to strong binding to the fibril.¹⁵

2.4. Effect of Hep-SPION on A β -Induced Cytotoxicity

Although it remains debatable whether A β peptides cause AD, the toxicity of A β on neuronal cells is an important contributing factor to the pathology of the disease. Among the various forms of A β , the soluble A β oligomers are considered to be the most toxic.^{4, 34} Shifting the equilibrium between the oligomers and fibril towards the more benign fibrils can potentially decrease the adverse effects of A β .^{35–36} As Hep-SPION can reduce the proportions of A β oligomers (Figure 3A), we hypothesized that Hep-SPION could protect neuronal cells from A β induced toxicity.

To test the effects of A β and Hep-SPION on cells, cell viability assays were performed with SH-SY5Y neuroblastoma cells, a common model utilized in A β toxicity studies.^{36–37} Various concentrations of A β were incubated with SH-SY5Y cells in a 96-well cell culture plate. Cells in each well were collected and then mixed with 7-aminoactinomycin D (7-AAD), a fluorescent stain specific to dead cells. The numbers of live and dead cells were counted via fluorescence-activated cell sorting (FACS) with the percentage of live cells without any treatment set as 100%. A β peptide exhibited dose dependent cytotoxicities (Figure S3) with about 75% cell viability when treated with 5 μ M A β . The SH-SY5Y cells were then incubated with A β (5 μ M) in the presence of increasing concentrations of Hep-SPION. As shown in Figure 5, Hep-SPION could protect the cells from A β induced toxicity with 0.01 mg/mL of Hep-SPION enough to fully mitigate the effect of A β on the cells. Hep-SPION by itself did not have a significant impact on viability of the cells, demonstrating the biocompatibility of the nanoparticles. The protective effect of Hep-SPION can potentially be due to two factors: 1) the nanoparticles can induce the transformation of A β into the more

benign fibril form; and 2) by binding with A β , the Hep-SPION can serve as a sink to reduce the amount of A β available for interactions with the neuronal cells.

3. Conclusion

We demonstrated that Hep-SPION could bind A β and heparin was essential for the interaction. Furthermore, Hep-SPION promoted the transition of A β into the more benign fibrils. This in turn could protect neuronal cells from A β induced cytotoxicity. As iron oxide nanoparticles have been widely applied as MRI contrast agents and drug carriers,^{23–25} Hep-SPION can potentially be a useful platform for future imaging and drug delivery studies targeting A β .

4. Experimental Section

4.1. Preparation of A β

A β peptide (0.5 mg) was dissolved in spectroscopy grade 99.9% 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (1.5 mL), sonicated for 15 min, and lyophilized for 72 h. The thin film was then dissolved in 0.22 μ m filtered solution of 10 mM NaOH solution (0.25 mL). The pH of the solution was adjusted to 6 with 10 mM HCl solution and diluted with deionized water to a total volume of 1.0 mL (the concentration of A β stock solution was 100 μ M). For experiments that needed A β fibrils, the stock solution was incubated at 37°C for 48 h.

4.2. ELISA Assay

A β fibrils (100 nM) along with Hep-SPION at different concentrations (0.008, 0.016, 0.031, 0.062, 0.125, 0.25, 0.50, 1.0 μ g/mL) were added into a 96-well plate (100 μ L/well) and incubated at 22°C overnight. All wells were washed with 300 μ L PBST three times and blocked with 1% BSA (300 μ L/well) at 22°C for 1 h. After washing with 300 μ L PBST three times, anti A β 1–16 IgG (6E10) monoclonal antibody (100 μ L/well, 0.82 nM, 1 : 4000 in 1% BSA containing PBS) was added and then incubated at 37°C for 1 h. The solutions were then discarded and washed again with 300 μ L PBST three times. The goat anti-mouse HRP-conjugated secondary antibody (100 μ L/well, 5.1 nM, 1: 6000 in 1% BSA containing PBS) was added into each well and incubated at 37°C for 1 h followed by washing with 300 μ L PBST three times. To a freshly prepared 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (5 mg of TMB was dissolved in 2 mL of DMSO and then diluted to 20 mL with citrate phosphate buffer), 20 μ L of H₂O₂ was added. This mixture (150 μ L/well) was immediately added to the plate and a blue color was allowed to develop for 20 min. The reaction was then quenched by 0.5 M H₂SO₄ (50 μ L/well) and the absorbance was measured at 450 nm on an iMark microplate reader.

4.3. Cell Viability Assay

Different concentrations of Hep-SPION solutions (0.002, 0.02, 0.2, 1 mg/mL) were pre-incubated with or without A β fibrils for 24h and then added into 96-well plate (50 μ L/well). In each well, 2*10⁴ cells were added in 4% serum solution. The final solutions in those wells are A β (5 μ M), Hep-SPION (0.001, 0.01, 0.1, 0.5 mg/mL) in 2% serum (100 μ L/well).

The plate was incubated for 24 h at 37°C. All media were collected in separate eppendorf tubes. Trypsin (50 µL) was then added into each well to digest cells and 4% culture media (200 µL*2) were used to wash wells and combined with original media in the eppendorf tubes. Cells were pelleted by centrifugation and resuspended with FACS buffer (300 µL) in FACS tubes. 7-AAD (3 µL) was added into each tube, followed by incubation at 0°C for 10 min. All solutions were then analyzed by a flow cytometer to evaluate the cell viability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Heparin-coated superparamagnetic iron oxide nanoparticles have been synthesized.
- Heparin nanoparticles can bind with β amyloid and accelerate the formation of fibril.
- Heparin nanoparticles can protect neuronal cells from A β induced cytotoxicity.

Figure 1.
(A) TEM characterization of Hep-SPION; (B) TGA of SPION and Hep-SPION.

Figure 2.

A β binding to plate decreased with increasing concentrations of Hep-SPION. The bound A β was detected by an anti-A β IgG mAb 6E10, followed by addition of HRP-conjugated anti-IgG secondary antibody and the TMB substrate.

Figure 3.

(A) PAGE gel of A β only (lane 1) or A β (25 μ M) incubated with 0.0078 mg/mL (lane 2), 0.0156 mg/mL (lane 3), 0.0312mg/mL (lane 4) and 0.125 mg/mL (lane 5) of Hep-SPION.

(B) Percentage of low-molecular-weight A β oligomer in total A β in presence of various concentrations of Hep-SPION. The percentage was calculated by dividing the intensity of the low molecular weight oligomer band by the sum of the intensities of all bands in the specific lane.

Figure 4.

The intensities of ThT fluorescence at 489 nm ($\lambda_{\text{ex}} = 440$ nm). From left to right: 1) ThT; 2) ThT + A β (25 μM); 3) ThT + A β (25 μM) + Hep-SPION (0.0020 mg/mL); 4) ThT + A β (25 μM) + Hep-SPION (0.0078 mg/mL); 5) ThT + A β (25 μM) + Hep-SPION (0.0312 mg/mL); 6) ThT + Hep-SPION (0.0312 mg/mL). Incubation of A β in the presence of Hep-SPION significantly enhanced ThT fluorescence.

Figure 5.

Cell viability assay of SH-SY5Y cells. Addition of Hep-SPION protected SH-SY5Y cells from A β induced cytotoxicity. Incubation of cells with Hep-SPION (0.5 mg/mL) did not exhibit any cytotoxicity indicating the high biocompatibility of the nanoparticles.

Scheme 1.

Synthesis of heparin coated magnetic nanoparticles by A) the thermal decomposition and ligand exchange method; and B) the co-precipitation method.