Curcumin-conjugated magnetic nanoparticles for detecting amyloid plaques in Alzheimer's disease mice using magnetic resonance imaging (MRI)

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Diagnosis of Alzheimer’s disease (AD) can be performed with the assistance of amyloid imaging. The current method relies on positron emission tomography (PET), which is expensive and exposes people to radiation, undesirable features for a population screening method. Magnetic resonance imaging (MRI) is cheaper and is not radioactive. Our approach uses magnetic nanoparticles (MNPs) made of super-paramagnetic iron oxide (SPIO) conjugated with curcumin, a natural compound that specifically binds to amyloid plaques. Coating of curcumin-conjugated MNPs with polyethylene glycol-polylactic acid block copolymer and polyvinylpyrrolidone by antisolvent precipitation in a multi-inlet vortex mixer produces stable and biocompatible curcumin magnetic nanoparticles (Cur-MNPs) with mean diameter < 100 nm. These nanoparticles were visualized by transmission electron microscopy and atomic force microscopy, and their structure and chemistry were further characterized by X-ray diffraction, thermogravimetric analysis, X-ray photoelectron spectroscopy, time-of-flight secondary ion mass spectrometry, and Fourier transform infrared spectroscopy. Cur-MNPs exhibited no cytotoxicity in either Madin–Darby canine kidney (MDCK) or differentiated human neuroblastoma cells (SH-SY5Y). The P app of Cur-MNPs was 1.03 × 10^{-6} cm/s in an in vitro blood–brain barrier (BBB) model. Amyloid plaques could be visualized in ex vivo T2*-weighted magnetic resonance imaging (MRI) of Tg2576 mouse brains after injection of Cur–MNPs, and no plaques could be found in non-transgenic mice. Immunohistochemical examination of the mouse brains revealed that Cur-MNPs were co-localized with amyloid plaques. Thus, Cur–MNPs have the potential for non-invasive diagnosis of AD using MRI.

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1. Introduction

Worldwide, dementia afflicts about 40 million patients and costs approximately US$0.7 trillion annually [1,2]. Alzheimer’s disease (AD) comprises 60–80% of all dementia cases. AD is a progressive neurodegenerative disorder with no means yet known for prevention or cure. The disease initially affects cognitive abilities, and in the advanced stage, patients lose motor function and require the assistance of others to perform basic activities. Even though the causes of AD have not been clearly identified, brain atrophy, especially around the hippocampal region, is commonly found in AD patients. Soluble amyloid β (Aβ) is normally secreted by brain cells and then cleared from the brain, but in abnormal conditions Aβ aggregates as oligomers and amyloid fibrils. These form plaques, which attract reactive astrocytes and microglia. Aggregates of Aβ may contribute to neuronal damage [3–5]. A potential
difficulty in preventing irreversible brain degeneration in AD, even if effective drugs are developed, is the subtlety of the cognitive changes that occur early in the disease. At the time of diagnosis, AD is usually already at a mild to moderate stage. Current treatments cannot stop progression of the dementia, and therefore recent therapeutic approaches are shifting to early intervention that might halt neurodegeneration before irreversible damage accumulates. This requires more sensitive diagnostic techniques. Since amyloid β plaques can be found in the brain before any cognitive changes [6–8], early AD diagnosis or screening may be aided by methods of visualizing Aβ plaques in vivo, and several studies have been reported in mouse models [9,10].

Current brain imaging of plaques in humans is based on positron emission tomography (PET) [11–13]. In 2012, the FDA first approved a commercially available radiotracer for PET imaging of amyloid plaques in the brain [14]. However, PET scans are limited by low spatial resolution such that individual plaques cannot be visualized. Other drawbacks of PET are high cost, limited availability, and exposure to radiation.

In contrast, MRI does not involve ionizing radiation, and it has superior spatial resolution and is widely available in the clinical setting. However, MRI without contrast agent or amyloid specific probe was reported to lack the sensitivity for early AD diagnosis. In an AD mouse model, only large and mature senile plaques could be visualized, and even those required a high magnetic field (9.4 T) and very long scanning time (several hours) [15–17]. A probe that could specifically bind to Aβ plaques and visualize them in MRI might make MRI a feasible early detection tool for AD.

Recent studies [18–22] show that an amyloid binding tracer conjugated with ultrasmall superparamagnetic iron oxide (USPIO), co-injected with blood–brain barrier (BBB) permeability enhancer (mannitol), can bind to Aβ plaques and enable their visualization by MRI in AD transgenic mice. However, the amyloid β specific tracer that was used, Aβ1–42 peptide, may be neurotoxic and could initiate further Aβ deposition. Another potential difficulty was that the USPIO-Aβ1–42 could not penetrate the BBB, thus mannitol was needed to induce temporary opening of the BBB. These factors may hinder application of USPIO-Aβ1–42 in future clinical trials. The authors have introduced a novel nano formulation using a less toxic fragment of Aβ peptide [23] and co-linking with polyethylene glycol (PEG) to replace co-injection of mannitol [24]. The Aβ peptide and PEG require a special chemical linker (EDC/NHS) to attach to the USPIO surface. The coupling process involves additional steps and chemicals that might introduce cytotoxic effects. As the Aβ conjugated USPIOs were not stabilized or shielded by non-immunogenic molecules, they may have a tendency to agglomerate and be cleared by the RES and spleen filtering [25,26].

In this project, we developed a novel curcumin-conjugated SPIO stabilized by amphiphilic block copolymer (PEG-PLA). The PEG layer can prolong the circulation of nanoparticles in the blood and enhance their penetration through the BBB [27–32]. Curcumin is a natural product extracted from turmeric, the root of the Curcuma longa plant. It has been studied for potential treatment of various diseases, such as cancer, AD and inflammation [33–36]. Curcumin demonstrated safety in a human clinical trial which resulted in no adverse chronic effect during 6 months of high dose consumption (4 g/day) [37]. It also possesses the ability to bind both amyloid β plaques ($K_m = 0.2$ nM) [38,39] and iron [40] by different regions of the molecule. Curcumin naturally binds to the SPIO surface by intermolecular hydrogen bonds, without the need for chemical linkers. Therefore, curcumin is a good candidate for locating amyloid plaques in AD brain. SPIO is a clinically approved contrast agent for MRI, and its safety profile has been well established [41–43]. It shortens the T2 and T2* signals and provides darker color as compared to unlabeled tissues [44]. It provides a high signal to noise ratio and detection sensitivity. Using a multi-inlet vortex mixer (MIVM) and flash nanoprecipitation, curcumin conjugated iron oxide complex selectively partitioned inside the core of a PEG-PLA block copolymer layer in milliseconds. Due to the high energy generated during the rapid mixing, and due to the presence of a hydrophilic barrier on their surfaces, the curcumin magnetic nanoparticles (Cur−MNP) were prevented from aggregating [45–47] and were recovered as stable nanoparticles with mean diameter of <100 nm.

In the current project, Cur−MNP were subjected to a series of characterization studies to determine their size, morphology, structure, and chemistry. These techniques included dynamic light scattering (DLS), atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (ToF-SIMS). Tests were carried out in vitro on Madin–Darby canine kidney (MDCK) and differentiated human neuroblastoma (SH-SY5Y) cells for cytotoxicity and on an MDCK cell monolayer model for blood–brain barrier penetration ability. Optimized Cur−MNP were injected into Tg2576 (Tg) AD mice and age-matched controls and then were subjected to ex-vivo T2* MRI. After MRI scanning, all harvested brains were sectioned, and immunohistochemical staining was applied to sections to correlate with MRI results.

2. Materials and methods

2.1. Materials

Iron (II) sulfate heptylhydrate (≥99%), iron (III) chloride hexahydrate (≥99%), potassium thiocyanate (≥99%), sodium hydride (≥98%, anhydrous pellets), ammonium persulfate (≥98%), polyethylene glycol (MW: 4000, Pharmaceutical grade) and L-ascorbic acid (≥99%) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Curcumin (purity >95%) was synthesized by and purchased from Yung Zip Chemical (Taiwan). PEG-PLA (2 k–8 k) block copolymer was purchased from SRI Biomaterials Inc (USA). Polyvinylpyrrolidone (PVP), BP grade, was from Wing Hing Chemical Company Ltd. (Hong Kong). Organic solvents and reagents used, dimethylformamide (DMF) (reagent grade 99%), ethyl acetate (EA) (reagent grade 99%) and hydrochloric acid (HCl) (analytical grade 37%) were purchased from J&K Chemical Company Ltd. (Hong Kong). Solvents and reagents were used as received. Purified water from a Millipore Direct Q3 ultra-pure water system was used throughout the experiments.

2.2. Synthesis of superparamagnetic nanoparticles

SPIO nanoparticles were synthesized by the reverse co-precipitation method [48], which produced smaller crystals than did normal co-precipitation. The iron solution was made with 1.35 g of FeCl3·6H2O and 0.7 g of FeSO4·7H2O (2 mol Fe3+ : 1 mol Fe2+) dissolved in 20 ml of deoxygenated purified water in a 50 ml round bottom flask in a nitrogen environment and stirred at 900 rpm by a magnetic stirrer. 1.3 g of sodium hydride was dissolved in 20 ml of deoxygenated purified water, and the solution was added drop-wise into the iron solution while stirring at 1200 rpm. Dark black precipitate was formed while the NaOH was mixed with the iron solution. After the addition of all sodium hydride solution, the mixture was kept stirring at the same speed overnight under nitrogen. The resulting black precipitate was washed four times by water, re-suspended in 30 ml DMF, and stored at 4°C.

2.3. Adsorption isotherm of curcumin and iron oxide

The study is based on a published protocol [49] and is briefly described as follows. Iron oxide at pH 5.0 in DMF was mixed with curcumin in DMF at initial concentrations of 2 mg/ml iron oxide and 0.015–1 mg/ml curcumin. The mixture was continuously shaken for 24 h at room temperature while it reached equilibrium. The adsorption capacity of iron oxide for curcumin, $q_v$ (mg/mg), can be calculated from the mass balance by Eq. (1).

$$ q_v = \frac{(C_o - C_f)}{m} V $$

$C_o$ is the initial curcinium concentration in solution (mg/ml), and $C_f$ is the curcumin concentration in solution when the mixture system reaches equilibrium (mg/ml). $V$ is the total solution volume of the mixture (ml), and $m$ is the total mass of iron oxide. The dependent variables, $q_v$ and $C_f$, were found through the experiments, and data were fit to the Langmuir adsorption model (Eq. (6)) in order to determine the adsorption behavior and maximum uptake of curcumin onto iron oxide nanoparticles.
2.4. Synthesis of curcumin magnetic nanoparticles (Cur-MNPs)

2.4.1. Preparation of curcumin conjugated PEG-supported SPIO nanoparticles

Since pure MNPs tend to agglomerate and form larger particles, a small amount of PEG is needed to prevent this and enhance dispersibility of MNPs. An aliquot of MNPs suspension was ultrasonicated (Misonix Microson XL 2000) at amplitude 10 for 2 min. PEG in DMF was then added to 45 (mole ratio with iron oxide) and ultrasonication was resumed for another 3 min. The desired amount of curcumin was dissolved in DMF, added to the PEG-supported SPIO mixture, and sonicated for another 5 min [50]. The solution was then adjusted to a slightly acidic pH (~5.5) using acetic acid. The amount of curcumin loaded onto SPIO was calculated based on the adsorption isotherm results. The maximum mole ratio of curcumin to iron oxide for which curcumin could form a monolayer surface was estimated at 0.05:1, and this ratio was defined as 1X loading, while 4 times this ratio was denoted as 4X (0.2:1), and 8 times was denoted as 8X (0.4:1). The whole process was performed in a nitrogen purged environment, and the particles were stored at 4 °C in order to prevent further oxidation of MNPs. The particle size and zeta potential were monitored by a dynamic light scattering (DLS) analyzer (DelsaNano C, Beckman Coulter, USA) throughout the process.

2.4.2. Coating of curcumin conjugated MNPs with polymers

Curcumin conjugated MNPs were stabilized by coating with amphiphilic polymers, which were deposited on nanoparticles by using the antisolvent principle. Details of the theory and procedures were as described previously with some modifications [51]. Rapid mixing was performed in a multi-inlet vortex mixer (MIVM) with four inlet streams [47]. Two of the inlet streams were de-ionized water, one was organic solvent, and one was PVP in water (concentration ~ 0.43 mg/ml). The organic stream consisted of 80.4 mg of curcumin conjugated SPIO dispersed in water stirred at 600 rpm at 4 °C. The suspension was placed inside a polymer dialysis bag (molecular mass cut-off be- tween 2.4 and 7 kDa) (Spectra/Por, Spectrum, USA). The organic stream consisted of 80.4 mg of curcumin conjugated SPIO dispersed in water stirred at 600 rpm at 4 °C. After dialysis, the nanoparticle size distribution and zeta potential were measured using a dynamic light scattering (DLS) analyzer (DelsaNano C, Beckman Coulter, USA). The particle size distribution and zeta potential of nanoparticles were monitored by a dynamic light scattering (DLS) analyzer (DelsaNano analyzer from Beckman Coulter, USA).

2.4.3. Removal of free solvent and curcumin

Organic solvent was removed from the Cur-MNP suspension by dialysis. The suspension was placed inside a polymer dialysis bag (molecular mass cut-off between 2.4 and 7 kDa) and completely immersed in 24 ml of de-ionized water in a nitrogen purged environment, and the particles were stored at 4 °C in order to prevent further oxidation of MNPs. The particle size and zeta potential were monitored by a dynamic light scattering (DLS) analyzer (DelsaNano analyzer from Beckman Coulter, USA).

2.4.4. Curcumin and MNP loading and encapsulation efficiency

The protocols for measuring curcumin and MNP in the final product are similar to those previously described [51]. Cur-MNP particles were extracted by ultrafiltration. A 0.5 ml sample of Cur-MNP suspension from each of two different stages (freshly made and after dialysis) of the process was placed in the inner tube of a 0.5 ml ultrafiltration tube device (Amicon Ultra-0.5 centrifugal filter 30 kDa) with a 30 kDa cut-off. The filtration tube was centrifuged at 16,000 g for 15 min. The solution, together with non-encapsulated curcumin or MNPs, was filtered from the outer to the inner tube, where Cur-MNPs were larger and were retained. The total curcumin and MNP concentrations of the filtrate (solution filtered out), the concentrate (retained inside the inner tube), and the unfiltered Cur-MNP suspension were measured using UV spectrophotometry at 420 nm for curcumin and 490 nm for MNP [52,53]. The encapsulation efficiency was calculated by Eq. (2).

\[
E(\%) = \frac{\text{unfiltered drug} - \text{free drug in filtrate}}{\text{unfiltered drug}} \times 100
\]

For the MNP and curcumin loading, only one product was tested and lyophilized: after-dialysis Cur-MNPs (8X). The suspension was frozen at –80 °C overnight prior to freeze drying at ~40 °C and 50 μbar in a vacuum dryer (Labconco FreeZone 12L) for 1 day. Dried Cur-MNPs were weighed and tested using a UV spectrophotometer as described previously for determining curcumin or MNP content per unit weight of dried powder. The loading of curcumin and MNP was calculated by Eq. (3).

\[
\text{Curcumin or MNP loading (mg/wt%) = } \frac{\text{mass of curcumin or MNP in dried powder}}{\text{total mass of dried powder}} \times 100
\]

2.5. Characterization of Cur-MNPs formulation

2.5.1. Physical characterization

Lyophilized Cur-MNPs were tested by X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, and thermogravimetric analysis (TGA). XRD was measured using a powder X-ray diffractometer system (PW1830, Philips, Almelo, The Netherlands). Powder samples were evenly dispersed on a grid and were kindly supplied by Prof. Mary Waye (The Chinese University of Hong Kong, Hong Kong). Both cells were cultured and maintained in monolayer conditions in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U penicillin/ml and 10 mg streptomycin/ml) (PS), all supplied by Invitrogen (USA), at 37 °C in an atmosphere of 5% CO2 in 55 cm2 cell culture dishes (Greiner Bio-one, Germany). When cells reached ~80% confluence, subculture was performed by trypsinization with 0.25% trypsin-EDTA (Invitrogen, USA). Three days prior to the cytotoxicity test, 25 μM retinoic acid (Sigma–Aldrich, USA) was added to SH-SY5Y cells to induce differentiation and promote neurite outgrowth [57].
2.6.1. Cell viability test

Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays [58]. Briefly, cells were seeded onto a flat-bottom 96-well plate (Corning Life Science, USA) at a density of 1.5 x 10^4 cells/well. Seeded plates were incubated at 37°C for 24 h. The medium was withdrawn from each well and replaced with 150 μl of desired concentrations of each sample in medium: pure MNPs (120, 75, 30, 15, 6, 3 μg/ml), curcumin (40, 20, 8, 4, 2, 1 μg/ml), 8X Cur-MNPs (167, 125, 50, 25, 10, 5 μg/ml) and blank nanoparticles (particles formed using block copolymer and PVP without curcumin conjugated iron oxide core) (334, 250, 100, 50, 20, 10 μg/ml). Cell culture medium was used as negative control. All samples were dispensed in triplicate for 4 h incubation at 37°C. Then, all medium was withdrawn and replaced with 170 μl MTT solution for 2 h. Finally, all MTT solution was removed, and 200 μl of DMSO was added to each well to dissolve the remaining cells. MTT metabolites were measured by microplate spectrophotometer (Multiskan FC microplate photometer, Thermo Scientific, USA) at 595 nm. The cell viability was calculated by Eq. (4).

\[
\text{Viability} \times 100 = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100\% \tag{4}
\]

The inter- or intra-group results were further analyzed by one-way ANOVA, with p < 0.05 considered to be significant.

2.6.2. MDCK cell monolayer permeability assay

The experimental setup was based on one previously reported [51], with some modifications. Briefly, MDCK cells were cultured, seeded onto Transwell™ (Corning Inc., USA) permeable supports, and grown for four days to reach confluence. To check that the monolayer did not leak, transepithelial electrical resistance (TER) was measured using chopstick electrodes. 8X Cur-MNPs, 8X Cur-MNPs (no polymer coating), 1X Cur-MNPs, 1X Cur-MNPs (no polymer coating) and pure MNPs were tested for transport across the cell monolayer. Initially, 1.5 ml of each sample at concentration 167 μg/ml was loaded in each apical compartment, and 2.6 ml of plain DMEM was placed in the basal compartment. Samples of 500 μl were collected from each basal well at 0, 30, 60, 90, 120, 150 and 180 min while the Transwell™ discs were incubated in a CO2-supplied incubator at 37°C. The cell monolayer and permeable membrane were collected at the end of the experiment (180 min). The concentration of MNPs was measured using UV spectrophotometry.

The apparent permeability coefficient (P_app) was calculated using Eq. (5).

\[
P_{\text{app}} = \frac{\frac{\text{dQ}}{\text{dt}}}{A \times C_0} \tag{5}
\]

The rate of appearance of the drug in the basolateral is dQ/dt. The area of the filter membrane is A, while C_0 is the initial drug concentration on the donor side (apical well).

2.7. In vivo test in AD mouse model

Tg2576 transgenic mice [59] were used for in vivo tests throughout the study. Mice purchased from Taihoku Farms were housed by the Laboratory Animal Services Center of The Chinese University of Hong Kong. The protocols of the animal experiment were approved by the Department of Health of the government of the Hong Kong Special Administrative Region, China. Animal experiments were conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, according to the spirit of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International’s expectations for animal care and use/ethics committees (http://www.aaalac.org/education/module_1.cfm).

For ex vivo MRI scanning and immunohistology analysis, Cur-MNPs were injected into 4 Tg2576 mice (18 months old) and 2 age-matched wild-type control mice. Mice were anesthetized (5 ml/kg body weight) with a mixture of 100 mg/ml ketamine and 10 mg/ml xylazine in phosphate-buffered saline (PBS). Cur-MNPs suspension was diluted with PBS (pH 7.4) to a dose of 0.17 mmol Fe/kg of body weight immediately before injection. A total of 150 μl of diluted Cur-MNPs was injected through the right femoral vein.

2.7.1. Ex vivo MRI brain imaging

All mice were sacrificed 4 h after intravenous injection of Cur-MNPs, and then their brains were extracted. The 4 h timeframe was based on another experiment, in which Cur-MNPs were injected into wild-type control mice, and their iron content was measured in different organs such as liver, kidneys and brain. The results (data not shown) showed that the maximum uptake of iron in brain was between 3 and 5 h, in agreement with a previous report [24]. Mice were first anesthetized and perfused with ice-cold PBS via the right ventricle of the heart. Then the extracted brains were soaked in 4% paraformaldehyde at 4°C overnight. Four brains per batch were fixed inside a plastic tube using 1% agarose gel prior to T7 MRI imaging on an MRI PharmaScan™ 7.0/16.7–10 T (300 MHz 1H) (Bruker Biospin GmbH, Germany). The imaging parameters were set as follows: TR/TE = 464 ms/25 ms, FA = 20°, FOV = 2 cm x 2 cm, Matrix = 400 x 400, in-plane resolution = 50 microns, slice thickness = 0.55 mm, NEX = 80, imaging time = 180 min. The MRI images were analyzed by ImageJ software (version 1.47v), where the proportion of the area occupied by labeled Aβ amyloid plaques in eight serial sections of each mouse brain was measured by a blinded operator. The results were examined by one-way ANOVA (SPSS version 20, IBM) followed by Bonferroni post-hoc test. P < 0.05 represents a significant difference between compared groups.

2.7.2. Immunohistology

After ex vivo imaging, brains were removed from the agarose gel and soaked in 30% sucrose at 4°C until the tissue sank. Brain tissues were sectioned at 40 μm thickness using a Cryotome™ FSE cryostat and were mounted on Super Frost Plus glass slides (Thermo Fisher Scientific, USA). The sections were double stained in order to locate iron oxide and β-amyloid deposition, using red alkaline phosphate substrate kit (Vector Laboratories, USA) for β-amyloid and Prussian blue stain for iron oxide. All sections were first stained overnight with a combination of monoclonal anti-Aβ antibodies, 4G8 and 6E10 (Covance, USA), each at a dilution of 1:50. Then, the immunolabeled sections were treated with alkaline phosphate labeled goat anti-mouse IgG secondary antibodies (Sigma–Aldrich, USA) at 1:50 dilution for 1 h. Sections were further treated with a working solution of alkaline phosphate substrate for 30 min in the dark to ensure better color development. Sections were rinsed with water and further stained by Prussian blue. Briefly, a mixture of 10% potassium ferrocyanide and 20% hydrochloric acid solution was applied to each section for 30 min. Sections were further treated with a working solution of alkaline phosphate substrate for 30 min and dried. The sections were double stained in 0.1% silver nitrate for 30 min to ensure better color development. Sections were rinsed with water and further stained by Prussian blue. The silver nitrate solution was applied to each section for 30 min, and the sections were double stained in 0.1% silver nitrate for 30 min. Sections were further treated with a working solution of alkaline phosphate substrate for 30 min and dried.

3. Results

3.1. Particle size distribution of Cur-MNPs

Table 1 and Fig. 1A show the mean hydrodynamic particle size, distribution and polydispersity index (PDI) for Cur-MNPs using different amounts of conjugated curcumin, either freshly made or after dialysis. Results revealed that MNPs without curcumin had a large mean particle size (~222 nm). For 1X and 4X curcumin loading
Curcumin to iron mole ratio of 0.05:1 and 0.2:1, respectively). The freshly made mean particle size was less than 80 nm, but after 24 h of dialysis, the size exceeded 200 nm, similar to MNPs without curcumin. The size distributions of the above formulations were broad (PDI > 0.2). On the other hand, particles made with 8X curcumin loading (curcumin to iron mole ratio of 0.4:1) maintained their size during dialysis (<100 nm) and showed a narrow size distribution (PDI ~ 0.14).

The particle size data suggest that the curcumin loading is a critical factor for the stabilization of the nanoparticle. Fig. 1B shows the TEM images of MNPs with or without curcumin. The individual iron oxide core was ~9 nm whether or not curcumin was added, demonstrating that the iron oxide core would not aggregate or change in size throughout the synthesis process.

AFM images (Fig. 1C) show the morphology, height and phase differences of Cur-MNPs (8X). The height in AFM was >50 nm (1C, left), in the same range as the diameter measured by dynamic light scattering (DLS): ~90 nm. The phase image (1C, right) revealed that the TEM images of MNPs with or without curcumin. The individual iron oxide core was ~9 nm whether or not curcumin was added, demonstrating that the iron oxide core would not aggregate or change in size throughout the synthesis process.

AFM images (Fig. 1C) show the morphology, height and phase differences of Cur-MNPs (8X). The height in AFM was >50 nm (1C, left), in the same range as the diameter measured by dynamic light scattering (DLS): ~90 nm. The phase image (1C, right) revealed that particles consisted of three distinctive layers of materials of different relative “hardness”: a soft outer rim and a hard core.
Fig. 2. Physical characterization of curcumin-magnetic nanoparticles. (A) Adsorption isotherm of Cur-MNPs and fitting to the Langmuir isotherm model (inset). (B) X-ray diffraction (XRD) patterns of Cur-MNPs at different stages of formulation. The MNP core size is ~9 nm, which can be calculated by the Scherrer equation. (C) Fourier transform infrared spectra with some specific bond peaks for pure curcumin, pure MNPs, MNPs physically mixed with curcumin and curcumin conjugated iron oxide complex. (D) Fourier transform infrared spectra with some specific bond peaks for pure polyvinylpyrrolidone (PVP), polyethylene glycol poly(lactic acid) diblock copolymer (PEG-PLA) and Cur-MNPs. (E) Thermogravimetric analysis (TGA) shows the percentage of total weight loss of pure iron oxide, curcumin conjugated MNPs and Cur-MNPs.
sandwiching a layer of intermediate hardness. Fig. 1D shows the step-wise process of making the Cur-MNPs and the final layout of each coating.

3.2. Curcumin and MNP entrapment efficiency and loading

Table 1 lists the entrapment efficiency of curcumin and MNPs in Cur-MNPs for various curcumin loading conditions and stages of formulation. For freshly made nanoparticles, entrapment efficiency was over 90% for either curcumin or MNPs regardless of the curcumin loading. However, after dialysis, the entrapment of both MNPs and curcumin dropped considerably, except for the highest curcumin loading (8X).

Since Cur-MNP (8X) was the most stable formulation, only this formulation was analyzed for the loading of MNPs and curcumin. The calculated loading of MNPs and curcumin was 15% (w/w) and 8% (w/w), respectively.

3.3. Stability of nanoparticles

The zeta potential of fresh Cur-MNPs (Table 1) was nearly zero but became quite negative after dialysis, except for high curcumin loading (8X). Correspondingly, some degree of particle precipitation was found at 4°C storage after 7 days, except for 8X curcumin loading. Since the Cur-MNP (8X) formulation was the most stable, this formulation was selected for further characterization and for in vitro and in vivo tests, unless stated otherwise.

3.4. Physical characterization of Cur-MNPs

3.4.1. Adsorption isotherm and binding affinity between curcumin and MNPs

Fig. 2A shows the adsorption isotherm for curcumin and MNPs. The MNP adsorption capacity for curcumin increased asymptotically to a plateau at ~0.8 mg/ml. Regression analysis of the curcumin partition ratio (i.e., curcumin aqueous concentration divided by concentration on MNP solid at equilibrium) versus the curcumin aqueous concentration at equilibrium for the Langmuir isotherm model (Fig. 2A inset) produced high linearity (R² = 0.996). The Langmuir isotherm model is expressed by the following Eq. (6):

\[
\frac{C_e}{q_e} = \frac{1}{Q_m b} + \frac{1}{Q_m} \frac{C_e}{q_e}
\]

where \(C_e\) (mg/ml) and \(q_e\) (mg/mg) are the equilibrium concentrations of curcumin in aqueous solution and on MNP surface phases, respectively. \(Q_m\) (mg/g) is the maximum monolayer coverage of curcumin on MNPs and is governed indirectly by the strength of adsorption.

The adsorption of curcumin on MNPs follows the Langmuir isotherm model indicating adsorption of curcumin on homogeneous surface sites of MNPs. The maximum curcumin uptake was calculated to be ~75 mg of curcumin adsorbed per gram of MNPs. According to Saha et al. [49], the binding affinity between curcumin and MNPs is categorized as a “favorable binding.”

3.4.2. X-ray diffraction of MNPs and Cur-MNPs

The characteristic XRD peaks for MNPs with or without curcumin (Fig. 2B) were found at 2θ = 30.1°, 35.5°, 43.2°, 53.6°, 57.0°, and 62.6°, belonging to (220), (311), (400), (422), (422), and (511) Bragg reflection of Fe₃O₄ standards from a JCPDS file (PDF no 65–3107). The Fe₃O₄ nanoparticles had a cubic spinel structure that was unchanged throughout the fabrication process. The absence of 31° peaks, which represent γ-Fe₂O₃ and α-Fe₂O₃, demonstrate that these were not produced from oxidation of magnetite (Fe₃O₄) [60]. The purity of Fe₃O₄ in the core of Cur-MNPs was further confirmed by these XRD results.

3.4.3. FTIR and TGA analysis of nanoparticles

Cur-MNPs and physically mixed curcumin and MNPs showed distinctive FTIR spectra (Fig. 2C). The physical mixture mainly displayed the corresponding peaks of curcumin and MNPs: the broad stretching hydroxyl peak between 3500 and 3000 cm⁻¹ for both curcumin and Fe₃O₄, the characteristic sharp peak of C–O/C=O stretching at 1628 cm⁻¹, the peak at 1603 cm⁻¹ of symmetric aromatic C–C, enolic COH peaks of curcumin at 1429 and 1376 cm⁻¹, and the strong Fe–O peak at 575 cm⁻¹.

In curcumin conjugated MNPs, however, the peaks at 1628, 1603, 1429 and 1376 cm⁻¹ were diminished, and a strong peak at 1654 cm⁻¹ appeared, representing a conjugated C–O bond [50,61,62]. The O–H peak broadened and shifted to 3422, meaning that more hydroxyl bonds formed in the conjugated product, while the enolic (COH) peaks were not found, consistent with the C=O...
and COH in the central part of curcumin molecules participating in forming C=O conjugated bonds with Fe$_3$O$_4$ by hydrogen bonding. The Fe–O peak was not altered and remained intact during the process.

Fig. 2D shows the spectra of the final, polymer-coated product, Cur-MNPs, and the polymers PVP and PEG-PLA (2k–10k). The Cur-MNPs inherited the characteristic peaks of PVP, PEG, and PLA, such as N–C=O at 1654 and C–H at 2955 cm$^{-1}$ for PVP, O–C==O at...
1752 cm$^{-1}$ for PLA, and C–O–C at 1094 cm$^{-1}$ for PEG. The strong Fe–O peak had a blue shift from 575 to 583 cm$^{-1}$, which was due to the finite size of Fe$_3$O$_4$ [63]. These results showed that the encapsulation process indeed prevents the agglomeration of MNPs by protecting them inside the polymer coating.

TGA (Fig. 2E) left 94% of the weight of MNPs without curcumin. This 94% represents the net iron oxide content. The remaining 6% comes from other constituents such as adsorbed moisture and organics on iron oxide surface. For curcumin conjugated MNPs, the net iron oxide content was 36%, and the remainder was other constituents as above, plus curcumin and PEG. For the final product, Cur-MNP (8X), net iron oxide content was 14%. The remaining 86% was from curcumin, PEG, and the polymeric coatings that consist of 2k–10k PEG-PLA block copolymer, PEG and PVP.

### 3.4.4. XPS: A bottom-up approach to verify the surface coating of Cur-MNPs

XPS confirmed the expected coating of Cur-MNPs. Fig. 3A shows the binding energy spectrum of pure Fe$_3$O$_4$: the 2p$^{3/2}$ and 2p$^{1/2}$ peaks for iron oxide, located at 710.9 and 724.5 eV, respectively, conform to the spectrum of magnetite, as reported earlier [64,65]. There is only a minimal amount of carbon: the 1s peak located at 285 eV. The carbon content comes from organic contaminants in ambient air.

Fig. 3B shows the intermediate product, curcumin and PEG conjugated on the iron oxide surface. The carbon peak intensity was much higher while the iron peak was lower than that of pure iron oxide nanoparticles, due to the presence of more organic materials (curcumin and PEG) on the surface.

XPS of the final product, Cur-MNPs, showed (Fig. 3C) no iron oxide peak, as it was encapsulated within the block copolymer (2k–10k PEG-PLA) and PVP. Because the final product revealed a peak for nitrogen at 1s (399 eV), which was not detected in the pure iron oxide or intermediate samples, and because the only nitrogen-containing component was PVP, PVP was present mostly on the surface of Cur-MNPs. The peak for carbon at 1s (285 eV) was much higher than that of the intermediate product shown in Fig. 3B, consistent with having more organic compounds on the iron oxide surface.

The atomic concentrations (%) of different stages of product were measured by XPS (Table 2). The Fe signal decreased from high to minimal as pure Fe$_3$O$_4$ was converted to Cur-MNPs, while the carbon signal increased as the organic coating was added layer by layer. A detectable amount of nitrogen was only found in Cur-MNPs.

### 3.4.5. ToF-SIMS: a top-down approach to verify the surface coating of Cur-MNPs

The ToF-SIMS results in Fig. 4 represent samples of pure iron oxide, intermediate (curcumin and PEG conjugated iron oxide) and final product (Cur-MNPs), respectively. The positive ion mass data were used to analyze the depth profile of all samples. Fig. 4A shows no other ion mass signals except Fe, m/z = 55.9. As the sputtering deepened, the Fe intensity decreased somewhat and then stabilized, reflecting the roughness of the sample.

For the intermediate product (Fig. 4B), the characteristic ion mass signals of PEG, m/z = 45.0, and curcumin, m/z = 177.1, decrease with depth while the Fe signal increases. These data show that curcumin and PEG are found predominantly on the surface, while iron oxide lies deeper, with the Fe signal reaching a plateau at a depth of ~15 nm.

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Atom peak, atomic concentration (%)</th>
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<tr>
<td></td>
<td>Fe 2p (710.9 eV)</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>36.9</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ + Cur + PEG</td>
<td>18.5</td>
</tr>
<tr>
<td>Cur-MNPs</td>
<td>0.1</td>
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</tbody>
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Fig. 5. In vitro tests of Cur-MNPs and different components. (A) Cytotoxicity test of curcumin, pure MNPs, empty nanoparticles (polymer formed nanoparticles without payload), Cur-MNPs (1X) and Cur-MNPs (8X) on wild type MDCK and (B) Differentiated human SH-SY5Y (bottom) cells. Different components with matched concentration of Cur-MNPs are tested. Cell viability <80% indicates toxicity. (C) In vitro cell monolayer permeability test of 8X Cur-MNPs, 8X Cur-MNPs (no polymer coating), 1X Cur-MNPs, 1X Cur-MNPs (no polymer coating) and pure MNPs. Values shown are iron mass concentrations in receiver compartment.
In the final product (Fig. 4C), the ion mass signals of PEG, curcumin and iron oxide have patterns similar to those of the intermediate product. Moreover, the mass signal profiles of PVP, m/z = 112.1, and PLA, m/z = 560.0, (from block copolymer), which were only introduced in the final product, follow those of PEG and curcumin: decreasing with depth. These findings clearly demonstrate that the PVP, block copolymer PEG-PLA and curcumin are coated on the iron oxide core surface, following exactly the engineered design.

3.5. Cur-MNPs tested in vitro

3.5.1. Cell toxicity test in wild type MDCK and SH-SY5Y human neuroblastoma cells

The toxicity of different stages of preparation of Cur-MNPs was tested on wild type MDCK and human neuroblastoma SH-SY5Y cells (Fig. 5A and B). The concentrations of MNPs and curcumin that were tested match the concentrations of MNP and curcumin in Cur-MNPs. For both cell lines, even the highest concentrations of MNPs (120 μg/ml), curcumin (40 μg/ml), Cur-MNPs 8X or 1X (167 μg/ml) or nanoparticles made by polymer without curcumin and MNPs (blank NPs) (167 μg/ml) were non-toxic, as defined by cell viability falling between 80 and 120% of the viability of untreated cells. One-way ANOVA showed p > 0.05 between and within groups.

3.5.2. Cell monolayer permeability test

Fig. 5C shows the concentration of Fe versus time in the receiver compartment of an in vitro MDCK cell monolayer model (r² of calibration curve for Fe was 0.9999 (n = 10). The limit of quantification (LOQ) of Fe was 0.39 μg/ml in PBS). From the plot, only Cur-MNPs (8X) produced detectable Fe in the receiver compartment, while none was detected in 1X formulation, pure MNPs or Cur-MNPs without polymeric encapsulation. For those formulations without detectable Fe in the receiver compartment, the calculated Papp was the same age after Cur-MNP injection. No iron oxide signal was detected by MRI (left). As expected, no amyloid plaques (red) or iron (blue) were detected on the matched histology section (right).

3.6. In vivo test on Tg2576 and control mice

3.6.1. Ex vivo magnetic resonance images

The brains of 18 month old Tg2576 transgenic APP and littermate control mice, injected with Cur-MNPs (8X), were harvested and scanned by 7-T MRI. Fig. 6A shows serial coronal magnetic resonance images around the middle part of the brains for a control mouse (left) and a Tg2576 mouse (right). Many dark spots were found in Tg2576 brain, while almost no spots were found in the control brain.

Fig. 6B shows the fraction of brain area labeled by Cur-MNPs under MRI. Eight serial coronal sections from around the middle part of each brain were examined and showed that all Tg mice significantly differed from controls, with p values ranging from less than 0.0001 to 0.028.

3.6.2. MRI and immunohistochemistry of amyloid plaques

Fig. 6C shows a typical brain section of a Tg2576 mouse injected with Cur-MNPs. The red spots indicate amyloid plaques stained by a mixture of 4G8 and 6E10 monoclonal antibodies to Ab peptide, and blue indicates iron oxide. The magnified view demonstrates that iron oxide was in close proximity to an amyloid plaque.

Fig. 6D shows a matched MRI (left) and double-stained Tg2576 mouse brain section (right). Many of the dark spots found in MRI co-localized with immunolabeled amyloid plaques (red) and iron oxide (blue). The inset is a 40x magnification of the selected area, showing amyloid plaques (red) and iron (blue) in the bright field image (left) and plaques (orange) in the fluorescent image (right). The fluorescence was orange because curcumin emits at 520 nm, while the fluorescent label used for immunolabeling the plaques emits at 570 nm. The combination of these yellow and red emitted signals results in a bright orange color. The images show that Cur-MNPs are able to bind amyloid and can be visualized by MRI or immunohistochemistry.

Fig. 6E shows a section of a non-transgenic control mouse of the same age after Cur-MNP injection. No iron oxide signal was detected by MRI (left). As expected, no amyloid plaques (red) or iron (blue) was detected on the matched histology section (right).

Brain sections of Tg2576 mice injected with Cur-MNPs (Fig. 6F) were observed for co-localization of curcumin, iron oxide and amyloid plaques. In the insets, the bright views (left) show iron oxide in blue, and the fluorescent views (right) show amyloid plaques in red and curcumin in yellow (or orange for co-localized amyloid and curcumin).

4. Discussion

4.1. Stability and physical characterization of Cur-MNPs

Recent reports describe a number of curcumin-modified magnetic nanoparticle formulations [50,60,66–68]. These formulations have successfully loaded curcumin onto MNPs through different chemical linkages or adsorption onto a hydrophobic medium. However, they have a number of flaws, such as 1) treating curcumin as a payload which is released from the MNP surface rather than as a linker between target and MNP, 2) not protecting MNPs by inert material encapsulation to prevent rapid clearance by the reticuloendothelial system (RES) [26] or curcumin release before reaching the target sites, 3) not stabilizing MNPs with surfactant, producing unstable particles whose sizes are difficult to control and which are susceptible to coalescence [69–71] to form large aggregates [72], 4) using chemical linkers which may incur cytotoxicity, and 5) not targeting penetration of the blood–brain barrier.

An ideal nanoparticle formulation should be stable and small (<100 nm) in order to circulate in the smallest capillaries and escape from filtration by the spleen [25,73]. The delivery package should be protected from opsonin protein uptake in order to enhance the half-life of nanoparticles in blood circulation. In addition, all the materials involved should be biocompatible. Most
Fig. 6. Ex vivo $T2^*$ MRI and histochemical staining of 18 month old Tg2576 and control mice injected with Cur-MNPs (8X). (A) Cur-MNPs label plaques in Tg2576 mice (left) but not control mice (right). (B) Statistical analysis of the MRI images for area occupied by Aβ plaques (%) in control and Tg mice. All Tg mice showed significantly different plaque area than age-matched controls. One way ANOVA examined differences between groups, resulting in $p < 0.0001$, and post-hoc test (Bonferroni) found individual $p$ values between each control and each Tg mouse (shown in the figure). (C) Bright view of histochemically labeled mouse brain section. Immunohistochemically labeled amyloid plaques (red) and Prussian blue stained iron oxide (blue). Inset: 40X magnification of a region displaying co-localization of iron oxide with a plaque. (D) Match between the black dots of MRI (left) and plaques labeled immunohistochemically (red) and by Cur-MNPs (blue). Left inset: iron (blue) anchored on plaques (red). Right inset: curcumin (fluorescent) co-localization on the plaques. (E) Matched brain section of MRI and histochemically labeled aged control mouse. No dark spots or amyloid/iron are seen in the sections. (F) Triple-labeled amyloid plaques in Tg mouse brain section. Insets: 100X magnification of plaques. Immunohistochemically labeled amyloid plaques (red), curcumin (yellow fluorescence in insets) and iron (blue) co-localized in some plaques. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
important, the nanoparticles should be able to pass through the blood–brain barrier and specifically target amyloid plaques.

The novel curcumin-conjugated MNPs described in this work have the desired attributes. Characterization of the Cur-MNPs revealed details of their architecture as well as chemistry, providing indication of their stability under stringent physiological conditions.

A critical factor in making Cur-MNPs is the binding affinity between curcumin and MNPs. Studies by Saha et al. [49] showed that MNPs adsorb dyes containing hydroxyl groups at specific ranges of pH and concentrations. Since curcumin contains hydroxyl groups, it was postulated that it can bind to an MNP surface. The adsorption isotherm study (Fig. 2A) confirmed that curcumin preferentially adheres (following a Langmuir model) to MNPs without the need of chemical linkers. The enolic and dione groups of curcumin participated in forming a C—O conjugated bond with MNPs (Fig. 2C) [50]. Once curcumin bonds to MNPs, the MNPs become hydrophobic.

Since the antisolvent principle is used to flash nanoprecipitate the polymer (as an outer layer) onto a curcumin-conjugated MNP core, the surface coverage of curcumin on MNPs, and thus their
were coated (Fig. 3A) and PVP, which has no other components in Cur-MNPs contains nitrogen. The Fe 2p peak decreased and the C 1s peak increased as the nanoparticles coated. One of the major findings: 1) the coating materials completely covered the iron oxide core, 2) PVP was on the outer layer of the Cur-MNPs [76].

4.2. Analysis of the surface coating of Cur-MNPs by XPS (bottom-up approach)

Since XPS is a sensitive quantitative technique to measure the surface elements of composite samples, when the surface materials are very different than the core (i.e., oxidation states) or form specific bonds, XPS can be very sensitive. The N 1s peak belongs to PVP as no other components in Cur-MNPs contain nitrogen. The Fe 2p peak decreased and the C 1s peak increased as the nanoparticles coated (Fig. 3A–C and Table 2). Also, the coating on the surface was uniform, covering the entire iron oxide core surface. These demonstrated two major findings: 1) the coating materials completely covered the iron oxide core, 2) PVP was on the outer layer of the Cur-MNPs [76].

4.3. Depth profile of Cur-MNPs by ToF-SIMS (top-down approach)

XPS can only explain the nanoparticle properties near the surface (depth ~5–10 nm). ToF-SIMS sputter depth profiling can provide additional information on the ensemble. However, several studies attempted to use ToF-SIMS sputter depth profiling to reveal coating patterns, but problems emerged, such as: 1) coating layers fused with the core of the nanoparticle during ion bombardment, preventing the characteristic mass fingerprint of each material from being clearly differentiated, and 2) many nanoparticle formulations used insulating or uncharged materials, which destabilize the sputtering rate, resulting in an inconsistent profile [56].

In our case, the iron oxide core is inorganic and very hard, and it is not infused with organic layers during ion bombardment. As a result, the sputter depth profile exhibited two distinctive groups of mass spectra, which were iron mass fragments of the inorganic core and organic layers. Also, the iron oxide core is a conductor; thus, no charge compensation was required during measurement, and the sputtering rate was consistent among all samples. In addition, the iron oxide core functions as a supporting layer, hence, no particles emerged during ion bombardment when high energy was applied. If the nanoparticle contained only soft organic materials, fusion between particles could happen during the ion sputtering process, introducing artifacts.

In general, the molecular ion mass fingerprint of each compound was identified. The depth profile clearly showed that the components of the organic layer, consisting mainly of PVP, PEG-PLA and curcumin, were abundant on the surface and sparse towards the interior. On the other hand, the Fe intensity was low on the surface and abundant towards the interior, where organic materials were absent (Fig. 4). The pure iron oxide depth profile (Fig. 4A) showed a gradual decrease in mass intensity with increasing depth. This phenomenon may be explained by the uneven distribution of the iron oxide surface: when the incident ion beam penetrated deeper, the iron oxide surface became more even, resulting in constant mass intensity. The results also indicate that, in encapsulated nanoparticles, the iron core is more evenly distributed than in non-encapsulated iron oxide nanoparticles, suggesting that the polymeric stabilizers effectively encapsulated the nanoparticles.

Both the bottom-up and top-down analysis approaches point to a structure of Cur-MNPs in which the polymeric layers are on the exterior of the nanoparticles while curcumin is on the iron oxide surface. Thus, the iron oxide core is well embedded inside the polymeric shell and protected from coalescence, which is a common problem in nanoparticle formulation due to their high surface energy [69–71]. As we have previously published, the stability of our nanoparticles is based not on electrostatic repulsion but on the neutral molecules PEG and PVP providing steric hindrance to nanoparticle fusion [51].

4.4. In vitro toxicity and monolayer permeability test

Cell toxicity tests demonstrated that no individual raw materials or finished products at two different curcumin loading ratios, 8X or 1X Cur-MNPs, were toxic to wild type MDCK or human neuroblastoma SH-SYSY cells. These results confirm that each component, PEG-PLA block copolymer, PVP, curcumin and MNPs, is biocompatible when used alone [71,78–80] or in the final product, Cur-MNPs. From our previous study [51] on a nanoparticle formulation of curcumin, nanoparticles of curcumin had significantly higher permeability than did ordinary curcumin. Nanoparticles of the current formulation, Cur-MNPs (8X), exhibited \( P_{\text{app}} = 1.03 \times 10^{-6} \) cm/s. Wang et al. [81] studied central nervous system drugs on an MD-R-MDCK cell line and a rat brain perfusion model, concluding that compounds with \( P_{\text{app}} > 3 \times 10^{-6} \) cm/s had high brain uptake potential, while compounds with \( P_{\text{app}} < 1 \times 10^{-6} \) cm/s were unable to penetrate the BBB. According to our findings, Cur-MNPs (8X) would be categorized as able to pass through the BBB.

Neither the non-encapsulated curcumin-conjugated MNPs nor the unstable 1X formulation of Cur-MNPs could be detected in the receiver compartment of the monolayer model. Thus, two factors may govern the permeability of Cur-MNPs: 1) the polymeric surface hydrophobicity, is critical for stabilizing the nanoparticles. This effect is observed in the studies on nanoparticle size, zeta potential and entrapment efficiency. These factors deteriorated when the loaded curcumin was decreased from 8X to 1X (Table 1). The trends reveal that at least an 8-fold excess of the minimum amount of curcumin needed to coat the surface in a monolayer must be loaded to fully encapsulate the curcumin-conjugated MNP core within a polymeric shield. Consistent with this, TGA results show that the curcumin coating comprises 7.3–8% (w/w) of the Cur-MNPs.

The XRD, FTIR, XPS and ToF-SIMS results provide evidence that the coating layers fused with the core of the nanoparticle during ion bombardment. This demonstrates that during the storage and fabrication process, entry of oxygen into the system was effectively minimized. Since magnetite is well documented to be more biocompatible than maghemite [74], ensuring that the core of the nanoparticle consists of magnetite enhances its biocompatibility. In addition, magnetite, due to the difference of its spinel structure from that of maghemite, has higher net spontaneous magnetization. At 300 K, magnetite is 92 emu/g while maghemite is 78 emu/g [75].

PVP and PEG-PLA (block copolymer) coated the curcumin-conjugated MNP core but did not appear to form direct chemical bonds to the core, as indicated by the absence of new bonds. The characteristic bonds such as N=C=O and C=H for PVP, O=C=O for PLA and C=O=C for PEG (Fig. 2D) reside mostly on the surface, consistent with encapsulation. Moreover, the overall content of PVP, PEG-PLA and iron oxide are much higher than curcumin (shown in Fig. 2E, TGA results), thus the signature peaks of curcumin were masked by them. Specifically, the conjugated C=O peak at 1654 cm \(^{-1}\), which appeared for both curcumin-conjugated MNP (Fig. 2C) and PVP (Fig. 2D), could not be clearly differentiated by FTIR. Therefore, FTIR has its limitations for characterizing the surface composition of nanoparticles. Thus, XPS and ToF-SIMS were used to analyze the surface coating of Cur-MNPs. By building up the layers one after another and examining by XPS, the elemental composition of each layer can be explored (bottom-up approach). On the other hand, the final product can be examined by depth profile using ToF-SIMS (top-down approach).
encapsulation, and 2) the stability of nanoparticles. Non-encapsulated nanoparticles or Cur-MNPs (1X) appear as precipitates or aggregates of particles and thus can undergo neither transcytosis nor paracellular passive diffusion through tight junctions. On the other hand, the polymeric shield of Cur-MNPs (8X) was intact, and these particles were suspended in the medium without aggregating. Thus, these small particles (<10–20 nm) may be able to penetrate the cell monolayer, perhaps by transcytosis or via a paracellular pathway by passive diffusion.

The P_{app} of Cur-MNPs was 1.03 × 10^{-6} cm/s, which is similar to the P_{app} we had previously reported for nanocurcumin, 1.8 × 10^{-6} cm/s [51]. In that study, we concluded that nanocurcumin was transported into cells, either by transcytosis and/or endocytosis. The similarity of the P_{app} values for Cur-MNPs and nanocurcumin suggests that Cur-MNPs may follow the same penetration pathway, entering cells by transcytosis or endocytosis.

In summary, the 8X formulation of Cur-MNPs exhibited a much greater brain uptake potential than all other tested formulations. The mechanism by which the nanoparticles penetrate the cell monolayer may be transcytosis and/or passive diffusion through tight junctions.

4.5. Ex vivo MRI and immunohistochemistry

The ex vivo magnetic resonance images (Fig. 6A, D, and E) and immunohistochemical staining of the same brains demonstrate that the dark spots found in MRI of Tg mice are amyloid plaques labeled with Cur-MNPs. The area occupied by plaque in MRI differed significantly between Tg and control mice, providing evidence that Cur-MNPs can pass through the BBB, bind specifically to Tg mice, and differentiate them from controls. Further evidence for this is the co-localization of curcumin, iron, and amyloid in Tg mouse brains (Fig. 6D and F). On the other hand, MNPs (without curcumin or polymers) are unable to pass the BBB [82].

These results suggest two possible mechanisms by which Cur-MNPs reach the brain: passing through the possibly disrupted BBB of Tg mice or penetrating BBB endothelial cells by transcytosis. Evidence suggests an intact BBB in AD model Tg mice [83,84]. Wadghiri et al. [24] found that USPIO and USPIO-Aβ1-42 reached the brain of APP/PS1 transgenic mice only if they were co-injected with a BBB permeation enhancer, suggesting the integrity of the BBB in that model. If the BBB of our mice were compromised, Cur-MNPs may be expected to be distributed irregularly, following the distribution of BBB damage rather than the distribution of plaques, but MRI showed co-localization with plaques. By contrast, several studies of USPIO distribution in neuroinflammatory or BBB damaged rat models resulted in a more heterogeneous pattern of particles inside the brains [85–87]. Therefore, the mechanism by which Cur-MNPs enter the brain likely does not involve a disrupted BBB in AD model mice.

Another possible mechanism is transcytosis, which is facilitated by properties of Cur-MNPs including their small size and their PEG layer, which prolongs blood circulation and which interacts with BBB endothelial cells. Studies [25,88,89] showed that particles <100 nm exhibited significantly greater bioavailability than larger particles. Thus, our final nanoparticle size distribution (<100 nm) was suited for brain targeting. In addition, a number of studies [26,90,91] have revealed that PEG coating of nanoparticles reduces the opsonization process in which nanoparticles associate with opsonin proteins and are then recognized by the RES system and cleared through the liver and/or spleen. The opsonin proteins bind poorly to PEGylated nanoparticles, which, as a result, become “stealthy” and remain circulating inside the body. However, longevity in blood circulation is not the only factor that determines whether nanoparticles can pass through the BBB. Calvo et al. [27,28] reported on a polymer for which having a longer circulation time did not necessarily result in higher brain uptake. He compared poloxamine 908 coated nanoparticles versus PEGylated nanoparticles, and even though the former circulate much longer in blood than do the latter, the poloxamine NP still could not penetrate the BBB and reach the brain. Therefore, the ability of PEG to interact with BBB endothelial cells and mediate transcytosis may be independent of its ability to keep NP circulating a long time. Our Cur-MNPs inherited these abilities from PEG, making Cur-MNPs suitable for brain targeting.

In our design, curcumin functions as a linker between amyloid plaques and iron oxide. Adsorption isotherm results of curcumin and iron oxide show that they preferentially bind each other. Published results showed a high binding affinity between curcumin and Aβ (1–40) aggregates (K = 0.2 nM) [38]. From our immunohistostaining and ToF-SIMS results, it can be seen that curcumin remained on the MNP surface throughout the procedure. Even after Cur-MNPs were injected into mice, curcumin did not delaminate but remained on the particles and eventually anchored them to amyloid plaques.

When comparing our nanoparticles with those designed by Wadghiri et al. [18–22,24] using a similar approach, it was reported that most of the nanoparticle formulations, except one recent design which incorporated PEGylated USPIO, require co-injection of mannitol in order to enhance the passage of the nanoparticles through the BBB. In their formulation, Aβ1-42 peptide or the non-toxic fragment Aβ1-30, rather than curcumin, is used as the plaque ligand, and it is chemically anchored to the USPIO surface [23]. Because our particles do not require such a chemical linkage, they are simpler to synthesize. The components-polymer stabilizers, excipients, curcumin, and MNPs—are biocompatible and widely used in therapeutic applications [37,78,80,92]. Furthermore, in vitro toxicity results demonstrate that Cur-MNPs were not cytotoxic even at a very high concentration (167 μg/ml). Another advance of this novel nano formulation is the ability of PEG in the block copolymer PEG-PLA to prolong circulation time and minimize RES uptake and spleen filtering. Most important is that PEG interacts with BBB endothelium cells and initiates transcytosis of nanoparticles through the BBB. Thus, it is not necessary to co-inject BBB permeability enhancing agents with our formulation. This may have improved uptake of particles into the brain and thus permitted a shortened MRI scanning time.

5. Conclusions

We have demonstrated that curcumin can naturally bind to the MNP surface and that the curcumin conjugated MNPs can be further stabilized by PEG-PLA block copolymer and PVP polymer. Cur-MNPs have a narrow size distribution (PDI = 0.14) and a mean particle size under 100 nm. The particles show low cytotoxicity and exhibit BBB penetration potential in an in vitro monolayer cell permeability test. In vivo, the particles can penetrate the BBB of both Tg2576 AD model and non-transgenic mice. Cur-MNPs bind amyloid plaques in mouse brains, as shown by multiple detection methods. T2* ex vivo MRI reveals more dark spots in Tg than control mice. Many of the dark spots aligned with amyloid plaques on immunohistochemically stained sections matched with magnetic resonance images. Iron staining, fluorescence, and immunohistochemistry revealed co-localization of MNPs and curcumin on amyloid plaques. Therefore, Cur-MNPs are novel nanoparticles with potential use for visualizing amyloid plaques in AD patients. Further in vivo MRI testing in AD mice models is required to further elucidate the potential of Cur-MNPs for early diagnosis of AD.


