Targeted Magnetic Nanoparticles for Remote Manipulation of Protein Aggregation

by

Colleen Loynachan

Submitted to the
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Abstract

Local heat delivered by magnetic nanoparticles (MNPs) selectively attached to their target proteins can be used to manipulate and break up toxic or obstructive aggregates. We applied this magnetic hyperthermia treatment to the amyloid beta (Aβ) peptide, which unnaturally folds and self-assembles forming amyloid fibrils and insoluble plaques characteristic of amyloidogenic diseases such as Alzheimer’s disease. We demonstrate remote disaggregation of Aβ aggregates using heat dissipated by ferrite MNPs in the presence of an alternating magnetic field (AMF). Specific targeting was achieved by MNP functionalization with a targeting peptide sequence that binds a hydrophobic domain of Aβ. AMF parameters and MNP composition and size were tailored to maximize hysteretic power losses. Transmission electron microscopy image analysis and thioflavin T fluorescence spectroscopy were used to characterize the morphology and size distribution of aggregates before and after AMF stimulus. We found that the AMF stimulus is effective at destabilizing Aβ deposits and causing a reduction in aggregate size. This targeting scheme has potential as a therapy for amyloidosis and as a minimally invasive tool for analyzing and controlling protein aggregation.

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Chapter 1

Introduction

Proteins participate in virtually every cellular process. Therefore if their function is impaired, the consequences can be devastating. The misfolding of proteins such as amyloid beta peptide, prion protein, a-synuclein, and their subsequent fibrillization and aggregation is the hallmark of over forty major human diseases. These range from neurodegenerative diseases, such as Alzheimer’s disease (AD) and Parkinson’s disease, to non-neuropathic diseases, such as Type II diabetes and amyloid heart disease. In this thesis, we examine the aggregation of the amyloid beta (Aβ) peptide, which has been widely studied due to its links to Alzheimer’s disease.

The largest group of protein misfolding diseases is associated with the conversion of proteins from their soluble functional states into highly organized fibrillar aggregates. It is generally accepted that the aggregation process plays a central role in the pathogenesis of these diseases and that the accumulation of amyloid deposits has toxic consequences leading to cellular damage. Currently, there are no effective treatments for these types of amyloid diseases. Antibodies against Aβ have been shown to be effective at facilitating the clearance of amyloid plaques in older mice and have prevented plaque formation in young mice through opsonization and subsequent microglial-mediated phagocytosis of the plaque. Two recent phase II and phase III clinical trials of bapineuzumab and solanezumab, which attempted to disrupt amyloid protein aggregation, failed to show significant cognitive improvement or prevention of cognitive decline. Unfortunately, active immunization of patients with AD using these immunotherapy strategies revealed
antibodies crossing the blood–brain barrier causing meningoencephalitis-like inflammation in some patients and clinical trials with these drugs have been discontinued. Thus, there is still a clinical need for developing techniques to disaggregate misfolded protein plaques.

We are interested in developing minimally invasive technological platforms to interface with neural systems. To that end, we have developed a method to remotely disassemble amyloid protein aggregates by employing an alternating magnetic field (AMF) method as seen in Fig 1.1. In this method, we target ferrite magnetic nanoparticles (MNPs) to Aβ aggregates. In the presence of AMF, MNPs undergo hysteretic power loss and dissipate heat. We hypothesize that the loss power of MNPs is sufficient to disrupt hydrophobic interactions and hydrogen bonds between fibrils and effectively destabilize the aggregate.

![Figure 1.1 Method for remote protein disaggregation using AMF and MNPs.](image)

In this method the MNPs act as transducers, converting energy stored in the AMF to heat. We carefully tuned materials chemistry to control size and composition of ferrite materials to tailor the magnetic properties of MNPs for biomedical applications. For suggested therapeutic purposes, the ferrite MNPs would have to be administered at the
lowest concentrations possible. In order to maximize heating rates of these particles in the presence of AMF, we maximized their heat dissipation efficiency, commonly referred to as specific loss power (SLP). We use AMF to drive disaggregation of proteins because magnetic fields can penetrate deeply into the body with no attenuation of signal because of the low conductivity and magnetic susceptibility of tissue.7

Previous studies have demonstrated the possibility of remotely manipulating Aβ aggregates using the local heating of gold nanoparticles in microwave fields.8–10 However, microwave fields may have deleterious effects on the body. In contrast, AMF provides a form of energy that is safe to the tissue.7

We use ferrite materials because they show a low cytotoxic response. The concept of local heating of iron oxide nanoparticles has been employed in cancer hyperthermia studies for over fifty years to trigger necrosis and apoptosis of tumor cells,11 recently this method has been shown effective in phase II clinical trials to treat glioblastoma.7

Focusing on three major design considerations, in this thesis we present a method to remotely manipulate protein aggregation using the local heat dissipated by MNPs in the presence of AMF. First, in Chapter 2 we examine the power dissipation of MNPs and optimize materials chemistry to synthesize MNPs that are highly efficient at dissipating heat. In Chapter 3, we discuss the functionalization of MNPs to stabilize them in physiological conditions and augment them with the capability of targeting Aβ. In Chapter 4, we characterize the model protein misfolding system, Aβ. Finally, in Chapter 5, we quantify the effect of the AMF stimulation on Aβ aggregates using electron microscopy and fluorescence spectroscopy.
Chapter 2

Materials for maximizing heat dissipation

2.1 Theoretical basis for hysteretic power loss of MNPs

In the presence of an alternating magnetic field, the magnetic moment of individual single domain MNPs must overcome an anisotropy energy barrier to realign with an applied field and reduce its configurational energy.\textsuperscript{12,13} The hysteretic power loss of the MNPs in the presence of AMF is dissipated as heat.

If an assembly of MNPs is placed in AMF of frequency $f$ and field amplitude $\mu_0H_{\text{max}}$, the amount of heat $A$ released by the MNPs during one cycle of the magnetic field simply equals the area of their hysteresis loop, which can be expressed as

$$A = \int_{-H_{\text{max}}}^{+H_{\text{max}}} \mu_0 M(H) dH$$

where $M(H)$ is the MNP magnetization. The MNP power dissipation rate per gram, or SLP, is then

$$\text{SLP} = Af$$

and is expressed in W/g.\textsuperscript{12} The amount of heat released, $A$, depends on the MNPs’ effective anisotropy, their volume, the temperature, and the frequency and amplitude of the AMF. For therapeutic purposes, the MNPs would be administered at the lowest concentration possible, and the product of the AMF frequency $f$ and the amplitude $H_o$ should be less than $5*10^9$ Am$^{-1}$s$^{-1}$. This field-frequency product limits nonspecific heating of healthy tissue \textit{via} eddy currents induced by the applied AMF.\textsuperscript{14} To satisfy this
condition, the AMF parameters are usually limited to amplitudes of $5 - 30 \text{ kA m}^{-1}$ and frequencies below 1 MHz. To maximize heat dissipation under the constraint of the field-frequency product, SLP should be maximized.

Guided by the dynamic hysteresis model of coherent magnetization reversal in single domain MNPs, our group previously varied saturation magnetization and the effective anisotropy energy barrier for AMF of a given amplitude and frequency to maximize SLP. Optimization of SLP is critical for our application because we plan to deliver a selective supply of energy into the Aβ system to disrupt the bonds that stabilize the aggregate.

### 2.2 Selecting AMF parameters to maximize SLP

To illustrate the origin of heat dissipation in MNPs, we first calculate hysteresis loops for magnetite Fe$_3$O$_4$ with varying diameters with applied AMFs at amplitude $H_0 = 30 \text{ kA m}^{-1}$ and frequency $f = 103.9 \text{ kHz}$ (Fig. 2.1). We assume that the MNPs are effectively uniaxial and their easy-axes aligned with the applied field for this numerical simulation.
Figure 2.1 Field-dependent magnetization curves from numerical simulations for Fe$_3$O$_4$ MNPs of diameters varying between 9.1 and 33.8 nm.

For Fe$_3$O$_4$ ($K_{\text{eff}} = 1.4 \times 10^4$ J m$^{-3}$) MNPs with diameters less than 13.1 nm (at $f = 100$Hz), the hysteresis loop appears almost reversible due to the negligible anisotropy barrier for the given temperature and frequency. This superparamagnetic behavior results in low SLP (Fig. 2.2). SLP depends on the relationship between the effective uniaxial anisotropy energy constant ($K_{\text{eff}}$) and the configurational energy associated with the MNP in AMF. As the energy barrier scales with MNP volume, $E_a \propto K_{\text{eff}} \cdot d^3$, the area of the hysteresis loop becomes significantly larger and displays a ferromagnetic shape at diameters above 16.6 nm. The hysteresis loop with the largest area corresponds to the maximum value for SLP at the chosen AMF parameters. As the MNP diameter increases beyond 33.8 nm, the anisotropy energy increases such that the field amplitude no longer
exceeds the coercive field and only minor hysteresis loops can be accessed, leading to a decrease in overall heat dissipation.

We characterized the heat dissipation of ferrite MNPs of varying sizes and compositions by performing calorimetry to measure SLP. We found that 20 nm Fe$_3$O$_4$ nanoparticles exhibited some of the highest SLPs measured for synthetic materials for the therapeutically relevant operating conditions of field amplitude 30 kA m$^{-1}$ and frequency of 100 kHz (Fig. 2.2).

![Figure 2.2 Experimental calorimetry data from Christiansen et al. for SLP vs. magnetic diameter at AMF of frequency of 100 kHz and amplitudes 15-65 kA m$^{-1}$ measured for Fe$_3$O$_4$ single domain MNPs in aqueous solutions (~2 mg Fe/mL), with vertical error bars representing the standard deviation over five trials. Star at 340 W/g represents field conditions selected for experiments.]

While we expected larger MNPs to perform better at the chosen AMF parameters from the numerical simulations (Fig. 2.1), we found that these larger MNPs were not as easily accessible from a synthetic point of view and their saturation magnetization was not as high due to aggregation effects. From experimental calorimetry, we found that SLP
reaches its maximum value of about 340 W/g for Fe$_3$O$_4$ MNPs with 20 nm diameters in the chosen AMF parameters. Since the 20 nm particles display maximum hysteretic power loss, we synthesized these materials to investigate their use in disrupting Aβ aggregates.

2.3 Nanoparticle synthesis

Thermal decomposition of organometallic precursors allows us to prepare monodisperse and uniform MNPs with high yield and reproducibility. SLP is intimately related to the material’s anisotropy energy, which is dependent on the material’s composition and size. Using this method allows us to prepare high quality magnetic materials that efficiently dissipate power in AMF. The MNPs should have a tight size-distribution, since polydispersity has degradative influence on the heating rates achieved. For this thesis, monodisperse spherical Fe$_3$O$_4$ MNPs were synthesized via thermal decomposition of metal-oleate precursors and made water soluble as outlined previously in Chen et al. The concentrations of the MNP solutions were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES, ~2 mg Fe/mL for all samples).

2.4 AMF Experimental set-up

We constructed a custom AMF coil driven by a 200 W amplifier (Electronics & Innovation, Inc.) to perform field test experiments, in which we apply AMF to Aβ and MNP solutions. The coil used to produce AMFs at 100 kHz consisted of a ferrite toroid
(Ferroxcube, 3F3) machined to include a 0.75 cm gap and wrapped with 1050 strand 40 gauge litz wire (MWS Wire Industries) (Fig. 2.3). An RLC resonance circuit (see A1 for circuit diagram) focuses AMF within the gap, which is large enough to accommodate three, 500 μl volume sample tubes. The field magnitude was measured by a custom-built inductive field probe employing a pickup loop and an oscilloscope (TDS2022C, Tektronix). Thermal effects of resistive power loss were offset by a simple cooling system circulating ice water to the coil via silicone tubing. To ensure that we were maintaining physiological conditions, the temperature of the sample solution was monitored throughout the experiment using an AMF insensitive fiber optic temperature probe (Omega Engineering).

Figure 2.3 In the experimental set up, a magnetic field was applied by inserting the sample into the gap of a custom-built coil with a soft ferromagnetic core driven by an RLC resonance circuit.
Chapter 3

Biocompatibility and functionalization of MNPs

3.1 Peptide selection

Specific targeting of MNPs to amyloid aggregates was achieved through MNP functionalization with a targeting sequence that binds a hydrophobic domain of Aβ. Prior to that, the as-synthesized MNPs undergo a high-temperature phase transfer process to drive the coordination of poly(acrylic acid) (PAA) onto the MNP surface to stabilize the particles in water as outlined in Chen et al. However, we found that these electrostatically dispersed particles would not be useful under physiological conditions due to charge screening and the formation of salt bridges.

Carbodiimide chemistry was used to graft bi-functional polyethylene glycol (PEG) chains onto the PAA-coated MNPs to stabilize the particles through steric repulsion and impart an additional biocompatibility to the system, since PEGylation is the most commonly used method for reducing premature clearance of NPs from circulation in vivo. In this cross-linking chemistry, the carboxylic group of PAA may be reacted to N-hydroxysuccinimide (NHS) in the presence of a carbodiimide such as ethyl(dimethylaminopropyl) carbodiimide (EDC), resulting in a semi-stable NHS ester, which may then be reacted with primary amines on the bi-functional PEG to form amide crosslinks.

The PEG coated MNPs were further functionalized with a peptide sequence that targets Aβ using the same NHS/EDC chemistry outlined in §3.2 (Fig. 3.1). The peptide
sequence Leucine-Proline-Phenylalanine-Phenylalanine-Aspartic acid (LPFFD) binds a hydrophobic domain on the Aβ structure (amino acids 17-20 of the hydrophobic core of Aβ). Proline is a well-known β-sheet blocker and decreases Aβ’s propensity to adopt a β-sheet structure. Aspartic acid was added at the end of the targeting peptide to increase solubility. LPFFD is a β-sheet breaker sequence that in high ratio concentrations with Aβ is able to block amyloid fibril growth. LPFFD was synthesized using Intavis Model MultiPep multiple peptide synthesizer and purified using high performance liquid chromatography (HPLC). In this study, we targeted Aβ using LPFFD, however, to use this technique to target and disaggregate other protein misfolding systems, alternative targeting peptides must be engineered.

![Diagram](image)

**Figure 3.1** Functionalization of MNPs for stabilization in physiological conditions (PEG) and selective binding to amyloid beta (LPFFD).

### 3.2 Conjugation chemistry
The MNPs stabilized with PAA were suspended in $10^{-2}$ M EDC and $10^{-2}$ M NHS solution at pH 5.6 for 30 min to activate carboxylic groups. For PEG (10 kDa) immobilization, MNPs were precipitated by centrifugation at 13,000 rpm for 10 min and resuspended in a solution of 5 mg/mL of bi-functional PEG (NH$_2$-PEG-COOH) in a $10^{-2}$ M EDC and $10^{-2}$ M NHS solution at pH 8.6 under mechanical stirring for 24 h. For LPFFD grafting, PEG-MNPs were incubated in a solution of 1 mg/mL of LPFFD in a $10^{-2}$ M EDC and $10^{-2}$ M NHS solution at pH 8.6 under mechanical stirring. After 24 h, the MNPs were rinsed 5 times with PBS. Conjugation was confirmed using gel electrophoresis (see A2). A 5 nm thick PEG-LPFFD corona surrounds the conjugated MNPs (Fig 3.2).

![Figure 3.2 TEM image of functionalized MNPs with ~5 nm protein corona.](image)

The PEG coating was effective at stabilizing the MNPs under physiological conditions. The 20 nm MNPs functionalized with PEG-LPFFD coating are more
dispersed in phosphate buffered saline (PBS) than particles functionalized with LPFFD only (Fig. 3.3).

![TEM images of MNPs functionalized with PEG-LPFFD (right) are more dispersed in physiological conditions than MNPs functionalized with just the targeting peptide (left).](image)

**3.3 LPFFD functionalized MNPs target Aβ**

Transmission electron microscopy (TEM) image analysis was used to examine the interactions of targeted and un-targeted MNPs with Aβ (Fig. 3.4). The “targeted” MNPs are functionalized with LPFFD, the peptide sequence that has been shown to specifically bind Aβ²² (see A3 for additional TEM images of MNP targeting).

![TEM images of the interactions of untargeted (left) and targeted (right) MNPs with Aβ.](image)
We used ImageJ\textsuperscript{23} software to set a threshold for TEM image analysis, which we used to extract the MNP coverage of the aggregates for 16 images. To calculate the area of the fibrils from the TEM images, we set the upper threshold by finding the mean pixel value of the background of the image and subtracting two standard deviations from this mean (2\(\sigma\)). A similar process involving the mean pixel value and 2\(\sigma\) of the fibrils was used to calculate the area of the MNPs. There is a significant increase in MNP coverage of amyloid aggregates for MNPs that have been functionalized with the targeting peptide sequence as determined by a two-sample t-test using the "ttest2" function in Matlab, not assuming equal variances (\(p = 0.0016\)) (Fig. 3.5).

![Figure 3.5 Fraction of MNP coverage of Aβ aggregates for MNPs that are untargeted (green) and targeted (red).](image)

**Figure 3.5** Fraction of MNP coverage of Aβ aggregates for MNPs that are untargeted (green) and targeted (red).

**TEM sample preparation:**

Negative-staining TEM was used to visualize Aβ. 10 \(\mu\)l of each sample was adsorbed for 2–4 min onto carbon-coated copper grids (Ted Pella, Inc.) and gently wicked away with filter paper. 10 \(\mu\)l of freshly filtered 2% uranyl acetate staining solution was then
adsorbed for 2 min onto the grid and gently wicked off. Grids were allowed to dry in a light-protected environment for several minutes before being viewed on a Tecnai G2 Spirit TWIN electron microscope at 120 kV.
Chapter 4

Amyloid beta fibril formation pathway

Now that we have established an MNP materials system that is able to target amyloid aggregates and dissipate heat efficiently, we must examine the self-assembly characteristics of Aβ.

4.1 Aβ fibril formation

Alois Alzheimer first defined the pathology of Alzheimer’s disease (AD) in 1907 when he observed “senile plaques” and “tangles” in stained sections from the postmortem brain of his first diagnosed patient. The plaques were composed of fibrils formed by the aggregation of 42 amino acid peptides (Aβ1-42), which are part of a much larger transmembrane amyloid precursor protein (APP). In patients with AD, these peptides are unnaturally cleaved from APP. When the Aβ1-42 is liberated from APP it misfolds and aggregates to form a β-sheet fibrillar structure. For aggregation prone proteins, such as Aβ1-42, the misfolded state can be more stable (energetically favorable) than the functional structure under certain conditions.

The monomer of Aβ is a 42 amino acid peptide sequence (~ 4.5 kDa, see A4 for sequence) that is unstructured in solution and follows a nucleation and growth process to form insoluble fibrils with a characteristic cross-β-sheet structure. The time course of the conversion of a peptide into its fibrillar form typically includes a lag phase followed by exponential growth. The lag phase is the time required for “nuclei” to form. Once nuclei...
are formed, fibril growth proceeds by further association of monomers or oligomers with the nucleus.\textsuperscript{2-4} The pathway leading to Aβ fibril formation and the various spectroscopic techniques that can be used to examine the aggregation states of Aβ from monomers, to insoluble fibrils to large-scale aggregates, across multiple length scales are presented in Fig. 4.1.

![Fibril formation pathway diagram]

**Figure 4.1** Aβ fibril formation pathway. Aβ monomers follow a nucleation and growth process to form fibrils with a β-sheet structure that self-assembles into insoluble aggregates.

### 4.2 Aβ preparation

Numerous protocols for Aβ aggregation have been reported.\textsuperscript{26-29} Before starting an aggregation time course, a homogeneous solution, free from aggregates must be prepared. Here we adapted methods by Zagorski \textit{et al.}\textsuperscript{28} and Hellstrand \textit{et al.}\textsuperscript{27} to produce seedless
Aβ (monomeric form) for aggregation studies.

Following Zagorski et al.'s protocol,28 Aβ1-42 (GenScript, β-Amyloid (1-42), Human sequence) was lyophilized and stored at -20°C until use. In the same vial where it was lyophilized, Aβ1-42 was treated with trifluoracetic acid (TFA) at an approximate 1:1 ratio (mg/mL) in order to eliminate any preexisting aggregates. The solution was sonicated for 15 min during which additional portions of TFA were added until the peptide dissolved completely. TFA was then evaporated under a stream of nitrogen. To thoroughly remove TFA, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added and evaporated under a nitrogen stream. This last step was repeated three times and the sample was left overnight in a desiccator. The desiccated aliquot was carefully resuspended in 18 μl of 10 mM NaOH per 100 μl of final sample. Finally, 10 μl of 200 mM phosphate buffered saline (PBS) was added per 100 μl of final sample. All solutions were filtered through a 0.22 μM pore size filter (Millipore). At the end of the process Aβ1-42 was at 20 μM, but was further diluted in PBS to 5 μM for certain aggregation time course experiments. The solution was divided into aliquots and stored at -80°C until use. Samples prepared with this method were used for investigating the targeting capability of functionalized MNPs.

We found that the above method of preparation, although useful for targeting studies, did not sufficiently remove all seed nuclei, so the kinetic data for amyloid fibrillization was not consistently reproducible. Therefore we adopted another method for initial disaggregation using a protocol by Hellstrand et al.27 20 μM seedless Aβ1-42 was provided by Tiernan O’Malley from the Laboratory for Neurodegenerative Research at Brigham and Women’s Hospital. Aβ1-42 was expressed in Escherichia coli and purified via gel filtration as outlined in Hellstrand et al. and frozen as identical 200 μl aliquots. These
samples yielded highly reproducible amyloid fibrils and aggregates due to several critical preparation steps: (i) complete degassing and filtering of the buffer used to prepare the samples, (ii) gel filtration of Aβ_{1-42} in the degassed experimental buffer and collection of the monomer on ice just prior to starting the experiment, (iii) careful pipetting on ice without introducing air bubbles, (iv) minimized air-water interface area relative to sample volume, and (v) absence of any cosolvents or substances other than Aβ and the components of the buffer chosen for the study.\textsuperscript{27}

The purified seedless sample was incubated in low protein binding tubes at 37°C in PBS at pH 7.4 to form the desired aggregation state. Fibril growth was monitored using TEM (Fig. 4.2).

![Figure 4.2 TEM images of the formation of Aβ fibrils (6-12 h) and aggregates (24+ h) incubated under physiological conditions.](image_url)
After 6 h of incubation, the fibrils are flexible and consist of globular structures similar to “beads on string,” referred to as protofibrils (this constitutes the lag phase). After 12 h, fibrils are interacting with other fibrils and the globular units form the beginning of a long smooth fibril. At 24 h, fibrils are smooth, very long, and straight, but intertwined to form “nests.” By 48 h, we observe the formation of micrometer scale aggregates.

4.3 Aβ structural and kinetic analysis

4.3.1 Circular Dichroism (CD) spectroscopy

To confirm the aggregation state of Aβ, an Aviv Model 202 CD spectrometer was used to probe the secondary structure of Aβ. The amyloid sample was aggregated at physiological conditions for seven days and was placed in a 1 mm pathlength quartz cuvette (Hellma cells) for CD analysis (Fig. 4.3).

Figure 4.3 (A) CD data for aggregated Aβ sample reveals characteristic β-sheet structure. (B) Schematic representation of Aβ (1-40) of a single molecular layer, or cross-β unit from Petkova et al. The yellow indicates the direction of the long axis of the fibril, which coincides with the direction of intermolecular backbone hydrogen bonds. The cross-β unit is a double-layered structure, with parallel β-sheets formed by residues 12-24 (orange ribbons) and 30-40 (blue ribbons).
We conclude from the CD data that the amyloid sample has a characteristic β-sheet conformation consistent with literature, exhibited by the positive peak around 196 nm and negative peak around 217 nm.31

4.3.2 Thioflavin T (ThT) fluorescence assay

In addition to TEM, the aggregation kinetics of amyloid was monitored using ThT, a dye that displays enhanced fluorescence when it binds β-sheet rich structures, such as Aβ.32-34 2 mM ThT (Sigma) was prepared in purified water, filtered through a 0.22 μM filter and aliquots prepared and frozen at -20°C as a 100x stock. To prepare samples for fluorescence measurements, a 1:100 dilution of ThT stock was made into the amyloid sample. Amyloid samples were incubated under physiological conditions to a desired aggregation state. Immediately before fluorescence measurement, ThT was added to the sample and each sample was pipetted into multiple wells of a 96-well half-area plate of black polystyrene with black bottom and nonbinding surface (Corning 3993), 50 μl per well. ThT fluorescence was measured from the top of the plate in a Tecan Infinite Pro 2000 microplate reader. Since ThT has some fluorescence on its own, the fluorescence intensity of PBS buffer with the same concentration of ThT, was subtracted from the fluorescence intensities of amyloid samples. The fluorescence intensity data for Aβ samples aggregating over seven days is presented in Fig. 4.4.
Figure 4.4 Kinetic traces of Aβ_{1-42} aggregation by ThT fluorescence for 4 replicates at concentration 5 μM, gain is 150 for all time points and trials.

The Aβ sample exhibits the characteristic lag time, elongation, and plateau of ThT fluorescence, corresponding to the β-sheet conformation adopted as fibril formation and aggregation proceeds cf. §4.1. Using ThT, a particular aggregation state can be linked with fluorescence intensity.
Chapter 5
Disaggregation during AMF

In chapter 4, we analyzed the amyloid system and established a reproducible method to form Aβ aggregates and study aggregation kinetics. In this chapter, we investigate the possibility of interfering with the amyloid aggregation process by mixing the functionalized MNPs discussed in chapters 2 and 3 with the amyloid system described in chapter 4. We hypothesized that when functionalized MNPs bind to amyloid aggregates, in the presence of AMF, the local heat dissipated by the particles has the potential to disrupt hydrogen bonds between fibrils and destabilize the aggregate.

5.1 Sample preparation and controls for AMF test

For all AMF experiments, Aβ₁₋₄₂ was aggregated in PBS for seven days, and immediately before performing the AMF test, functionalized MNPs were added to the solution. To test the effectiveness of the MNP field stimulation, we prepared three controls: Aβ + H₂O, Aβ + MNP-PEG, and Aβ + MNP-PEG-LPFFD. Samples were prepared by mixing 108 μl of 5 μM Aβ solution with 12 μl of either H₂O or 2.5 mM functionalized MNPs (to a final concentration of 250 μM MNP in the amyloid solution). Half of the volume (60 μl) was then pipetted into each of the three low protein binding eppendorf tubes that were placed into the gap of the toroid coil for the AMF experiment. The remaining 60 μl of each sample was placed back into the 37°C incubator for the 6 hour test (see A5 for photo of experimental set-up).
An AMF insensitive fiber optic temperature probe was secured to the eppendorf tubes inside the gap to monitor the temperature throughout the AMF experiment and maintain it at 37 ± 3°C (to control for possible bulk heating of the sample). The AMF experiment was performed at the therapeutically relevant operating conditions of field amplitude 30 kA m⁻¹ and frequency of 100 kHz, chosen to maximize the hysteretic power loss of 20 nm Fe₃O₄ particles cf. §2.2.

5.2 Quantifying effects of AMF

TEM image analysis and fluorescence spectroscopy were used to determine the morphology and size distribution of aggregates exposed to 0 or 6 h of AMF stimulation.

5.2.1 TEM image analysis – binning of aggregate sizes

TEM image analysis was used to compare AMF-tested and non AMF-tested Aβ + MNP-PEG-LPFFD samples (Fig. 5.1, see A6-8 for TEM images of other controls).

Figure 5.1 Representative TEM images of Aβ + MNP-PEG-LPFFD, treated with (left) no AMF, (right) 6 hrs of AMF.
In samples not treated with AMF, we observed large aggregates profusely decorated with MNPs. After AMF, we observed small fibrils, amorphous aggregates, and many MNPs detached from fibrils. In ImageJ 1.47c, the “Analyze Particles...” command under the Analyze tab on the toolbar was used to bin the sizes of aggregates in TEM images from each test group. First, a threshold was set by finding the mean pixel value of the background of the image and subtracting 2σ. Using the particle analysis tool, and neglecting particles with areas smaller than 9000 nm$^2$, over 700 Aβ aggregates were examined. The “randi” function in Matlab was used to extract 37 uniformly distributed pseudorandom integers from each test group, and these randomly selected areas were binned (Fig. 5.2).

The data was not normally distributed so we performed non-parametric statistics for analysis. The AMF-tested and non AMF-tested samples had significantly different distributions as determined by a two-sample test.

Figure 5.2 (A) Histogram of the areas of 37 randomly selected aggregates from AMF-tested (orange) and non AMF-tested samples (green). (B) Areas re-binned into smaller intervals to examine distribution of AMF-tested samples. Bin “0” contains aggregates with sizes between 9000 nm$^2$ and $10^7$ (A) or $10^5$ nm$^2$ (B).

There is a clear shift to smaller aggregate sizes for AMF treated samples, as all of the AMF tested aggregate areas (orange) lie in the first bin, whereas the non AMF-tested samples are distributed across larger area bins.
Kolmogorov Smirnov test (p < 0.001). To further analyze the data, a Wilcoxon rank-sum was performed to test for equal medians. The medians were found to also be significantly different (p < 0.001).

5.2.2 Fluorescence spectroscopy

Fluorescence intensity can be linked to a certain aggregation state using ThT. **Fig 5.3** presents the data for fluorescence spectroscopy of the three control samples: Aβ + H2O, Aβ + MNP-PEG, Aβ + MNP-PEG-LPFFD, with and without AMF stimulation.

![Fluorescence spectrum graph](image)

**Figure 5.3** Fluorescence spectroscopy of AMF-tested (orange) and non AMF-tested (green) samples. A decrease in fluorescence is observed for Aβ + targeted MNPs after AMF, which is linked to the disruption of the aggregate, gain is 177, p = 0.003, n = 3.

For the Aβ sample with no MNPs, an increase in fluorescence is exhibited after the AMF test due to accelerated aggregation that occurs at elevated temperatures during the course of the experiment. This is consistent with other ThT studies of Aβ aggregation. For the Aβ sample with non-targeted MNPs, the fluorescence does not significantly change. The natural tendency for the Aβ would be to continue aggregating over time and
exhibit an increase in fluorescence during the AMF test. However, some of the MNPs become nonspecifically trapped in the aggregate structure and upon AMF stimulation, the local heating of the MNPs inhibits further aggregation. The sample with targeted MNPs exhibits an overall decrease in fluorescence following AMF stimulation. This correlates with the disruption of hydrophobic interactions and hydrogen bonds by the local heating of the MNPs.

5.3 Mechanism of disaggregation

We found that MNPs attached to Aβ aggregates have a significant effect on Aβ aggregate size after AMF stimulation demonstrated by TEM image analysis and fluorescence spectroscopy data. However, it is unclear whether the local heating of the MNPs in the presence of AMF is the sole or even primary cause of disaggregation. The disaggregation of the amyloid deposits is likely due to a collective effect. From calculations using the SLP of the Fe$_3$O$_4$ MNPs in the relevant field conditions (340 W/g), the loss power per particle was calculated to be $10^{-15}$ J/s. The strength of a hydrogen bond, or amyloid binding energy is $10^{-20}$ J. Therefore, every $10^{-5}$ seconds a MNP releases enough energy to break a fibril bond. However, not all of the MNP energy goes into breaking bonds. It is generally assumed that significant heating occurs only in the very close vicinity of the MNP surface. Since the surface-to-volume ratio for the 20 nm diameter particles is very high, they dissipate energy to the surrounding environment rapidly. With the concentration of MNPs administered, the collective power loss of the particles may be sufficient to disrupt bonding and cause the changes in aggregate size.
observed in TEM images in §5.2. While bulk heating of proteins has been shown to cause accelerated aggregation by increasing the number of collision events that lead to binding, the targeted MNP approach allows for a selective supply of energy to be delivered to the system to allow for manipulation of aggregation in a controlled manner.\textsuperscript{8,36}

In the TEM images in §5.2.1, the MNPs are not always evenly distributed across aggregates, and large clusters of MNPs can be seen in certain regions. Keblinski \textit{et al.} found that sustained heating of a large number of dispersed MNPs can produce a significant global temperature rise, even if the local temperature rise at each particle’s surface is negligible.\textsuperscript{37} Therefore, for an ensemble of MNPs, which we see clustered on the Aβ aggregates, we can consider the superposition of the temperature fields from all MNPs, which leads to a temperature rise at the center of the macroscopic region.\textsuperscript{37} The collective effect of MNP agglomerate heating could be sufficient to denature Aβ.\textsuperscript{40,41} Finally, any small physical rotations of the MNPs in solution would lead to mechanical forces that could also disrupt the aggregate structure. We plan to augment our experiments with numerical modeling to further elucidate the mechanism of disaggregation.
Chapter 6

Conclusion & Outlook

In this thesis, we demonstrate for the first time, the ability to locally and remotely heat and disaggregate amyloid deposits of Aβ1-42, a small protein involved in Alzheimer’s disease, via the combined use of AMF and ferrite MNPs, without any bulk heating. First, we tuned the materials chemistry to synthesize ferrite MNPs with some of the highest loss powers measured for synthetic materials. We then functionalized these particles with PEG and a hydrophobic peptide sequence that targets Aβ. Finally, we quantified the effect of AMF stimulation using TEM image analysis and fluorescence spectroscopy.

Further investigations should seek to tune the AMF parameters to shorten the AMF test time to a therapeutically relevant time scale. Additionally, we plan to optimize other parameters, such as the concentrations of Aβ:MNPs. We intend to tune these parameters so that we can administer the lowest dose of MNPs for the shortest AMF exposure time and still have the effect on aggregate sizes seen in §5.2. We also aim to investigate the samples at time points following AMF to see if the small aggregates and fibrils reform over time, or if the MNPs successfully interfere with further aggregation.

For some protein misfolding diseases, such as Alzheimer’s disease, the pre-fibrillar species are hypothesized to be more toxic than the aggregates.² If this hypothesis is true, we may be able to apply our remote targeting to the pre-fibrillar species and prevent the formation of fibrils and aggregates with AMF. We plan to use the technology presented in this thesis to interfere with the dynamics of fibril growth.
We can further validate our results by performing other experiments such as atomic force microscopy (AFM) to compare the thickness or height of aggregates for AMF-tested and non AMF-tested samples. Additionally, we can use Raman spectroscopy to characterize the changes in secondary structure of the proteins after AMF stimulation.

We demonstrated this new technology for disaggregation of the amyloid system, but it has the potential to be applied more broadly to other protein misfolding systems in which disaggregation could be a beneficial therapy, and it can also be used as a noninvasive tool for analyzing and controlling protein aggregation.
Bibliography


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Appendix

A1. RLC resonance circuit drives custom-built toroid coil for AMF experiments.

A2. Gel electrophoresis was performed on 1.5% agarose gels in 1X TEA buffer. The unconjugated MNPs migrate faster than the MNPs conjugated to PEG-LPFFD. This indicates the success of the conjugation since the functionalized MNPs have a larger molecular weight and thus run slower on the gel.
A3. TEM images of Aβ decorated with MNPs that have been functionalized to bind the hydrophobic core of Aβ.

A4. The 42 amino acid peptide sequence of Aβ1-42 (human)

{ASP} {ALA} {GLU} {PHE} {ARG} {HIS} {ASP} {SER} {GLY} {TYR} {GLU} {VAL} {HIS} {HIS} {GLN} {LYS} {LEU} {VAL} {PHE} {PHE} {ALA} {GLU} {ASP} {VAL} {GLY} {SER} {ASN} {LYS} {GLY} {ALA} {ILE} {ILE} {GLY} {LEU} {MET} {VAL} {GLY} {GLY} {VAL} {VAL} {ILE} {ALA}
A5. AMF test set-up. Three, 500 µl volume low protein binding eppendorf tubes are placed in the gap of a custom-built coil.

A6. Representative TEM images of Aβ with no AMF exposure and 6 h AMF. For Aβ not incubated with MNPs, the average aggregate area increases for samples that undergo AMF test. This is consistent with the ThT fluorescence measurements. Accelerated aggregation occurs when the experimental conditions allow for temperature fluctuations that exceed 37°C.
A7. Representative TEM images of Aβ + MNP-PEG with no AMF exposure and 6 h AMF. For Aβ incubated with untargeted nanoparticles, some smaller aggregates and fibrils are observed for AMF-tested samples; however there are also still many large aggregates remaining after AMF stimulation. Aggregates may be broken up in AMF when MNPs get nonspecifically trapped in the amyloid aggregate, even if they are not directly targeted to it. In the presence of AMF, these MNPs will undergo hysteretic power loss that is sufficient to break hydrogen bonds and disassemble some of the aggregates. However the effect is not as pronounced as for the MNPs that are selectively targeted to the aggregate (A8). Further TEM analysis should be performed.
A8. TEM images of Aβ + MNP-PEG-LPFFD with 6 h AMF exposure. For Aβ incubated with targeted MNPs, we found many short fibrils and small aggregates after the AMF test. Small aggregates appeared as if they had been cut or "broken" off from larger aggregates. MNPs in the AMF-tested samples were mostly found near the ends of fibrils. Additionally, many of the MNPs were detached from fibrils following AMF stimulation indicating fibril scission at these points of local power loss.