CHARACTERIZATION OF MAGNETIC NANOPARTICLE MOBILITY IN BIOLOGICAL MATRICES AS A FUNCTION OF MAGNETIC RELAXATION MEASUREMENTS

By

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To my family and my beloved Venezuela
No matter the distance, time or circumstance that separated us…
  I love you more than anything.
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The success of nanotechnology-based therapies depends on the mobility and lengths over which nanoparticles can move through biological barriers to reach their target site. Current efforts to reduce the uncertainty in clinical translation of magnetic nanoparticles for biomedical applications are intimately related to the study of the absorption, distribution, metabolism and elimination patterns of these nanocarriers. However, there is a lack of understanding of how biological barriers can reduce the diffusion of magnetic nanoparticles due to Derjaguin, Landau, Vervey and Overbeek (DLVO) and non-DLVO interactions, which can impact the nanoparticle’s colloidal stability and mobility in complex milieu, increasing the risk of toxicity and compromising intended use. This is in part because the available instrumentation to perform such studies are based on optical approaches, which are limited to optically transparent samples. A less explored approach to study particle mobility and stability in situ involves the dynamic magnetization response of magnetic nanoparticles to an alternating magnetic field, which can be used to monitor changes in the local environment of the magnetic nanoparticles with precision and sensitivity. In this dissertation, the use of dynamic magnetic susceptibility (DMS) measurements to study the colloidal stability and
mobility of nanoparticles in protein suspensions, blood, and tumor explants was established. The studies presented here are the first to assess nanoparticle mobility in highly concentrated protein solutions, blood and tumor stroma \textit{in situ} using time-dependent magnetic susceptibility measurements. By continuously studying magnetic nanoparticle mobility in complex milieu via DMS measurements, as a useful assessment tool to design nanoparticles for biomedical applications without the limitation of optical transparency in sample evaluation, innovative design features of magnetic nanoparticles can be rationally incorporated. Considering these features one can design new generation of magnetic nanotherapeutics with enhanced colloidally stability and minimal interactions with blood, plasma proteins and tumor-stroma microenvironment. We expect that this technique will contribute to assessing nanoparticle diffusion rates, thus helping design particles with high mobility in biological environments.
CHAPTER 1
BACKGROUND AND SIGNIFICANCE

1.1 Characterization of Magnetic Nanoparticle Mobility in Biological Matrices to Enhance Particle Design for Biomedical Applications

It is almost a century since Heilbrunn and Seifritz [1] first used nickel microparticles in 1920s with the aim to determine rheological properties of non-living jellylike tissue. This was followed by Crick and Hughes [2], who in 1950 performed uptake experiments of colloidal iron oxide particles in chicken cell embryos in order to study intracellular rheology, revealing no toxic effects of iron oxide particles in those cells in contrast to nickel particles. Currently, the clinical applications of iron oxide magnetic nanoparticles (MNPs) include (i) oral and intravenous iron-dextran complex therapy in iron deficiency anemia since 1947 [3], (ii) the use of superparamagnetic iron oxide MNPs as a negative contrast agent for liver tumor enhancement in magnetic resonance imaging since 1988 [4], and (iii) local magnetic hyperthermia for cancer treatment in lymph nodes, postulated by Gilchrist et al.[5] in 1957, and currently used in clinical trials for the treatment of glioblastoma multiforme and prostate adenocarcinoma in Europe and United States [6]. Even today, interest in the use of iron oxide magnetic nanoparticles continues to increase because of advances in particle synthesis, development of suitable surface modification strategies with biocompatible polymers and controlled bioconjugation strategies to enhance their in vivo pharmacokinetics and biodistribution profiles of MNPs [7-9]. Also, the ability to manipulate MNPs in the presence of an alternating magnetic field [10] along with research efforts to understand nanoparticle-protein interactions, and the development of innovative methods for the characterization of MNPs in biological environments [11-15] have played a key role in engineering magnetic nanoparticles for biomedical applications. Inorganic magnetic
nanoparticle cores embedded in several biocompatible polymers have been prepared and characterized for potential medical applications ranging from targeted magnetic fluid hyperthermia [16, 17], drug delivery [18], magnetically triggered cargo release [18-20], optical and magnetic particle imaging [21], magnetic analyte capture (biomarkers, circulating tumor cells, and human pathogens) [22-24], gene delivery [25-31], nanowarming of cryopreserved tissue [32] and cell receptor activation [33-36].

In the biomedical applications mentioned earlier, colloidally stable, well-defined and safe magnetic nanoparticles have been characterized in water, saline solutions, biological buffers and complete cell culture media, but not in fluids mimicking the complex biological environment. Therefore, there is a lack of understanding of how MNPs interact with biological barriers such as blood and other body fluids (e.g. cerebrospinal fluid, epithelial lining fluid or mucus, peritoneal fluid, and synovial fluid) or with dense connective tissue structures such as tumor stroma or vitreous humor. Understanding such interactions and the ability of MNPs to remain colloidally stable to achieve an efficient targeting to specific tissue organs and cells vis a critical step in the rational design of MNPs for biomedical applications. Strategies to design MNPs for use in therapeutic applications should also be based on understanding the stability and mobility patterns of MNPs in the biological milieu, which consists of confined and crowded conditions of macromolecules and ions in the nanometer to micrometer range. Principally, the stability and mobility patterns of MNPs can be altered by nanoparticle-protein interactions and those interactions can influence immune cell uptake and distribution in the mononuclear phagocytic system (monocytes, macrophages, and
dendritic cells), thus affecting particle clearance and biodistribution in vivo, which directly impacts nanotechnology-based targeted therapies.

1.2 The Importance of Studying Magnetic Nanoparticle Mobility in Crowded Biological Environments

In crowded biological environments, semi-empirical corrections considering a self-crowding factor, $\kappa$, have been developed to represent experimental data and estimate the bulk viscosity. As an example, Ross and Minton in 1977 [37] described a generalized semi-empirical form of Mooney’s equation to quantify the viscosity of concentrated protein solutions, shown in Equation (1-1)

$$
\eta_{Rheo} = \eta_0 \exp \left( \frac{\nu\phi}{1 - \kappa\phi} \right),
$$

where $\eta_0$ is the viscosity of the suspending medium, $\phi$ is the volumetric fraction of the suspended proteins, and $\kappa$ estimated by Mooney to be between 1.35 and 1.91 for hard spheres. However, this model potentially fails at high protein (> 150 mg/mL) and salt concentrations where interactions between proteins become relevant such as charge and hydrophobic interactions become relevant [38]. This is important to address because [39] the shear-rate dependent viscosity in protein solutions is a complex phenomenological phenomenon that depends on the protein molecular weight, volume fraction, friction factor, hydration factor, pH-dependent net charge, conformational state, and intermolecular potential between proteins.

Correlations to study the concentration-dependent viscosity of protein solutions are valuable in the pharmaceutical industry [40-44]. To test the stability of concentrated monoclonal antibody (mAb) therapeutics, in the order of 80 mg/mL or higher [45], which relies on detection of protein aggregates using for example, viscosity measurements are
made as a function of concentration, pH, ionic strength and excipient species present in the formulation. This is in part because, the presence of protein aggregates may severely compromise drug efficacy and safety, inducing immunogenic responses [46]. The stability of proteins at high protein concentrations under different conditions are typically required for subcutaneous administration of mAb (<1mL - 1.5 mL) [47]. However, process control via experimental validation is time-consuming and often requires the use of large volumes of protein suspensions that are not available during drug discovery. For example, bulk rheology characterization requires 0.750 mL – 10 mL, or complex instrumentation such a capillary rheometer and microfluidic devices (e.g. m-VROC; RheoSense Inc.) require 10 µL – 750 µL of protein suspension. Thus, by monitoring magnetic particle mobility, and demonstrating that the rotational diffusivity of tracer MNPs in concentrated protein solutions follows the predictions of the SE relation under specific conditions, the protein concentration-dependent viscosity can be determined in different protein systems, and protein aggregation may be evaluated precisely in short times using a small volume of sample (< 200 µL) via magnetic relaxation measurements at different buffered conditions.

1.3 Translational and Rotational Mobility of Nanoparticles in Biological Matrices

Water serves as the primary milieu for life’s processes and hence, it is necessary to understand the factors that determine rates of molecular movement in aqueous environments [48]. Most biological processes occur in an environment that is predominately water, with macromolecules in a crowded-like environment, surrounded by ions. The cell, as the basic unit of life, contains 70-85% v/v water and the extracellular space of most tissues is 99% v/v water [48].
Nanoparticles with different arrangements, compositions and therapeutic effects can be introduced into a biological matrix within the body in a variety of ways [49-52]. However, the success of nanotechnology-based therapies depends on the mobility and extents to which nanoparticles can move through tissue structures to arrive at a target site. The rates of diffusive transport of nanoparticles can vary between biological matrices (e.g., whole blood, tissues and cells) due to macromolecular crowding effects, which are an important but neglected aspect of the extracellular and intracellular environment. Crowding effects cannot be neglected when blood contains about 80 mg/mL of protein or knowing that the concentration of total protein inside a cell is on average ~250 mg/mL [53]. In such complex biological environments, mass transport equations are fundamental for the study of nanoparticle diffusion-convection patterns. This can be defined as follow [54]

\[
\frac{\partial C}{\partial t} = \nabla \cdot (D_{\text{eff}} \nabla C) - \nabla \cdot (vC),
\]

where the effect of molecular diffusion on mass transport and the contribution of convection are linked. In non-flow systems, only the molecular diffusion accounts for the mass conservation equation, thus, effective diffusivity \(D_{\text{eff}}\) becomes a critical transport parameter by means of connecting the translational and rotational degrees of freedom for a molecule.

1.3.1 Diffusion in Biological Systems

Diffusion is the random motion of the solute molecules driven by collisions with solvent molecules because of translational and rotational kinetic energy. In a dilute system, collisions between colloidal particles are negligible, which makes the driving force for diffusion the thermal molecular motion of solvent molecules. Indeed, in a living
cell diffusion is highly efficient for internal transport. Mainly, in crowded biological environments diffusion is dominant for the transport of small molecules, macromolecules and particles [55] such as oxygen in tissues \((R = 0.12 \text{ nm})\), drugs in the vitreous cavity of the eye [56], and drugs in solid tumors, for example anti-VEGF drugs such as ranibizumab \(R = 2.8 \text{ nm}\), aflibercept \(R = 3.7 \text{ nm}\), bevacizumab \(R = 4.6 \text{ nm}\) or liposomal Doxorubicin \(R \sim 50 \text{ nm}\) [57].

In 1905, Einstein [58] showed that the diffusivity \(D\) of a particle in suspension in thermal equilibrium can be related to its hydrodynamic mobility according to the with the equation

\[
D = \frac{k_B T}{6\pi \eta R},
\]

where \(k_B T\) is the thermal energy of the system, and \(\eta\) is the shear viscosity of the solvent. This equation was obtained based on the assumption that the concentration distribution of the particles is uniform. Unless an external force acts on each particle, the diffusion flux is balanced by a negative convective flux at low Reynolds (Re) number which, is defined as

\[
\text{Re} = \frac{\rho \nu D_{\text{hydraulic}}}{\eta},
\]

where \(\rho\) is the density of the fluid, \(\nu\) is the mean velocity of the fluid, and \(D_{\text{hydraulic}}\) is the hydraulic diameter at the section normal to the flow. Also, it was assumed that the force is exerted along in the \(x\)-axis, and the particle concentration is dilute. In fluid dynamics, the expression \(6\pi \eta R\) is denominated as the translational friction factor \((f_T)\), which is used to quantify the drag or resistance to particle motion in the surrounding medium. On the other hand, when the colloidal sphere exchanges angular momentum with the
solvent, such as when the particle probe is actively driven within the solvent by a magnetic force in an oscillatory magnetic field, the rotational frictional coefficient \( f_\mu \) is considered.

1.3.2 Frictional Coefficients for Spheres

In biological systems, convection typically transports molecules over distances for which diffusion is too low. When a fluid is in motion the character of the flow changes dramatically above a critical value of the Re number, depending on which the flow can be laminar or turbulent. Fluid flow in the cardiovascular system and tissues differs from the steady laminar flow of a Newtonian fluid mainly because blood is Non-Newtonian, its flow is pulsatile, blood vessel entrance lengths are not long enough to form a fully developed flow, and Re numbers range between 20-1,500. In branched vessels flow turbulences are common at normal physiological conditions. In contrast, flow turbulences are rare in confined biological systems over the dimension of a cell (10 µm). Certainly, cells are typically crowded macromolecular environments in which diffusion is highly efficient for internal transport, and is usually modeled by the Stokes equation for small Reynolds numbers (Re << 1). In this respect, low Reynolds number flows around a sphere are relevant in several biomedical applications, and transport within cells, proteins, and microfluidic devices [55]. Respectively, considering a solid non-interacting sphere of radius R surrounded by a large volume of quiescent fluid and using the continuity equation for an incompressible flow, and the corresponding components of the Navier-Stokes equation in spherical coordinates; we can obtain the translational friction factor and the rotational friction factor for the sphere as follows [55, 59].
1.3.2.1 Translational friction factor \((f_t)\)

Assuming that the sphere slowly translates and does not rotate in steady flow, the continuity equation is simplified to Equation (1-5)

\[
0 = \frac{1}{r^2} \frac{\partial (r^2 u_r)}{\partial r} + \frac{1}{r \sin \theta} \frac{\partial (v_\theta \sin \theta)}{\partial \theta}
\]  

\((1-5)\)

Neglecting variations in pressure \((p)\) due to gravity around the sphere and neglecting inertial terms in the corresponding Navier-Stokes equations, the \(r\) and \(\theta\) components of the Navier-Stokes equation are given by Equation (1-6) and (1-7)

\[
0 = \frac{\partial p}{\partial r} + \eta \left( \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial v_r}{\partial r} \right) + \frac{1}{r^2 \sin \theta} \frac{\partial}{\partial \theta} \left( \sin \theta \frac{\partial v_r}{\partial \theta} \right) - 2 \frac{v_r}{r^2} - 2 \frac{v_r}{r^2} \frac{\partial v_\theta}{\partial \theta} - 2 \frac{v_\theta}{r^2} \cot \theta \right) 
\]

\((1-6)\)

\[
0 = -\frac{1}{r} \frac{\partial p}{\partial \theta} + \eta \left( \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial v_\theta}{\partial r} \right) + \frac{1}{r^2 \sin \theta} \frac{\partial}{\partial \theta} \left( \sin \theta \frac{\partial v_\theta}{\partial \theta} \right) + 2 \frac{v_r}{r^2} \frac{\partial v_r}{\partial \theta} - \frac{v_\theta}{r^2 \sin^2 \theta} \right) 
\]

\((1-7)\)

With the boundary conditions based on the behavior of the velocity components far from the sphere

\[
v_r = v_0 f(r) \cos \theta \\
v_\theta = -v_0 g(r) \sin \theta \\
v_\phi = 0
\]

and,

\[
v_r = v_\theta = 0 \quad \text{as} \quad r \to R \quad \text{with} \quad f(r) = g(r) = 0
\]

\[
f(r) = g(r) = 1 \quad \text{as} \quad r \to \infty
\]

The resulting expressions for the velocities and the pressure are

\[
\frac{v_r}{v_0} = \left[ 1 - \frac{3}{2} \frac{R}{r} + \frac{1}{r^2} \frac{R^3}{r^3} \right] \cos \theta
\]

\((1-8)\)
\[ \frac{v_\theta}{v_0} = \left[ 1 - \frac{3}{4} \frac{R}{r} + \frac{1}{4} \frac{R^3}{r^3} \right] \sin \theta \] (1-9)

\[ p = p_\infty - \rho g x - \frac{3\eta v_0}{2R} \left( \frac{R}{r} \right)^2 \cos \theta \] (1-10)

At the infinity, the pressure in the uniform flow is

\[ p \bigg|_{r=R} = p_\infty - \rho g R \cos \theta - \frac{3\eta v_0}{2R} \cos \theta \] (1-11)

Then, the relevant component of the shear stress is

\[ \tau_{r\phi} \bigg|_{r=R} = -\frac{3\eta v_0}{2R} \sin \theta \] (1-12)

The total force \( F \) on the sphere is the integral of stress over the whole sphere surface

\[ F_z = \int_0^{2\pi} \int_0^{\pi} p \bigg|_{r=R} \cos \theta R^2 \sin \theta d\theta d\phi + \int_0^{2\pi} \int_0^\pi \tau_{r\phi} \bigg|_{r=R} \sin \theta R^2 \sin \theta d\theta d\phi \] (1-13)

\[ F_z = 6\pi \eta v_0 R \] (1-14)

Finally, the translational friction factor of the sphere in a pure viscous fluid is given by the Equation (1-15)

\[ f_T = 6\pi \eta R \] (1-15)

1.3.2.2 Rotational friction factor (\( f_r \))

Assuming that the sphere slowly rotates at a constant angular velocity \( \omega \) around the z-axis, the torque \( T_z \) required to maintain the sphere’s rotation defines the rotational friction factor as \( T_z = f_r \omega \). Under these conditions the Stokes equation becomes:

\[ 0 = \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial v_\phi}{\partial r} \right) + \frac{1}{r^2 \sin \theta} \frac{\partial}{\partial \theta} \left( \sin \theta \frac{\partial v_\phi}{\partial \theta} \right) - \frac{v_\phi}{r^2 \sin^2 \theta} \] (1-16)

With the boundary conditions

\[ v_\phi = 0 \quad \text{as} \quad r = \infty \]

\[ v_\phi = R \omega \sin \theta \quad \text{as} \quad r = R \]
To finally obtain the velocity profile relation by a rotating sphere

\[ v_\phi (r, \theta) = \omega R \left( \frac{R}{r} \right)^2 \sin \theta \]  

(1-17)

Then, the relevant component of the shear stress is

\[ \tau_{r\phi} = -\eta r \frac{\partial}{\partial r} \left( \frac{v_\phi}{r} \right) \]  

(1-18)

and the torque needed to integrate the tangential force \( \tau_{r\phi}(r = R)dS \) exerted on the fluid by a solid surface element \( dS \) will correspond to

\[ T = \int \tau_{r\phi} R \sin \theta dS \]  

(1-19)

\[ T = \int_{0}^{2\pi} \int_{0}^{\pi} (3\eta \omega \sin \theta) (R \sin \theta) R^2 \sin \theta d\theta d\phi \]  

(1-20)

\[ T = 6\pi \eta R^3 \omega \int_{0}^{2\pi} \sin^3 \theta d\theta = 8\pi \eta R^3 \omega \]  

(1-21)

To close, the rotational friction factor of the sphere in a pure viscous fluid is given by Equation (1-22)

\[ f_R = 8\pi \eta R^3 \]  

(1-22)

1.3.2.3 The Stokes-Einstein and the generalized Stokes-Einstein relations applied to complex biological environments

Rotational and translational diffusion rates of small compounds, proteins and lipid molecules undergoing Brownian motion are responsible for the completion of a wide variety of biological functions and cellular processes. For example, it has been demonstrated that diffusive oxygen transport is determined only by translational diffusion in comparison with the diffusive hydrogen proton transport which appears only be based on rotational diffusion [60]. Bigger molecules such as proteins, drugs, viruses, bacteria and nanoparticles with a radius \( R \) in confined environments diffuse following
the Stokes-Einstein (SE) predictions, in which the diffusion coefficient $D$ is inversely correlated with the viscosity of the medium and the nanoparticle’s hydrodynamic diameter. The diffusion coefficients by translation ($D_{T-SE}$) and rotation ($D_{R-SE}$) are related to the particle’s mobility as translational and rotational displacements according to the follow equations [61]

$$D_{T-SE} = \frac{k_B T}{6\pi \eta R}$$ (1-23)

$$D_{R-SE} = \frac{k_B T}{8\pi \eta R^3}$$ (1-24)

For spherical particles moving in a medium of proportionally small molecules the SE equations still hold. Moreover, these relations have been employed for estimating the size of particles and biomolecules in dilute suspensions and the viscous properties of complex fluids by a variety of characterization techniques based on, for example, dynamic light scattering (translation) or fluorescence depolarization (rotation).

In concentrated and dilute dispersions with significant particle interactions, particle diffusion can be slowed down due to electrostatic and hydrodynamic particle interactions with highly structured gel-like connective tissue or crowded intracellular environments. As a consequence, the SE relation may no longer represent the particle diffusivity. However, the SE relation still holds for a wide range of solvents, temperatures, and pressures with $R$ being of the order of the molecular radius with some exceptions at the nanoscale, for example, supercooled water [62], polymer melts [63] and polymer solutions [64]. Indeed, Tuteja and Mackay [15] studied the diffusion coefficients of 5 nm diameter nanoparticles in polymer melts by using X-ray photon correlations spectroscopy, and demonstrated that the SE relation between viscosity and
diffusion coefficient was not valid for such small particles. On the other hand, for particle suspensions in semidilute polymer solutions, nanoparticles can be trapped by the surrounding polymer mesh and deviate from predictions based on bulk solution viscosities. According to Poling-Skutvik et al.[64], this is in part because of the heterogeneities present in polymer solutions over length scales analogous to the polymer radius of gyration ($R_g$) and the correlation length ($\xi$).

Due to the importance of understanding how particle mobility in biological complex environments towards improving the design of nanomaterials intended for biomedical applications such as targeted delivery of drugs in biological tissues, many research efforts have been directed to evaluate a generalized Stokes-Einstein (GSE) relation. GSE relations have been used to study the passive or driven diffusion by external applied magnetic fields or optical tweezers, to probe mechanical properties at nanoscale and microscale by correlation of a diffusional property with rheological properties such as complex viscosity and the complex shear modulus [61]. In order to avoid positive or negative deviations from the SE relation, the length scale of the nanoparticle probes intended to measure the mechanical properties of the surrounding fluid should be considerable larger than the length scale of the molecules in the fluid [65]. Mainly, this is to facilitate that the particle probes measure the average of the bulk rheological characteristics of the complex fluid, and can detect microscale heterogeneities and fluctuations.

Magnetic nanoparticles (MNPs), due to their ability to be manipulated by external applied magnetic fields, are promising to assess mechanical properties of complex environments or detect changes in their dispersion state upon protein adsorption or
nanoparticle-protein interactions in gel-like biological environments. This can be made possible by monitoring the magnetic relaxation of a liquid particle system as it approaches an equilibrium state condition after removal of the applied magnetic field. Under specific conditions, the relaxation time can be related to the hydrodynamic diameter of the particle probe and the viscosity of the carrier fluid and thus their rotational diffusivity. Understanding factors affecting the diffusion of magnetic nanoparticles in complex biological fluids using magnetic relaxation measurements is particularly important for biomedical applications because this technique requires small sample sizes (less than 1 mL) and can be used for optically opaque samples. The opacity of biological complex fluids limits colloidal stability assessments of MNPs using traditional optical techniques. Consequently, there is a need to address this, to improve and clinically translate the use of MNPs in biomedical applications.

Several therapeutic applications of magnetic nanocarriers can be improved by using magnetic nanomaterials with enhanced colloidal stability features to diffuse through viscoelastic biological fluids, gels, and membranes. This can be made possible by monitoring colloidal stability of MNP probes through magnetic relaxation measurements in complex biological matrices at time windows comparable which those intended for use in the following applications i) systemic delivery of targeted drug-loaded or gene-loaded nanocarriers to treat solid tumors [66], ii) intravenous injection or inhalation of nanocarriers for antibiotics to treat cystic fibrosis [26], iii) intraocular injections of gene-loaded nanomedicine for the treatment of degenerative diseases in the eye [67], and iv) delivering therapeutic agents across the intact tympanic membrane (ear drum) to treat short term inflammatory ear conditions [68]. The complex biological
matrices to take into account in the application scenarios described above are the blood which is composed of proteins, electrolytes and other nano-sized molecules; tumor stroma constituted by extracellular matrix components such as collagen, fibronectin, hyaluronan, fibrin, and proteoglycans produced by fibroblasts in the case of solid tumors [66]; thick mucus, rich with heavily glycosylated mucins in the airways of the lungs; and collagen-polymer chains in humor vitreous gel from the eye [56], along with the tympanic membrane.

1.3.3 Colloidal Stability of Magnetic Nanoparticles in Biological Environments

In contact with biological environments, nanoparticles can lose their designed features due to electrostatic, steric and hydrodynamic interactions with macromolecules and ions, which can lead to aggregation or sedimentation. Therefore, controlled surface chemistry with biocompatible polymers for stabilization, and suitable bioconjugation strategies play a fundamental role in particle colloidal stability for biomedical applications. Indeed, the stability of a colloidal dispersion of nanoparticles is defined by the particles remaining suspended in a liquid medium after a given experimental observation time. A stable suspension can remain dispersed in solution for long periods of time (days to months) without a significant change in hydrodynamic diameter and without visible aggregation. Fundamental research using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory to predict the total energy potentials has been employed to provide a better understanding of the effects of polymer coating thickness on the stability of iron oxide magnetic nanoparticles in water under different ionic strength conditions (varying NaCl concentration) and pH, considering van der Waals, electrostatic, steric, and magnetic interparticle forces [69, 70].
Overall, biological environments are more complex systems than water, having different ionic strength and pH. For this reason, extended research in colloidal stability and nanoparticle-protein interactions after nanoparticle incubation with biological buffer solutions and biological entities have been widely performed in the last decade [71]. For iron oxide MNPs, colloidal stability studies have been performed in conditions that mimic biological environments, for example, saline solution, isotonic buffers (PBS or Hank’s balanced salt solution), incomplete and complete cell culture media, diluted blood, serum and plasma, dilute fetal bovine serum (FBS) or fetal calf serum, diluted cationic (histone, lysozyme) or anionic (e.g. human or bovine serum albumin, fibrinogen, transferrin and immunoglobulins) protein suspensions, sodium bicarbonate/ MES or HEPES buffers or even sodium citrate buffer (mimicking acidic lysosomal compartments)[71-84]. These studies have shown protein-corona formation, nanoparticle aggregation, nanoparticle dissolution, or irreversible desorption of anchoring ligands [7]. Moreover, the competitive adsorption of proteins onto metal oxide, silicon oxide, and polymer surfaces in plasma was described extensively by Vroman [85, 86] and is therefore commonly referred to as the “Vroman effect” [87, 88].

Although the first biological barrier that MNPs will encounter after intravenous injection is blood, there are limited studies in blood and crowded macromolecular environments to assess colloidal stability and nanoparticle-protein interaction in situ. Plasma proteins can absorb onto the surface of magnetic nanoparticles as a function of particle physicochemical properties, thus causing aggregation and activating immune response in the blood stream, and in tissue by local phagocytes. These nanoparticle-
blood interactions may result in altered pharmacokinetics and biodistribution, compromising magnetic nanoparticle therapeutic effects [89, 90].

According to Vogler [91], protein adsorption onto nanoparticles can be studied using two general experimental approaches. One approach measures the fraction of protein that remains adsorbed on the nanoparticles after an adsorbent-rinsing protocol. Because of the rinsing process, it is expected that weakly-bound proteins are removed and what remains are strongly bound proteins, hence representing the “hard protein corona”. Most often nanoparticles are incubated with proteins in isotonic conditions and are subsequently separated in order to determine which proteins, if any, were adsorbed [92]. The most common method for isolating unbound proteins from protein-nanoparticle complexes is centrifugation [93, 94]. Depending on the size and colloidal stability of the nanoparticles, centrifugation times can be long and/or high centrifugal forces may be applied, potentially leading to co-settling of un-adsorbed proteins and nanoparticles. Because the fraction of proteins adsorbed unto the nanoparticles is determined by spectrophotometric measurement of the protein in solution, co-settling of un-adsorbed proteins can lead to an overestimation of the extent of protein adsorption. In some cases this process can be further complicated due to interference by the nanoparticles with the spectrophotometric technique being used to quantify free proteins. This is particularly the case with magnetic iron oxide nanoparticles, where it can be alleviated by iron quantification of the supernatant and accounting for its effect on the spectrophotometric reading [95]. The second approach described by Vogler[91], corresponds to characterizing in situ the proteins that are tightly and loosely bound to the nanoparticles, the so-called hard and soft protein coronas [96].
1.4 *In Situ* Methods to Study Nanoparticle Colloidal Stability, Nanoparticle Mobility and Adsorption of Proteins

At present, techniques used to perform colloidal stability and nanoparticle-protein interaction assessments *in situ* of magnetic nanoparticles are based on optical methods, sedimentation through a density gradient, and magnetic measurements. All these methods are connected by using the Stokes-Einstein relation.

1.4.1 Optical Methods

Dynamic light scattering (DLS), photon correlation spectroscopy (PCS), zeta potential measurements, nanoparticle tracking analysis (NTA), and fluorescent correlation spectroscopy (FCS) are common optical methods used for colloidal stability and interaction studies. In DLS and zeta potential measurements, the speed at which the particles diffuse due to Brownian motion is measured by correlating the rate at which the intensity of the scattered light fluctuates, which is proportional to the sixth power of the particle diameter, thus making this technique very sensitive to the presence of large aggregates. NTA uses a laser scattering microscope and a charge-coupled device camera to record extremely dilute nanoparticle suspensions with an aim to identify and track individual nanoparticles moving under Brownian motion and uses the conventional mean squared displacement (MSD) method to estimate the translational diffusivity of particles in solution. Hence the hydrodynamic diameter can be estimated using the SE equation [97].

FCS measures the fluctuation of the fluorescence intensity produced by particles diffusing by Brownian motion in a very small volume (femtoliter). Röcker and collaborators [98] used human serum albumin (HSA) along with polymer coated FePt nanoparticles decorated with the dye DY-636 for quantitative protein adsorption analysis
and, determined that it only takes 100 seconds to form a protein corona of about 3.3 nm of thickness. This study was carried out using 4 nM nanoparticle solutions with HSA concentrations up to 65 mg/mL. Due to high sensitivity to large aggregates, several autocorrelation functions were measured, averaged and analyzed to properly relate the estimated translation diffusion coefficient with the SE equation and calculate the hydrodynamic diameter of particles in contact with HSA. Unfortunately, drawbacks of this technique are the use of fluorescent labels which can interfere with protein adsorption on the nanoparticle surface [99, 100] and can be quenched in proximity of the metal cores [101], and very low particle concentrations required for analysis [102]. For all the aforementioned optical techniques, a common limitation is the optical transparency required for sample evaluation. This restricts the use of whole blood as carrier fluid due to its complex nature (mixture of water, serum proteins, red blood cells and white blood cells) and study of high particle concentrations (> 10 mMFe).

1.4.2 Differential Centrifugal Sedimentation

To study patterns of aggregation, differential centrifugal sedimentation (DCS) has been employed to produce high resolution size distributions of particles between 10 nm to 50 um, made of several materials whose density is different from that of the solvent [81, 103, 104]. With knowledge of solvent viscosity and particle density, and using the Stokes equation, hydrodynamic diameter distributions in different solvents can be determined in situ by monitoring the time required for the particles to settle a certain distance. The concentration of particles at each size is determined by turbidity measurements which are converted to a weight distribution using Mie’s light scattering theory for spheres [105]. This information is used to correlate the protein corona thickness with the apparent size change of the nanoparticle-protein complexes
compared to the particles without proteins. Using DCS, Amiri et al.[106] demonstrated that protein corona thicknesses between 3-5 nm affect the relaxivity and magnetic resonance imaging contrast efficiency of magnetic nanoparticles by evaluating in situ protein corona formation of negatively and positively charged dextran coated MNPs in the presence of FBS (10%) at a concentration of 7 mMFe (1 hour incubation at 37°C). The major disadvantage of this technique is that nanoparticle-protein suspensions need to be diluted in a sucrose gradient which may disrupt the protein corona interface, and the turbidity data cannot be corrected based on particle density and fluid density.

1.4.3 Magnetic Measurements

Dynamic magnetic measurements can be used to evaluate changes in the local environment and size of the magnetic nanoparticles with extreme precision, and can be linked to changes in particle mobility due to particle aggregation or re-dispersion in different colloidal systems. Currently, techniques such as magneto-optical birefringence, temperature-dependent AC-susceptometry, dynamic magnetic susceptibility measurements, and magnetic particle spectroscopy (MPS) have become relevant to study colloidal stability or particle degradation as a function of changes in magnetization or magnetic relaxation with specific reproducibility in biological environments. A detailed explanation of these magnetic measurements is presented in the following sections.

1.4.3.1 Magneto-optical birefringence

Dynamic magneto-optical probing is achieved when in the presence of an external magnetic field thermally blocked particles experience a torque, causing the particle’s magnetic dipole to align along the field direction. Thus, the MNPs impart optical birefringence to the surrounding medium. After removing the magnetic field, the MNPs randomly disorient and the magneto-optical birefringence relaxes with a
characteristic time related to the rotational diffusivity of particles dispersed according to Debye’s equation and Perrin’s equation, respectively [107-109]. In the past, this relaxation technique has been used to quantify antibody adsorption and particle aggregation as a function of pH [110, 111]. In addition, Lartigue et al. [77] monitored changes in hydrodynamic sizes of negatively charged maghemite nanoparticles (10 mMFe) incubated with rat plasma (0-100%), BSA suspensions (up to 65 mg/mL) and Roswell Park Memorial Institute (RPMI) medium, showing that protein corona formation as a function of time affects macrophage uptake. Although highly concentrated protein suspensions can be assessed using this technique, the fluid carrier should be transparent because the decay of the birefringence is detected as decreasing light intensity. Thus, whole blood may be a challenge to assess. Also, for an increase in signal, particles with elongated structures (shape anisotropy) are preferred instead of spherical nanoparticles [112], which limits particle evaluation.

1.4.3.2 Temperature-dependent AC-susceptometry

To study particle degradation in complex environments, temperature-dependent magnetic properties at low frequencies have been studied using a superconducting quantum interference device (SQUID) based AC-susceptometry. This information has been used to evaluate biodistribution and degradation of iron oxide magnetic nanoparticles by monitoring the out-of-phase susceptibility component as a function of temperature [79, 113, 114]. The temperature dependence of the in-phase and out-of-phase susceptibility components during particle degradation has shown that the shift of the peak temperature location towards lowers temperatures over time is associated with particle size reduction in acidic environments. Conversely, in animal tissue samples it is
difficult to distinguish between reductions in the particle size from a possible decrease in particle-particle interactions or breaking of magnetic particle clusters.

1.4.3.3 Dynamic magnetic susceptibility measurements

The third option corresponds to the use of dynamic magnetic susceptibility (DMS), in which the complex magnetic susceptibility of MNPs is measured as a function of frequency. The response of MNPs, such as ferrimagnetic cobalt ferrite, thermally blocked iron oxide, and iron oxide with combined relaxation mechanisms can deliver precise information about the size distribution and local viscosity of a liquid suspension. To achieve this from the susceptibility spectra, DMS measurements are fitted to the Debye model of AC susceptibility by considering the polydispersity of the particles using a lognormal size distribution. From this analysis, the hydrodynamic diameter distributions of particles interacting with biofluids can be extracted to evaluate particle mobility, and rotational diffusivity of MNPs.

A sensor based on measurements of rotational relaxation time of magnetic nanoparticles was proposed by Connolly and St. Pierre in 2001 [115], wherein the change in hydrodynamic diameter of the nanoparticles upon binding to a targeted analyte would result in a change in the characteristic rotational relaxation time of the particles, measurable through DMS measurements as a function of frequency. This sensor principle was demonstrated to detect specific binding of prostate specific antigen to the surface of magnetic nanoparticles[116] and to detect S-protein and biotinylated T7 bacteriophage [117]. A similar concept has been applied to monitor aggregation of nanoparticles in aqueous suspensions [118] and melt polymers [119], to monitor gelation triggered by temperature [120], to obtain nanoscale viscosity measurements [121, 122] and to demonstrate the existence of a critical molecular weight for a
polyethylene glycol (PEG) melt, below which the Stokes-Einstein (SE) relation precisely describes the rotational diffusivity of PEG coated MNPs and above which the SE relation no longer applies [123]. Recently, Soukup et al. [124] used DMS measurements to follow the in situ magnetic response of superparamagnetic and thermally blocked iron oxide MNPs, stabilized electrostatically by citric acid coating, after their intracellular internalization and posterior release by freeze-thaw lysis. The findings obtained by Soukup et al. may have significant implications in the design of MNPs for magnetically mediated energy delivery systems [125] and confirmed the potential of DMS measurements in monitoring in situ mobility of MNPs in cells, a crowded macromolecular environment.

1.4.3.4 Magnetic particle spectroscopy

As a fourth option, magnetic particle spectroscopy (MPS) measurements have shown that changes in the magnetization of magnetic nanoparticles are linked to their colloidal stability in cell culture media. The derivative of magnetization as a function of the magnetic field has been measured under typical magnetic particle imaging (MPI) conditions to determine aggregation patterns, and to provide insights for the enhanced design of MNPs for MPI applications [126]. Also, changes in the magnetization of MNPs were monitored to detect re-dispersion of MNP aggregates mediated by the proteolytic cleavage with trypsin or MMP-2 proteases in cell culture supernatants. These MNP aggregates were formed due to the high affinity between neutravidin-coated MNPs and biotinylated peptides and served as an assay platform for detection of cancer-specific proteases [127]. Although MPS represents a sensitive magnetic tool to study colloidal stability of MNPs, and the current theoretical description of MPS/MPI spectra allows the calculation of the particle core size distribution, for a more precise calculation of the
complex MPS spectra better models including appropriate expressions for the magnetic-field dependence of the Néel time constant are required [128].

1.5 Magnetic Relaxation Measurements to Study Mobility of Magnetic Nanoparticles in Biological Matrices

The magnetization equation is a phenomenological equation describing the behavior of magnetic nanoparticles suspended in a nonmagnetic medium. The magnetization vector \( \mathbf{M} \) of the particles changes according to the magnetization equation derived by Shliomis [129, 130]:

\[
\frac{\partial \mathbf{M}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{M} = \mathbf{\omega} \times \mathbf{M} - \frac{1}{\tau} \left( \mathbf{M} - \mathbf{M}_{eq} \right)
\]

(1-25)

Here the second term on the left-hand side corresponds to the convective derivative of the magnetization for linear motion. On the right-hand side, the first term represents changes in magnetization due to “rotation” of the medium and the second term represents change in magnetization \( \mathbf{M}_{eq} \) via various “relaxation” processes, with combined time constant \( \tau \) [131].

When the fluid is at rest in a stationary unidirectional magnetic field, the equilibrium magnetization \( \mathbf{M}_{eq} \) is given by the Langevin function. According to the Langevin function, in the low magnetic field limit, the magnetization is approximately linear with \( H \) and has constant magnetic susceptibility \( \chi_0 \). The relation between \( \mathbf{M} \) and \( H \) is expressed as \( \mathbf{M}_{eq} = \chi_0 H \). \( \chi_0 \) is in general dependent on suspension composition and temperature, in addition to particle and suspending fluid properties. This relation is given explicitly for weakly interacting particles by [132]

\[
\chi_0 = \frac{\phi \mu_0 \pi M^2 d^3}{18k_B T},
\]

(1-26)
where $\phi$ is the volume fraction of the magnetic particles, $\mu_0$ is the permeability of free space, $M_d$ is the domain magnetization, $d_c$ is the core diameter, and $k_BT$ is the thermal fluctuation energy. This low magnetization limit is appropriate when the Langevin parameter $\alpha_L$ is much less than unity

$$\alpha_L = \frac{\pi \mu_0 M_d H d_c^3}{6 k_BT} = \frac{mH}{k_BT} \ll 1 \quad (1-27)$$

here $H$ is the magnetic field within the fluid, and $m$ is the magnetic moment of a single particle.

The commonly accepted relaxation processes for practical ferrofluid suspensions are rotational Brownian motion, and Néel relaxation [132]. When the reorientation of the magnetic moment of a particle suspended in a liquid is tied to the rotation of the particle itself, the Brownian relaxation time of rotational diffusion is given by Equation

$$\tau_B = \frac{\pi \eta D_h^3}{2k_BT} = \frac{3V_h \eta}{k_BT}, \quad (1-28)$$

where $\eta$ is the viscosity of the suspended fluid and $D_h$ is the hydrodynamic particle diameter and $V_h$ is the hydrodynamic volume.

The Néel theory of superparamagnetism explains that a spontaneous change of the magnetization direction commonly occur in single-domain particles under the influence of thermal fluctuations, thus leading to the following expression [133]

$$\tau_N = \frac{1}{f_0} \exp \left( \frac{KV}{k_BT} \right) = \tau_0 \exp \left( \frac{\Delta E}{k_BT} \right), \quad (1-29)$$

where $K$ is the anisotropy constant, $V$ is the volume of the particle, and $f_0$ is the frequency factor of the order of $10^{-9}$ s.
In consequence, the relative contribution of the Brownian and Néel relaxation times is related as

$$\frac{1}{\tau} = \frac{1}{\tau_B} + \frac{1}{\tau_N} \quad (1-30)$$

thus, relaxation time $\tau$ of the magnetization of the suspension is obviously determine by the shorter of the times $\tau_N$ and $\tau_B$.

Since we considered MNPs suspended in a quiescent fluid, in the magnetization equation (1-25) the convective and rotational terms are neglected. Also, MNPs are assumed to align along the applied magnetic field $H = H_0 \cos \Omega t$ (uniaxial magnetic field). Considering all the previous assumptions the time dependent magnetization equation can be summarized as follow

$$\frac{dM}{dt} = -\frac{1}{\tau} \left[ M - M_{eq} \right]. \quad (1-31)$$

Equation (1-31) is the magnetization relaxation equation for the particle suspension described by the simplest Debye-like equation in which any deviation of $M$ from its equilibrium value $M_{eq}$ decays exponentially $\left[ M - M_{eq} \right] = e^{-\frac{t}{\tau}}$. Thus, the time-dependent magnetization response is given by [134]

$$M(t) = \chi(\Omega) \cdot H \quad (1-32)$$

The complex frequency-dependent magnetic susceptibility, $\chi(\Omega)$, depends on its real and imaginary components as follows: $\chi(\Omega) = \chi' - i\chi''$, where $\chi'$ and $\chi''$ are the real and imaginary components of the susceptibility

$$\chi' = \frac{\chi_0}{1 + \Omega^2 \tau^2} \quad (1-33)$$

$$\chi'' = \frac{\Omega \tau \chi_0}{1 + \Omega^2 \tau^2} \quad (1-34)$$
Debye’s theory of dielectric relaxation of non-interacting polar molecules under the influence of their rotational Brownian motion and a time-varying applied field of frequency $\omega$ has been applied widely to ferrofluids [135, 136]. Specifically, Debye’s theory for MNPs featuring Brownian-like behavior, also called thermally blocked MNPs, relates the characteristic Brownian relaxation time $\tau_B$ of the MNPs to the viscosity of the suspension medium, and the hydrodynamic diameter of the nanoparticle [137]. Typically, the out-of-phase susceptibility component in a Debye plot for a Brownian MNP reaches a maximum at the condition where $\Omega_{\text{peak}} \tau_B = 1$ in the Equation (1-34), whereas the effective relaxation time can be obtained from the inverse of the frequency corresponding to a peak in $\chi''$ and this allows to determine an average particle diameter when the viscosity of the system is known.

Dynamic magnetic susceptibility (DMS) measurements can be employed to determine frequency-dependent diffusion coefficient of a system of interacting Brownian relaxing MNPs. Thus, the medium viscosity can be estimated by correlating the susceptibility response of the MNPs with their rotational diffusivity when the magnetic nanoparticle size distribution is known [123]. Also, when it is assumed that the interactions between the particle probes and the polymer chains in the complex fluid are relevant (e.g. electrostatic, steric, hydrophobic and hydrodynamic interactions within a matrix of a particular mesh size), DMS data can be used to assess mobility of MNPs and to determine the nanoparticle size distributions due to nanoparticles-protein interactions in dilute protein suspensions [78]. This mobility assessment tool, knowing the viscosity of the complex fluid or assuming a reference value ($\eta_{\text{water}-25^\circ\text{C}} = 0.001$ Pa.s), can be linked to the colloidal stability of the magnetic nanoprobe. The technique
can be applied to relatively small sample sizes (< 200 µL) and even optically complex and opaque fluids, with a low concentration of MNPs (~0.06%-0.2% v/v) [78, 121, 138]. The equations required to determine nanoparticle size distributions or particle rotational diffusivity are presented in the sections which follow.

1.5.1 Analysis of Dynamic Magnetic Susceptibility Spectra to Obtain Nanoparticle Size Distributions

A superconducting quantum interference device (SQUID) based AC-susceptometer and commercially available AC-susceptometers are commonly used to perform dynamic magnetic susceptibility measurements by applying a small AC magnetic field \( H = H_0 \cos \Omega t \) that may be superimposed on a DC field. This causes a time-dependent rotation of a collection of MNPs where the magnetic dipoles undergoes a rotation in the same direction as the applied field. The measured signal is obtained through an induced voltage in a receive coil. Assuming that the particles follow a lognormal volume distribution of hydrodynamic diameter \( n_v(D_h) \), \( \chi'' \) is defined as [78]

\[
\chi'' = \frac{1}{\pi \eta D_{hpv}^3} \int_0^\infty \frac{\Omega \tau_{hpv} y^3}{1 + \Omega^2 \tau_{hpv}^2 y^6} n_v(D_h) dy; \quad y = \frac{D_h}{D_{hpv}};
\]

\[
\tau_{hpv} = \frac{\pi \eta D_{hpv}^3}{2k_B T} = \frac{1}{2D_{R,DMS}},
\]

where

\[
n_v(D_h) = \frac{1}{\sqrt{2\pi D_h \ln \sigma_g}} \exp \left[ -\frac{\ln^2 y}{2\ln^2 \sigma_g} \right],
\]

\( \chi_{0, hpv} \) and \( \tau_{hpv} \) are the volume weighted median diameter of the distribution, and \( \ln \sigma_g \) is the geometric deviation of the lognormal distribution. In Equation (1-35) \( \chi_{0, hpv} \) and \( \tau_{hpv} \) are the volume
weighted average initial susceptibility and the relaxation time, respectively. The DMS spectra can be fitted to the Debye’s model in Equation (1-35) using the probability density of the particles described in Equation (1-36), through nonlinear regression in order to determine the lognormal volume diameter distribution and a geometric deviation as the fit parameters of the particles in a fluid of known viscosity. The arithmetic mean diameter, $D_p$, and standard deviation, $\sigma$, of the resulting diameter distributions can be calculated as given

$$D_p = \exp\left( \ln D_{\text{hg},v} + \frac{\ln^2 \sigma_g}{2} \right),$$  \hspace{1cm} (1-37)

$$\sigma = \sqrt{\exp\left( \ln^2 \sigma_g - 1 \right)}$$  \hspace{1cm} (1-38)

1.5.2 Analysis of Dynamic Magnetic Susceptibility Spectra to Obtain Particle Rotational Diffusivity(1-39)

DMS measurements can be employed to determine the local diffusivity of particles in the surrounding environment. Independent values of the hydrodynamic diameter in a reference solvent (e.g. water or an isotonic buffer) can be used in the non-linear regression to Equation (1-35) to obtain the viscosity experienced by the nanoparticles as the fit parameter. Then, the viscosity and particle size distribution are related to the particle rotational diffusivity by Equations (1-40) and (1-41).

$$D_{R-\text{DMS}} = \int_0^{\infty} \frac{k_B T}{D_{\text{h},v} \eta_{\pi}} n_p(D_h) dD_h = \frac{k_B T}{D_{\text{h},v} \eta_{\text{DMS} \pi}} \exp\left( \frac{9}{2} \ln^2 \sigma_g \right),$$  \hspace{1cm} (1-40)

$$\eta_{\text{DMS}} = \frac{k_B T}{\pi D_{\text{h},v} D_{R-\text{DMS}}} \exp\left( \frac{9}{2} \ln^2 \sigma_g \right)$$  \hspace{1cm} (1-41)

Through this analysis, the rotational diffusion and solution viscosity can be effectively determined.
1.6 Dissertation Outline and Objectives

In this work, it is hypothesized that MNPs can be engineered with enhanced colloidal stability in biological matrices by characterizing nanoparticle-protein interactions and mobility patterns of MNPs in complex biological environments *in situ* monitoring magnetic relaxation measurements. These relaxation measurements can be obtained by dynamic magnetic susceptibility spectra generated by the oscillatory response of the magnetic dipoles in a small amplitude alternating magnetic field. The complex susceptibility data is directly connected to the rotational diffusion coefficient of the magnetic nanoparticles by applying the Debye model of susceptibility in conjunction with the Stokes-Einstein relation.

1.6.1 Objectives

The purpose of this dissertation is to systematically study colloidal stability as a function of polymer coated magnetic nanoparticles mobility in relevant biological buffer solutions, protein suspensions, blood and tumor tissue ex-vivo using magnetic relaxation measurements as a complementary characterization technique for the design of MNPs for biomedical applications. The technique focuses on measurements of the rotational relaxation time of MNPs to provide information of particle mobility; wherein, a change in the local environment affecting the characteristic rotational relaxation time of the particles can be detected through dynamic magnetic susceptibility measurements as a function of frequency. The polymer coatings carboxymethyl dextran (negatively charged), hydroxyethyl starch and polyethylene glycol (neutral charged polymers), and branched polyethyleneimine (positively charged) evaluated in this work, represent commonly used polymers for biomedical applications. Inorganic cores used were thermally blocked magnetic nanoparticles (cobalt ferrite MNPs) and commercial
thermally blocked iron oxide nanoparticles. Moreover, it was demonstrated that the mobility assessment can be extended to systems of particles relaxing by Brownian and Néel relaxation mechanisms which are typically obtained by chemical co-precipitation of ferric and ferrous salts in alkaline medium. Specific aims were to:

1. Monitor nanoparticle-protein interactions in situ by dynamic susceptibility measurements
2. Estimate rotational diffusivity of magnetic nanoparticles in concentrated protein solutions
3. Determine the mobility of polymer coated magnetic nanoparticles in blood and tumor tissue ex-vivo using magnetic relaxation measurements

1.6.2 Chapters Overview

In Chapter 2, the use of dynamic susceptibility measurements to study nanoparticle-protein interactions in situ was demonstrated. Briefly, to illustrate the technique data relating the effect of surface charge in carboxymethyl dextran coated cobalt ferrite MNPs and their interaction with model anionic and cationic proteins is presented. Proteins such as bovine serum albumin (BSA), immunoglobulin G (IgG), fibrinogen (FIBR), apo-transferrin (TRANS), lysozyme (LYZ), and histone (HIS) were studied in a protein concentration range between 0-10 mg protein/mL whereas BSA was studied up to 45 mg protein/mL to mimic albumin concentration in blood. Through this study, it was confirmed that nanoparticle-protein interactions were dominated by electrostatic repulsion between like-charged particle-protein pairs and attraction between oppositely charged protein-particle pairs.

Once the technique was established, measurements of the rotational diffusivity were made for neutral polyethylene glycol (PEG) coated cobalt ferrite MNPs in
concentrated BSA protein suspensions (0-200 mg protein/mL) at different pH, with the aim of mimicking the crowded intracellular environment. These results were reported and compared with the values estimated using the Stokes-Einstein relation along with bulk rheological measurements in Chapter 3. Rotational diffusivity was shown to be consistent with the Stokes-Einstein relation in water suspensions below and above the BSA isoelectric point. However, deviations were observed in the presence of salts and chelating agents.

In Chapter 4, we extended these studies based on DMS measurements to study the colloidal stability and mobility of nanoparticles in whole blood and tumor tissue. The polymer coatings evaluated were carboxymethyl dextran, hydroxyethyl starch, PEG and branched polyethyleneimine (PEI). Inorganic cores such as cobalt ferrite and iron oxide magnetic nanoparticles were used. It was confirmed that the mobility of particles in blood and particle distribution in tumor tissue depends strongly on electrostatic interactions and the nature of the coating.

Dynamic susceptibility measurements show that the dynamic response of MNPs in an alternating magnetic field can be used to study colloidal stability as a function of particle mobility in situ in highly concentrated protein suspensions, blood and tumor tissue environments. This can take place without the limitation of optical transparency in sample evaluation, and provides a useful assessment tool to design nanoparticles for biomedical application with enhanced colloidally stability and minimal interactions with blood, plasma proteins and tumor-stroma microenvironment. Finally, Chapter 5 provides a research summary with conclusions and future directions to study colloidal stability and nanoparticle-protein interactions in situ as a function of the mobility of polymer
coated magnetic nanoparticles in relevant biological environments using magnetic relaxation measurements.
CHAPTER 2
MONITORING NANOPARTICLE-PROTEIN INTERACTIONS IN SITU BY DYNAMIC SUSCEPTIBILITY MEASUREMENTS

2.1 Introduction

Interactions between nanoparticles and opsonin proteins can play a determining role in the biocompatibility and fate of nanomaterials used for in vivo applications.[139] The competitive adsorption of proteins onto metal oxide, silicon oxide and polymer surfaces in plasma was described extensively by Vroman [85, 86] and is therefore commonly referred to as the “Vroman effect” [87, 88]. Depending on the physicochemical properties of nanoparticles, proteins can adsorb to the nanoparticle surface forming a so-called protein corona,[96] which can be either quite stable (the so-called “hard protein corona”) or fragile (the so-called “soft protein corona”)[140] and which potentially changes over time [141]. The adsorption of proteins onto the surface of nanomaterials can result in their specific and/or non-specific uptake by cells [142-144], can lead to their aggregation or to their stabilization[145], could screen functional groups on the surface of the nanoparticles, such as targeting ligands, and prevent their action, thus ultimately impacting their distribution and fate in an organism. As such, it is of great importance to characterize and understand the interaction of nanoparticles and proteins.

In this contribution, we report the use of dynamic magnetic susceptibility measurements to study nanoparticle-protein interactions in situ. The technique consists of measuring the rotational diffusivity of thermally blocked magnetic nanoparticles in

diluted protein solutions. To illustrate the technique, we studied the effect of nanoparticle zeta potential in carboxymethyl dextran coated magnetic nanoparticles and their interaction with model anionic and cationic proteins, such as albumin (BSA), immunoglobulin G (IgG), fibrinogen (FIBR), apo-transferrin (TRANS), lysozyme (LYZ), and histone (HIS) in a range of protein concentrations.

2.2 Materials and Methods

Iron (III) chloride hexahydrate (FeCl₃·6H₂O, 97%), cobalt (II) chloride hexahydrate (CoCl₂·6H₂O, 98%), sodium hydroxide (≥98%) pellets, iron (III) nitrate nonahydrate (≥98%), dimethyl sulfoxide (DMSO, ≥98%), chloroacetic acid (≥99%), dextran from *Leuconostoc mesenteroides* with average molecular weight of 9,000-11,000 Da, and N-hydroxysuccinimide (NHS) purchased from Sigma Aldrich (ACS reagents) were used. The following reagents, tetramethylammonium hydroxide ((CH₃)₄NOH, 10%), acetic acid (99.8%, for analysis), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), glycerol (99+% extra pure) were purchased from Fisher scientific. 3-aminopropyltriethoxysilane (NH₂(CH₂)Si(OC₂H₅)₃) (APS) was purchased from TCI America.

The proteins selected in this study were obtained from Sigma Aldrich and used as received without further purification. The purity percentages are detailed as follow: bovine Serum Albumin (BSA, ≥98%), lysozyme from chicken egg white (LYZ, ≥90%), IgG from bovine serum (IgG, ≥95%), fibrinogen from bovine plasma protein (FIBR, 65-85%), apo-transferrin bovine (TRANS, ≥98%), and histone from calf thymus (HIS) which was a natural mixture of histones H1, H2A, H2B, H3, and H4.
Differences between histones correspond to the fraction lysine rich (H1), fractions slightly lysine rich (H2A and H2B), and fractions arginine rich (H3, H4), respectively.

2.2.1 Synthesis of CMDx with Different Degrees of Substitution

For the purpose of these studies, CMDx with nominally 5, 23, and 31 carboxylic acid molecules per dextran chain were prepared following a procedure described by Ayala et al.[146]. Modified polymers were prepared by carboxymethylation reaction of dextran from Leuconostoc mesenteroides by reacting with monochloroacetic acid in the presence of sodium hydroxide at 70 °C for 1 hour. The degree of carboxymethylation was controlled by adjusting the concentration of sodium hydroxide. In general, 20 g of dextran were dissolved in 100 mL of distilled water. Sodium hydroxide (NaOH) was dissolved in 34 mL of distilled water to obtain a final concentration of 1M, 2M or 3M, depending on the desired degree of substitution of -COOH groups per dextran chain. The solutions were cooled in an ice bath to 4°C. Afterwards, NaOH was added dropwise to the dextran solution. Then, 29 g of solid monochloroacetic acid was added and dissolved using a small rotor. After the reaction, the mixture was neutralized to pH 7 with acetic acid. The product was precipitated with ethanol overnight. A solid ambar paste was recovered, suspended in water (20 mL) and dialyzed until a conductivity of ≤ 10 µS/cm was obtained. Finally, the CMDx was concentrated using rotary evaporation and dried at 60°C. The dried sample was powdered and stored at 4°C.

2.2.2 CMDx Acidimetric Titration

After reaction, the CMDx was obtained as a sodium salt. To determine the degree of substitution (DS) of the –COOH groups by acidimetric titration it is required to convert the CMDx to the free acid form by washing it with an acid reagent [147]. In brief,
1 g of CMDx was washed with 14 mL of a solution of anhydrous methanol and nitric acid 70 %v/v. This reaction mixture was mechanically stirred at 150 rpm overnight at room temperature. The acid reagent was removed by vacuum filtration and the CMDx was finally washed several times with ethanol to remove traces of the acid reagent, and dried in a vacuum oven at 60°C. To accomplish the acid titration, a solution at 1% w/v of the CMDx in free acid form was prepared in a distilled water/acetone mixture (1:1) and 5 mL to 10 mL of NaOH 0.0121 N were added (n = 3 per each CMDx prepared). This solution was titrated with hydrochloric acid 0.0121N, using phenolphthalein as indicator.

2.2.3 Preparation of CMDx Coated Cobalt Ferrite Nanoparticles

The cobalt ferrite particles synthetized via co-precipitation [148] were surface modified with HNO₃ 2 M and hydrothermally treated with ferric nitrate according to Gomes et al.[149] MNPs were then peptized using tetramethylammonium hydroxide (TMAO) for further surface modification using condensation of APS molecules to covalently attach CMDx. Indeed, peptized nanoparticles were functionalized with APS to graft functional amine groups (-NH₂) onto the nanoparticle surfaces following a procedure described by Herrera et al.[150]. For this, peptized nanoparticles were suspended in DMSO, 10 mL APS, 1.25 mL of water, and 100 μL of acetic acid. The reaction mixture was mechanically stirred at 150 rpm for 72 hours at room temperature. Nanoparticles were washed four times with ethanol, and dried at room temperature to obtain a layer of black solid APS coated nanoparticles (ICO-APS). ICO-APS nanoparticles were functionalized with CMDx via EDC/NHS chemistry in water [73]. This reaction was carried out by dissolving 4 g of CMDx in 40 mL deionized water (pH 4.5-5), with 100 mg of EDC and 60 mg of NHS. The CMDx solution was mixed with the ICO-APS solution (0.4 g ICO-APS per 40 mL of deionized at the same pH) and stirred at 150
rpm for 36 hours at room temperature. Finally, CMDx coated nanoparticles were washed three times with ethanol (1:3) followed by centrifugation at 7500 rpm for 15 min, and dried at 60°C in a vacuum oven. The nanoparticles were stored at 4°C for future experiments. This procedure was followed for each of the prepared CMDx (CMDx-A, CMDx-B, and CMDx-C).

2.2.4 Characterization of Magnetic Nanoparticles

To prepare samples for TEM, particles were suspended in water at a concentration of 1 mg particles/mL, 3 μL of particle solution was placed in formvar coated copper grids and dried in a vacuum oven for 30 min. A JEOL 1200EX Transmission Electron Microscope (TEM) was used to image the particles and the images were analyzed to estimate the size distribution of magnetic cores. The number weighted mean diameter \( \langle D_{pgw} \rangle \) and geometric deviation \( \ln \sigma_g \) were determined by fitting a histogram of nanoparticle diameters determined using image analysis (ImageJ, distributed by NIH) to a log-normal size distribution [151].

A Brookhaven Instruments Zeta Plus was used to determine the hydrodynamic diameter of the nanoparticles and their zeta potential. The hydrodynamic diameter of ICO-A, ICO-B and ICO-C was measured at 37°C by suspending particles (0.9 mg core/mL) in water and filtering the suspension with 0.2 μm nylon syringe filters. For DLS measurement the data was interpreted using the lognormal distribution of an average of three separate volume weighted measurements, providing a histogram of particle size and their corresponding relative intensity. Zeta potential measurements were performed at 37°C, at 0.9 mg core/mL, in a mixture water:glycerol (40%wt glycerol).
A Mettler Toledo STARe TGA/DSC1 Thermogravimetric Analyzer was used to estimate the amount of CMDx attached to the magnetic cores. Samples were dried at 115 °C in order to remove adsorbed water and solvent traces. A temperature ramp from 25°C to 800°C was used, with a heating rate of 10°C/min in air. Samples were analyzed in triplicate to obtain an average of the remnant weight.

A Quantum Design MPMS XL-7 SQUID magnetometer was used to measure the dynamic magnetic susceptibility of the suspended nanoparticles at 37°C. In order to identify Brownian behavior of cobalt ferrite magnetic nanoparticles synthesized by co-precipitation method (ICO), dynamic magnetic susceptibility measurements of bare ICO particles were realized before modification with CMDx. The solution concentration was 14 mg core/mL, estimated by thermogravimetric (TGA) measurements. The ferrofluid solution was mixed with 80% wt. glycerol to perform DMS measurements.

2.2.5 Dynamic Magnetic Susceptibility Measurements in Protein Suspensions

As illustrated in Figure 2-1, adsorption of protein or protein induced aggregation would lead to a change in the effective hydrodynamic diameter of the particles and hence in their rotational diffusivity and Brownian relaxation time. To illustrate the technique, we consider nanoparticles coated with carboxymethyl substituted dextrans (CMDx) of varying degrees of carboxylic acid substitution (which we call CMDx-A, CMDx-B and CMDx-C and which have approximately 3, 23 and 31 carboxylic groups per dextran chain), resulting in particles with varying levels of negative zeta potential. Here, it was hypothesized that nanoparticle rotational diffusion will be affected by interaction with proteins in a manner consistent with electrostatic attraction/repulsion depending on the charge of the protein of interest, and at concentrations of up to 45 mg protein/mL.
For nanoparticle-protein interaction studies the field amplitude used in the dynamic magnetic susceptibility measurements was 2.0 Oe, with a frequency range between 0.1 Hz to 1,300 Hz.

The nanoparticles were suspended at a concentration of 0.9 mg core/mL (0.2% v/v) in a water:glycerol (40%wt. glycerol) mixture filtered with 0.2μm nylon syringe filters. Proteins at different concentrations were suspended in particle solutions by using sonication in an ice bath before experiments were carried out (1 min). It has been shown that at high concentrations glycerol prevents protein aggregation, a fact that is attributed to preferential protein hydration in water-glycerol mixtures [152, 153], hence we can be sure that for our experiments the proteins should not be significantly affected by the addition of glycerol. Experiments were carried out at 37°C mimicking biological conditions.

2.3 Results and Discussion

Acidimetric titration showed that the number of carboxylic acid groups per chain were 3.1 ± 0.7, 23 ± 1, and 31 ± 9, for CMDx-A, CMDx-B, and CMDx-C respectively. The hydrodynamic diameter of bare cobalt ferrite nanoparticles as-synthesized by the co-precipitation method was calculated through dynamic magnetic susceptibility measurements (Figure 2-2) and the Debye model, resulting in a hydrodynamic diameter of 49 nm (ln σ = 0.49) by fitting the in-phase susceptibility component and a hydrodynamic diameter of 49 nm (ln σ = 0.46) according to fitting the out-of-phase susceptibility component. MNPs cluster formation due to the co-precipitation synthesis method and posterior stabilization via peptization was confirmed as a result of observation of a larger hydrodynamic diameter relative to the physical diameter obtained by TEM analysis.
The core diameter of the nanoparticles after CMDx conjugation was determined by TEM analysis, which demonstrates that the modified particles have a mean diameter of 14 nm (with a geometric deviation ln σ = 0.45), 11 nm (ln σ = 0.47), and 11 nm (ln σ = 0.43), for particles coated with CMDx-A (ICO-A), CMDx-B (ICO-B), and CMDx-C (ICO-C). TEM images and histograms are shown in Figure 2-3, and it can be noticed that MNPs have a wide size distribution. Note that although particles are seen in clusters there is space between them due to the grafted CMDx.

DLS measurements at 37 °C demonstrated that ICO-A, ICO-B, and ICO-C particles have a volume weighted hydrodynamic diameter of 68 nm (ln σ = 0.48), 86 nm (ln σ = 0.46) and 98 nm (ln σ = 0.44), respectively (Figure 2-4). No aggregation was observed after 1 week of suspension stored at 4 °C. The hydrodynamic diameter of the particles was also calculated using the DMS spectra of the particles suspended in 60:40 %wt water:glycerol mixtures and a nonlinear regression fit to the Debye model weighted by the lognormal size distribution.

As can be observed in Table 2-1, the hydrodynamic diameter distributions obtained from fitting the Debye model weighted by a lognormal size distribution to the dynamic magnetic susceptibility spectra are in good agreement with the distribution obtained from dynamic light scattering in the 60:40 %wt water:glycerol mixtures in the absence of protein.

A concentration of 0.9 mgcore/mL (0.2% v/v of MNPs) was determined to be optimal for the dynamic magnetic susceptibility experiments such that the in-phase and out-of-phase susceptibilities were within the SQUID magnetometer detection limits with no particle aggregation effects. This particle concentration was used for all subsequent
experiments in protein suspensions. When the particles were suspended in 60:40 %wt water:glycerol mixtures the resulting pH was close to 6, hence we expect that all the proteins retain their surface charge (negative or positive depending on their isoelectric points). The addition of glycerol is common practice in preparing protein solutions for crystallography [153] and does not have an impact in protein conformation. Afterwards, the protein experiments were designed in order to develop a characterization technique to study nanoparticle-protein interactions in situ, and the use of buffers and salts was avoided in order to avoid electrostatic interactions from ions that can screen nanoparticle-protein interactions in the evaluated particle platform.

Table 2-1. Hydrodynamic diameter distributions values obtained from dynamic light scattering (lognormal volume weighted) and DMS measurements at 0.9 mg core/mL

<table>
<thead>
<tr>
<th>Particles</th>
<th>DLS</th>
<th>AC Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dh, [nm]</td>
<td>Ln σ, [A.U]</td>
</tr>
<tr>
<td>ICO-A</td>
<td>68</td>
<td>0.48</td>
</tr>
<tr>
<td>ICO-B</td>
<td>86</td>
<td>0.46</td>
</tr>
<tr>
<td>ICO-C</td>
<td>98</td>
<td>0.44</td>
</tr>
</tbody>
</table>

The zeta potentials of the particles at 37 °C were -30 ± 1 mV, -45 ± 3 mV, -54 ± 2 mV, for ICO-A, ICO-B, and ICO-C particles, respectively. Also, no aggregation or settling was observed during zeta potential analysis and after 1 week of suspension in the 60:40 %wt water:glycerol mixtures at 4 °C, indicating that the CMDx coated MNPs are stable for at least 1 week at the experimental conditions used. The CMDx coating is covalently bonded to the particles and it is expected to stay in place, rather than being displaced by other macromolecules as can happen when the coating is adsorbed to the particle surface [73, 154]. The fact that the particles used here have a stable coating and are colloidally stabilized by steric repulsion is a key factor in interpreting the experiments discussed below as it allows us to attribute the observed changes in
relaxation time and hydrodynamic diameter to adsorption and aggregation induced by
the presence of proteins.

Thermogravimetric analysis of bare, ICO-A, ICO-B and ICO-C magnetic
nanoparticles indicates that the cobalt ferrite cores correspond to 93.7 ± 0.3 %, 6.9 ±
0.8 %, 13.4 ± 0.5 % and 19.4 ± 0.4 %, respectively, of the total mass per particle
(Figure 2-5).

During the experimental design, proteins that possess either an acidic or basic
isoelectric point were chosen to compare nanoparticle-protein interactions from the
point of view of electrostatic interactions. The bovine counterpart of human serum
albumin, Bovine Serum Albumin (BSA), immunoglobulin G (IgG), apo-transferrin
(TRANS), and fibrinogen (FIBR), were negatively charged at the experimental
conditions, while lysozyme (LYZ) and histone (HIS) were positively charged. These
model proteins were employed for the analysis of nanoparticle-protein interactions using
ICO-A, ICO-B, and ICO-C particles. For ICO-A particles a LYZ and HIS concentration
range between 0.1-10 mg of protein/mL was used. HIS concentration for ICO-B and
ICO-C was 10 mg of protein/mL. BSA concentration used for all particles was between
1-45 mg of protein/mL. For all other proteins, we used a concentration of 10 mg/mL.

2.3.1 Nanoparticle-Protein Interactions Between ICO Nanoparticles and Negatively
Charged Proteins

It was hypothesized that negatively charged proteins would repel from the
negatively charged nanoparticles, hence there would be no changes in the Brownian
relaxation time or hydrodynamic diameter. This expectation was confirmed by the
hydrodynamic diameter distributions obtained from fitting the Debye model to the
dynamic magnetic susceptibility measurements, shown in Figure 2-6.
Carboxylate groups in aspartic acid and glutamic acid confer negative surface charge to BSA, IgG, TRANS and FIBR proteins in the experimental conditions evaluated. According to Hermanson [155], both aspartic and glutamic acids contain carboxylate groups that have similar ionization properties with pK\(_a\) lower than 5. For this reason at pH above 5, these groups are generally ionized to negatively charged carboxylates resulting in a net negative surface charge. Thus electrostatic repulsion between particles and these anionic proteins avoids adsorption and aggregation, and the particles can freely rotate under the action of the alternating magnetic field. This maybe can be the reason for not observing an increase in the hydrodynamic diameter distributions at these experimental conditions (Figure 2-6a and 2-6b).

The hydrodynamic diameter distributions obtained from fitting the Debye model to the dynamic magnetic susceptibility measurements for ICO-B particles in proteins are presented in Figure 2-6c and 2-6d. As with the ICO-A particles the hydrodynamic diameter distributions obtained from fitting the Debye model are in good agreement with the distribution obtained from dynamic light scattering in the 60:40 %wt water:glycerol mixtures in the absence of proteins, indicating that there is little adsorption of proteins or aggregation induced by the presence of the proteins.

The hydrodynamic diameter distributions of the ICO-C particles seemed to decrease slightly in the presence of anionic proteins as shown in Figure 2-6e and Figure 2-6f. From a size of 89 nm (ln \(\sigma = 0.28\)), hydrodynamic diameter and polydispersity was reduced to 86 nm with ln \(\sigma = 0.29\) in 1 mg BSA/mL, 87 nm (ln \(\sigma = 0.26\)) in 10 mg BSA/mL, 82 nm (ln \(\sigma = 0.37\)) in 10 mg FIBR/mL, 84 nm (ln \(\sigma = 0.13\)) in 10 mg IgG/mL,
and 85 nm ($\ln\sigma = 0.16$) in 10 mg TRANS/mL from fitting the Debye model weighted by a lognormal size distribution to the out-of-phase susceptibility spectra. These shifts in hydrodynamic diameter relative to the diameter obtained by fitting the Debye model in the 60:40 %wt water:glycerol mixture in the absence of proteins are relatively small. At 45 mg BSA/mL the hydrodynamic diameter obtained from fitting the Debye model to the dynamic magnetic susceptibility was 96 nm ($\ln\sigma = 0.25$), a value that is close to the DLS measurement. However, at such high protein concentrations it is expected that the viscosity experienced by the particles may increase[156], hence our use of the viscosity of 60:40 %wt water:glycerol mixture in determining the hydrodynamic diameter from the dynamic magnetic susceptibility measurements may introduce an error that tends to over-estimate the size of the particles at high protein concentrations.

2.3.2 Nanoparticle-Protein Interactions between ICO nanoparticles and Positively Charged Proteins

In the case of interaction with positively charged proteins, we hypothesized an increase in hydrodynamic diameter and perhaps aggregation induced by electrostatic interactions with the negatively charged particles. In addition, it is expected that these effects would be more pronounced with increasing negative zeta potential of the nanoparticles. Interestingly, experiments with LYZ and HIS indicated that although both are positively charged and have similar molecular weight their interaction with the nanoparticles were different. Figure 2-7 illustrates these interactions for ICO-A and both cationic proteins, in terms of the hydrodynamic diameter distributions. Figure 2-7a, 2-7b, 2-7d, and 2-7e show dynamic magnetic susceptibility spectra for selected concentrations of LYZ and HIS. In the case of LYZ no changes in hydrodynamic
diameter were observed at concentrations of up to 10 mg LYZ/mL (Figure 2-7c). In contrast, we observed a decrease in hydrodynamic diameter for a concentration of 0.46 mg HIS/mL (Figure 2-7f). Above this concentration, the particles were observed to precipitate completely after experiments. We suspect that the reason for the observed decrease in hydrodynamic diameter is aggregation and precipitation of the larger particles in the distribution. This was consistent with the observed decrease in the magnitude of the low frequency in-phase susceptibility and with the observation of what appears to be a high-frequency tail at the low end of the frequency range of the out-of-phase susceptibility.

At higher concentrations of HIS (9-10 mg HIS/mL) the Brownian relaxation peak reappeared even though the particles did not re-disperse completely. This observation would be due to formation of a protein coating, which can now stabilize the nanoparticles. For ICO-A it was observed: i) a minimum HIS flocculation concentration of 0.46 mg HIS/mL, ii) flocculation and precipitation for concentrations between 7-9 mg HIS/mL, and iii) partial stabilization for higher concentrations. The differences between aggregation patterns of ICO-A particles in the presence of LYZ or HIS are due to differences in protein structure, including distribution of the positive charges and hydrophobic sites.

In contrast to ICO-A nanoparticles, we observed significant changes in the dynamic magnetic susceptibility spectra of ICO-B and ICO-C particles interacting with LYZ, as would be expected on the basis of their greater negative charge and the fact that the protein is cationic. The hydrodynamic diameter distributions are shown in Figure 2-8. It is seen that for ICO-B and ICO-C nanoparticles the dynamic magnetic
susceptibility spectra change significantly for concentrations of 7 mg LYZ/mL for ICO-B and 9 mg LYZ/mL for ICO-C. As with ICO-A in contact with HIS, at higher concentrations the Brownian relaxation peak re-appeared even though the particles did not re-disperse completely.

We only tested dispersion of ICO-B and ICO-C nanoparticles in HIS at a concentration of 10 mg HIS/mL (Figures 2-8b and 2-8d). For both particles, we observed dynamic magnetic susceptibility spectra consistent with extensive particle aggregation (Figures 2-8a and 2-8c) where for the greater the increase in hydrodynamic diameter, the greater is the frequency shift in the imaginary component of the susceptibility. The nanoparticles in these samples were observed to completely settle out of solution after a few hours.

2.4 Conclusions

The experiments described here prove that dynamic magnetic susceptibility measurements seem an appropriate in situ technique to study nanoparticle-protein interactions that lead to adsorption of proteins or aggregation of particles. The observed patterns of aggregation, flocculation, and some re-dispersion are consistent with nanoparticle-protein interactions being dominated by electrostatic interactions. The advantage of these measurements is the ability to assess changes in nanoparticle hydrodynamic diameter in the presence of proteins without having to separate the particles from the protein solutions disrupting the so-called protein-corona which provides the biological identity to the polymer coated MNPs. The method is fast and easy; it requires relatively small volume sample (<200 μl) and a minimal amount of MNPs. Optical transparency is not required, so that the technique is applicable even in optically complex and opaque fluids such as blood. A limitation of this approach is that
in protein mixtures the dynamic magnetic susceptibility measurements can only
determine if the proteins result in a change in particle hydrodynamic diameter, but
cannot indicate which protein is responsible for such changes. For this reason, the
reported technique should be viewed as complementary to other approaches to
characterize nanoparticle-protein interactions. Also, collection of magnetic nanoparticles
tracers must possess a Brownian fraction of MNPs that can be fitted to the Debye
model of susceptibility [157].

For the experiments reported, the choice of water: glycerol mixtures as a carrier
fluid with viscosities adjusted was made for convenience, to make sure that the
Brownian relaxation peak would lie in the frequency range of our SQUID based AC-
susceptometer, which is limited to a maximum frequency of 1,300 Hz. For particles
suspended in aqueous solutions without added glycerol it is expected that the Brownian
relaxation peak would appears at much higher frequencies and if measurements are
carried out using a SQUID AC-susceptometer, there is a chance that the lack of a
Brownian peak would introduces some uncertainty in the measurements. However, AC-
susceptometers with much wider frequency range can be built [158] and are
commercially available [159]. A susceptometer that operates at higher frequencies (> 1300 Hz) should be suitable for similar experiments without the need for glycerol, albeit
with the disadvantage of lower sensitivity compared to SQUID based AC-
susceptometers. It is expected that the observations reported here in terms of
nanoparticle-protein interactions can be extrapolated to the same systems in aqueous
solutions without glycerol, isotonic buffers, blood and other biological complex fluids.
Figure 2-1. Monitoring nanoparticle-protein interactions though rotational Brownian relaxation
Figure 2-2. DMS spectra of bare cobalt ferrite (CoFe$_2$O$_4$), showing their Debye behavior in a 60:40 %wt water:glycerol mixture. In-phase susceptibility component (○) and out-of-phase susceptibility component (●).
Figure 2-3. Representative Transmission Electron Microscope (TEM) images for CMDx coated cobalt ferrite nanoparticles with different degrees of carboxylic acid substitution and lognormal core size distributions by weighted number. Scale bar 100 nm.
Figure 2-4. Hydrodynamic diameter distributions of the particles in the absence of proteins determined from dynamic light scattering (volume weighted distributions) and dynamic susceptibility measurements.
Figure 2-5. Representative thermogravimetric analysis of CMDx coated cobalt ferrite nanoparticles. The first mass loss corresponds to physisorbed water.
Figure 2-6. Hydrodynamic diameter distributions of magnetic nanoparticles interacting with negatively charged proteins at different protein concentrations. a), b) ICO-A MNPs in presence of BSA up to 45 mgBSA/mL, and FIBR, IgG and TRANS at 10 mg protein/mL. c), d) ICO-B MNPs in presence of BSA up to 45 mgBSA/mL, and FIBR, IgG and TRANS at 10 mg protein/mL. e), f) ICO-C MNPs in presence of BSA up to 45 mgBSA/mL, and FIBR, IgG and TRANS at 10 mg protein/mL
Figure 2-7. Representative dynamic magnetic susceptibility spectra and hydrodynamic diameter distributions of ICO-A particles interacting with positively charged proteins at different concentrations. a), b) representative DMS spectra of ICO-A MNPs with lysozyme showing the Debye pattern at 0.1 mgLYZ/mL and 10 mgLYZ/ml, c) diameter distributions of ICO-A nanoparticles at different lysozyme concentrations, d), e) representative DMS spectra of ICO-A MNPs with histone showing the Debye pattern at 0.1 mgHIS/mL and 0.46 mgHIS/mL, f) diameter distributions of ICO-A nanoparticles at 0.1 mgHIS/mL and 0.46 mgHIS/mL
Figure 2-8. Diameter distributions of ICO-B and ICO-C particles in contact with different cationic protein concentrations at 37°C. a) ICO-B MNPs in presence of lysozyme protein solutions from 0.1 mgLyz/mL to 10 mgLYZ/mL, b) loss of the Brownian-like behaviour of ICO-B MNPs in presence of histone at 10 mgHIS/mL, c) ICO-C MNPs in presence of lysozyme protein solutions from 0.1 mgLyz/mL to 10 mgLYZ/mL, and d) Loss of the Brownian-like behaviour of ICO-C MNPs in presence of histone at 10 mgHIS/mL.
CHAPTER 3
ROTATIONAL DIFFUSIVITY OF MAGNETIC NANOPARTICLES IN CONCENTRATED PROTEIN SOLUTIONS

1.1 Introduction

Nanoparticles intended for biomedical applications must navigate biological milieu that consists of a complex, crowded, and confined mixture of structures and molecules that span length scales from the nanoscale to the microscale. The transport properties of nanoparticles in such complex and crowded fluid environments is expected to deviate from those found in the simple fluids in which they are typically characterized in the laboratory (e.g., water, saline solutions, and simple cell culture media). As such, the characterization of nanoparticle transport properties in fluids mimicking the biological environment is of interest in the development of nanoparticles for biomedical applications. Understanding the mobility of particles in complex environments such as biological fluids and highly concentrated environments plays a pivotal role in optimizing particle physico-chemical properties (e.g. size, shape, surface coating, graft density, colloidal stability) for successful application. Important biological fluids such as the cell cytoplasm and blood serum consist of concentrated (60-300 mg/mL) [53] solutions of mixtures of proteins and other macromolecules.

The rotational diffusivity of nanoparticles can be estimated experimentally by knowing the hydrodynamic diameter of the particles and the viscosity of the media, but there is still concern in terms of evaluating small aggregates, nanoparticle-protein interactions and protein-protein interactions that are typically ignored with conventional rheological measurements. Magnetic particle rheology, which helps determine the rotational diffusivity of the nanoparticles and hence mechanical properties of the particle medium such as viscosity through magnetic susceptibility measurements in complex
fluids [138], is ideally suited for highly concentrated protein solutions, where scattering of light by the proteins might interfere with optical methods of measuring nanoparticle diffusion.

In this contribution, we report measurements of the rotational diffusivity of magnetic nanoparticles suspended in concentrated solutions of bovine serum albumin (BSA), as a function of protein concentration and solution pH. The method used to measure the rotational diffusivity of the nanoparticles is based on magnetic excitation of nanoparticle rotation and magnetic measurement of the resulting response which is analyzed using the Debye model. We compared the rotational diffusivity obtained using the magnetic nanoparticles with the rotational diffusivity obtained using macromolecular rheological measurements. Below we report on the physical and colloidal properties of the nanoparticles used in these studies and the rotational diffusivities obtained for the nanoparticles in concentrated solutions of BSA as a function of pH. We also report the viscosities determined for the BSA solutions using the Stokes-Einstein expression for the rotational diffusivity, rheological measurements in a double gap viscometer and the hydrodynamic size of the nanoparticles.

3.2 Materials and Methods

Bovine serum albumin (BSA, Sigma-Aldrich, A7906) was dialyzed before experiments. Nitric acid (HNO₃, 0.1M), potassium hydroxide (KOH, 0.1M), phosphate buffer saline modified without calcium chloride and magnesium chloride (PBS, Sigma-Aldrich, D5652, 137 mM NaCl), iron (III) nitrate nonahydrate, (Fe(NO₃)₃·9H₂O, ≥98%), tetramethylammonium hydroxide ((CH₃)₄NOH, 10%), oleic acid, Mono-methoxy PEG (mPEG), isopropyl alcohol, sodium sulfate nonahydrate (Na₂O • 9H₂O), and acetic acid were purchased from Sigma-Aldrich and used as received. 3-aminopropyl
triethoxysilane (NH$_2$(CH$_2$)Si(OC$_2$H$_5$)$_3$) was purchased from TCI America and used as received. Sodium citrate dehydrate (Fisher BP226-500) and citric acid anhydrous (Fisher BP339-500) were used as received. Diethyl ether anhydrous, 0.2 µm nylon syringe filters, 0.22 µm polyethersulfone (PES) syringe filters (MILLEX GP filter unit), 10 kDa MWCO centrifugal filters (Millipore Amicon Ultracell-15 UFC901024) were purchased from Sigma-Aldrich. Slide-A-Lyzer dialysis flasks, 10 kDa MWCO, 250 mL (Thermo Fisher Scientific, 87762).

### 3.2.1 Synthesis of Magnetic Nanoparticles

The cobalt ferrite particles synthetized via co-precipitation [148] were surface modified with HNO$_3$ 2 M and hydrothermally treated with ferric nitrate according to Gomes et al.[149]. Then, MNPs were peptized using tetramethylammonium hydroxide (TMAO) for chemisorption of oleic acid to further perform a ligand exchange with PEG-Silane. The adsorption reaction to obtain cobalt ferrite nanoparticles coated with oleic acid was carried out as described by Hubbard et. al.[160]. Briefly, 7g of dried peptized particles were suspended in deionized water (280 mL) and placed in a sonicating water bath (90 seconds, Fisher Scientific Mechanical Ultrasonic Cleaner FS60) to allow particles to suspend. Then, particle colloid was placed for 20 min in a high-intensity ultrasonic processor (Sonics Vibra-Cell VCX 750) to break aggregates in solution. Oleic acid (~2% v/v) was added to the solution which was again placed in the sonicated water bath for 10 min for homogenization. This reaction mixture was transferred in a 500 mL cylindrical reaction vessel and was stirred at room temperature for 15 min. Then, temperature was increased to 80°C at a heating rate of 4°C/min. The reaction was carried out at 80°C for 1 hour and oleic acid was adsorbed onto the inorganic cobalt ferrite cores. To remove free oleic acid, nanoparticles were washed once with ethanol
(1:3 volume ratio) via centrifugation at 7500 rpm for 15 min. Magnetic separation was used to discard the supernatant and collect the particle precipitate. Particles were placed in a vacuum oven at 60 °C overnight to remove ethanol. The dried oleic acid coated nanoparticles were stored in a refrigerator at 4°C until further use.

Mono-methoxy PEG (mPEG, 2 kDa) was converted to oxidized PEG (mPEG-COOH) using Jones reagent [70] a strong oxidizing agent that converts the terminal hydroxyl group in mPEG to carboxylic acid. For this, 50 mmol of mPEG was dissolved in 400 mL of acetone followed by the addition of 17 mL of Jones reagent. The mixture was stirred at room temperature for 24 hours and quenched by addition of 5 mL of isopropyl alcohol. To remove chromium salt by-products of the reaction, charcoal was added to the mixture (10% w/w with respect to polymer mass). Afterwards, the mixture was vacuum-filtered several times until a clear acetone solution was obtained. The obtained solution was concentrated to a viscous liquid using a rotary-evaporator and then dried in a vacuum oven at 60 °C overnight. Dried polymer was suspended in water (20 g per 10mL water) and dialyzed with a membrane 1000 Da MWCO to complete the purification process. Further, this solution was concentrated to a viscous liquid using a rotary-evaporator and then dried in a vacuum oven at 60°C overnight to obtain a white sticky polymer. To synthesize PEG-Silane, mPEG-COOH MW 2 kDa was reacted with 3-aminopropyl triethoxysilane in a 1:1 molar ratio under nitrogen atmosphere at 180 °C for 2 hours. A brown paste was obtained and stored in a desiccator. Ligand exchange with PEG-Silane was performed as described by Barrera et. al.[70] using 200 mg of oleic acid coated cobalt ferrite nanoparticles suspended in toluene (~ 6 mg PEG-Silane/mL) in the presence of acetic acid as catalytic agent.
PEG coated nanoparticles were precipitated using 500 mL of diethyl ether anhydrous and magnetic decantation. Particles were re-suspended in 50 mL of acetone and sonicated for 10 minutes followed by diethyl ether precipitation (150 mL) and magnetic decantation three times to remove free polymer excess. Finally, particles were dried at 60 °C for 2h in the vacuum oven to remove traces of organic solvents, and stored at 4 °C for further experiments.

3.2.2 Characterization of Magnetic Nanoparticles

A stock solution of magnetic particles was prepared by dissolving dry powder PEG-Silane coated nanoparticles in deionized water, and sonicating using a Fisher Scientific Mechanical Ultrasonic Cleaner FS60 (15 min). This resulted in a stable and homogeneous stock solution at a concentration of 11 mg core/mL based on thermal gravimetric analysis. The nanoparticle solution was then passed through a 0.2 um nylon filter twice and stored in the refrigerator at 4°C. For characterization, the particle stock solution was diluted up to 0.5 mgcore/mL for dynamic light scattering and zeta potential measurements. The hydrodynamic diameter and zeta potential of the PEG coated cobalt ferrite nanoparticles was characterized using dynamic light scattering (DLS, Brookhaven Instruments ZetaPlus), while the dynamic magnetic susceptibility was measured using a susceptometer (Dynomag, Imego), in a frequency range from 10 Hz to 160 kHz at an applied field amplitude not higher than 0.5 mT (5 G). Direct imaging of the polymer coated nanoparticles was done using transmission electron microscopy with an acceleration voltage of 100 kV (Hitachi H-7600 TEM).

The percentage of inorganic core of the oleic acid and PEG coated MNPs in dry powder form was estimated using thermal gravimetric analysis (TGA). A Mettler Toledo STARe TGA/DSC1 Thermogravimetric Analyzer was used to estimate the amount of
oleic acid and PEG-Silane attached to the magnetic cores. Samples were dried at 115 °C in order to remove adsorbed water and solvent traces. A temperature ramp from 25°C to 800°C was used, with a heating rate of 10°C/min in air. Samples were analyzed in triplicate to obtain an average of the remnant weight.

The magnetic core size and saturation magnetization of MNPs stock in water was determined by measuring the response of the equilibrium magnetization under the application of a DC field at 300 K in a magnetic range from 6 to -6T using a Quantum Design MPMS-3 Superconducting Quantum Interference Device (SQUID) magnetometer.

3.2.3 Unbuffered and Buffered, Salted and Non-Salted BSA Stock Solutions at pH = 2.7 and pH = 7.4

Proteins are considered polyampholytes, due to presence of both acidic and basic functional groups, with pH, salt, and concentration-dependent conformations [161]. Hence, protein dynamics are governed by the surrounding buffer pH, ionic strength, and the volume fraction of the proteins at the studied conditions. Indeed, protein solutions can contain protein monomers in dynamic equilibrium with reversible clusters, and also form nonequilibrium irreversible clusters [162]. Based on this information, the preparation of BSA protein suspensions was carried out with extreme care using a standardized protocol based on literature review of work using protein colloidal systems at high concentrations [46, 156, 162-166], including globular proteins and molecular antibodies to find the optimal approach. Also, observable physical changes were documented to connect these experimental annotations with further results using bulk rheology and DMS measurements.
Solutions were prepared between 4°C-10°C during most of the preparation stages. Sterile filtration of buffers and protein stock solutions, and dilutions were carried out in a laminar flow hood. Also, according to the supplier, the solubility of BSA in water is 40 mgBSA/mL, thus BSA was dissolved under the buffered conditions tested in a concentration of ~35 mgBSA/mL which is lesser than the solubility value. The pH values selected in this study for DMS and bulk rheology measurements were below and above the BSA isoelectric point (~pI = 5.1)[46, 161]. A three-step procedure was followed to prepare the concentrated BSA stock solutions at different buffer conditions.

First, a BSA solution at 35 mgBSA/mL (~480 mL) was prepared by dissolving protein powder at different buffered solutions that were pre-filtered with 0.22 um bottle top filters (PES membrane). Complete dissolution of the BSA powder in appropriate buffer conditions occurred with occasional and gentle stirring at 4°C for about 1 day (quasi-quiescently). Briefly, ~17 g of BSA (added to the buffer slowly during about 1 day) were suspended in i) ultrapure deionized water at adjusted pH of 2.7 and 7.4, respectively using KOH 0.1 M and HNO3 0.1 M (unbuffered and non-salted), ii) ultrapure deionized water with 137 mM NaCl pH = 2.7 (unbuffered and salted), iii) PBS pH = 7.4 (buffered and salted), iv) sodium acetate + citric acid 0.1 M pH=2.7 (unbuffered and non-salted), and v) sodium acetate + citric acid 0.1 M + 137 mM NaCl pH=2.7 (unbuffered and salted).

Second, protein suspensions per buffer condition were divided into two dialysis flasks (10 kDa MWCO), and exhaustively dialyzed into each buffer condition to reduce any ionic impurities or remove fatty acid impurities left in the BSA overnight at 4°C. The
pH was monitored and adjusted using KOH 0.1 M and HNO₃ 0.1 M, if it was required during the dialysis process.

Third, the BSA solutions were subsequently filtered through 0.22 um syringe filters (PES membrane) to remove any undissolved material, and concentrated by ultrafiltration with Amicon devices (100 kDa MWCO) using a fixed angle rotor centrifuge (Eppendorf 5430R – Rotor: F-35-6-30) at 5,000 rcf and 10°C for about 1 day, with gentle pipetting every 30 min to avoid protein caking in the membrane due to concentration polarization. Then, protein solutions were centrifuged at 5,000 rcf for 30 min at 10°C to keep the supernatant as the final protein stock solution. This centrifugation step was performed to remove residual particulates, and microscopic bubbles immediately before protein concentration determination, DMS measurements and bulk viscosity measurements. Protein concentration determination was performed with the collected supernatant after centrifugation by using A280 method with an extinction coefficient for BSA of 0.667 cm²/mg for a 1% dilution [46]. BSA stock solutions were diluted to the desired concentration with the respective buffer (8 points per buffer condition), and pH was monitored after measurements up to 24 hours. After dilution, protein concentration were again validated by using the A280 method.

3.2.4 Bulk Viscosity Measurements and DMS Measurements

For bulk rheological measurements, 7.5 mL of the diluted protein solutions were mixed with 2.5 mL of the appropriate buffer per condition to obtain a final target BSA concentration. DMS measurements were performed with diluted BSA suspensions (300 µL) mixed with a freshly prepared stock solution of MNPs in the corresponding buffer condition (100 µL). This resulted in a total particle concentration of ~0.007% v/v within the same target BSA concentration used for rheological measurements.
A stress-controlled rheometer MCR301 (Anton Paar) with double-gap (GP) geometry was used to perform bulk rheological measurements. The viscosity of each sample was measured twice at a constant set temperature of 25°C. Every time the sample went through one cycle of shear rate sweep ramping up from 1 s⁻¹ to 1000 s⁻¹ and then ramping down from 1000 s⁻¹ to 1 s⁻¹. The average value was obtained by calculating the average viscosity of the shear rate sweep of two independent measurements per sample in the linear viscosity range between 20 s⁻¹-1000 s⁻¹.

The dynamic magnetic susceptibility of protein solutions containing MNPs was measured using a susceptometer (Dynomag, Imego) in a frequency range from 10 Hz to 160 kHz at an applied field amplitude not higher than 0.5 mT (5 G). Measurements at different protein conditions were performed in triplicate at 25°C with 200 µL of protein-nanoparticle solutions. The rotational diffusivity of the PEG coated MNPs was estimated by assuming that the SE relation for the rotational diffusivity applies in BSA solutions, and that the particle’s hydrodynamic diameter in the BSA solutions are the same as those measured in the buffer solutions without proteins using DMS measurements, which are in close agreement with DLS measurements.

As illustrated in Figure 3-1, DMS measurements represent a unique technique to evaluate the rotational dynamics of magnetic nanoparticles in complex biological environments. This is accomplished by sensing the particle rotation opposed by the fluid resistance using time-dependent magnetic torques through the application of an alternating magnetic field. Thus, the resistance due to molecular crowding would lead to a change in the Brownian relaxation time of the nanoparticle, which is inversely proportional to the rotational diffusivity of the nanoparticle. Protein adsorption or protein-
induced aggregation, also can lead to a change in the Brownian relaxation time of the particle due to a change in the effective hydrodynamic diameter.

### 3.3 Results and Discussion

In the experiments reported here, polyethylene glycol (PEG) was chosen as the polymer coating grafted onto the inorganic cobalt ferrite cores, due to its relevance in biomedical applications for preventing the adsorption of proteins via steric hindrance, with a typically neutral charge, which reduces electrostatic interactions. Thus, PEG coating was used to reduce adsorption of bovine serum albumin (BSA) onto the surface of MNPs at high protein concentration and to obtain the frequency-dependent diffusion coefficient of a system of interacting Brownian particles using dynamic magnetic susceptibility (DMS) measurements. The physical characterization results of the PEG coated MNPs are presented from Figure 3-2 to Figure 3-5. TGA measurements (n = 3) presented in Figure 3-2, demonstrated that approximately 82% ± 1% of the weight loss represents the PEG layer onto the surface of the cobalt ferrite nanoparticles.

The mean core diameter of the nanoparticles, estimated by fitting the number weighted diameter histogram obtained using TEM to a lognormal size distribution was 14 ± 1 nm. The size distribution histogram (weighted by volume) corresponding to Figure 3-3a is included in Figure 3-3c for comparison with the hydrodynamic size distributions estimated from DMS and DLS measurements. Despite particle polydispersity, well separated particle clusters of ~40 nm in diameter can be recognized. Particle polydispersity and cluster formation is a result of synthesis by the co-precipitation method, and after the surface modification procedure using PEG-Silane polymers synthesized by the method described by Barrera et al. [70, 167] due to the formation of APS oligomers.
DMS spectra (Figure 3-3b) shows that the in-phase and out-of-phase components of the magnetic susceptibility cross almost at the peak of the out-of-phase susceptibility, and that both approach zero at high frequency, as expected for particles with a single relaxation mechanism of response to magnetic fields following the Debye model. The Brownian peak, characteristic of thermally blocked MNPs, was well defined regardless of the polydispersity and quasi-spherical shape of the cobalt ferrite MNPs within clusters. This is because of the well-defined polymer shell covalently bonded to the magnetic nanoparticles after the ligand exchange procedure, which provides steric repulsion in the particle system. In Figure 3-3c (solid black line), the arithmetic mean diameter and the standard deviation of the hydrodynamic size distribution estimated from fitting the DMS spectra to the Debye model was 44 ± 10 nm.

DLS measurements were used to obtain the hydrodynamic diameter distribution of the nanoparticles and for further validation of the DMS measurements described above. As can be seen in Figure 3-3c (dashed red line), the lognormal volume-weighted distribution is broader than the hydrodynamic distribution obtained by fitting the DMS spectrum to the Debye model. At a pH of 7.4, the arithmetic mean diameter and the standard deviation of the particles was 37.0 ± 15 nm, which is lower than the hydrodynamic diameter obtained from DMS measurements, likely due to the DMS diameter being influenced by larger particles. The difference in the size distributions obtained by TEM in comparison with DMS and DLS values is due to the presence of inorganic clusters embedded in dense PEG-Silane brushes.

The characterization of the DMS spectra and hydrodynamic diameter distribution of the magnetic nanoprobes in water over a wide range of pH showed that the particles
are colloidally stable, relax through the Brownian magnetic relaxation mechanism, and experience negligible changes in hydrodynamic diameter over the studied pH range based on lognormal weighted distributions (Figure 3-4a).

Figure 3-4b shows that DLS distributions remain unchanged across pH values between 2.7 and 10.0 in water with exception at pH = 4.7, consistent with the observation that the DMS spectrum is unaffected by pH. Table 3-1 shows a summary of the buffer conditions used to prepare BSA suspensions at different protein concentrations below (pH = 2.7) and above (pH = 7.4) the isoelectric point of BSA (∼pH = 5.1). Note that each condition has a sample name that will be used consistently throughout this entire chapter.

Table 3-1. Buffer conditions employed for bulk rheology and DMS measurements of BSA at different concentrations

<table>
<thead>
<tr>
<th>Sample name</th>
<th>pH</th>
<th>Solvent</th>
<th>Buffer</th>
<th>Ionic strength</th>
<th>DMS – Hydrodynamic size (lognormal weighted by volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-NB-NS</td>
<td>2.7</td>
<td>Water</td>
<td>n.a</td>
<td>0 mM NaCl</td>
<td>68 nm (lnσ = 0.47)</td>
</tr>
<tr>
<td>A-NB-S</td>
<td>2.7</td>
<td>Water</td>
<td>n.a</td>
<td>137 mM NaCl</td>
<td>38 nm (lnσ = 0.27)</td>
</tr>
<tr>
<td>A-B-NS</td>
<td>2.7</td>
<td>Water</td>
<td>Citric acid + sodium citrate 0.1 M</td>
<td>0 mM NaCl</td>
<td>39 nm (lnσ = 0.22)</td>
</tr>
<tr>
<td>A-B-S</td>
<td>2.7</td>
<td>Water</td>
<td>Citric acid + sodium citrate 0.1 M</td>
<td>137 mM NaCl</td>
<td>74 nm (lnσ = 0.44)</td>
</tr>
<tr>
<td>N-NB-S</td>
<td>7.4</td>
<td>Water</td>
<td>n.a</td>
<td>137 mM NaCl</td>
<td>68 nm (lnσ = 0.33)</td>
</tr>
<tr>
<td>N-B-S</td>
<td>7.4</td>
<td>Water</td>
<td>Phosphate buffered saline</td>
<td>137 mM NaCl</td>
<td>55 nm (lnσ = 0.36)</td>
</tr>
</tbody>
</table>

Sample names were denoted according to the following order: A and N for acidic (∼pH = 2.7) and neutral conditions with respect to the BSA isoelectric point (∼pH = 7.4) – NB and B were designated for no buffered (unbuffered) and buffered conditions – NS and S were intended for no salted or salted (137 mM NaCl) conditions.
The colloidal stability analysis of nanoparticles in the buffer conditions selected for protein suspension were performed using DLS and DMS measurements. There were observed changes in hydrodynamic diameter distributions in selected buffers and ionic strength conditions, as seen by a displacement of the Brownian peak by up to one order of magnitude in Figure 3-5. However, PEG coated MNPs exhibited Brownian relaxation even though slightly aggregated in buffered and salted conditions. Figure 3-5a and 3-5b are representative examples of DMS spectra at pH = 7.4. Agreement between DLS and DMS measurements in all the buffer conditions tested was observed. MNPs did not precipitate over a period of 1 week, except for particles suspended in citric acid + sodium citrate 0.1 M 137 mM, which precipitated after 72 h. These results confirmed that the particles synthesized are suitable to study their rotational dynamics at different BSA concentrations. This is because, DMS measurements are conveniently short (~20 min) and studies were carried out per buffer condition in triplicate in about a day.

Figure 3-5d and Figure 3-5e are representative examples of hydrodynamic diameter distributions of PEG coated nanoparticles in neutral conditions, whereas in the presence of salts at pH = 7.4 MNPs have shown a slight increase in hydrodynamic size in contrast with deionized water at pH = 7.4 (Figure 3-4b). The close agreement between the hydrodynamic diameters obtained by DLS and DMS measurements indicates that the magnetic dipoles in PEG coated particles suspended at the buffer conditions in this study, respond to an alternating magnetic field by physical particle rotation.

Despite the presence of aggregates, PEG coated MNPs remain with a hydrodynamic diameter less than 100 nm. The existence of this aggregation pattern can
be explained by electrostatic interactions between free unprotected amine groups on the surface of the PEG coated MNPs, and chloride, citrate and phosphate ions. Indeed, the pKa of primary amines is usually high (pKa = 10), thus at pH values under pH = 10 primary amines from unreacted APS are protonated contributing to an overall positive surface charge in the MNPs. First, zeta potential measurements at pH = 7.4 revealed that PEG coated MNPs are slightly positive ~ 11 mV. This positive value did not change significantly in a wide pH range (from pH 2 to pH 10), which confirms the presence of free amine groups. Second, sodium chloride (NaCl) and PBS constituent ions may have a screening effect on the surface charge of the MNPs because chloride and phosphate ions in water can interact electrostatically with protonated amine groups. Finally, citrate ions act as buffering agents, acidifiers, and chelators with metals forming coordination compounds, which make them suitable for example, in blood anticoagulation applications. Interestingly, citrate ions are known for being the least chaotropic ions in the Hoffmeister series for salting or desalting proteins [168] but may favor bridging interactions and selective particle aggregation due to electrostatic interactions of exposed protonated amine groups onto the surface of PEG coated MNPs, and carboxylate groups in citrate buffer used in this study. These interactions can lead to particle hydrodynamic diameter increases. Also, citrate ions are well known as chelating agents contributing on the degradation kinetics of MNPs which can compromise particle stability if the polymer coating onto the magnetic nanoparticles is not stable or covalently attached. In our experiments, particle degradation was not found in acidic environments as the MNPs were coated with covalently grafted PEG brushes. However, an increase of particle hydrodynamic diameter was observed in a time window of 72 h in
acidic environments (pH = 2.7) in contrast to neutral environments (pH = 7.4), which agrees with the idea of electrostatic interactions favor bridging interactions and selective particle aggregation.

Equilibrium magnetization measurements at 300 K for aqueous PEG coated MNPs, demonstrated superparamagnetic behavior (Figure 3-6) in the particle stock. By fitting the data to the Langevin function using the procedure established by Chantrell [169], the magnetic diameter was 14 ± 6, while the volume fraction (\(\phi\)) of the dilute stock solution was 0.02%.

For clarification, to study particle mobility and the mechanical properties of highly concentrated protein solutions depending on the buffer and salt concentrations, the rotational diffusivity will be estimated using the hydrodynamic size distribution obtained via DMS measurements in each buffer condition without proteins added, which will be used below to interpret DMS measurements in concentrated BSA solutions using the Debye model of susceptibility along with the Stokes-Einstein relation.

### 3.3.1 Protein Solutions and Potential Aggregates/Cluster Formation

In this study, biophysical characterization of the BSA protein suspensions was assessed by quantifying BSA concentration (A280) in BSA protein solutions, and measuring the bulk viscosity in each protein solution. Following it, the particle mobility of MNPs via particle rotational dynamics using alternating magnetic fields was studied. Potentially, this technique will allow sensing the changes in the surroundings of the MNPs at nanoscale due to protein aggregation, protein crowding, and/or protein adsorption onto the surface of the magnetic nanoparticles because of DLVO or non-DLVO interactions.
Figure 3-7 shows protein suspensions in acidic conditions at ~35 mgBSA/mL after dialysis, and their respective pH stability graphs about 1 day of observation. Buffered acidic conditions in the presence of citric acid + sodium citrate 0.1 M (pH = 2.7) showed opalescence because of protein aggregation. This physical observation agrees with Sarangapati et al. [161], who observed for BSA protein solutions up to 200 mgBSA/mL in citric acid + sodium citrate buffer (pH = 3) at low ionic strength (20 mM), changes in the Small-Angle Neutron Scattering (SANS) data attributed to BSA conformational changes.

Before centrifugal concentration in Amicon devices (100 kDa MWCO), samples were filtered to remove protein aggregates, as shown Figure 3-8a to Figure 3-8d. During protein concentration, it was observed that BSA gels were formed at the bottom of the centrifugal filters. Thus, these gels were separated out from the concentrated BSA stocks solutions (citric acid + sodium citrate 0.1 M) by centrifugation. As a representative example, Figure 3-8e shows the supernatant collected from a BSA stock solution buffered in citric acid + sodium citrate 0.1 M salted with 137 mM NaCl, and Figure 3-8f shows the bottom left as a BSA gel. It was observed that without the addition of salts the BSA gel became more solid-like and homogeneous. We therefore expect to observe a difference in the mobility patterns of the MNPs in these BSA protein suspensions.

During protein sample preparation, it was observed that at acidic conditions (~pH = 2.7) without NaCl, gelation of BSA was triggered by the addition of citric acid + sodium citrate 0.1 M buffer (A-B-NS condition). This was detected under visual inspection during protein concentration at low temperatures using centrifugal filtration.
devices where BSA concentrations higher than 100 mg/mL were obtained. In contrast, salted protein solutions in citric acid + sodium citrate 0.1 M buffer (A-B-S condition) showed a reduced gel formation, which means that gelation can be inhibited by addition of NaCl at physiological concentrations (137 mM). On the other hand, acidic suspensions of BSA in ultrapure deionized water (A-NB-S and A-NB-NS conditions) did not show this pattern of gel-like conformation of BSA in solution.

Esue et al. [170] have shown carboxylate-dependent gelation of monoclonal antibodies, in which carboxylates could act as a bridge to crosslink antibody monomers. However, they stated that for the monoclonal antibody (mAb) studied, gel assembly was not activated by high ionic strength but with multivalent carboxylates. These observations were made based on sodium citrate as a multivalent carboxylate. In recent work, Esue and collaborators [171] found that positively charged histidine play a pivotal role in the thermodynamics of interactions between citrate and mAbs, followed by other hydrophobic and aromatic amino acid residues that may induce assembly and stabilization of molecular structures leading to gelation of mAbs.

The findings reported by Esue and collaborators with monoclonal antibodies [170, 171] may be extended to globular proteins such as BSA. This is in part because, the gelation patterns observed in our experiments agreed with their results. Based on our physical observations, we can suggest that the citrate buffer has a determinant role in the gelation of BSA, which can be reduced by addition of sodium chloride like previous studies with mAbs have shown. The buffered conditions tested in our studies lead to changes in the mechanical properties of concentrated BSA suspensions, due to conformational changes of BSA in solution (solution pH above BSA pI) to BSA gel-like
solid (solution pH below BSA pI). By monitoring conformational changes of BSA in solution using dynamic magnetic susceptibility measurements, our qualitative observations will become quantitively relevant. Hence, we will predict changes in the rotational diffusivity of MNPs in BSA solutions and tie those variations with changes in the mechanical properties of BSA suspensions such as BSA protein solution viscosity.

3.3.2 Dynamic Magnetic Susceptibility Measurements and Rheological Analysis

Dynamic magnetic susceptibility measurements performed for PEG coated MNPs in BSA suspensions indicated the particles had a single magnetic relaxation mechanism consistent with the Debye model for all the buffer conditions tested. This is consistent with the nanoparticles remaining well dispersed and colloidally stable in all the protein solutions. Figure 3-9 shows a representative picture of two dilution series of concentrated BSA from 103 to 0 mg/mL without (top image) and with PEG coated MNPs (bottom image) after 48 h in acidic conditions with an ionic strength of 137 mM NaCl (citric acid + sodium citrate 0.1 M). By simple observation, none of the nanoparticle-BSA solutions showed evidence of aggregation or precipitation for about one week, with the only exception being PEG coated MNPs particles that precipitated in the case when no protein was added (0 mgBSA/mL) at 72 h as shown Figure 3-9. In other buffer conditions, all solutions remained brilliant and with no evidence of remarkable particle or protein aggregation or sedimentation.

As seen in Figure 3-10, the frequency corresponding to the peak in the out-of-phase susceptibility decreases with increasing BSA concentration (A-B-NS condition). This is consistent with an increase in the particle relaxation time and corresponding decrease in rotational diffusivity. However, this reduction in rotational diffusivity based on macromolecular crowding effects can also occur because of particle aggregation or
protein adsorption. We discarded particle aggregation because Debye-like features remain in the DMS spectra of MNPs in all the protein conditions tested. To rule out a protein adsorption effect which can lead to aggregation and precipitation of MNPs, the initial susceptibility value for all the obtained DMS spectra remained almost constant. This means that the magnetic volumetric fraction of PEG coated MNPs was the same at different protein concentrations per buffer condition and thus particles were able to respond by physical rotation to an externally applied magnetic field.

DMS measurements are consistent with a decrease in rotational diffusivity with increasing concentration and decreasing pH value from 7.4 to 2.7. We can infer that the decrease in peak frequency as concentration increased indicates that the local environment around the magnetic nanoparticles is becoming more crowded, resulting in hindered particle rotation. Then, if the Stokes-Einstein relation for the rotational diffusivity is assumed to apply and the nanoparticle’s hydrodynamic diameter is assumed to be the same in all the protein solutions, this decrease in diffusivity can be interpreted as an increase in the viscosity of the solution in which the nanoparticles are suspended.

However, a dramatic reduction in the rotational diffusivity of the MNPs was observed in sodium citrate buffer without added salt (A-B-NS condition). In Figure 3-11 (filled blue squares), this severe reduction in rotational diffusivity can be linked to a change in the BSA conformation, which produces a gel-like BSA structure. This is because of the presence of citrate, a multivalent carboxylate ion which can trigger crosslink and bunding of protein networks. In the DMS spectra of BSA solutions at acidic conditions buffered with citric acid + sodium citrate buffer 0.1 M without salts (A-
B-NS condition), particle mobility is restricted probably due to interfilamentous interactions or steric hindrance as was shown in recent research work using spindle-shaped MNPs in hydrogels samples [172]. It is interesting to note that this pattern is not present at the same buffered conditions with addition of 137 mM NaCl (A-B-NS condition). Most probably, this is because salts can screen the carboxylate ions in solution, reducing the formation of bridges between proteins.

It is important to point out that gelation of BSA is commonly completed at high temperatures with the presence of salts over a wide range of pH. However, in our BSA experiments, acid-induced cold gelation of BSA between 4°C-10°C was triggered by sodium citrate ions without the need of an increase in temperature. As an example, acid-induced cold gelation at 25°C of globular proteins such as whey protein isolate (WPI) has shown the formation of a protein network by physical interactions, which is subsequently stabilized by the formation of disulfide bonds [173]. Physical interactions can occur due to protein conformational changes – α-helices changing into β-aggregates, and disulfide bond formation is specific for each globular protein depending on the number of cysteine groups per protein chain. Indeed, this acid-induced cold gelation of WPI is not triggered by the crosslinking of citrate ions, and it seems to be relevant for comparisons with our BSA results at unbuffered acidic conditions tested (A-NB-NS and A-NB-S conditions).

The rotational diffusivity \( (D_{R,DMS}) \) was calculated first by fitting the DMS spectra to the Debye model weighted by the known lognormal size distribution at zero protein concentration to obtain the viscosity experienced by the particles at the tested
conditions, and second using the obtained viscosity value along with the lognormal size distribution at zero protein concentration with the Stoke-Einstein equation.

Examination of the rotational diffusivity patterns in Figure 3-11 suggests that BSA gel-like solutions in citric acid + sodium citrate 0.1 M salted (A-B-S condition) generated restriction of MNPs mobility upon application of an alternating magnetic field for all the acidic conditions tested (ionic screening), in comparison to BSA gel-like solutions in citric acid + sodium citrate 0.1 M no salted (A-B-NS condition), which showed the highest restriction in particle mobility (carboxylate bridges).

Gelation of gelatin solutions triggered by temperature has been studied by Barrera and collaborators [120] through DMS susceptibility measurements using PEG coated cobalt ferrite MNPs suspended in gelatin from bovine skin type B. This was possible by monitoring sharp transitions in the out-of-phase component of the complex susceptibility, and correlating them to observations of gelation through conventional bulk rheology. The studies reported here demonstrated that tighter three-dimensional gels restricted rotation and eventually trapped the MNPs. This was observed at the nanoscale when no Debye-like peak was observed at 10°C, which was below the gelation temperature for gelatin from bovine origin. Then, translating these observations to our BSA studies, the gelation network formed in the BSA suspensions was not entangled enough to totally restrict particle rotation, for this reason well defined Debye-like peaks were observed during gelation triggered by citrate ions with and without added salt.

The bulk rheological measurements ($\eta_{\text{Rheo}}$) made for BSA protein solutions were performed at 25°C showing apparent Newtonian behavior at shear rates between 10-
1000 1/s. This is a characteristic pattern in the rheology of surfactant-free protein solutions previously demonstrated by Sharma et al.[156] in BSA protein solutions. Figure 3-12 shows representative flow curves of BSA protein solutions at acidic conditions in citric acid + sodium citrate 0.1 M not salted.

An increase in viscosity was observed as a function of protein concentration in all the buffer conditions using bulk rheology. Indeed, Figure 3-13 indicates that the solution viscosity increases more rapidly at pH = 2.7 than at pH = 7.4. This could be due to changes in the conformation of BSA at low pH values, resulting in changes in protein-protein interactions due to cold-acidic gelation at the studied conditions or triggered gelation by citrate ions.

In addition, the viscosity of concentrated BSA solutions obtained according to magnetic nanoprobes (\( \eta_{DMS} \)) was estimated assuming the Stokes-Einstein relation for the rotational diffusivity applies. The particle’s hydrodynamic diameter in BSA solutions was assumed to be the same as those measured in water at different pH using DMS measurements. The viscosity of the BSA solutions was calculated by fitting the Debye model weighted by a lognormal size distribution to the out-of-phase DMS spectra. The change in viscosity as a function of BSA concentration is seen in Figure 3-14 for each buffer condition studied at pH = 2.7 and pH = 7.4. Like in bulk rheological measurements, it was observed that the solution viscosity increases more rapidly at lower pH values that at higher pH values. As we expected, Figures 3-13 and 3-14 show a dependence between BSA concentration and increase in viscosity under the presence of salt and buffers for both rheological measurements and DMS estimations. Overall, a concentration increase is associated with a viscosity increase.
Particularly, the results obtained via bulk rheology and DMS measurements shown agreement in almost all the buffer conditions studied for BSA with the exception for acidic conditions in citric acid + sodium citrate 0.1 M buffer without salt (A-B-NS condition). Conversely, an order of magnitude difference was found between the macromolecular viscosity and the viscosity experienced by the MNPs in their local environment in this buffer condition as can be seen in Figure 3-15. This may be explained by solid-like BSA gels formation. Hence, entangled protein filaments may be restricting particle rotation, and eventually trapping the MNPs.

Furthermore, BSA conformation has been reported to transition from a closed so-called “normal” (N) state to an open molecular structure (referred to as the “fast” state, F) as pH decreases from neutral to acidic in the range of pH 7.4 to ~4.7-4 [174-177]. This transition is characterized by an opening of the molecular structure and corresponding increase in protein dimensions, asymmetry, and increased exposure of hydrophobic groups. As pH decreases from 4.7 to 2.7, BSA further unfolds to an “expanded” (E) state, increasing in size and asymmetry [174, 175]. In addition, other states have been observed, such as the “basic” (B) state seen at pH 8-10, which is associated with structural fluctuations with a loosening of molecular structure and loss of rigidity, and an “aged” (A) state at pH values greater than 10 [175, 176]. However, this picture is not without controversy, as some studies claim that the most critical change in volume and exposure of hydrophobic groups is during the N-F transition, followed by a decrease in volume as pH is lowered to the E state [178].

The fact that at pH = 2.7 in citric acid + sodium citrate 0.1 M buffer without salts, the solution viscosity becomes increasingly sensitive to increasing protein concentration
in contrast with the other buffer conditions studied at acidic and basic scenarios could be consistent with an expansion of BSA and/or with increased protein-protein repulsion due to acquired charge. First, as the BSA molecule expands, a given mass of BSA occupies a greater volume fraction in solution, thus resulting in greater suspension-scale viscosity. Similarly, greater protein-protein repulsion would lead to a higher effective volume fraction at a given mass concentration, again resulting in increased viscosity. These two possible explanations for the pH dependence of the viscosity observed using DMS measurements in Figure 3-15 are consistent with the above discussion of changes in the charge and conformation of BSA by changing pH.

3.3.3 Rotational Diffusion and its Deviation from the Predictions Based on the Stokes-Einstein Relation

From the previous section, it was observed that viscosity values using DMS evaluations under acidic conditions increased in comparison with those values at neutral conditions. This is in part because of gelation of BSA due to protein conformational changes as was discussed in previous sections, which were more significant at buffered conditions with citrate ions. Figure 3-16 shows that the rotational diffusion coefficient estimated using DMS measurements negatively deviate from the Stokes-Einstein relation at acidic conditions. However, at neutral conditions (pH = 7.4) the relation still applies because there is no conformational change in the BSA structure (no BSA gel formation). The rotational diffusion predicted by the SE relation (\( D_{R,SE} \)) was estimated by using the bulk viscosity along with the lognormal size distribution at zero protein concentration.

Our results agree with the observations made by Saraganpati et al. [161], who concluded that protein-protein interactions increase in importance at high protein and
salt concentrations. Hence, charge distribution and surface patchiness, relative orientation, hydrophobic interactions, etc., potentially become relevant to describe the viscosity of protein solutions.

According to reported DMS and DLS measurements, the particles do not appreciably change in size or aggregate at the buffer conditions studied in the absence of proteins except for pH = 2.7 buffered at salted conditions after 72 h. Thus, an explanation for the tendencies observed in Figure 3-16, especially at acidic pH are not a result of particle aggregation. In the case of particle aggregation this will result in distortions in the out-of-phase susceptibility spectrum, which were not observed in all the buffer condition evaluated in this study. Furthermore, because the rotational diffusivity is inversely proportional to the hydrodynamic diameter, protein adsorption and particle aggregation can count for the observed patterns in concentration and pH dependence of the rotational diffusivity. However, since initial susceptibility values remain mostly constant for all the buffered conditions studies increase in hydrodynamic diameter distributions followed by particle aggregation and precipitation were discarded. Therefore, a simpler justification could be that the observed changes in rotational diffusivity are due to the dependence of the viscosity of the BSA solutions on BSA concentration at pH value below and above the isoelectric point of BSA where BSA is sensitive to conformational changes affecting the local environments of the MNPs.

3.4 Conclusions

The results reported herein demonstrate that under the buffer conditions studied, the rotational diffusivity of magnetic nanoparticles in concentrated BSA protein solutions follows the predictions of the Stokes-Einstein (SE) relation only at neutral buffer conditions (pH = 7.4), which implies that the viscosity used is the concentration-
dependent protein solution viscosity. However, when there is evidence of conformational changes in BSA such as gelation patterns at acidic conditions, a negative deviation from the rotational diffusion coefficient predicted by the SE relation was observed. Our observations are important because they lend confidence to the use of the Stokes-Einstein relation when predicting the dynamics of nanoparticles in protein solutions at pH values above the isoelectric point of the protein itself mimicking physiological conditions. These predictions serve to illustrate how magnetic nanoparticle probes can be used to study particle mobility and colloidal stability in crowded protein environments and to extract the mechanical properties of concentrated protein solutions, which can be negligible via conventional bulk rheology measurements.

Because the reported method requires small volumes (< 200 µl) of sample, it is potentially suitable for the study of solution containing difficult to obtain proteins (e.g. monoclonal antibodies) and other biological fluids (e.g., mucus, synovial fluid, etc.).

In generalizing our observation of rotational diffusivity consistent with the values obtained using the SE relation we point out that the particles used herein consisted of cobalt ferrite clusters embedded in a covalently-bonded dense brush of poly (ethylene glycol). We had previously reported how similar coated nanoparticles are colloidally stable due to steric repulsion in a wide range of pH and in the presence of buffers and proteins [167]. We therefore expect our observation of rotational diffusivity consistent with the SE relation to be applicable to other similarly-stabilized inorganic particles exhibiting Brownian relaxation and possessing dense polymer coatings, and which prevent protein adsorption and/or nanoparticle aggregation in complex environments.
Furthermore, the results reported in this chapter provide insight into the concentration and pH dependence of concentrated protein solutions, particularly from the perspective of a probe entity with the size in a nanoscale range. The observed increase in sensitivity of solution viscosity with increasing protein concentration was also consistent with other reports of expansion of BSA molecule with decreasing pH below BSA isoelectric point.

Finally, it was observed that acid-cold gelation of BSA at acidic conditions was triggered by the presence of sodium citrate ions, and the gelation pattern could be reduced by increasing ionic strength in protein solutions using sodium chloride.
Figure 3-1. Particle mobility and colloidal stability in concentrated protein solutions can be studied by means of monitoring magnetic particle rotation in an alternating magnetic field and calculating the rotational diffusivity of magnetic nanoparticles.
Figure 3-2. Thermogravimetric analysis of oleic acid coated cobalt ferrite nanoparticles and PEG-Silane coated MNPs (n = 3)
Figure 3-3. Physical, magnetic, and colloidal characterization of the PEG-Silane coated cobalt ferrite nanoparticles. a) Representative TEM micrograph shows a physical diameter of ~14 nm (scale bar 100 nm). b) DMS spectra at pH = 7.4 in ultrapure deionized water showing characteristic Debye’s trend for thermally blocked MNPs. c) Size distributions for the inorganic core, obtained by TEM (blue line), and for the hydrodynamic diameter, determine from DLS (red line) and DMS measurements (black line)
Figure 3-4. Stability of MNPs at pH values between 2.7 and 10 in water. a) Out-of-phase susceptibility data for PEG-cobalt ferrite MNPs. The peak position of the normalized out-of-phase susceptibility data does not vary significantly between changes in pH, indicating that the particle rotational dynamics is not modified by changes in pH. b) DLS measurements showing that particles remain polydisperse at different pH values, along with more common particle population remaining within the same hydrodynamic diameter.
Figure 3-5. Particle stability at the buffer conditions used to study PEG-cobalt ferrite MNPs mobility at higher BSA concentrations. a) Illustrative DMS spectra of MNPs at basic conditions, no buffered, and salted (137 mM NaCl) showing the characteristic Debye relaxation pattern. b) Illustrative DMS spectra of MNPs at basic conditions, buffered (PBS), and salted (137 mM NaCl) showing the characteristic Debye relaxation pattern. c) Out-of-phase susceptibility data for MNPs at the buffer conditions used to monitor MNPs mobility at high BSA concentrations, whereas the peak position of the normalized out-of-phase susceptibility data vary an order of magnitude between changes in pH in the presence of salts, indicating that the particle rotational dynamics is modified due to buffer and salt electrostatic interactions with protonated amine groups onto the surface of MNPs.
Figure 3-6. Equilibrium magnetization curves at 300 K for the PEG coated MNPs show a magnetization value about 100 A/m without mass normalization.
Figure 3-7. BSA protein solutions in acidic conditions at 4°C. a) BSA at pH = 2.7, no buffered, no salted, BSA at pH = 2.7, no buffered, salted, BSA at pH = 2.7, buffered, no salted, and BSA at pH = 2.7, buffered, salted after continuous dialysis for 24 h. b)-e) BSA protein pH stability at different protein concentrations.
Figure 3-8. BSA solutions showing aggregates/cluster and gel formation. a)-d) BSA in acidic conditions before (left) and after (right) filtration using a 0.22 um syringe filter. e) BSA in citric acid + sodium citrate 0.1M salted 137 mM NaCl supernatant after protein concentration and posterior centrifugation to remove protein gel structures. f) BSA gel bottom after protein concentration and posterior centrifugation. g) BSA in basic conditions buffered in PBS (pH = 7.4) before (left) and after (right) filtration using a 0.22 um syringe filter. No aggregation was observed before and after dialysis, and gelation was observed after protein concentration.
Figure 3-9. Decreasing concentration of BSA at pH = 2.7 with an ionic strength 137 mM NaCl (sodium citrate + citric acid 0.1 M buffer) in the AC-susceptometer vials (200 µL). The top image corresponds to protein suspension without MNPs, and bottom image corresponds to protein suspension with PEG coated MNPs after 48 h of exposure.
Figure 3-10. Normalized out-of-phase dynamic magnetic susceptibility for nanoparticles in BSA at pH = 2.7 buffered using citric acid + sodium citrate 0.1 M. 137 mM NaCl
Figure 3-11. Rotational diffusivity of PEG coated MNPs in acidic and basic conditions with and without the presence of salts. \( D_0 \) is the rotational diffusivity of PEG coated MNPs in buffer without added proteins.
Figure 3-12. Bulk rheological measurements at pH = 2.7 buffered and no salted (A-B-NS conditions)
Figure 3-13. Viscosity measurements of BSA in buffered and salted conditions at pH = 2.7 and pH = 7.4 obtained via bulk rheological measurements
Figure 3-14. Viscosity measurements of BSA in buffered and salted conditions at pH = 2.7 and pH = 7.4 obtained via DMS measurements.
Figure 3-15. Comparison between viscosity values of BSA protein solutions in buffered and salted conditions at pH = 2.7 and pH = 7.4 obtained via macromolecular rheology and DMS measurements.
Figure 3-16. Deviation of the Stokes-Einstein relation in BSA solutions below and above the BSA isoelectric point.
CHAPTER 4  
DETERMINING THE MOBILITY OF POLYMER COATED NANOPARTICLES IN  
BLOOD AND TUMOR TISSUE USING MAGNETIC RELAXATION MEASUREMENTS  

4.1 Introduction  
Depending on the inorganic metal core (e.g. iron oxide or cobalt ferrite), magnetic nanoparticles may possess either a freely rotating magnetic dipole (Néel relaxation mechanism) or a dipole fixed along a crystal direction (Brownian relaxation mechanism). These relaxation mechanisms represent the theoretical foundation to understand how the response of magnetic nanoparticles subjected to alternating magnetic fields can provide useful information in terms of nanoparticle-protein interactions that lead to adsorption of proteins or aggregation of particles in complex biological environments. In this work, we tested a method to determine hydrodynamic diameter distributions of magnetic nanoparticle suspensions in blood and tumor tissue by using the Debye model applied to dynamic magnetic susceptibility measurements to assess nanoparticle-protein interactions and colloidal stability prior in vivo clearance experiments. These colloidal stability measurements were incorporated as a complementary technique to predict clearance of magnetic nanoparticles after intravenous injection in rodent models and provide a systematic route to design long term circulation magnetic nanoparticles for biomedical applications.  

4.2 Materials and Methods  
DMEM-high glucose power media (D5648, Sigma-Aldrich), fetal bovine serum (F6178, Sigma-Aldrich), L-asparagine (A4159, Sigma-Aldrich), 10% L-Glutamine (25030081, Thermo Fisher), penicillin-streptomycin (15140122, Thermo Fisher), 175 cm² culture flasks (431466, CorningTM), Lithium-Heparin tubes (365965, BD).
Iron (III) chloride hexahydrate (FeCl₃·6H₂O, 97%, ACS reagent), cobalt (II) chloride hexahydrate (CoCl₂·6H₂O, 98%), oleic acid, mono-methoxy PEG MW 2000, sodium hydroxide (NaOH, ≥98%) pellets, nitric acid (HNO₃, 70%), iron (III) nitrate nonahydrate (≥98%), dimethyl sulfoxide (DMSO, ≥98%), N-hydroxysuccinimide (NHS), polyethyleimine, branched average Mw ~25,000 by LS and CM-Dextran sodium salt Ave. MW 10,000-20,000 were purchased from Sigma Aldrich. Tetramethylammonium hydroxide ((CH₃)₄NOH, 10%), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sulfo-N-hydroxysuccinimide (Sulfo-NHS), sulfo-N-hydroxysuccinimide-acetate (sulfo-NHS-acetate), and CBQCA protein quantitation kit were purchased from Thermo Fisher Scientific. 3-aminopropyltriethoxysilane (NH₂(CH₂)₃Si(OCH₃)₃) (APS) was purchased from TCI America. Bionized NanoFerrite particles with nominal sizes of 80nm and 100nm (BNF-Starch MNPs) were purchased from Micromod Partikeltechnologie GmbH and used as received. 2µm nylon syringe filters and 100 kDa centrifugal filters (Amicon) were purchased from Sigma-Aldrich.

4.2.1 Animals and Tumor Model

A subline of MDA-MB-231 cells [179] (a gift by Dr. Dietmar W. Siemann, Department of Radiation Oncology, University of Florida, College of Medicine) were cultured in vitro in DMEM-high glucose power media supplemented with 10% fetal bovine serum, 2.5 wt. % L-asparagine, 10% L-Glutamine and 10% penicillin-streptomycin. Cells were cultured and incubated at 37°C in a 5% CO₂ atmosphere. A cell suspension of ~6 x 10⁶ MDA-MB-231 cells in 200 µL phosphate buffered saline was injected subcutaneously into the abdominal fat pad of 6-week old female NOD SCID mice (Envigo). After six weeks post cell inoculation, a tumor ~ 1000 mm³ in volume was formed. Tumor growth was monitored every three days using caliper measurements.
Animal handling and procedures were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of The University of Florida.

4.2.2 Synthesis of Cobalt Ferrite Magnetic Nanoparticles

Thermally blocked cobalt ferrite magnetic nanoparticles were synthetized by thermal decomposition of an organometallic precursor as described by Herrera et. al.[180] and by the co-precipitation method by controlling the flow rate addition of metal ions in sodium hydroxide under boiling conditions,[148] respectively.

4.2.2.1 PEI coated cobalt ferrite MNPs

Oleic acid coated cobalt ferrite MNPs obtained by thermal decomposition were used for subsequent aqueous phase transfer mediated by the cleavage of the double bond in the oleic acid to form a carboxylic terminal [181]. Then surface modification of carboxylated MNPs was carried out as described by Cruz-Acuña et. al.[182] with some modifications. In acidic conditions, coupling with branched 25 kDa PEI via EDC/ sulfo-NHS chemistry was performed to obtain PEI 25 kDa coated cobalt ferrite (tICO-PEI-25kDa) MNPs. Briefly, 4 mg EDC and 0.5 mg sulfo-NHS were dissolved with 5 mL of carboxylated MNPs at pH 5 (10 wt. % in deionized water) and left to react 10 min for carboxylic activation. Then, a solution of branched PEI in deionized water (20 wt. % 25 kDa MW, pH 4.5-5) was mixed with the activated carboxylic MNPs and left to react under a high-intensity ultrasonic processor (XL2020, Misonix Inc.) to break aggregates (1 h in an ice bath). After reaction, MNPs were dialyzed into ultrapure deionized water using 100 kDa centrifugal filters (Millipore) centrifuged at 5000 rcf for 10 min at 4°C three times.
4.2.2.2 CMDx coated cobalt ferrite MNPs and PEG coated cobalt ferrite MNPs

The cobalt ferrite particles synthetized via co-precipitation[148] were surface modified with HNO₃ 2 M and hydrothermally treated with ferric nitrate according to Gomes et al.[149]. Then MNPs were peptized using tetramethylammonium hydroxide (TMAO) for further surface modification using either condensation of 3-aminopropyl triethoxysilane (APS) molecules or oleic acid chemisorption i) to covalently attach CMDx or ii) to perform a ligand exchange with PEG-Silane.

Bohorquez et. al. [78] described the procedure to coat cobalt ferrite MNPs synthetized by the co-precipitation method with CMDx after APS condensation. To coat cobalt ferrite nanoparticles with oleic acid, an adsorption reaction was carried out as described by Hubbard et. al.[160] Thus, ligand exchange with PEG-Silane MW 2 kDa was performed as described by Barrera et. al.[70] using as-synthetized oleic acid coated cobalt ferrite nanoparticles suspended in toluene. To remove free polymer excess, polymer coated MNPs were dialyzed against deionized water using 100 KDa centrifugal filters (Millipore) centrifuged at 5000 rcf for 10 min at 4°C three times.

4.2.3 Iron Oxide Magnetic Nanoparticles

Iron oxide MNPs exhibiting a combination of Brownian and Néel relaxation mechanisms were synthetized by chemical co-precipitation of ferric and ferrous salts in alkaline medium and using TMAO as a peptizing agent.[183] CMDx and PEG coatings were grafted onto the surface of the iron oxide nanoparticles with slightly modifications to the described procedures developed by Herrera et. al[150] and Barrera et. al[70] for iron oxide MNPs. A ligand exchange with PEG-Silane MW 2kDa was performed with iron oxide cores modified with oleic acid via adsorption at 80°C as was described for cobalt ferrite MNPs synthetized by using the co-precipitation method.
4.2.4 Surface Modification, Amine Functionalities and Amine Quantification

4.2.4.1 PEG coated MNPs – amine functionalities

PEG-Silane polymers synthetized to obtain colloidally stable PEG coated MNPs, contain some traces of APS molecules with free amine groups. Then, after ligand exchange PEG particles possess exposed amine groups that can vary from batch to batch for a fixed PEG molecular weight. Previously, it has been demonstrated that for low molecular weight PEG (0.75 kDa - 1 kDa), amine groups are exposed contributing to an overall positive surface charge whereas particle coated with higher molecular weight PEG were almost neutrally charged (up to 5 kDa).[167] Therefore, a molecular weight of 2 kDa was selected for particle mobility studies in whole blood and tumor tissue using DMS.

4.2.4.2 CMDx coated MNPs and capped CMDx coated MNPs – amine functionalities

EDC-mediated coupling reaction between amine groups in APS coated MNPs and CMDx polymer chains proceeds with low conjugation efficiency, then unreacted amine groups can remain covered by the CMDx polymer shell after reaction. Domenech et al.[184] were able to conjugate the dye BODIPY-FL to the free amine groups in cIO-CMDx via EDC/NHS reaction, suggesting the presence of unreacted amine groups. For DMS measurements, free amine groups in cIO-CMDx MNPs were acetylated with sulfosuccinimidyl acetate (sulfo-NHS-acetate) to irreversible cover primary amine groups and avoid undesirable particle aggregation due to electro-viscous phenomena mediated between a polyelectrolyte particles (particle with both amino and carboxylic functionalities) in PBS, blood, and tumor tissue. To remove the free polymer excess and unreacted EDC/NHS, CMDx coated MNPs were dialyzed against deionized
water using 100 KDa centrifugal filters (Millipore) centrifuged at 5000 rcf for 10 min at 4°C three times. For Capped CMDx coated MNPs, MNPs were loaded into MWCO 10 kDa dialysis cassettes (Slide-A-Lyzer™, Thermo Fisher) after acetylation reaction and dialyzed against PBS under mild agitation at 100 rpm for 24h.

4.2.4.3 Quantification of amine groups in the surface of the magnetic nanoparticles

The number of amine groups per particle was estimated using 3-(4-carboxybenzoyl) quinolone-2-carboxaldehyde (CBQCA) which reacts to primary amines in the presence of thiols or cyanide at basic conditions. The fluorescent product of CBQCA has an absorption peak excitable at 430-490 nm with maximum emission at around 560 nm. NTA measurements were used to determine particle concentration per each particle stock solution. First, the linear dynamic range of CBQCA response was studied under three fixed peptized iron oxide particle suspensions (1.69 mgFe/mL, 1 mgFe/mL and 0.25 mgFe/mL) using ethanolamine as calibration curve standard. Then, a post validation of particle interference under a fixed ethanolamine concentration in CBQCA assay was performed in a particle concentration range between 0-2 mgFe/mL. From these conditions the optimal fluorescence signal vs particle concentration was 0.25 mgFe/mL. This is in part because the dynamic range was the highest between 0-1.5 μg ethanolamine increasing assay sensitivity. Afterwards, CBQCA unknown samples (500 μL) were prepared from particles stocks in water diluted up to 0.25 mg Fe/ml with 0.1 M sodium borate at pH = 9.3. Then, the derivatization reaction was carried out by mixing 130 μL of CBQCA unknown sample with 5 μL of 20 mM KCN and 10 μL of a 4 mM CBQCA working solution for 2 hours in a 96-well plate microplate for analysis in a fluorescence microplate reader. Amine groups were quantified using an
ethanolamine calibration curve prepared under the same previous reaction conditions as follow, 130 µL of peptized iron oxide MNPs in 0.1 M sodium borate at pH = 9.3 (0.25 mgFe/mL) were mixed with 1 µL ethanolamine standard solutions, 5 µL of 20 mM KCN and 10 µL of a 4 mM CBQCA working solution. Amine quantification was performed by triplicates per particle used in this study.

4.2.5 Characterization of Magnetic Nanoparticles

Infrared spectroscopy in a Varian 800 Transform Infrared (FTIR) equipment was used to study changes in functional groups obtained in the synthesized coated MNPs. Powder samples were placed on a Pike attenuated total reflectance (ATR) stage with ZnSe window. The results are presented in a plot of transmittance (dimensionless units) vs. wavenumber (cm⁻¹).

A Hitachi 7600 transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA) equipped with a MacroFire slow-scan CCD camera (Optronics, Goleta, CA) and AMT Image capture software (Advanced Microscopy Techniques, Danvers, MA) operating at 120 kV acceleration voltage was used to image the nanoparticles. Stock samples at 0.5 mgFe/mL (5 µL) were applied onto a ultrathin Formvar-carbon-coated nickel grid and air dried. The physical diameter of the nanoparticles has been determined from the TEM pictures using ImageJ and counting at least 200 particles. The histogram of particle size distribution was fitted to a lognormal size distribution, to estimate the number-weighted mean diameter and the geometric deviation of the MNPs.

The hydrodynamic size distributions of the synthetized MNPs were determined by dynamic light scattering (DLS) and dynamic magnetic susceptibility (DMS) measurements in water with adjusted ionic strength using 1 mM KNO₃ at pH 7.4 at
25°C. DLS and zeta potential measurements were performed using a particle analyzer (Zeta PALS, Brookhaven Instruments). The nanoparticles stock solutions were prepared in water and then filtered through 0.2 µm nylon syringe filter twice prior to measurements and then diluted to ~0.1 mgFe/mL. Volume-weighted size distributions were fitted to a lognormal distribution and the arithmetic mean diameter and standard deviation were calculated along with zeta potential measurements (10 runs per sample).

DMS measurements using a commercial AC susceptometer (Dynomag system, Acreo Swedish ICT) were performed at room temperature. During measurements, the amplitude of the excitation field varied between 0-0.5 mT, and the frequency interval was evaluated in a range of 10 Hz to 160 kHz. All DMS measurements were fitted to the generalized Debye’s model assuming that the particles follow a lognormal size distribution and the viscosity of the fluid carrier assumed as water (0.001 Pa.s).

Nanoparticle tracking analysis (NTA) was performed using a Nanosight LM-10 system (Malvern Instruments) with 405 nm laser excitation. Particle stock solutions in water were diluted in KCl 0.1 M by a 10^6 dilution factor and filtered through 0.2 µm nylon syringe filter. Samples for analysis were injected into the sample chamber using a sterile syringe, and video capture was initiated immediately. All measurements were collected at room temperature with the camera level to the maximum value. For each measurement, three videos were taken with a video acquisition time auto defined and analyzed (50-100 particles per frame, 10^8-10^9 particles per mL) using NTA 3.1 software. Number-weighted size distributions were fitted to a lognormal distribution and converted to volume-weighted size.
For iron oxide particle stocks in water, the iron content was determined using a colorimetric assay based on the complexation of Fe$^{+2}$ with 1,10 phenanthroline. Ten microliters of particle stocks in water were digested with 1 mL of 70% nitric acid (Optima Grade, Fisher Scientific) in a dry bath at 101°C overnight. Then, 10 µL of digested sample were evaporated at 115°C for 1 hour, followed by addition of 46 µL of deionized water and 30 µL of hydroxylamine hydrochloride (8 M). After 1 hour of reaction, reduction from Fe$^{+3}$ to Fe$^{+2}$ was completed and 49 µL of sodium acetate (1.2 M) were added, followed by 75 µL of 1,10-phenanthroline monohydrate (13 mM). Afterwards, 100 µL of each sample were transferred into a 96-well quartz microplate for absorbance analysis in a microplate reader at 508 nm. Absorbance data was used to determine the iron concentration against an iron standard calibration curve. Samples and calibration standards were run in triplicate.

The magnetic core size and saturation magnetization of MNPs stocks in water (~0.5 mgFe/mL) were determined by measuring the response of the equilibrium magnetization under the application of a DC field at 300 K in a magnetic range from 6 to -6T using a Quantum Design MPMS-3 Superconducting Quantum Interference Device (SQUID) magnetometer. The Langevin equation weighted using a lognormal size distribution was used to calculate the magnetic diameter of the nanoparticles and the saturation magnetization was obtained by averaging 10 points in the maximum saturation region and normalized by iron quantification.

**4.2.6 Blood Collection and Tumor Harvesting**

Mice were randomly selected for particle mobility assessments when tumors reached a size between 800-1000 mm$^3$ in volume. Blood was collected by cardiac puncture using sodium citrate-wetted syringes (0.1 v/v %) and 25G needles and
transferred into capillary blood collection tubes. Then blood collection tubes were placed in a tube rotator with slow rolling wrist motion to avoid clot formation and hemolysis. Whole blood was used immediately to perform DMS measurements. Tumors were harvested under sterile conditions, kept on ice and used the same collection day. Processing consisted of transferring the tumor into a Petri dish containing PBS and cutting fragments using a disposable biopsy punch (10-12 fragments of 6 mm per tumor) to: i) assess colloidal stability in tumor tissue using DMS measurements, ii) perform Prussian blue staining, and iii) nanoparticle-tumor tissue visualization using transmission electron microscopy (TEM).

4.2.7 Assessment of Particle Mobility Using Dynamic Magnetic Susceptibility Measurements

Colloidal stability as a function of particle mobility was assessed using a commercial AC susceptometer (Dynomag system, Acreo Swedish ICT) at room temperature. The amplitude of the excitation field varied between 0-0.5 mT, and the frequency interval was evaluated in a range of 10 Hz to 160 kHz. A representation scheme for the sample preparation during particle mobility assessments is shown in Figure 4-1. Blood and harvested tumor tissue hydrated with PBS were used immediately. Briefly, 10 µl of polymer coated MNPs (~39 µgFe) suspended in PBS were mixed gentle with 90 µL of whole blood and immediately tested in the AC susceptometer (t = 0 h). For tumor tissue sample evaluation, a tumor fragment was placed in a polycarbonate capsule size 4 and a hole was opened in the closed capsule using a 26G needle. Then 10 µL of polymer coated MNPs (~39 µgFe) suspended in PBS were loaded in a 25 µL Hamilton syringe coupled with a Hamilton needle 26G 0.75” 12° bevel and slowly injected in the tumor fragment placed inside the
polycarbonate capsule. After infusion needle was left inside the tissue for 5 min post-injection to optimize nanoparticle distribution and then removed carefully. The tissue fragment was immediately tested in the AC susceptometer (t = 0 h). Next, whole blood and tumor tissue samples were measured at t = 24 h as a second evaluation time point. One mouse was used per replicate. Three replicate were evaluated per condition.

Dynamic magnetic susceptibility data was fitted to the generalized Debye’s model assuming that the particles follow a lognormal size distribution and the viscosity of water (0.001 Pa.s), obtaining as the fit parameters the lognormal diameter distribution and geometric deviation which represents a measure of the particle mobility in PBS, mice blood and tumor tissue. Along with the obtained diameter distributions, initial susceptibility values of MNPs in mice blood and tumor-tissue were normalized with initial susceptibility values in PBS at t = 0 h, and the arithmetic mean diameter and standard deviation were estimated to evaluate particle polydispersity in biological millennium as a function of time.

4.2.8 Tissue Preparation for Electron Microscopy

Tissue processing and embedding into Epon resin for examination was performed as follows: harvested tumor tissue fragments injected with MNPs (~1 mm³) were fixed in a mixture of 4% formaldehyde – 2% glutaraldehyde in PBS overnight (primary fixation). Afterwards, samples were washed with PBS in order to remove fixation cocktail and a post fixation treatment with 1% osmium tetroxide/ 2-mercaptoethanol (2-ME) in 0.1M Na cacodylate was performed. Samples were washed, dehydrated through a graded series of ethanol, and embedded with Quetol 651 resin following procedures that have been previously published [185]. Ultrathin sections of samples (70 nm) containing well-preserved ducts were mounted on uncoated copper
grids. Sections with and without uranyl acetate (alcoholic 8%)/ Venable’s lead citrate stains were examined using a Hitachi 7600 transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA) equipped with a MacroFire slow-scan CCD camera (Optronics, Goleta, CA) and AMT Image capture software (Advanced Microscopy Techniques, Danvers, MA) operating at 120 kV acceleration voltage.

4.3 Results and discussion

The particle coatings studied herein were selected due to their relevance in several biomedical applications. First, a non-viral gene transfection mediator, branched polyethylenimine (b-PEI), was studied for being a polycation with a large buffering capacity at physiological conditions [186]. PEI coated MNPs have been used in magnetofection to improve DNA transfection efficiency [187] and for targeted gene delivery [188, 189]. The second selected coating was carboxymethyl dextran (CMDx). CMDx coated MNPs have been used extensively for magnetic cell labeling [190], magnetic resonance imaging[191] and hyperthermia studies for cancer treatment [16, 184, 192-194]. They are well known to have non-specific interactions in biological systems due to overall negative surface charge, phagocytic cell uptake, and biodegradability. As third coating alternative, we selected hydroxyethyl starch (BNF-Starch). This is a non-ionic starch derivative biodegradable stabilizer in BNF-Starch commercial MNPs used mostly for magnetic fluid hyperthermia applications [195]. Finally, as a fourth coating we chose polyethylene glycol (PEG), which provides immune evasion and opsonization resistance [139] for magnetic nanoparticles in biological environments, a suitable option for targeted therapeutics. Other surface coatings or modifications included as reference were amino-propyl-triethoxysilane (APS) condensation and sulfosuccinimidyl acetate (sulfo-NHS-acetate) induced acetylation in
CMDx coated MNPs. APS was studied due to its highly positive surface charge, and resistance to enzymatic attack in lysosomes used in earlier magnetic fluid hyperthermia studies [196], and significance as an intermediate step in preparation of covalently bound CMDx coated MNPs [73]. To determine the impact of controlled surface chemistry after CMDx immobilization onto APS coated MNPs, unreactive primary amines were acetylated (capped CMDx coated MNPs) and particle mobility tested. Acetylation of primary amine groups performed with sulfosuccinimidyl acetate forms an amide bond under basic conditions (R-NH$_3^+$ groups are converted to R-NH-CO-CH$_3$). It has been shown that partial acetylation of lysine residues reduce intraprotein crosslinking/aggregation [197] and we tested this approach to cover unreactive primary amine groups in cIO-CMDx MNPs that could interfere with particle stability in biological systems.

The FTIR spectra of five representative coated MNPs used here are presented in Figure 4-2. For the PEG coated nanoparticles, the Si-O-Fe and Fe-OH peaks were detected between 800-900 cm$^{-1}$. The peak at 1095 cm$^{-1}$ is characteristic of −COC vibrations from the ether group in PEGSilane. Also, a strong band at 2780 cm$^{-1}$ was attributed to C-H stretching vibrations. A band appeared at 1635 cm$^{-1}$, characteristic of the −C(=O)−NH vibration in PEG-Silane [198, 199].

Silanized MNPs formed small particle aggregates as a result of co-precipitation synthesis and APS condensation which lead to the formation of nanoclusters embedded in siloxane shell (Figure 4-3). Table 4-1 shows a summary of the physicochemical characterization of the MNPs in water at pH ~7.4 containing 1 mM potassium nitrate (KNO$_3$) or 1 mM potassium chloride (KCl) to control ionic strength. PEI coated MNPs
and APS coated MNPs showed the expected positive surface through zeta potential measurements. Differences in hydrodynamic diameter distributions for both particles are due to the employed synthesis method and surface modification procedure. The cationic PEI coated MNPs made by the thermal decomposition method were stabilized via electrostatic repulsion and steric hindrance, showing a narrow size distribution in comparison with APS grafted MNPs.

Table 4-1. Physicochemical characterization of coated magnetic nanoparticles used in this study.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inorganic core</th>
<th>Coating</th>
<th>$\zeta$-potential, [mV]</th>
<th>$D_H$ (DLS), [nm]</th>
<th>$D_H$ (NTA), [nm]</th>
<th>$D_H$ (DMS), [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>tICO-PEI</td>
<td>Cobalt ferrite</td>
<td>b-PEI 25kDa</td>
<td>23 ± 1</td>
<td>37 ± 16</td>
<td>64 ± 22</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>cICO-CMDx</td>
<td>Cobalt ferrite</td>
<td>CMDx 10 kDa</td>
<td>-30 ± 2</td>
<td>72 ± 24</td>
<td>81 ± 31</td>
<td>79 ± 23</td>
</tr>
<tr>
<td>cICO-PEG</td>
<td>Cobalt ferrite</td>
<td>PEG-Silane 2 kDa</td>
<td>0 ± 1</td>
<td>52 ± 20</td>
<td>171 ± 81</td>
<td>54 ± 16</td>
</tr>
<tr>
<td>BNF-Starch 80 nm</td>
<td>Iron oxide</td>
<td>Hydroxyethyl starch</td>
<td>-4 ± 1</td>
<td>103 ± 24</td>
<td>-</td>
<td>102 ± 23</td>
</tr>
<tr>
<td>BNF-Starch 100 nm</td>
<td>Iron oxide</td>
<td>Hydroxyethyl starch</td>
<td>-6 ± 1</td>
<td>121 ± 38</td>
<td>-</td>
<td>129 ± 34</td>
</tr>
<tr>
<td>cIO-APS</td>
<td>Iron oxide</td>
<td>APS</td>
<td>34 ± 2</td>
<td>69 ± 28</td>
<td>139 ± 63</td>
<td>89 ± 52</td>
</tr>
<tr>
<td>cIO-CMDx</td>
<td>Iron oxide</td>
<td>CMDx 10 kDa</td>
<td>-38 ± 1</td>
<td>73 ± 22</td>
<td>64 ± 22</td>
<td>68 ± 39</td>
</tr>
<tr>
<td>Capped cIO-CMDx</td>
<td>Iron oxide</td>
<td>CMDx 10 kDa</td>
<td>-16 ± 1</td>
<td>70 ± 26</td>
<td>64 ± 21</td>
<td>64 ± 32</td>
</tr>
<tr>
<td>cIO-PEG</td>
<td>Iron oxide</td>
<td>PEG-Silane 2 kDa</td>
<td>4 ± 0</td>
<td>59 ± 31</td>
<td>59 ± 19</td>
<td>49 ± 16</td>
</tr>
</tbody>
</table>

All CMDx coated MNPs presented a negative surface charge. However, capped cIO-CMDx particles showed a reduction in the absolute zeta potential value (from -38 ± 1 mV to -16 ± 1), which confirms that acetylation modified the $\zeta$-potential in the interfacial double layer. Due to amide bonds formation after acetylation reaction, amide...
groups should not contribute with a positive surface charge at physiological conditions, thus no signal by using the CQBCA assay. This change in ζ-potential could be attributed to electrostatic screening effect after dialysis against phosphate buffered saline (PBS) for capped cIO-CMDx MNPs. Increase in salt content leads to lower zeta potential values due to the increased impact of electrostatic shielding. Differences in hydrodynamic size distributions were not noticed between cIO-CMDx and capped cIO-CMDx MNPs based on optical and magnetic measurements.

The ζ-potential measurements for BNF-Starch MNPs remained almost neutral at the studied pH and ionic strength conditions. Size distribution estimations via DLS and DMS measurements were in good agreement. PEG coated MNPs showed a minimal ζ-potential value which confirms that steric stabilization is predominant in this colloidal system.

Nanoparticle tracking analysis (NTA) measurements showed a slight increase in hydrodynamic size distributions in comparison with DLS and DMS analysis for most of the particles used in this study with the exception of positively charged MNPs. This is in part because, NTA can measure single particles and absolute particle concentration thus it is more prone to detect a wider range of particle sizes per sample. For cationic particles, the deviation was more dramatic on converting number-weighted distributions to volume-weighted distributions. DMS measurements confirmed that particles present relax predominantly by the Brownian relaxation mechanism exhibiting a well-defined susceptibility spectrum that follows the characteristic Debye’s model.

To confirm the presence of free amine groups, shielded by the longer polymer chains of CMDx and PEG, but which may still cause nanoparticle-blood or nanoparticle-
tissue interactions, a chromatographic derivatization reagent was used to quantify accessible primary amines in solution. Figure 4-4 shows a comparison between a) zeta potential measurements and b) amine quantification results using 3-(4-carboxybenzoyl) quinolone-2-carboxaldehyde (CBQCA) for the particles used in this study. Number of particles per particle stock solution were estimated using NTA measurements.

Amine quantification was carried out under basic conditions (pH ~9.3). The reaction occurs in presence of potassium cyanide (KCN), starting with a nucleophilic substitution between the aldehyde terminal in CBQCA and the cyanide ion (\(-\text{C}≡\text{N}\)).

Then primary amino groups react with this intermediate product to form a highly fluorescent isoindole derivative [200, 201]. Important features of CBQCA as a labeling agent are that i) it does not fluoresce unless it reacts with primary amino groups (excess of CBQCA does not need to be removed from sample) and ii) the stability of the derivatization reaction [202].

Branched PEI has primary, secondary and tertiary amino groups in a ratio of 1:2:1, and up to 25% of these amino groups are protonated under physiological conditions[203]. However, the primary amine groups in b-PEI were covalently coupled to the carboxylic groups onto MNPs using EDC and sulfo-NHS. Then, primary amine groups did not contribute for the overall \(\zeta\)-potential value obtained, and they were unable to be detected via CBQCA as shown Figure 4-4a and 4-4b.

For cICO-CMDx MNPs and cICO-PEG MNPs we calculated 49 ± 2 amine groups/MNP and 296 ± 67 amine groups/MNP, respectively. No amine groups were expected in BNF-Starch MNPs, as was confirmed via CBQCA detection. The highest value obtained was 458 ± 83 amine groups/MNP for cIO-APS MNPs as expected, then
after CMDx coupling the number of free amine groups was reduced to 167 ± 5 amine groups/MNP which signifies that 64% of the free amine groups in the APS coated MNPs were utilized at the reaction conditions employed for CMDx immobilization. In order to control the remaining free amine groups, the acetylation reaction via sulfo-NHS-acetate modification for iron oxide MNPs showed a capped efficiency of 100% with no amine signal contribution. Finally, for cIO-PEG MNPs it was calculated 166 ± 17 amine groups/MNP.

Equilibrium magnetization measurements at 300 K in aqueous solutions demonstrated that all MNPs exhibited superparamagnetism. As a representative equilibrium magnetization curve, Figure 4-5 shows the magnetic response of PEI coated MNPs. By fitting the data to the Langevin function using the procedure established by Chantrell [169], the magnetic diameter was 11 ± 4, while the volume fraction ($\phi$) of the dilute stock solution was 0.03%.

4.3.1 Particle Mobility in PBS, Whole Blood and Tumor Tissue Using Magnetic Relaxation Measurements

MNPs in PBS, blood and tumor tissue were subjected to DMS measurements to track particle mobility. The following graphs show the particle mobility patterns obtained for i) polymer coated cobalt ferrite MNPs (Figures 4-6 and 4-7), ii) thermally blocked polymer coated iron oxide MNPs (Figures 4-8 and 4-9), and iii) polymer coated iron oxide nanoparticles with mixed relaxation (Figure 4-10 and 4-11). MNPs predominantly followed the behavior of the Debye model. However, iron oxide nanoparticles as-synthesized by co-precipitation method shows a mixture of magnetic relaxation components [157]. DMS spectra evaluating changes in peak frequency from the out-of-phase susceptibility have provided sufficient information to understand what was
happening in the local environment of the MNPs upon contact with biological milieu for 24 hours.

In Figure 4-6a we observed that upon contact with tumor tissue PEI MNPs deviate from the Debye model and the Brownian peak almost disappeared may be due to possible particle aggregation and/or posterior particle precipitation. In blood some particle mobility still remains but it is limited in comparison with particle mobility in PBS. Hydrodynamic diameter distributions obtained via DMS susceptibility measurements revealed that particle polydispersity increased in tumor tissue environments (Figure 4-6b). We can attribute this to electrostatic interactions of particle with blood proteins playing a significant role in particle aggregation. Relative retardation of particle mobility due to electrostatic interactions has been observed with cationic particles suspended in hyaluronic acid solutions and bovine vitreous humor ex-vivo using single particle tracking (SPT) microscopy analysis [204]. Positively charged particles have shown a heterogeneous diffusion pattern in vitreous humor, where a small fraction remains mobile and the majority of particles immobilized were attached to collagen fibrils in the vitreous humor, which may be a close representation of what is happening in our tumor tissue experiments.

Figure 4-7 shows that PEI coated particles dramatically lose their magnetic response after 24h of incubation in mouse blood and tumor-tissue (normalized initial susceptibility values decreased to 0.2). Electrostatic interactions seem to determine particle aggregation in this system and we cannot involve other interaction component such as hydrophobicity with the information collected for this experimental design. This
could happen due to negative charge contribution of proteins in blood and several fibrous connective tissues in tumor stroma interacting with positively charged MNPs.

In contrast, CMDx and PEG coated nanoparticle experienced mobility up to 24h of evaluation in PBS and blood because of no reduction in the normalized initial susceptibility values, which mean that particles remained stable in size responding by physical rotation to an applied external magnetic field. In contrast, in tumor-tissue explants differences were observed in particle mobility between CMDx and PEG coatings. Cobalt ferrite coated CMDx MNPs slightly aggregate when interacting with tumor-tissue fragments (t = 0h), and after 24h the out-of-phase Brownian peak shifts to a lower frequency range as a result of an increase of particle aggregation and/or particle immobilization upon particle contact with tumor tissue explants.

BNF-Starch 80nm MNPs remain stable in blood and tumor tissue in comparison with BNF-Starch 100 nm MNPs (Figure 4-8). Particle mobility restriction close to 90% was observed for BNF-Starch 100nm MNPs in tumor tissue after 24h (Figure 4-9). BNF-Starch 80nm and CMDx MNPs showed partial mobility in blood and tumor tissue after 24h. However, BNF-Starch 100nm MNPs have shown lesser magnetic response after 24h according to the dynamic susceptibility spectra evaluation. An explanation of this could be particle diffusion inside the tumor matrix. Since BNF-Starch 100 nm particles are bigger particles and less colloidaly stable than CMDx and CMDx-Blocked MNPs, they diffuse slowly until coating desorbs or clusters get internalized by cells still alive in the time window of this study. CMDx-blocked nanoparticles even though are smaller and diffuse faster, their specific surface modification allows them to not attach in the cell matrix, helping them diffuse freely. If there was the case of MNPs internalization in cell
compartments during the time window studied, in less than 24h particles could be already discarded to the extracellular space maintaining a hydrodynamic size less than 100 nm, which can be related to observe a well-defined Brownian component or a constant initial susceptibility value measured using the AC susceptometry.

Iron oxide CMDx coated nanoparticles due to free amine groups became highly polydisperse after CMDx conjugation (Figure 4-10). However, this can be tuned by blocking amine groups using sulfo-NHS acetate, an inert molecule. This degree of polydispersity is detectable by optical measurements such as DLS and NTA but becomes more relevant using dynamic magnetic susceptibility measurements in order to study colloidal stability in blood and tumor tissue due to the complexity of these biological environments. These findings demonstrate that surface modification needs to be monitored and improved specifically in targeting strategies and sensing applications to avoid confusing the electrostatic interactions with particles in bio-environments instead of targeting-ligand interactions.

Since this set of iron oxide nanoparticles was synthesized by using the coprecipitation method, their magnetic relaxation mechanism is a combination between Brownian and Néel relaxation mechanisms, hence we cannot rely on the analysis of the normalized initial susceptibility values with respect to PBS to estimate the amount of MNPs in suspension after 24 h. However, we can monitor the value of $\chi''$ at the peak-frequency of the Brownian component of the MNPs in order to evaluate if nanoparticle-protein interactions are restricting the rotation of the MNPs in solution during the experimental time window of 24 h as shown in Figure 4-11.
APS coated MNPs interact with blood and tumor stroma environments due their positive charge and poor colloidal stability forming aggregates. Hence, rotation of particles is restricted which results in the disappearance of the Brownian peak from the frequency range used in this study. In contrast, for CMDx, capped CMDx and PEG coated MNPs the Brownian peak does not disappear, suggesting that the physical rotation of particles relaxing by the Brownian mechanism remains unaffected. This can be explained by electrostatic repulsion between negatively charged blood proteins and tumor stroma, and the negatively charged surface of the CMDx polymer shell. PEG coated nanoparticles are neutral, hence do not interact with the charged biological environment.

4.3.2 Visualization of Nanoparticle-Tumor Interactions Using Transmission Electron Microscopy

To characterize cellular distribution of the particles in tumor tissue ex-vivo, we performed transmission electron microscopy after 30 min of particles incubation with tumor tissue fragments. For these studies five MNPs from the DMS susceptibility studies, namely positively charged PEI MNPs, commercial BNF-Starch 100 nm (neutral), CMDx coated iron oxide MNPs (negative charged), capped CMDx coated iron oxide MNPs (negative), and PEG coated iron oxide MNPs (neutral) were selected.

PEI MNPs showed a strong aggregation pattern in tissue after particle contact ex-vivo (Figure 4-12). PEI coated MNPs were found in dark necrotic disintegrating cells. These results agree with the DMS response studied in tumor tissue (no particle rotation). There was no evidence of particle uptake in cancer cells or macrophages.

For the rest of the MNPs studied the distribution patterns in tumor tissue were slightly different under TEM. Particularly, in the case of PEG coated particles (cI0-
PEG), which were distributed into the tumor stromal tissue and cell bodies in a less clustered form in comparison than the rest of the particles in this study.

We observed an agreement between TEM and DMS measurements in the case of PEG coated MNPs (Figure 4-13) and BNF-starch MNPs (Figure 4-14) and which showed endocytosis of MNPs after 30 min. It seems like BNF-Starch 100nn MNPs were taken up by tumor associated macrophages and internalized in vesicles and lysosomal compartments. CMDx and capped CMDx coated MNPs did not show agreement with our previous DMS measurements (Figures 4-15 and 4-16 respectively). They look aggregated but they are not restricted inside cell structures, which could be the reason to observe particle mobility via DMS measurements after 24 h. These particles were identified in several cancer cell structures and in dark necrotic disintegrating cells but not in tumor associated macrophages.

4.4 Conclusions

The response of MNPs to an alternating magnetic field can be used to study colloidal stability as a function of particle mobility in situ in blood and tumor tissue environments via magnetic relaxation measurements. It was confirmed that the mobility of particles in blood and particle distribution in tumor tissue depends strongly on electrostatic interactions. First, it was identified that cationic particles aggregated in tissue avoiding particle diffusion, which was observed as a reduction in the initial susceptibility component as a function of time. Second, PEG coated nanoparticles as-synthesitized by the co-precipitation method remained stable up to 24 h observation in blood and tissue with a size less than 100 nm. This agreed with previous biodistribution results in our research group. Third, less interaction patterns were observed with CMDx coated MNPs modified with sulfo-NHS-acetate in contrast with CMDx coated MNPs.
Fourth, BNF-Starch MNPs aggregated in both blood and tumor tissue environments. The results highlight that the dynamic response of MNPs in an alternating magnetic field can be used to study colloidal stability as a function of particle mobility *in situ* in blood and tumor tissue environments.
Figure 4-1. Particle mobility assessment in blood and tumor tissue ex-vivo. 1) Cancer cell inoculation in a mice model, 2) tumor harvest and blood collection 30 days after cancer cells inoculation, 3) blood sample preparation, 4) tumor tissue sample preparation, and 5) dynamic magnetic susceptibility measurements at room temperature
Figure 4-2. FTIR measurements for cI0-APS, cI0-CMDx, capped cI0-CMDx and cI0-PEG magnetic nanoparticles
Figure 4-3. Representative TEM micrographs and DMS spectra for PEI, CMDx, starch and PEG coated MNPs. TEM images show particle clusters and DMS measurements reveal that particles respond in the presence of an alternating magnetic field by physical rotation resulting in a Debye-like susceptibility plot.
Figure 4-4. Comparison between polymer coated particles selected in this study. a) \( \zeta \)-potential measurements and b) amine quantification results.
Figure 4-5. Equilibrium magnetization curves at 300 K for the PEI coated magnetic nanoparticles (tICO-PEI) show a magnetization value about 125 A/m without mass normalization.
Figure 4-6. DMS spectra and lognormal diameter distributions for thermally blocked PEI, CMDx and PEG coated MNPs in PBS, whole blood and tumor tissue.
Figure 4-7. Initial susceptibility values normalized with respect to samples in PBS and arithmetic mean hydrodynamic diameter – PEI, CMDx, PEG coated thermally blocked cobalt ferrite MNPs
Figure 4-8. DMS spectra and lognormal diameter distributions for thermally blocked BNF-Starch 80 nm and 100 nm MNPs in PBS, whole blood and tumor tissue.
Figure 4-9. Normalized initial susceptibility values with respect to PBS – BNF-Starch thermally blocked iron oxide nanoparticles
Figure 4-10. DMS spectra and lognormal diameter distributions for cIO-MNPs as-synthesized by co-precipitation method with different surface functionalities – particles with mixed magnetic relaxation mechanisms.
Figure 4-11. Monitoring peak frequency displacement to the lower frequencies area upon contact with PBS, blood and tumor tissue for MNPs with magnetic relaxation mixtures.
Figure 4-12. TEM micrographs of PEI coated cobalt ferrite nanoparticles in tumor tissue ex-vivo. Highly electron dense black dots correspond to PEI coated nanoparticles. There were observed disintegrating cells (D), vesicles (V), and a swollen mitochondria (s). a) PEI coated MNPs adjacent to a cancer cell, b) Zoom in of an area full of MNPs (right) and delimitating disintegrating cells from the cancer cell cytoplasm (left)
Figure 4-13. TEM micrograph of PEG coated iron oxide nanoparticles in tumor tissue *ex-vivo*. Highly electron dense black dots correspond to PEG coated nanoparticles. a) Perinuclear membrane (Pm) with PEG coated MNPs, and a section with apoptotic cell bodies (A), b) Cancer cell showing part of the cell nucleus (N), mitochondria (Mi) and swollen mitochondria (S) and a lysosome (L) (left). Right side section shows a zoom in the lysosomal compartment with a highly electron dense area which may correspond to internalized PEG coated MNPs.
Figure 4-14. TEM micrographs of commercial BNF-Starch magnetic nanoparticles in tumor tissue ex-vivo. Highly electron dense black quasi-squares correspond to BNF-Starch 100nm nanoparticles. a) Vesicle (V) with colocalized BNF starch MNPs (left), and zoom in of MNPs inside vesicles (right).
Figure 4-15. TEM micrographs of CMDx coated iron oxide nanoparticles in tumor tissue ex-vivo. Highly electron dense black quasi-spheres correspond to CMDx coated nanoparticles. a) Cancer cell (C) with some areas marked in squares showing CMDx coated MNPs, cell nucleolus (N) and lysosomal bodies (L) (left), in the right side a zoom of the left top area is shown. b) Stroma tissue (St) with CMDx coated MNPs.
Figure 4-16. TEM micrographs of Capped CMDx coated iron oxide nanoparticles in tumor tissue ex-vivo. Highly electron dense black dots correspond to capped CMDx coated nanoparticles. a) Perinuclear membrane (Pm), a cancer cell, a lysosome (L) and Capped CMDx coated MNPs can be distinguished (left), b) zoom in of Capped CMDx coated MNPs.
CHAPTER 5
SUMMARY

This dissertation described the study of nanoparticle-protein interactions \textit{in situ} via dynamic magnetic susceptibility (DMS) measurements in optically transparent water-glycerol mixtures at 37°C using SQUID-AC susceptometry with carboxymethyl dextran (CMDx) coated cobalt ferrite MNPs. Due to the promising results using DMS measurements in determining the colloidal stability of nanoparticles as a function of nanoparticle-protein interactions, the use of this technique was extended to estimate the rotational diffusion of highly concentrated protein solutions and particle mobility in whole blood and tumor tissue using a commercial AC-susceptometer as follow:

5.1 Estimating Rotational Diffusivity of Magnetic Nanoparticles in Concentrated Protein Solutions

The rotational diffusivity of particle probes with reduced non-specific interactions was monitored in concentrated protein solutions at different pH and salt conditions, mimicking crowded biological environments. In this sense, particles were capable to sense protein gelation at acidic conditions in the presence of sodium citrate ions.

5.2 Determining the Mobility of Polymer Coated Magnetic Nanoparticles in Blood and Tumor Tissue \textit{ex-vivo} Using Magnetic Relaxation Measurements

Nanoparticle-blood interaction and nanoparticle-tumor stromal tissue interaction studies were carried out with nanoparticles coated with different polymers to correlate surface charge with the magnetic relaxation profile in the complex milieu, by detecting changes in the hydrodynamic size of magnetic particles or aggregation patterns using DMS measurements and transmission electron microscopy in tumor tissue fragments. The polymer coatings evaluated represent commonly used polymers in biomedical applications such as carboxymethyl dextran (negatively charged), branched
polyethyleneimine (positively charged), hydroxyethyl starch and modified polyethylene glycol (neutral polymers). Inorganic cores used in this study were thermally blocked magnetic nanoparticles (cobalt ferrite core) and commercial thermally blocked iron oxide nanoparticles. It was further demonstrated that this technique can be extended to particle systems with a mixture of magnetization relaxation mechanisms.

5.3 Concluding Remarks

Nowadays, the design of new strategies to evaluate colloidal stability in complex bio-environments have become valuable in targeted nanotherapeutics, since it has been demonstrated that after systemic injection nanoparticle-blood interactions can accelerate macrophage uptake with implications on solid tumor targeting and thus limiting particle tumor accumulation. We expect that DMS measurements can be taken into account more often for colloidal stability evaluations of polymer coated iron oxide nanoparticles due to its reliability in situ by getting rid of artifacts associated to free proteins at high protein at particle concentrations in contrast to the existent optical techniques. This can take place without the limitation of optical transparency in sample evaluation, and provides a useful assessment tool to design nanoparticles for biomedical application with enhanced colloidally stability and minimal interactions with blood, plasma proteins and tumor-stroma microenvironment.
REFERENCES


BIOGRAPHICAL SKETCH

Ana Carolina Bohorquez was born in Caracas, the capital city of Venezuela. She pursued her Bachelor of Science in Chemical Engineering at the Central University of Venezuela. As an undergraduate student, she received a scholarship from the Gran Mariscal de Ayacucho foundation (Fundayacucho) to complete her undergraduate studies. During her undergraduate studies, she did an internship in the Department of New Business Development and Evaluation for the Orinoco Oil Belt at the Venezuelan Petroleum Corporation (CVP), a division of Petroleos de Venezuela, S.A. (PDVSA) which is the Venezuelan stated-owned oil and natural gas company. She was awarded the Best Undergraduate Honor Thesis Distinction for her thesis project, “Feasibility Study of Petroleum Coke Gasification Technologies for the Production of Electricity, Hydrogen, and Liquid Hydrocarbons” in 2009. This distinction in her undergraduate studies facilitated her to travel to San Salvador, El Salvador, where she was awarded 2nd place in the Oral Presentation Competition during the XV Latin American Congress of Chemical Engineering Students 2009.

After acquiring some professional experience in Belcorp Cosmetics S.A as a Warehouse Logistics Supervisor, she decided to pursue a Master of Science in Chemical Engineering degree at the Universidad de Puerto Rico, Recinto Universitario de Mayagüez in 2010. During her studies in Mayagüez, she conducted research involving the synthesis of magnetic nanoparticles for biomedical applications under the guidance of Dr. Magda Latorre-Esteves and Dr. Carlos Rinaldi. In fall 2012, she started her doctoral degree in Biomedical Engineering at the University of Florida under the supervision of Dr. Carlos Rinaldi with the aim to understand interactions of magnetic materials with biological environments under the application of alternating magnetic
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