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NANOPARTICLE BEHAVIOR IN BIOLOGICAL GELS AND BIOFLUIDS: THE IMPACT OF INTERACTIONS WITH CHARGED BIOGELS AND THE FORMATION OF PROTEIN CORONAS ON NANOPARTICLES

Xiaolu Zhang
University of Kentucky, luluchem@hotmail.com

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Xiaolu Zhang, Student
Dr. Jason DeRouchey, Major Professor
Dr. Dong-Sheng Yang, Director of Graduate Studies
NANOPARTICLE BEHAVIOR IN BIOLOGICAL GELS AND BIOFLUIDS: THE IMPACT OF INTERACTIONS WITH CHARGED BIOGELS AND THE FORMATION OF PROTEIN CORONAS ON NANOPARTICLES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Art and Science at the University of Kentucky

By
Xiaolu Zhang

Lexington, Kentucky

Director: Dr. Jason DeRouchey,
Assistant Professor of Department of Chemistry

Lexington, Kentucky

2015

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ABSTRACT OF DISSERTATION

NANOPARTICLE BEHAVIOR IN BIOLOGICAL GELS AND BIOFLUIDS: 
THE IMPACT OF INTERACTIONS WITH CHARGED BIOGELS AND THE 
FORMATION OF PROTEIN CORONAS ON NANOPARTICLES

With the rapid growth of nanotechnology, situations where nanomaterials will interact with biological systems will unquestionably grow. Therefore, it is increasingly understood that interactions between nanomaterials and biological environments will play an essential role in nanomedicine. Biological polymer networks, including mucus and the extracellular matrix, serve as a filter for the exchange of molecules and nanoparticles. Such polymer networks are complex and heterogeneous hydrogel environments that regulate transport processes through finely tuned particle-network interactions. In chapters 3 and 4, we investigate the role of electrostatics on the basic mechanisms governing the diffusion of charged molecules inside model polymer networks by using fluorescence correlation spectroscopy (FCS). In chapter 3, we show that particle transport of charged probe molecules in charged hydrogels is highly asymmetric and that the filtering capability of the gel is sensitive to the solution ionic strength. Brownian dynamics simulations are in quantitative agreement with our experimental result. In chapter 4, we focus on hyperbranched cationic dendrimer macromolecules (polyamidoamine, PAMAM) which differ from probes in size, charge density and chain flexibilities. Our results show PAMAM has strongly reduced mobility in like charge gels and greatly enhanced apparent diffusivity in oppositely charged gels. Further studies with salt suggest that the oppositely charged polymer network acts as a giant counterion enhancing the mobility of PAMAM by changing its conformation to a more compacted state.

Due to their large surface areas, nanomaterials in biological fluids are modified by adsorption of biomolecules, mainly proteins, to form so called “protein coronas”. These coronas ultimately define the biological identity of the nanoparticles and dictate the interactions of cells with the protein-NP complex. We have studied the adsorption of human transferrin and bovine serum albumin on the surface of sulfonated polystyrene nanoparticle. In chapter 5, we show the formation of multi-layered protein coronas and compare to established adsorption models. In addition we followed for the first time the protein binding kinetics as a function of pH and salt. Through these studies, we aim to
gain quantitative knowledge of the dynamic rearrangement of proteins on engineered nanomaterials.

KEYWORDS: Nanoparticle, FCS, diffusion, PAMAM, Protein corona
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By

Xiaolu Zhang

________________________
Dr. Jason DeRouchey
Director of Dissertation

________________________
Dr. Dong-Sheng Yang
Director of Graduate Studies

________________________
Dec. 17th 2015
Date
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Chapter 1 Background and Introduction

In recent years, significant effort has been devoted to the field of nanotechnology in which functional structures are designed at the atomic or molecular scale and constructed with at least one characteristic dimension measured in nanometers. Nanoparticles (NPs) often demonstrate unique physical and chemical properties compared to bulk materials, and hence been therefore extensively investigated for a variety of biological and biomedical applications.¹

1.1 Advantages of nanoparticles over bulk materials

In general, engineered nanomaterials (ENMs) are materials molecularly designed with at least one dimension ≤ 100 nm to produce new materials, structures and devices.² There are two major reasons why many nanoscaled materials have unique properties compared to bulk materials. First, nanoparticles have a very large surface area to mass (volume) ratio resulting in a significant increase in the percentage of atoms at the surface of a material. For example, if we compare the same mass of carbon in either 60 nm or 60 µm particle form, the nanoparticles would have 1000 times the surface area the microparticles resulting in the surface area for chemical reaction being enhanced 1000-fold.³ The large functional surface areas inherent to nanoparticles enable them to very efficiently bind, adsorb and carry other compounds, such as drugs, probes or proteins, while maintaining the small size required to successfully penetrate physiological barriers and translocate within living organisms.⁴ The second main reason for the unique properties observed in nanomaterials is due to new quantum effects that arise due to the particle size. When the size of a particle is decreased into the nanometer scale, comparable to the wavelength of electrons, the motion of randomly moving electrons in the material is restricted to specific energy levels (discreteness) giving rise to quantum confinement effects.⁵ This phenomenon can give rise to unique optical and electronic properties in nanoparticles that are inaccessible in bulk materials. An example of a class of materials that clearly exploits quantum effects is quantum dots (QDs) — synthesized semiconductor nanomaterials. By simply changing the size of CdSe quantum dots, their emission can be effective tuned from red (520 nm) to the near infrared (NIR) (700-900
nm).\textsuperscript{6} This ability to molecularly manipulate properties increases the possibilities for designing tailored QDs for specific imaging, cellular tracking and diagnostic applications.\textsuperscript{7}

There are many types of nanoparticle platforms with differing size, shape, composition and functionality under investigation for use \textit{in vivo}. Most commonly, nanostructures are engineered to either carry therapeutics for safe delivery to targeted sites or use as a contrasting agents for various imaging techniques.\textsuperscript{8} Nanoparticles commonly consist of inorganic materials, including metal, metal oxides and semiconductors, or a wide range of organic particles such as polymeric particles, liposomes, and dendrimers.\textsuperscript{1-2, 9} These materials can be further chemically modified to create desirable surface properties like charge, hydrophobicity or incorporation of targeting agents for specific applications. Conceptually, such flexibility in designing and modularity enables nanoparticles to be tailored into highly versatile therapeutic agents capable of performing complex functions within physiological systems (Figure 1.1).\textsuperscript{9}

![Figure 1.1 Illustration of different type of nanoparticle platforms. Nanoparticles can be modularly assembled from different materials composition with different physical and chemical properties and functionalized with a myriad of ligands for biological targeting.](image.png)
1.2 Nanoparticles in biological applications

1.2.1 Nanoparticles in biosensors

A biosensor is an analytical device with biological recognition elements used to measure biological processes or physical changes.\textsuperscript{10} The unique physicochemical properties of nanomaterials make them promising candidates for designing new and improved biosensors tailored to address specific analytical needs. Different kinds of nanoparticles may play different roles in sensing systems based on their unique properties. Generally, metal nanoparticles act as “electronic wires” for electron transfer enhancement or as catalysts to increase electrochemical reactions based on their excellent conductivity and catalytic properties. Oxide nanoparticles are often used in the immobilization of biomolecules due to their biocompatibility, while semiconductor nanoparticles are often used as labels or tracers for electrochemical analysis.\textsuperscript{11-12}

As an example, gold nanoparticles are particularly popular for biosensor research because of their intriguing surface chemistry.\textsuperscript{11} One method of using gold nanoparticles as signal transduction amplification tags is the bio-barcode method developed by the lab of Dr. Chad Mirkin.\textsuperscript{13-14} This is an ultrasensitive method for DNA and protein detection and shows great promise for the diagnosis of genetic and pathogenic diseases.\textsuperscript{15-16} In the general procedure of bio-barcode detection, gold nanoparticles are modified with single-strand oligonucleotides that hybridize with target sequences resulting in not only a binding but also a controlled aggregation of Au-oligonucleotide probes. Based on the unique optical property of the gold nanoparticles, the color of the solution changes from red to blue when the interparticle distance is decreased to less than the diameter of gold nanoparticles.\textsuperscript{17-18} Therefore the signal of hybridization process can be assessed by this simple colorimetric sensing strategy. Compared with traditional radioactively labeled probe detection methods, this method has no problem with sample disposal and no requirement of specially trained personnel.\textsuperscript{13} In addition, this method allows for the detection of oligonucleotides at sub-picomolar level without the assistance of polymerase chain reaction (PCR).\textsuperscript{19}
1.2.2 Nanoparticles in bioimaging

Medical imaging techniques, such as magnetic resonance imaging (MRI), optical imaging (OI), computed tomography (CT), ultrasound imaging (USI), and positron emission tomography (PET), create visual presentation of *in vitro* and *in vivo* biological specimens and thus play critical roles in disease detection, prognosis, and treatment planning.\(^1\) Engineered nanomaterials, especially inorganic nanoparticles with their intrinsic quantum mechanical properties, have proven to be remarkable contrast agents and are used in various imaging techniques. First, nanoparticles improve the sensitivity of imaging due to their large surface area to volume ratio thus allowing for the loading of a large amount of imaging agents in a single dosing. Second, nanoparticles are able to accumulate in tumor tissue more efficiently than they do in normal tissues due to the enhanced permeability and retention (EPR) effect resulting in an increasing of the local concentration of contrast agent.\(^20\) This selective accumulation of nanoparticles in tumor cells makes significant improvements on cancer diagnosis and treatment.\(^21\) In addition, the high capacity for nanoparticle chemical modification enables them to be engineered to deliver several different types of imaging agents simultaneously. Development of multifunctional nanoparticles allows for more reliable and accurate detection of disease sites through synergetic multimodal imaging.\(^22\) For instance, there are currently dual-functional nanoparticles with integration of optical and magnetic properties for simultaneous imaging by OI and MRI.\(^23\)

One of the nanoparticle platforms with outstanding optical properties, quantum dots (QDs), have been facilitating the development of *in vivo* optical imaging.\(^24\) Compared to conventional fluorophores, QDs have advantages like high quantum yields, broad absorption spectra, narrow and size-tunable emission spectra, and strong resistance to photobleaching.\(^24\)-\(^26\) Since emission spectra of QDs can be tuned into the NIR region (700-900 nm), the autofluorescence from tissues can be greatly reduced providing a clear target window for *in vivo* optical imaging.\(^27\) As an example, Cai *et al.* reported the targeting and imaging of tumor vasculature in living mice by using arginine-glycine-aspartic acid (RGD) peptide-conjugated NIR QDs.\(^28\)
1.2.3 Nanoparticles in drug delivery

The efficient delivery and release of therapeutics to the target site remains a crucial challenge in the treatment of many diseases. Various nanoparticle-based drug delivery vectors are proposed to improve the therapeutic outcome based on one or more of the following aspects:

1. To improve the bioavailability of poorly water-soluble drugs;
2. To protect drug molecules from undesirable biodegradation;
3. To transport drugs across tightly controlled epithelial and endothelial barriers;
4. To deliver drugs at a specific location with controlled release;
5. To reduce the toxicity of drugs while maintaining the therapeutic effect;
6. To deliver more than one type of drug or therapeutic modality for combination therapy;
7. To visualize the location of drug delivery by integrating therapeutic agents and imaging modalities.

In nanoparticle-based drug delivery, drug molecules need to first be either covalently or non-covalently bound to nanocarriers. The nanocarriers are required to efficiently transfer drug molecules to the target site and ensure their stability and functionality during the transport. Targeted drug delivery can be approached from two strategies: active or passive targeting. Active targeting requires carriers to be attached with customized ligands (antibodies, peptides, aptamers or small molecules) to specifically bind to the receptors on target cells. For example, folic acid or methotrexate are selectively used for cancer treatment because the folate receptors are highly expressed on the cancer cell surface but not on normal cells. In the case of passive targeting, selective accumulation of carriers in the targeted cells or tissues is based on physicochemical or pharmacological factors. One of the unique pathophysiological characteristics of tumor vessels, the EPR (enhanced permeability and retention) effect enables nanoparticles to accumulate preferentially in tumor tissues. After the carriers reach the target cells or tissues, drug molecules then need to be released from the nanoparticles. This releasing process can be triggered either externally by heat or light or exploit the local chemical environment of the target, such as pH, hydrophobicity or concentration of specific ions. In a more advanced delivery system, multifunctional nanoparticles are able to perform as imaging agents and therapeutic agents simultaneously. Successful integration of these different modalities in one delivery system, a “super” particle, may enable simultaneous
diagnosis, therapy and monitoring of therapeutic response.\textsuperscript{35}

1.3 The challenge of applications of nanoparticles in biomedicine

As described above, nanoparticles provide tremendous potential advantages in drug delivery, discovery of biomarkers and molecular diagnostics. The growing use of nanotechnology in biological and biomedical applications also brings safety concern to human health. The unique properties of nanoparticles which make them so attractive in medicine, may also contribute to the toxicological profile of nanoparticles in biological systems.\textsuperscript{36} For example, the nanosize of these particles potentially allows them to cross various biological barriers within the body and translocate to different compartments of organs, tissues or cells. For example, while nanoparticles with the potential ability to cross the blood-brain-barrier (BBB) may open new means for the diagnosis and treatment of brain diseases, at the same time, nanoparticles penetrating the BBB may also cause unintended detrimental health effects on the brain and nervous system not seen presently with conventional drug delivery systems.

As nanotechnology rises, the opportunities for biological systems to interact with engineered nanomaterials, intentionally or unintentionally, will only increase. There is a growing concern therefore about how these nanomaterials behave once inside the body. The potential toxicity of certain nanoparticles may result in the generation of disorders such as inflammation, immunoreaction, or even cancer.\textsuperscript{37} The mechanisms of these effects are not well understood yet, but might be due to concentration-dependent side effects like cell injury or death or the undesirable accumulation in vital organs like liver, lungs or kidneys.\textsuperscript{37-39} In many nanoparticle-based drug delivery studies, a reduction of toxicity is obtained from the optimization of the drug molecules, whereas the potential toxicity caused by nanocarriers is often not in consideration.\textsuperscript{4} To date, understanding of the possible reactivity of nanoparticles \textit{in vivo} and the basics of the interaction between nanoparticle and biological systems has been lacking yet is desperately needed for developing safe nanomaterials for biomedical applications.\textsuperscript{4,40}

1.4 Research motivation and introduction to specific projects

It is obvious that for effective diagnosis or therapy a sufficient concentration of
nanoparticles and their functional surface chemistry need to be maintained. To balance the biocompatibility and toxicity of the nanoparticles, we are interested in the study of the transport processes and biodistribution of nanoparticles in biological systems. Extensive studies have been done to characterize the physicochemical characteristics of nanoparticles themselves, however little is known about how these nanoparticles interact with biomacromolecules inside living systems which ultimately determines the destiny, distribution and bioaccumulation of the nanoparticles in vivo.

In the first two projects described here, we have studied the transport of charged probe molecules and charged, hyperbranched dendrimer macromolecules in neutral and charged biological hydrogels, in chapter 3 and 4 respectively. Recent studies have shown that the movement of particles in biogels, such as mucus, the extracellular matrix or the nuclear pore complex, can depend on the charge and size of the particles as well as the properties of the network itself.\textsuperscript{41-42} There is growing evidence that more complex interactions between the diffusing particle and the polymer matrix result in a more intricate selection process called interaction filtering, which allows some particles to pass through the network and others to be kept out.\textsuperscript{43-45} Particle-gel interactions in vivo may include many specific and nonspecific interactions, such as electrostatics, hydrophobic interactions, and chemical binding. To gain insight into these complex interactions, it is instructive to examine transport properties within simpler model systems such as water-soluble polymer networks. In chapter 3, we examine the transport properties of charged probe molecules in neutral and charged hydrogels. Our results show that the probe transport in the charged hydrogels is highly asymmetric, with diffusion slowed down much more by electrostatic attraction than by repulsion. In addition, we show that the filtering capability of the hydrogel is sensitive to the solution ionic strength in quantitative agreement with Brownian dynamics simulation results. In chapter 4, we examine the diffusion of hyperbranched charged PAMAM dendrimer molecules within neutral and charged hydrogels. As expected, diffusion of the PAMAM is hindered in neutral gels due to steric. Like the probe molecules, diffusion is even more strongly hindered in gels with the same charge (electrostatic repulsion). This reduced transport of charged particles in like-charged networks is most likely due to a “caging” of the molecules due to the repulsive particle-network electrostatics. Surprisingly when the
dendrimer and hydrogel have opposite charge, i.e. electrostatic attraction, the apparent mobility of the dendrimer increases significantly even surpassing the diffusion coefficient of the dendrimer in pure water. Additional experiments examining the effects of added salt suggest the flexible dendrimers are able to undergo large conformational change resulting in a significantly more compacted particle at high ionic strength with a smaller diffusion coefficient. Our results suggest the oppositely charge hydrogel acts like a large counterion resulting in the dendrimer compacting within the network even without additional salt present. Taken together these studies reveal the essential role of electrostatic interaction in governing the nanoparticle transport in biological hydrogels and may lead to better understanding of how the biological barriers filter and control the exchange of molecules between organelles and cells and their environments.

When nanoparticles are introduced into biological fluid, the large surface area of the nanoparticles (NPs) result in the particles being rapidly modified via the adsorption of biomolecules such as proteins or lipids. The end result is a nanoparticle coated with a “corona” of biomaterial. This corona formation affects the biodistribution of nanoparticles and their fate in vivo by influencing the biological response of the particle with the body. Understanding the fundamental processes of protein corona formation is therefore necessary for control and manipulation of protein binding and engineering of nanoparticles with favorable bioavailability. In Chapter 5, we examined the interaction of proteins with polymeric nanoparticles by fluorescence correlations spectroscopy (FCS). Specifically, we studied human transferrin (Tf) and bovine serum albumin (BSA) adsorption on 50 nm diameter sulfonated polystyrene (PS) beads. FCS analysis uniquely allows us to quantitatively determine both the size and number of fluorescently labeled particles in solution. Appropriate labeling of the protein or nanoparticle, allows for sensitive real-time monitoring of the assembly of protein-NP complexes over time under a variety of conditions. By detecting size and changes in free and bound protein fractions, FCS allows for the quantitative determination of the absolute number of bound proteins in the corona and their exchange dynamics in body fluid. Our results show significant differences in the resulting thickness of the corona between Tf-PS and BSA-PS complexes at the same protein/NP stoichiometry ratios. Kinetic studies show the binding rates of Tf-PS are strongly affected by the solution pH which BSA-PS are significantly
less affected. The solution ionic strength for both protein-PS systems does not show a strong dependence of the binding rate; however, the binding affinity is highly dependent on salt. Such studies may lead to a deeper understanding of how local physicochemical properties govern the protein corona formation and complete the pharmacokinetic profiles of nanoparticles.
Chapter 2 Fluorescence Correlation Spectroscopy

To study the dynamics of nanoparticle transport in biological environments, we employed fluorescence correlation spectroscopy (FCS), which offers non-invasive and direct monitoring of chemical kinetics on a single-molecule level. FCS is able to extract dynamic information from observing the intensity fluctuations of small ensembles of fluorescent molecules in thermal equilibrium. The fluorescence fluctuations are autocorrelated to quantify the characteristic diffusion time of labeled molecules and have been shown to provide information on important molecular properties such as particle concentration, translational and rotational diffusion, chemical kinetics, binding reactions, and molecular aggregation in solution as well as the characteristic lifetimes of the fluorophore.46-48

Due to the focused laser beam and confocal pinhole setup, FCS has high spatial and temporal resolution that allows extremely low sample consumption and short sampling time. Compared to other techniques commonly used to measure the dynamics of nanoparticle diffusion, FCS has unique advantages.47-49 FCS is a technique that evolved from dynamic light scattering (DLS). FCS is able to measure the size distribution profile of small particles and their states of motion in solution by analyzing the scattering light.50 While DLS is very sensitive to contaminants such as dirt, FCS measures fluctuations only of fluorescently labeled particles and has less problems with contaminants. Another popular technique, Fluorescence Recovery After Photobleaching (FRAP), measures the diffusion rate by monitoring fluorescence recovery within photobleached spots.51-52 Limitations of FRAP include not being able to measure the concentration, inability to distinguish between single and multiple component diffusion, and the need for a high intensity laser that can cause photodamage to some biological samples like living cells. Single-particle tracking (SPT) obtains transport parameters by imaging the pathway of single molecule. The analysis, however, can be complicated and severely affected by noise. The image noise affects the number of obtained trajectories as well as the accuracy of the particle coordinates.53 Due to the shortcoming of other methodologies, FCS has quickly become a favorable method to probe the molecular diffusion and transport
processes of particles \textit{in vitro} and \textit{in vivo} for biophysical, cell biology and analytical chemical research.

2.1 History of FCS

FCS was devised in 1972 by D. Magde, E. Elson and W.W. Webb. One of the first applications of FCS was to measure the kinetics of the binding of a fluorescent molecule (ethidium bromide, EtBr) to double stranded DNA.\textsuperscript{54} EtBr is a small dye molecule whose fluorescence quantum yield increases 20 times after it intercalates between DNA base pairs. Therefore the binding-debinding process caused the fluorescence fluctuation that allowed accessing the dynamic parameters of this equilibrium reaction.\textsuperscript{54-55}

In the early 1970s, the original FCS setup had a laser excitation of 6 kW cm\textsuperscript{-2} at 514 nm. The scattered excitation light was filtered by a saturated K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} solution, and fluorescence was collected by a parabolic reflector then detected with a photomultiplier tube.\textsuperscript{55} Nowadays, excitation light can be conveniently and efficiently separated from fluorescence light by using dichroic mirrors and interference filters. In the 70’s, the dimension of observation volume of FCS was 5 mm transversally and 150 mm longitudinally and about 10\textsuperscript{4} molecules were in the field of view.\textsuperscript{55} These parameters are several orders of magnitude larger compared to a typical modern FCS setup. Although the original FCS had relatively low sensitivity, it proved that chemical kinetics were assessable through the analysis of fluorescence fluctuation.

FCS was not widely applied to biotechnology or biological sciences until the spatial resolution was greatly enhanced in the early 1990’s. In 1993, Rigler \textit{et al} first introduced the confocal illumination scheme in FCS which used a pinhole in the detection path to minimize the scattered light from background.\textsuperscript{56} With the adaption of a confocal setup, the signal to noise ratio dramatically increased. With this increased sensitivity, FCS was finally capable of monitoring concentrations down to the nanomolar range and conducting the measurements on the single-molecule level.
In the 21st century, with the integration of diverse types of optical approaches, such as dual-color cross correlation and multi-photon excitation, FCS is able to describe multiple dynamic processes occurring in complex environments and has been effectively applied to both *in vitro* and *in vivo* systems.\(^{49,57-58}\) Nowadays FCS is becoming a versatile tool used to screen drug molecules; investigate the conformational changes in biomolecules; evaluate diffusion inside cells and on the cell membrane; and characterize adsorption kinetics and enzyme kinetics.\(^{59}\)

2.2 Theory of FCS

2.2.1 Autocorrelation

FCS records and correlates the spontaneous fluorescence signal fluctuation to reveal the dynamics in the sample under study. Fluctuations in fluorescence signals are caused by fluorophores moving in and out of the observation volume by Brownian motion and also can be induced by the photodynamic property change from molecules during dwell time.\(^{60}\)

The intensity of fluorescence at certain time \(I(t)\) is measured against the average intensity over time \(<I(t)>\). The intensity fluctuation is calculated as:

\[
\delta I(t) = I(t) - <I(t)>
\]  

with

\[
<I(t)> = \frac{1}{T} \int_0^T I(t) dt 
\]

Autocorrelation is used to describe the self-similarity of a time signal. The time dependent fluctuation can be quantified by the autocorrelation function \(G(\tau)\) which is normalized as:

\[
G(\tau) = \frac{<\delta I(t) \cdot \delta I(t+\tau)>}{<I(t)>^2}
\]
where $I(t + \tau)$ is fluorescence intensity at delay time $\tau$. The signal self-similarity is analyzed in lag time $\tau$.

### 2.2.2 Single-component diffusion

Parameters of interest are determined after fitting the autocorrelation curve to the appropriate mathematical model. Here we start with the simplest situation that has one chemical species in the observation volume. In the most common model, the observation volume is assumed to be an ellipsoidal Gaussian resulting in the autocorrelation function derived as $^{60-61}$

$$G(\tau) = \frac{1}{CV} (1 + \frac{\tau}{\tau_D})^{-1} (1 + \frac{1}{(\frac{r_0}{r_D})^2 \tau_D})^{1/2}$$

or

$$G(\tau) = \frac{1}{N} (1 + \frac{\tau}{\tau_D})^{-1} (1 + \frac{1}{(\frac{r_0}{z_D})^2 \tau_D})^{1/2}$$

where $C$ is the average concentration, $V$ is an effective observation volume and $\tau_D$ as the mean dwell time of the particle in the observation volume. $V = \pi^{3/2}r_0^2z_0$ where $r_0$ and $z_0$ are the lateral and axial dimensions of laser beam. $N$ is the average number of particles in the defined observation volume and the local concentration can be determined from $N$. When in the limit $\tau \to 0$, $G(0)$ is inversely proportional to the number of particle in sampling volume. As the number of particles increases, the relative fluctuation of a single molecule on total fluorescence decreases thus the amplitude of autocorrelation decreases. To maintain the sensitivity of measurement, it is important to minimize the concentration of sample between subnanomolar to submicromolar for the femtoliter sized illumination volume.

The dwell time $\tau_D$ is the characteristic time that a molecule resides in the illumination volume.
\[ \tau_D = \frac{r_0^2}{\alpha \cdot D} \]  \hspace{1cm} (6)

where \( D \) is the translational diffusion coefficient and \( \alpha \) is a constant, which equals 4 for one-photon excitation and 8 for two-photon excitation.

### 2.2.3 Multi-component diffusion

When there are two species in the system but no interaction between them, the diffusion times for each of the species are independent. Considering the contribution of particles for each component, \( n_1 \) and \( n_2 \), to total fluorescence, the correlation function leads to\(^6\)\(^0\),\(^6\)\(^2\)

\[ G(\tau) = \frac{n_1^2}{(n_1 + n_2)^2} \cdot G_1(\tau) + \frac{n_2^2}{(n_1 + n_2)^2} \cdot G_2(\tau) \]

\[ = \frac{Q_1^2 N_1}{(Q_1 N_1 + Q_2 N_2)^2} \left( 1 + \frac{\tau}{\tau_{D1}} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_{D1}} \right)^{-\frac{1}{2}} + \frac{Q_2^2 N_2}{(Q_1 N_1 + Q_2 N_2)^2} \left( 1 + \frac{\tau}{\tau_{D2}} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_{D2}} \right)^{-\frac{1}{2}} \]  \hspace{1cm} (7)

where \( Q_1 \) and \( Q_2 \) are the quantum yields for individual component, and \( N_1 \) and \( N_2 \) are average numbers of particles of each component. \( \tau_{D1} \) and \( \tau_{D2} \) are the independent diffusion times of two components crossing the illumination volume described by its structure parameter \( \omega \) (\( \omega = r_0^2 / z_0^2 \)).

Similar to the equation above, it is not hard to derive the correlation function for the case of multiple non-interaction species as

\[ G(\tau) = \frac{Q_j^2 N_j}{\sum Q_k N_k} \left( 1 + \frac{\tau}{\tau_{Dj}} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_{Dj}} \right)^{-\frac{1}{2}}. \]  \hspace{1cm} (8)
2.2.4 Triplet state

To ensure the single-molecule level detection, a high turnover between the excited singlet state $S_1$ to ground state $S_0$ is required. There is a possibility that a part of excited molecules exist in the triplet state. According to the Jablonski diagram shown as figure 2.1, the fluorescence emission occurs from the first excited level of electronic state $S_1$ to the lowest vibrational level of the ground state $S_0$. When the transition of energy undergoes an intersystem crossing process in which state the electron spin is parallel to the spin in its ground state, it is the so called triplet state (T).

![Jablonski diagram](http://www.tissuegroup.chem.vt.edu/chem-ed/quantum/jablonsk.html)

**Figure 2.1** Jablonski diagram.

The triplet state gives rise to phosphorescence, but also is able to revert back to the first excited singlet state $S_1$ to emit fluorescence. The transition between excited singlet state and triplet state is radiationless and much slower than the relaxation time from $S_1$ to $S_0$ therefore causing a delay on emitting fluorescence. Thus considering this triplet-excited state, the expression of correlation function can be simplified as
\[ G(\tau) = \frac{1}{N} (1 + \frac{\tau}{\tau_D})^{-1} \left( 1 + \frac{1}{(\frac{\tau}{\tau_D})^2 \tau_D} \right)^{1/2} \left( 1 + \frac{\exp\left(-\frac{\tau}{\tau_T}\right)}{1-T} \right) \]  

(9)

where \( T \) is the fraction of molecules in the triplet state and \( \tau_T \) is the triplet relaxation time which can be determined by the forward and backward transition rate from the singlet to triplet state. For more than one fluorescent dye molecule having the triplet state, equation 9 can be further modified to include a triplet correction term for each fluorescent species.

### 2.2.5 Unimolecular Isomerization and Cross-correlation

In more realistic situations, there exists interactions or chemical reactions between different species. Referring to the experiment did by Magde et al (1974), the reaction can be expressed like:

\[ k_{AB} \]

\[ A \text{ (fluorescent)} \quad \Rightarrow \quad B \text{ (non-fluorescent)} \]

\[ k_{BA} \]

The fluorescence property of EtBr changes between binding and non-binding state. This unimolecular isomerization case is very similar to triplet state with two processes involved, diffusion and isomerization, but the diffusion rate will not influenced by isomerization process. Thus the correlation function leads to

\[ G(\tau) = \frac{1}{N} (1 + \frac{\tau}{\tau_D})^{-1} \left( 1 + \frac{1}{(\frac{\tau}{\tau_D})^2 \tau_D} \right)^{1/2} \left( 1 + \exp\left(-\frac{\tau}{\tau_l}\right) \right) \]  

(10)

where \( K \) is the equilibrium constant of reaction \( K = \frac{k_{AB}}{k_{BA}} \). \( \tau_l \) is the relaxation time of chemical reaction equal to \( (k_{AB}+k_{BA})^{-1} \).

The interactions between two species can also be measured by labeling them with two different fluorescent dyes, like red and green, with independent excitation and detection but shared illumination volume. If two species move independently, the autocorrelation
can be analyzed for each individual species but no cross-correlation between two species will be observed. Only if two species couple and move as the same entity, will the cross-correlation be measured. Assuming that the illumination volumes generated from red and green lasers are perfectly overlapped and the emission spectra are fully separable, the normalized cross-correlation function is defined for two fluorescent species, the independent green (G) and red (R) channels, as follows\textsuperscript{46, 65}:

\begin{equation}
G_G(\tau) = \frac{<C_G>Diff_i(\tau) + <C_GR>Diff_i(\tau)}{V_{GR}(<C_G> + <C_GR>)^2} \tag{11}
\end{equation}

\begin{equation}
G_R(\tau) = \frac{<C_R>Diff_i(\tau) + <C_GR>Diff_i(\tau)}{V_{GR}(<C_R> + <C_GR>)^2} \tag{12}
\end{equation}

And the cross-correlation function is derived as

\begin{equation}
G_{GR}(\tau) = \frac{<C_GR>Diff_i(\tau)}{V_{GR}(<C_G> + <C_GR>)(<C_R> + <C_GR>)} \tag{13}
\end{equation}

where \(C_G, C_R\) and \(C_GR\) are the concentrations of green-labeled, red-labeled and coupled components. \(V_{GR}\) is the overlapped effective illumination volume. The amplitude of \(G_{GR}(\tau)\) is inversely proportional to the concentration of complex. The motion-related term of the correction function is

\begin{equation}
Diff_i(\tau) = \frac{1}{(1+\frac{\tau}{\tau_{DI}})(1+\frac{r^2\tau}{\tau_{DI}})^{\frac{3}{2}}} \tag{14}
\end{equation}

which describes the diffusion time of each component in the observation volume. Particularly, the diffusion time of coupled two-color fluorescent species \((\tau_{D,GR})\) is defined by the structure parameters from both the green \((w_G)\) and red \((w_R)\) beams under the two-photon excitation condition and expressed as
$$\tau_{D,GR} = \frac{w_D^2 + w_R^2}{8D_{GR}}.$$  

(15)

2.3 Experimental Setup

The most common modern setup of FCS is shown as Figure 2.2. The light source is one or more parallel setting commercial lasers with different wavelengths. The laser light passes through a beam expander and is reflected by dichroic mirrors into an objective with high numerical aperture. The fluorescent molecules in the confocal volume (observation volume) being excited originate fluorescence and then the emission signal is collected by the same object and transmitted through dichroic mirrors, an additional bandpass filter and a pinhole. Finally the fluorescence photons directly impinge on the detector that converts the emission signal into electronic pluses. The intensity fluctuation and autocorrelation curves will be calculated and displayed by the computer software.

Figure 2.2 Schematic experimental setup for FCS measurements. (Reprinted from reference: Elke Haustein and Petra Schwille. Annu. Rev. Biophys. Struct. 2007. 36: page 153.)
2.3.1 Laser source: One-photon excitation and two-photo excitation

In the last couple of decades, laser diodes have been commonly used as a light source in standard spectrofluorometers. In FCS, the main requirement for the light source is high intensity stability because the intensity fluctuation introduced by the laser cannot be separated from the fluorescence fluctuation from sample emission. In addition, a highly focused illumination volume is needed to limit the number of particles excited at any one time.

Generally, fluorophores absorb the energy from one photon to reach the excitation state shown as one-photon excitation in Figure 2.3 on the left side. In the 1990s, Denk, Strickler and Webb applied the multi-photon method (a concept first described by Maria Goeppert-Mayer in 1931) and developed two-photon excitation fluorescent microscopy.66 Basically, two photons with each carrying approximately half the energy of one-photon excitation can be absorbed simultaneously and excite the fluorophore in a single quantum event.

![Jablonski diagrams for one-photon excitation and two-photon excitation.](https://sites.middlebury.edu/durst/research/)

**Figure 2.3** Jablonski diagrams for one-photon excitation and two-photon excitation. (Reprinted from Biomedical Optics Lab in Middlebury College http://sites.middlebury.edu/durst/research/)
For one-photon excitation FCS, low laser power (<1 mW) is sufficient for measurement. Argon ion (\(\lambda = 488\) nm) and HeNe (\(\lambda = 633\) nm) laser diodes are the most commonly used laser sources for green and red fluorophore measurement. Two-photon excitation FCS requires a near-infrared laser with high instantaneous photon flux densities (~\(10^{32}\) photons/cm\(^2\)) that sufficient for two-photon absorption. Most two-photon systems use the titanium-sapphire laser which covers a wide range of wavelength from 690 to 1050 nm and provides high average power (about 1 KW), high repetition frequency (80 MHz), and short pulse width (~100 fs).\(^67\)

The two-photon excitation applied in conventional FCS offers several advantages including (i) the scattered light from background can be greatly minimized because of the wide separation between excitation and emission wavelength and (ii) the out-of-focus photobleaching can be reduced because the laser with infrared wavelength is usually not absorbed by sample.\(^68\) With deeper penetration and reduced photodamage, two-photon FCS is ideally suited for a study of biological samples like living cells or tissues.

### 2.3.2 Confocal microscopy

As discussed above, a clear requirement of FCS is a small, well-defined illumination volume to reduce the number of fluorophores in observation at a given time point. Therefore, high fluorescent intensity with high signal to noise ratio is required. To achieve this, the confocal illumination scheme is applied to FCS and that brings great improvement on the spatial resolution by reducing the background fluorescence.

Figure 2.4 shows the principle of a confocal microscope. The excitation laser beam gets reflected on a dichroic mirror and focused into the sample by a high Numerical Aperture (NA) microscope objective. In an ideal case, fluorescence arises from a unique focal plane and is collected by detectors. However, when the sample is thicker than the focal plane, there will be out-of-plane fluorescence emission generated in the whole illumination volume. This scattered excitation light from different depths within the sample can also reach the detectors giving rise to background noise. To reduce this noise, confocal microscopy takes advantage that the lights from different depths are spatially
separable. By placing a pinhole at the proper position conjugated to the illumination focal plane, the light originating from out-of-focus regions can be blocked. The scattered light reaching the detector is thus greatly attenuated.  

![Illustration of the operational principle of a confocal microscope.](image)

**Figure 2.4** Illustration of the operational principle of a confocal microscope.

### 2.3.3 Detectors: PMT and ADP

In a modern FCS setup, the most commonly used detectors are a photomultiplier tube (PMT) and avalanche photodiode (ADP). Shown in Figure 2.5, PMTs produce an output signal following the processes: an incoming photon liberates an electron from the photocathode, the photoelectrons are then accelerated in an electric field and pass through a focusing electrode to hit on the dynodes. Dynodes are electrodes with each one held at a more positive potential, by about 100 Volts, than the previous one. Therefore the photoelectrons generated from preceding dynodes will be attracted to the next one and generate more electrons. Afterwards a chain reaction starts from the first dynode to the last so that a cascade of electrons is finally collected and converted into voltage. In this
manner, the signal from an individual photon can be greatly multiplied thus the device is described as a photomultiplier. ⁶⁹

Figure 2.5 Schematic diagram of Photomultiplier tubes. (Reprinted from Australian Microscopy & Microanalysis Research Facility http://www.ammrf.org.au/myscope/confocal/confocal/lasers.php.)

ADP is essentially the equivalent of the PMT but electron amplification occurs in a semiconductor based internal structure. As shown in Figure 2.6, the incident photons first generate electron-hole pairs in the silicon photodiode layer. With applied strong electric field, these electron-hole pairs move towards the respective PN junctions with high speed (up to 105 m/s) and thus increase the electric field between electrodes. This gain in electric field strength ionizes more electron-hole pairs to cause further gains in ionization. So this avalanche process significantly amplifies the input photon signal. Compared to PMTs, advantages of ADPs include smaller size, higher speed, lower capacitance and better linearity. ADPs have higher signal-to-noise ratio in the longer wavelength regions of the spectrum (> 500 nm) but lower sensitivity in the blue wavelength region (~400 nm). ⁶⁸
2.4 Biological and chemical applications of FCS

2.4.1 Translational diffusion and size studies

The most direct measurement performed with FCS is the measurement of translation diffusion. The major advantages in using FCS rather than other techniques are, first the observation volume is very small which gives direct local measurement with minimized disruption from biomolecular environment. And more importantly, the measurement is non-invasive which reduces the phototoxicity for biological samples like a living cell. To study the diffusivity, molecules of interest must be labeled with a fluorescent dye, and their diffusion-based motion results in the autocorrelation curve with the characteristic diffusion times and their diffusion coefficients are then determined.

Molecular size is another interesting parameter that can be predicted by diffusivity study. According to the Stokes-Einstein equation, the hydrodynamic radius $R_h$ of a spherical particle can be estimated:
\[ R_h = \frac{kT}{6\pi\eta D} \]  

(16)

where \( D \) is the translational diffusion coefficient, \( k \) is the Boltzman constant, \( T \) is the temperature, \( \eta \) is the viscosity of medium. In this way, the size of particles in solutions can be better characterized compared with other techniques like electron microscopy since the particles have an additional hydration shell in solution. Also the apparent size in different aqueous or organic solvents can also be characterized by knowing the viscosity.

### 2.4.2 Local concentration

As the amplitude of the autocorrelation curve is inversely proportional to the average number of particles in the observation volume, the concentration and change of concentration of fluorescently labeled molecules can easily be determined. With the high spatial resolution resulting from small sampling volume, FCS can be used to investigate the local concentration in an adsorption process or a ligand-receptor binding process. As the local concentration is much higher than the highly diluted bulk concentration under this circumstance, a direct measurement is therefore crucial to determine exact binding coefficients.\(^{47}\)

### 2.4.3 Molecular interactions

Interactions between biomolecules, like protein-protein and receptor-ligand binding, are also well-suited for FCS studies. Typically a small fluorescently labeled compound binds onto a larger object resulting in a complex with a significantly slower translational diffusion coefficient. When the chemical kinetics of binding/debinding processes is much slower than the diffusion kinetics of the molecules across the observation volume, the two-component autocorrelation function is used to resolve the two contributions: the fast diffusion of free small molecules and slow diffusion of bound complexes. Furthermore, by analyzing the correlation function using a two-component model, the fractions of both bound and unbound molecules can be determined and thus the binding stoichiometry is determined. Figure 2.7 is a schematic showing how FCS autocorrelation curves change from a pure single component to a pure complex of greater size.\(^{70}\)
**Figure 2.7** Theoretical FCS autocorrelation curves expected for study a series of binding processes by using FCS. In the intermediate binding states, two diffusion components are detectable (red, green and blue curves). (Reprinted from Ly, S., et al., *Quantifying Interactions of a Membrane Protein Embedded in a Lipid Nanodisc using Fluorescence Correlation Spectroscopy*. Biophysical Journal, 2014. 106(2): p. L5-L8.)

In some situations, like enzyme binding with substrate, the size difference between free species and bound complex is small. This makes the change in diffusion times hard to distinguish by FCS since the dynamics of diffusion is typically on a logarithmic time scale. In this case, dual-color Fluorescence Cross Correlation Spectroscopy (FCCS) was developed to solve this drawback. The principle of FCCS is mentioned in chapter 2.2.5. In FCCS, two components are separately labeled with distinct dyes with minimal overlap in fluorescence emission. Two emission signals are acquired simultaneously while separately by two channels. A cross-correlation of the two channels will only be observed for complexes containing both molecules. If there is no interaction between two labeled species, the channel 1 and 2 will present the diffusion of each individual component, respectively, showing no cross-correlation signal (Figure 2.8 A). If interaction occurs between the two-labeled species, a cross-correlation signal is observed (Figure 2.8B).
amplitude of the cross-correlation function directly corresponds to the concentration of the complexes carrying both fluorophores. Thus, FCCS allows for the determination of the number and kinetics of molecular interactions, like association or dissociation processes, despite the indistinct size difference."

Figure 2.8 Schematic of using FCCS to determine the interaction between green and red labeled species. (A) represents that there is no interaction between two labeled species with showing no cross-correlation. (B) represents that interaction occurs between the two-labeled species and a cross-correlation signal is observed. (Reprinted from Stowers Microscopy Center http://research.stowers.org/microscopy/external/Technology/FCS/index.htm)

2.4.4 More in vivo applications with advanced FCS (TIR-FCS AND RICS)

In recent years, FCS has been combined with alternative detection schemes to extend its application to measurement of the dynamics in a heterogeneous system and living biological systems. As many essential processes occur on cell membranes, like immune
response, ion transfer or receptor-ligand interactions, it is important to learn the dynamics
on the cellular membrane. FCS has the potential to be utilized in the study of diffusion
behavior of membrane-bound molecules and also the binding and unbinding process to
and from the membrane.\textsuperscript{71-72} However, the thickness of the lipid bilayer is generally less
than 10 nm, which is several orders of magnitude smaller than the axial length of the
typical FCS observation volume. As a result, the measurement of membrane-bound
molecules will be compromised by the freely diffusing molecules above and below the
membrane of interest.\textsuperscript{72} This problem can be addressed by applying the Total Internal
Reflection principle to FCS (TIR-FCS). In TIR-FCS, the laser approaches to the interface
between media (from high refractive index to low refractive index) with an appropriate
angle to make the light totally reflected. The total internal reflection allows some energy
to penetrate through the interface to form a \(~10\) nm thin layer excitation region, giving
rise to an evanescent field. The evanescent beam is only able to excite the fluorescent
molecules close to the surface of interface therefore the detection volume is narrowed
down to attoliters \((\sim10^{-18}\text{L})\) and axial resolution is greatly enhanced.\textsuperscript{59,73} The depth of the
evanescent field can be tuned by the angle of the incoming beam. This refinement makes
TIR-FCS ideal to investigate the diffusion of molecules in the membrane, the interactions
with the membrane and the kinetics of other cellular processes.

For some slow-moving biological samples, like protein clusters, with limited complex
diffusion the incorporation of laser scanning techniques has allowed for the measurement
of aggregation kinetics within samples. Raster Image Correlation Spectroscopy (RICS)
can repetitively move the focal point to obtain images of different sections within a
sample. The fluorescent intensity at one pixel is correlated in time with the intensity at
the same pixel in following frames. In this matter, spatial-temporal information of the
labeled species is able to be extracted from the images.\textsuperscript{74-75} Nowadays, RICS has been
commonly applied to separate the immobile from mobile fraction, distinguish the
diffusion from binding and study the diffusion in complex biological environments such
as cell interiors.

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Chapter 3 Probe molecule transport in hydrogels

3.1 Introduction

To reach specific loci in target cells, molecules of interest must traverse complex surroundings consisting of a crowded, interacting environment of biomacromolecules. Molecule diffusion through biological gels such as the cytoplasm, mucus, nuclear pore complex, or the extracellular matrix (ECM) is dictated by the local environment and critical for proper functioning of cell processes.\textsuperscript{41-42, 76-78} Due to their importance as protective barriers against viruses, bacteria, and toxic agents, there has been substantial research in recent years to obtain a better understanding of the transport processes governing the diffusion and penetration of particles through biogels. Although free diffusion of molecules is well understood physically, how the biomacromolecules in these crowded environments affect the mobility and transport of nanometer-sized particles is a key aspect of biology that is not yet fully understood.

3.1.1 Biological hydrogels have complicated filtering strategies

Biological hydrogels are networks that surround biological entities like cells, tissues, organs or entire organisms. They consist of protein-polysaccharide chains and typically contain 90%-99% water. In addition to their mechanical properties, these biogels can also act as selective barriers that control the exchange of molecules between different compartments.\textsuperscript{44} Despite this important barrier function, little is currently known about the structure, dynamics and molecular interactions that allow these biogels to selectively filter particles.

The diffusion of nanoparticles in polymer gels has been studied extensively both experimentally and theoretically. Typically for biological applications, the range of interest is when the particle size is on the order of the gel correlation length or mesh size, $\xi$. From steric arguments alone, one would anticipate that as nanoparticle size approaches $\xi$, the transport of the molecule through the gel will be inhibited, resulting in size filtering; i.e., large particles move more slowly.\textsuperscript{79} However, recent studies have shown that movement of particles in biogels, including the extracellular matrix and nuclear pore complex, can depend on the charge and size of the particles as well as the properties of the network.\textsuperscript{41-42, 44-45, 80-81} There is growing evidence that more complex interactions
between the diffusing particle and the polymer matrix result in a more intricate selection process called interaction filtering, which allows some particles to pass through the network and others to be kept out.\textsuperscript{43-45, 80, 82-83} For instance, a recent study found that 100 nm coated polystyrene beads were much more strongly immobilized inside undiluted human mucus than were 200 and 500 nm polystyrene beads with the same coating.\textsuperscript{84} This directly contradicts the idea that the finite mesh size of cross-linked hydrogels is solely responsible for hindered diffusion in bionetworks.

3.1.2 FCS to measure dynamic processes inside hydrogels

Particle-gel interactions in vivo may include many specific and nonspecific interactions, such as electrostatics, hydrophobic interactions, and chemical binding.\textsuperscript{82, 84-85} To gain insight into these complex interactions, it is instructive to examine transport properties within simpler model systems such as water-soluble polymer networks. Although most experimental methods do not allow for the direct measurement of diffusion coefficients in turbid media, it has been shown that fluorescence correlation spectroscopy (FCS) effectively measures the dynamic processes of small molecules in polymeric systems, hydrogels, and tissues.\textsuperscript{79, 86-93} In FCS, the diffusion coefficient, $D$, of fluorescent particles is calculated from the autocorrelation of the recorded intensity fluctuations through a defined illumination volume. Some of the advantages of FCS over alternative methods are that only a small illumination volume (~fL) and low concentrations of fluorescence particles, typically nanomolar, are required minimizing particle-particle interactions. In this chapter, we will discuss FCS experiments performed to determine the translational diffusion coefficients ($D$) of a charged probe molecule (Alexa488, -2 charge at near neutral pH) in uncharged and charged polymer solutions. Both positive and negative polymer networks are used to compare the effects of attractive and repulsive charge interactions. We find that particle transport through hydrogels is highly charge asymmetric. Repulsive charge interactions are not effective, whereas attractive probe-gel interactions are very effective in significantly hindering particle diffusion through the gel. Addition of salt increases particle diffusivity and for large salt concentrations renders the diffusivity the same as in a corresponding neutral polymer network, demonstrating the importance of electrostatic interactions, in agreement with biological findings in mucus\textsuperscript{77} and the nuclear pore complex\textsuperscript{41-42}. The salt concentration
range where these interactions are screened occurs at a characteristic crossover salt concentration, indicating the potential of salt to function as a gating switch in these hydrogel systems.

3.1.3 Modeling and Brownian dynamic simulations

Although free diffusion of particles and the effect of steric and hydrodynamic interactions between particle and gel are reasonably predicted theoretically, electrostatic effects on diffusion of charged particles in charged gels are still poorly understood. There have been a number of different attempts at devising a useful simulation model consisting of a cubic and periodic environment suitable for Brownian dynamics (BD) simulations.43, 94-98 Early work focused on steric effects94, 97-98, whereas more recent studies have begun to include some form of electrostatic interaction43, 95-96. The aim of such models is to approximate the specific interaction inside a real hydrogel in a manner that can be readily implemented in simulations and is of reasonable computational cost. The need for such models is driven by experimental results that suggest that particle-matrix interactions other than steric effects are most influential on the diffusivity of particles inside biological hydrogels. These interactions seem to be of an electrostatic nature, since the mobility is highly dependent on the particle surface charge42, 44, 80, 84-85, 99-100 and the salt concentration44-45, 80, 101-102.

To better the understanding of nanoparticle diffusion in charged biomacromolecular gels, we collaborated with Dr. Roland Netz’s group (Department of Physics, Free University of Berlin) to propose a simple theoretical model that approximates a real polymer network as a cubic and periodic lattice of connected polymer chains. This cubic lattice is comprised of rigid, straight chains that interact either attractively or repulsively with the diffusing particle. BD simulations were implemented for spherical tracer particles to understand the key factors influencing the diffusivity of the particles inside the polymer network. BD simulations were performed by the Netz lab and will only be discussed briefly here for quantitative comparison to the FCS experiments performed at UK. Using BD simulations, we examined the dependence of particle diffusion on short-range screened electrostatic interactions, mesh size, and particle size within the hydrogels. Simulation outcomes are in quantitative agreement with our experimental results of
diffusion of charged probe molecules within charged networks. To our knowledge, this is the first work showing quantitative comparison between measurements and theory of these asymmetric charge effects. Both theory and experiments show that probe transport within charged hydrogels is governed by both the sign of the interaction potential and the ionic strength. The significant decrease in diffusion for attractive charge interactions is revealed by BD simulations to be due to the charged particles sticking at the vertices of the oppositely charged matrix polymer network.

3.2 Materials and methods

3.2.1 Materials

Polyethylene glycol (PEG) (20 kDa), dextran (15-25 kDa) dextran (500 kDa), carboxymethyl-dextran (CM-dextran(-), 15-20 kDa), and diethylaminoethyl-dextran (DEAE-dextran(+), 500 kDa) were purchased from Sigma Aldrich (St. Louis, MO). CM-dextran is negatively charged, whereas DEAE dextran is positively charged and pure dextran is charge-neutral. A variety of water-soluble fluorescent probe molecules were purchased for FCS measurements. Rhodamine 110 (R110) (absorbance/emission (Abs/Em) maxima, 496/520 nm) was purchased from Fisher Scientific (Waltham, MA). Rhodamine 6G (R6G) (Abs/Em maxima, 530/556 nm) was purchased from Sigma Aldrich. Alexa Fluor 488 succinimidyl ester dye (Alexa488) (Abs/Em maxima, 495/519 nm) and the fluorescent protein R-phycoerytherin (Abs/Em maxima, 546/578 nm) were purchased from Invitrogen (Carlsbad, CA). All of the fluorescent probe molecules were used without further purification.

3.2.2 Preparation of PEG and dextran solutions

PEG and dextran (neutral dextran, CM-dextran(-), and DEAE-dextran(+)) stock solutions of 10% w/v were prepared by weighing out the appropriate amount of polymer powder as received, dissolving in milli-Q water (Millipore, Billerica, MA), and mixing thoroughly. These solutions were subsequently dialyzed overnight against a large excess of milli-Q water to remove additional salts. After dialysis, solutions were lyophilized back to dry powder. The dialyzed polymers were subsequently dissolved at 10% w/v in MES buffer (10 mM, pH=6.4), stirred thoroughly, and incubated at room temperature overnight. Subsequent dilutions with MES were made from the stock solutions, resulting
in final concentrations of 1–8% w/v polymer solutions. All polymer solutions were allowed to incubate for 1 day before use.

For FCS experiments, fluorescent probe molecules were prepared and mixed with polymer (PEG or dextran) solutions to achieve a final probe concentration of ~5–10 nM. Samples of probe molecules in polymer solutions were mixed thoroughly then incubated at room temperature for > 6 h to ensure uniform dispersion of the probe molecules through the sample. From each solution, 500 µL of sample was loaded into NUNC LabTek 8-well microscopy chambers (Nalge Nunc, Penfield, NY) and measured directly by FCS at room temperature.

3.2.3 FCS setup and data analysis

FCS experiments were made using a commercial ISS Alba confocal fluorescence fluctuation system coupled to a Nikon Ti-U microscope equipped with a 60/1.2 NA water-immersion objective lens. The illumination source was a continuous-wave 488 nm laser diode passed through a 514 nm longpass edge filter before detection. Emission signal was recorded by two separate photomultiplier tube (PMTs). All FCS measurements were performed in NUNC LabTek 8-well microscopy chambers (Nalge Nunc) with a final volume of ~500 µL. Determination of the focal volume was established via calibration against an aqueous solution of rhodamine 110 with known diffusion coefficient \( D = 440 \, \mu \text{m}^2 \, \text{s}^{-1} \). FCS results are an average of at least 20 measurements, taken at various positions within the polymer solutions to ensure homogeneity, with typical sampling times of 30 s. All results are expressed as the mean ± SD of the measured diffusion coefficient. FCS measurements were analyzed using VistaVision 4.0 software (VWR, Radnor, PA).

As the basic principles of FCS have been described in detail in chapter 2, we give only a brief overview here. FCS measures the fluorescence fluctuations emitted from labeled molecules moving in and out of a small confocal volume (~1 fL). The size of the effective illumination volume is fixed by the confocal detection optics and the excitation profile of the focused laser beam and characterized by measurements against a standard of a known diffusion constant. For ideal particles of uniform size diffusing by Brownian
motion, dynamic information can be determined from the intensity fluctuations by means of a time autocorrelation given by

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{1}{\langle r_0^2 \rangle / \tau_D} \right)^{1/2}. \]  

(1)

Here, \( N \) is the average number of particles in the illumination volume and the structure parameters \( r_0 \) and \( z_0 \) are the axial and radial dimensions of the excitation beam determined by calibration measurements against a known standard. The autocorrelation can then be normalized by

\[ G_{\text{Norm}}(\tau) = \frac{G(\tau)}{G(0)}. \]  

(2)

The diffusion time, \( \tau_D \), is related to the translational diffusion coefficient \( D \) by the simple relationship

\[ \tau_D = \frac{r_0^2}{aD}, \]  

(3)

where \( D \) denotes the translational diffusion coefficient of the molecules in solution and is calculated from the lateral dimensions of the focused incident beam and the experimentally determined \( \tau_D \). For spherical particles, the diffusion coefficient \( D \) follows from the hydrodynamic radius, \( r_h \), in solution and can be calculated by the Stokes-Einstein relation \( D = k_B T / 6\pi \eta r_h \), where \( k_B \) is the Boltzmann constant, \( T \) is the temperature, and \( \eta \) is the viscosity of the medium.

### 3.2.4 BD simulation

Brownian dynamic (BD) simulations were performed in the laboratory of Professor Roland Netz (Free University Berlin). In a BD simulation the random walk of a diffusing particle follows from the Langevin equation

\[ \dot{r}_i(t) = -\mu_o \nabla_i U(\vec{r}(t)) + \zeta_i(t), \quad (i = x, y, z) \]  

(1)

where \( \dot{r}_i, \nabla_i \) and \( \zeta_i \) are the time derivative of the particle position, the spatial derivative, and a random velocity in \( i \)-direction, respectively. \( U \) is the potential, \( t \) the time and \( \mu_o \) the
bulk sphere mobility. The random velocity $\zeta_i$ is a stochastic variable, modeled with Gaussian white noise to simulate the random collisions of the particle with solvent molecules:

$$\langle \zeta_i(t) \rangle = 0,$$

$$\langle \zeta_i(t) \zeta_j(t') \rangle = 2\mu_o k_B T \delta(t - t') \delta_{ij}.$$  

In a BD simulation, the Langevin equation is evaluated stepwise to create the diffusive motion of the particle inside the model system. At every time step, the mobility forces acting on the particle are calculated and a Gaussian distributed random displacement is added.

A simple cubic lattice comprised of polymer chains is used to model a cross-linked hydrogel. Figure 3.1 shows a unit cell of this the lattice, including particle distributions for a) attractive and b) repulsive electrostatic interactions. Here, the “rods” of the lattice represent the polymer chains and the size of a single cell b for “box size” corresponds to the average mesh size of the hydrogel. The symmetry of the system allows for the usage of periodic boundary conditions. The diffusing particle experiences a total potential

$$U = \sum_{i=1}^{M} (U_i^e + U_i^s)$$

where the subscripts e and s denote electrostatic and steric interaction, respectively. The index $i$ denotes the contributions to the total potential by the individual rods. For computational efficiency the summation is limited to a suitably chosen finite number of $M$ nearest neighbor rods.

The steric effect is included in the form of a truncated Lennard-Jones (LJ) potential similar to the one used by Zhou and Chen:  

$$U^s(R) = 4\epsilon \left[ \left( \frac{p}{2R} \right)^{12} - \left( \frac{p}{2R} \right)^6 + \frac{1}{4} \right] \text{, } R \leq R_c$$

$$U^s(R) = 0 \text{, } R > R_c$$

where the energy depth is $\epsilon = 1 \ k_B T (\approx 4.1 \cdot 10^{-21} \text{J at } 25^\circ \text{C})$, $p$ the particle diameter and $R$ the distance between the particle center and the rod. The potential is truncated at the...
cutoff distance $R_c = 2^{-5/6} \rho$, which corresponds to the value of $R$ at which the LJ potential has its minimum.

To include the electrostatic interaction between a diffusing particle and a polymer chain, an exponential interaction potential is used:

$$U^e(R) = U_o \exp \left( \frac{-R}{k} \right)$$

where $R$ is the radial distance between the particle and the rod, $k$ the range of the potential and $U_o$ is the potential strength. The potential only acts between particle and rod, since the individual rods are static and do not interact with each other. For negative $U_o$ the potential is attractive and for positive $U_o$ it is repulsive. A simple exponential potential has been chosen in a generic form to represent a short-range interaction, where the range $k$ can be understood as the Debye screening length of the potential, given by

$$k^2 = \frac{\varepsilon \varepsilon_0 k_B T}{2 \varepsilon I}$$

where $\varepsilon$ is the elementary charge and $I = \frac{1}{2} \sum_j n_j z_j^2$ the ionic strength, $z$ the valence of the salt ions and $n$ their bulk number densities. The strength of the potential $U_o$, scaled with $k_B T$, can be interpreted as the product of the particle charge and polymer charge density. It is important to note the relative units of the variables. All spatial variables will be given in units of $b$, i.e. relative to the box size. Hydrodynamic effects are disregarded.

Furthermore, the model is designed as a strong simplification of the realistic scenario of particle diffusion in hydrogels, with a focus on electrostatic interaction between hydrogel and particle.

In the long-time limit, the mean square displacement (MSD) of the particle becomes proportional to the diffusion parameter

$$\lim_{t \to \infty} \left\langle (r(t) - r(0))^2 \right\rangle = 6Dt$$

The diffusivity $D$ of the particle is obtained by linearly fitting the MSD in the long-time limit, where it approaches a constant value. The diffusion parameter for particle diffusion without any interactions is $D_o$, i.e. the free diffusion coefficient in water, which
follows from the Einstein equation $D_o = \mu_o k_B T$. Most of the simulation results are focused on the relative diffusivity $D/D_o$ to show how much the particle diffusion is inhibited inside the hydrogel. Values of the relative diffusivity lie in the interval $0 \leq D/D_o \leq 1$, where $D/D_o = 0$ corresponds to complete immobilization of the particle and $D/D_o = 1$ to free diffusion.

![Simulation unit cells including 2000 particle-position snap-shot obtained during one simulation run at an attractive interaction potential with strength $U_0=-5k_BT$ (left) and a repulsive interaction potential with strength $U_0=5k_BT$ (right). In both cases, the interaction range is set to $k=0.3b$ and the effective particle diameter is $p=0.1b$. The length $b$ denotes the box size. (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)]

3. 3 Results and Discussion

3.3.1 Probe diffusion in uncharged polymer solutions

To disentangle steric and electrostatic effects, we first investigated the diffusion of a variety of small fluorescent probe molecules and the larger fluorescent protein R-phycoerythrin in uncharged PEG solutions to determine steric contribution. Figure 3.2 shows the scaled translational diffusion coefficients $(D/D_o)$ as determined by FCS for three similar-sized probe molecules (Rh110, Rh6G, and Alexa488) and R-phycoerythrin
as a function of PEG polymer concentration (0–8% w/v). $D_0$ is the translation diffusion coefficient of each molecule in buffered water without polymer as determined by FCS. FCS autocorrelation curves show an increase in the characteristic diffusion times of these probe molecules with increasing PEG concentration. All autocorrelation curves are well described by a single-component fit, allowing for the extraction of $N$ and $\tau_D$ from Equation 1. Translation diffusion coefficients ($D$) can be calculated from the autocorrelation curves using Equation 3. Using the Stokes-Einstein relationship, we obtain hydrodynamic radii of $r_h \sim 0.6$ nm for each of the fluorescent dyes and $r_h \sim 5.6$ nm for the phycoerythrin protein in water, consistent with published data. FCS is also very sensitive to aggregation. No signs of large aggregates or decrease in the number of molecules are observed, suggesting that probes freely diffuse within the polymer networks. Similar results are observed in uncharged dextran solutions, indicating that these probe molecules do not interact (e.g., immobilization, aggregation, or photobleaching) significantly with the sugar backbones of the dextran network.

Several different physical models based on hydrodynamic interactions have been used in recent years to model probe diffusion in uncharged networks. These theories posit that hydrodynamic and steric interactions are the dominant force determining particle transport in the gels. Despite developing from different physical mechanisms, most models derive equations to describe probe diffusion that can be expressed in the form of a stretched exponential. The solid lines in Figure 3.2 depict best fits to the data using $D = D_0 \exp(-\alpha c^n)$, where $D_0$ is the probe diffusion coefficient in pure solvent, $c$ is the polymer concentration, and $\alpha$ and $n$ are constants dependent on the probe-gel system. Typically, $n$ reflects the polymer solvent quality and $\alpha$ is dependent on other relevant system parameters (such as probe radius, molecular mass, etc.) contingent on the specific model. Measured $\alpha$ and $n$ values are listed in Table 3.1 and are in good agreement with previously published data of probe molecule diffusion in poly(vinyl alcohol) solutions. All $n$ values are in the range 0.68–0.88, where $n = 0.75$ has been suggested to correspond to water being a good solvent for the polymer. Values of a range from ~0.2 for the rhodamine and Alexa probe molecules to ~0.5 for the larger phycoerythrin in PEG. Similar values of $\alpha$ and $n$ are obtained in uncharged dextran solutions.
Figure 3.2 Scaled diffusion coefficients of various probe molecules as a function of PEG concentration. Solid lines are fits of the data with the stretched exponential $D = D_0 \exp(-\alpha c^n)$, where $\alpha$ and $n$ are fitting parameters. Values of the parameters (see Table 3.1) are consistent with previously measured values in poly(vinyl alcohol). (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)

Table 3.1 Exponent and prefactor values determined from best fits of $D = D_0 \exp(-\alpha c^n)$ to data.

<table>
<thead>
<tr>
<th>Probe</th>
<th>$\alpha$</th>
<th>$n$</th>
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</thead>
<tbody>
<tr>
<td>R110</td>
<td>0.20</td>
<td>0.80</td>
</tr>
<tr>
<td>R6G</td>
<td>0.17</td>
<td>0.83</td>
</tr>
<tr>
<td>Alexa488</td>
<td>0.26</td>
<td>0.65</td>
</tr>
<tr>
<td>R-Phycoerythrin</td>
<td>0.48</td>
<td>0.88</td>
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3.3.2 Probe diffusion in charged polymer solutions

To examine the role of electrostatic interactions on nanoparticle transport in polymer networks, we next used FCS to determine probe translational diffusion coefficients within charged dextran solutions. Here, we focus on one probe molecule, Alexa488, which has a net charge of -2 at neutral pH. We looked at diffusion of Alexa488 in CM-dextran(-) (molecular mass ~20 kDa) and DEAE-dextran(+) (molecular mass ~500 kDa). We use the notation dextran(-) and dextran(+) from here on to more easily distinguish the network charge in these systems. Dextran(-) has approximately one negative charge per five glucoses, whereas dextran(+) has approximately one amine group per three glucoses. These two dextrans had very different molecular mass, so for comparison, we also examined the probe diffusion in both 20 kDa and 500 kDa uncharged dextran solutions (dextran20 and dextran500, respectively). To ensure that Alexa488 was uniformly distributed throughout the dextran solution, FCS measurements were performed at various spots within the solution and averaged.

Normalized autocorrelation functions of the negatively charged Alexa488 in solutions of dextran(-) and dextran (+) as a function of polymer concentration are shown in figure 3.3, A and B, respectively. Figure 3.3 A reveals that in gels with the same charge as the probe molecule, the characteristic diffusion time, $\tau_D$, of Alexa488 increases only weakly with increasing dextran(-) concentration. Translation diffusion coefficients, $D$, as determined from Equation 3, are plotted in Figure 3.4 for dextran (-) from 0 to 8 wt % dextran. As can be seen, the measured diffusion coefficients in dextran(-) are nearly the same as $D$ observed in uncharged dextran of comparable molecular mass (dextran20). In comparison, Figure 3.3 B shows the normalized correlation functions measured by FCS for the negatively charged Alexa488 probe in dextran(+). Here, we see large shifts in the characteristic diffusion time, $\tau_D$, that are highly dependent on the dextran(+) weight percentage. The largest shift in the characteristic diffusion time, or, equivalently, the largest decrease in the translation diffusion coefficient, $D$, is observed between the no-polymer-network state (or 0 wt % dextran) and 1 wt % dextran(+). Increasing the
dextran(+) concentration further resulted in an observed decrease in \( \tau \) or, equivalently, an increase in the apparent \( D \). This decrease in \( \tau \) is likely due to a higher counterion concentration adding to the ionic strength of the solution in the higher-weight-fraction dextran(+) networks. Figure 3.4 shows the measured translational diffusion coefficients for both dextran(+) and the comparable neutral-molecular-mass dextran (dextran500).

We anticipated that our negative Alexa probe would be slowed in neutral dextrans with increasing weight percent due primarily to steric interactions. With the introduction of electrostatics, we expected repulsive interactions (i.e., negative probe in negative gel) to slow the probe down due to the probe particles avoiding the gel polymers, whereas attractive interactions (negative probe in positively charged gel) would hinder the probe transport the most because the probe would stick to the gel. Instead, as shown in Figure 3.4, Alexa488 diffusion in dextran20 is nearly identical to that in dextran(-), both \( \sim20 \) kDa mol wt polymers, and diffusion in both is comparable to that in dextran500. However, attractive interactions severely reduce probe transport. Comparing dextran500 and dextran(+), we see significant decreases in the Alexa488 transport through the oppositely charged dextran polymer network due to electrostatics alone.
Figure 3.3 (A) Representative plots of normalized FCS autocorrelation curves of Alexa488 NHS ester (net charge -2) in 10 mM MES buffer, pH 6.4, and 1, 3, and 5 wt % dextran(-) solutions. (B) Normalized FCS autocorrelation curves for Alexa488 NHS ester in 10mM MES buffer, pH 6.4, and 1, 3, and 5 wt % dextran(+) solutions. In both A and B, solid lines represent fits to the experimental data by Eq. 1. (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)
Figure 3.4 FCS measured diffusion coefficients of Alexa488 as a function of charged and uncharged dextran concentrations. Negatively charged CM-dextran and positively charged DEAE-dextran solutions were compared directly to neutral dextrans of comparable molecular mass (20 kDa and 500 kDa, respectively). Significant changes were observed only for the negatively charged probe in positively charged dextran solutions. (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)

3.3.3 Salt effects on probe-network interactions

Next, we investigated the effect of added NaCl salt concentration on the electrostatic interactions between the probe and the network. Any electrostatic effects would be expected to have a strong salt dependence due to the Debye screening of the electrostatic interactions. Plotted in Figure 3.5 are the diffusion coefficients, D, for Alexa488 probe molecules in 1 wt % charged and uncharged dextran solutions as a function of added NaCl salt concentration. Diffusion of Alexa488 in 1 wt % dextran500, dextran20 (not shown), and dextran(-) solutions is essentially unaffected by added salt. Electrostatic interactions between probe and polymer solutions are not critical for these systems.
contrast, the measured translational diffusion of negatively charged Alexa488 in dextran(+) was highly sensitive to added salt. With added salt, D is observed to increase greatly, reaching a plateau consistent with that for other dextran solutions, after which D is found to be independent of further added salt. From Figure 3.5, we see that the attractive electrostatic interactions between the negatively charged Alexa probe and the positively charged DEAE-dextran can be effectively screened out at ~125 mM added NaCl concentration. This salt range appears to be a characteristic crossover salt concentration, suggesting that the system is near instability, and may function as a gating switch with ionic strength changes.

**Figure 3.5** FCS measured diffusion coefficient of Alexa488 in 1 wt % neutral and charged dextran solutions as a function of added NaCl salt concentration. Significant salt screening of the hindered diffusion of Alexa488 in dextran(+) is observed above ~125 mM NaCl. (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)
3.3.4 Comparison of simulation and experiments

Since Alexa488 has a net negative charge, the diffusion of Alexa488 particles in positively charged Dextran(+) can be compared to simulated particle diffusion in an attractive potential (i.e. $U_0<0$). Equivalently, the diffusion of Alexa488 in Dextran(-) can be compared to the simulation results with a repulsive potential ($U_0>0$). In order to do this, the interaction range $k$ needs to be converted to an ion concentration $C_{\text{ion}}$ by use of equation (8). Furthermore, we need to gauge the simulation parameters $p$, the particle size, and $b$, the mesh size. There is, to our knowledge, no definitive experimental data on the mesh size of a dextran hydrogel. Hence, we chose the particle diameter to be $p=0.1$ b in relative units and $p=2.3$ nm in absolute units. This value corresponds to the width of an Alexa488 molecule (1.5 nm), plus a water molecule (0.4 nm) and the width of a dextran monomer (0.4 nm) and it leads to an approximated mesh size of $b=23$ nm which is in the same order as reported estimations for the dextran mesh size at concentrations of around 7 to 11 (w/v)$^\%$.

Figure 3.6 shows a comparison between simulation and experimental data. Experimental data from FCS measurements is indicated by unconnected, filled symbols and the simulation data by connected points, where the lines are included to guide the eye. One can see that the simulation data and the experimental data are in good agreement. With an attractive potential of strength $U_0 = -8kbT$, the theoretical model is in quantitative agreement with our experimental results for negatively charged Alexa488 in positively charged Dextran(+). At low ionic strength, the attractive force dominates greatly reducing diffusivity. As ion concentration increases, these attractive interactions are screened and the diffusivity increases up to a “saturation” point at around 100 mM ion concentration, where the diffusivity approaches that of the repulsive interaction data. To draw a similar comparison to the repulsive case of Alexa488 in negative dextran, we note that Dextran(-) has approximately one negative charge per 5 glucoses while the Dextran(+) has one positive amine group per 3 glucoses. This 60 % difference in charge density implies that the interaction potential strength should be taken as smaller for the repulsive case. To approximate this, we set the potential strength in the repulsive case to
$U_0 = +5kT$, which corresponds to about 60% of the potential strength of the attractive case. In Figure 3.6 one can observe that the simulation and experimental data agree for all ion concentrations except towards small ones. Where in the experimental data $D/D_{\text{uncharged}}$ remains constant towards decreasing $C_{\text{ion}}$, the simulation data shows a slight decrease. The discrepancy could be caused by residual attractive hydrophobic or hydrogen-bonding interaction between the probe molecule and the polymer chain, which cancel the repulsive charge interactions, or by the polymer flexibility which is not included in the simulation model.

**Figure 3.6** Comparison between experimental results (unconnected, filled symbols) and simulation (connected points). The diffusivity is shown over the total added ion concentration including the 10 mM due to the MES buffer and another 6 mM and 10 mM to take into account the ionic strength of the mobile ions which enter the solution upon addition of 1 wt% Dextran(-) and Dextran(+), respectively. Data was scaled by $D_{\text{uncharged}}$, the diffusion coefficient measured at 1 wt% in the comparable molecular weight uncharged Dextran solution (D20 or D500). Simulations have been carried out at attractive ($U_0 = -8 \text{kT}$) and repulsive potentials ($U_0 = 5 \text{kT}$) for particles of diameter $p = 0.1 \text{ } b = 2.3 \text{ nm}$. Quantitative agreement between experiment and theory is observed. (This figure is adapted from: Biophysical Journal Volume 108 February 2015 530–539 with the permission from Biophysical Journal.)
3. 4 Conclusion and future perspective

The aim of this work was to examine nanoparticle diffusion in cross-linked hydrogels under consideration of nonsteric interactions between particle and hydrogel both experimentally and theoretically. FCS is a reliable tool for measuring diffusion of probe molecules in polymeric solutions. Transport properties of probe molecules in charged and uncharged gels were examined and compared to a simple theoretical model implementing a rigid, cubic periodic lattice designed to model the hydrogel structure and short range nonsteric interactions via an exponential potential between hydrogel polymer chains and diffusing particles. This model made it possible to perform BD simulations to examine key parameters in our system, including particle size, interaction strength, and interaction range. We found, both experimentally and theoretically, that the filtering capability of a hydrogel is strongly influenced by the sign of the interaction potential. Using a reconstituted model polymer system, we have shown that electrostatic interactions play an important role in governing the transport of charged probe molecules within charged hydrogels in a highly asymmetric fashion. This asymmetry with respect to charge is consistent with naturally occurring systems where the sign of the charge is significant in dictating the successful transport of molecules of interest through biogels.\textsuperscript{41-42} Attractive electrostatic forces between probe and network are much more effective than repulsive forces in hindering particle diffusion. In vivo, these particle-network interactions are likely to be significantly more complicated and are expected to depend on particle size, charge density, local charge distribution, and other factors, as has been suggested previously based on experiments of micron-size particles and charged peptides in reconstituted hydrogels.\textsuperscript{45, 80, 100, 114} Using a simple lattice model to perform BD simulations, we find quantitative near agreement between simulations and the experimentally determined diffusion of probe molecules in model charge networks. Our simulations reveal that the asymmetry between attractive and repulsive electrostatic interactions is due to the efficient sticking of particles to the vertices of the polymer network in the case of an opposite charge between particle and network. These results are also consistent with experiments on natural hydrogel networks, where nature appears to
use this charge asymmetry to filter and control the exchange of molecules between organelles and cells and their environments.\textsuperscript{42, 45, 100}

One limitation of our model is the rigidity of the network compared to the experimental hydrogel systems. Simulations predict a smaller but visible decrease in particle transport for similar charged probe-and-network systems that was not observed experimentally. A possible explanation for this is that a flexible network, unlike a rigid network, would allow the chains to bend away from the diffusing particle, thus reducing the effect on probe diffusion. The inclusion of flexible polymer chains will be addressed in future work, although we note that considerably longer simulation times will be necessary, making it more difficult to span the complete parameter space of such a more complex model. Previous experiments on the ECM, mucins, and the nuclear pore complex have shown significant differences in particle penetration depending on charge, and suggested electrostatic charge may enhance diffusion in biological hydrogels.\textsuperscript{41-42, 45, 100} Our data suggest that electrostatics alone will not enhance diffusion through the network, indicating that charge-charge interactions are only part of the story for interaction filtering processes. To further refine our model, other nonelectrostatic interactions, including hydrodynamic and hydrophobic interactions, need to be included. Tuning these nonsteric interactions to control particle transport inside hydrogels may lead to greater understanding of molecular transport in vivo, as well as to more effective gel-penetrating therapeutics.
Chapter 4 Diffusion of PAMAM dendrimers in neutral and charged hydrogels

4.1 Introduction

4.1.1 Nanocarrier transport in biological gels

Many nanomaterials have been designed for use as carriers to deliver molecular therapeutics to their intended targets. Nanomaterials offer several advantages as therapeutics due to their small size, large surface-to-volume ratio, ease of chemical modification and flexibility in molecular design. Extensive efforts have been made to investigate the properties of nanocarrier-drug complexes to improve their solubility, permeability or targetability.\textsuperscript{115-116} For efficient use as a carrier, nanomaterials capable of reducing the toxicity and improving the solubilities of the drugs they bind are used. Typically excess nanomaterial is used to ensure complete drug encapsulation. Nanomaterial properties can be highly tailored; however, there are complex questions remaining regarding how these nanocarriers interact with biological systems. Surprisingly, only recently was there an appreciation that the nanocarriers themselves may increase possible risks to the patient due to their interactions in vivo. There is a need therefore to understand the interactions between the carrier molecules and biological systems to optimize and guide the carrier design for therapeutic purposes.

One major barrier in gene and drug delivery is that the engineered nanocarriers must traverse through complex crowded environments to reach specific loci in the target cells. The barrier functions of these biological hydrogels, including mucus, extracellular matrix and nuclear pore complex, present a tremendous challenge in achieving optimized effective treatment. Currently it is not fully understood how biomacromolecules in the system impact the mobility and transport of nanocarriers or nanocarriers-drug complexes. The physicochemical properties of nanocarriers and their interactions with biological hydrogels can be critical to determine the in vivo fate of the nanomedicines. Therefore a detailed knowledge of the diffusion behavior is required for engineering the next generation of delivery vehicles.

In this chapter, we focus on a common biocompatible nanocarrier system; polyamidoamine (PAMAM) dendrimers. PAMAM dendrimers are hyperbranched
cationic polymers which have shown great promise as a water soluble, polymeric carrier for a broad range of biomedical applications. Nanoparticle-biogel interactions may include many specific and nonspecific interactions; including electrostatics, chemical binding and hydrophobic/hydrophilic interactions. To gain insight into these complex systems, we will focus here on examining the transport properties within simpler model water-soluble polymer networks. PAMAM dendrimers are at the boundary between traditional polyelectrolytes and charged colloids. The flexibility of the PAMAM polymer chains could influence their transport properties in ways inaccessible to hard colloids or the charged probe molecules studied in Chapter 3. Here we will discuss biophysical studies performed to better understand the importance of electrostatics on PAMAM transport in a reconstituted model hydrogel system that allows us to modulate the range and sign of the charge interactions. FCS experiments are performed to determine translational diffusion coefficients of a fluorescently labeled PAMAM (Alexa488-PAMAM) in neutral and charged polymer solutions. FCS is unique in its ability to measure dynamic processes of small molecules in polymeric systems typically inaccessible by traditional diffusion measurements such as dynamic light scattering (DLS). Since FCS only requires nanomolar concentration, another key advantage is the minimization of particle-particle interactions between dendrimers as well as the intrinsic viscosity of the dendrimer solution.

### 4.1.2 Unique properties of dendrimers

Dendrimers represent a class of macromolecules with unique chain architecture and properties. These unique attributes of dendrimers have made these molecules long considered one of the most promising nanocarriers for different therapeutic categories of bioactives. The term dendrimer is derived from the Greek word *Dendron* which means “tree.” Dendrimers are hyperbranched spherical macromolecules that are radially symmetrical around a core. As shown in Figure 4.1, the structure of dendrimer consists of three major components: (i) a central core, typically a molecule having at least two identical reactive chemical functions, (ii) interior dendritic branches extend outward from the core and are traditionally grown in a step-wise fashion. Every subsequent reaction step produces a new ‘generation’ of dendrimer resulting in an approximate doubling of
the molecular weight as well as a doubling of the surface functional groups. The interior branch structure also includes internal void spaces, or ‘cavities’, which affect the host-guest properties, (iii) an exterior surface populated with functional, terminal surface groups. The surface groups vitally determine the properties of dendrimers as well as the molecular interactions of dendrimers with drugs or biological environments. They are also active sites for further modifications.\textsuperscript{118}

\textbf{Figure 4.1} Schematic representation of a third generation (G3) dendrimer. Dendrimers consist of three major components: a central core, an interior dendritic branch structure (including internal void spaces or cavities), and an exterior surface populated with functional surface groups. Dendrimers are grown in a step-wise manner from the core with each subsequent reaction step producing a new generation of dendrimer. Each successive generation results in a dendrimer roughly twice the molecular weight of the previous generation with twice the number of surface functional groups (S).
Dendrimers were first introduced by two different groups; Buhleier et al.\textsuperscript{119} and Tomalia et al.\textsuperscript{117} in the late 1970s and early 1980s. PAMAM dendrimers are not only one of the first synthesized dendrimers, but also the first commercialized dendrimer and therefore represent the most well-known and studied dendrimer family.\textsuperscript{120} With the precisely controlled architecture and tunable surface groups, dendrimers offer a huge potential to be nanoengineered with favorable bioactivities. Compared to other nanoscale synthetic structures like traditional linear polymers, metallic nanoparticles or carbon nanotubes, dendrimers have unique physical and chemical properties which offer significant advantages as discussed below.

4.1.2.1 Monodispersity

Unlike traditional linear polymers, dendrimers have well-defined molecular structures and nearly monodisperse molecular weights. Their monodisperse nature has been widely verified by mass spectrometry, size exclusion chromatography, gel electrophoresis and transmission electron microscopy.\textsuperscript{121} PAMAM dendrimers, with appropriate synthesis and purification, have remarkably low dispersity for low dendrimer generations (e.g. G0-G5). Even at higher generations, the molecular weight distribution still remains very narrow (i.e. polydispersity index, PDI = 1.05) even though imperfections or "branching defects" occur more frequently due to incomplete reaction products.\textsuperscript{120} Dendrimers therefore offer a controllable and consistent molecular weight parameter not typically possible in other synthetic nanomaterials, which is crucial to the run-to-run reproducibility. The ability to tailor surface functionality through dendrimer generation as well as post-synthesis modification also makes dendrimers highly promising nanocarrier candidates.

4.1.2.2 Nanoscale size and shape

The size of dendrimers ranges from several nanometers to tens of nanometers increasing systematically with the generation number similar to many biomacromolecules. For instance, the diameters of PAMAM are calculated to range from 1.5 nm for G0 PAMAM to 13.5 nm for G10. PAMAM therefore represents a series of well-defined macromolecules that increase in diameter ~ 1 nm for each generation.\textsuperscript{122} Dendrimers’
nanoscale sizes make it possible for them to cross biological barriers like membranes with the reduced risk of being prematurely eliminated by the immune system. The size of dendrimers is also relevant to their 3D shapes. Dendrimers are starburst polymers. Early generations of PAMAM (G0-G3) are flexible and open structures without well-defined interior characteristics. Intermediate PAMAM generations (G4-G7) are thought to be more globular structures with defined interior structure due to geometric closure yet still containing a semi-flexible surface. These PAMAM dendrimers are believed to have accessible interiors to encapsulate drug molecules that are determined by the dendrimer generation number and properties of the surface groups. At higher generations (i.e. >G8), deGennes dense packing is severe and the interior structure is very rigid. With the highly dense packed branches, the interior of high generation dendrimers allows little access except for very small guest molecules.

Based on the systematic size-scaling properties, dendrimers have been used as globular protein mimics. For instance, G3 PAMAM is the same size as insulin (diameter ~ 3.0 nm) and G5 PAMAM is approximately the same size and shape as hemoglobin (Hb) (diameter ~5.5 nm). With the precise control of size, structure and surface chemistry, dendrimers are referred as artificial proteins in a variety of applications including diagnostics, gene delivery, molecular weight calibrators, enzyme mimics and site isolation.

4.1.2.3 Surface functional groups: density, charge and functionalization

Another key feature of dendrimers is the high density of surface functional groups. The number of terminal groups increases exponentially with each generation. Dendrimers may possess positive, neutral or negative charged surface functional groups for different biological applications. For instance, cationic dendrimers such as PAMAM can form complexes with negatively charged nucleic acids (NA) for use as a non-viral gene delivery vector. Positively charged dendrimer-NA complexes, or dendriplexes, have been shown to interact with biological membranes to enhance cellular uptake for the purpose of intracellular delivery while protecting the NA from extra- and intracellular nucleases. Further engineering and modification of the surface charge of the cationic
PAMAM through acetylation has also shown to be effective in tuning the ability of the carrier to deliver NAs as well as reduce cell toxicity.\textsuperscript{116,129} Neutral or negatively charged dendrimers, such as PAMAM with PEGylated or carboxylated surface groups, can be engineered for binding of hydrophobic or cationic drug molecules, respectively, for use as a nanotherapeutic.

The high density of functional surface groups facilitates multiple simultaneous interactions between the dendrimer and its environment. This polyvalency of dendrimers is very important for biomedical applications as the multimeric binding of dendrimers is highly cooperative compared to monovalent systems.\textsuperscript{130-131} The multiple end groups can be precisely tailored and functionalized with various chemistries. The surface engineering strategy provides endless possibilities to design dendrimer as an engineered nanomaterial with favorable properties such as increased binding specificity and affinity.\textsuperscript{120}

**4.1.3 Dendrimers in therapeutic applications, biomedical applications and diagnostic applications**

Dendrimers with their easily controllable features like size, shape, and surface functionality, are considered to be one of most promising polymers in therapeutic, biomedical and diagnostic applications. Dendrimers are prepared with a level of control not attainable with most linear polymers, making them an ideal delivery vehicle candidate for explicit study of the effects of size, charge and composition on biologically relevant properties such as cell uptake, cytotoxicity and biodistribution.

**4.1.3.1 Dendrimers as drugs and as carriers for drug delivery**

Based on the large number of peripheral groups, dendrimers alone have been investigated for their antimicrobial and antiviral properties. Cationic dendrimers and their derivatives are reported to show notable antimicrobial activity.\textsuperscript{132} With the interrelationship between generation number and dendrimer size and number of surface groups, dendrimers offer the possibility to explicitly study the effects of particle size and charge to optimize activity against prokaryotic membranes while minimizing eukaryotic toxicity.\textsuperscript{133} For instance, Cai \textit{et al.} reported that G5 PAMAM showed high antibacterial
activity when exposed to Gram-negative bacteria strains (PA19660 and the clinical strain PA2219). Furthermore, reduced cation charge through partial surface functionalization of the PAMAM with PEG exhibited the same antimicrobial activity while greatly reducing the cytotoxicity of the dendrimer to human epithelial cells.132 Some dendrimers derivatized with peptides or anionic groups have also been shown to inhibit viral binding. One of the most successful examples is a sulfonated polylsine dendrimer called VivaGel that is used against HIV and currently undergoing phase I/II clinical trials.134

Because of their unique physicochemical properties, dendrimers are also well suitable as nanocarriers either encapsulating guest molecules in the interior or covalently binding drugs on the periphery.135 Dendrimers are known to act as unimolecular micelles, encapsulating hydrophobic drug molecules in their interior void space, therefore enhancing the solubility and bioavailability of drug compounds. Many common small molecule drugs are under risk of being filtered out by kidneys. The increase in particle size upon complexation in the dendrimer-drug complex helps the drug to exceed the renal threshold and extend circulation times in the bloodstream.134 Additionally, dendrimers show the potential to perform targeted drug delivery. For instance, dendrimers have been shown effective in the delivery of chemotherapeutic agents to passively targeted tumor cells by taking advantage of ‘the enhanced permeation and retention’ effect; the increasing uptake of large macromolecules in tumor tissue. Dendrimer-drug conjugates also exhibit slower release of the therapeutic, producing higher accumulation in solid tumors, and reduced systemic toxicity compared to the free anticancer drug.136 For instance, N. Malik et al. conjugated G3.5 PAMAM dendrimer to the classic anticancer drug cisplatin. The resulting dendrimer-platinate complexes exhibited slower release of the drug, higher accumulation in solid tumors and 3~5 fold less toxicity compared to bare cisplatin.137

4.1.3.2 Dendrimers as gene delivery vectors

Dendrimer-based gene delivery is under active investigation in nonviral gene therapy approaches. Cationic dendrimers function as potential synthetic vectors that interact with plasmid DNA, oligonucleotides, or siRNA, to form complexes that protect genetic materials from degradation and improve transfection efficiency into the nucleus.130, 138
The formation of nucleic acid (NA)-dendrimer complexes, or dendriplexes, is based on the ability of cationic dendrimers to condense the negatively charged nucleic acid (NA) phosphates. Dendriplexes are subsequently taken up by cells by endocytosis and transferred from cytoplasm to nucleus followed by NA release resulting in transcription processes and target protein translation. Cationic dendrimers, particularly amine terminated dendrimers such as PAMAM or polypropylenimine (PPI), or polylysine dendrimers, have been reported to enhance the transfection efficiency both in vitro and in vivo. Generally, high generation dendrimers with sufficiently high nitrogen to phosphate (N/P) charge ratios correlate with better cellular uptake. For PAMAM, efficient transfection is generally limited to generations > 4. Like most polyamines, the high surface charge of the dendrimers does result in a charge- and concentration-dependent cytotoxicity profile. Surface modifications, such as grafting polyethylene glycol (PEG) groups or acetylation, which result in a partial reduction in the PAMAM surface charge have been shown to reduce the cytotoxicity while maintaining transfection efficiency. For instance, D. Luo et al showed that G5 PAMAM modified with 3400 Da PEG had low toxicity yet was able to produce a 20-fold increase in transfection efficiency compared with partially degraded dendrimer controls.

4.1.3.3 Dendrimers as diagnostic agents

Through linking to various ligands, dendrimers are also promising candidates for diagnostic applications such as molecular detection, radiotherapy and as imaging agents. For example, dendrimers have been used as contrast agents in magnetic resonance imaging (MRI) - a standard method of imaging associated with cancer diagnoses. Gadolinium chelates like Gd(III)-diethylenetriaminepentaacetic acid(Gd-DTPA) are currently the most widely applied contrast agents for clinical MRI since they have a different relaxation time in diseased and normal tissue. The biggest shortcoming of these agents is that they are excreted from the body very rapidly because of their low molecular weight. To improve upon this drawback, macromolecules like poly(amino acids), polysaccharides, and proteins have been conjugated to Gd(III) to enhance the circulation time and improve the image contrast. The limitation of many of these macromolecular agents however is their potential toxicity from the accumulation of
released Gd(III) ions in the body. Hence, dendrimers, with the appropriate, well-defined, and controllable structure, are ideal macromolecules to form complex with Gd. Dendrimer-based Gd(III) chelates have been reported to have strongly increased relaxivity and circulation time.\textsuperscript{144} Moreover, the undesired retention time and potential risk of Gd release can be reduced by modification of surface functional groups through dendrimer PEGylation.\textsuperscript{145}

### 4.1.4 Challenges and our research motivation

To date, dendrimers have achieved significant success and shown great promise in a variety of pharmaceutical and biomedical applications. Dendrimers offer a very high degree of molecular control and represent ideal carriers for structure-activity studies to determine the explicit effect of polymer size, charge and composition on biologically relevant properties. Most uses require that these dendrimers, or their dendrimer-therapeutic complexes, traverse complex biological hydrogels such as the ECM to reach their cell targets. Despite extensive studies of the physicochemical properties of dendrimers, little is currently known about the structure and dynamics of dendrimers inside biological gels.\textsuperscript{146} Many important questions remain: How do the dendrimers transport themselves or their cargos in biological fluids? How do the dendrimer-gel interactions influence their diffusion behavior or the permeability of biological gels? To begin to address these questions, we used FCS experiments to determine the translational diffusion coefficient of PAMAM dendrimers in uncharged and charged polymer solutions. Such mechanistic and systematic studies to determine the effects of particle-network interactions on dendrimer dynamics \textit{in vivo} may lead to a greater understanding of how to tune these nonsteric interactions to optimize molecular transport and molecular release of dendrimer-based medicines and produce more effective gel-penetrating therapeutics.

### 4.2 Methods and materials

#### 4.2.1 PAMAM characterization and labeling

Polyamidoamine (PAMAM) dendrimers (Generation 5, ethylenediamine core, amine-terminated and 5 wt\% solution in methanol) were obtained from Sigma Aldrich (St Louis,
MO). Before use, methanol was removed under reduced pressure at room temperature using a Labconco Centrivap Centrifugal Vacuum Concentrator. G5-PAMAM were subsequently dissolved in deionized water and buffered with 10 mM MES buffer and titrated with acid or base to the desired final pH of 6.4. This pH is sufficient to maintain the full protonation of the PAMAM surface functional groups.

For FCS experiments, PAMAM was labeled with amine reactive Alexa Fluor®488 Succinimidyl Ester purchased from Invitrogen. To minimize the effect of the dye molecule on the PAMAM dynamic behavior the reactants mole ratios were controlled to result in a PAMAM:Dye ratio of ~1:1. Following the protocol, the labeling chemical reaction was carried out in sodium bicarbonate buffer solution (0.2M pH=7.5). Labeled G5 PAMAM was purified by disposable PD-10 Desalting Columns (GE Health care Life Sciences) to remove the free dye and buffered subsequently in MES. Slightly higher labeling ratios were examined (PAMAM: Dye of ~1:2 and 1:4) with no significant effect on the FCS measured translational diffusion coefficients and hydrodynamic radius.

4.2.2 Preparation of polymer solutions

To study PAMAM diffusion in polymer gels, charged and uncharged dextran polymer solutions were used. Dextran (MW = 15-25 kDa), Dextran (MW = ~500 kDa), Carboxymethyl-dextran (CM-dextran(-))(MW = 15-20 kDa) and diethylaminoethyl-dextran (DEAE-dextran(+))(MW=~500 kDa) were all purchased from Sigma Aldrich. To remove salts and contaminants, all polymer solutions were extensively dialyzed against Milli-Q water and then lyophilized before use. Purified polymer solutions were then redissolved in MES buffer (10 mM, pH 6.4) to make the desired percent w/v dextran solutions and stirred overnight. Fluorescently labeled dendrimer was added into dextran solutions and the final concentration was retained to be ~10 nM. All the samples were incubated at room temperature for more than 12 hours to ensure the fluorescent molecules had enough time to disperse uniformly throughout the sample. 500 µL sample from each individual solution was loaded into 8 well NUNC sample holders and measured by FCS at room temperature.
4.2.3 FCS setup and data analysis

FCS experiments were made using an ISS Alba confocal fluorescence fluctuation system (Urbana, IL) coupled to a Nikon Ti-U microscope equipped with a 60×1.2 NA 1.2 water immersion objective lens. The illumination source was a continuous-wave 488 nm laser diode. The emission signal for the 488 nm was passed through a 514 nm long-pass edge filter before detection. Emission signal was recorded by two separate photomultiplier tubes (PMTs). All FCS measurements were performed in NUNC LabTek 8-well microscopy chambers (Nalge Nunc) with a final volume of ~500 µL. Determination of the focal volume was established via calibration against an aqueous solution of rhodamine 110 \( (D = 440 \mu m^2 s^{-1}) \). FCS results are an average of at least 20 measurements, taken at various positions within the polymer solutions to ensure homogeneity, with typical sampling times of 30 s. All results are expressed as the mean ± SD of the measured diffusion coefficient. FCS measurements were analyzed using VistaVision 4.0 software. 3D-Gaussian one-component and two-component fitting models, as described in chapter 2, were used for data analysis.

As the basic principles of FCS have been described in detail in chapter 2, we give only a brief overview here. FCS measures the fluorescence fluctuations emitted from labeled molecules moving in and out of a small confocal volume (~1 fL). The size of the effective illumination volume is fixed by the confocal detection optics and the excitation profile of the focused laser beam and characterized by measurements against a standard of a known diffusion constant. For ideal particles of uniform size diffusing by Brownian motion, dynamic information can be determined from the intensity fluctuations by means of a time autocorrelation given by

\[
G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{1}{\frac{r_0}{z_0} \tau_D} \right)^{1/2}.
\]

Here, \( N \) is the average number of particles in the illumination volume and the structure parameters \( r_0 \) and \( z_0 \) are the axial and radial dimensions of the excitation beam determined by calibration measurements against a known standard.

When there are two species in the system but no interaction between them, the
diffusion times for each of the species are independent. Considering the contribution of particles for each component the correlation function leads to\textsuperscript{60,62}

\begin{equation}
G(\tau) = \frac{N_1}{(N_1+N_2)^2} \left(1 + \frac{\tau}{\tau_{D1}}\right)^{-1} \left(1 + \frac{\tau}{\omega^2 \tau_{D1}}\right)^{-\frac{1}{2}} + \frac{N_2}{(N_1+N_2)^2} \left(1 + \frac{\tau}{\tau_{D2}}\right)^{-1} \left(1 + \frac{\tau}{\omega^2 \tau_{D2}}\right)^{-\frac{1}{2}}
\end{equation}

(2)

which \(N_1\) and \(N_2\) are average numbers of particles of each component. \(\tau_{D1}\) and \(\tau_{D2}\) are the independent diffusion times of two components crossing the illumination volume described by its structure parameter \(\omega\) (\(\omega = r_0^2/\zeta_0^2\)).

The autocorrelation can then be normalized by

\begin{equation}
G_{Norm}(\tau) = \frac{G(\tau)}{G(0)}
\end{equation}

(3)

The diffusion time, \(\tau_D\), is related to the translational diffusion coefficient \(D\) by the simple relationship

\begin{equation}
\tau_D = \frac{r_0^2}{x_D}
\end{equation}

(4)

where \(D\) denotes the translational diffusion coefficient of the molecules in solution and is calculated from the lateral dimensions of the focused incident beam and the experimentally determined \(\tau_D\). For spherical particles, the diffusion coefficient \(D\) follows from the hydrodynamic radius, \(r_h\), in solution and can be calculated by the Stokes-Einstein relation \(D = k_B T/6 \pi \eta r_h\), where \(k_B\) is the Boltzman constant, \(T\) is the temperature, and \(\eta\) is the viscosity of the medium.

### 4.2.4 DLS and zeta potential measurement

PAMAM was dissolved into 10 mM MES buffer to a final concentration of 1 mg/mL for hydrodynamic measurements by dynamic light scattering (DLS). DLS measurements to determine the hydrodynamic radius of the dendrimers were performed with a
NanoBrook 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation) at 25°C with a fixed scattering angle of 90°. Zeta Potential was measured in MilliQ water with a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation) at room temperature. Each data point was an average of at least 4 runs and the results are expressed as mean ± SD.

4.3 Results and discussion

4.3.1 Dendrimer labeling and purification

To make the PAMAM molecules suitable for FCS measurements, we chose Alexa Fluor 488 succinimidyl ester as a label to react with the primary amine groups on the surface of PAMAM. It is important to examine if the dendrimer molecules have been successfully labeled and sufficiently purified as well as ensuring no undesired side reactions, such as aggregation, occurred during the labeling reaction; both measurements ideally suited for FCS investigation.

Labeled PAMAM was separated from free dye by gel chromatography using a double PD-10 column. Fractions of 200 µL were collected and examined by FCS. There were two detectable fractions: free dye (f1) and labeled PAMAM (f2) with their respective diffusion coefficients (Df1 and Df2). Listed in Table 4.1 are the experimentally determined diffusion coefficients and hydrodynamic radii for the various fractions. All fractions were fit with either a one-component or two-component fit resulting in a best fit for the measured autocorrelation function. rh represents hydrodynamic radius calculated from the Stokes-Einstein relation. From Table 4.1, it is clear that the first two fractions from size exclusion separation are purified, Alexa labeled PAMAM. The autocorrelation curves are best described with a one-component fit resulting in diffusion coefficients and rh values in good agreement with the theoretical G5 PAMAM radius (2.7 nm). No evidence of significant unreacted free dye is observed. Subsequent separation fractions are only described by a two-component fit with a mixture of a fast component (unreacted free dye) and a slow component (labeled PAMAM). The final separation fractions show only unreacted free dye molecules and again have measured autocorrelation functions best described by a one-component model (equation 1). Representative FCS autocorrelation
curves (symbols) with their corresponding fits (solid lines) are shown in Figure 4.2. The labeled PAMAM has a much longer diffusion time indicating its distinct size difference from free dye. The fraction 7 sample is identical to the standard Alexa488 solution measured independently. The fraction 1 sample is well described by a one component fit suggesting that labeled PAMAM was successfully purified from excess free dye molecules. No significant aggregation is observed by FCS.

**Table 4.1** Translational diffusion coefficients ($D$) and calculated hydrodynamic radii ($r_h$) of each fraction after PD-10 column purification. $D_{f1}$ represents diffusion coefficient for free dye. $D_{f2}$ represents the diffusion coefficient for labeled PAMAM. For fraction 1,2,5,6 and 7 the diffusion coefficients were determined by using one-component model as equation 1. For fraction 3 and 4, the $D_{f1}$ and $D_{f2}$ were determined by using two-component fitting model as equation 2.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$D_{f1}$ (µm$^2$/s)</th>
<th>$r_h$(nm)</th>
<th>$D_{f2}$ (µm$^2$/s)</th>
<th>$r_h$(nm)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>91.8</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
<td>78.6</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>267.6</td>
<td>0.8</td>
<td>85.9</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>240.9</td>
<td>0.9</td>
<td>87.6</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>280.5</td>
<td>0.8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>286.0</td>
<td>0.8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>294.5</td>
<td>0.8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
To minimize the effect of the dye labeling on the dynamic properties of the PAMAM molecules and its interactions with biological gels, we needed to carefully control the labeling ratio. G5 PAMAM has 128 primary amine groups on the surface which offer 128 active sites for labeling reaction. By tuning the molar ratios of PAMAM:dye, we were able to approximately control the amount of labeling per PAMAM. PAMAM:Alexa488 ratios of 1:1, 1:2 and 1:4 were synthesized. Only slight changes in the PAMAM diffusion time, and thus hydrodynamic radii, were observed by FCS with increasing ratio of dye molecules. This is shown from the normalized autocorrelation curves in Figure 4.3. However, all were clearly distinguishable from free dye showing that all reactions did result in labeled PAMAM. The measured diffusion coefficient and calculated hydrodynamic radii are listed in Table 4.2. Reactions resulting in approximately one dye per PAMAM had the least increase on the measured PAMAM size compared to the

Figure 4.2 Normalized FCS autocorrelation curves for Alexa488 solution (open square), fraction 1 (open triangle) and fraction 7 (open circle) from Alexa488 labeled PAMAM after purification on a PD-10 column. Solid lines represent fits to the experimental data by equation 1.
theoretical radius. With more dye attached, the measured size of PAMAM gets slightly larger. To achieve minimal interference on PAMAM surface, the 1:1 labeling reaction samples were used for all the following experiments.

![Normalized FCS autocorrelation curves for standard Alexa488 solution and G5 PAMAM with different labeling ratios. Solid lines represent fits to the experimental data by one-component model as equation 1.](image)

**Figure 4.3** Normalized FCS autocorrelation curves for standard Alexa488 solution and G5 PAMAM with different labeling ratios. Solid lines represent fits to the experimental data by one-component model as equation 1.

**Table 4.2** Diffusion coefficients ($D$) and calculated hydrodynamic radius ($r_h$) of alexa488 labeled G5 PAMAM with different labeling ratios.

<table>
<thead>
<tr>
<th>Labeling ratio</th>
<th>$D$ ($\mu m^2/s$)</th>
<th>$r_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>89.2±2.9</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>1:2</td>
<td>81.5±4.4</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>1:4</td>
<td>75.3±3.9</td>
<td>3.0±0.1</td>
</tr>
</tbody>
</table>
4.3.2 PAMAM diffusion in uncharged polymer solutions

To study the steric effects on dendrimer diffusion in hydrogels, we first investigated the diffusion of G5 PAMAM in uncharged polymer. The polymer networks were maintained in 10 mM MES (pH 6.4) to ensure complete protonation of all of the G5 PAMAM primary amines on the dendrimer surface. Figure 4.4 shows the scaled translational diffusion coefficients ($D/D_0$) as determined by FCS as a function of three different uncharged polymer solution concentration. $D_0$ is the translation diffusion coefficient of G5 PAMAM in MES buffer without the polymer. As expected, the diffusion coefficients ($D$) decrease with increasing polymer concentration due to steric effects. Diffusion is hindered the most in the highest molecular weight, and thus most viscous, polymer solution (500 kDa dextran). PAMAM diffusion in the similar ~20 kDa PEG and dextran solutions are nearly identical.

Several different physical models based on hydrodynamic and steric interactions have been used to model particle diffusion in uncharged networks. While the different models start with different physical phenomena, most models derive equations that describe nanoparticle diffusion in the form of a stretched exponential. Solid lines in Figure 4.4 depict best fits to the data using $D=D_0 \exp(-\alpha c^n)$ where $D_0$ is the particle diffusion in pure solvent, $c$ is the polymer concentration and $\alpha$ and $n$ are constants dependent on the particle-gel system. As discussed in chapter 3, $n$ is thought to reflect the polymer solvent quality while $\alpha$ is dependent on system parameters such as particle size and molar mass. Fitting parameters $\alpha$ and $n$ are listed in Table 4.3. All the $n$ values are in a narrow range close to $n=0.75$ which corresponds to good polymer solvent quality in water. $\alpha$ values for PAMAM in 20 kDa PEG and dextran are very similar but significantly increases in value for PAMAM in 500 kDa dextran solution suggesting that $\alpha$ has a dependence on the polymer molecular weight in agreement with the Phillis’s model.\textsuperscript{93} Compared to the results from probe diffusion project (Table 2.1), the $\alpha$ and $n$ values for PAMAM diffusion are more similar to the values for larger macromolecules like phycoerythrin rather than small probe dye molecules. This simply suggests that these large macromolecules experience different local dynamics from small probe molecules as we would anticipate for particles that have sizes closer in size to the gel correlation lengths.
Figure 4.4 Scaled diffusion coefficients of G5 PAMAM as a function of the concentration of different molecular weight non-charged polymer solutions (PEG 20 kDa, dextran 20 kDa and dextran 500 kDa). Solid lines are fits of the data with the stretched exponential $D = D_0 \exp(-\alpha c^n)$, where $\alpha$ and $n$ are fitting parameters.

<table>
<thead>
<tr>
<th>Gel solution</th>
<th>$\alpha$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 20kDa</td>
<td>0.33</td>
<td>0.81</td>
</tr>
<tr>
<td>Dextran 20kDa</td>
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<td>0.84</td>
</tr>
<tr>
<td>Dextran 500kDa</td>
<td>0.56</td>
<td>0.72</td>
</tr>
</tbody>
</table>
4.3.3 PAMAM diffusion in charged polymer solutions

To investigate the electrostatic interaction on PAMAM transport in polymer networks, we next measured the translational diffusion coefficients of G5 PAMAM within charged polymer solutions. G5 PAMAM has 128 primary amine groups (pKa~9.2) on the surface. In MES buffer (pH 6.4), we would therefore anticipate each dendrimer molecule to carry a net +128 charged sites. Here, the charged polymer solutions are dextran(+) (DEAE-dextran) and dextran(-) (CM-dextran) as discussed in chapter 3. The charged dextran solutions have significant molecular weight differences and are thus compared to PAMAM diffusion in similar MW uncharged dextran.

Normalized autocorrelation curves of the cationic Alexa488-PAMAM in the cationic DEAE-dextran are shown in Figure 4.5 as function of the polymer concentration. Comparable with probe molecules studied in chapter 3, Figure 4.5 reveals that in gels with the same charge as the PAMAM molecules, the characteristic diffusion time (τ) of the PAMAM increases significantly compared to MES buffer. This shift in the diffusion time reveals that in gels of the same charge as the dendrimer, the mobility of the PAMAM is significantly reduced. τ shows only weak dependence on increasing dextran(+) concentration. Translational diffusion coefficients, D, as determined by equation 4, are shown in Figure 4.6 for G5 PAMAM in 1-8 w/v% Dextran (+) solution. Compared with values of PAMAM diffusing in uncharged dextran of comparable molecular weight (500 kDa Dextran), the measured diffusion coefficients are greatly reduced due to electrostatic repulsions rather than steric effects. Based on our previous Brownian dynamic simulations of probe molecules in like-charged gels, we hypothesize that the highly positively charged PAMAM molecules in this repulsive regime would undergo a “trapping” effect where the like charges of the polymer network changes confine the PAMAM particles within smaller regions inside the hydrogel. The repulsive forces form a “cage effect” limiting the space available for the PAMAM molecules to access thus slowing down their diffusion within the biogels.
Figure 4.5 Normalized autocorrelation curves of G5 PAMAM in 10mM MES buffer, 1%, 4% and 8% DEAE-Dextran(+) solutions. Solid lines represent fits to the experimental data by one-component model as equation 1.

Figure 4.6 Diffusion coefficients determined by FCS of G5 PAMAM as a function of DEAE-Dextran(+) solution concentration.
Figure 4.7 shows the normalized autocorrelation curves of the cationic PAMAM in buffer, 1%, 4% and 8% dextran(-) solutions; an attractive electrostatic regime. Here, we anticipated the PAMAM molecules to “stick” to the polymer network leading to a stronger immobilization of the dendrimer molecule and ultimately a significantly greater reduction in the particle dynamics within the gels. Instead, however, we observe that the characteristic diffusion time ($t$) of the PAMAM decreases even compared to MES buffer for all Dextran(-) concentrations. Figure 4.8 shows the diffusion coefficients of G5 PAMAM as a function of Dextran(-) solutions. Surprisingly PAMAM does not appear to have hindered diffusion due to sticking like the smaller, hard sphere, probe molecules but rather the apparent diffusivity of PAMAM is significantly enhanced in this oppositely charged system.

![Normalized autocorrelation curves of G5 PAMAM in MES buffer, 1%, 4% and 8% CM-Dextran(-). Solid lines represent fits to the experimental data by one-component model as equation 1.](image-url)

**Figure 4.7** Normalized autocorrelation curves of G5 PAMAM in MES buffer, 1%, 4% and 8% CM-Dextran(-). Solid lines represent fits to the experimental data by one-component model as equation 1.
Together, these results suggest the electrostatic interactions between PAMAM molecules and polymer matrix clearly impact the dynamic behavior of PAMAM. Moreover, PAMAM showed completely opposite behaviors in positively and negatively charged hydrogels. To further understand these significant differences we next will investigate the effect of added salt on the PAMAM diffusion.

4.3.4 Salt effect on PAMAM-hydrogel interactions

Any electrostatic effects between diffusing particle and network would be expected to be strongly dependent on salt due to the Debye screening of the electrostatic interactions. It is therefore important to know how the ionic strength affects the transport properties of PAMAM in these charged gel networks. In Figure 4.9, the diffusion coefficient \(D\) of G5 PAMAM in 1 wt % dextran(+) is plotted as a function of added NaCl concentration. As
shown in Figure 4.5, 1 wt% dextran(+) was sufficient to greatly reduced the translation diffusion coefficient compared to MES buffer alone. Here, we see the measured translational diffusion coefficient of the cationic PAMAM is highly sensitive to added salt. It increases greatly, reaching a plateau at about 75 mM NaCl in solution, after which $D$ is seen to be independent of further added salt. This diffusion coefficient at the plateau is approximately the same as the measured $D$ of PAMAM in a similar molecular weight uncharged dextran solution. This suggests the hindered diffusion of cationic PAMAM in dextran(+) is due to electrostatic repulsion within the network. As expected, this “caging” effect is eliminated with the addition of added salt up to a characteristic crossover salt concentration as the repulsive electrostatics between PAMAM and network are screened out.

![Figure 4.9](image)

**Figure 4.9** Diffusion coefficients of G5 PAMAM in 1% DEAE-Dextran solution as a function of added NaCl concentration.

In the case of attractive electrostatics, here where the cationic PAMAM is diffusing in dextran(-), Figure 4.10 shows the diffusion coefficient of cationic PAMAM in 1 wt %
dextran(-) solution as a function of salt concentration. As shown in Figure 4.7, 1 wt % dextran(-) results in a significant increase in the translation diffusion coefficient of PAMAM faster even than diffusion in buffer alone. Here, we see with added salt the measured diffusion coefficient $D$ is unaffected by adding salt. This suggests the mobility of PAMAM in an electrostatically attractive network is independent of charge. It is noted that the measured diffusion coefficients of PAMAM, in both positively and negatively charged networks with high salt concentration, are greater than it in MES buffer solution consistent with steric effects due to the presence of the 1% polymer solution.

![Figure 4.10](image)

**Figure 4.10** Diffusion coefficients of cationic G5 PAMAM in 1% CM-Dextran(-) solution as a function of added NaCl concentration.

To further parse the effect of salt on PAMAM diffusion we measured salt effects on PAMAM dynamics in uncharged polymer as well as buffer. Figure 4.11 shows the measured diffusion coefficients (squares) of G5 PAMAM in 10 mM MES buffer (pH 6.4)
as a function of added salt concentration. The corresponding hydrodynamic radius (circles) are also plotted in Figure 4.11 as calculated from Stokes-Einstein equation. Here we see both $D$ and $R_h$ plateau at a critical salt concentration of $\sim$30-40 nM added salt. The reduction in the apparent hydrodynamic radius suggests a conformation change of the PAMAM in the presence of salt to a much more compacted form. Such compaction of the PAMAM has been predicted previously from MD simulations due to the ability of the PAMAM chains to fold into the internal cavities of the dendritic structure.\textsuperscript{148-150}

![Figure 4.11](image)

**Figure 4.11** The measured translational diffusion coefficient, $D$, of G5 PAMAM in 10 mM MES buffer as a function of added NaCl concentration. $R_h$ is the corresponding hydrodynamic radius calculated by using Stokes-Einstein equation.

We examined PAMAM diffusion in uncharged dextran solutions comparable to the charged dextran molecular weights (20 kDa and 500 kDa respectively). Figure 4.12 shows the dependence of $D$ for G5 PAMAM in neutral dextran as a function of added salt. Again the translational diffusion coefficient measured by FCS increases with added salt.
reaching a plateau at about ~60-70 mM added NaCl similar to the crossover salt concentration observed in charged dextrans. This result confirms the increase in $D$ is independent of any steric effects though the maximal $D$ reached depends on the molecular weight of the Dextran.

![Figure 4.12](image)

**Figure 4.12** The diffusion coefficients of PAMAM in 20 kDa and 500 kDa uncharged dextran solutions as a function of added NaCl concentration.

To characterize the potential property change of PAMAM with addition of salt, we applied zeta potential analysis to examine the surface charge of PAMAM molecules. Measured zeta potential ($\zeta$) plotted as a function of added salt concentration is shown in Figure 4.13. G5 PAMAM in MES buffer solution (10 mM, pH=6.4), gives high positive zeta potential values (>25 mV) indicating that PAMAM molecules carry significant positive charge on the surface and remain stable in the solution. The measured zeta potential decreases significantly with added NaCl reaching ~ 0 by 60-70 mM NaCl concentration. This result suggests the surface charge of PAMAM is effectively screened,
or partially screened, by chloride ions. When the salt concentration reaches to ~60mM, the surface net charge is approximately zero.

**Figure 4.13** Zeta potential of G5 PAMAM in 10 mM MES buffer solution (pH 6.4) with different sodium chloride concentrations.

In MES buffer (pH=6.4), the primary amine groups on the surface of PAMAM are all protonated and repel each other to form more like an open and spherical structure. This repulsive force can be screened out by adding salt into the system and makes the branches on the dendrimer more flexible and results in a compaction of the dendrimer to a significantly smaller hydrodynamic radius compared to the normal charged PAMAM. Thus, the chain flexibility of the PAMAM gives rise to new behavior not observed in hard sphere particles. PAMAM in polymer networks of the same charge can get caged by electrostatic repulsions between the chain and polymer and show a slowing down of their diffusion coefficients. This electrostatic effect can be screened out with salt. PAMAM in an attractive electrostatic network shows a great increase in the PAMAM
diffusion coefficient. This increase in $D$ is independent of added salt. PAMAM in uncharged networks and in buffer also show an increase in $D$ with added salt suggesting a conformational collapse. The final $D$ measured by FCS for PAMAM in uncharged polymer networks is very similar to the $D$ measured PAMAM/Dextran(-) measurements. Combined these results suggest that PAMAM size is highly dependent on salt concentration. In addition, these results suggest the increase in $D$ observed for PAMAM in an oppositely charged polymer network arises due to the network itself acting as a large counterion to the PAMAM molecule resulting in a situation equivalent to PAMAM in salt.

4.4 Conclusions and future perspective

The purpose of this study was to investigate the role of electrostatic interactions in the transport of a highly charged and flexible PAMAM macromolecule in hydrogels. The diffusion coefficients of G5 PAMAM in both uncharged and charged polymer networks were measured by FCS and the diffusion behaviors are distinct. PAMAM diffusion in neutral gel is hindered due to steric. PAMAM transport in like-charged polymer networks results in a much more hindered diffusion of the particles. This diffusion hindrance was screened out with the addition of salt suggesting it is electrostatic in nature. Most likely this scenario is a similar “caging” effect observed in Brownian dynamic simulations of probe molecules in like-charge polymer networks. For PAMAM in gels of opposite charge, an attractive electrostatic situation, a new behavior is observed. The diffusion coefficient of the PAMAM increases significantly and is faster than the diffusion of the PAMAM in water. This increased diffusion is independent of added salt concentration. Studies of salt effects on PAMAM diffusion in buffer and in neutral gels also show an increased diffusion coefficient with added salt. This suggests that with added salt, the repulsions of the surface functional groups are screened and the PAMAM collapses to a more compacted conformation with a significantly reduced hydrodynamic radius. Several recent theoretical studies suggest the conformation of PAMAM is rigid and cannot be modulated by electrolytes with low salt concentration or changes in pH. These studies are consistent with recent small-angle neutron scattering (SANS) measurements, performed at relatively high PAMAM concentrations, that showed little
change in PAMAM size with pH. However, other recent experimental studies have shown the swelling and deswelling of PAMAM, especially of intermediate generation number, at different pH consistent with early theoretical studies. These studies argue that PAMAM is flexible with the deprotonation of surface groups able to cause the surface branches to back-fold resulting in a more compacted state with a smaller hydrodynamic radius and greater diffusivity.\textsuperscript{154-158} Our FCS results of PAMAM in buffer and in neutral polymers, at very low dendrimer concentrations not typically accessible experimentally, also suggest a significant compaction of the PAMAM with added salt up to a critical salt concentration where no further compaction is observed. We note that the increased diffusivity of the PAMAM in oppositely charged polymer networks results ultimately in a diffusion coefficient nearly identical to PAMAM diffusing in a comparable molecular neutral dextran polymer solution. We hypothesize that the oppositely charged polymer network may therefore be acting as a large counterion resulting in a similar PAMAM compaction within the hydrogel. Taken together, our results reveal the essential role of electrostatic interaction in nanoparticle transport through biological hydrogels especially for highly charged and flexible polyelectrolytes. The potential of dendrimers as drug/gene delivery vectors suggest this behavior is essential to understand how best to optimize the therapeutic benefits of dendrimers as nanocarriers especially inside biogels.

As the structural compaction is believed to be dependent on the PAMAM generation number, future studies should include looking at the generation dependence on the salt-induced compaction of PAMAM. Low generation PAMAM would have short branches with open, ill-defined internal structure and would not be expected to compact greatly. High generation PAMAM would also not be expected to be able to compact significantly due to the highly rigid dense packed structures. Maximal compaction would be anticipated for the intermediate generations (like G5) used in this study. In addition, we used only one type of PAMAM functional surface chemistry; an amino group. Comparison of PAMAM behavior modified with COOH groups (- charge) or PEG (neutral) would further elucidate the details of the electrostatic interactions in controlling PAMAM-biogel interactions. Our current results, suggest we should see the same results for the negative charged PAMAM in either attractive or repulsive networks. We would anticipate only steric effects for neutral PEGylated PAMAM. As discussed in the
introduction, the high level of molecular control of dendrimers make them ideal candidates for nanomedicines including drug and gene delivery vehicles. Additional future studies could examine transport of PAMAM-gene or PAMAM-drug complexes to determine the role of electrostatics on complex penetration as well as therapeutic release within charged polymer networks. Such experiments would help further elucidate the mechanism and kinetics of the release within dendrimer nanocarriers and what role the biogel plays on this release.
Chapter 5 Corona formation and binding kinetics of albumin and transferrin to polystyrene nanoparticles

5.1 Introduction

It is increasingly accepted that with the rapid growth of nanotechnologies, situations where engineered nanomaterials (ENMs) will interact with biological systems will continue to rise. Interactions between nanomaterials and living matter play an essential role in governing their potential efficiencies and toxicities. Hence, understanding the molecular interactions of ENMs with living matter is emerging as a key objective for their safe and efficient biomedical application. In the biological milieus, nanoparticles typically are modified by adsorption of biomolecules, commonly proteins, due to their large surface areas. This protein layer or ‘protein corona’ ultimately defines the biological identity of the nanoparticle surface. In general, the nature of ENM surfaces (material character) dictates the formation of the corona – or, in a biological context, “what the cell sees” during, e.g., drug delivery. Thus we predict the corona therefore governs the biological response of the body to the ENM and thus also ultimately determines the biodistribution and in vivo fate of the nanoparticles.159-163

In the body, the formation of the protein corona is a complex, dynamic process and depends on many parameters. There is no one “universal” plasma protein corona for all nanomaterials. The amount and presentation of the proteins on the surface of the nanoparticles is not correlated simply to the protein relative abundances in the biological fluid and appears to be unique to each type of ENM and the physiological conditions.164 Factors that control the structure and composition of the corona include (i) the physicochemical properties of the nanoparticle such as size, shape, terminal groups and surface charges, (ii) the exposure time of nanoparticles in biological fluids and (iii) nature of the physiological environment.160, 164 Protein-nanoparticle interactions govern the formation of the protein corona that then endows the nanoparticles with new biological identities that differ greatly in surface composition from their engineered surface in the pristine state.161, 165 It is increasingly believed that the identity of ENMs, and their in vivo fate and effects, are ultimately determined by the biomolecular corona formed as they are exposed to complex media. Controlled transport distribution of ENMs through biological
fluids and in cells is crucial to many potential applications. There is a critical need therefore for a quantitative knowledge of the creation of protein coronas on nanoparticles as well as the dynamic and kinetics of the corona formation process.

5.1.1 Hard and soft protein corona

Due to their high free energy, ENM surfaces in contact with biological media are rapidly covered by biomacromolecules that form a corona. In blood the coating is proteins, in lung it might be surfactants, and in the environment it might be natural organic matter. Focusing on blood, the adsorption of proteins on the surface of nanoparticles is governed by protein–nanoparticle binding affinities and protein–protein and protein–NP interactions. Proteins with high binding affinity that are tightly adsorbed to nanoparticle surfaces are referred to as the “hard” corona. The hard corona therefore is typically believed to consist of a single protein layer coating the nanoparticle surface. More loosely bound proteins with low binding affinity to the nanoparticle may be connected through weak protein-protein interactions and are referred to as the “soft” corona (Figure 5.1). Generally, the hard corona is believed to consist of proteins that show irreversible protein binding to the nanoparticle or very low protein exchange rates. The soft corona is thought to have high exchange rates and can be easily replaced through competition with other biomolecules in a given biological environment. Over time, proteins that have a higher affinity for the ENM should predominate to form a more stable corona similar to the Vroman effect of small molecule protein adsorption. There is some evidence that the hard protein corona may be responsible for directing cell recognition and the subsequent interaction pathways for some nanomaterials. Due to the transient nature of the soft corona, usually there is little to no information gained about the possible role of the soft corona on NP-cell interactions. Indeed, to date there is still very little known about the true biologically relevant corona composition due to the difficulties of identifying the soft corona in vivo.
80

5.1.2 The biological impact of the protein corona

The nature of ENM coronas is determined by interactions with local environments. These interactions are dynamic and rapid; their time course is poorly understood. The long residence times of the hard corona suggests these proteins remain adsorbed on ENMs even through processes such as transport through the blood or cell endocytosis. Proteins may adsorb and detach at any time suggesting a corona composition that changes over time in response to its biological environment. This is consistent with the ‘Vroman effect’ that suggests while the total amount of adsorbed protein may remain constant, the exact composition may change over time. We may imagine therefore that the corona composition depends not only on the current biological fluid the ENM is
exposed to as it traverses the body but potentially on all the environments the ENM has moved through. Little is known about these exchange dynamics but if it is slow, relative to the particle pharmacokinetics, the corona may retain proteins from their previous environments while travelling to different locations within organisms.\(^{163, 171-172}\)

Internalization, biodistribution and cell response to nanoparticles \textit{in vivo} have been posited to be most critically affected by the composition of the hard corona. For example, the adsorption of certain proteins, such as IgG and fibrinogen, has been shown to allow macrophages to recognize the nanoparticles more easily and promotes early inflammation and clearance from the system.\(^ {173-174}\) Alternatively, adsorption of albumin has been reported to reduce adhesion of the nanoparticles to cell membranes and promote prolonged circulation times in the blood.\(^ {175-177}\) Others report that the binding of specific proteins may allow for the targeted delivery of nanoparticles. For example, the adsorption of apolipoprotein E on the surface of nanoparticles has been utilized for improving the distribution of nanoparticles across the blood brain barrier (BBB) thus enabling potential drug delivery to the brain.\(^ {178}\)

People are still debating on the benefits or disadvantages of the protein corona.\(^ {179}\) In some cases, protein binding can be used to direct delivery or target the nanoparticle to a particular area of the body and even trigger the nanoparticle-cell recognition or initiate alternative cell signal transduction.\(^ {180-181}\) However, the formation of protein corona also can cause the premature clearance of nanoparticles or the rapid accumulation of NPs into the liver and spleen thus increasing the risk of toxicity.\(^ {179}\) It has been shown that, in some cases, the cytotoxicity of nanoparticles can be mitigated by the presence of a protein corona that protects cells from direct exposure to bare nanoparticle surfaces.\(^ {182-183}\) The immune cell response can also be activated depending on the type of corona formation.\(^ {184}\) For example, when NPs are exposed to blood, the plasma corona formation can mark nanoparticles as antigens and promotes phagocytosis to elicit an immune response. Here, the protein corona promotes the immunotoxicity by causing inflammation and damage to the host.\(^ {185-187}\) Considering the biosafety of ENMs, we believe that a deep understanding
of protein corona formation and kinetics is vital to predicting effect on physiological systems and regulating the resulting immune responses and cell toxicities.

5.1.3 Model proteins: bovine serum albumin (BSA) and human transferrin (Tf)

Nanoparticles for different biomedical applications are commonly given via intravenous administration. This route results in pristine or treated NPs being first exposed to blood after IV injection. The complete human blood-plasma proteome may contain as many as 3700 different proteins with 12 order of magnitude difference in their concentrations. Among these thousands of proteins, serum albumin is the most abundant protein accounting for ~ 60% of total proteins in blood plasma. Many researchers have demonstrated that serum albumin can interact with various nanomaterials and affect cellular-level events, including nanoparticle binding, internalization, and transport. Therefore, we chose bovine serum albumin (BSA) as one of our model proteins to investigate in our experiments.

We also chose to study human transferrin (Tf) as our second protein model. Transferrin is an abundant human plasma glycoprotein which has a molecular weight of around 80 KDa and contains two specific high-affinity Fe$^{3+}$ binding sites. It binds to the Tf-receptor (TfR) in iron-bound form and activates receptor mediated endocytosis to control the level of free iron in cells. It has been reported that tumor cells have a higher demand for iron and generally overexpress the TfR. Although the precise cellular mechanism is not clear, it is known that transferrin can increase delivery of nanoparticles across the blood-brain-barrier (BBB) through receptor mediated transcytosis of Tf-TfR. Therefore Tf has been widely used to functionalize many nanomaterials for site-specific drug delivery and cancer treatments.

5.1.4 Research motivation

Since the composition and kinetics of protein adsorption on nanoparticles are likely to be important biologically to transport process and cellular responses, a detailed characterization of the formation and kinetics of protein corona formation is necessary to understand the beneficial applications and risks. As new information is gained about the pharmacokinetics of ENMs in vivo, understanding the kinetics of corona
formation and exchange is even more critical. The formation of protein corona can be determined by a variety of parameters such as particle size, surface charge, colloidal stability of NPs, as well as type of proteins. However, the knowledge of protein corona is still far from comprehensive. To gain sufficient knowledge on this complex topic, huge datasets are required. Moreover, many studies were done on very unique systems which are sufficiently different from each other as to not allow for easy comparison. Therefore, we need new methods to quantitatively determine the equilibrium and kinetic parameters of protein corona formation.

Corona formation is a dynamic process and there are continuous protein association and dissociation events before reaching binding equilibrium. Understanding the time evolution of the protein corona formation in a given biological fluid, enables predictions of the life time and biological fate of nanoparticles. Compared to the study of characterization of protein corona, relatively little work has been done on determining the kinetics of corona formation. Many studies use “immediately”, ”rapidly” or “ several minutes to several hours” to describe the time-scale on corona formation. This may be due to the limitations of most methods that have not allowed for time-dependent analysis.

Many techniques used to measure the protein corona are ex situ and require isolation of the nanomaterial with its bound protein from the physiological environment. Isolation is typically performed using either differential centrifugation (DC) or size exclusion chromatography (SEC). It is believed that isolation biases subsequent analysis towards the hard corona and removes evidence of the soft corona. The quantitative identification of adsorbed proteins on the nanoparticle surface is often performed by using poly(acrylamide) gel electrophoresis (PAGE) followed by mass spectrometry. By using these methods, samples have to be treated with high temperatures, high salt concentrations, detergents, and enzymes and thus make the proteins under the risk of to be denatured.

FCS is a powerful method for the in situ study of protein corona formation without some of the shortcomings of other methods. FCS allows for measurements under diluted
NP concentration more representative of in vivo conditions. Another advantage of FCS is that measurements can be carried out in situ without separation of unbound excess proteins from the system and thus measurements in real equilibrium are possible.\textsuperscript{202} By using FCS, the precise fractions of protein-NP complex and unbound protein are provided which allow us to characterize both the thickness and density of protein corona based on the stoichiometry ratio. FCS also enables us to continuously track the evolution of protein corona on the ~minute time scale after nanoparticles are introduced into a protein solution. In this manner, we can monitor the change in bound protein fraction as a function of exposure time in protein solutions. These measurements can also be carried out under different conditions to study how physiological environments influence the kinetic parameters of corona formation.

It is not easy to investigate the fundamentals of binding processes with a complex mixture of proteins, such as blood plasma where competitive adsorption may occur. It is therefore instructive to use more model systems to examine protein binding to NPs. In this chapter, we will examine protein adsorption to 50 nm diameter sulfonated polystyrene (PS) beads in solutions containing simply buffer and one protein (serum albumin (BSA) or human transferrin (Tf). Using these simple model conditions, we can establish the binding profile for specific proteins with high affinity. Comparable to previous studies, we show the formation of multi-layered protein coronas on PS beads and compare the corona formation to established adsorption models. The thicknesses of these coronas are dependent not only on concentration but the identity of the adsorbing protein. To the best of our knowledge, we also for the first time measured by FCS the protein binding kinetics as a function of pH and salt. Our results show that BSA adsorption kinetics is only slightly affected by either pH or salt. In contrast, Transferrin shows significantly enhanced protein binding rates with decreasing pH. In addition, we show that though the rate of binding is unaffected by salt, the binding affinities for BSA and Tf are greatly enhanced by the presence of additional salt. These results begin to shed light on how coronas are formed on nanomaterials under different conditions. Through these studies, we aim to gain quantitative knowledge of the dynamic rearrangement of proteins on engineered nanomaterials. Such studies can also be used to
guide our biophysical modelling of the in vivo conditions for corona formation to engineer optimized nanoparticles for biomedical applications.

5.2 Materials and Methods

5.2.1 Sample preparation

Polystyrene latex beads, sulfonated modified PSOSO$_3$H (nominally 50 nm) were purchased from Polysciences Inc. (Warrington, USA). Purified fluorescently labeled proteins: Alexa488 conjugated Human transferrin (Tf) and Alexa488 conjugated bovine serum albumin (BSA) were purchased from Invitrogen by Life Technology (Carlsbad, CA). Unlabelled proteins were dissolved with 1x PBS (Sigma Aldrich (St. Louis, MO)) into 1mg/ml stock solutions as recommended by the supplier and then further diluted to the required concentration/buffer conditions as needed. For protein-nanoparticle binding equilibrium experiments, polystyrene beads were added into protein solutions and equilibrated for at least one hour before measurements. To study the kinetic of binding, the measurements started right after the polystyrene beads were mixed into protein solutions.

5.2.2 FCS setup and data analysis

FCS experiments were made using an ISS Alba confocal fluorescence fluctuation system coupled to a Nikon Ti-U microscope equipped with a 60×1.2 NA water immersion objective lens. The illumination source was a continuous-wave 488 nm laser diode. The emission signal for the 488 nm was passed through a 514 nm long-pass edge filter before detection. Emission signal was recorded by two separate photomultiplier tubes (PMTs). All FCS measurements were performed in NUNC LabTek 8-well microscopy chambers with a final volume of ~500 µL. Determination of the focal volume was established via calibration against an aqueous solution of rhodamine 110 ($D = 440$ µm$^2$ s$^{-1}$). FCS results are an average of at least 20 measurements with typical sampling times of 30-60 s. All results are expressed as the mean ± SD of the measured diffusion coefficient. FCS measurements were analyzed using VistaVision 4.0 software.
3D-Gaussian one-component and two-component fitting models, as described in chapter 2, were used for data analysis.

The basic principles of FCS are described in detail in chapter 2. Here we give just a brief overview. The measured signal in FCS is the fluorescence intensity I(t) emitted by the observed particles moving in and out of a small, femtoliter illumination volume. The size of the effective illumination volume is fixed by the confocal optics and the excitation profile of the focused laser beam is characterized by measurements against a standard of a known diffusion constant. For ideal particles of uniform size diffusing by Brownian motion, dynamic information can be determined from the intensity fluctuations by means of a time autocorrelation given by

\[
G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_B} \right)^{-1} \left( 1 + \frac{\tau}{(r_0/z_0) \tau_B} \right)^{1/2}.
\]

Here, \(N\) is the average number of particles in the illumination volume and the structure parameters \(r_0\) and \(z_0\) are the axial and radial dimensions of the excitation beam determined by calibration measurements against a known standard.

To determine the fractions of unbound protein and protein-NP complex, a two-component fitting model is applied to analyzing the measured autocorrelation function. As only the protein is labeled, fluorescence observed from both the fast and slow components come from the same dye. The quantum yield term can thus be cancelled and the equation can be simplified to:

\[
G(\tau) = \frac{1}{N} \left[ (1 - \alpha) \left( 1 + \frac{\tau}{\tau_{D1}} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_{D1}} \right)^{-1/2} + \alpha \left( 1 + \frac{\tau}{\tau_{D2}} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_{D2}} \right)^{-1/2} \right]
\]

where \(\tau_{D1}\) and \(\tau_{D2}\) are the independent translational diffusion times of fast (free protein) and slow (protein-NP complex) components crossing the illumination volume. \(\omega\) is the structure parameter equal to the ratio of \(r_0\) and \(z_0\). \(\alpha\) is the fraction of particles with
slower diffusion time ($\tau_{D2}$) of the total number of fluorescent particles which is expressed as $N$. The autocorrelation can then be normalized by

$$G_{norm}(\tau) = \frac{G(\tau)}{G(0)}$$

(3)

The total number of protein molecules in the FCS confocal volume is $N_{total} = N_{free} + N_{bound}$ where we assume that each observed NP has at least one fluorescently labeled protein bound and that quenching of chromophore on PS beads is insignificant. The fraction of bound protein can be expressed as

$$\alpha = \frac{N_{bound}}{N_{total}}$$

(4)

To compare the protein corona formation in different systems, we focused on the normalized fraction of bound protein and defined it as

$$f = \begin{cases} 
1 & \text{if } x \leq x^*_c \\
\frac{x^*_c}{x} & \text{if } x > x^*_c
\end{cases}$$

(5)

where $x$ represents the molar ratio of protein to nanoparticles that $x = [\text{protein}]/[\text{NPs}]$. $x^*_c$ is the critical molar ratio of a saturated monolayer coverage; in other words, the average number of proteins required to form a single monolayer (hard corona) per nanoparticle.

### 5.3 Results and discussion

#### 5.3.1 Formation of hard and soft corona layers

Before examining the kinetics of protein corona formation, we first examined the equilibrium formation of a protein corona on our nanomaterials. Two Alexa488 labeled proteins, bovine serum albumin (BSA) and human transferrin (Tf), were examined by FCS for their ability to form protein corona layers on 50 nm diameter sulfonated polystyrene (PS) beads. The adsorption of protein is detected by a change in the diffusion
coefficient of protein bound to the NP and the fractions of free proteins and bound proteins are determined by a two-component fit to the time autocorrelation function from FCS. One unique aspect of FCS is that it not only provides information about hydrodynamic size but also gives information related to the average number of fluorescent-labeled particles or proteins in solution. Figure 5.1A shows FCS autocorrelation curves for BSA at four PS concentrations. The amplitude of the time autocorrelation $G(0)$ is inversely proportional to the number of fluorescent species, $N$ and thus concentration. With increasing PS bead concentration, $G(0)$ increases while simultaneously shifting to larger values for the dwell time ($\tau$) or diffusion time of the particle. At each concentration, samples were mixed thoroughly and allowed to equilibrate for at least one hour. Since all the samples were made with the same initial Alexa488-BSA concentration, the sharp increase in the amplitude, $G(0)$, of the autocorrelation curves indicated a significant decrease in the measured number of labeled particles due to adsorption to the PS beads. The increase in the observed diffusion time to larger values is best seen in the normalized autocorrelation curves shown in Figure 5.1B.

Without the PS nanoparticles in the system, the autocorrelation of free protein is well described by a one component fit with a single mean dwell time, $\tau$. In the presence of PS beads, measured autocorrelation functions are best described by a two-component fit where the fast component (free protein) is fixed at the experimentally determined value. This two-component fit to $G(\tau)$ allows us to directly quantify the fractions of slow (protein-NP complex) to fast (free protein) in solution. Experimentally the slow fraction was observed to be near constant diffusion time and consistent with the measured hydrodynamic radius of a fully coated PS bead by DLS. Diffusion times for both the slow and fast fractions were therefore fixed to these values and only the ratio of slow/fast was allowed to vary in the plotted fits (solid lines).

Similar results are observed for Tf binding to the same 50 nm PS nanoparticles. The experimentally determined autocorrelation functions and normalized autocorrelation functions for Tf-PS at varying PS concentrations are shown in Figure 5.2A and 5.2B, respectively.
Figure 5.2 (A) Autocorrelation functions of BSA binding to 50 nm diameter PSOSO$_3$H NPs in DI water at different NP concentrations. Four NP concentrations are shown. Solid lines represent the best fits from a two-component model described by equation 2. (B) Normalized autocorrelation curves of BSA-NP exhibit a systematic shift to longer correlation times indicating an increasing fraction of bound protein with increasing NP concentration.
Figure 5.3 (A) Autocorrelation functions of Tf binding to 50 nm diameter PSOSO$_3$H NPs in DI water at different NP concentrations. Solid lines represent the best fits from a two-component model described by equation 2. (B) Normalized autocorrelation curves of Tf-NP exhibit a systematic shift to longer correlation time indicating an increasing fraction of bound protein with increasing NP concentration.
The two component fit to $G(\tau)$ allows us to quantify the amplitude of the slow (bound) and fast (free) component in the data and thus determine the fraction of bound proteins in solution. To improve the robustness of the fitting, the diffusion time of free protein and the size of fully coated protein-PS complex are kept as fixed parameters. The $\tau_D$ is related to the translational diffusion coefficient $D$ by the simple relationship $D = \frac{r_h^2}{4\tau_D}$. The diffusion coefficients of free BSA and Tf as determined by FCS were $68.4 \pm 6.1 \, \mu m^2/s$ and $59.6 \pm 6.2 \, \mu m^2/s$. The corresponding hydrodynamic radius calculated from Stokes-Einstein equation are $3.4 \pm 0.3$ nm and $3.9 \pm 0.4$ nm; in good agreement with the values obtained for BSA and Tf by dynamic light scattering (DLS). Likewise, the calculated hydrodynamic radius of nanoparticles fully covered with proteins are in good agreement with the protein coated nanoparticles measured by DLS. All the relevant values are listed in Table 5.1 for comparison.

Table 5.1 Measured hydrodynamic radius ($r_h$) of free proteins and protein-NP complexes by FCS and DLS. The first row shows the hydrodynamic radius of the commercial Polystyrene (PS) beads, the unlabeled BSA and Tf as well as protein-PS complexes measured by dynamic light scattering (DLS). The second row gives the corresponding values as determined by FCS. The free protein $r_h$ values marked by * are reported values from the literature.

<table>
<thead>
<tr>
<th>$r_h$ (nm)</th>
<th>PS-50</th>
<th>BSA</th>
<th>Tf</th>
<th>BSA-PS</th>
<th>Tf-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS</td>
<td>24.4±0.9</td>
<td>3.48*</td>
<td>3.72*</td>
<td>27.5±0.4</td>
<td>29.7±0.5</td>
</tr>
<tr>
<td>FCS</td>
<td>N/A</td>
<td>3.4±0.3</td>
<td>3.9±0.4</td>
<td>28.1±2.6</td>
<td>30.8±3.3</td>
</tr>
</tbody>
</table>

Next, we examined the fraction protein bound, $f$, as a function of the PS bead concentration. Figure 5.3 shows the normalized fraction of bound protein as a function of nanoparticle (PS) concentration for two BSA protein concentrations. Figure 5.4 shows similar measurements for Tf measured at the same two protein concentrations. As expected with increasing NP concentration, the fraction of bound protein, $f$, increases reaching a plateau of 100% bound at a critical NP concentration. However, the adsorption is seen to depend on the amount of protein present with more nanoparticles.
required to bind all the proteins in solution for samples with higher initial protein concentration. The dashed lines in Figure 5.3 and 5.4 represents best fits to the experimental data assuming a Langmuir adsorption isotherm. This model is used to describe the adsorption equilibrium with the assumption that each binding site is equivalent to all the adsorbate. This behavior is also referred as the Hill equation in biochemistry. The protein adsorption to nanoparticle surface can be modeled by a Langmuir isotherm given as:

\[ N = N_{max} \cdot \frac{1}{1 + \left( \frac{K_D'}{c(P)} \right)^n} \]

Here, \( N_{max} \) is the maximum number of proteins binding to the nanoparticle. \( c(P) \) is the concentration of free protein. \( K_D' \) is the dissociation constant. \( n \) is the Hill coefficient. In this interpretation, the Hill coefficient describes how the bound proteins influence the adsorption of further free protein onto the same nanoparticle. When the Hill coefficient, \( n > 1 \), proteins already bound to a nanoparticle facilitate the binding of further proteins to the remaining vacant site. This is described as cooperative binding. When \( n < 1 \), the bound proteins lower the tendency for additional proteins to bind and is described as anti-cooperative binding.\(^{202}\) The results of our fits have \( n > 1 \) for Tf-PS but \( n < 1 \) for BSA-PS system (Table 5.2) indicating that BSA and Tf may undergo different adsorption mechanism to the sulfonated PS bead surface.

**Table 5.2** Hill coefficients of protein adsorption isotherms (at different initial protein concentrations) fit by Hill equation (Langmuir isotherm).

<table>
<thead>
<tr>
<th>Hill coefficient</th>
<th>BSA (2.5 µg/ml)</th>
<th>BSA (20 µg/ml)</th>
<th>Tf (2.5 µg/ml)</th>
<th>Tf (20 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>0.85</td>
<td>0.95</td>
<td>1.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Figure 5.4 Normalized bound BSA protein fraction, $f$, determined by FCS measurements, as a function of increasing PS bead in DI water. Experiments were performed at two fixed concentrations of BSA. Dashed lines are best fits to a Langmuir adsorption isotherm.

Figure 5.5 Normalized bound Tf protein fraction, $f$, determined by FCS, as a function of increasing PS bead in DI water. Experiments were performed at two fixed concentrations of Tf. Dashed lines are best fits to a Langmuir adsorption isotherm.
In Figure 5.5 and 5.6, the normalized bound fraction \( f \) is equal to 1 up to the critical molar [protein]/[NP] value indicative of full surface coverage and consistent with a strong binding model of protein to NP. Above the saturation concentration \( (x_c^*) \), \( f \) decreases due to the presence of unbound protein. We note that the bound fraction does not decrease as rapidly as predicted for a simple strong binding model indicating that at higher protein/NP concentrations more than a single monolayer of protein is binding to the nanoparticles. \( x_c^* \) represents a protein concentration sufficient for a complete monolayer formation on the nanoparticles for a given system. This concentration will be used later for kinetic studies. We determined \( x_c^* \sim 110 \) proteins per bead for BSA-PS and \( \sim 130 \) proteins per bead for Tf-PS. A 50 nm diameter PS bead has a surface area of roughly 7900 nm\(^2\). The surface area occupied by each protein can be estimated by knowing \( x_c^* \). From the determination of \( x_c^* \), we can estimate that a single BSA protein occupies about 71 nm\(^2\) on the surface of PS while Tf occupies about 60 nm\(^2\). These values are slightly larger than the surface area of bare protein (BSA=50 nm\(^2\), Tf=42 nm\(^2\))\(^{168,190}\) as estimated from their structure in solution.
**Figure 5.6** The normalized fraction of bound protein, $f$, as a function of ratio of BSA to NPs. The vertical dash line indicates the ratio of full surface coverage which is around 110. The solid line is the simple single layer strong binding curve.

**Figure 5.7** The normalized fraction of bound protein, $f$, as a function of ratio of Tf to NPs. The vertical dash line indicates the ratio of full surface coverage which is around 130. The solid line is the simple single layer strong binding curve.
To characterize the thickness of protein corona, we use the model described previously by Milani et al.\textsuperscript{168} In Figure 5.7 and 5.8, we plot the surface coverage $\Gamma$ as a function of the molar ratio of proteins to nanoparticles for BSA and Tf respectively. $\Gamma$ represents the number of protein molecules bound per nanoparticle and is calculated from the measured fraction of bound proteins from FCS and the known total number of proteins per NP as

$$\Gamma = \begin{cases} 
\frac{[\text{Tf}]}{[\text{NPs}]} & \text{if } x \leq x^*_c \\
\frac{[\text{Tf}](f - f_{\text{HC}})}{[\text{NPs}]} & \text{if } x > x^*_c 
\end{cases}$$

where $f$ is the fraction bound from experimental data. $f_{\text{HC}}$ is the calculated fraction bound to saturate the monolayer ‘hard’ corona from the known saturation concentration (vertical dash line). The horizontal dash lines indicate the theoretical number of proteins in each corona layer calculated from the known surface area of the PS bead and the occupation area of each protein molecule. We observe for both BSA and Tf the tendency to form multi-layer coronas on the PS nanoparticles. This is an estimate and it should be noted that the adsorption of proteins may be more heterogeneous than assumed in this model. The existence of multi-layer protein coronas on PS beads has been reported previously as measured by other methodologies such as AFM.\textsuperscript{208}
Figure 5.8 Surface coverage, $\Gamma$, in number of BSA molecules bound per NP. The number of BSA bound beyond full coverage was inferred from experiments by subtracting the hypothetical first monolayer of molecules. Theoretical surface coverage for BSA molecules per layer calculated from the known bead surface area and occupation area per BSA molecule are given by the horizontal dashed lines.

Figure 5.9 Surface coverage, $\Gamma$, in number of Tf molecules bound per NP. The number of Tf bound beyond full coverage was inferred from experiments. Theoretical surface coverage for Tf per layer calculated from the known bead surface area and occupation area per Tf protein are given by the horizontal dashed lines.
5.3.2 The kinetics of protein corona formation

For kinetic studies, we will focus on conditions sufficient to form a complete monolayer of protein per molecule; i.e. $x^*_c$ for a given protein-NP system. Figure 5.9 shows the normalized autocorrelation functions of Alexa488-Tf as a function of exposure time with the 50 nm sulfonated PS nanoparticles. The curves clearly shift to longer diffusion times due to protein-NP complex formation as a function of the incubation time. The complex formation occurs on a time scale on the order of minutes and is readily resolved by FCS. Similar results were observed for BSA-PS complex formation.

![Normalized autocorrelation curves of Alexa488-Tf at different incubation times after adding PS nanoparticles.](image)

Figure 5.10 Normalized autocorrelation curves of Alexa488-Tf at different incubation times after adding PS nanoparticles. 4 μg/ml Tf was mixed with PS under 130:1 molar ratio in 1mM MES buffer. The black curve represents the free Tf solution before adding PS. The blue, green and red curves represent the measurements at 5, 10 and 30 mins after adding PS, respectively.

The dynamics of protein binding reactions can be affected by various parameters. First we examined the binding kinetics as a function of protein concentration. Under the conditions of FCS, we are most sensitive at low ~nM concentrations of protein, so we
focused on lower protein concentrations equivalent to 1x, 2x, and 3x the saturated molar protein/NP concentration ($x^*_c$) determined for the protein. From the results shown in Figures 5.10 and 5.11, we see the duration to reach binding equilibrium (where $f=1$) has little dependence on the protein concentration in this concentration regime. Figure 5.11 may suggest Tf-PS binding kinetics has a weak concentration dependence, especially compared to BSA-PS (Figure 5.10) which shows almost no change. As will be shown below, this weak dependence is minimal compared to the dependence of the binding kinetics for other parameters such as pH and salt.

**Figure 5.11** Concentration dependence of the fraction of bound protein, $f$, as a function of incubation time for BSA-488 interacting with PS beads. BSA was mixed with PS at molar ratios equivalent to 1x, 2x, and 3x the saturated [protein/NP] concentration ($x^*_c$) determined previously.
Figure 5.12 Concentration dependence of the fraction of bound protein, $f$, as a function of incubation time for Tf-488 interacting with PS beads. Tf was mixed with PS at molar ratios equivalent to 1x, 2x, and 3x the saturated [protein/NP] concentration ($x_s^*$) determined for Tf-PS previously.

5.3.3 pH and salt effect on protein binding rate and binding affinity

The polystyrene nanoparticles used in this study are sulfonated and known to contain a slight anionic charge from sulfate ester terminal groups (PSOSO$_3$H) which helps prevent agglomeration and maintain the beads in aqueous solution. The degree of protonation of the PS terminal groups can thus change the surface chemistry of the PS beads and the interactions with proteins. To examine the effects of surface charge, we measured in Figures 5.12 and 5.13, the BSA and Tf fraction of bound protein, $f$, as a function of low concentrations of MES (pH 6.4) buffer. We believe in this buffer concentration range (10 µM to 1 mM), we are spanning a range from only partial buffering of the NP solution to a more complete buffering of the NPs. BSA and Tf have similar net protein charge and both are slightly negative. BSA does differ from Tf in being a fairly hydrophobic protein and is known to strongly bind to hydrophobic surfaces.$^{209-211}$ For the hydrophobic BSA protein, the binding rates to PS do not change significantly over this buffer concentration range. There is a slight shift to faster adsorption (~75 mins to 60 mins) by 1 mM MES vs. 10µM MES. In contrast, the binding
of Tf to PSOSO$_3$H changes dramatically over this buffer concentration range. At $x^*_T$, in 10 µM MES, the formation of the hard corona takes approximately 50-60 minutes similar to what we observe in DI water. Increasing the MES concentration to 100 µM and 1 mM greatly increasing the binding kinetics to ~20 and 10 minutes respectively. Under these conditions, both Tf and BSA are negatively charged since their pI are 6.1 and 4.7, respectively.$^{212-213}$ Due to the pKa of the proteins, we would not expect the buffer to be significantly modifying the charge of the protein. Therefore, we speculate that this concentration of buffer is sufficient to modify the PSOSO$_3$H surface charge resulting in significantly faster protein corona formation. We hypothesize that the hydrophobic BSA is more attracted to the hydrophobic PS surface and less influenced by the small changes in surface charge caused by the MES buffer.

Figure 5.13 The fraction bound of BSA, $f$, as a function of incubation time with PS NPs in thee different concentrations of MES buffer (pH 6.4). BSA-488 was mixed with PS NPs at the critical molar ratio ($x^*_c$) with a sufficient concentration of protein to form the hard corona monolayer.
Figure 5.14 The fraction bound of Tf, $f$, as a function of incubation time with PS NPs in three concentrations of MES buffer (pH 6.4). Tf-488 was mixed with PS at the critical molar ratio ($\chi_c^*$) for a sufficient concentration of protein to form the hard corona monolayer.

Changing buffer concentration over this range may be effecting the ionic strength concentration of the solution. To disentangle the role of pH and salt, we examined the protein corona formation kinetics for the same concentration of two different buffers and a neutral pH NaCl salt solution. Again all these experiments were performed at the critical molar concentration $\chi_c^*$ where there is sufficient protein to form a single monolayer on all the PS nanoparticles present. These results are shown in Figures 5.14 and 5.15. Protein binding to PS was examined at 100 µM solutions of MES (pH 6.4), PBS (pH 7.4) and NaCl solution (pH 7). For the hydrophobic BSA, all three sample conditions showed similar protein corona formation kinetics (Figure 5.14). For Tf, however, we see a significant shift to a much faster equilibrium binding time only at low pH. Tf binding to PS is nearly identical in buffered pH 7.4 solution as in 100 µM neutral NaCl salt solution. These results indicate the increase in the rate of protein binding at
this buffer concentration is most likely not due to ionic strength effects but rather due to modifications of the PS surface charge at low pH.

Figure 5.15 The bound protein fraction of BSA, $f$, as a function of incubation time with PS NPs at the critical molar ratio ($x^*_c$) to form monolayer corona.
The bound protein fraction of Tf, $f$, as a function of incubation time with PS NPs under the critical ratio ($x^*_c$) to form a monolayer corona.

In addition, we examined the effect of added salt at neutral pH on protein binding kinetics to our sulfonated PS beads. Low salt concentrations must be used as higher salt concentrations lead to an aggregation of the PS beads even in the absence of protein. Again, PS nanoparticles were incubated with the experimentally determined critical molar concentration ($x^*_c$) sufficient to form a monolayer corona. Protein binding kinetics was examined at three NaCl salt concentrations ranging from 0 to 1 mM added salt. The normalized protein binding fractions, $f$, as a function of incubation time are shown in Figures 5.16 and 5.17, for BSA-PS and Tf-PS respectively. Similar to previous results, we see no significant change in the binding kinetics for these protein-PS systems over this salt concentration range. Salt does not appear to make the protein binding any faster; the protein-PS samples appear to reach the equilibrium of hard corona formation at equal rates. Although the rate of protein binding is not affected by the ionic strength, when we look at the nominal fraction of bound protein ($\alpha$) determined by FCS we see that the fraction of bound protein under these [protein]/[NP] concentrations is highly dependent
on salt. This is shown for BSA-PS and Tf-PS in Figures 5.18 and 5.19. This indicated that the protein binding affinities for PS are altered with the addition of added salt. Again, the effect of salt is significantly less for the hydrophobic BSA protein compared to the more hydrophilic Tf protein. Over this NaCl salt concentration range, we see the absolute amount of BSA increase from ~46% to 55% bound at $x^*_c$. In contrast, total Tf binding to PS increases from 60% to nearly 100% at 1 mM added NaCl.

**Figure 5.17** The fraction bound of BSA, $f$, as a function of incubation time with PS NPs under the critical ratio ($x^*$) to form monolayer corona. The experiments were performed in pure deionized water and salt solutions with different concentration at pH=7.0.
The fraction bound of Tf, $f$, as a function of incubation time with PS NPs under the critical ratio ($x^*$) to form monolayer corona. The experiments were performed in pure deionized water and salt solutions with different concentration at pH=7.0.

The FCS measured fraction of bound BSA ($\alpha$) as a function of incubation time with PS NPs under the critical ratio ($x^*$) to form a monolayer corona. The experiments were performed in pure deionized water and salt solutions with different concentration at pH=7.0.
Figure 5.20 The FCS measured fraction of bound Tf (α) as a function of incubation time with PS NPs under the critical ratio ($x^*_c$) to form a monolayer corona. The experiments were performed in pure deionized water and salt solutions with different concentration at pH=7.0.

We hypothesize that the binding of BSA, a hydrophobic protein, to PS nanoparticles is driven primarily by the hydrophobic protein-NP interactions. Despite being slightly sulfonated, it is reasonable to assume that much of the PS bead surface is fairly hydrophobic similar to bulk PS. As this interaction dominates, the binding kinetics of BSA to PS is nearly independent of the pH or salt consistent with our experimental results. It is known that Transferrin is capable to reversibly bind with ferric irons and this binding process is very sensitive to the pH of the surrounding. It has been reported that the affinity of transferrin for Fe$^{3+}$ is extremely high ($10^{23}$ M$^{-1}$) at pH 7.4 but iron begins to dissociate from transferrin at acidic pH (pH = 6).$^{214-215}$ Moreover, the processes of binding and releasing Fe$^{3+}$ are associated with a significant conformational change in the 3D structure of transferrin.$^{216-217}$ Control of the Tf-PS interactions appears to be governed by the electrostatics in the system and results in a significant increase in the rate of corona formation. As Tf, like our PS beads, has a slightly negative charge, a reduction
in the charge of the PSOSO$_3$H bead surface with low pH may explain the increased kinetics. This may be the result of an increased hydrophobicity of Tf at low pH. We also hypothesize that corona formation may follow a multi-step process where there is first an approach of the protein to the NP followed by a second slower step, or ‘hardening’, where the protein binds in a nearly irreversible manner forming the hard corona. We see an increase in the binding affinity, but not binding kinetics, for both systems as a function of added salt. The added salt concentrations are low and not expected to significantly alter the Debye screening length of the PS bead. This increase in binding kinetics may then suggest that the process of hardening is enhanced by the presence of the added salt resulting in a much higher affinity for the protein to the bead.

5.4 Conclusion and future perspective

In this study, we investigated the protein corona formation and the binding kinetics of two proteins (BSA and Tf) to 50 nm sulfonated PS beads by FCS. We characterized both the complex size and corona thickness for these systems. Our results, consistent with previous results on 100 nm PS beads, show multilayer corona formation consisting of both a hard and soft corona. Total corona thickness was dependent on the protein with BSA forming a thicker soft corona layer compared to Tf at equivalent [protein]/[NP] concentrations. More importantly, we were able to measure the kinetics of the protein corona formation process for both protein-PS systems. Our results show that the binding rate of Tf to PS can be enhanced significantly at lower pH. BSA-PS binding rates were not strongly affected by solution pH. Added salt for both protein-PS systems did not significantly change the binding kinetics; however, the binding affinities of the protein were greatly enhanced even at relatively low (≤ 1 mM) added NaCl salt concentration. Tf-PS binding was especially sensitive with nearly 100% binding affinity achieved at 1 mM NaCl. We hypothesize that BSA binding to PS is mostly dominated by hydrophobic interactions and less influenced by salt or pH. Tf, being significantly less hydrophobic, shows much more sensitivity to surface binding on PS depending on the charge state of the surface. This may be due to transferrin having a higher hydrophobicity at low pH that drives the protein adsorption. The hydrophobicity of BSA is then relatively independent of the pH variation in this study. The increased binding
affinities of the proteins to PS occur at salt concentrations not likely to affect Debye screening lengths. This may suggest the effect of added salt is to enhance “hardening” processes which drives more complete corona formation under these conditions.

We have established FCS as not only a useful tool for the study of protein adsorption on NPs capable of distinguishing the presence of hard and soft coronas, but also as a means to study the dynamics of the corona formation process. Although many qualitative studies of protein-NP interactions have been done in recent years, the protein adsorption equilibria and rates on NPs not been well characterized. There is a critical need for quantitative data of equilibrium and kinetic parameters of protein-ENM systems in both model systems and more realistic, biological-relevant systems. This data is needed to develop a better understanding of underlying principles of nanoparticle-cell interactions \textit{in vivo}. Future studies will focus on the extension of our methodologies to metallic nanoparticles. In addition, the effects of surface treatments and particle aging need to be explored for their impact on corona formation, corona thickness and kinetics of assembly. In addition, we are working with collaborators to calculate from the surface coverage determined by FCS if a protein mass coverage can be determined to assess the completeness and density of the hard corona formed under different conditions. The capacity of FCS to measure dynamics in polymer or biological solutions is promising for looking at more realistical biologically relevant solution conditions. Through such studies we may be able to guide the synthesis of chemically modified nanoparticles for in vivo application that mitigate undesired side effects or toxicities.
Summary

Due to the rapid development of nanomedicine and the growing concern about the behavior of engineered nanomaterials (ENMs) in the body, in this dissertation we focused on the study of transport of nanoparticles with different hydrogel environments and the effects of protein interactions with nanoparticle surface. Using fluorescence correlation spectroscopy (FCS), we were able to quantitatively determine the dynamics of molecular transport within neutral and charged hydrogels as well as monitor the kinetics of protein corona formation in situ.

In chapter 3 and 4, we investigated the role of electrostatics on the basic mechanisms governing the diffusion of charged probe molecules and dendrimer macromolecule inside model polymer networks. Our results suggest the mobility of nanoparticles in charged biological network is highly charge asymmetric and dependent on the properties of the particles such as size, structure flexibility and surface charge density. Small probe molecules and larger NPs that generally used as nanocarriers show totally different diffusion behavior within the same charged networks. Combined with simulation results, our study reveals the essential role of electrostatic interaction in particle transport in biological hydrogels, and the complexity of interaction filtering strategy in biological gels.

In chapter 5, we have quantitatively characterized the formation of a multi-layered protein corona on the surface of polystyrene nanoparticles. FCS also allowed us to probe the kinetics of protein corona formation in situ. Our results suggest that the thickness of protein corona, the assembly rate of protein-NP complex and the protein binding affinities on PS beads are dependent on the properties of proteins as well as the local physicochemical properties. These studies begins to shed light on how coronas are formed on ENMs under different conditions and can also be used to guide biophysical modeling of the in vivo conditions for corona formation to engineer optimized nanoparticles for biomedical applications.
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Vita

Xiaolu Zhang was born in the city of Hohhot, Nei Mongol province, P.R.China.

Education
Bachelor, Pharmaceutical Science and Technology, Tianjin University, Tianjin, 2010

Publication

Conference presentations
2013 The 245th ACS national meeting & exposition, New Orleans, LA.
2013 The 2nd Bluegrass molecular biophysics symposium, Lexington, KY
2014 The 66th Southeastern Regional Meeting of the American Chemical Society, Nashville, TN.
2014 The 3rd Bluegrass molecular biophysics symposium, Lexington, KY

**Professional Affiliations**

Member of American Chemical Society (ACS) 2013–present
Graduate student in Department of Chemistry, University of Kentucky 2010–present