Characterizing the Role of Nanoparticle Design on Tumor Transport and Stability in the Extracellular Environment

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials & Biomedical Engineering
University of Toronto

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Abstract

Nanotechnology has emerged as an exciting strategy for the delivery of diagnostic and therapeutic agents into established tumors. Advancements in nanomaterial synthesis have generated an extensive number of nanoparticle designs made from different materials. Unfortunately, it remains impossible to predict a design’s effectiveness for in vivo tumor accumulation. Little is known about how a nanoparticle’s morphology and surface chemistry affect its interactions with cells and proteins inside the tumor tissue. This thesis focuses on the development of in vitro experimental tools to evaluate how nanoparticle design affects transport in a three-dimensional tumor tissue and stability in the tumor microenvironment. Nanoparticle transport was evaluated using a novel 'tumor-on-a-chip' system where multicellular tumor spheroids were immobilized in a microfluidic channel. This setup created a three-dimensional tumor environment displaying physiological cell density, extracellular matrix organization, and interstitial flow rates. The tumor-on-a-chip demonstrated that accumulation of nanoparticles was limited to diameters below 110 nm and was improved by receptor targeting. Nanoparticle stability in the tumor microenvironment was evaluated using media isolated from different tumor cell lines. Nanoparticle diameter and surface chemistry were important determinants of stability in cancer cell-conditioned media. Small nanoparticles with unstable surface chemistries adsorbed cellular proteins on their surface and were prone to aggregation. Nanoparticle aggregation altered cellular interactions leading to changes in cell uptake. Using a novel technique to generate different aggregate sizes possessing a uniform surface composition, it was determined that aggregation can change receptor affinity, cell internalization mechanisms and sub-cellular sequestration patterns. Data from this thesis characterize the behavior of nanoparticles within
modeled tumor environments and provide some preliminary design guidelines for maximizing nanoparticle tumor accumulation. This work highlights the importance of characterizing nano-bio interactions for engineering successful nanomaterial-based delivery systems.
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List of Abbreviations

AuNP: gold nanoparticle
BSA: bovine serum albumin
Ci: citrate
DLS: dynamic light scattering
ECM: extracellular matrix
FBS: fetal bovine serum
ICP-AES: inductively coupled plasma atomic emission spectroscopy
LC-MS/MS: liquid chromatography and tandem mass spectrometry
MUA: 11-Mercaptoundecanoic acid
NP: nanoparticle
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PdI: polydispersity index
PEG: polyethylene glycol
SERS: Surface-enhanced Raman scattering
TEM: transmission electron microscopy
Tf: transferrin
UV-Vis: ultraviolet-visible (spectra)
1 General Introduction

When Craig Venter was sequencing the human genome at the end of the 20th century, he proclaimed that the 21st century would be the ‘century of biology’ [1, 2]. He predicted that the next century would generate immense progress in biology mirroring the great leaps in physics during the 20th century and chemistry in the 19th century. Advancements in DNA sequencing, protein structure analysis, biomacromolecule synthesis, and bioinformatics, are poised to transform the face of medicine in the next century. The convergence of biology with disciplines such as chemistry, physics and engineering has generated numerous technologies that will bring forth a new generation of diagnostic and therapeutic strategies. Several promising concepts have emerged from the multi-disciplinary crosspollination of ideas such as personalized medicine, localized drug delivery, stem cell-based regenerative medicine and nanomedicine. Unfortunately, these technologies remain several decades removed from widespread clinical use until fundamental research can characterize and optimize each system. The application of nanotechnology to medicine-related challenges, known as nanomedicine, is one of the most promising fields to arise from multi-disciplinary collaborative science. Nanomedicine aims to utilize the physicochemical properties of new nanomaterials to improve current diagnostic and therapeutic strategies.

1.1 Nanotechnology

Nanotechnology is defined as the manipulation of matter with at least one dimension in the 1-100 nm range [3]. At the nanoscale, some materials possess unique properties not present in bulk materials [4]. These unique nanoscale properties arise in some metals and semi-conductors due to the large ratio of surface atoms compared to bulk materials. To illustrate this concept, only 0.00001% of atoms in a 1 cm iron cube are on found on the surface. In contrast, surface atoms represent 10% of the iron in a 10 nm cube and 100% of a 1 nm cube [5]. In this size regime, small changes in a nanomaterial’s dimensions will significantly alter surface-to-volume ratio and produce tunable optical, physical and electric properties [5]. Some examples of nanoscale properties include gold’s ability to absorb light at specific wavelengths and some semi-conductors’ ability to emit fluorescence at high quantum yields [5] (Figure 1.1). In the last 30
Figure 1.1: Size-dependent properties of nanomaterials. 

(a) Solutions of spherical gold nanoparticles with various diameters demonstrating size-dependent changes in color. Scale bar = 100 μm. Figure adapted with permission from [6]. Copyright (2005) Nature Publishing Group.

(b) Absorbance of spherical gold nanoparticles with different diameters. Peak absorbance wavelength is proportional to nanoparticle diameter. Adapted from [7].

(c) Quantum dot fluorescence emission (Em.) as a function of diameter (D). Adapted from [7].

(d) Absorbance (black line) and emission spectra of quantum dots presented in panel c. Adapted with permission from [7]. Copyright (2008) American Chemical Society.
years, tunable nanomaterials have provided a new resource for resolving countless technological challenges across various fields such as electronics [8, 9], computing [10], solar energy [11], cosmetics [12], and drug delivery [13].

Early work with metal and semi-conductor nanomaterials revolved around the correlation of optical, electrical, and magnetic properties with a material’s structural and compositional features. Initially, metallic nanomaterials were developed for electronic, computer and ex vivo diagnostic applications [14]. Later studies made efforts to render nanomaterials biocompatible and have since opened up the possibility of developing accurate diagnostics and novel therapies for patients [4, 13, 15, 16]. In the last two decades, the development of stable surface chemistries has generated a multitude of biocompatible nanomaterials [17, 18]. Spherical nanoparticles are the most common form of nanomaterials due to their relatively simple synthesis and uniform surface properties. Nanoparticles diameters in the 1-100 nm size range possess numerous advantages for drug delivery. First, their large surface-to-volume ratio allows for the transport of numerous surface-bound molecules. Second, nanoparticles are in the same size regime as proteins and small viruses making them able to cross several physiological barriers inside the body and access most of the body’s cells through blood-borne transport [4].

1.1.1 Nanoparticles and Tumor Delivery

Nanoparticles are often used for blood-borne transport of therapeutic and diagnostic agents into a target tissue [19, 20]. In this scenario, a nanoparticle is designed, synthesized and administered into the patient. Once inside the blood, the nanoparticle will recognize its target tissue, exit the blood vessel, diffuse through the tissue, bind to target cells, and deliver its cargo (Figure 1.2). Nanoparticles have generated a lot of interest in recent years because they offer the potential for localized drug delivery. The ability to deliver drugs directly into a target tissue with minimal off-target accumulation can greatly reduce unwanted toxicity and side-effects. The reduction in off-target toxicity also allows clinicians to administer higher effective doses directly to the tumor with better therapeutic outcomes. Nanoparticle-based delivery seems especially promising for tumor delivery. In 1985, Maeda and colleagues discovered that a tumor’s leaky vasculature and poor lymphatic drainage produce the enhanced permeability and retention of larger particulates in the blood [21]. Nanoparticles fall within the size regime of solutes retained by tumors in vivo.
To date, nanoparticles have been used to target cancer cells in pre-clinical animal research and in patients during clinical trials [13].

Nanoparticles have been hailed as a ‘magic bullet’ in tumor treatment given their potential to target cancer cells while avoiding other tissues [22, 23]. Unfortunately, several challenges must be addressed before nanotechnology can make a significant impact on cancer patient care [19, 24, 25]. One of the biggest disappointments with current nanoparticle designs is that less than 5% of the administered dose ultimately reaches the tumor. Since the majority of nanoparticles are retained in the liver and spleen, ‘localized tumor drug delivery’ is a considerable misnomer. Another challenge with tumor delivery is that nanoparticle accumulation mechanisms are largely unknown and produce a heterogeneous distribution inside the tumor leaving a large fraction of tumor cells unexposed to nanoparticles. To improve nanoparticle tumor accumulations, there are two important objectives: decrease off-target accumulation and increase tumor accumulation. Improving nanoparticle design to achieve these objectives has been difficult because the factors restricting nanoparticle tumor accumulation remain largely unknown [19, 24]. This has lead to a widespread trial-and-error approach for designing tumor-targeting nanoparticles. The failure to design effective tumor targeting nanoparticles is due to a poor knowledge of nanoparticle-tumor interactions caused by ineffective experimental models. At present, nanoparticle formulations are evaluated either in cell culture or animal models. In vitro cell culture provides a convenient setup to analyze nanomaterial-cellular interactions and track sub-cellular localization. Unfortunately, the homogenous distribution of a monoclonal cell population over a flat surface often lacks the necessary spatial organization to accurately model in vivo interactions. Animal models are a better predicator of nanoparticle performance in human patients and they provide an experimental system to quantify pharmacokinetics and tissue distribution. However, animal models are still considered ‘black box’ systems where nanomaterials are administered and data is collected with little knowledge of underlying accumulation mechanisms. The gap between oversimplified in vitro cell culture and highly variable black box in vivo models must be bridged by new experimental models and approaches. New models must be designed to facilitate the analysis of nanoparticle-biological interactions in highly controllable conditions to identify the obstacles restricting efficient tumor accumulation.
Figure 1.2: Challenges of nanoparticle delivery. Blood borne nanoparticles must avoid clearance by the liver and spleen’s mononuclear phagocyte system (MPS). The nanoparticles then extravasate through the large fenestrations in between the endothelial cells lining the leaky tumor vasculature. Once they exit the blood vessel, nanoparticles interact with cells and the extracellular matrix inside the tumor. The nanoparticles are eventually internalized by tumor cells and sorted into different organelles via intracellular trafficking mechanisms. Figure adapted with permission from [20]. Copyright (2013) American Chemical Society.
Before designing the next generation of successful tumor-targeting nanomaterials, it will be necessary to identify current limitations and define specific objectives for improving tumor delivery. The analysis of nano-bio interactions is complicated by the large number of interactions occurring between the nanomaterials, serum proteins, the extracellular matrix, cell receptors and different cell types. The complexity of biological environments requires accurate tracking of well characterized and stable nanomaterials in controllable experimental conditions to successfully extricate the different interactions occurring at the nano-bio interface. The focus of this thesis is to develop new in vitro experimental models to evaluate nano-bio interactions during tumor accumulation. Ultimately, drug delivery comes down to nanoparticle-cell interactions. However, numerous factors directly upstream of cell contact can alter nanoparticle properties and alter cellular responses. The goal of this work is to determine how the tumor’s extracellular environment can affect nanoparticle transport, stability and cellular interactions.

1.1.2 Gold Nanoparticles

This thesis will utilize gold nanoparticles as a model system to study nano-bio interactions since they offer numerous advantages. An important advantage of gold nanoparticles over other nanomaterials is that their synthesis is achievable through relatively simple high-yield techniques which produce a homogenous population of nanoparticles. Gold nanoparticles with diameters between 2 and 250 nm are usually synthesized through the chemical reduction of metal salts in solution. Using the Turkovich-Frens method, sodium citrate is added to a boiling solution of chloroauric acid (H[AuCl₄]) [26, 27]. This reaction causes the reduction of Au³⁺ ions to neutral Au atoms which assemble into clusters via metal bonding. As clusters of gold atoms continue to grow and fuse together, the surface area of gold atoms decreases. The reaction reaches completion when all gold atoms are reduced and the negatively-charged sodium citrate anions are capable of preventing additional nanoparticle growth. Thus, the sodium citrate also serves as a capping agent since its negatively-charged carboxylic groups bind to the electron-avid nanoparticle surface and prevent nanoparticle aggregation. The citrate anions ensure electrostatic inter-particle repulsion and prevent the attractive Van der Waals forces from inducing aggregation. The Turkovich-Frens synthesis method is used to produce citrate-capped gold nanoparticles 10-20 nm in diameter depending on the citrate-to-gold ratio used [26, 27].
Citrate-capped gold nanoparticles can also be used to generate larger nanoparticles using seed-mediated growth techniques where gold is deposited onto ‘seed nanoparticles’ in order to generate larger diameters. Hydroquinone is incapable of reducing ionic gold in the absence of seed nanoparticles at room temperature and constitutes an excellent reducing agent for seed mediated growth [28]. Hydroquinone-mediated nanoparticle synthesis typically uses ~15 nm citrate-capped ‘seed nanoparticles’ to produce nanoparticles between 30 to 250 nm in diameter with very narrow size distributions. Synthesis requires seed nanoparticles, chloroauric acid, hydroquinone and sodium citrate as a capping agent. Nanoparticle diameter is controlled by maintaining the concentrations of all reagents while changing the number of seeds added to the reaction. For a fixed amount of ionic gold, the number of seeds will dictate the average thickness of deposited gold per nanoparticle. A small number of seeds will accumulate large deposits of gold and produce larger nanoparticles whereas a high number of seeds will show small increases to their diameters. The nanoparticles synthesized by hydroquinone-mediated seed growth are extensively washed with sodium citrate in order to produce a population of large citrate-capped nanoparticles.

Citrate-capped nanoparticles offer two important advantages: stability and simple functionalization. Nanoparticles capped with citrate are stable in solution and can be stored for several months. The nanoparticles can also be functionalized with many types of molecules including ions, poly-electrolytes [29], surfactants [30], polymers [31], lipids [32], proteins [33], DNA [34], and RNA [35]. Electrostatic and hydrophobic interactions can lead to the uncoordinated adsorption of these molecules onto the gold nanoparticle surface. Coordinated functionalization is possible through ligands containing terminal thiols, phosphines or amines [14]. Terminal thiols are the most common surface functionalization strategy since the sulfur atom shares two unpaired electrons with gold and forms a stable sulfur-gold coordinate bond (~45kJ/mol) [36]. When thiol-terminated molecules encounter gold surfaces, they quickly organize into a self-assembled monolayer of molecules [36]. The density and composition of self-assembled monolayers are controllable by adjusting the concentration and molar fraction of thiolated molecules. Thus, gold nanoparticles offer the possibility to generate homogeneous populations of nanomaterials while providing exquisite control over size and surface functionalization. These advantages have made gold nanoparticles an important tool in analyzing the role of nanomaterial design on nano-bio interactions [37, 38].
1.1.3 Plasmon Resonance

In addition to their synthesis and functionalization advantages, gold nanoparticles also possess unique optical properties arising from their nanoscale dimensions. This is evident when comparing the metallic yellow of bulk gold to a ruby red solution of nanoparticles. The characteristic color of gold nanoparticles is caused by a phenomenon called ‘surface plasmon resonance’ (Figure 1.3) [6, 14]. Gold is a conductor and thus possesses both a valence band where electrons are strongly associated with the atomic nucleus and a conduction band where electrons at higher energy levels can move freely [5]. Nanoparticles with 5-200nm diameters are large enough to display the conductive properties of bulk gold but smaller than the wavelength of visible light (400-750 nm). In this size range, the surface plasmon resonance is caused by the coherent (or coordinated) excitation of free electrons in the conduction band on the nanoparticle surface [5, 6]. The electric field of incoming light causes the synchronized oscillation of electrons in a negatively charged ‘electronic cluster’ and a corresponding cluster of positively charged gold ions which are missing their conduction band electrons. The oscillation of these two poles produces a net charge difference on the nanoparticle surface and results in surface plasmon resonance and the absorption of specific light wavelengths [39-41]. In gold nanoparticles, the surface plasmon resonance produces a strong absorbance of green-blue light (~530 nm) while red light (~700 nm) is reflected resulting in a rich red color. Nanoparticle diameter directly affects plasmon absorption where increasing particle size produces a wider peak width due to loss of coherent electron motion and a spectral red shift towards longer wavelengths. For example, a 15 nm nanoparticle will show an absorbance peak at ~520 nm whereas 90 nm nanoparticles will exhibit a peak at ~540 nm (Figure 1.1).

The size, shape, surface chemistry and surrounding medium all contribute to the optical properties of gold nanoparticles. For example, rod-shaped nanoparticles, called nanorods, possess two plasmon bands: a transverse plasmon in the visible spectrum (~520 nm) and a longitudinal in the near-infrared infrared (700-800 nm) [5, 42]. The combination of these plasmon peaks produces a brownish color. More complex geometries such as prisms and stars
Figure 1.3: Surface plasmon resonance. Schematic of plasmon oscillation for a sphere showing the displacement of the electrons in the conduction band relative to incident light. A dipole is generated which oscillates with the electric field of the incident light. Figure adapted with permission from [43]. Copyright (2003) American Chemical Society.
also possess various plasmon bands corresponding to their morphology [44, 45]. Gold nanoparticle aggregation or assembly can also change the plasmon frequency by a coupling effect. In this scenario, two adjacent nanoparticles can produce a longitudinal plasmon [46]. This causes a red-shift in the plasmon frequency and changes the nanoparticle solution from red to purple, blue or grey depending on the extent of aggregation. The exquisite sensitivity of the surface plasmon resonance band allows researchers to monitor the configuration, aggregation and surface composition of nanoparticles by simply measuring sample absorbance spectra via spectrometry. This concept has been applied to generate gold nanoparticle-based colorimetric detection platforms where the presence of a given solute will trigger aggregation and alter the solution from red to purple [47-49].

1.2 Nanoparticle-Cell Interactions

The highly controllable synthesis, surface functionalization and characterization of gold nanoparticles make them an important model system for studying nano-bio interactions. Initially, nanoparticles were considered inert carriers capable of delivering a molecular payload. It is now widely accepted that their morphology and surface topography also contribute to how the payload interacts with a biological system. At the moment of cell contact, several factors determine the fate of a nanoparticle such as its morphology, its surface chemistry, the cell receptors engaged and the cell’s phenotype (Figure 1.4).

1.2.1 Surface functionalization with Bio-ligand

The most common approach to target nanoparticles for cell uptake is to decorate their surface with proteins, peptides, DNA aptamers or other bioligands which can bind to cell receptors. In general, when these molecules are bound to the nanoparticle surface they demonstrate an improved stability and an increased binding avidity. Both DNA and proteins have demonstrated a significant increase in molecular stability when bound to nanoparticle surfaces [50, 51]. Anchoring and packing molecules on a nanoparticle’s surface has been shown to improve their stability by limiting enzyme-mediated degradation. For nanoparticle-bound DNA, the high
Figure 1.4: Nanoparticle-cell interactions. List of factors which can influence nanoparticle-cell interactions at the "nano-bio" interface.

1. Size, shape, charge
2. Ligand density
3. Receptor expression levels
4. Internalization mechanism
5. Cell properties (phenotype, location)
concentration of sodium counter-ions required to neutralize the negative phosphate backbone disables degradative enzymes [52]. For proteins, high density packing may sterically hinder the binding of degradative enzymes. Another advantage of fixing ligands onto the nanoparticle surface is the multivalent effect when multiple ligands on the nanoparticle interact with multiple receptors on the cell. The binding strength of the complexed ligands is more than the sum of individual affinities and is measured as the ‘avidity’ for the entire complex. The nanoparticle-mediated multivalent enhancement has resulted in higher ligand avidities for Herceptin antibody [53], RGD peptide [54], folate [55], ICAM-1 binding peptide [56], angiogenesis regulation peptides [57], and transferrin [58]. In most cases, nanoparticles will improve the probability of ligand-receptor binding by assembling high avidity complexes and this can lead to improved cell signaling and more potent biological responses than free ligands in solution [53, 57].

Nanoparticle morphology will dictate the organization of ligands on its surface. Nanoparticle size and shape will determine the total available surface area, maximum ligand density and interactions with the cell membrane. For spherical nanoparticles, the diameter determines their volume, area and surface curvature. Smaller nanoparticles possess a smaller volume, smaller surface area and higher surface curvature. The surface curvature has an important effect on the maximum packing density of surface molecules (Figure 1.5). For thiol-terminated ligands such as oligonucleotids and polythelyne glycol (PEG), a high surface curvature leads to increased packing densities [59, 60]. The higher curvature produces larger deflection angles between ligands and allows more thiol-gold interactions per unit of surface area. At lower curvatures and on flat surfaces, inter-molecular repulsive forces decrease the density of thiol-gold interactions. In contrast, a high surface curvature restricts the overall ligand density during protein adsorption. Herceptin antibody [53], fibrinogen [61], and lysozyme [62] all show decreased surface densities when decreasing the nanoparticle’s diameter. Uncoordinated gold-protein interactions usually require a large contact area with the nanoparticle surface compared to terminal thiol groups. Interactions occur with different amino acids side chains through sulfur bonding (cysteine [63]), electrostatic attraction through amine groups (lysine [64]) and hydrophobic attractions (tryptophan [65]) must produce enough attractive forces to anchor the protein on the nanoparticle surface. Thus, a high surface curvature limits the orientation of each molecule and restricts protein-gold interactions [53]. To illustrate this effect, the Herceptin antibody displays a binding affinity of $10^{-10}$ M for its receptor ErbB2 in its soluble form. When Herceptin is bound to
Figure 1.5: Effect of surface curvature on the adsorption of thiolated molecules and proteins. (a) At high surface curvatures, a higher deflection angle between thiolated ligands leads to less steric hindrance and higher loading density. (b) At lower surface curvatures, increased steric hindrance between the thiolated ligands decreases overall ligand density. (c) At high surface curvature, proteins are restricted from making proper contact with the nanoparticle surface and produce a lower packing density. (d) At low surface curvature, proteins can interact with the nanoparticle surface more readily and pack at higher density.
nanoparticles, it produces a binding avidity of $5.5 \times 10^{-12}$ M on a 10 nm nanoparticle, and $1.5 \times 10^{-13}$ M on a 70 nm nanoparticle [53]. The reduced surface curvature of the 70 nm nanoparticles increases the packing density of Herceptin and leads to a four-fold increased binding avidity. Thus, tunable nanoparticle size and ligand avidity provide additional design freedoms when synthesizing cell-targeting nanoparticles.

### 1.2.2 Effect of Nanoparticle Design on Cell Uptake

Nanoparticle morphology is also important in dictating how affixed ligands are presented to cell receptors. The shape of a nanomaterial is thought to directly influence cell uptake where rods > spheres > cylinders > cubes when dimensions are larger than 100 nm [66]. In studies with nanomaterials smaller than 100 nm spherical nanoparticles show higher uptake than rods [33, 67, 68]. In fact, increasing the aspect ratio of nanorods seems to decrease total cell uptake [67, 68]. There have been limited studies on non-spherical nanoparticles thus far but their interactions with cells may be more complex. Ligand-coated rod shaped nanoparticles may present to the cell with two different orientations. On the short-axis, there would be fewer multivalent interactions in comparison to the long-axis [33]. For irregularly shaped nanoparticles such as gold nanurchins, the location of the ligand on the spikes would be presenting ligands to the target molecules differently than when it is located between the spikes [69]. For spherical nanoparticles, diameter is the main determinant of cell uptake. A 50 nm diameter seems to be optimal for maximizing mammalian cell uptake of gold nanoparticles, silica nanoparticles, and quantum dots [33, 70, 71]. Although size can influence a number of parameters including surface curvature [59], ligand density [72], sedimentation rate [73] and diffusion speed [74], it remains unclear how these phenomena are responsible for producing the increased uptake of 50 nm nanoparticles.

In HeLa cells, the number of transferrin-coated gold nanoparticles per cell after 8h was ~500 for 14 nm, ~1500 for 30 nm, ~2500 for 50 nm, and 2000 for 74 nm. These trends reveal a ‘sweet spot’ for the uptake of nanoparticles with diameters around 50 nm. One theory for this preferential uptake proposes that the binding of nanoparticle-bound ligands to their target receptor will produce a localized decrease in the Gibbs free energy, which causes the membrane to wrap around the nanoparticle forming a closed vesicle [75]. A 5 nm nanoparticle coated with a 50 kDa protein may only interact with 1 or 2 cell receptors, while a 100 nm nanoparticle will
have >100 proteins and bind many receptors. In this scenario, multiple 5 nm nanoparticles must bind to the cell membrane in close proximity to each other in order to generate enough free energy to drive membrane wrapping. Thus, entry of 5 nm nanoparticles requires clustering at the cell membrane prior to entry [75, 76]. Thermodynamically, a 40-50 nm nanoparticle is capable of recruiting and binding enough receptors to successfully produce membrane wrapping. Above 50 nm, nanoparticles bind such a large number of receptors, that uptake is limited by the redistribution of receptors on the cell membrane via diffusion to compensate for local depletion [72]. Mathematical modeling of this phenomenon has demonstrated that optimal endocytosis occurs when there are enough ligands on the nanoparticle and no localized receptor shortage on the cell surface [72]. This ‘sweet spot’ occurs in nanoparticles 30 to 50 nm in diameter because ligand density is optimal. In theory, larger nanoparticle uptake can be improved by reducing ligand density to prevent local receptor depletion.

1.2.3 Nanoparticle Endocytosis

Once a given nanoparticle-ligand formulation encounters the cell membrane, it can undergo one of many fates including: (1) ligands bind to cell receptors and induce or block a signaling cascade with no internalization; (2) ligands bind to receptors, enter the cell and then exit the cell; (3) ligands bind to receptors, enter the cell and target sub-cellular structures; (4) nanoparticles interact non-specifically with the membrane and are subsequently internalized (Figure 1.6). Once bound to the cell surface membrane, cell internalization or endocytosis can occur depending on the receptors engaged. It is hypothesized that the majority of nanoparticle uptake is due to receptor-mediated endocytosis [53, 68, 75]. Although nanoparticle morphology can influence cell uptake, ligand-receptor interactions are another important determinant of uptake. When biological ligands bind their receptors, a receptor-specific internalization pathway is usually engaged. When ligands are added to nanoparticle surfaces, they will often employ the same pathways as their soluble counterparts. Transferrin is an iron transport protein that binds to its receptor CD71 then enters the cell through a clathrin-dependent pathway [77]. Transferrin-coated gold nanoparticles also utilize clathrin-dependent mechanisms and demonstrate similar internalization rates [33, 78]. The internalization pathways of Shiga toxin and ricin also remain unaffected when surface-bound to 30 nm nanoparticles [78]. Once internalized, sub-cellular processing, trafficking and exocytosis can be retarded or inhibited when ligands are bound to nanoparticles [33, 78].
Figure 1.6. Ligand-coated nanoparticles interacting with cells. (1) The ligand-coated nanoparticles can interact with cells and induce a signaling cascade. (2) The nanoparticles can be internalized and exocytosed by the cell, without ever leaving the vesicle. (3) Internalized nanoparticles can escape the vesicle and interact with various organelles. (4) Nanoparticles can interact non-specifically with the cell surface membrane.
Although general conclusions are tempting, there is little evidence supporting some universal effect when fixing bioligands onto nanoparticles. Some studies demonstrate that nanoparticle size and morphology may change endocytosis mechanisms but these differences are only observed when a material’s size goes from the nanoscale to the microscale [79, 80]. It is difficult to compare these studies since they use different nanoparticles, different ligands and different cell lines. It is also important to consider that two different ligands targeting the same receptor may utilize different uptake mechanisms. In one study, it was demonstrated that targeting the transferrin receptor CD71 with transferrin protein or a monoclonal antibody leads to unequal cell uptake and drug delivery [58]. Each ligand’s unique binding site and receptor affinity produces a different therapeutic outcome. The cell line used in each experiment will affect nanoparticle interactions and change the uptake trends. There is some evidence to suggest that size-dependent uptake trends may be cell-specific [33, 81-83]. Although most studies demonstrate that 30-50 nm nanoparticles show the highest uptake in different cell lines, the total number of internalized nanoparticles can vary greatly. Different cell lines can demonstrate preferential uptake of specific morphologies and utilize unique internalization pathways. One example is the preferential uptake of nanorods by neurons versus the preferential uptake of nanostars by microglia cells [84]. Another example is the distinct intracellular trafficking pathway of quantum dots by closely related prostate cancer cell lines [85]. These differences are likely related to varying receptor expression, cell metabolism, and internalization pathways. After a decade of nanoparticle-cell studies, very few broad conclusions can be made regarding the interactions occurring between cells and nanoparticle. One of the biggest concerns with these initial studies is the lack of material characterization and a general oversight in regards to nanoparticle stability in biological environments. In these early studies, the stability of nanomaterials in cell culture conditions was overlooked and may have lead to data misinterpretation and erroneous conclusions. With the development of new tools and methods to accurately monitor nanoparticle size, charge, dispersion and surface composition the conclusions from some of these early studies may have to be revisited to confirm their validity.

A good example of data misinterpretation in earlier nano-bio studies caused by nanoparticle instability is the original hypothesis behind the increased uptake of positively charged
nanoparticles. Early studies falsely assumed that positively charged nanoparticles entered the cells more readily due to electrostatic attractive interactions with the negatively charged surface membrane [86, 87]. This hypothesis was based on earlier work with small cationic molecules and peptides showing higher affinity to the negatively charged cell membrane [88, 89]. However, it is now apparent that cationic nanoparticles will adsorb different proteins than neutral or anionic nanoparticles which likely interact with a different set of cell receptors [90]. Thus, the studies attributing the higher cell uptake of cationic nanoparticles to their surface charge are only half correct. The positive surface charge is screened by the adsorbed proteins yet these specific proteins are responsible for increased uptake. Thus, it is not the surface charge itself but the recruitment of specific proteins that produces increased cell uptake of cationic nanoparticles. By deciphering the complexities of nano-bio interactions, future studies may be capable of elucidating some universal mechanisms underlying nanoparticle-cell interactions.

1.3 Nanoparticles in Vivo

*In vitro* nanoparticle-cell studies are important for understanding the nanoparticle-cell interactions which dictate biological outcomes. These studies are necessary to design cell-specific nanoparticles with desired binding avidities, drug release kinetics and cytotoxicity. Once a suitable nanoparticle has been designed, *in vivo* studies are required to evaluate whether the nanoparticles can reach their target cells and produce a therapeutic effect. Before reaching their destined target, nanoparticles must navigate the through various physiological compartments, organs and tissues. Similar to cell uptake, systemic interactions will also depend on the size, shape and surface chemistry of a nanoparticle. Pharmacokinetic studies looking at nanoparticle’s tissue distribution are important because they determine the effectiveness, toxicity and overall therapeutic potential of a given design. Nanoparticles are predominantly administered intravenously in the majority of pharmacokinetic studies. However, some studies administer the nanoparticles *via* intratumoral injections to circumvent interactions with the circulatory system and to achieve increased accumulation [91-93]. Unfortunately, intratumoral injections have limited clinical relevance since feasibility is limited patients with accessible tumors. In animal experiments, nanoparticles are usually injected intravenously, blood pharmacokinetics are evaluated and organs accumulation is quantified at the experimental end-point.[94]
1.3.1 Blood Serum Interactions

The blood half-life of a nanoparticle will depend on its size, shape and surface chemistry because these affect interactions with serum proteins [95, 96]. Once in the blood, nanoparticles immediately start to interact with serum proteins such as immunoglobulin, lipoprotein, complement and coagulation factors, acute phase proteins, metal-binding, and sugar-binding proteins [97, 98]. These proteins instantaneously bind onto the nanoparticle surface and form a protein corona which dictates the long-term fate, metabolism, clearance and immune response [99]. The adsorption of proteins onto the nanoparticle surface produces a corona thickness of ~20 nm on 50 nm citrate-capped gold nanoparticles [100], ~25 nm on 50 nm SiO$_2$ nanoparticles [101] and ~35 nm on 200 nm PSOSO$_3$ nanoparticles [101]. The corona thickness is much larger than the diameter of any single serum protein and is likely composed of multiple layers. The organization of the protein corona is complex but can be described as hard and soft corona layers. The hard corona contains proteins that are strongly adsorbed to the nanoparticle surface ($K_d \sim 10^{-6}$ to $10^{-8}$ M) [102] while the soft corona layer contains serum proteins weakly interacting with the hard corona layer [103, 104]. This outermost layer is likely to be dynamic and could vary during the course of the nanoparticle’s life in vivo. The nanoparticle’s initial surface features are important in selecting the specific proteins which will make up the corona. Anionic nanoparticles surface were shown to bind plasma proteins with isoelectric points pI > 5.5 and cationic surfaces bind proteins with pI < 5.5 [90]. Usually, protein coronas will produce a surface charge (zeta potential) of -10 to -20 mV regardless of initial surface charge [105-107]. Hydrophobic surface coatings can also adsorb a different set of proteins than hydrophilic coatings [108, 109]. Surface curvature has also been demonstrated to affect protein corona, with high curvature restricting protein-nanoparticle interactions (Figure 1.5). The most abundant serum proteins show a significantly reduced affinity for nanoparticle surfaces when the diameter is decreased from 100 nm to 5 nm [110].

Multiple studies have demonstrated that charged nanoparticle bind more serum proteins and have shorter blood residence times than neutral formulations. Cationic nanoparticles cause hemolysis, platelet aggregation and are often rapidly cleared from the blood [111-114]. The quicker blood clearance is due to the presence of opsonin proteins in the cationic nanoparticle’s protein corona. Opsonin proteins such as immunoglobulins, complement factors and fibrinogen will flag circulating materials as a foreign body and promote uptake by macrophages both in vitro and in
In the body, opsonins trigger the rapid removal of nanoparticles from circulation by the mononuclear phagocyte system (MPS) inside the liver and spleen [119]. Most nanomaterials, when administered into the blood, are quickly taken up by the phagocytic cells of the MPS within minutes or hours. Rapid clearance can be avoided by adding polyethylene glycol (PEG) to the surface of nanomaterials. The addition of PEG drastically increases the blood half-life of all nanomaterials by preventing opsonization. Generally, increasing the molecular length and grafting density of PEG improves the blood half-life of nanoparticles by increasing the thickness of the protective layer [94, 119]. The addition of PEG can increase the blood half-life of a nanomaterial over 100-fold by decreasing MPS uptake [98, 120, 121]. PEG and other molecules have also been used to produce long-circulating “stealth” nanoparticles and improve tumor accumulation [122-124].

1.3.2 Nanoparticle Biodistribution

For circulating nanoparticles, tissue accumulation will depend on the morphological features of the vasculature’s endothelial lining in different regions of the body [125-127]: continuous endothelium is present in the majority of healthy blood vessels; the fenestrated endothelium found in the vasculature of various glands and kidneys possesses 60 nm gaps; discontinuous endothelium found in the liver and spleen’s vasculature contains ~100-150 nm gaps; the unregulated growth of new blood vessels inside the tumor produces large fenestrations between 100-1000 nm in size.[25, 128, 129] Thus, circulating nanoparticles accumulate mostly in tissues possessing permissive endothelium such as the liver, spleen and tumor. Nanoparticles diameter is an important determinant of pharmacokinetics and biodistribution. Nanoparticles smaller than 8 nm are quickly eliminated from the body since they can pass through the kidney’s glomerular capillary walls and get excreted through urine [130, 131]. When nanoparticle diameters increase above 100 nm, there is increased uptake by liver and spleen [94, 132-134].

In regards to tumor accumulation, a liposome study demonstrated that diameters in the 70-300 nm size range produce the highest accumulation [135]. For gold nanoparticles, diameters between 60 and 80 nm nanoparticles show the highest tumor accumulation [94]. However, a study with polymeric micelles demonstrates that tumor accumulation size-dependent trends may be directly related to the type of tumor used [136]. Permeable tumors produced similar accumulation for all nanoparticles in the 30-100 nm size range whereas poorly permeable tumors
only allowed the accumulation of 30 nm nanoparticles. Although tumor type will impact overall accumulation, multiple experiments have demonstrated that nanoparticle accumulation significantly decreases when diameter is increased beyond 100 nm [94, 136-138]. Thus taking into account kidney filtration, MPS clearance and tumor accumulation, most nano-based delivery systems are typically in the 10-100 nm size range when administered intravenously.

1.3.3 Nanoparticle Tissue Transport

Once inside the tumor, nanoparticle diameter also affects accumulation and penetration depth [94]. After exiting the blood vessel, the nanoparticle must navigate through the interstitial space between the cancer cells. This space is filled with a fibrilar network of proteins and polysaccharides called the extracellular matrix. The structural integrity of the extracellular matrix is dictated by the collagen and elastin which are responsible for the architectural organization and mechanical properties of a tissue [139]. Hyaluronan and proteoglycans are large brush-shaped macromolecules composed of sulfated carbohydrates attached to protein cores which fill the gaps in the collagen-elastin scaffold, bind water molecules and produce a hydrogel-like consistency [139]. Laminin and fibronectin are the extracellular matrix’s adhesion proteins and bind to the cells’ integrin receptors [139]. Transport through the tumor’s extracellular matrix can occur by diffusion or convective flow (Figure 1.7). In highly vascularized tumor regions, interstitial fluid will flow from the capillaries through the extracellular matrix in a process known as interstitial flow. In poorly vascularized regions, the elevated interstitial pressure leaves diffusion as the sole mode of transport in the extracellular matrix [140]. The tumor tissue’s lack of functional lymphatic drainage, abnormal vasculature and irregular extracellular matrix composition make it difficult to predict the transport of molecules, antibodies and nanoparticles.

For nanoparticles travelling through the tumor’s extracellular matrix, size is an important determinant of total accumulation and tissue distribution. When nanoparticles diffuse through the extracellular matrix, Brownian motion causes continuous collisions with protein fibers and cells in the tissue. According to Stokes’ law, diffusion will be inversely proportional to nanoparticle diameter due to increased frictional force for larger particles [141]. Nanoparticle diameter can also affect convective transport given that solutes larger than 70 kDa have a reduced velocity in the extracellular matrix [142]. Tumor accumulation studies have demonstrated that nanoparticles with diameters below 30 nm readily diffuse into the tumor tissue whereas diameters above 100
Figure 1.7: **Transport through the tumor.** Transport of solutes from blood vessel to the tumor can occur through diffusion or convection through the basement membrane and interstitial space. Figure adapted with permission from [143]. Copyright (2011) Annual Reviews.
nm remain immobile [94, 136, 137, 144]. In a study by Perrault et al. [94], 20 nm PEG-coated gold nanoparticles were able to travel deep into the tumor tissue but were somewhat cleared by 24h whereas 100 nm PEG-coated nanoparticles did not extravasate far beyond the blood vessel (Figure 1.8). Highest accumulation was observed for 60-80 nm nanoparticles at 24h because this size did not migrate as far as 20 nm but remained in the tumor after 24h. In addition to size, nanoparticle surface chemistry also affects tumor transport. Surface charge can lead to electrostatic interactions with charged components of the extracellular matrix and can accelerate or restrict nanoparticle transport [145, 146]. Nanoparticle shape is also likely to affect tumor tissue transport however this has not been systematically studied to generate conclusive findings.

1.3.4 Modeling Nanoparticle Transport in Vitro

Several groups have analyzed nanoparticle transport kinetics using multicellular tumor spheroids. These three-dimensional micro-tissues are produced by various methods which include rotating culture systems and culture on non-adherent concave surfaces. In most cases, cells spontaneously assemble into spheroids with diameters from 200-1000 µm [147]. When the diameter of a spheroid > 500 µm, the diffusion of nutrients and efflux of metabolites is severely restricted leading to the formation of a necrotic core [147]. The spheroid’s necrotic core is thought to mimic avascular regions of the tumor where low concentrations of oxygen and nutrients restrict proliferation and accumulating metabolites cause cytotoxicity. Surrounding the necrotic core is a quiescent region where cells are viable but non-proliferative. The surface of the spheroid contains proliferative and metabolically-active cells which are analogous to proliferating tumor cells adjacent to blood vessels. Spheroids have been used to evaluate the affinity, diffusivity, distribution and toxicity of various molecules [148-152]. Generally, small molecule drugs and nanoparticles are less effective at killing cancer cells when they are arranged into a spheroid than a monolayer [153-155]. This may be due to a number of factors which include restricted solute diffusion into the spheroid, heterogeneous cell metabolism and altered gene expression. In should be noted that the behavior of cells is significantly different in spheroids compared to monolayers more closely mimicking the gene expression profile of in vivo tumors [156-158].
Figure 1.8: Particle size-dependent permeation of the tumor interstitial space. (a-i) Histological samples were obtained for 20, 60, and 100 nm particle sizes at 1, 8, and 24 h post-injection. Tumor isolates were treated with silver development kit to visualize the position of nanoparticles relative to blood vessels (marked by arrows) (scale bar = 40 μm). (a-c) At 1h, all particle sizes are localized in the perivascular space. (d-f) At 8 h, a size-dependent trend emerges with 20 nm particles migrating far into the interstitial space, 60 nm particles showing limited migration, and 100 nm particles localized at the perivascular space. (g-i) At 24h, few 20 nm particles remain in the region surrounding the blood vessel, the 60 nm particles are seen migrating from the blood vessel, and 100 nm particles have very limited migration. Figure adapted with permission from [94]. Copyright (2009) American Chemical Society.
In regards to nanoparticle uptake and distribution, some spheroid studies have demonstrated that nanoparticle diffusion is size-limited [138, 159]. Nanoparticles larger than 100 nm mostly accumulate in the spheroid periphery (Figure 1.9). Disruption of the extracellular matrix using collagenase greatly increased accumulation of all nanoparticles 20-200 nm in diameter. However, accumulation of 100 and 200 nm nanoparticles was still limited to the spheroid periphery even with the disrupted collagen network suggesting an upper limit to extracellular matrix diffusivity. Multicellular spheroids have had somewhat of a resurgence during the last few years especially in studies evaluating nanoparticle tissue accumulation in vitro. The continued use of spheroids to screen nanoparticle performance can be attributed to their ability to accurately predict accumulation trends in vivo [160, 161].

1.3.5 Active Targeting

It is still uncertain whether the addition of a targeting moiety onto a nanoparticle will improve tumor delivery. Some studies have demonstrated improved tumor accumulation upon addition of a targeting ligand [162, 163]. However, these increases seem limited to nanoparticles < 30 nm and may be exclusive to specific regions of the tumor possessing vasculature with slow blood flow [163]. Generally, addition of a targeting moiety only produces a modest increase in tumor accumulation. This seems to occur with nanoparticles targeting other organs as well. Nanoparticles functionalized with a peptide targeting infarcted regions of the heart only show slight increase compared to nanoparticles functionalized with the scrambled peptide [164]. Although improvements in total accumulation are modest, the addition of a targeting moiety does seem to have a significant effect on intra-tumoral localization. When looking at the distribution of nanoparticles inside the tumor tissue, cell targeting appears to increase cell binding and cell uptake [162, 165]. Multiple studies report that addition of a targeting moiety will shift nanoparticle location from vacant regions of the extracellular matrix to cell membranes [162, 166-169]. This increased association of nanoparticle with tumor cells has been demonstrated to increase cell internalization up to six-fold. Studies looking at a therapeutic effect such as gene knockdown or tumor size reduction, active targeting produced significant improvements [170, 171]. Although these trends are apparent in several studies, it is important to note that specific outcomes depend on the targeting moiety selected. In one study with gold nanorods, peptides
Figure 1.9: Effect of nanoparticle diameter on spheroid distribution. Fluorescence microscopy images of spheroids co-incubated with various sizes of fluorescently-labeled nanoparticles with ("collagen treated"), or without ("not treated") 0.1 mg/mL collagenase. Scale bar represents 200 μm. Figure adapted with permission from [138]. Copyright (2007) DOVE Medical Press.
targeting the tumor vasculature, tumor cells or stromal cells each produced a unique distribution inside the tumor tissue [169].

The binding between nanoparticles and the cells inside the tumor is likely to follow some of the rules of nanoparticle-cell interactions studied in vitro. However, the organization of cells into three dimensional structures creates some problems in vivo. As noted previously, ligand density will dictate avidity for cell receptors and this can slightly improve tumor accumulation [172, 173]. However, if binding affinity is too high it becomes restrictive and decreases tumor accumulation [172]. When the nanoparticle’s binding avidity is too strong, it will bind to the first available receptors it encounters when entering a tissue. This produces a binding-site barrier effect and restricts accumulation in deeper regions of the tissue. This is a common problem with antibody therapies [148, 174], but has also been observed with nanoparticles [162]. An overly high avidity is also restrictive for transport through the tissue’s cells. One study with gold nanoparticles demonstrated that surface density of a targeting ligand could control the rate of transcytosis. When the binding avidity was too high, nanoparticles remained bound to the receptors of peripheral cells and did not enter the target tissue [175]. At lower avidities, nanoparticles bound to cell receptors were internalized by peripheral cells, released into the tissue and internalized by the tissue’s deeper cells. Thus, nanoparticle avidity must be within a specific range to ensure nanoparticle tissue retention while permitting tissue penetration.

Another challenge with in vivo tumor targeting is ensuring that nanoparticle-bound ligands can properly bind to their intended receptors. While PEG improves blood circulation times by blocking the binding of opsonin proteins, it can also block the binding of a targeting moiety to its receptor in vivo [176-178]. The adsorption of serum proteins onto the nanoparticle surface can also restrict ligand-receptor binding and negate the effect of active targeting [179]. Serum proteins can also directly interact with targeting moieties and increase blood clearance by phagocytic cells [169]. Thus, determining the correct concentration, coordination and composition of ligands bound to nanoparticle surfaces is still required to improve the effect of active targeting in vivo.
Based on studies from the last decade, the emerging picture is that active targeting may be a misnomer [19, 24, 180]. Nanoparticles with blood half-lives longer than 6h (PEGylated 10-100 nm) accumulate into the tumor via the EPR effect. Ligands probably do not have an effect at this stage of nanoparticle transport since ligand-receptor attraction requires proximity at the nanometer scale. Once inside the tumor, ligands may improve nanoparticle retention, tumor cell uptake and therapeutic outcomes. Unfortunately, previous studies use different experimental models and obtain contradictory findings making it difficult to properly assess the effect of active targeting in vivo. Similar to the other important questions that remain unanswered in nano-bio studies, it is difficult to draw significant conclusions about in vivo receptor targeting without utilizing new strategies and better experimental models. To improve nanoparticle design and establish the effect of receptor targeting, a more systematic approach is needed where nanoparticle transport and accumulation is evaluated under controlled conditions. It is difficult to draw general conclusions from studies using different nanoparticles, ligands, and tumor models. It is also difficult to determine the mechanism of tumor accumulation given the complex overlapping interactions between nanoparticles, proteins, tissues and cells inside the body.

1.4 Improving Tumor Delivery

1.4.1 Current State of the Field

The main conclusion regarding nanoparticle interactions with biological systems is that nanoparticle design influences the final physiological outcome. Unfortunately, there is still no consensus on what design strategy is most suitable for tumor delivery. Questions such as “what is the best diameter for maximum accumulation?” or “is active targeting necessary to improve accumulation?” remain unanswered. Unfortunately, these questions cannot be addressed using conventional in vitro cell monolayer experiments, because a monolayer configuration of cancer cells exposed to nanoparticles produces a large contact area between the two. At best, the nanoparticle-cell interactions produced in vitro represent the final step in the delivery process. At worst, these in vitro data are meaningless because they have no physiological relevance. The goal of this work is to improve in vitro cell studies by addressing the complexities of tumor cell delivery. This was done by considering the nanoparticle delivery process slightly upstream of cell contact. Prior to encountering a cancer cell, the nanoparticle must travel through the tumor
environment. Tumor transport causes two important concerns in regard to delivery: unequal nanoparticle distribution and nanoparticle instability.

As previously mentioned, tumor distribution of nanoparticles is heterogeneous due to several factors including disordered vasculature, dysfunctional or absent lymphatic drainage, irregular extracellular matrix composition, and varied hydrostatic pressure gradients. In healthy tissues, vasculature is well-organized and follows a hierarchal architecture, supplying cells with nutrients and eliminating metabolic waste. Under physiological conditions, cells are within 100 to 200 μm of the nearest capillary to ensure the proper diffusive exchange with the blood [181, 182]. In tumors, blood vessels are disorganized and branch with unequal diameters leaving large portions of the tissue with no vasculature [183]. The irregular organization of tumor tissue produces many distinct microenvironments where solute transport occurs by a combination of diffusion, convection and transcytosis. A solute’s diffusivity will also vary within different tumor regions since extracellular matrix composition is irregular and denser regions can limit transport [25]. These factors produce heterogeneous accumulation patterns throughout the tumor which are determined by the nanoparticle’s properties and the tumor’s features. In stark contrast, cell culture experiments use a cell monolayer exposed to a uniform dose of nanoparticles. Thus, nanoparticle transport to inaccessible regions is an important physiological concern that must be included in a good experimental model. Evaluating receptor targeting nanoparticles must consider tissue transport in addition to receptor avidity.

Poor tumor vascularization not only affects nanoparticle transport into the tumor, but also creates a unique tissue microenvironment where nutrients and oxygen are depleted and metabolites accumulate. Hypoxia and acidosis are typical features of the tumor environment [184-186]. The prolonged interaction of nanoparticles with the tumor’s microenvironment, extracellular matrix and cells are likely to change their properties. Inside the tumor, destabilization of the nanoparticle surface can lead to degradation, aggregation and remodeling of the protein corona. The final properties of the nanoparticle in the tumor microenvironment constitute its ‘biological identity’ because they will determine receptor binding and cellular interactions. The biological identity of a nanoparticle will depend on its initial properties and the molecules in its tumor environment [187, 188]. By ignoring the potential nanoparticle instability in the tumor tissue, in vitro experiments with fresh cell culture media can produce a different biological identity than
nanoparticles inside the tumor. These different biological identities may produce distinct biological outcomes with little correlation between *in vitro* and *in vivo* models.

1.4.2 Thesis Overview

At this point in the development of nanomedicine, *in vitro* systems are still necessary to analyze the effect of individual parameters on nano-bio interactions. Therefore, this thesis will utilize strategically designed *in vitro* systems to analyze nanoparticle-cell interactions in experimental conditions which are more physiologically relevant than conventional techniques. Analysis of nanoparticle-cell interactions using improved experimental models will provide better insight into nano-bio interactions inside the tumor tissue. These findings can be used to improve the design of tumor-targeting nanoparticles. Since conventional *in vitro* cell uptake experiments represent the final step in nanoparticle delivery, this thesis will focus on characterizing upstream tumor transport and exposure to the tumor microenvironment (*Figure 1.10*). *Chapter 2* will outline the optimization of a novel technique that simultaneously quantifies gold nanoparticles and cell number in a given sample [189]. This technique was necessary to obtain accurate nanoparticle uptake data in the rest of the thesis. *Chapter 3* will present the development of an *in vitro* system called the ‘tumor-on-a-chip’ which allows the study of nanoparticle transport inside a three dimensional tissue [190]. *Chapter 4* looks at the effect of nanoparticle design on stability in the tumor microenvironment by analyzing the impact of cancer cell media conditioning on nanoparticle aggregation and protein corona composition. *Chapter 5* presents a novel technique to synthesize nanoparticle aggregates with customizable size and surface chemistry. *Chapter 6* employs the aggregation synthesis technique to analyze the effect of nanoparticle aggregation on cell uptake and toxicity [191]. Finally, *Chapter 7* will provide a brief summary of the thesis’ findings and suggest some future experiments for the continuation of this work.
Figure 1.10: Transport of nanoparticles in tumor. (1) The nanoparticles must first be transported through the tumor’s extracellular matrix before reaching their target cells. (2) The nanoparticles are exposed to the tumor’s microenvironment which contains uncleared cellular metabolites. The extracellular environment alters nanoparticle properties through adsorption of proteins and the formation of aggregates. (3) The modified nanoparticles then interact with tumor cells differently than the initial nanoparticles.
2 Simultaneous quantification of cells and nanomaterials by inductive-coupled plasma techniques

2.1 Introduction

When investigating nano-bio interactions, nanoparticles are usually administered to cells \textit{in vitro} or administered systemically \textit{in vivo}. At the experimental end point, total accumulation of nanoparticles in cells or tissues is quantified using a variety of techniques including fluorescence and radio labeled markers [192, 193]. Inductively-coupled plasma (ICP) techniques are the most frequently used approach for quantifying metal or semi-conductor nanoparticle in biological specimens. ICP is coupled with either with atomic emission spectroscopy (ICP-AES) or mass spectrometry (ICP-MS). These techniques represent the gold standard for measuring the amount of nanoparticles in biological sample when nanoparticles are composed of rare physiological atoms such as gold, silver, cadmium, iron or other metal atoms [68, 194-197]. ICP techniques use electromagnetic induction to produce an argon plasma whose temperature can range from 6,000-10,000K, which is enough to break most molecular and ionic bonds. For ICP-AES, a sample’s atoms are excited by the plasma and a spectrometer is used to resolve and quantify the electromagnetic radiation emitted by the various atoms. In ICP-MS, the plasma is used to atomize and ionize the sample and the mass-to-charge ratio is obtained for each ion using a mass spectrometer.

When using ICP techniques on cell culture samples [33, 53, 68], a solution of nanoparticles is applied to a cell monolayer and incubated for a given amount of time. The nanoparticles are removed, the cells are washed, and detached from plastic culture plates using a trypsin solution. Cells must then be counted with a hemocytometer using viability dyes such as propidium iodide or Trypan blue. Samples are then acid digested and analyzed by ICP-based techniques. When performing an experiment, using two separate techniques to quantify cell number and nanoparticle concentration is a relatively slow process and leads to significant experimental variability between the replicates. In order to improve experimental accuracy, previous studies
have added rare elements such as uranium into biological samples to determine cell number using ICP-MS [198]. In this chapter, I investigated whether the cell’s endogenous ions (e.g., magnesium, sulfur, calcium, etc.) could be used to quantify cell number using ICP-AES. This technique would improve current gold nanoparticle quantification protocols since it allows for the simultaneous quantification of nanomaterials and total cell number using one sample and one technique.

2.2 Material and Methods

2.2.1 Analysis of Cell’s Elemental Content

HeLa and MDA-MB-435 cells were cultured in DMEM and RPMI, respectively, supplemented with 10% fetal bovine serum. The day of the experiment, cells were detached from cell culture flasks with a cell scraper and counted using Trypan blue exclusion assay. Cells were added at specific numbers into separate tubes, centrifuged at 400g for 5 min at 4°C and washed with PBS. For serum starvation experiments, 2 x 10^6 cells were seeded in 6-well plates overnight and the following day, serum supplemented media was removed and cells were incubated with media containing 0.2% bovine serum albumin (BSA).

2.2.2 ICP-AES Analysis

Prior to ICP-AES analysis, samples were lysed with 500µl nitric acid, transferred into 1.5 mL conical tubes and incubated at for 30 min in a 70°C water bath. Samples were then chilled on ice for 1 min, pulse centrifuged to collect all the liquid and diluted in 3 mL double-distilled ultra pure water. All conditions were performed in triplicate. Samples were analyzed using a Perkin-Elmer Optima 3000 ICP-AES system. Sample introduction conditions were: Teflon Mira Mist nebulizer, 0.5 L/min auxiliary argon flow, 1.1 L/min sample flow, 0.8 L/min nebulizer gas flow, high purge gas flow. Plasma was set to 1500W with a gas flow of 15 L/min. Measurements were performed with a read delay of 45s, an integration time of 5-10s and performed in triplicate. Aqueous calibration curves of Au, Mg, and Cd were prepared from certified elemental standards (High Purity Standards, Charleston SC, USA).

2.2.3 Nanomaterials Synthesis

575nm-emitting CdSeS/ZnS quantum dots (Trilite™) were purchased from Cytodiagnostics (Burlington, ON Canada). The quantum dots were rendered water-soluble via a ligand-exchange
procedure with thioglycolic acid adapted from Chan et al [17]. The 15 nm gold nanoparticles were synthesized using the Frens method [26] with reagents purchased from Sigma-Aldrich. The 70, 85 and 90 nm gold nanoparticles were synthesized using hydroquinone-mediated growth of the 15 nm seeds [28]. Nano-urchins were prepared using hydroquinone-mediated growth of the 15 nm seeds described by Li et al [199]. Nanoparticle and nano-urchin diameters were confirmed by dynamic light scattering and electron microscopy. The hydroquinone synthesized nanomaterials were washed thrice using a solution of 3.4 mM sodium citrate tribasic (Ci). Protein adsorption was performed using 0.34 mM Ci and 4 mg/mL transferrin. After 1h incubation at 37°C, bovine serum albumin (BSA) was added to the transferrin-coated AuNPs at a 0.1% concentration to ensure AuNP stability during centrifugation steps. The AuNPs were washed twice in a 0.1% BSA solution and used in cell uptake experiments.

2.2.4 Cell Uptake Studies

The cell uptake assays were performed on cells pre-incubated overnight in 6-well plates at 85% confluency at the start of experiment. The following day, media was removed and cells were exposed to nanomaterials in serum-free media (RPMI + 0.2% BSA). At the end of the experiment, cells were washed thrice with PBS and frozen as a monolayer until analysis by ICP-AES. For gold nanoparticle studies, we added $1.2 \times 10^{11}$ 15 nm nanoparticles in 1 mL of media and compared it to the equivalent volume of gold for 70 nm ($2.80 \times 10^9$) and 90 nm ($1.32 \times 10^9$) AuNPs. For nano-urchin studies, 5 pM ($3 \times 10^9$ total) of either urchins or size-matched nanoparticles were added to A549 cells. Total number of urchins and nanoparticles was determined by calculating their molecular weight. The molecular weight of spherical nanoparticles was calculated using the average diameter measured by electron microscopy, material density and atomic weight. The number of gold atoms per nano-urchin was calculated by dividing the total number of gold atoms required for synthesis by the total number of 15 nm gold nanoparticles added as seeds for hydroquinone-mediated growth.

2.3 Results & Discussion

2.3.1 Evaluating Intracellular Elemental Content

We evaluated nine elements – Ca, Cu, Fe, K, Mg, Na, P, S, and Zn – for quantifying cell numbers using ICP-AES (Figure 2.1). These elements were chosen since they all possess well-
known roles in cell function and general metabolism. To determine how each of these elements correlated with total cell numbers, we prepared nitric acid digestates ranging from 0.25 to $5 \times 10^6$ MDA-MB-435 or HeLa cells. Of these nine elements, Mg showed the highest correlation with cell number followed by S and Zn (Table 2.1). All three elements showed strong correlation ($R^2 > 0.96$) with total cell number in both cell lines. Interestingly, correlation was consistently higher in HeLa cells for all the elements tested. Certain elements like Ca and Cu showed poor correlation and this may be due to quantities below the detection limit of the ICP-AES. Other elements such as Na and K also show poor correlation due to the presence of these elements in the phosphate-buffered saline used to wash cells prior to acid digestion.

2.3.2 Stability of Intracellular Elemental Content

It was important to evaluate whether change in the cell’s metabolic state could impact the intracellular content of Mg, S, or Zn since this could affect cell quantification. To evaluate the effect of cellular metabolism, cells were serum-starved for up to 6 hours. Over this time, intracellular content of Mg, S and Zn showed some slight variation (Figure 2.2). By 6h, Zn content increased < 5%, Mg increased 5-10% and S increased 20-30%. The large change in S suggests a time-dependent increase of albumin in the digestates. Each albumin molecule contains 35 cysteines and by extension 35 S atoms. Both the Zn and Mg showed small but significant increases, which may be due to a change in cellular metabolism.

2.3.3 Validation of Magnesium as a Cell Marker

These three elements would be suitable for ICP-AES-based cell quantification. However, measuring all three would be redundant and we proceeded to pick the most advantageous candidate for nanomaterial-cell studies. Unfortunately, ZnS is often used to cap the surface of quantum dots and this eliminates both S and Zn as markers for cell numbers. Also, the abundance of S atoms in many proteins makes this element a poor candidate for cell quantification. Since Mg is not present in many nanomaterials and showed the highest
Table 2.1: Slopes and goodness of fit values for data in Figure 1.

<table>
<thead>
<tr>
<th>Element</th>
<th>MDA-MB-435 Slope ±</th>
<th>R²</th>
<th>HeLa Slope ±</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca 317.933</td>
<td>149.0 ± 55.74</td>
<td>0.3938</td>
<td>209.4 ± 60.93</td>
<td>0.5415</td>
</tr>
<tr>
<td>Cu 327.393</td>
<td>31.14 ± 10.54</td>
<td>0.421</td>
<td>38.70 ± 3.163</td>
<td>0.9374</td>
</tr>
<tr>
<td>Fe 238.204</td>
<td>40.18 ± 3.629</td>
<td>0.9109</td>
<td>166.5 ± 3.636</td>
<td>0.9953</td>
</tr>
<tr>
<td>K 766.490</td>
<td>148234 ± 16716</td>
<td>0.8676</td>
<td>169361 ± 16313</td>
<td>0.9151</td>
</tr>
<tr>
<td>Mg 279.077</td>
<td>227.0 ± 6.272</td>
<td>0.9909</td>
<td>297.8 ± 2.069</td>
<td>0.9995</td>
</tr>
<tr>
<td>Na 589.592</td>
<td>217761 ± 274735</td>
<td>0.04975</td>
<td>317095 ± 265701</td>
<td>0.1247</td>
</tr>
<tr>
<td>P 178.221</td>
<td>75.04 ± 7.647</td>
<td>0.8892</td>
<td>174.4 ± 8.770</td>
<td>0.9754</td>
</tr>
<tr>
<td>S 180.669</td>
<td>48.61 ± 2.047</td>
<td>0.9792</td>
<td>82.58 ± 0.7493</td>
<td>0.9992</td>
</tr>
<tr>
<td>Zn 213.857</td>
<td>51.80 ± 2.759</td>
<td>0.9671</td>
<td>130.3 ± 4.478</td>
<td>0.9883</td>
</tr>
</tbody>
</table>
Figure 2.1: Elemental content of cells. Signal in counts per minute for each element analyzed by ICP-AES in MDA-MB-435 (black boxes) and HeLa (open circles) cells.
Figure 2.2: Effect of nanomaterial uptake on intracellular magnesium. Change in elemental composition of Mg, S and Zn as quantified by ICP-AES. MDA-MB-435 (black boxes) and HeLa (open circles) cells were serum starved for various amounts of time and analyzed. Data presented as the spectral counts per minute detected using ICP-AES.
correlation with cell number, we tested whether this element was a suitable for cell quantification. Mg plays an important role in biological systems due to its catalytic properties. Mg\(^{2+}\) is one of the most abundant divalent metal ions in cells, participating in many cellular processes and the regulation of hundreds of enzymatic reactions [200]. For example, Mg ions bind to polymerases and act as a cofactor in DNA and RNA synthesis. Also, intracellular Mg concentration is similar for both apoptotic and intact cells [201]. The high cellular content of Mg and its stable intracellular concentration make this element suitable for cell quantification by ICP.

To validate this proposed technique, cell standards were prepared from 5 different cell lines and compared to an Mg standard (Figure 2.3). All cells showed an excellent correlation with Mg concentration \((R^2 > 0.99)\) albeit with different slopes (Table 2.2). When comparing the calculated slope for each cell line, we noticed a correlation with each cell’s volume (Table 2.2) [196, 202-204]. Generally, smaller cells, such as the RAW 264.7, possessed a smaller slope (0.0398) compared to the much larger SK-BR-3 cells (0.1917). However, Hep2G and SK-BR-3 cells have similar slopes and are drastically different sizes, suggesting that cell phenotype also influences the total content of Mg per cell type. These findings demonstrate excellent correlation between cell number and Mg concentration in acid-digested samples. Since each cell type possesses its own intracellular concentration of Mg, reference samples are necessary for accurate quantification of each cell line tested. Based on the serum starvation studies, it is necessary to prepare the reference samples with cells exposed to the same experimental conditions used in order to account for small but significant fluctuations in intracellular Mg concentrations.

### 2.3.4 Effect of Nanoparticle Uptake on Intracellular Elemental Content

Once Mg-based cell quantification was validated, it was important to verify whether incubation with nanomaterials would alter intracellular elemental concentration. Two frequently used nanomaterials are gold nanoparticles and CdSeS/ZnS quantum dots. To determine the effect of nanomaterial uptake on intracellular Mg concentration, we incubated cells with these nanomaterials for 6h and monitored the differences in Mg concentration. Based on previous experiments, 6h incubation was long enough to ensure > 80% of maximum nanomaterial uptake
**Table 2.2**: Characterization of various cell lines and their correlation between number and Mg content.

<table>
<thead>
<tr>
<th>cell line</th>
<th>slope</th>
<th>( R^2 )</th>
<th>cell volume ((10^3 \text{ um}^3))</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sk-Br-3</td>
<td>0.1971</td>
<td>0.999</td>
<td>21.9</td>
<td>mammary gland adenocarcinoma</td>
</tr>
<tr>
<td>Hep2G</td>
<td>0.1747</td>
<td>0.995</td>
<td>2.6</td>
<td>liver hepatocellular carcinoma</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>0.1198</td>
<td>0.999</td>
<td>3.5</td>
<td>melanoma cell</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.1183</td>
<td>0.997</td>
<td>2.4</td>
<td>cervix adenocarcinoma</td>
</tr>
<tr>
<td>Raw 264.7</td>
<td>0.0398</td>
<td>0.998</td>
<td>1.68</td>
<td>leukaemic monocyte macrophage</td>
</tr>
</tbody>
</table>


Figure 2.3: Quantification of cells using magnesium. Correlation between magnesium content and cell number for multiple cell lines using ICP-AES.
We tested 15, 70 and 90 nm gold nanoparticles coated with transferrin protein and incubated these nanoparticles with three different cell lines. The inclusion of transferrin on the surface of nanoparticles was to promote receptor-targeted endocytosis, a common strategy for nanomaterials uptake. The three cell lines we used expressed the transferrin receptor [191, 205] and demonstrated significant uptake of gold nanoparticles without any changes to cellular Mg content (Figure 2.4a). All three cell lines show much higher uptake of the 90 nm nanoparticles than 15 nm. Uptake of the 70 nm nanoparticles was similar to 15 nm in SK-BR-3 and HeLa cells but significantly higher in MDA-MB-435 cells, demonstrating some cell phenotype-based variability in uptake. Similar findings were also observed for thioglycolic acid-capped 575 nm-emitting CdSeS/ZnS quantum dots. When Hep2G and RAW264.7 cells were incubated with quantum dots for 6h, some cell uptake was apparent after 6h with no change in cellular Mg content (Figure 2.4b). Similar to gold nanoparticles, the quantum dots also show distinct uptake quantities based on cell phenotype. In regards to cell quantification, our data show that uptake of gold nanoparticles and quantum dots do not significantly alter intracellular Mg concentrations. These findings confirm the accuracy of this cell quantification technique in nanomaterial uptake studies.

2.3.5 Application of the Technique on Nano-Urchin Uptake

To highlight the advantages our technique, we decided to assess the effect of nano-urchin (or nano-star) shape on cell uptake. These novel nanostructures possess interesting optical properties which make them excellent candidates for the design of surface-enhanced Raman scattering (SERS) probes and hyperthermia-based therapies [206]. However, their characteristic protrusions render their surface asymmetrical and pointy. Since most uptake studies focus on relatively smooth nanomaterials (spheres, cubes, prisms), we wanted to determine whether the spikes on the surface of a gold nanosphere would alter cellular uptake. We used the A549 lung carcinoma cell line to compare the uptake of transferrin-coated nanoparticles and nano-urchins over various time points. Cells were incubated with the nanomaterials, washed and lysed directly on the plate with nitric acid. In addition to the cell digestates, we prepared five Au standards, five Mg standards and two duplicate A549 references counted by Trypan Blue exclusion assay (1x10^6 and 2x10^6 cells).
Figure 2.4: No effect of nanomaterial uptake on intracellular magnesium concentration. Cells were incubated with transferrin-coated gold nanoparticles (a) or thioglycolic acid-coated CdSe QDs (b) for 6h and both Mg and Au was quantified in samples by ICP-AES. Data shown is the average and standard deviation of three replicates.
Based on our elemental standards and cell references, we were capable of quantifying both the number of nanostructures and total cell number per digestate utilizing only ICP-AES (Figure 2.5). The Mg content of most samples did not change with any statistical significance, except for the 15h time point where we noticed a ~10% increase in Mg likely due to cell proliferation. For a given set of experimental replicates, normalizing Au concentration to Mg reduced the standard error compared to the Au values alone. The gold uptake studies revealed a decreased internalization rate for the nano-urchins compared to nanoparticles during the first 2h (170.5 vs. 343 cell\(^{-1}\) h\(^{-1}\)). Beyond the 2h time point, no significant differences were detected. The differences observed in the early time points leads to a different uptake half-life for the nanoparticles (3.18h) compared to nano-urchins (4.35h). These findings reveal a decreased uptake of nano-urchins by A549 cells during short exposure times, likely due to irregular morphology.

2.4 Conclusion

Our findings confirm that Mg can be used to quantify cell numbers in a given sample. Using this approach, it is possible to resuspend the nanomaterials-treated cell monolayer with nitric acid directly in the cell culture plate, eliminating the need for trypsinization. Our method provides a label-free ‘internal standard’ to ensure that the quantity of nanomaterials detected in a given sample is for a specific number of cells. This technique reduces the variation coefficient between replicates by minimizing sample loss-related errors and will speed up the analysis of nanoparticle interaction with cells. The quantification of cellular elements such as Mg or Zn can also be used to assess the effect of nanoparticles on the state of a cell. For example, during long experiments, this technique can account for either cytotoxicity or cell proliferation and eliminate any sample-to-sample bias caused by a change in cell numbers. This technique will be employed in Chapters 4 and 6 to quantify in vitro cell uptake of nanomaterials.

2.5 Author Contributions

This chapter was adapted from [189]. The proposed technique and experiments were conducted by A. Albanese. Magnesium content and quantum dot uptake data from Raw 264.7 and HepG2
cells was obtained from K. Tsoi. The original manuscript was written by A. Albanese and edited by W. Chan.
Figure 2.5: Cell uptake of nano-urchins. (a) UV-Vis spectra of 15 nm gold nanoparticle seeds, 80 nm nanoparticles and urchins. (b) Electron microscope images of urchins and 80 nm nanoparticles. Scale bar = 100 μm. (c) A549 cells were incubated with 5pM transferrin-coated 80 nm gold nanoparticles (sphere) or nano-urchins (urchin). *p<0.05, ***p<0.001
3 Tumor-on-a-chip provides an optical window into nanoparticle tissue transport

3.1 Introduction

The previous chapter looked at cell-nanoparticle interactions using highly simplified in vitro conditions. Transferrin-coated nanoparticles were incubated with a cell monolayer expressing the CD71 transferrin receptor. This type of configuration is typically inaccurate at predicting the in vivo performance of tumor-targeting nanomaterials. Improving the design of tumor-targeting nanomaterials requires a better understanding of how nano-sized blood-borne carriers enter their target tissue and distribute at the cellular level. In vivo animal models are useful for assessing general pharmacokinetics but make it impossible to evaluate tumor tissue transport independent of blood clearance rate, vascular permeability, tissue clearance and lymphatic drainage [150]. Animal studies are also hindered by the limited resolution of whole-animal imaging techniques which makes it difficult to visualize target tissues at the cellular level [163]. As a result, our knowledge of nanoparticle-cellular interactions is predominantly derived from in vitro cell monolayer experiments [38]. These studies typically characterize the binding affinity, uptake, cellular responses, and toxicity of these agents [53]. However, the lack of three-dimensional tissue architecture and dynamic flow conditions eliminate tissue transport from the experimental conditions. Tissue transport produces heterogeneous distribution of nanocarriers and each cell will be exposed to a different concentration. By excluding tissue transport, traditional in vitro experiments are inaccurate at predicting in vivo outcomes.

Microfluidic devices offer a highly customizable microscale platform to evaluate complex cellular configurations using microliter amounts of reagents under controllable flow conditions [207]. Microfluidic devices can be used to culture cells [208], create microenvironments [209], mimic organs [210] or evaluate organ tissue ex vivo [211]. Several in vitro setups have been used to evaluate drug transport mechanisms but not for studying the role of drug-carrier design in tumor transport. These studies do not provide a definitive conclusion on how the physicochemical properties of a molecule or nanoparticle will affect transport through the tumor tissue because these studies have mostly utilized small organic drug molecules (e.g. doxorubicin,
paclitaxel). Although there are various permutations of microfluidic chip designs for mimicking tumor systems and for analyzing drug transport, none have been validated using animal models, and therefore, it is difficult to assess the accuracy of these microfluidic devices for predicting in vivo outcome. Nevertheless, such studies highlight microfluidics as potential strategy for the rapid and cost-effective pre-screening of drugs while also providing sufficient data to model the transport process prior to full animal studies [207]. The optimal microfluidic design to mimic in vivo tumor systems remains unclear but is a very active research area.

In this chapter, the objective was to develop and apply a tumor-on-a-chip microfluidic platform to study the transport of synthetic carriers through a complex three-dimensional tissue environment. The tumor-on-a-chip technology was used to address an important question in nanotechnology: “how does the size and surface chemistry of a nanoparticle influence transport within the tumor tissue under various flow conditions?” While it is well-recognized that nanoparticle size, shape, and surface chemistry can affect tumor accumulation [38], the transport kinetics, retention and the role of interstitial flow rate remain unclear. This tumor-on-a-chip experimental platform was used to address these questions and data was validated using a murine human tumor xenograft model. This chapter demonstrates that the tumor-on-a-chip provides an accurate in vitro method to predict nanoparticle transport in vivo.

3.2 Methods

3.2.1 Fluorescent Nanoparticle Synthesis

The 15 nm gold nanoparticles were synthesized by citrate reduction method. The 30, 70, and 100 nm gold nanoparticle were synthesized using hydroquinone-mediated growth of the 15 nm seeds [28]. Size and polydispersity was verified for each batch using a Malvern Zetasiszer instrument. Nanoparticles were washed thrice using 340µM sodium citrate tri-basic (Ci) and functionalized with PEG (Laysan) or transferrin (Tf) (Sigma). PEG-NPs were prepared by adsorbing a mixture of 15% NH$_2$-PEG-5k-SH and 85% CH$_3$-PEG-5k-SH to 15 nm NPs for 1h at 60°C [212]. For 30, 70 & 100 nm, we used the same conditions with 15% NH$_2$-PEG-10k-SH and 85% CH$_3$-PEG-10k-SH. The longer PEG was necessary to prevent gold nanoparticle-mediated quenching of fluorescence [212]. PEG-NPs were centrifuged and washed twice with 0.02% Tween-20 + 340µM Ci buffer (Sigma), washed once with 340µM Ci buffer and incubated with Cyto633-NHS ester (Cytodiagnostics) overnight at 4°C. PEG-NPs were centrifuged and washed three times as
described in the previous step. The initial intent was to normalize nanoparticle dose to administer the same surface area as 25 nM of 15 nm nanoparticles for the larger sizes. However, due to higher scattering and overall optical density of the nanoparticle solutions it was difficult to detect signal fluorescence. When we diluted the nanoparticles ~1/200, we were able to achieve high fluorescence and used these dilutions indicated in (Table 3.1).

3.2.2 Fluorescently-Labeled Transferrin Nanoparticles

Cyto633-transferrin was prepared by incubating Cyto633-NHS ester (Cytodiagnosics) at a 10-to-1 molar excess with transferrin in 200 mM carbonate buffer pH 8.3. The reaction was incubated at 4°C overnight and purified twice using a NAP-5 desalting column (GE Healthcare) equilibrated in 0.1X PBS. The dye-to-protein ratio was ~5 as determined by UV-Vis spectrometry according to the manufacturer’s instructions. Tf-Cyto-633 conjugate was still functional as it could bind to Tf-receptor expressing cells as assessed by flow cytometry. Binding of the Tf-Cyto-633 conjugate was blocked by native Tf protein at a 1:1 molar ratio. The Tf-NPs were functionalized by passive adsorption with Cyto633-labelled transferrin for 1h at 37°C and washed three times in 340µM Ci buffer + 0.1% serum albumin. We checked for the stability of Tf-NPs by incubating the nanoparticles in imaging media for 1h and did not detect and desorption of Tf from the nanoparticle surface.

3.2.3 Cell Culture Conditions

Spheroids were prepared with MDA-MB-435 (ATCC) cells as previously described [213]. Briefly, round bottom 96-well plates were coated with Polyhydroxyethyl methacrylate (Sigma), 750 to 1500 cells were placed in each well and centrifuged at 800g for 10 min. Spheroids were grown for 3d in RPMI supplemented with 10% fetal bovine serum and 2.5% basement membrane extract (BD Matrigel) and measured using optical microscopy. Spheroids measuring 260-280 µm were loaded into microfluidic devices and used for experiments.
Table 3.1: Characterization of *in vitro* nanomaterials. The hydrodynamic diameter and polydispersity index as obtained from dynamic light scattering.

<table>
<thead>
<tr>
<th>Surface</th>
<th>HD * (nm)</th>
<th>PDI</th>
<th>zeta † (mV)</th>
<th>TEM ‡ (nm)</th>
<th>Dose (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40nm PEG-NP</td>
<td>39.53</td>
<td>0.132</td>
<td>-17.50 ± 0.70</td>
<td>14.10 ± 1.05</td>
<td>25</td>
</tr>
<tr>
<td>Tf-NP</td>
<td>31.07</td>
<td>0.136</td>
<td>-28.33 ± 2.42</td>
<td>14.10 ± 1.05</td>
<td>25</td>
</tr>
<tr>
<td>70nm PEG-NP</td>
<td>72.27</td>
<td>0.12</td>
<td>-16.53 ± 0.81</td>
<td>29.30 ± 5.14</td>
<td>0.3</td>
</tr>
<tr>
<td>100nm PEG-NP</td>
<td>114.8</td>
<td>0.042</td>
<td>-23.40 ± 0.36</td>
<td>70.04 ± 4.69</td>
<td>0.0078</td>
</tr>
<tr>
<td>150nm PEG-NP</td>
<td>149.8</td>
<td>0.002</td>
<td>-22.70 ± 0.17</td>
<td>96.24 ± 8.66</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

* Hydrodynamic diameter (HD) and polydispersity index (PDI) were obtained using dynamic light scattering

† Zeta potential was obtained using dynamic light scattering

‡ Nanoparticle core diameter was obtained using transmission electron microscopy (TEM) and is presented as mean ± standard
3.2.4 Microfluidic Device Fabrication

Microfluidic devices were fabricated with polydimethylsiloxane (PDMS) (Dow-Corning) as previously described [214]. The cured mold was removed from the epoxy resin master and irreversibly bonded to a 24x50 mm cover slip (VWR Scientific) using oxygen plasma (Harrick Scientific). Tygon tubing was inserted directly into the cored-out port holes. The microfluidic device was designed with a channel height of 250 µm to slightly compress the ~280 µm spheroids against the glass cover-slip to ensure adequate immobilization and optical definition. The channel entrance was 600 µm wide and widened further to 1200 µm at the imaging chamber to reduce the fluid linear velocity and ensure gentle motion of spheroids before arriving at the dam wall to minimize physical damage. The width of the imaging chamber also allowed solution to flow around the spheroid inducing a pressure drop across the tissue and generate convective flow through the tissue [214]. Spheroids were gently loaded into the microfluidic device using gravitational flow of imaging media (0.2% bovine albumin, 125 mM NaCl, 5.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES, pH 7.4) [215]. Interstitial flow rates were quantified by measuring the time required to fill the interstitial space with 0.1 mM 10kDA Dextran-568 (Invitrogen). An image was taken every second while dextran was flowed into the device. We measured the time between the appearance and disappearance of the non-fluorescent “tail” exiting the spheroid [214]. Administration of dextran at the various flow rates was performed on three separate spheroids. For extracellular matrix staining we used 10 µg mL⁻¹ anti-Laminin-FITC (Abcam).

3.2.5 Confocal Microscope Conditions

The microfluidic device was mounted on a microscope stage inside a 37°C incubator (Okolabs). Flow rate was controlled using a syringe pump. Nanoparticle accumulation images were taken using a 40X or 63X oil-emersion objectives using 633 nm excitation and 650-700 nm emission on a Zeiss LSM710 confocal microscope. Images were taken at 10 µm depth intervals every 5 or 10 min for 1h. Fluorescence distribution was analyzed in ImageJ (freeware, NIH) by drawing a contour around the spheroid freehand and a macro was developed to measure the mean fluorescence at various depths throughout the spheroid (1.38 µm increments of radial
contraction). This produced a ‘fluorescence vs. radial depth’ graph and nanoparticle accumulation was obtained by taking the mean fluorescence of the spheroid’s first 75 µm and normalizing it to the fluorescence of the surrounding media (administered dose). For each spheroid four separate Z-plane images were averaged per time point. All experiments were performed on at least 3 spheroids.

3.2.6 Silver Staining Procedure

For each experimental condition, 20-30 spheroids were harvested from 96-well plate into 1.5 mL tubes and treated with 25 nM PEG-NP or Tf-NP. Spheroids were washed once with PBS, fixed with 10% buffered formalin solution and embedded in low melt 3% agarose solution. Spheroids were then embedded in paraffin, sectioned, and were stained with hematoxylin, eosin and a silver staining kit (Ted Pella). Samples were analyzed by optical microscope and nanoparticle accumulation was determined by obtaining the sum intensity of silver stain in each spheroid section.

3.2.7 Interstitial Flow Rates

Image analysis reveals that ~15% of a spheroid’s volume is its interstitial space. For a spheroid 280 µm in diameter, the volume of the interstitial space is $1.72 \times 10^{-12}$ m$^3$. Because the interstitial spaces are heterogeneous in shape and dimensions, it is impossible to obtain the area of each space’s cross section. Instead, we modeled the interstitial space as a network of 1 µm channels that pass through the centre of the spheroid. The length of each “interstitial channel” in this model is equal to the spheroid diameter (280 µm). This organization will produce 100% flow through channels in parallel with the microfluidic flow and 0% in perpendicular channels. This concentric symmetry will produce an average flow efficiency of 50%. In a sphere, we need to consider all three planes, so the overall flow efficiency is 50% in each plane totaling 12.5%.

Assuming that the shape and orientation of the interstitial channels remain constant regardless of the pump’s flow rates, the cross-sectional area (A) of each channel is:

$$A = (3.1416) \left(1 \times 10^{-6} \text{ m} \div 2\right)^2 = 7.854 \times 10^{-13} \text{ m}^2$$

The volume (V) of each channel is:

$$V = A \times \text{length} = (7.854 \times 10^{-13}) \times (280 \times 10^{-6}) = 2.2 \times 10^{-16} \text{ m}^3$$
In our spheroids, interstitial space represents \(1.72 \times 10^{-12} \text{ m}^3\) and this translates into 7,836 channels with 12.5% flow efficiency \((E)\). The average flow rate \((Q)\) through each channel will be:

\[
Q_{\text{IS channel}} = \frac{\text{Efficiency} \times Q_S}{\# \text{ channels}}
\]

where \(Q_S\) is the flow through the spheroid which was determined experimentally using 10 kDA Dextran-568. Once the flow rate through the interstitial channels is obtained, it is possible to convert it to a velocity \((V)\) using the following formula:

\[
V = \frac{Q_{\text{IS channel}}}{A}
\]

This allowed us to produce Table 3.3 and Figure 3.1c.

### 3.2.8 Calculating the Rate of Nanoparticle Accumulation

Flow of nanoparticles in the microfluidic channel where a spheroid is held stationary causes a diffusion of nanoparticles into the tissue. Once in the spheroid, the nanoparticles can accumulate in or leave the tissue. In this context, the spheroid can be viewed as one functional unit with inputs and outputs.

A mathematical model was developed that accounts for the incoming and outgoing activities in the spheroid. It can be assumed that the rates at which nanoparticles enter \((k_{\text{IN}})\) and exit \((k_{\text{OUT}})\) the spheroid are proportional to the concentrations of nanoparticles present in inlet reservoir \((\text{dose or } C_D)\) and spheroid \((C_S)\), respectively. Therefore, the time dependent value of nanoparticle accumulation in the spheroid is the resultant of the inputs and outputs. Putting these notions together yields:

\[
\frac{dC_s}{dt} = k_{\text{IN}} C_D - k_{\text{OUT}} C_s
\]  
\text{(Eq. 1)}
The concentration of nanoparticles in the inlet reservoir \((C_D)\) remains constant independent of time; one can therefore simplify the equation for \(C_S\) by normalizing it to \(C_D\) (dividing both sides of Eq. 1 by \(C_D\)), as shown by Equation 2:

\[
\frac{dC_S}{dt} = k_{IN} - k_{OUT}C_S \tag{Eq. 2}
\]

Where \(C_S\) is the dimensionless concentration of nanoparticles accumulated in the spheroid normalized to dose \((C_D)\), \(k_{IN}\) is the rate constant at which the NPs enter the spheroid \((\text{min}^{-1})\), \(k_{OUT}\) is the rate constant at which the nanoparticles leave the spheroid \((\text{min}^{-1})\), \(t\) is the independent variable, time (min). Equation 2 is a linear first order differential equation with one dependent variable with respect to time, \(C_S\). Solving Equation 2 via the integrating factor, \(\exp(\int k_{OUT} \, dt)\) or \(\exp(k_{OUT} \, t)\) yields:

\[
C_S = \frac{k_{IN}}{k_{OUT}} [1 - \exp(-k_{OUT}t)] \tag{Eq. 3}
\]

Equation 3 has two rate constants. To solve for them empirically, one can substitute two experimental data points for \((C_S, t)\) into Equation 3 and solve for the resulting set of two linear equations with two variables, \(k_{IN}\) and \(k_{OUT}\).

### 3.2.9 In Vivo Tumor Model Specifications

To inoculate tumors, MDA-MB-435 cells collected by cell scraping, concentrated by centrifugation, resuspended in 50% Matrigel (BD), and \(3 \times 10^6\) cells in 150µL were injected subcutaneously into the mammary fat pad on the flank of 6-week old female CD1 nude athymic mice (Charles River) and allowed to grow for three weeks. Mice bearing tumors with a 1 cm cross-sectional diameter were tail-vein injected with each nanoparticle formulation (Table 3.2). The quantity of nanoparticles injected was normalized to achieve a surface area of \(7 \times 10^{15} \text{ nm}^2\). Tumor fluorescence was then monitored by whole animal fluorescence using a Carestream In Vivo FX Imager under isofluorane-enriched oxygen. Dorsal images of mice were taken at 0.17, 2, and 48h post-injection at a 10 min exposure with excitation and emission wavelengths of 750nm and 830nm respectively. Tumor accumulation of nanoparticles was assessed using a method published by Chou et al. [212] Briefly, mean fluorescence of the tumor and opposing
Table 3.2: Characterization of *in vivo* nanomaterials. The hydrodynamic diameter and polydispersity index as obtained from dynamic light scattering.

<table>
<thead>
<tr>
<th>Surface</th>
<th>HD (nm) *</th>
<th>PDI</th>
<th>zeta † (mV)</th>
<th>TEM ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% 10kDa PEG-Alexafluor750 50nm PEG-NP</td>
<td>46.3</td>
<td>0.1</td>
<td>-26.1 ± 13</td>
<td>14.10 ± 1.05</td>
</tr>
<tr>
<td>85% 5kDa mPEG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3% OPSS-PEG-5k-holo-Tf 50 nm Tf-NP</td>
<td>49.5</td>
<td>0.1</td>
<td>-1.1 ± 7</td>
<td>14.10 ± 1.05</td>
</tr>
<tr>
<td>82% 5kDa mPEG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15% 10kDa PEG-Alexafluor750 160nm PEG-NP</td>
<td>166.0</td>
<td>0.1</td>
<td>-6.0 ± 4</td>
<td>96.24 ± 8.66</td>
</tr>
<tr>
<td>85% 5kDa mPEG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Hydrodynamic diameter (HD) and polydispersity index (PDI) were obtained using dynamic light scattering
† Zeta potential was obtained using dynamic light scattering
‡ Nanoparticle core diameter was obtained using transmission electron microscopy (TEM) and is presented as mean ± standard
mouse flank were measured and normalized to their initial fluorescence at 0.17h post-injection. Normalized flank fluorescence was then subtracted from normalized tumor fluorescence.

Tumors were harvested from mice 48h post-injection, fixed with formalin, sectioned at a thickness of 4 µm and stained with haemotoxylin-eosin as well as silver enhancement kit to visualize tissue features and NPs [216], respectively. Stained sections were analyzed by bright-field at 20x on an Olympus IX-51 with an Amscope MU500 camera. Nanoparticle distribution expanding radially away from blood vessels was analyzed in ImageJ by drawing a contour around blood vessels freehand and a macro was developed to measure mean nanoparticle content at various radii into the tissue (0.5µm increments of radial expansion).

### 3.2.10 Statistical Analysis Parameters

All values in this chapter are presented as “mean value ± s.d.” The data in figures is presented as the average of at least three spheroids with standard error. Statistical significance was determined using ANOVA followed by the Dunnet post-test comparing all sizes to 40 nm or all flow rates to 50 µl h⁻¹.

### 3.3 Results

#### 3.3.1 Tumor-on-a-Chip Characterization

Our tumor-on-a-chip system consists of a multi-cellular spheroid immobilized in the channel of a two-layer PDMS microfluidic chip (Figure 3.1a-c). The spheroid contains a heterogeneous distribution of MDA-MB-435 cells embedded in an extracellular matrix forming a tortuous network of interstitial spaces similar to the tumor tissue. Spheroids are frequently used to screen the therapeutic efficiency of drugs, antibodies and nanoparticles in a static environment [150, 217, 218]. Incorporation of the spheroid into a microfluidic chip holds the micro-tissue stationary in moving solution, generates controllable flow conditions, and provides an optical window for non-disruptive real-time analysis using confocal microscope imaging [214]. First, we prepared 280 µm MDA-MB-435 melanoma cell spheroids and gently loaded them into the PDMS chip using gravity-driven flow. Cells inside the spheroid remained viable, and measured 10.69±2.67 µm in diameter at the rim and 12.61±2.63 µm in deeper layers. Interstitial spaces between the cells measured 1.00±0.52 µm and represented 16.23±2.47% of the total spheroid.
volume. A non-uniform layer of extracellular matrix containing laminin protein surrounds the spheroids and forms a physical barrier between the cells and their surrounding medium (Figure 3.1c). Regulating the volumetric flow rate into the microfluidic device creates the unique flow properties necessary for precise control of the pressure gradient, shear stress, and fluid velocity in the channel (Figure 3.1d & e). Flow rates between 50 and 450 μL h⁻¹ produced a 75 to 675 μm s⁻¹ fluid velocity, which is comparable to blood flow in tissue capillaries [219, 220]. At 450 μL h⁻¹, flow remained laminar with the pressure drop at 9 Pa and shear stress at 0.06 Pa in the imaging chamber where the spheroid is immobilized. When flow rate is raised from 50 to 450 μL h⁻¹, accumulation kinetics of 10 kDa dextran demonstrate that convective flow increases from 0.4 to 1.41 μm s⁻¹ (Figure 3.1f and Table 3.3). These values are similar to interstitial flow inside a tumor, which can vary between 0.1 and 3 μm s⁻¹ [221, 222]. The tumor-on-a-chip therefore provides an experimental platform with controllable interstitial flow through a three-dimensional tissue.

3.3.2 Effect of Nanoparticle Size on Tissue Accumulation

With the tumor-on-a-chip we addressed a series of questions concerning nanoparticle tumor delivery that have been difficult to address using conventional animal models or cell culture experiments. In the first set of experiments, we examined the effect of nanoparticle size on tissue transport kinetics. Fluorescently-labeled PEGylated gold nanoparticles [212] possessing a hydrodynamic diameter of 40, 70, 110, or 150 nm (Table 3.1) were administered to spheroids in the microfluidic chip for 1 h at 50 μl h⁻¹ (Figure 3.2) and accumulation was quantified. Of note, the distance-dependent quenching effect of gold surfaces on fluorescent molecules was previously characterized and referenced to ensure adequate fluorescence of the nanoparticles designed for this study [212]. The 40 and 70nm NPs were visible inside the spheroid’s interstitial spaces within 10 min and showed an entry rate of 0.00400 min⁻¹ and 0.00396 min⁻¹, respectively (Figure 3.2e). In contrast, the 110 and 150 nm NPs were excluded from interstitial spaces and accumulated significantly less. Efflux of the nanoparticles from the spheroid tissue occurred rapidly and confirms that nanoparticle PEGylation inhibits binding to cells and extracellular matrix proteins (Figure 3.2f). Electron microscopy and spheroid silver staining were used to confirm the presence of PEG-NPs. However, the multiple washes required for sample preparation removed PEG-NPs from the spheroid and made it impossible to quantify accumulation.
Table 3.3: Interstitial flow rates. These values were calculated using the mathematical model described in the methods section.

<table>
<thead>
<tr>
<th>Q (µL h⁻¹)*</th>
<th>Qₛ (µL h⁻¹)†</th>
<th>Qₛ.Channel (µL h⁻¹)‡</th>
<th>V= Qₛ.Channel A⁻¹ (µm s⁻¹)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.073</td>
<td>1.16 x 10⁻⁶</td>
<td>0.41</td>
</tr>
<tr>
<td>100</td>
<td>0.103</td>
<td>1.64 x 10⁻⁶</td>
<td>0.58</td>
</tr>
<tr>
<td>150</td>
<td>0.138</td>
<td>2.20 x 10⁻⁶</td>
<td>0.78</td>
</tr>
<tr>
<td>300</td>
<td>0.177</td>
<td>2.82 x 10⁻⁶</td>
<td>0.98</td>
</tr>
<tr>
<td>450</td>
<td>0.25</td>
<td>3.98 x 10⁻⁶</td>
<td>1.41</td>
</tr>
</tbody>
</table>

* Flow rate of media into the device
† Flow rate through the spheroid modeled as porous media
‡ Spheroid interstitial flow rate assuming 1µm channels
§ Spheroid interstitial flow rate converted to µm s⁻¹
Figure 3.1: Schematic and characterization of the tumor-on-a-chip. (a) Schematic of the PDMS microfluidic device placed onto a microscope stage. (b) Scheme of the microfluidic device demonstrating that channel width is 600µm at the inlet then widens to 1200 µm in the imaging chamber where the spheroid is immobilized. Channel height is 250 µm and then drops to 25 µm at the end of the imaging chamber forming a dam. (c) Image of the microfluidic device; scale bar = 1000 µm. On the right, a spheroid stained with anti-laminin-FITC for 10 min and then flushed with imaging media for 5 min; scale bar = 100 µm. (d) COMSOL Computational Fluid Dynamics (CFD) simulation values for the pressure drop and shear stress in the imaging chamber prior to the dam. (e) COMSOL Computational Fluid Dynamics (CFD) simulation values for the Reynolds number and velocity in the imaging chamber prior to the dam. (f) Flow rate versus media exchange (left axis) which represents the amount of time required for the non-fluorescent media inside the spheroid to be filled with 10kDa Dextran-568. On the right axis, are displayed the corresponding interstitial flow rates. Error bars represent s.e.m. where n = 3.
Figure 3.2: Effect of nanoparticle size on tissue accumulation. (a) 40nm fluorescent PEG-NPs administered for 1 h at 50 µL h\(^{-1}\) enter the spheroid and accumulate in the interstitial spaces (arrows). Scale bar = 100 µm (b) The 110 nm fluorescent PEG-NPs were administered for 1 h at 50 µL h\(^{-1}\) and were excluded from the spheroid. Images represent an overlay of fluorescence (excitation: 633 nm, emission: 650-700 nm) and differential interference contrast images. Scale bar = 100 µm. (c) Tissue accumulation of different 40 (red), 70 (blue), 110 (green) and 150 nm (orange) PEG-NPs administered to the spheroid at 50 µL h\(^{-1}\). (d) Tissue accumulation of PEG-NPs after 1 h at 50 µL h\(^{-1}\) and after flushing with clear solution for 30 min at 50 µL h\(^{-1}\). Tissue accumulation represents the average fluorescent intensity from the spheroid’s perimeter to a depth of 75 µm normalized to the medium’s fluorescence. (e) Rate constants were determined by fitting data from 2c fit using Eq. 3. (f) Efflux data was obtained by flushing spheroids with blank imaging buffer at 50 µL h\(^{-1}\) for 30min. Because there was no external fluorescence to normalize values, all data was obtained as a percentage of t=0 min. These values were then multiplied by the final accumulation value of the spheroid before flushing. Data is presented as the average values from 3-5 spheroids with s.e.m. *** p<0.001 using ANOVA.
3.3.3 Effect of Receptor Targeting on Tissue Accumulation

Next, we used the tumor-on-a-chip to compare passive and active targeting nanoparticles. Passive nanoparticles are predominantly conjugated with PEG to minimize interactions with cell receptors and other proteins. They are designed to evade blood clearance and progressively diffuse into tumors [94, 223]. The 40 nm PEG-NPs were used as models of passive targeting nanoparticles and they diffused into the surrounding extracellular matrix reaching $27.37\pm6.02\%$ of the administered dose after 1h. Average fluorescence in the spheroid was significantly lower at $4.98\pm0.23\%$ and mostly limited to interstitial spaces (Figure 3.3 a-b). With active targeting nanoparticles, the addition of a receptor-targeting biomolecule can increase accumulation compared to diffusion alone. Nanoparticles functionalized with the iron-transporting transferrin (Tf) protein were used as a model of active targeting nanoparticles and formed a concentrated region at the spheroid perimeter $287.1\pm101.2\%$ of the administered dose (Figure 3.3 c-d). This highly fluorescent “binding-site barrier” is caused by an elevated accumulation of nanoparticles in the outermost extracellular matrix and Tf receptor-expressing cells. Inside the spheroid, Tf-NPs co-localized with the cells and formed visible punctate regions caused by receptor binding [224] (Figure 3.3c). Tf-NPs showed a 15-fold increase inside the spheroids compared to PEG-NPs similar to certain tumor regions in vivo [163]. Flushing produced a slight decrease in peripheral extracellular matrix fluorescence but not in the spheroid fluorescence. Blocking the cell receptors with soluble Tf protein reduced accumulation inside the spheroid by 50% but did not affect the surrounding extracellular matrix (Figure 3.4). This suggests that a combination of specific interactions with cell receptors and non-specific protein interactions with extracellular matrix proteins anchor the Tf-NPs in the tissue preventing efflux. Electron microscopy and spheroid silver staining confirmed the presence of a binding-site barrier with Tf-NPs bound to cell membranes and the surrounding extracellular matrix. However, minimal Tf-NPs was detected inside the cells. These findings illustrate that the addition of Tf to the 40 nm nanoparticle surface improves accumulation and retention in the tissue through receptor-specific and non-specific protein interactions.
Figure 3.3: Effect of nanoparticle surface chemistry on accumulation. (a) Top: Intensity map of PEG-NP fluorescence taken at 40X after 1 h of flow at 50 µL h⁻¹. Bottom: 63X image of PEG-NP fluorescence in the interstitial spaces (I.S.). Scale bars = 50 µm. (b) Mean fluorescence of surrounding matrix [ECM] and spheroid [sphr] treated with PEG-NPs at 50 µL h⁻¹. (c) Top: intensity map of Tf-NP fluorescence taken at 40X after 1 h of flow at 50 µL h⁻¹. Bottom: 63X image of Tf-NP punctuate fluorescence co-localizing with cell membranes. Scale bars = 50 µm. (d) Mean fluorescence of surrounding matrix and spheroid treated with Tf-NPs at 50 µL h⁻¹.
Figure 3.4: Blocking transferrin receptors on spheroids. Images of spheroids treated with 25nM Tf-NP without (a) or with (b) 6.41µM Tf for 30min at 50 µL h⁻¹. Accumulation (c) and tissue distribution (d) of Tf-NPs without (black) or with (grey) 6.41µM Tf.
3.3.4 Effect of Flow Rate on Tissue Accumulation

Finally, the effects of interstitial flow rates on nanoparticle accumulation were investigated. Variable convection is a common feature of the tumor environment and can be attributed to heterogeneous vascularization, interstitial pressure, and lymphatic drainage. These conditions produce rapid interstitial flow at the tumor perimeter and no convection in the centre [222]. Augmenting the flow rate in our microfluidic device from 50 to 450 µL h⁻¹ produced a two-fold increase in both PEG-NPs and Tf-NPs accumulation (Figure 3.5). We originally hypothesized that faster flow rates would increase convection, allowing nanoparticles to penetrate deeper in the tissue. However, it was the amplitude of tissue fluorescence distribution that increased with no detectable change in the average penetration depth (Figure 3.5). Instead, faster flow rates produced a higher accumulation of nanoparticles on the spheroid’s outer layer and increased the total number of nanoparticles diffusing into the tissue (Figure 3.6). These findings demonstrate that interstitial flow determines the number of nanoparticles accumulated at the tissue interface. Once at the interface, the nanoparticles are immobilized by the extracellular matrix fibers and diffuse into the tissue over time.

3.3.5 In Vivo Tumor Accumulation

We used a murine xenograft MDA-MB-435 tumor model [94, 212] to evaluate whether the tumor-on-a-chip system predicted the tissue transport of nanoparticles through the same tumor cells in vivo (Figure 3.7). We intravenously injected two different sized fluorescently-labeled PEG-NPs (50 vs. 160 nm) and a fluorescently-labeled 50 nm Tf-NPs into tumor-bearing mice. Whole animal imaging reveals minimal tumor accumulation for 160 nm PEG-NP whereas 50 nm PEG-NPs and 50 nm Tf-NPs progressively accumulate between 2 and 48h (Figure 3.7a & b). At 48h post-injection, we isolated the tumor tissue, silver stained to highlight nanoparticle location, and measured the penetration depth of the different nanoparticle designs. Histological analysis demonstrates that 160 nm PEG-NPs are seldom found in the tumor tissue and do not penetrate into the tissue surrounding tumor vasculature. This is in agreement with tumor-on-a-chip data where nanoparticles larger than 100 nm did not penetrate into the spheroid tissue after 1h (Figure 3.2b). For 50 nm nanoparticles, the addition of Tf increased accumulation
Figure 3.5: Effect of flow rate on nanoparticle accumulation. Mean PEG-NP accumulation at 60 min (a), accumulation kinetics (b), fluorescence distribution (c), and penetration depth (d) of spheroids at various flow rates. Mean Tf-NP accumulation at 60 min (e), accumulation kinetics (f), fluorescence distribution (g), and penetration depth (h) of spheroids at various flow rates. Data is presented as the average values from 3-5 spheroids with s.e.m. *p<0.05, **p<0.01 using ANOVA.
Figure 3.6: Flow rate controls the concentration of nanoparticles in the first layer of the tissue. (a) Scheme of spheroid showing that accumulation can be subdivided into multiple cell layers (L1, L2, L3, etc.) To determine whether faster flow rates can increase the penetration depth of NPs, we can look at the ratios between L2 vs. L1 or L3 vs. L2. (b) Accumulation of PEG-NPs in the first layer or “reservoir”. (c) Accumulation ratios between the two adjacent layers demonstrate that flow rate does not change the ratios. (d) Accumulation of Tf-NPs in the first layer or “reservoir”. (e) Accumulation ratios between the two adjacent layers demonstrate that flow rate does not change the ratios. Data for each time point is presented as the mean ± s.e.m. (n=3).
of NPs in the tissue surrounding the blood vessel (Figure 3.7c). Statistical analysis demonstrates a significant increase in the accumulation of 50 nm Tf-nanoparticles within a 7 μm radius of tumor blood vessels. These findings confirm the tumor-on-a-chip results which demonstrate that nanoparticle design is an important determinant of tissue transport. The accumulation is limited to nanoparticles with diameters that are <110 nm and significantly increased by active targeting in MDA-MB-435 tumors for the 40 nm nanoparticles.

In our microfluidic studies, we demonstrated that properties of the tumor microenvironment such as interstitial flow rates can also affect nanoparticle transport into the tissue. Our in vitro findings revealed that the number of nanoparticles at the spheroid-media interface reflected total tissue accumulation (Figures 3.5 & 3.6). To validate if a similar relationship was apparent in vivo, we took advantage of the large variability in tissue accumulation of nanoparticles between different blood vessels. For a given tumor, nanoparticles can accumulate around blood vessels at different quantities (Figure 3.7d). Using histology data from tumors treated with 50 nm Tf-NPs and 50 nm PEG-NPs, we compared the accumulation of nanoparticles at the blood vessel-tumor tissue interface with accumulation in deeper cell layers surrounding the blood vessel. We defined the blood-vessel interface or ‘perivascular region’ as the region within a 5 μm radius of the blood vessel and determined its relationship with the accumulation within a 25 μm radius. Figure 3.7e demonstrates a linear trend between the perivascular region and tumor tissue accumulation. We used the F test to confirm that the positive slope was significantly different from the null hypothesis (p<0.0001). This finding is in agreement with our tumor-on-a-chip results demonstrating that higher flow rates increased the concentration of nanoparticles at the spheroid surface. However, because we cannot directly characterize the flow, permeability, pressure differential, porosity, and other properties of the tumor microenvironment in vivo, these conclusions remain correlative in nature.

3.4 Discussion

Our tumor-on-a-chip system allowed us to investigate the transport mechanism of nanoparticles through a model tissue. The peripheral matrix surrounding the spheroid is analogous to the laminin-rich basement membrane through which plasma must diffuse prior to entry into the tissue. Similarly, liquid in the tumor-on-a-chip travels at physiological rates, encounters the peripheral matrix and produces convection through the spheroid’s interstitial spaces. Our data
Figure 3.7: *In vivo* behavior of nanoparticles. (a) Top: representative images of tumor fluorescence from mice injected with NPs in the tail vein at 48h post-injection. Bottom: sample images of tumor histological sections treated with a silver enhancement kit to visualize NPs around blood vessels. The 50 nm Tf-NPs and 50 nm PEG NPs diffuse into the tissue, while 160 nm PEG-NPs remain inside the blood vessel. Scale bars = 50µm. (b) Quantification of animal fluorescence at 2 and 48h using whole animal images (n=3; *p<0.05, **p<0.01 using two-way ANOVA). (c) Distribution of NPs in tumor blood vessels quantified using image analysis of silver-stained histological sections (n=33 for 50 nm NPs and n=15 for 160 nm NPs; **p<0.01 using two-way ANOVA). (d) Representative image of two distinct blood vessels in the same tumor treated with 50nm PEG-NP demonstrating variability in extravasation and tissue penetration. Image threshold was set to isolate silver-stained NPs in the tissue. Red highlighted area corresponds to the perivascular region. Scale bars = 25µm (e) Correlation between accumulation of NPs in the perivascular region (5µm radius) and the tumor tissue (25µm radius) around the blood vessel.
demonstrate that both nanoparticle physicochemical properties and the tumor microenvironment can affect tissue accumulation. Our study illustrates that tissue transport requires nanoparticles to embed into the tissue-fluid interface and progressively diffuse into the tissue. For passive targeting, the reservoir at the tissue-fluid interface is responsible for dictating the number of nanoparticles diffusing into the tissue. For active targeting, the binding-site barrier will saturate available binding sites and allow additional nanoparticles to diffuse into the tissue. Our findings in vitro and in vivo reveal that the concentration of nanoparticles in the reservoir or the binding site barrier is an important determinant of tissue transport kinetics. These conclusions are in agreement with models of inconsistent nanoparticle delivery due to the variable tumor vasculature [137]. The tissue-fluid interface appears to predict nanoparticle tumor accumulation in vivo. Interestingly, a review of earlier spheroid studies reveals a time-dependent increase in nanoparticles at the spheroid surface which correlates with overall tissue accumulation [159, 218].

The tumor-on-a-chip reveals that interstitial flow rate, cell receptor binding and nanoparticle diameter all influence accumulation at the tissue-media interface. At the interface, nanoparticles <110 nm can diffuse through the extracellular matrix and interact with tumor cells. Above 110 nm, nanoparticles are excluded from the MDA-MB-435 spheroids and the tumors because they are larger than the extracellular matrix’s effective pore size and cannot embed into the protein matrix. Our findings are in agreement with previous studies demonstrating the limited tissue penetration of larger nanoparticles [94, 136-138]. A recurring 100 nm cutoff may reflect the upper limit of extracellular matrix diffusivity.

The addition of Tf to the nanoparticle surface produced a 15-fold increase in the accumulation of Tf-NPs relative to PEG-NPs. Unfortunately, receptor-targeting does not benefit from such an impressive advantage in vivo. Systemically administered nanoparticles show better tumor transport kinetics around blood vessels, but this did not translate into a statistically significant increase in tumor fluorescence. This may be due to several factors including large inter-animal variability, the addition of PEG to the 50 nm Tf-NPs to prevent blood clearance, and numerous nanoparticle-biological interactions upstream of tumor accumulation. Our data demonstrate that the challenges of receptor targeting include non-specific binding to the extracellular matrix and formation of a binding-site barrier. Previous work has demonstrated that that receptor targeting can produce a moderate increase in tumor accumulation [162] but may only benefit nanoparticles
<40 nm when entering specific “slow flow” regions of the tumor [163]. When nanoparticles are directly administered into the tumor, receptor-targeting can improve therapeutic outcome [170]. Whether active targeting is only useful in certain regions of the tumor or whether it impedes upstream events such as blood clearance and extravasation remains an important question in drug delivery. In regards to tissue transport, short term experiments with systems like the tumor-on-a-chip seem to predict the long-term tissue transport of nanoparticles when coupled to highly sensitive detection techniques such as confocal microscopy. With rapid 1h in vitro experiments, we successfully predicted nanoparticle transport in MDA-MB-435 in vivo tumors. It must still be confirmed whether this approach can predict drug carrier behavior in vivo using other tumor cell lines and other diseased tissue models.

The tumor-on-a-chip improves on previous in vitro work where spheroids remained in a static solution for several hours [138, 150, 217, 218] and required disruption before [209] or after [138, 150, 217, 218] incubation with drug carrier systems. The real-time imaging of intact spheroids using confocal microscopy improved sensitivity and provided a window into early nanoparticle-tissue interactions. These improvements offer an unprecedented look at early nanoparticle-tissue interactions under controllable flow conditions. Our experimental setup permits the rapid assessment of how a nanoparticle’s physicochemical properties affect its transport through tumors and potentially, other tissues. Based on this initial study, early nanoparticle-tissue interactions monitored using microfluidic-based tissue models may predict the long-term behavior of nanoparticles in a tissue in vivo. With recent advances in the commercialization of microfluidics and nanomaterials, researchers can simply insert their specific tumor model into a purchased device. This will facilitate the evaluation of multiple nanoparticle designs in different tumor systems and will validate microfluidic-based tissue models as versatile platforms to evaluate the performance of drug carriers prior to animal studies.

3.5 Author Contribution

This chapter has been adapted from [190]. A. Albanese, A.K. Lam, J.V. Rocheleau and W.C.W. Chan designed the experiments and optimized the method. The manuscript was predominantly written by A. Albanese with microfluidic device fabrication and characterization sections written by A.K. Lam. Edits and rewrites were provided by J.V. Rocheleau and W.C.W. Chan. A. Albanese and A.K. Lam performed the experiments and analyzed the data. E.A. Sykes
preformed *in vivo* experiments for comparative analysis. The published manuscript contains supplementary information and figures.
4 The Extracellular Environment Alters Nanoparticle-Cell Interactions

4.1 Introduction

In the last chapter, the tumor-on-a-chip provided a detailed look into the transport of nanoparticles in a modeled tumor environment. Originally, we wanted to develop a microfluidic device which could immobilize spheroids with diameters above 500 μm. These larger spheroids can mimic the different regions of tumors since they possess limited exchange of nutrients and metabolic waste products [147]. The nutrient and waste gradients produce a necrotic core, quiescent region and proliferative surface which are comparable to avascular, semi-vascularized and vascularized regions of the tumor. Unfortunately, the channel height of the microfluidic device could not be increased above 250 μm due to limitations with the photoresist coating thickness during device fabrication. Thus, small 260-300 μm spheroids were used since they fit into the device. These smaller spheroids demonstrate a good exchange with their surrounding media but lack a necrotic core making them good models for tumor tissue adjacent to blood vessels.

Since we could not use the tumor-on-a-chip to study the effect of the tumor microenvironment in necrotic regions, conventional in vitro cell culture techniques were used to analyze the role of extracellular environment separately. Cell cultures were used to generate metabolites and cell-secreted proteins to mimic the cancer microenvironment. The incubation time between the cells and media was used to control the concentration of cell-secreted molecules and to mimic the various states of the tumor’s extracellular environment.

When nanoparticles are transferred into a biological fluid such as blood or cell culture medium, biomolecules in solution interact with the nanoparticle surface. These interactions can lead to protein adsorption, surface destabilization and aggregation [16, 96]. In protein-rich biological environments, proteins can bind to the nanoparticle surface and form a ‘protein corona’ which will alter the surface’s chemistry and can increase its total hydrodynamic diameter by 10-40 nm depending on surface chemistry [95, 225, 226]. The destabilization of the nanoparticle surface can also cause nanoparticles to aggregate into large asymmetrical structures that interact with
cells differently than monodisperse nanoparticles [16, 67, 110, 227-230]. Thus, in biological media, protein adsorption and aggregation both alter the nanoparticle’s original physicochemical properties and generate a new ‘biological identity’ that is distinct from their initial ‘synthetic identity’ post-synthesis. Ultimately, it is the ‘biological identity’ that is ‘seen’ by cells and governs downstream physiological responses [225, 231]. Previous studies have analyzed protein corona and aggregation separately; however, both should be considered when characterizing a nanoparticle’s biological identity. The thorough characterization of a nanoparticle’s biological identity is necessary to elucidate which of the nanoparticle’s properties produces a given physiological response.

Several factors can influence a nanoparticle’s biological identity such as its initial physicochemical properties upon synthesis [187], the composition of the surrounding biological media [188], and the incubation time between the two [232]. It has also been observed that the translocation of nanoparticles from one biological environment into another can change the protein corona’s composition [233]. These studies imply the possibility of a labile biological identity for nanoparticles that travel through compositionally different biological environments. In various biological systems, cell populations continuously alter their microenvironment by changing the concentration of proteins, nutrients, small solutes and ions [234]. Cells will internalize soluble biomolecules, produce metabolites and secrete extracellular matrix proteins, enzymes, growth factors, hormones, and cytokines [235]. When the extracellular environment is not replenished in avascular tumor regions or in cell cultures, the cells will ‘condition’ their environment and change its composition over time. How the composition of the extracellular environment affects a nanoparticle’s biological identity and downstream cellular interactions has yet to be evaluated. Characterizing the interplay between the extracellular environment, biological identity and cell uptake is necessary to improve nanoparticle targeting both in vitro and in vivo. In this chapter, we investigate whether media conditioning by cancer cells can induce nanoparticle aggregation, remodeling the protein corona and changes in cell uptake for multiple nanoparticle designs.
4.2 Methods

4.2.1 Nanoparticle Preparation

The 15 nm gold nanoparticles were prepared by citrate reduction according to the Frens-Turkovich method [26]. Nanoparticles were then modified with thiol-terminated methoxyPEG-5000 or 11-mercaptoundecanoic acid (MUA) using standard ligand-exchange procedures [236]. The 15 nm gold nanoparticles were also used as seeds for the preparation of 30, 60 and 90 nm nanoparticles by hydroquinone-mediated growth [28]. The nanoparticles were synthesized, washed three times via centrifugation with 0.01% (w/v) sodium citrate tribasic and incubated in PBS containing 10% (v/v) fetal bovine serum (FBS) for 1h at 37°C. The nanoparticles were centrifuged and added to cells or conditioned media at 5 nM (3 x 10^{12} nanoparticles/mL) for 15 nm nanoparticles. For larger nanoparticles, concentrations were normalized to achieve the same surface area as the 15 nm nanoparticles (2.1 x 10^{15} nm^2/mL).

4.2.2 Cell Experiments

Cell experiments were conducted with A549 human lung epithelial carcinoma cells, HeLa human cervix epithelial adenocarcinoma cells, RAW 264.7 murine leukemia virus-transformed monocyte cells and MDA-MB-435 human melanocyte cells. Nanoparticle experiments were conducted in phenol-free DMEM (Gibco) containing 25 mM HEPES and supplemented with 10% FBS (Gibco). Twelve-well plates were seeded with enough cells to achieve ~85% confluency at the start of the experiment. Either 8x10^5 MDA-MB-435, 8x10^5 NIH 3T3, 8x10^5 HeLa cells, 1.6x10^5 RAW 264.7 cells or 3x10^5 A549 cells were seeded overnight. The following day, media was removed and 835 µl of fresh culture media was added to wells and incubated for 1 to 24h. When using other types of culture plates, such as 48-well or 6-well, we normalized cell numbers and media volume to the growth surface’s area to maintain identical cell-to-media ratios. For direct cell exposure experiments nanoparticles were directly added to cells along with fresh media. For indirect exposure experiments, nanoparticles were incubated with pre-conditioned media for 4h at 37°C. A 4h incubation time between nanoparticles and conditioned media was chosen based on preliminary experiments demonstrating that nanoparticle aggregation reached a plateau at 4h.
4.2.3 Characterization of Nanoparticle Aggregation

After exposure to cells or pre-conditioned media, nanoparticles were kept on ice and analyzed by UV-Vis (Shimadzu) to assess aggregation by quantification of the plasmonic red-shift. We used the 620/520 nm, 620/527 nm, 700/544 nm, and 700/577 nm absorbance ratios as the ‘aggregation index’ for 15, 30, 60, and 90 nm nanoparticles, respectively. Aggregation index values were subtracted by the aggregation index of nanoparticles in fresh medium. When comparing the aggregation of 15 nm NPs in various cell cultures and supernatants, we determined the rate of aggregation using the following hyperbolic equation:

\[ y = y_{\text{max}} (1 - e^{-kx}) \]

where \( y \) is the aggregation index (620/520 nm), \( y_{\text{max}} \) is the maximum aggregation index at 24h, \( x \) is time in hours and \( k \) is the aggregation rate. Conditions were set to \( y_{\text{max}} < 1.2 \) since this is the maximum possible value using our experimental conditions. All points were fit to the equation producing fits at \( R^2 > 0.88 \). The curves of individual replicate series were obtained and compared to other conditions to determine statistical differences using ANOVA.

Some nanoparticle samples were also analyzed by dynamic light scattering (Malvern) to measure the hydrodynamic diameter and by transmission electron microscopy to measure the size of aggregates. Electron microscope images were analyzed using an ImageJ macro to quantify the number of nanoparticles per aggregate.

4.2.4 Characterization of the Protein Corona

After incubation in serum-containing media, gold nanoparticles were purified from unbound protein by repeated centrifugation and resuspension in phosphate buffered saline. Bound protein was stripped by incubating nanoparticles in 2% (w/v) sodium dodecyl sulfate (SDS) and 100mM dithiothreitol for 1h at 70°C. An aliquot of the protein isolates was drawn and resolved on a NuPAGE 4-12% Bis-Tris poly(acrylamide) gel (Invitrogen) in MOPS running buffer (Invitrogen) according to the manufacturer’s protocol. The gels were stained using Krypton fluorescent protein stain (Pierce) and imaged on a Typhoon laser scanner (Amersham). Remaining protein isolates were repeatedly precipitated to remove SDS, as described previously [60]. Proteins pellets were reduced and alkylated before being digested with trypsin. Protein
digests were analyzed on the Orbitrap Velos (Thermo) as described previously [60]. The percentage (w/w) relative abundance of each of each identified protein was estimated using the following formula:

\[ RA(n)_{\% (w/w)} = \frac{SpC(n)}{\sum_{i=1}^{m} SpC(i)} \]

where \( RA(n)_{\% (w/w)} \) is the relative abundance of protein \( n \), and \( m \) is the total number of identified proteins. \( SpC(i) \) is the total number of spectral counts recorded for protein \( i \).

4.2.5 Cell Uptake

Cells were prepared the day before by seeding 12-well plates with \( 3 \times 10^5 \) A549 cells or \( 1.67 \times 10^5 \) RAW264.7 cells overnight. Each cell type was seeded in numbers to achieve 85% confluency at the start of experiment. The 15 nm citrate nanoparticles were exposed to A549 cell-conditioned media for 4h then added to A549 cells and incubated for 1h at 37°C for uptake studies, for 1h at 4°C for membrane binding studies and for 1h at 4°C followed by 4h in fresh media at 37°C for retention studies. For experiments with larger nanoparticles, nanoparticle formulations were exposed to either A549 cell-conditioned media for 4h then incubated with A549 cells for 1h at 37°C or exposed to RAW264.7 cell-conditioned media for 4h then incubated with RAW264.7 cells for 1h at 37°C. After incubation with nanoparticles, cells were washed three times with PBS + 0.5% bovine serum albumin and frozen until elemental analysis. Samples were thawed, digested with 0.5 mL nitric acid for 30 min at 70°C, diluted in 3 mL Millipure water and analyzed by ICP-AES. Samples were analyzed for Au and Mg content. The Mg content was used to quantify the number of cells per sample in reference to cell standards [189]. The ICP-AES data was converted to obtain gold quantification as ng/cell and multiplied by the average number of RAW264.7 or A549 cells in a given experiment (to minimize variability caused by differences in cell number). The nanoparticle exposure dose was determined by measuring an aliquot of nanoparticles by ICP-AES. The percentage dose was defined as:

\[ \frac{[\text{ng gold in all cells}]}{[\text{ng of nanoparticles added}]} \times 100\% \]
4.3 Results & Discussion

4.3.1 Nanoparticle Aggregation

As a model system, we used gold nanoparticles because they can be easily synthesized within a narrow distribution in the 15 to 200 nm size range [28] and readily functionalized with various surface ligands. We prepared 15 nm gold nanoparticles with three distinct surface chemistries: citrate-coated as a model non-functionalized surface, 11-mercaptoundecanoic acid (MUA)-coated as a model anionic surface, and polyethylene glycol (PEG)-coated as a model non-fouling surface. These ligands represent some of the most common surface chemistries used in biological applications. Following synthesis, the nanoparticles were incubated with 10% (v/v) fetal bovine serum to produce a protein corona that simulates the nanoparticle’s initial exposure to blood. The nanoparticles were then incubated with cell monolayers for up to 24h and aggregation was measured in the supernatants by using UV-Vis spectrometry. The ‘aggregation index’ measures the degree of aggregation by comparing the absorbance intensity at 620 nm (aggregates) to 520 nm (monodisperse nanoparticles) [237]. Cell exposure induced aggregation of citrate-coated nanoparticles at 1h in all cell lines tested (Figure 4.1a-c). All cell lines produced similar aggregation of the citrate nanoparticles by 24h, except the MDA-MB-435 cells which induced less aggregation. The PEG-coated nanoparticles showed some slight aggregation in A549 and HeLa cells whereas MUA-coated nanoparticles showed very little aggregation by 24h. Our findings highlight that a nanoparticle’s surface chemistry and a cell’s phenotype both contribute to nanoparticle aggregation during cell culture experiments.

To determine whether cell conditioning was responsible for nanoparticle aggregation in cell cultures, conditioned media was collected from cells between 1 and 24h. The conditioned media was then incubated with nanoparticles between 0.5 and 8h to establish the optimal incubation time for our experiments. The longer nanoparticles were exposed to conditioned media, the more aggregation we observed. We settled on a 4h incubation time with conditioned media since it produced ~75% of the aggregation produced with an 8h incubation. Conditioned media was collected from the different cell lines and incubated with our three nanoparticle designs (Figure 4.1d-f). For 15 nm citrate-coated nanoparticles, A549 and RAW 264.7 cell-conditioned media
Figure 4.1: Aggregation of nanoparticles exposed to conditioned media. (a) Scheme of nanoparticles directly exposed to cells. (b) Aggregation index assessed by UV-visible spectrometry of nanoparticles directly exposed to A549 cell cultures. (c) Aggregation index after 24h exposure to various cell lines. (d) Scheme of nanoparticles exposed to pre-conditioned media. (e) Aggregation index of nanoparticles incubated with A549 cell-conditioned media for 4h. (f) Aggregation index after exposure to 24h cell-conditioned media for 4h. (g) Aggregation rate of 15nm citrate nanoparticles exposed to cells or conditioned media in various cell lines. *p< 0.05, ***p<0.001 using two-way ANOVA (h) Hydrodynamic diameter of nanoparticles exposed to A549 cells or cell-conditioned medium. Data presented as the average with s.e.m. from at least three separate experiments.
produced slower aggregation kinetics culminating in the same plateau at 24h (Figure 4.1f & g). MDA-MB-435 and HeLa cell-conditioned media caused less aggregation than direct cell contact experiments. PEG-coated nanoparticles did not aggregate in conditioned media demonstrating that their aggregation requires prolonged cell contact. Dynamic light scattering and electron microscopy validated spectrometry data and confirmed the presence of aggregates in samples possessing large aggregation indices (Figure 4.1g & 4.2). Our findings demonstrate that media conditioning is at least partially responsible for nanoparticle aggregation resulting from cell exposure. The three nanoparticle formulations illustrate that surface chemistry is an important determinant of nanoparticle stability in conditioned medium.

Next, we synthesized a second set of 30, 60 and 90 nm citrate-coated gold nanoparticles to evaluate the effect of nanoparticle diameter on stability since surface curvature is well known to affect nanoparticle interactions with serum proteins [60, 61, 188]. We looked at nanoparticle aggregation media conditioned by two different cell types: the RAW-264.7 leukemia virus-transformed monocyte cell line and the A549 lung epithelial carcinoma cells. In both cell lines, larger nanoparticles demonstrated improved stability when exposed to cell-conditioned media (Figure 4.3). These findings demonstrate that cell conditioning of the culture medium can lead to nanoparticle aggregation over time independently of cell contact in poorly stabilized nanomaterials. A nanoparticle’s susceptibility to aggregation was dependent on its diameter, its initial surface composition, cell phenotype and medium conditioning time.

4.3.2 Nanoparticle Protein Corona

Next, we investigated the stability of the protein corona in conditioned media. For these experiments, nanoparticles were initially incubated with 10% serum and then transferred into fresh or 24h conditioned media for 4h. The protein corona of nanoparticles was compared using polyacrylamide gel electrophoresis (PAGE). Exposure of 15 nm citrate nanoparticles to 24h conditioned media alters the initial protein corona established upon serum exposure (‘start’ lane). In contrast, exposure to fresh medium did not significantly alter the protein corona composition.
Figure 4.2: Electron microscope images of aggregates in conditioned media. (a) Transmission electron microscopy (TEM) was used to confirm the aggregation of 15-Ci NPs exposed to conditioned media. (b) Average number of nanoparticles per aggregate as determined using image analysis of TEM data.
Figure 4.3: Aggregation of different nanoparticle sizes. UV-Visible spectra of 15 nm (a), 30 nm (b), 60 nm (c), or 90 nm (d) incubated with fresh media (grey area), 24h A549 cell-conditioned medium (blue) or 24h RAW 264.7 cell-conditioned medium (red). The dotted lines demonstrate the wavelengths used to calculate the aggregation index for each nanoparticle size. (e) Aggregation index of citrate-coated nanoparticles of different diameters exposed to A549-cell conditioned media. (f) Hydrodynamic diameters of nanoparticles exposed to fresh or 24h conditioned A549 cell culture medium. (g) Aggregation index of citrate-coated nanoparticles possessing different diameters exposed to RAW 264.7 cell-conditioned media. (h) Hydrodynamic diameters of nanoparticles exposed to fresh or 24h conditioned RAW264.7 cell culture medium. UV-Visible spectra are a representative of three separate experiments, aggregation index and DLS data presented as the average with standard error from three independent experiments.
Specific differences depended on the cell line which conditioned the medium (Figure 4.4a). One similarity between the various cell lines was the increased intensity of a large protein band (>220 kDa). Additional experiments reveal that the appearance of this >220 kDa band correlates with the length of media conditioning (Figure 4.4b & c). The addition of PEG or MUA to the nanoparticle surface produces visible changes in the protein corona composition (Figure 4.4d). In conditioned media, MUA nanoparticles show little change in protein corona while PEG nanoparticles show a slight increase in the overall quantity of adsorbed proteins. Experiments with larger nanoparticles confirm that diameter can also affect corona composition. Exposure of larger nanoparticles to conditioned media also leads to the appearance of the >220 kDa band upon exposure to conditioned media (Figure 4.4e & f). This protein band’s intensity was inversely proportional to the nanoparticle diameter and correlated with the extent of nanoparticle aggregation (Figure 4.3e). As a whole, our PAGE results demonstrate that nanoparticle size and surface chemistry influence the initial protein corona composition. Cell phenotype and media conditioning time will determine how the protein corona evolves upon exposure to conditioned medium.

4.3.3 Mass Spectrometry Analysis of the Protein Corona

We employed liquid chromatography and tandem mass spectrometry (LC-MS/MS) to characterize the compositional changes within the protein corona exposed to conditioned media. We focused our analysis on the evolving population of 15 nm citrate nanoparticles exposed to A549 conditioned media. Of the 581 proteins identified by LC-MS/MS, 113 were in sufficient abundance to ensure accurate quantification via spectral counting. Our data reveal that the extent of protein corona remodeling is proportional to medium conditioning time. The coronae of nanoparticles exposed to 1h, 8h and 24h conditioned media demonstrate an 89%, 84% and 81% similarity with nanoparticles incubated in fresh media. This trend demonstrates that the corona deviates from its original composition as cell conditioning alters the biological environment.
Figure 4.4: Protein corona of nanoparticles exposed to conditioned media. (a) Krypton stained PAGE gel showing protein corona isolates from 15 nm citrate-coated gold nanoparticles after initial serum exposure (start) and after exposure to fresh (0h) or 24h cell-conditioned media for 4h. Dashed box included to highlight a large (>220 kDa) protein which shows visible change after exposure to conditioned media. Image (b) and densitometry (c) of >220 kDa protein from 15 nm citrate nanoparticles after exposure to media conditioned by A549 between 1 and 24h. (d) Krypton stained PAGE gel of 15 nm citrate-capped (15-Ci), MUA-capped (15-MUA) or PEG-capped (15-PEG) gold nanoparticles exposed to 24h A549 conditioned media for 4h. Image (e) and densitometry-based quantification (f) of the >220kDa band when different nanoparticle sizes were exposed to 24h A549 cell-conditioned medium. (n=3; ** p<0.01 using two-way ANOVA).
Proteins with similar trends were divided into six groups by unsupervised hierarchical clustering (Figure 4.5a). Groups 3 and 4 made up ~60% of the protein corona’s mass. Proteins from group 3 decreased as media conditioning time increased. Group 3 proteins represented ~40% of the corona in fresh medium and decreased upon exposure to conditioned media to ~20% in 24h conditioned medium. In contrast, the relative abundance of proteins in group 4 increased proportionally with media conditioning time. The most abundant proteins in group 3 included vitronectin, thrombin, plasma serine protease inhibitor, complement C3, and coagulation factor 5. The most abundant proteins from group 4 include thrombospondin-1, antithrombin III, apolipoprotein B, talin-1, and myosin-9.

In our experiments, we used bovine serum in the medium and human-derived A549 cells which allowed us to identify the origin of each protein based on the species-dependent differences in the peptide sequence (bovine vs. human). Cell-derived proteins were identified in group 4 and account for at least 4.8% of the protein corona after exposure to 24h conditioned medium. Human fibronectin, myosin-9, GAPDH and thrombospondin-1 were the most abundant cell-derived proteins in the nanoparticle corona. Statistical analysis of the correlation between protein abundance and medium conditioning time reveal that the most significant changes in relative abundance occurs for proteins from groups 3 and 4 (Figure 4.5b). MS-LC/LC identified fibronectin as the most abundant cell-derived protein in the corona of nanoparticles exposed to conditioned media. While the total amount of fibronectin within the corona remained constant, human fibronectin increased proportionally with cell-conditioning time progressively replacing serum-derived fibronectin (Figure 4.5c). To confirm these findings, we performed a Western Blot with an anti-human fibronectin antibody (Figure 4.5d). Although the antibody demonstrated some cross-reactivity with bovine fibronectin, we observed an increase in human fibronectin proportional to conditioning time. As a whole, the LC-MS/MS and Western Blot data demonstrate that the protein corona is significantly affected by cell-conditioning of the extracellular environment. This causes the progressive accumulation of cell-secreted proteins into the protein corona of nanoparticles.
Figure 4.5: Quantification of protein corona composition of nanoparticles exposed to conditioned media. (a) Heat map and dendrogram of protein expression for 113 proteins isolated from 15 nm citrate-capped gold nanoparticles identified by mass spectrometry. Each row of the heat map corresponds to a protein, and each column corresponds to the duration of A549 cell-mediated conditioning of the media. The intensity of the yellow color is related to the relative abundance (by mass) of a given protein when exposed to various conditioned media. Proteins were clustered into one of six groups based on their relative abundance in correlation with media conditioning time. A dendrogram is presented on the left of the heat map outlining protein grouping. The relative abundance trend for each protein group is displayed on the right of the heat map. (b) List of proteins showing largest change in abundance with reference to
cell-conditioning time. A higher slope value represents a larger increase of protein within the corona relative to conditioning time. Negative values represent a decrease in protein within the corona with respect to conditioning time. Grey columns are used for proteins from bovine origin and black columns are used for proteins of human origin. Proteins shown are thrombospondin (THBS), bovine C4b binding protein (C4b-BP), fibronectin (FINC), myosin-9, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), coagulation factor xii (CF xii) and kininogen-1 (KNG). (c) The total amount of fibronectin as a fraction of the corona (top) and the proportion of fibronectin from human and bovine origin using MS-LC/LC. (d) Western Blot using anti human fibronectin antibody staining of protein corona isolates from 15 nm citrate-capped gold nanoparticles exposed to A549 cell-conditioned media. Control wells contain human serum (HS) as a positive control, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) as negative controls.
4.3.4 Aggregation Mechanism

We noticed that the abundance of human fibronectin in the protein corona correlated with nanoparticle aggregation (Figure 4.1e vs. Figure 4.5d). This suggested a possible mechanistic link between the adsorption of cell-derived proteins and nanoparticle aggregation. To determine if cell-secreted proteins were causing nanoparticle aggregation, we filtered conditioned media through 10 kDa Amicon filters and compared the stability of nanoparticles in unfractionated medium, sub-10 kDa filtrate and the >10kDa retentate. PAGE analysis of our fractionation reveals that there are no detectable proteins in the sub-10kDa filtrate while the retentate produces a band pattern indistinguishable from unfractionated medium (Figure 4.6a). Incubation with 15 nm citrate-coated nanoparticles reveals that the sub-10kDa filtrate contained the biomolecules responsible for nanoparticle aggregation (Figure 4.6b & c). In A549 cells, sub-10kDa filtrate produced significantly more aggregation than unfractionated medium (two-way ANOVA, p<0.001). In RAW 264.7 cells, sub-10kDa biomolecules produced similar aggregation as unfractionated medium. In both cell lines, the >10 kDa retentate produced no significant aggregation (two-way ANOVA, p<0.001). These findings suggest that small cell-released biomolecules accumulate in the extracellular environment and cause nanoparticle destabilization. This results in nanoparticle aggregation, protein corona remodeling and an overall change in the nanoparticle’s biological identity.

4.3.5 Biological Identity and Cell Uptake

The progressive aggregation and protein corona remodeling will produce significant alterations to a nanoparticle’s biological identity. The exposure of nanoparticles to conditioned media is likely to impact its uptake into cells. Previous studies have demonstrated that nanoparticle aggregation and changes in the protein corona can both independently affect cell association and internalization [227, 238-240]. Cell uptake of 15 nm citrate-coated nanoparticles exposed to media conditioned between 1 and 24h was quantified by inductively-coupled plasma atomic emission spectroscopy (ICP-AES) and our data demonstrate that longer cell conditioning leads to significantly increased nanoparticle uptake (Figure 4.7a). Unfortunately, elemental analysis
Figure 4.6: Filtration of the conditioned media. (a) Krypton stained PAGE gel demonstrating the protein content of whole medium, <10 kDa filtrate and >10 kDa retentate fractionated using a 10 kDa Amicon centrifuge filter. Aggregation index of 15 nm citrate-capped nanoparticles exposed to media, filtrate and retentate of A549 (b) or RAW 264.7 (c) cell conditioned media. Data represent the mean and s.e.m. of three independent experiments.
Figure 4.7: Cell uptake of nanoparticles exposed to conditioned media. (a) ICP-AES-based cell uptake of 15 nm citrate-capped nanoparticles exposed to A549 conditioned media between 1 and 24h. Nanoparticles were incubated with cells for 1h at 37°C or 4°C. Data represents the mean and standard error from three independent experiments. (b) Average size of nanoparticle aggregates exposed to A549 cells (extracellular) or imaged in the intracellular vesicles (internalized). Data was obtained by analysis of electron microscope images. (c) Retention of membrane-bound 15nm citrate nanoparticles incubated for 1h at 4°C, washed and incubated at 37°C for 4h. Data represents the mean and standard error from three independent experiments; *p<0.05, **p<0.01 using ANOVA.
cannot distinguish between internalized and membrane-bound nanoparticles. However, electron microscopy confirmed that the majority of nanoparticles were internalized. Electron microscope image analysis reveals that as media conditioning increases nanoparticle aggregation over time, the size of internalized aggregates also increases (Figure 4.7b). Additional uptake experiments were performed at 4°C to prevent internalization in order to compare nanoparticle binding avidity. These studies demonstrate that cell conditioning increases the binding avidity of nanoparticles to the cell surface (Figure 4.7a). Membrane-bound nanoparticles were exposed to fresh media for 4h at 37°C to allow release, internalization, and exocytosis. Nanoparticles modified by the conditioned media showed significantly higher retention in cells during this 4h period (Figure 4.7c). Increased uptake and retention of nanoparticles may be due to a combined effect of increased aggregate size and the presence of cell-derived proteins in the corona. By virtue of their size, aggregates are likely to bind more receptors [72, 191] and display decreased exocytosis rates [33] compared to monodisperse nanoparticles. The addition of cell-secreted proteins inside the corona may increase binding to cell receptors [58] or target new receptors. The exposure of the 15 nm citrate-coated nanoparticles to A549 cell-conditioned media causes accumulation of thrombospondin, talin-1 and fibronectin all of which are involved in cell-cell or cell-matrix binding. It is possible that the progressive accumulation of these proteins increases nanoparticle binding to highly expressed cell receptors. These findings confirm that time-dependent changes in the nanoparticle’s biological identity can directly alter cell uptake. The co-evolution of medium composition and a nanoparticle’s biological identity can therefore generate data susceptible to misinterpretation.

Exposure to extracellular environments such as conditioned media does not affect all nanoparticles equally. To illustrate how transport through the conditioned media affects individual nanoparticle designs differently, cell uptake of citrate-coated 15, 30, 60, and 90 nm nanoparticles was quantified using non-phagocytic A549 cells and in phagocytic RAW 264.7 cell models (Figure 4.8). For A549 cells, uptake trends of nanoparticles exposed to fresh media reveal the preferred uptake of 60 nm nanoparticles. When nanoparticles were exposed to A549-conditioned media, we observed a significant increase in the uptake of 15 and 60 nm nanoparticles. With RAW 264.7 cells, we observed the similar uptake of all nanoparticles incubated in fresh media. However, exposure to RAW 264.7 cell-conditioned media dramatically increased the uptake of all nanoparticle sizes between 3- to 9-fold. In conditioned media, the
Figure 4.8 Effect of conditioned media on size-dependent uptake. (a) A549 cell uptake of 15, 30, 60 and 90 nm citrate nanoparticles exposed to fresh or 24h A549 cell-conditioned media. (b) RAW 264.7 cell uptake of 15, 30, 60 and 90 nm citrate nanoparticles exposed to fresh or 24h RAW264.7 cell-conditioned media. Cells were incubated with nanoparticles for 1h at 37°C and uptake was quantified using ICP-AES. Numbers indicate the fold increase in uptake of conditioned media exposed nanoparticles relative to their counterparts in fresh medium. Comparisons were performed at 0 and 24h using ANOVA where *** p<0.001.
RAW 264.7 cells show the preferential uptake of 60 nm nanoparticles. These findings demonstrate that experimental conditions such as nanoparticle design, cell line used, and media conditioning time will influence a nanoparticle’s biological identity prior to cell uptake. By ignoring the evolving media composition and its continuous effect on the nanoparticle’s biological identity, conclusion about size-dependent uptake based on the nanoparticles’ ‘synthetic identities’ would be erroneous because they ignore the roles of aggregation and protein corona remodeling.

4.4 Conclusion

This study highlights the dynamic nature of the cell culture microenvironment and its effect on the nanoparticle biological identity (Figure 4.9). A nanoparticle’s initial exposure to a biological environment will produce its initial biological identity and corresponding cell uptake. However, the progressive secretion of cell-derived proteins and metabolites will alter the extracellular environment and may cause nanoparticle instability. The cell-conditioned environment can cause nanoparticle aggregation and protein corona remodeling leading to changes in downstream cell interactions. The co-evolution of the extracellular environment and a nanoparticle’s biological identity can ultimately cause a drift in cell uptake trends leading to data misinterpretation. This study demonstrates that ‘basic studies’ looking at the importance of nanoparticle size, shape and surface chemistry may contain an unexamined layer of complexity when considering the biological identity [33, 67, 68, 241-243]. The co-evolution of nanoparticles with their biological environment is important to consider during both in vitro experiments and in vivo tumor delivery. As the nanoparticle travels through avascular regions of the tumor where cell metabolites and secreted proteins accumulate abundantly, the biological identity of the nanoparticle is subject to change. Instability causes aggregation, protein corona deposition, protein corona remodeling and will affect tumor cell nanoparticle uptake. Data from this chapter make it unclear how protein corona and aggregation independently affect nanoparticle-cell interactions. The role of nanoparticle aggregation is especially uncharacterized in the literature. Therefore the subsequent chapters of this thesis will focus on characterizing the effect of gold nanoparticle aggregation on cell uptake.
Figure 4.9: Evolution of nanoparticles in the media changes cell uptake. The figure demonstrates how initial exposure of the nanoparticle to a biological environment creates an initial biological identity through the formation of a protein corona or nanoparticle aggregation (not shown). Cell secretion of metabolites and proteins can destabilize the nanoparticle surface leading to protein corona remodeling and nanoparticle aggregation. As the biological identity of nanoparticles evolves over time so does cell uptake.
4.5 Author Contributions

This chapter has been adapted from a manuscript currently in revision with ACS Nano. A. Albanese, C. Walkey and W.C.W. Chan designed the experiments. Experiments were performed by A. Albanese (aggregation index and cell uptake experiments) and C. Walkey (protein corona characterization) with mass spectrometry work performed by J.B. Olsen, H. Guo and A. Emili. The manuscript was written by A. Albanese with mass spectrometry analysis by C. Walkey. Edits and rewrites by C. Walkey and W.C.W. Chan.
5 Synthesis of Size-Tunable Gold Nanoparticle Aggregates

5.1 Introduction

In the last chapter, it was concluded that the composition of a tumor’s extracellular environment played an important role in determining a nanoparticle’s biological identity prior to cell contact. The interactions between a nanoparticle and its environment can lead to aggregation and changes in the protein corona. Aggregation is an important concern with all nanoparticle designs since surface destabilization in any environment often leads to nanoparticle aggregation. Biological environments are especially a concern for nanoparticle stability because of their high concentration of proteins, biomolecules and ions. Several studies have demonstrated the rapid aggregation of poorly-stabilized nanoparticles in cell culture media [67, 110, 228, 229, 244] and in vivo [245]. In several cases, aggregation occurs when the van der Waals attractive forces between particles are greater than the electrostatic repulsive forces produced by nanomaterial’s surface [246, 247]. First, high concentration of ions in biological media can decrease the screening length of charged chemical groups on the nanoparticle surface. Second, the high protein content will eventually cause a thermodynamically-favored replacement of surface-associated molecules with serum proteins [188]. The destabilization of the nanoparticle surface by both these mechanisms will produce a population of aggregates in biological environments.

The process of aggregation can be simplified into a two-step process requiring nanoparticle encounter and adherence. In aqueous solution, diffusion (Brownian motion) is the predominant force driving nanoparticle movement and can be used to calculate aggregation kinetics using Smoluchowski’s coagulation equation [248, 249]. Diffusion-limited aggregation is a well characterized random process which produces predictable fractal structures [250]. Adherence, the second step, depends on the sum of two important inter-particle forces: van der Waals attractive forces and electrostatic repulsion. The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory combines these forces into a model where the nanoparticle’s surface charge must produce an energy barrier greater than the attractive forces to ensure particle stability [246, 247].
Nanoparticle aggregates can arise from two major mechanisms. The first involves neutralization of electrostatic forces on the nanoparticle surface to drive van der Waals-mediated assembly. The second requires linker molecules to drive self-assembly of nanoparticles through high-affinity molecular interactions. In biological environments, ions can neutralize surface charges on the nanoparticles while biomolecules can crosslink nanoparticles via inter-molecular forces.

The abundance of proteins in biological environments makes it difficult to differentiate how the protein corona and aggregate morphology independently affect cellular responses. Previous studies have not been able to successfully isolate the role of aggregate morphology because protein corona formation and remodeling typically occur in parallel with aggregation. To isolate the effects of aggregation, it was necessary to develop a synthesis technique capable of generating nanoparticle aggregates of different sizes while maintaining a consistent protein corona. Several techniques have been used to generate nanoparticle aggregates: cross-linking with aliphatic α,ω-dithiols [251, 252], light induced self-assembly [253], solution phase assembly [254, 255], DNA-directed assembly [256-261], and DNA crosslinking [262]. Most of these methods utilize inter-particle linking agents to drive aggregate assembly and prevent the addition of a bioligand to the nanoparticle surface for cell targeting. Thus, it was necessary to develop a new synthesis protocol which would allow for the addition of receptor-targeting proteins on the nanoparticle surface. In this chapter, a novel synthesis technique for the production of transferrin-coated nanoparticles is presented. Aggregates were synthesized by treating citrate-capped gold nanoparticles with NaCl and transferrin (Tf). By simultaneous addition of NaCl and Tf it is possible to generate aggregates whose size depends on NaCl and Tf molecular ratios. By limiting the nanoparticle surface composition to transferrin alone, it will be possible to study the effect of aggregation on a CD71 transferrin receptor-mediated endocytosis in Chapter 6.

5.2 Material and Methods

5.2.1 Instrumentation and Measurements

For absorbance spectra a Shimadzu UV-1601 UV-Vis spectrophotometer was used. For aggregation studies using absorbance, a TECAN Sunrise plate reader was used to scan 450-700 nm wavelengths. Transmission electron microscopy (TEM) images were obtained by deposition
of a dilute particle solution onto carbon-coated copper grids and imaged using Hitachi HD2000 STEM at 200 kV (Hitachi Corp). Hydrodynamic diameter of different samples was measured by dynamic light scattering (DLS) on a 633 nm laser Nano ZS Zetasizer (Malvern).

5.2.2 Aggregate Synthesis

Citrate-capped 16 nm gold NPs were synthesized using the Frens method [26] and sized using TEM. For aggregate preparation, a 10X solution of NaCl and transferrin (Tf) was added to 16 nm AuNPs under vigorous vortex mixing. After 1 min, additional Tf was added to achieve a final concentration of 100 µg/mL. After 10 min at room temperature, 0.1% bovine serum albumin (BSA) was added and incubated for 10 minutes and centrifuged at 6,500x g for 10 min at 4°C. After centrifugation, the supernatants were removed and pellets were resuspended in nanoparticle buffer solution (10 mM Tris, 0.1% BSA, pH 7.5) and sonicated to aid resuspension. All concentrations listed in this chapter indicate the final protein and NaCl concentrations.

5.2.3 Transmission Electron Microscope Image Analysis

For TEM, 10 µl aliquots of aggregation preparations were placed on formvar type B grids (Ted Pella), left for 15 min and then wicked off. For each sample, 10-30 images were taken at 55,000X. The images were analyzed using Image J software using the following macro: Gaussian blur = 8, make binary, crop image, analyze particles (Figure 5.2). Data shown represents the maximum diameter (Ferret’s diameter) for each cluster of nanoparticles.

5.2.4 Absorbance Index

For absorbance index comparison experiments, freshly synthesized nanoparticles (2.5 nM) were centrifuged at 15,000x g for 10 min at 4°C and concentrated to 20X (50 nM). Gold nanoparticle concentration was determined using UV-Vis spectroscopy and diluted using the supernatant of centrifuged samples. Aggregates were prepared in 96 well plates, mixed for 10 minutes, and BSA was added at 0.1% to quench aggregation. Absorbance was measured from 500 to 700 nm on an ELISA plate reader and the OD700 was divided by peak absorbance value (OD_{MAX}).

5.2.5 Density Gradient Centrifugation

A solution of 64% sucrose was diluted in ddH2O to prepare 50, 40, 30 and 20% solutions. The samples were then centrifuged at 2,500x g for 45 min at 20 °C with minimum breaking. After
centrifuging, each layer was carefully harvested using a P1000 pipette into 1.5 mL conical tubes. Samples were centrifuged at 6,500x g for 10 min at 20 °C and washed 3 times using the NP buffer.

5.3 Results

5.3.1 Aggregate Synthesis and Characterization

To synthesize aggregates, the reactants were 16 nm gold nanoparticles (AuNP) coated with citric acid, NaCl, and transferrin. When NaCl is added to water, it dissociates into Na⁺ and Cl⁻, Na⁺ binds to the carboxylic acid functional groups on the citric acid groups on the AuNP surface, neutralizes the surface charge, and induces the instantaneous and irreversible aggregation of NPs into large structures that sediment out of solution as a dark grey precipitate. The use of NaCl in aggregate synthesis has already been demonstrated for both gold and other types of nanoparticles [263, 264]. However, it was important to stabilize early aggregates and restrict nanoparticle assembly into defined aggregate sizes. Limiting NaCl-induced aggregation is possible by adsorbing proteins onto the nanoparticle surface [265]. Through the simultaneous addition of NaCl and transferrin in various molar ratios, it was possible to obtain non-precipitating nanoparticle aggregates of various sizes. This technique makes it possible to adjust aggregate size by varying the rates of NaCl-mediated destabilization (k₁) and stabilization by transferrin (k₂) (Figure 5.1). When conditions are optimized so that k₁>k₂, the brief time between surface charge neutralization by Na⁺ and adsorption of transferrin creates a brief window for diffusion-limited aggregation. At the end of the aggregation, protein adsorption will stabilize AuNPs from further aggregation. To fully quench the aggregation reaction, additional transferrin was added after 1 min of reaction. It should be noted that although the words ‘agglomerate’ and ‘aggregate’ are often used interchangeably, ‘aggregate’ is used since the NaCl-induced agglomeration of AuNPs is to our knowledge irreversible. Since NaCl is the force behind aggregate assembly and our experiments reveal the relatively long stability of the synthesized aggregates (vide infra) we can confidently describe our nanomaterials as ‘aggregates’.
Figure 5.1: Scheme of aggregate formation. NaCl neutralizes the stabilizing electrostatic forces ($E_{ES}$) on the citrate-capped NPs and the van der Waal forces ($E_{vdW}$) induce aggregate formation. When protein is added to this reaction, it can bind onto the gold surface of NP and aggregates, thereby preventing additional aggregation. The addition of NaCl and protein creates some small aggregates which are quickly stabilized by protein adsorption.
Aggregates were produced by fixing nanoparticle and transferrin concentrations at 2.6 nM (1.6 \times 10^{12} \text{ AuNP/mL}) and 64 nM (5 \mu g/mL), respectively, and varying the NaCl concentration between 0-400 mM. Nanoparticle aggregation occurred as evidenced by an instantaneous color change from red to purple (Figure 5.2a). Analysis of the UV-Vis absorbance spectra revealed a red shift in the main surface plasmon resonance band from 523 nm in samples with protein alone to 527, 528, 529 and 531 nm when NaCl concentration was 50, 100, 200, 400 mM, respectively (Figure 5.2b). Aggregation also leads to the appearance of a second red-shifted band at 700 nm as the concentration of NaCl increased. This second band is characteristic of the inter-particle coupling effect [46, 266] where metal nanoparticles in close proximity produce a longitudinal plasmon resonance band which absorbs light at longer wavelength. These dramatic changes in optical properties of aggregates have been used to monitor aggregation using UV-Vis spectroscopy in previous studies [254, 255, 267, 268]. Using absorbance at 700 nm, aggregation kinetics were measured in real time after addition of NaCl and Tf. The formation of aggregates was so rapid that the earliest possible measurements were already close to reaching plateau values. The earliest absorbance values were proportional to the concentration of NaCl added and demonstrated a slow but detectable increase over the subsequent 5 minutes. The biphasic kinetics suggests a rapid burst of aggregation followed by the slow growth of nanoparticle clusters. To minimize experimental variability, aggregate synthesis included an initial concentration of transferrin and after 1 minute additional transferrin was added to block the continued growth of nanoparticle aggregates.

Dynamic light scattering (DLS) was used to determine average aggregate size (Table 5.1). The AuNP hydrodiameter was increased by ~ 8 nm after addition of Tf due to the presence of a protein corona ~4 nm thick. Similar to absorbance, DLS data revealed a NaCl-dependent increase in aggregate size from ~44 nm to ~111 nm. The aggregates all possessed large polydispersity indexes suggesting a heterogeneous population. To confirm our observations, a TEM-based method was employed to determine aggregate size more precisely (Figure 5.2d & e). Although freshly synthesized citrate-capped AuNPs typically appear aggregated under TEM, Tf-coated aggregate size was determined by analyzing multiple TEM images using ImageJ in order to determine the maximum diameter and surface area of each aggregate structure. To validate our method, the first step was to determine how many ‘false aggregates’ were detected
**Table 5.1:** DLS-based size distribution of various nanoparticle-NaCl-Tf aggregate preparations

<table>
<thead>
<tr>
<th>Condition</th>
<th>d (nm)</th>
<th>Pdl</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF (post synth)</td>
<td>18.41</td>
<td>0.083</td>
</tr>
<tr>
<td>0mM NaCl + 5 ug/ml Tf</td>
<td>26.45</td>
<td>0.171</td>
</tr>
<tr>
<td>50mM NaCl + 5 ug/ml Tf</td>
<td>43.76</td>
<td>0.252</td>
</tr>
<tr>
<td>100mM NaCl + 5 ug/ml Tf</td>
<td>67.41</td>
<td>0.219</td>
</tr>
<tr>
<td>200mM NaCl + 5 ug/ml Tf</td>
<td>86.95</td>
<td>0.234</td>
</tr>
<tr>
<td>400mM NaCl + 5 ug/ml Tf</td>
<td>111.3</td>
<td>0.264</td>
</tr>
</tbody>
</table>
Figure 5.2: Synthesis of nanoparticle aggregates. The appearance (a) and UV-Vis absorbance spectra (b) of different aggregate preparations where the concentration NaCl varied while NPs and Tf concentrations remained at 1.2 nM and 64 nM (5 µg/mL), respectively. (c) Absorbance at 700 nm was measured at 1 s intervals for 5 min after addition of NaCl and 64 nM Tf to 1.2 nM NPs. The NaCl and Tf were rapidly mixed with nanoparticles and placed in the UV-Vis spectrophotometer. (d) Sample images outlining the different steps of the aggregate sizing macro developed with the ImageJ freeware. (e) Aggregate morphology and size distribution. TEM images taken at 55,000X and size determined using Image J. (Scale bars: 100nm)
in monodisperse transferrin-coated nanoparticle images. Multiple experiments revealed that even at high concentrations, transferrin-coated 16 nm nanoparticles do not form aggregates after air drying on the TEM grid. Once the TEM images were processed using our algorithm, ~5% of the monodisperse 16 nm nanoparticles were detected as aggregates due to small inter-particle distances. This reflects an accuracy of ~95% using this electron microscope image analysis technique. The assumption that near-neighbors on the TEM images are covalently linked when the sample is well diluted was previously used by Hussain et al. [254] and is validated by our data.

Under TEM, as expected, the aggregates possessed a fractal-like morphology consistent with diffusion-limited aggregation [248, 249, 263]. The aggregates constituted of a heterogeneous population of different sizes within a single preparation. Image analysis allowed us to estimate the maximum diameter of each aggregate. Although the heterogeneity of the samples resulted in large standard deviations, there was a significant trend towards larger aggregates as the NaCl concentration increased (p< 0.001, ANOVA). This large polydispersity is characteristic of diffusion-limited aggregation and has been present in previous aggregate synthesis studies [254, 269]. Further characterization of the aggregates using the sandwich ELISA technique revealed the presence of transferrin on the surface of aggregates in all preparations. Additionally, aggregates were found to be stable for at least 14 days when kept at 4 °C in buffer, with no significant change in size distribution. Aggregates were also found to be resistant to three rounds of centrifugation-resuspension-sonication. These findings indicate that nanoparticle clusters generated by NaCl-Tf addition are irreversibly aggregated, highly stable and coated with transferrin.

5.3.2 Characterization of the Aggregation Mechanism

Since the aggregation reaction requires the interaction of nanoparticles with both NaCl and proteins, varying the concentration of these reagents should affect aggregate size. To adequately compare different sample preparations, a high throughput and sensitive comparison method was required to quantify aggregation. Sample absorbance at 700 nm correlated with overall aggregate size but was dependent on the initial NP concentration. Therefore, in order to compare different nanoparticle concentrations, OD\(_{700}\) values were normalized to the peak absorbance value to obtain an aggregation index. Additionally, when nanoparticles precipitated out of solution, the
absorbance spectra appeared as a flat line and the aggregation index equaled 1. When nanoparticle concentration was varied, data points appeared to outline a hyperbolic trend similar to plots relating either one-site binding kinetics or enzyme reaction rates (Figure 5.3). Both the aforementioned phenomena have similar equations which can be adapted to aggregation:

$$A = \frac{A_{MAX} \times X}{EC_{50} + X}$$  \hspace{1cm} \text{(Eq.1)}

where A is the aggregation index as a function of NaCl concentration. Fitting the data with this equation allowed us to obtain values for maximum aggregation ($A_{MAX}$) and the NaCl concentration required for half of $A_{MAX}$ ($EC_{50}$). These values allowed us to quantify the plateau and relative slope of a given data set. This experimental setup can be used to monitor the effect of each reaction parameter on aggregation. Augmenting the concentration of nanoparticles increased $A_{MAX}$ and decreased $EC_{50}$ in a dose-dependent manner (Figure 5.3a). At 8 nM nanoparticle concentration, aggregation kinetics reached their maximum rate. Inversely, below 1 nM nanoparticles, aggregation is undetectable using 25-400 mM NaCl. Increasing nanoparticle concentration raises the frequency of inter-nanoparticle encounters in solution and depletes soluble protein molecules, both of which favor aggregation. These findings illustrate the importance of nanoparticle concentration during aggregate synthesis.

Protein concentration, on the other hand, will control nanoparticle stability and inhibit the aggregation reaction (Figure 5.3b). When transferrin is removed from the reaction, the aggregation index reaches ~1 at 12.5 mM NaCl, demonstrating poor nanoparticle stability in the absence of protein. Addition of Tf decreases the maximum achievable aggregation ($A_{MAX}$) in a dose-dependent manner. Increasing a protein’s concentration improves its adsorption kinetics onto the nanoparticles thereby limiting NaCl-nanoparticle interactions and reducing aggregation. After controlling the concentration of each reagent, we next evaluated experimental conditions by altering the pH and temperature of aggregate synthesis reactions. The reaction’s pH affected aggregation in either acidic or basic conditions (Figure 5.3c). At pH 5, the citrate ions are close to their isoelectric point and show reduced negative charge, which causes some aggregation before the addition of NaCl and produces a higher susceptibility to aggregation. When the
Figure 5.3: Optimization of aggregation synthesis. (a) Aggregation index of various samples when Tf concentration is fixed at 64 nM and nanoparticle concentration is varied. (b) Aggregation index of various samples when nanoparticle concentration is fixed at 2 nM and Tf concentration is varied. The tables below the graphs display the peak (AMAX) and EC50 calculated using Eq (1). (c) Effect of pH on aggregation using 1.2 nM nanoparticles and 64 nM Tf. (d) Effect of temperature on aggregation using 1.2 nM nanoparticles and 64 nM Tf. Lines are included as a visual aid.
pH > 9, the abundant OH- ions may displace the citrate anions on the nanoparticle surface, the weaker interactions hydroxyl ions and gold compared to citrate anions make the nanoparticles more susceptible to aggregation. In contrast, temperature did not seem to affect aggregate synthesis at 4°C or 37°C (Figure 5.3d).

5.3.3 Adaptability of Aggregation Reaction

Since the production of size-tunable aggregates depended predominantly on Tf protein’s ability to inhibit NaCl-mediated aggregation, it was important to elucidate whether this synthesis technique could be adapted to different proteins. Two proteins were selected to evaluate the adsorption of smaller and larger proteins: the 20kDa myoglobin protein and the 180 kDa bovine IgG protein. At pH7, myoglobin triggered the aggregation of nanoparticles in the absence of NaCl, similar to lysozyme [268]. At pH 8.5, aggregation was strictly dependent on NaCl concentration. IgG protein was similar to transferrin and was capable of preventing NaCl-mediated aggregation at pH 7. TEM analysis of myoglobin and IgG aggregates revealed the fractal arrangement of clusters and heterogeneous size distribution similar to Tf (Figure 5.4). Additional aggregate preparations were also obtained using BSA and 2 kDa thiol-terminated methoxy-PEG (Figure 5.4) to further illustrate adaptability of the aggregate synthesis protocol. These findings demonstrate that the aggregate synthesis protocol is adaptable to various proteins and thiol-terminated ligands for customizable surface functionalization. These preliminary experiments also reveal that certain proteins may require specific pH conditions to control aggregate size.

5.3.4 Fractionation of Aggregates

Given the heterogeneous nature of our aggregate preparations, a fractionation technique was developed to isolate separate aggregate populations within a given sample. Techniques such as gel filtration and density gradient ultracentrifugation can be used to separate different species of varying mass within a sample. However, the large mass and density of nanoparticles make these techniques incapable of discriminating between different nanoparticle aggregate sizes. After some optimization, successful aggregate fractionation was achieved using low speed centrifugation over dense sucrose gradients (Figure 5.5). This technique allowed the purification
Figure 5.4: Synthesis of aggregates with different surface molecules. TEM-based size distribution of aggregates prepared using 20 µg/mL myoglobin + 0.1M NaCl, 10 µg/mL bovine IgG + 0.2M NaCl, 0.63 µg/mL SH-mPEG-2000 + 400 mM NaCl, or 10 µg/mL BSA with 200 mM NaCl.

Figure 5.5: Fractionation of aggregate subsets using density gradient centrifugation. (a) Appearance of gradient after centrifugation. (b) Size of different aggregate fractions after 3 washes. (c) Size distribution of the various aggregate fractions compared to the sample pre-centrifugation.
of 7 separate fractions of varying purity and size distribution. Fractions 1 and 2 contain a narrow distribution of 40-60 and 60-100 nm aggregates, respectively, whereas fraction 6 is rich in aggregates ≥ 200 nm. Using this strategy our size distribution improved considerably in certain samples like fraction 1 where the constant of variation (CV) was ~33% compared to 147% for the starting sample. This fractionation demonstrates the ability to purify size specific nanoparticle clusters and illustrates the possibility of isolating both small nanoparticle doublets and larger clusters, depending on downstream applications.

5.4 Discussion

Aggregate synthesis was achieved using a simple technique that uses NaCl to reduce the inter-particle electrostatic repulsion allowing the van der Walls attractive forces to drive nanoparticle assembly. During NaCl-mediated aggregation of citrate-capped gold nanoparticles, addition of proteins or thiol-terminated molecules will adsorb onto the nanoparticle surface and slow down aggregate growth. A protein’s numerous functional groups and large size (relative to citrate) prevent salt-mediated aggregation by increasing the electrostatic repulsion between particles and by providing a steric barrier to nanoparticle-nanoparticle interactions. The presence of both NaCl and proteins in solution creates an equilibrium where small aggregates are produced and stabilized before growing into macroscopic structures (Figure 5.1). This unique approach to aggregate synthesis differs from previous attempts by its use of inter-particle forces to drive assembly instead of linking nanoparticles via surface chemistry. This approach is also likely to mimic the aggregation mechanism of nanoparticles in biological environments where ions and proteins are abundant.

An important challenge with this work was producing a suitable technique to determine the size of the synthesized aggregates. Due to their heterogeneity, methods such as dynamic light scattering (DLS) or UV-Vis spectroscopy can be unreliable. For example, the sensitive relationship between nanoparticle size and light scattering causes DLS data to overestimate aggregate sizes since larger clusters will skew the measured average. TEM was found to be the best option for aggregate sizing and was essential in confirming both the DLS and UV-Vis data. Surprisingly, this study appears to be the first to include the in-depth quantification of average aggregate size and sample distribution. UV-Vis absorbance spectra were an accurate albeit qualitative indicator of aggregation. The aggregation index was especially useful for high
throughput screening of reaction conditions to determine the concentration of protein and NaCl required for producing size-tunable aggregates.

Aggregates produced using this technique can be useful for various applications. Previous work has demonstrated that the physical and chemical properties of nanoparticle aggregates make them suitable for electromagnetic energy transport [270-272] and surface-enhanced Raman scattering (SERS) [273-275]. Nanoscale gaps between adjacent metal nanoparticles were shown to produce intense electromagnetic fields in response to optical excitation [276, 277]. These ‘hot-spots’ achieve large SERS enhancements when the inter-particle gap is below 10 nm [278, 279]. However, the motivation for this work was to generate multiple aggregate sizes possessing a similar surface functionalization in order to compare the effect of aggregate morphology on cell uptake and toxicity.

5.5 Author Contributions

This study was conceived by A. Albanese and W. Chan. All experiments were optimized, executed and analyzed by A. Albanese. This chapter was written by A. Albanese with additional edits and corrections by W. Chan.
6 Effect of Nanoparticle Aggregation on Cell Uptake and Toxicity

6.1 Introduction

The aggregate synthesis technique developed in the previous chapter permits the size-controlled synthesis of aggregates with customizable surface functionalization. These materials were generated to analyze the effect of aggregate morphology on nanoparticle-cell interactions. Aggregation is a widespread concern for nanoparticles exposed to biological environments in vitro and in vivo. Several studies have reported the rapid aggregation of uncoated or poorly-stabilized nanoparticles in cell culture media due to the high concentration of ions and proteins [67, 110, 228, 229, 244]. Nanoparticles are often stabilized with surface-bound PEG molecules, proteins, thiol-terminated DNA, etc. Although these stabilized nanoparticles do not aggregate in the short term, several studies have demonstrated eventual aggregation both in vitro [228] and in vivo [245].

Previous studies in nanotoxicology have demonstrated that nanoparticle aggregation or agglomeration will significantly affect both uptake and toxicity. The effect of aggregation varies from study to study depending on nanoparticle composition and the cell types used. However, published studies were mostly conducted in the context of environmental exposure to industrial byproducts such as cerium oxide [227], carbon nanotubes [240] or metal oxides [280]. To date, no study has focused on the effect of aggregation on nanoparticles designed for biomedical applications. Several obstacles have prevented this type of study in the past. First, most of the previous studies were done with agglomerated nanomaterials produced from ‘the top down’. In these studies, nanomaterials are originally agglomerated and are made into smaller agglomerates or monodisperse nanoparticles using sonication. These agglomerates are not likely to mimic aggregates occurring in vivo, but rather model nanoparticle that are aggregated/agglomerated prior to entering a biological environment. Second, previous aggregation studies did not use receptor-targeting nanoparticles in their studies. Therefore, the aggregates are likely to generate
distinct protein coronas in the extracellular environment than the monodisperse nanoparticles. This makes it difficult to distinguish between the effects of protein corona composition and aggregate morphology. In this chapter, different transferrin-coated aggregates are administered to multiple cell types in order to quantify changes in receptor-mediated uptake kinetics and cytotoxicity.

6.2 Methods

6.2.1 Gold Nanoparticles

Citrate-capped 16 nm gold nanoparticles were synthesized using the Frens method [26] and sized using TEM. The 30, 45 and 100 nm nanoparticles were synthesized using hydroquinone-mediated growth of the 16 nm seeds [28]. The 30, 45 and 100 nm nanoparticles were washed using a solution of 0.05% Tween-20 and 5 mM sodium citrate. Protein adsorption was performed using 5 mM citrate and 0.5, 1 and 4 mg/mL transferrin for 30, 45 and 100 nm nanoparticles, respectively. The different transferrin concentrations used for each nanoparticle size were determined experimentally as the minimum amount of transferrin required for full surface coverage. Full surface coverage was assessed by stability of transferrin-coated nanoparticles in 5% NaCl [265]. After 30 min incubation at 37°C, bovine serum albumin (BSA) was added to the transferrin-coated nanoparticles at a 0.1% concentration to ensure nanoparticle stability during centrifugation steps [53]. The nanoparticles were washed twice following transferrin adsorption in a 0.1% BSA solution.

6.2.2 Aggregate Synthesis

For aggregate preparation, a 10X solution of NaCl and transferrin was added to 16 nm nanoparticles directly after synthesis under vigorous vortex mixing. After 1 min, more Tf was added to achieve a final concentration of 50 µg/mL. After 10 min at room temperature, 0.1% BSA was added and incubated for an additional 10 min. Depending on the aggregation conditions, different centrifuge speeds were used. For 0 mM, samples were centrifuged at 15,000g for 15 min. For 25 mM, samples were centrifuged 6,500g for 10 min. For 100 mM, samples were centrifuged at 500g for 10 min, the supernatant was removed and centrifuged at 4,500g for 10 min. For 400 mM, samples were centrifuged 2,500g for 10 min. The aggregate pellets were then resuspended in 0.1% BSA, sonicated in a water bath for 1 min and centrifuged
under the same conditions to further improve size distribution. After this step, aggregates were characterized and used in cell uptake experiments. For cell experiments, aggregates were quantified using ICP-AES. Using the total concentration of gold in solution it was possible to calculate the concentration of monodisperse nanoparticles using the mass of a 16 nm gold sphere.

6.2.3 TEM Image Analysis

For TEM, 10 µl aliquots of aggregation preparations were placed on formvar type B grids (Ted Pella), left for 15 min and then wicked off. For each sample, 10-30 images were taken at 55,000X. The images were analyzed using Image J software using the following macro: Gaussian blur = 8, make binary, crop image, analyze particles. Data shown represents the maximum diameter (Ferret’s diameter) for each cluster of nanoparticles. The number of nanoparticles per aggregate was determined by dividing the surface area of each aggregate by the theoretical 2D surface area of a 16 nm nanoparticle ($\pi r^2$). Although the aggregates are 3D structures, we believe this method to be accurate for the measurement of small aggregates when nanoparticles are visible. For larger aggregates, their large three-dimensional structure makes it difficult to accurately determine the correct number of nanoparticles per cluster. However, this method does provide a good estimate to the average composition of aggregates. For TEM images of cell vesicles, cell sections were prepared by fixing cell pellets in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Quetol-Spurr resin. Sections that were 100nm thick were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate. For each sample, three separate tissue sections (taken at a different depth) were placed on a TEM grid and imaged. Cells were incubated with 1nM nanoparticles for 8h.

6.2.4 Cell Uptake and Toxicity

For nanoparticle uptake studies, HeLa and MDA-MB-435 cells were seeded 2 x 10^6 cells/well in 6-well plates and incubated overnight to allow cell attachment. The A549 cells were seeded at 1 x 10^6 cells/well under the same conditions. After the overnight incubation, cell media was removed and replaced with 1 mL RPMI (A549, MDA-MB-435) or 1 mL DMEM (HeLa) supplemented with 0.2% BSA. Cells were serum starved for 30 min and fresh media containing the nanoparticles and 0.2% BSA was added. Experiments were performed in the absence of
serum due to the large amounts of transferrin present in fetal bovine serum. Cells were incubated for 2, 4 or 8h in the presence of nanoparticles. At each time point, cell supernatant was removed and cells were washed 3 times with PBS. Samples were digested in 500µl nitric acid for 30 min at 70°C, diluted in 3mL ddH₂O. Gold uptake was quantified using ICP-AES using magnesium as an indicator of cell number. All conditions were performed in triplicate. The concurrent cell viability assay was performed using the XTT assay (Roche) in 96 well plates according to the manufacturer’s instructions.

6.2.5 Calculating the Aggregate Sedimentation Rate

Based on previous work by Limbach et al. [227], the settling velocity (m/s) of a fractal aggregate consisting of N nanoparticles in a liquid medium can be calculated using the following formula:

\[
v_{sed} = \frac{2(\rho_s - \rho_m)gN r^3}{9\mu R_g}
\]

(Eq. 1)

where \( \rho_s \) and \( \rho_m \) are the densities of gold (19,320 kg m\(^{-3}\)) and medium (1000 kg m\(^{-3}\)), respectively. Where \( g \) is the gravitational force (9.8 m s\(^{-2}\)), \( N \) is the number of nanoparticles per aggregate, \( r \) is the radius of nanoparticles (8 x 10\(^{-9}\) m), \( \mu \) is the viscosity of the medium (1.4 x 10\(^{-3}\) Pa s) and \( R_g \) is the radius of gyration (m). The only missing value in this equation is the \( R_g \) which is specific to each aggregate’s size.

using the following formula,

\[
N = \left( \frac{R_g}{r} \right)^{D_f}
\]

(Eq.2)

It is possible to determine \( R_g \) using the following formula:

\[
R_g = rN^{1/D_f}
\]

(Eq.3)

where \( r \) is constant, \( D_f \) is known to be 1.8 for diffusion-limited aggregation of gold colloids [263]. \( N \) is established by using the surface area of each aggregate in the ImageJ-processed TEM
images. The area of each aggregate is then divided by \( \pi (8)^2 \) which is the 2D surface area of a 16 nm nanoparticle under TEM. From the surface area of each aggregate, we calculate \( N \), \( R_g \) and finally \( v_{\text{sed}} \).

6.3 Results & Discussion

6.3.1 Characterization of Aggregates

Using the techniques developed in chapter 5, a series of transferrin-coated nanoparticle aggregates were generated by manipulating the kinetics and stoichiometry of salt, proteins, and gold nanoparticles. Aggregates were produced by fixing nanoparticle and Tf concentrations at 2.6 nM (1.6 x 10^{12} nanoparticles/mL) and 64 nM (5 µg/mL), respectively, and varying the NaCl concentration between 0-400 mM. For these in vitro studies, some important changes were made to the synthesis procedure. Of note, specific centrifugation speeds were used for each aggregate formulation in order to improve size distribution (details in the methods section). One example is for nanoparticles treated with 100 mM NaCl an initial 5 min centrifugation at 500g for 10 min. The supernatant was collected and centrifuged at 4,500g for 10 min and washed three times under those same conditions. The repeated wash steps at specific centrifuge speeds improved the size distribution of aggregates to an extent where we did not use sucrose gradient centrifugation-based purification to generate aggregates. The aggregates used for this study were synthesized and characterized in Figure 6.1 and Table 6.1. As expected, UV-Vis spectrometry, dynamic light scattering (DLS) and electron microscopy reveal a NaCl-dependent increase in the average aggregate size.

To work around the large polydispersity of our samples, we selected conditions that produced different size ranges. For example, 25 mM NaCl produced some doublets and triplets amongst a predominantly monodisperse population; 100 mM NaCl produced samples where > 80% of the sample was within the 20-80 nm size range; 400 mM NaCl generated aggregates where >60% aggregates were within the 80-200 nm size range. For simplification, each aggregate formulation was named after its mean diameter. Thus, the formulations used in these in vitro studies were either monodisperse (M) 15 nm nanoparticles or aggregate formulations.
<table>
<thead>
<tr>
<th>UV-Vis</th>
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<th>TEM</th>
</tr>
</thead>
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<td></td>
<td>Max WL</td>
<td>d (nm)</td>
</tr>
<tr>
<td><strong>AuNPs</strong></td>
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</tr>
</tbody>
</table>

Table 6.1: Characterization of nanomaterials used in this Chapter.
Figure 6.1. Characterization of aggregates. (a) Appearance of monodisperse (M; 0 mM NaCl), 26 nm (25 mM NaCl), 49 nm (100 mM NaCl) and 98 nm (400 mM NaCl) aggregates. (b) Gel shift assay of aggregates, electrophoresis was performed in 0.5% agarose migrated at 50V for 2h at 4°C. (c) UV-Vis spectra of aggregates which were prepared according to the protocol outlined in the methods section and washed twice with 0.1% BSA. (d) Appearance and size distribution of monodisperse, 26 nm, 49 nm and 98 nm aggregates. Data is presented as the percentage of all structures detected by TEM.
possessing mean diameters of 26, 49 or 98 nm. These different formulations were used to mimic the early and late stages of aggregation inside the tumor environment. At early time points, some doublets and triplets appear amongst a mostly disperse population of nanoparticles. At later time points, aggregation leads to formation of larger nanoparticle clusters. To ensure that the prepared aggregates did not continue to grow over time, long term characterization revealed good stability for at least 14 days in 0.1% BSA with no detectable change in UV-Vis spectra or hydrodynamic diameter. Aggregates were stable in cell culture media at 37°C in 5% CO₂ for at least 8h, which is the longest cell incubation time used in our experiments.

Sandwich enzyme-linked immunosorbent assays (ELISA) were used to confirm the presence of transferrin on the surface of aggregates (Figure 6.2a). It should be noted that an equal amount of nanoparticles were added to each well in the assay, however it was impossible to quantify how many nanoparticles were bound to the capture antibodies in each well. Also, the sandwich ELISA was designed to measure concentrations of soluble molecules, not proteins bound onto a surface. Thus, the ELISA data could not be considered quantitative and was simply used in a qualitative context to confirm the presence of transferrin. The aggregates demonstrate a decrease in detectable transferrin which may be due to less available surface area for protein adsorption since most nanoparticle surface is obstructed by inter-nanoparticle contact.

6.3.2 Cell Uptake of Aggregates

After preparing and characterizing our nanomaterials, aggregates were incubated with cultured cells and gold uptake was quantified using ICP-AES. Prior to incubation with cells, monodisperse nanoparticle and aggregates were quantified using ICP-AES in order to determine each material’s concentration. When comparing the nanoparticle formulations, we chose to use a constant number of nanoparticles in our cell culture experiments. We added 1 mL of 0.2 nM monodisperse nanoparticles (1.2 x 10¹¹ nanoparticle/mL) or the equivalent number of nanoparticles under various states of aggregation. Using this method, cells exposed to 100 individual nanoparticles would be compared to 50 two-nanoparticle aggregates or one aggregate made up of 100 nanoparticles. This approach generates experimental conditions where aggregate size is inversely proportional to concentration of structures in solution. However, these
Figure 6.2. Transferrin and CD71 transferrin receptor. (a) Detection of transferrin on the surface of monodisperse (M) and 26 nm, 49 nm and 98 nm aggregates using commercial sandwich ELISA kit (Bethyl Laboratories). As a negative control the Tf aggregates were compared to bovine albumin coated nanoparticles (BSA). Bars represent the mean and standard deviation of 3 replicate wells. Data is not quantitative since we cannot normalize data to number of bound nanoparticles per well. Reduced transferrin in the large aggregates may be due to less available surface for protein adsorption since most nanoparticles are part of large aggregates. (b) Expression of Tf receptor (CD71) for MDA-MB-435 (green), A549 (red) or Hela (blue) cells. Cells were harvested from culture and stained according to the manufacturer’s protocol with PE-conjugated anti-CD-71(eBioscience). (c) Amount of Tf-FITC bound by each cell line. Cells were serum-starved for 30 min, trypsinized, rinsed and stained with 10 µg/mL Tf-FITC for 15 min at 4°C. Values indicate the fold-increase in mean fluorescent intensity compared to unstained sample.
conditions mimic an experiment where 100 individual nanoparticles are injected into an animal and aggregate into a single large aggregate.

The monodisperse and aggregated nanoparticles were coated with the transferrin protein, which typically enters cells via the process of receptor-mediated endocytosis. We chose three commonly used human cell lines and quantified their expression of transferrin receptor (CD71) using flow cytometry. Results revealed that CD71 expression was highest in HeLa > A549 > MDA-MB-435 cells (Figure 6.2a). The amount of fluorescently-labeled transferrin bound to the cell surface also correlated with CD71 expression (Figure 6.2b). When we assessed the effect of aggregate size on uptake in HeLa cells, which express the highest levels of CD71, our data revealed that uptake was reduced by ~25% for 49 and 98 nm aggregates (Figure 6.3). The 25% reduction in uptake was apparent from 2h (p<0.05) and persisted until 8h (p<0.001). A549 cells also show reduced uptake of the 49 and 98 nm aggregates in comparison to single nanoparticles. The A549 cells also show the decreased uptake of the 26 nm aggregates at 8h. The earliest detectable differences in uptake occurred at 4h for A549 cells (p< 0.01). It should be noted that for A549 cells, detection of gold at 2h (13-19 µg/L) and 4h (17-30 µg/L) was slightly below our smallest measurement standard (25 µg/L). Thus, uptake differences may not be detectable at these early time points for A549 cells [281]. For these two cell lines it can be concluded that aggregation of transferrin-coated nanoparticles will significantly decrease cell uptake.

The MDA-MB-435 cells displayed a different uptake trend than the HeLa and A549 cells. Surprisingly, the 98 nm aggregates show approximately a two-fold increase in uptake (p < 0.001) in comparison to other formulations as early as 4h. Increased uptake was only apparent for the largest aggregates, suggesting that increased uptake only occurs above a certain size threshold not achieved by the 26 or 49 nm aggregate preparations. These findings were unexpected since theoretical models predict the optimal diameter for nanoparticle uptake to be ~50 nm in receptor-mediated endocytosis [75, 282, 283]. Since MDA-MB-435 cells possess the lowest expression of CD71 transferrin receptor, it is unlikely that large aggregates are using receptor mediated endocytosis to enter the cells. In theory, large aggregates require more receptor-ligand interactions in order to drive the thermodynamic process of membrane wrapping around the aggregates [72, 75, 284]. Thus, increased uptake in MDA-MB-435 cells may be due to alternate
Figure 6.3. Aggregate uptake in HeLa, A549 and MDA-MB-435 cells. Uptake of monodisperse (white), 26nm (grey), 49 nm (lines) and 98 nm (black) aggregates in HeLa (a), A549 (b) and MDA-MB-435 (c) cells. Cells were harvested at 2, 4 and 8h and analyzed for gold content using ICP-AES. Aggregate uptake was compared to monodisperse nanoparticles at each time point using ANOVA († p < 0.01, ‡ p < 0.001). Data represents average of three replicate wells with standard deviation. (d) Uptake of monodisperse (white) and 98 nm aggregates (black) in the presence of 1 mg/mL transferrin. Data is presented as the ‘% suppression’ when compared to controls where cells were incubated with nanoparticles in the absence of soluble transferrin.
internalization mechanisms. To validate this hypothesis, we compared the uptake of monodisperse single and 98 nm aggregates in the presence or absence of 1 mg/mL soluble transferrin. We hypothesized that in the presence of soluble transferrin, the nanoparticles would not enter the cells due to saturation of the CD71 receptors on the cell surface. As expected, in all three cell lines, high transferrin concentration was able to reduce uptake of monodisperse nanoparticles by 91% in HeLa cells, 85% in A549 cells and 73% in MDA-MB-435 cells (Figure 6.3d). Soluble transferrin was less efficient at blocking the uptake of nanoparticle aggregates. A549 and HeLa cells show 51% and 60% reduction in uptake of 98 nm aggregates, respectively. MDA-MB 435 cells only show a 10% reduction in uptake. These findings suggest that additional cell uptake mechanisms in addition to CD71-mediated endocytosis are involved in the internalization of 98 nm aggregates. Although these additional pathways play a minor role in A549 and HeLa cells, it is apparent that MDA-MB-435 cells are highly efficient at CD71 independent uptake. It remains unclear whether aggregates are entering MDA-MB-435 cells using a different cell surface receptor or are interacting non-specifically with the cell membrane.

6.3.3 Uptake of Aggregates Compared to Larger Nanoparticles

Next, we wanted to assess whether aggregate uptake trends observed were simply due to their larger size or their unique irregular morphology. To determine if uptake trends were caused by the aggregate’s larger diameter, we compared the uptake of aggregates with size-matched transferrin-coated nanoparticles possessing diameters of 30, 45, and 100 nm. To ensure accurate comparisons, all nanoparticle formulations were added to cells at concentrations that would produce the same volume of gold. Cells were analyzed by ICP-AES and data was calculated as the average mass of gold internalized per cell (pg/cell). Unfortunately, we were unable to adequately assess the internalization of 100 nm nanoparticles since they quickly settled to the bottom of 6-well plates by 2-3h due to their large mass. The rapid sedimentation speed of large gold nanoparticles increases their concentration near the cell membrane and alters uptake kinetics [285]. In this comparative study, aggregates did not follow the same uptake trends as their size-matched spherical counterparts. Each cell line showed an increased uptake of nanoparticles with larger diameter (Figure 6.4). These experiments allow us to conclude that aggregate uptake cannot simply be attributed to their larger diameter. It is likely that the
aggregates’ other properties and morphological features are also influencing cell uptake. Since aggregates are composed of 16 nm nanoparticles, the high surface curvature can lower the density of transferrin adsorbed onto the gold surface compared to larger nanoparticles [53]. Aggregates also possess irregular morphologies leading to a wide range of aspect ratios each capable of influencing endocytosis [67]. Unfortunately, addressing these variables is beyond the scope of this study. It should be noted that the asymmetrical structure of aggregates can also increase the complexity of interactions with a cell’s membrane. For example, a diamond shaped aggregate made of four nanoparticles can interact with a cell either at its tip where a single nanoparticle can bind with the cell receptors or at one of its edges where two nanoparticles can interact with the membrane (Figure 6.4b). In our example, a symmetrical diamond-shaped aggregate can interact with cell receptors in two different orientations. However, asymmetrical aggregates have a much larger number of configurations in which they can interact with cell surface receptors. In contrast, a spherical nanoparticle will possess a homogenous surface with a given curvature and a uniform density of bioligands. The complex morphology and numerous binding orientations of aggregates may partially explain why their uptake cannot be modeled by simply looking the uptake of larger nanoparticles.

6.3.4 Aggregates Uptake Dose Response

Our aggregates affected cell uptake in two distinct ways: a small reduction in aggregate uptake or a large increase in uptake of the biggest aggregates. Aggregate uptake in HeLa and A549 cells occurs mostly via the CD71 receptor but is less efficient compared to monodisperse nanoparticles. In contrast, MDA-MB-435 cells demonstrate a highly efficient receptor-independent uptake mechanism for aggregates. To better understand the role of transferrin receptor in aggregate uptake, we compared uptake of monodisperse nanoparticles and 98 nm aggregates at various concentrations to saturate receptor binding. We also simplified experimental conditions by simply comparing MDA-MB-435 and HeLa cells since each displayed a distinct response to aggregates. These experiments revealed that MDA-MB-435 cells maintained the increased uptake of 98 nm aggregates at all concentrations tested (0.2-1.0 nM). We also determined the percentage of nanoparticles taken up by the cells relative to the administered dose using the following equation:
**Figure 6.4:** Uptake of spherical transferrin-coated nanoparticles. (a) Amount of gold internalized per cell at 8h. The A549, HeLa and MDA-MB-435 cells were incubated with transferrin-coated spherical nanoparticles whose size was similar to average aggregate sizes. All data represents average of three replicate wells with standard deviation. (b) Scheme comparing uptake of 30 nm nanoparticle (left) with a diamond aggregate of similar size (right). Whereas the 30 nm nanoparticle has one way of interacting with the cell membrane, the aggregates has two possible interactions: tip or edge of the aggregate. When the aggregate’s tip interacts with cell membrane, there is not enough free energy produced by ligand-receptor binding to drive uptake.
For MDA-MB-435 cells, the data series for both disperse and aggregated nanoparticles did not produce a slope significantly different than zero. The percentage uptake of monodisperse nanoparticles and 98 nm aggregates was constant at 0.84% and 1.39%, respectively (Figure 6.5). In contrast, HeLa cells changed their uptake trends at higher concentrations. Above 0.6 nM, we could not detect any statistically significant differences in the uptake of monodisperse and aggregated nanoparticles. Below 0.6 nM, the difference in uptake between monodisperse and aggregated nanoparticles was inversely proportional to nanoparticle concentration. When comparing uptake as a percentage of total administered dose, HeLa cells consistently internalized ~ 2.5% of 98 nm aggregates, independent of the concentration. In contrast, monodisperse nanoparticles were internalized at 5.61% at 0.1 nM and this decreased to 4.36, 3.54 and 2.62% for 0.2, 0.4, and 0.6 nM, respectively (Figure 6.5d). These findings suggest that the high expression of CD71 transferrin receptor by HeLa cells produces a higher affinity for monodisperse nanoparticles than aggregates below 0.6 nM. Above 0.6 nM, the effect of high CD71 expression is saturated causing aggregated and monodisperse nanoparticles to behave similarly.

6.3.5 Aggregate Internalization

To better understand how MDA-MB-435 and HeLa cells differ in their nanoparticle uptake mechanism, we analyzed TEM images of cells incubated with monodisperse nanoparticles and 98 nm aggregates (Figure 6.6). These images confirm that both cell lines successfully internalize both monodisperse and aggregated nanoparticles into intracellular vesicles. No nanoparticles were detected in the cytosol suggesting that intracellular trafficking occurs predominantly through the endocytic pathway. In agreement with previous studies, the majority of monodisperse nanoparticles were aggregated inside the vesicles of both HeLa and MDA-MB-435 cells [33, 68]. Internalization of monodisperse nanoparticles produced aggregates in the intracellular vesicles with a mean size of 5.52 and 4.99 nanoparticles per aggregate in HeLa and MDA-MB-435 cells, respectively. The difference between these two cell lines was not significant and reveals a similar sequestration of monodisperse nanoparticle. When we analyzed
Figure 6.5: Aggregate dose response. Uptake of monodisperse nanoparticles (closed circles) and 98 nm aggregates (open squares) at various concentrations in MDA-MB-435 cells (a-b) and HeLa cells (c-d). Data is plotted either as number of nanoparticle per cell (a,c) or percentage of total dose (b,d). Data represents average of three replicate wells with standard deviation.
Figure 6.6: Size of internalized aggregates. (a) Average size of internalized nanoparticles aggregates inside vesicles of MDA-MB-435 and HeLa cells treated with monodisperse nanoparticles or 98 nm aggregates. Data represents mean and SD of n > 25. ‡ denotes p<0.001 using the statistical test ANOVA. Shown below are representative TEM images of HeLa cells incubated with monodisperse nanoparticles (b) or aggregates (c) and MDA-MB-435 cells treated with monodisperse (d) or aggregated nanoparticles (e). Bars represent 100 nm and arrows highlight internalized nanoparticle aggregates.
the internalization of the 98 nm aggregates, MDA-MB-435 cells contained noticeably larger aggregates inside the intracellular vesicles. HeLa cells had on average 14 nanoparticles per aggregate whereas MDA-MB-435 cells had 40 nanoparticles per aggregate. In HeLa cells, the size of internalized aggregates is similar to the average size of 98 nm aggregates post-synthesis (12.5 vs 14 nanoparticle/aggregate; Table 6.1). In contrast, aggregates internalized by MDA-MB-435 cells show a three-fold increase in size. Post-synthesis, 40 nanoparticle aggregates only represent ~10% of the entire population. MDA-MB-435 cells accumulate large aggregates in their vesicles by two possible mechanisms: either the largest aggregates are preferentially internalized or intracellular trafficking causes smaller aggregates to cluster into large structures inside the vesicles. To determine if sedimentation caused the preferential internalization of aggregates compared to monodisperse nanoparticles, we calculated the settling time of aggregates. The largest aggregate found using TEM consisted of ~130 nanoparticles and possessed a settling time of ~18h in our reaction volume compared to ~160h for a 16 nm nanoparticles (calculations are outlined in the methods section). Since 40 nanoparticle aggregates require a maximum of ~30h to settle, the settling velocity of larger aggregates may increase uptake in MDA-MB-435 cells and lead to some preferential accumulation of large intracellular aggregates.

6.3.6 Evaluating Aggregate Toxicity

After assessing the effect of aggregation on cellular uptake, it was important to determine whether the observed data trends were caused by aggregate-size related toxicity. Nanoparticle toxicity is always a concern in cell uptake studies depending on the materials used [286, 287]. To assess toxicity, cells were incubated with various disperse and aggregated nanoparticle formulations at multiple concentrations for 72h. Viability was quantified using the XTT assay where production of a colored substrate is dependent on dehydrogenase enzymes of metabolically active cells. Our results demonstrate that nanoparticles and aggregates at concentrations equal to or below 1 nM had no effect on cell viability (Figure 6.7). Thus, differences in cell uptake were not caused by changes in cell viability and instead related to internalization and sub-cellular distribution mechanisms.
Figure 6.7: Toxicity of aggregates. Effect of aggregates on cell viability after 72h incubation with 1 nM monodisperse (white), 26nm (grey), 49 nm (lines) and 98 nm (black) aggregates in three cell lines. Cell viability was determined at 72h, by replacing cell media containing nanoparticles with fresh media containing XTT and incubated for 2h. All conditions were performed in triplicate. Data represents average of three replicate wells with standard deviation.
6.4 Conclusions

The goal of this work was to determine the effect of nanoparticle aggregation independently of the protein corona’s composition. These initial experiments demonstrate that the morphology of aggregates can significantly change the uptake kinetics of transferrin-functionalized nanoparticles. Aggregation is capable of increasing and decreasing nanoparticle uptake in different cell lines. The aggregation of transferrin-coated nanoparticles reduces uptake via receptor-mediated endocytosis in HeLa and A549 cells. For MDA-MB-435 cells, aggregation causes nanoparticles to enter the cells independently of transferrin receptor via unknown mechanisms and this leads to the accumulation of large aggregates inside the cell’s vesicles. These findings suggest that the morphological features of the aggregates may produce unique uptake trends distinct from larger spherical nanoparticles. However, it is impossible to ignore the effects of aggregation on the sedimentation rate, diffusion speed, and concentration of administered nanomaterials. At present, it is impossible to determine how aggregation specifically changes cell uptake of nanoparticles. More fundamental studies will be required to produce predictive models which can determine how aggregation will affect cell uptake in different cell lines. It is likely that aggregate uptake will depend on the physicochemical properties of the nanoparticles, the biological properties of the bioligand used and the phenotype of the cell interacting with the nanoparticles [288].

The number of studies investigating the uptake of aggregates is too low to justify the possibility of a ‘universal aggregate uptake mechanism’. Current literature contains a handful of contradictory studies which make it difficult to draw any general conclusions. Some studies claim that aggregation produces no change in the cell uptake or inflammatory response compared to monodisperse nanoparticles [289, 290]. One study reports that aggregation increases the uptake of cerium oxide nanoparticles in lung fibroblasts through sedimentation [227]. However, two separate studies conclude that aggregates may bind to cell membranes but cannot be internalized as efficiently as monodisperse nanoparticles [291, 292]. It is difficult to compare the aforementioned studies since they each utilize different cells, nanomaterials and experimental conditions. This highlights the need for more cohesion when designing future uptake studies.
investigating the effect of aggregation during nano-bio interactions. Our work is the first to study the effect of aggregation on nanoparticles designed for biological application. Future studies will be necessary to confidently evaluate the long term effects and safety of engineered nanomaterials which are prone to aggregate. Considering the effect of aggregation will ultimately benefit the design of safe and effective nanoparticle platforms for biomedical applications.

6.5 Author Contributions

This study was conceived by A. Albanese and W. Chan. All experiments were optimized, executed and analyzed by A. Albanese. This chapter (adapted from [191]) was written by A. Albanese with additional edits and corrections by W. Chan.
7 Summary & Future Directions

7.1 Summary

The studies in this thesis provide insight into nanoparticle transport through the tumor’s extracellular environment using novel in vitro systems and approaches. The conclusions from this work demonstrate that transport through the tumor’s extracellular environment can affect downstream nanoparticle-cell interactions by dictating a nanoparticle’s tissue distribution and its biological identity. Interactions between the nanoparticles and the tumor microenvironment will depend on the nanoparticle’s initial properties and the composition of the extracellular environment. In Chapter 3, the tumor-on-a-chip system provided a look into early nanoparticle-tissue interactions. Tissue transport and accumulation depended predominantly on the nanoparticle’s diameter and surface chemistry. The nanoparticle’s design determined its diffusion through the interstitial spaces and interaction with cell surface receptors. For nanoparticles with diameters between 40 and 100 nm, transport through the tumor micro-tissues was diffusion-limited. A diameter above 110 nm restricted tissue accumulation in the extracellular matrix. Accumulation in the tissue’s periphery at the tissue-fluid interface was dependent on nanoparticle design and fluid velocity. This study was the first to demonstrate that accumulation of nanoparticles at the tissue interface and inside tumor spheroids within the first hour correlated with long term accumulation trends in vivo. The interplay between a nanoparticle’s design and the features of the tumor microenvironment ultimately dictate the spatiotemporal distribution of nanoparticles.

The accumulation of PEG-NPs exclusively in the interstitial spaces (Figure 3.2), the accumulation of Tf-NPs at the binding site barrier (Figure 3.3c) and the exponential decay patterns of nanoparticle fluorescence (Figure 3.5 c & g) all illustrate the unequal distribution of materials inside the tumor. The heterogeneous distribution of nanoparticles inside the tumor tissue creates numerous inaccessible regions. A cell’s distance from the nearest blood vessel will dictate when it encounters its first nanoparticle. In some regions of the tumor, it is likely that nanoparticles only reach cells 24 to 48h after administration. This prolonged exposure to the tumor microenvironment will lead to interactions between nanoparticles and biomolecules in the
extracellular environment. **Chapter 4** investigated the stability of nanoparticles in these tumor microenvironments and illustrated the importance of nanoparticle design in controlling interactions with extracellular proteins. Our study was the first to demonstrate that cell-secreted metabolites can provoke nanoparticle aggregation and trigger protein corona remodeling. Fibronectin, an extracellular matrix protein secreted by the cancer cells, was the most abundant cellular protein present in the corona of nanoparticles exposed to conditioned media. These results may provide an additional hypothesis for the non-specific binding between transferrin-coated nanoparticles and the spheroid’s extracellular matrix observed in **Chapter 3**. When nanoparticles enter the spheroid, the high concentration of extracellular matrix proteins may displace transferrin proteins and bind to the nanoparticle’s gold surface. Similar interactions will also occur during long-term tumor transport when nanoparticles are exposed to high concentrations of matrix proteins, secreted proteins and uncleared metabolites. This environment can destabilize nanoparticle surfaces, promote nanoparticle aggregation and remodel the protein corona.

Changes to a nanoparticle’s protein corona can change its cell uptake by interacting with a different set of cell receptors [238, 293]. However, the role of aggregation was mostly unknown prior to this work. Aggregation is an important concern since it is a prevalent problem for various nanoparticle designs and can occur in the extracellular environment or upon internalization into cellular vesicles where proton pumps produce an acidic environment. Nanoparticles aggregated in the extracellular environment or released from cellular vesicles will eventually interact with other cells leading to unexpected physiological responses. The aggregate synthesis technique presented in **Chapter 5** makes it possible to finally characterize the effects of aggregation on a well defined uptake pathway. This work provides the first evidence of aggregate size and morphology significantly influencing cell uptake. Interestingly, each cell line displayed a unique avidity, uptake and sequestration of the aggregates. In these experiments, aggregation could both increase and decrease nanoparticle uptake depending on the cell’s receptor expression and intrinsic phenotype.

From an engineering perspective, this work provides some preliminary guidelines for improving the design of tumor-targeting nanoparticles. Tumor transport requires nanoparticles with diameters below 110 nm for efficient diffusion through the extracellular matrix. The addition of a targeting moiety onto the nanoparticle surface drastically improves accumulation and retention
inside the tissue. If a given delivery strategy requires long term stability, functionalization of the nanoparticles with thiol-terminated molecules such as PEG or MUA can improve stability in the tumor microenvironment. Larger nanoparticles also demonstrate a greater stability in the presence of cell-secreted proteins and metabolites. An alternative delivery strategy may utilize the increased uptake of aggregates and of cell protein-remodeled coronas. Using this approach, nanoparticles would be designed to adsorb specific proteins from the tumor microenvironment to encourage cell uptake. Similarly, nanoparticles triggered to aggregate inside the tumor will have better tissue retention and can lead to increased cell uptake depending on the cancer cell’s phenotype and the number of receptors it expresses. Unfortunately, these design guidelines are only relevant for the final stages of delivery once the nanoparticles have entered the tumor tissue.

As mentioned in Chapter 1, a systemically administered nanoparticle must avoid kidney filtration, avoid blood clearance by phagocytic cells and extravasate through the tumor vasculature before arriving inside the tumor tissue. Each of these steps may require a specific set of properties to ensure optimal delivery. Choosing a specific diameter or surface functionalization scheme may benefit one stage of the delivery process, but is likely to impede another. For example, 40 nm PEG-coated nanoparticles possess a long blood half-life [94], but are quickly eliminated from the tumor tissue (Figure 3.3b). Since optimal drug delivery may require specific nanoparticle properties for each step of the delivery process, it is likely that nanomaterials with dynamic features will be required to improve tumor delivery. For example, using a strategy to induce aggregation of 40 nm PEG-coated nanoparticles inside the tumor would minimize tissue clearance. However, to develop successful design strategies it will be necessary to improve our understanding of nanoparticle interactions inside the tumor tissue. Elucidating the relationship between nanoparticles, their biological identity and tissue transport is necessary to identify current limitations and to determine effective delivery strategies.

7.2 Future Directions:

7.2.1 Improving the Micro-tissues

This thesis presents the development of a novel tumor-on-a-chip imaging platform which can be used to evaluate nanoparticle tissue transport. These initial studies demonstrated the platform’s capabilities and established techniques for data analysis. The tumor-on-a-chip offered an unprecedented look at early nanoparticle-tissue interactions and successfully predicted in vivo
tumor accumulation trends. This accurate prediction of nanoparticle performance in vivo highlights the tumor-on-a-chip approach as an important tool useful for multiple applications including high-throughput screening, tissue biopsy analysis and personalized medicine. The current ‘tumor-on-a-chip’ design can be used to evaluate the transport of various nanoparticle designs in different tissue types. Spheroids used in this initial study were generated from MDA-MB-435 cells, a cell line also used by our group to evaluate nanoparticle tumor accumulation in vivo. The initial tumor-on-a-chip studies used the MDA-MB-435 cells to help understand our in vivo tumor xenograft mouse model. However, there are many other cell lines capable of producing spheroids with different cell densities, tissue organization and extracellular matrix compositions. Comparing the accumulation of a single nanoparticle design in various spheroids can provide insight into the role of tissue structure in nanoparticle transport.

An important limitation with the MDA-MB-435 spheroids used in the tumor-on-a-chip is that they only contain one cell type. Inside the tumor, non-tumor stromal cells constitute up to 80% of total cells and include fibroblasts that synthesize the extracellular matrix, immune cells that suppress the inflammatory response, and endothelial cells that drive angiogenesis [294-297]. Thus, the incorporation of multiple cell types into the spheroids could increase the physiological relevance of the spheroid model and provide some insight into nanoparticle-cell interactions when multiple cell types are co-cultured. Cell labeling via fluorescent dyes such as carboxyfluorescein succinimidyl ester (CFSE) or genetic transfection with green fluorescent protein (GFP) will be necessary to identify specific cell populations during fluorescent imaging. Some co-culture models include the addition of fibroblast cells either directly into the spheroids or as a monolayer of feeder cells during spheroid growth in vitro. Endothelial cells can also be added to the surface of the spheroid to mimic extravasation through the blood vessel. The density of endothelial cells could be controlled to mimic different fenestration sizes of found in tumor vasculature. Macrophages can also be added into the spheroid to compare the internalization of nanoparticles between phagocytic tissue-resident cells and non-phagocytic cancer cells.

Instead of trying to improve the physiological relevance of the cultured spheroids, the current tumor-on-a-chip design can also be used to analyze biopsies from tumors and other organs. Biopsies from murine tumors or human patients can be immobilized in the microfluidic chip and analyzed. Unfortunately, the height of the microfluidic channel (250 µm) limits the size of the usable biopsies. However, a potential strategy is to employ a tissue slicer capable of producing
500 µm sections (Zivic Instruments) with a 300 µm diameter biopsy punch (Zivic Instruments) to generate microbiopsy cylinders 300 µm in diameter and 500 µm in height. For human biopsies, another strategy is to dissociate the biopsied tissue into single cells and generate spheroids via cell culture techniques. Initial studies with biopsies or biopsy-derived spheroids could be used to determine whether the tumor-on-a-chip is capable of predicting the accumulation of systemically-administered nanoparticles. Given the large variability in nanoparticle accumulation from mouse to mouse, it will be interesting to evaluate how accurately the tumor-on-a-chip predicts nanoparticle behavior in individual mice. It will be also interesting to compare how accurately the tumor-on-a-chip predicts accumulation of nanoparticles administered from different routes (intravenous vs. intratumoral). This strategy can eventually generate tools for personalized medicine to evaluate a patient’s responsiveness to nanoparticle based therapies. Pre-screening multiple nanoparticle designs ensures that physicians select the formulation that ensures the optimal therapeutic benefit for each patient.

7.2.2 Nanoparticle Design

The initial tumor-on-a-chip studies analyzed five nanoparticle designs: four possessing different diameters and one functionalized with transferrin. It will be important in future studies to analyze the effect of nanoparticle shape and surface chemistry on tissue transport. Simple questions that can be answered rapidly include: “how does active targeting affect the accumulation of larger nanoparticles?” and “how does tissue accumulation change when transferrin is replaced with another bioligand?” The design of receptor-targeting nanoparticles must also be improved in future experiments. Transferrin nanoparticles were prepared by passive adsorption onto gold nanoparticles. This technique produces stable nanoparticles with a high density of transferrin proteins which are ultimately unusable in vivo since they are rapidly cleared from the blood by the liver’s phagocytic cells. During the tumor-on-a-chip work, a nanoparticle synthesis protocol was developed to produce PEG-coated gold nanoparticles functionalized with transferrin. Synthesis of these nanoparticles requires the production of transferrin-PEG-thiol conjugates to coordinate surface anchoring and to allow for controllable surface density. Unoccupied surface can be backfilled with shorter length methoxy-PEG to improve nanoparticle stability without blocking transferrin-CD71 receptor binding. This synthesis technique was used to produce transferrin-coated nanoparticles for the in vivo validation of the tumor-on-a-chip findings. This
technique can be adapted for different biomolecules to generate libraries of nanoparticles functionalized at controllable densities.

Evaluating the performance of numerous nanoparticle designs is difficult using the current tumor-on-a-chip experimental setup. One way to increase throughput is to label individual nanoparticle design with unique fluorophores to enable the tracking of multiple designs simultaneously. The data presented in Chapter 3 employed a dye with the longest possible wavelength (Cyto633, 637/657 nm) to minimize photon absorbance by the tissue. Although many near-infrared dyes possess longer wavelength spectra, the excitation source (633 nm) and emission detector (400-700 nm) limited the selection of the fluorophore used. Preliminary experiments with yellow-green (505/515 nm) dye and Nile red dye (535/575 nm) polystyrene beads appear to work with the tumor-on-a-chip system. However, when new fluorophores will be linked to gold nanoparticles via PEG molecules, it will be necessary to assess fluorescence quenching by the gold nanoparticles and to determine whether spheroid tissue absorbance and scattering limits fluorescence detection. It is important to establish fluorescence standard curves in the deeper tissue layers to ensure that differences observed between nanoparticles are not affected by each fluorophore’s properties. By finding a combination of 3 or 4 dyes which are detectable on gold nanoparticles inside the spheroid and have enough spectral separation, it will be possible to analyze multiple nanoparticle designs in a single spheroid simultaneously, and increase the throughput of the assay.

7.2.3 Microfluidic Device Improvements

The tumor-on-a-chip device used in Chapter 3 was adapted from a device used to image pancreatic islets [211, 298]. The main difference with the islet-imaging device is an increase in the channel height from 125 µm to 250 µm to accommodate the larger spheroids. However, future studies would benefit from several improvements on the current design. Ideally, spheroids with diameters between 500-600 µm could be immobilized into a microfluidic device so we can assess the accumulation of nanoparticles in the necrotic, quiescent and proliferative regions of the tissue. However, this would require a different photoresist and a redesign of the imaging chamber to ensure that flow around the spheroid is not obstructed. It is important that a pressure gradient of sufficient magnitude is generated across the spheroid to drive the interstitial flow through the tissue. Other studies have incorporated large spheroid-based tissues into microfluidic
devices. A device developed in Neil Forbes’ lab uses a rectangular tissue reservoir seeded with multiple spheroids [209, 299]. The spheroids are flowed into the reservoir, blocked by pillars and allowed to grow for several days until they fill the reservoir volume (Figure 7.1). This produces a rectangular tissue up to 1000 µm deep which permits the analysis of one dimensional transport gradients in real time. The tissue contains regions of proliferating, quiescent and necrotic cells similar to >500 µm spheroids and tumor tissue. This system is good for evaluating diffusion over longer distances, through different tissue regions. Unfortunately, it does not produce convective flow through the tissue.

Another important improvement on the current tumor-on-chip design is the ability to increase throughput of experiments. The two best strategies for increased throughput involve parallelizing up to eight channels each containing a single spheroid and/or producing designs that can trap multiple spheroids in series within a single channel. While parallel channels allow the analysis of multiple conditions on one chip, spheroids in series can produce many experimental replicates with low volumes of nanoparticles. The challenge with the ‘in series’ strategy is designing systems which can trap multiple spheroids. One strategy is to use micropost trapping to sequentially capture multiple spheroids in a microfluidic channel (Figure 7.2) [300]. In this configuration, microposts create parallel flows through the trapping sites with the first vacant trapping site possessing the least hydraulic resistance. When a spheroid occupies this first trapping site, fluid flow is obstructed and reroutes to the adjacent trapping site. The pressure drop through the trapping site ensures spheroid immobilization and convection through the tissue. This design was published for 16 µm beads and will need to be optimized for much larger spheroids. COMSOL modeling will determine what the maximum length of the trapping site and the intra-site distance required to produce flow patterns necessary for spheroid capture. A similar strategy is to produce hydrodynamic traps using cup-shaped nozzles to trap pancreatic islets (Figure 7.3) [301]. In this design, multiple nozzles are linked via bypass channels and hydraulic resistance directs fluid through the empty nozzles. When an islet is captured by the first nozzle, flow is redirected through the bypass channel. This design reduces sheer stress around the islet while ensuring interstitial flow through the tissue. This design can be adapted for spheroids by increasing the channel height from 125 µm to 250 µm. A similar design has been used to trap ovarian cancer spheroids 500 µm in diameter [302]. Thus, it can be possible to trap multiple large spheroids possessing necrotic cores within a single microchannel. By parallelizing
Figure 7.1: Rectangular tissue trap. (a) Top view schematic of the microfluidic device demonstrating the tissue chamber where spheroids are loaded. Multiple spheroids enter the chamber and are packed against the cell retention filler through hydrostatic pressure. The packed spheroids are then left to grow for 24-48h and they form a tissue inside the imaging chamber. (b) Live (green) and dead (red) cell stain of spheroid and unstained cells are necrotic. Figure adapted with permission from [209]. Copyright (2013) Royal Society of Chemistry

Figure 7.2: Micropillar sequential capture technique. Modeled Fluid velocity field (a)–(c) and pressure field (d)–(f) simulations of 10 μm microbeads captured in the designated trapping sites between microposts. (a) Channel without any microbeads, (b) with 6 trapped microbeads, and (c) with microbeads trapped in all sites. (d) Pressure field simulations without any microbeads, (e) with 6 trapped microbeads, and (f) with microbeads trapped in all sites. Figure adapted from [300].
Figure 7.3: **Reverse nozzle hydrostatic trap**. (a) Design of the reverse nozzle hydrostatic trap for pancreatic islet cells. Bypass channel length was designed to produce hydraulic resistance favoring fluid flow through the nozzle. (b) Design of the microfluidic device with 10 reverse nozzles in series. Once the islet is captured in the reverse nozzle, flow is rerouted through the bypass channel. (c) Reverse nozzle with captured murine pancreatic islets. Scale bar = 100μm. Image adapted with permission from [301]. Copyright (2013) Royal Society of Chemistry.
microfluidic channels containing multiple spheroids, it will be possible to increase throughput. The development of high-throughput systems will be necessary for screening nanoparticle libraries to determine the optimal size, shape and binding avidity for specific tumor types.

### 7.2.4 Evaluating Extracellular Matrix Interactions with Nanoparticles

Nanoparticle interactions with the extracellular matrix are an important determinant of tumor transport as observed during the tumor-on-a-chip studies. The size-limited exclusion of nanoparticles (Figure 3.2) and the non-specific interactions with transferrin-coated nanoparticles (Figure 3.4) both illustrate how nanoparticle-matrix interactions ultimately dictate tissue accumulation. The abundance of cell-derived fibronectin in the corona of nanoparticles exposed to conditioned media (Figure 4.4) also illustrates downstream effects of nanoparticle-matrix interactions. Understanding how nanoparticle design influences interactions with the extracellular matrix proteins can improve tissue transport and increase tumor delivery. Microfluidics can provide a suitable platform for analyzing the transport and distribution of nanoparticles through the extracellular matrix. In future studies, the extracellular matrix can be isolated from tumors after de-cellularization using a 1% (wt/vol) solution of dodecyl sulfate. The resulting extracellular matrix will be rinsed, lyophilized, milled into a fine-grained powder, and rehydrated under acidic conditions [303]. The high concentration of collagen I in the tumor extracellular permits the rapid polymerization of rehydrated extracellular matrix solutions by increasing the pH with sodium bicarbonate. Extracellular matrix solutions can be polymerized around lithography-defined sacrificial gelatin channels [304, 305]. Gelatin channels can be micro-molded, placed onto a glass slide and encased inside a gasket. Acidic extracellular matrix precursor solutions will be administered into the gasket and solidified around the gelatin channels. Heating at 37°C will melt the gelatin and, after rinsing away the gelatin with saline, a network of microfluidic channels will be produced inside the extracellular matrix (Figure 7.4). These microfluidic devices can then be used to evaluate the transport kinetics of various nanoparticle formulations via diffusion and/or convection.

### 7.2.5 Characterizing Nanoparticle Biological Identity

Prolonged interactions with the tumor’s extracellular environment will determine diffusion/transport rates, but can also change the properties or ‘biological identity’ of the
Figure 7.4: Microfluidic device with sacrificial gelatin channel. (a) Fabrication of microfluidic gel with sacrificial gelatin channel. PDMS is stamped onto a glass slide to create the microfluidic network. Gelatin is administered into the channel and allowed to gel. The gelatin can be removed and embedded in an extracellular matrix precursor solution. The extracellular matrix is allowed to polymerize and gelatin is removed by flushing the channel at 37°C producing channels in the polymerized matrix. Reprinted with permission from [305]. Copyright (2007) Royal Society of Chemistry. (b) Design of a potential microfluidic device to analyze the transport of solutes through extracellular matrix (ECM) isolates. The scheme outlines regions of convective transport (conv.) and diffusion.
nanoparticles over time. Nanoparticle aggregation and protein corona remodeling were both affected upon exposure to cell-secreted proteins and metabolites. These alterations to the biological identity were shown to change downstream nanoparticle-cell interactions. For certain nanoparticle designs, the biological identity can evolve accordingly to the composition of their surrounding environment. Although the experiments presented were conducted in vitro, systemically administered nanoparticles must navigate through multiple biological environments in vivo. Although each of these environments actively maintains a state of homeostasis, they are subject to continuous compositional fluctuations. Plasma, interstitial fluid, mucus and saliva all demonstrate changes in their composition [306-309]. In turn, the biological identity of each nanoparticle may reflect its unique journey through different microenvironments and can produce a distinct cellular response. It would be interesting to analyze the aggregation and protein corona of blood-borne and tissue-resident nanoparticles post-injection in animal models. The biological identity of these nanoparticles can be obtained using conventional technique such as mass spectrometry and UV-Vis spectrometry. The challenge with this work is developing nanoparticle isolation protocols which will not disrupt the in situ biological identity. For example, the isolation of tissue-resident nanoparticles will require tissue disruption using mechanical, enzymatic and/or detergent-based methods. Once the tissue is disrupted and solubilised, nanoparticles must be isolated using techniques such as density gradient centrifugation or size-exclusion chromatography. It will be necessary to ensure gentle isolation and purification protocols to cause minimal disturbance to the nanoparticle biological identity. These studies are likely to reveal a time-dependent evolution of nanoparticle biological identity which reflects the composition of the tissue microenvironment where they reside.

7.2.6 Understanding the effect of Nanoparticle Aggregation

This thesis has proposed a novel technique to extricate the role of nanoparticle aggregation from protein corona remodeling. By synthesizing size-tunable aggregates with a fixed surface functionalization, it is possible to understand the role of aggregation on a nanoparticle’s biological identity. Chapter 6 presents the first look into the effect of aggregation on cellular responses. However, it is difficult to make any broad conclusions from this work since transferrin-coated aggregates were exposed to three cell lines. To better elucidate the effect of aggregation on cellular interactions, aggregates coated with different bioligands will be
synthesized to distinguish between cell-specific and receptor-specific trends. For example, the SK-BR3 cell line could be exposed to aggregates coated with either transferrin or Herceptin antibody. It would be interesting to see whether larger aggregates produce the same change in cell uptake depending if they bind to the CD71 transferrin receptor or the ErbB2 receptor. It would also be interesting to use pharmacological inhibitors that block individual cell uptake mechanisms to determine whether aggregation can change the internalization pathways used.

The ability to synthesize PEG-coated aggregates (Figure 5.4) also makes it possible to analyze the blood half-life and biodistribution of different aggregate sizes. These studies are important to accurately assess how aggregation can skew experimental data and affect final therapeutic outcome. These studies in conjunction with the characterization of blood- and tissue-isolated nanoparticles (vide supra) will help provide a better understanding of how aggregation can impede or improve nanoparticle tumor delivery. There already exists some evidence to suggest that systemically administered gold nanoparticle aggregates produce a different tissue biodistribution than their monodisperse counterparts [310]. The aggregates showed significantly increased accumulation in the lung and heart while showing decreased accumulation in bone marrow relative to disperse nanoparticles. Aggregates also appear to limit internalization to specific cells in contrast to disperse nanoparticles. However, the nanoparticles used in this study were not coated with stable surface ligands and were likely opsonised with serum proteins. Thus, it is impossible to relate the changes in biodistribution specifically to aggregation and not different protein corona between the aggregates and disperse nanoparticles. Future work will need to elucidate the effect of aggregation morphology independently of corona composition to improve understanding of nano-bio interactions.

### 7.3 Conclusion

The studies presented in this thesis and the proposed future experiments aim to improve nanoparticles tumor targeting. A better understanding of how the tumor’s extracellular environment affects nanoparticle transport and stability will aid the design of future nanomaterials. Good design requires an understanding of the required objectives and current constraints for achieving a desired outcome. For tumor targeting, the objective is to promote efficient nanoparticle transport throughout the tissue to ensure maximum cancer cell uptake. Some constraints include the extracellular matrix pore size, non-specific interactions with the matrix proteins, the formation of a binding-site barrier and nanoparticle stability. Improved
nanomaterials will require that future designs address these concerns. Nanoparticle stability is an important concern if the experimental objective is to model nanoparticle-cell interactions. For these types of experiments materials must maintain their properties in a biological environment or else conclusions will be erroneous. Characterizing the interactions occurring between stable nanomaterials and cells, tissues or organisms is necessary to establish the best size, shape and surface chemistry for a desired objective. In some circumstances, voluntary instability or ‘dynamic nanomaterials’, that can change their properties upon exposure to the tumor micronenvironment, may be a necessary design strategy. Dynamic nanomaterials can be designed to adsorb specific proteins from the extracellular tumor environment to promote cell uptake or designed to aggregate in the tumor to prevent tissue clearance. Additional strategies can also be used to promote nanoparticle properties that favor accumulation and tumor retention.

The recent burst of innovative design strategies for tumor-targeting nanomaterials has been met with excitement by academia, industry, governments and the general public [311]. However, the rush to publish novel proof-of-concept studies has occurred at the expense of fundamental nano-bio studies. This lack of fundamental knowledge is catching up to the field since we are currently unable to draw specific or general conclusions on the impact of size, shape, and surface chemistry-dependent interactions. Nano-bio interactions must be heavily investigated in the future to improve the design of nanoparticle-based tumor delivery systems and make a substantial impact on modern medicine. Hopefully nanoparticle research will shift from proof-of-concept studies to thorough fundamental studies characterizing how nanomaterials interact with cells, tissues and organisms. The publication of well-executed fundamental studies can potentially change how we engineer nanostructures in this century and could lead to novel nano-based biomedical applications.
References


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Appendix A:

Curriculum Vitae
ALEXANDRE ALBANESE

Education

Ph.D. Biomedical Engineering, University of Toronto 2014

- Thesis: “Characterizing the Role of Nanoparticle Design on Tumor Transport and Stability in the Extracellular Environment”
- Supervisor: Warren Chan
- Synthesis, functionalization and characterization of gold nanoparticles
- Evaluation of nanoparticle interactions with proteins, cells and micro-tissues
- Development of microfluidic model for tissue transport
- Supervised graduate and undergraduate students

M. Sc. Microbiology & Immunology, McGill University 2006

- Thesis: “Functional impact of the protective Idd3 allele on regulatory T cells and protection from type-1 diabetes”
- Supervisor: Ciro Piccirillo
- Evaluation of suppressor T cells
- Adoptive transfer of cells into mice and analysis of tissues with flow cytometry
- Supervised undergraduate student

B. Sc., Microbiology & Immunology, McGill University 2004

- Thesis: “Proteome Characterization of Regulatory T cells” with Ciro Piccirillo
- Graduated with first class honours, CGPA = 3.56/4

Research

Research Technician, Vasogen Inc. (Toronto, ON) 2008

- Worked on synthetic immune-modulating liposomes
- Designed and developed ELISA-based assay to monitor liposome-mediated immuno-suppression of murine leukocytes
- Responsible for reagent ordering

Associate Scientist, GSK Biologicals (Laval, Qc) 2006-2008

- Protein synthesis for vaccine development
- Extraction, purification and refolding of recombinant proteins
- Characterization of recombinant proteins
- Evaluation of epithelial cell inflammatory response using ELISA and flow cytometry

Research Assistant, McGill University (Montreal, Qc) 2006

- Supervisor: Samantha Gruenheid
- Worked with mouse model for Enterohaemorrhagic E. coli (EHEC)
- Administered bacteria by oral gavage and monitored bacterial load in the colon and feces
• Developed an organized system for product ordering

**Teaching Experience**

**Instructor**, University of Toronto (Toronto, ON) 2010-2012

• DaVinci Engineering Enrichment Program (DEEP) - Summer Academy
• Participated in yearly Instructor Training Conference
• Taught “Nanomedicine: Improving Medicine with Nanotechnology” to high school students (grade 10-11)
• Co-created, prepared and presented 25h course (slides, activities and experiments)
• Ranked highly on student satisfaction survey

**Teacher’s assistant**, University of Toronto (Toronto, ON) 2010-2012

• BME340: Biomedical Engineering Instrumentation & Technology
• Taught and supervised ~40 students how to use ELISA, Spectrometer, fluorescent microscope
• Designed slides for course instructor
• Corrected lab reports, evaluated poster presentations

**Workshop**, University of Toronto (Toronto, ON) 2010-2011

• Faculty of Applied Sciences & Engineering workshop for prospective students
• Organized and prepared the Nano-Forensics component

**Teacher’s assistant**, McGill, (Montreal, Qc) 2006

• MIMM413: Parasitology
• Taught and supervised group of 16 students throughout semester
• Corrected multiple reports and exams
• Rated highly by students (>90%) during their course evaluation

**Community**

**IBBME Podcast**, University of Toronto (Toronto, ON) 2012-2014

• Creator, producer, audio engineer and host of IBBME: Focal Point podcast
• Interviewed multiple faculty members to highlight research
• Working under mentorship of the Communications Officer
• Training and management of other contributors

**Reviewer**, ACS Nano (American Chemical Society Publications) 2013-

• Reviewed >5 manuscripts investigating nanoparticle-biological interactions

**Reviewer**, Scientific Reports (Nature Publishing Group) 2012-

• Reviewed a manuscript investigating nanoparticle-immune cell interactions

**Reviewer**, Journal of Nanoparticle Research (Springer) 2011-
• Reviewed >3 manuscripts investigating nanoparticle-biological interactions

**IBBME**, University of Toronto (Toronto, ON) **2012-2014**

• Participated in student recruitment event at McGill University (Montreal, Qc) in November 2013
• Member of the 2013 Scientific Day organizing committee
• Selected to participate in “2013 Departmental Website Re-design Committee”

**Publications**


Sykes EA, **Albanese A**, Chan WC. BIOPHOTONICS: Implantable waveguides (News & Views), Nature Photonics 2013 [impact factor = 27.254]

**Albanese A**, Lam AK*, Sykes EA, Rocheleau JV, Chan WC. Tumor-on-a-chip provides an optical window into nanoparticle transport, Nature Communications, 2013 Nov 1; 4:2718 [impact factor = 10.015]


**Albanese A**, Chan WC. Effect of gold nanoparticle aggregation on cell uptake and toxicity. ACS Nano. 2011 Jul 26;5(7):5478-89. [impact factor =12.062; citations =63]


Sgouroudis E, **Albanese A**, Piccirillo CA. Impact of protective IL-2 allelic variants on CD4+ Foxp3+ regulatory T cell function in situ and resistance to autoimmune diabetes in NOD mice. J Immunol. 2008 Nov 1;181(9):6283-92. [impact factor =5.520; citations =34]


**Presentation and Meetings**

**Three Minute Thesis 2013 (3MT)**, University of Toronto
• University of Toronto finalist
• Tumor-on-a-Chip: Screening Nanoparticles to Cure Cancer (Albanese A, Lam AK, Rocheleau JV, Chan WC.)

**BMES 2012 Annual Meeting**, Atlanta, GA
• oral presentation (peer reviewed)
• Effect of Active Targeting and Microfluidic Flow Rate on Nanoparticle Distribution in a Cell Spheroid. (Albanese A, Lam AK, Rocheleau JV, Chan WC.)

**IBBME Scientific Day 2011**, University of Toronto
• oral presentation (peer reviewed)
• The Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity (Albanese A & Chan WC.)
• Annual event organized by IBBME department

**BMES 2010 Annual Meeting**, Austin, TX
• Poster (peer reviewed)
• Synthesis of size-tunable protein-gold nanoparticle aggregates (Albanese A, Chan WC)

**BiopSys All-Network Meetings (#2-8) 2008-2012**, University of Toronto
• Poster (twice per year)
• Oral presentation in 2011
• NSERC Strategic Network for Bioplasmonic Systems

**Awards**

*NSERC Postdoctoral Fellowship Award*, National Award, 2014-2016
*Ontario Graduate Scholarship*, Provincial Award, 2012-2013
*SGS Conference Grant*, Institutional Award (U of T), 2012
*Ontario Graduate Scholarship*, Provincial Award 2011-2012
*Award for Best “Lightning Round Presentation (12 min)”,* Departmental Award, 2011
*Ontario Graduate Scholarship*, Provincial Award 2010-2011
*St. George’s Society Graduate Scholarship*, Institutional Award (U of T) 2010
*Ontario Graduate Scholarship*, Provincial Award 2009-2010
*Milligan Graduate Fellowship*, Institutional Award (U of T) 2008
*McGill Graduate Studies Award*, Institutional Award 2005
*McGill University Health Centre Award*, Institutional Award 2005
*F.C Harrisson* Institutional Award, (McGill) 2004