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PRESENTED BY
Sahil Kumar Rastogi

ACCEPTED BY THE DEPARTMENT OF BIOMEDICAL ENGINEERING

GE YANG, FACULTY ADVISOR

ROBERT D. TILTON, FACULTY READER

YU-LI WANG, DEPARTMENT HEAD

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Sahil K. Rastogi

B.Tech. Biotechnology, Indian Institute of Technology, Guwahati

Carnegie Mellon University
Pittsburgh, PA

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Abstract

The interaction between nanoparticles and cells has been studied extensively, especially for targeted cancer drug delivery. However, our understanding of the influence of particle size and surface modification on cellular uptake of nanoparticles remains rather limited. This study investigated the effect of particle size and surface modification on the cellular uptake of polystyrene nanoparticles into several cultured cell lines, including BSC1, HEK, and 3T3, as well as primary mouse hippocampus neurons. The results showed that overly small nanoparticles (25nm in diameter) as well as overly large nanoparticles (100nm in diameter) were not efficient for cellular uptake, whereas the 70 nm size range appeared to have the highest uptake efficiency. Coating the nanoparticles with cell penetrating peptide significantly improved their cellular uptake by the cell lines. However, no improvement in cellular uptake was observed when the nanoparticles were coated with epidermal growth factor. Different from the cell lines, in the case of primary mouse hippocampus neurons, there was a significant increase in the uptake of nanoparticles when coated with either epidermal growth factor or cell penetrating peptides. Together, these results revealed that the cellular uptake efficiency of nanoparticles depends strongly on particle size, surface modification as well as cell types.
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List of Abbreviations

PSB  Polystyrene bead
MNP  Magnetic nanoparticle
CPP  Cell penetrating peptide
TAT  Trans-activating transcriptional activator
PTD  Protein transduction domains
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
DNA  Deoxyribose nucleic acid
MRI  Magnetic resonance imaging
CT   Computed tomography
PCR  Polymerase chain reaction
RES  Reticuloendothelial system
QD   Quantum dots
CNS  Central nervous system
CME  Clathrin-mediated endocytosis
EDC  1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
FCM  Flow cytometry
DLS  Dynamic light scattering
TEM  Transmission electron microscopy
1 INTRODUCTION

All the living organisms are built of cells, which have a size range of typically 10~20 μm across. The cells comprise of much smaller cellular components that lie in the sub-micron size domain. Proteins, often referred to as the workhorses of the cell, have an average size of just 5 nm, which is comparable with the dimensions of smallest manmade nanoparticles [1]. It is therefore not surprising that nanoparticles have proved as very important probes for visualization and analysis of the cellular machinery [2]. However, our understanding of the interaction between manmade nanoparticles and the cells remains rather limited.

A strong driving force behind development of nanotechnology is the need to study and understand biological processes at the nanoscale [3]. The unique properties of nanoparticles such as modifiable platform character, surface to volume ratio, shape and size, and optical properties, have enabled their use as promising tools to study biological processes [4], as drug delivery systems [5], as contrast agents for bio-imaging [6], and as probes for bio-sensing [7]. Various nanoparticle platforms, such as polymeric, lipid based, magnetic nanoparticles etc. have been used for applications in biology and medicine. Diverse strategies, including active and passive targeting, electroporation etc., have been adopted to target the nanoparticles/nanomedicines to the desired site, the details of which are reviewed in the subsequent sections of this thesis.

Since in many applications the targets of nanoparticles are localized in subcellular compartments, modulation of nanoparticle-cell interactions for efficient cellular uptake through the plasma membrane remain a formidable challenge [8]. The nanoparticle-cell interactions are mainly dictated by the size and surface properties of nanoparticles. Therefore, an understanding of how different nanoparticle properties especially their size and surface chemistry, influence their interactions with cells, is essential. In the past, a few studies have been conducted to answer the questions regarding influence of surface coating and size of nanoparticles on the cellular uptake [9–11]. These studies have been conducted primarily on cancer cell lines such as HeLa and murine melanoma cell lines, and the size range of the particles being examined in those studies was not sufficient to provide the information required to fully understand the dependence of cellular uptake on nanoparticle size. Since these studies have focused on a limited size range and cell types, further investigation is required in order to get a better understanding of nanoparticle-cell interactions. Also, there is a need to determine the size range for the most efficient cellular uptake and the surface modification to improve the uptake of the nanoparticles.

The objective of this study is to determine how the size of the nanoparticle, in the range of 25nm to 100nm, and its surface chemistry influence its uptake efficiency by non-cancer mammalian cells. In particular, several basic questions will be addressed, including: what is the upper limit on nanoparticle size which could be easily taken up by the cells?; do smaller the nanoparticle have better the uptake efficiency?; how much does the surface modification improve the uptake of the nanoparticles?; and are there other ways in addition to surface modification to improve uptake efficiency of larger nanoparticles?

The research work for this thesis utilized 25nm, 50nm, 70nm and 100nm fluorescent polystyrene beads, and 100nm fluorescent magnetic nanoparticles. Using flow cytometry and fluorescence cell imagining, the relative cellular uptake efficiency of the polystyrene beads by the BSC1, HEK and 3T3 cell lines was studied. The BSC1, HEK and 3T3 cell lines have been referred to as non-cancer cell lines for this study
since these cell lines are immortalized forms of the normal mammalian cells such as fibroblast and kidney cells, but unlike HeLa cell lines they are not obtained from naturally occurring cancer. This difference in cell type origin leads to differences in properties such as cell membrane morphology, pore size, surface receptors/markers etc. between cancer cell lines and non-cancer cell lines. Since prior studies have been conducted primarily on cancer cell lines therefore this thesis work has focused on the non-cancer cell lines.

The results obtained from the experiments showed that nanoparticles in the 70nm size range were uptaken by the cells most efficiently. The nanoparticles were then conjugated with streptavidin to further attach the biotinylated cell penetrating peptides (CPPs) to the surface. The relative uptake efficiency was measured using the 25nm, 50nm, 70nm and 100nm polystyrene beads, with and without CPP conjugation. The results showed that even after conjugating the nanoparticles with CPPs, those with size of ~70nm was taken up by the cells most efficiently. The study also showed that conjugating the CPPs to the surface of nanoparticles in the size range of 25nm to 70nm, significantly improved the cellular uptake rate. Lastly, the results revealed that the conjugation of the nanoparticles with epidermal growth factor did not improve the cellular uptake by the non-cancer cell lines. However, the conjugation of nanoparticles with epidermal growth factor did increase the uptake of nanoparticles by the mouse hippocampus neurons, indicating that cellular update of such nanoparticles was dependent on specific cell types.
2 BACKGROUND

2.1 Nanoparticle applications in biology and medicine

Nanoparticles for medical applications are defined as particles with a size between 1 and 1000 nm [12], which offers them the possibility to interact with as well as influence cellular components and processes at their natural scale [13]. Also, the high surface to mass ratio and ease of introducing new properties or modifying already existing properties, give nanoparticle systems the potential of bringing significant advances in the prevention, diagnosis as well as treatment of various diseases [4].

2.1.1 Nanoparticles for studying basic biological processes

The size of nanoparticle cores can be tuned in order to provide a suitable platform for the interaction of nanoparticles with proteins and other biomolecules [14]. The interactions between nanoparticle and protein can be very crucial, for e.g., nanoparticles can be used to activate cell signaling pathways [15], and to induce protein synthesis [16]. Nanoparticles such as quantum dots have been extensively studied for many biological applications that use fluorescence. Some of their uses include immuno-staining of fixed cells and tissues, membrane proteins and cytoskeleton filaments [17, 18]. Recently, nanoparticles have also been used to visualize the molecular dynamics of individual molecules in live cells [19]. Nanoparticle platforms can also be used to locally perturb protein activities in cells at a subcellular scale. For example, magnetic nanoparticles can be coated with a biocompatible surface layer that can be functionalized with ligands to target specific cell-surface receptors. These receptors can then be activated remotely by applied magnetic fields. Active signaling proteins can be attached to magnetic nanoparticles and can be displaced by magnetic forces into different locations within the cell. In a recent study, this approach was used to study how nanoparticle mediated activation of specific signaling pathways can lead to changes in cellular responses [20].

2.1.2 Nanoparticles as drug delivery systems

Nanoparticles are very promising for intracellular delivery of drugs, stem cell differentiation biomolecules and cell activity modulators. Although initial studies in the area of intracellular drug delivery have been performed primarily in the delivery of DNA [21], there is an increasing interest in the use of other molecules to modulate cell activity because of the flexibility to tailor the nanostructure construction (drug release characteristics, low immunogenicity etc.) yielding improved treatment efficacy and reduced side effects [5, 22]. A nanoparticle drug delivery system is made by encapsulating a therapeutic agent within a nanocarrier or attaching it to a nanoparticle platform. The aim of nanoparticle drug delivery systems is to administer the drug through controlled delivery, such that an optimum amount reaches the target site, resulting in increased efficacy of treatment and avoiding toxicity to the surrounding cells. In particular, subcellular availability and accessibility of a target is important for the effective delivery of therapeutic and imaging agents [23]. Delivering agents to subcellular organelles can also potentially illuminate certain molecular processes and mechanisms that are still unknown in organelle biology.
2.1.3 Nanoparticle systems as contrast agents for \textit{in-vivo} bio imaging

The function of contrast agents is to improve the visibility of features by enhancing image contrast of solid or fluidic structures within the human body that otherwise would be difficult to detect. Compared to conventional molecular-scale contrast agents, nanoparticle systems offer the possibility of targeting to specific sites of interest and increased circulation time, which allows for prolonged imaging. This helps in developing diagnostic procedures to image pathogenic processes on a molecular level [24, 6]. This new diagnostic paradigm is called molecular diagnostics, whose aim is to diagnose diseases on a molecular level before the development of symptoms [6]. Non-invasive imaging modalities such as ultrasound, magnetic resonance imaging (MRI), X-ray and computed tomography (CT) can also benefit from the development of nanoparticle contrast agents [24]. Such molecular diagnostics might be essential both for early diagnosis as well as understanding the underlying mechanisms of many diseases. Also, the ability to image and track the fate of a nanomedicine \textit{in vivo} will be of great importance to the development of effective drug delivery systems.

2.1.4 Nanoparticles as probes for bio-sensing

Nanoparticle systems prove to be a good candidate for bio-sensing applications because of their unique physicochemical properties coupled with the inherent increase in signal-to-noise ratio provided by miniaturization [7]. One of the important applications of nanoparticles as biosensors involves the detection of specific oligonucleotide sequences to diagnose genetic and pathogenic diseases. This could be achieved through the fabrication of nanoparticles functionalized with single-stranded DNA. Upon addition of the target sequence the particles aggregate, changing the color of the solution. Using this method oligonucleotides can be detected at sub-picomolar level without the assistance of PCR [25]. Another application of nanoparticles as biosensors involves detection and separation of pathogens. One of the most common methods used for the detection of bacteria has been through the use of magnetic biosensors that involve direct immunological reactions using magnetic nanoparticles coated with antibodies against surface antigens [26, 27].

2.2 Types of nanoparticles

Various nanoparticle platforms have been developed for biomedical purposes such as polymeric, ceramic, magnetic, solid-lipid and viral-based nanoparticles, liposomes, micelles, dendrimers and carbon nanotubes [28, 29]. Nanoparticles have many unique properties which can be modified according to their size [30]. They also have visible properties as they are small enough to confine their electrons to produce quantum effects [30]. Since the nanoparticles can be made from a variety of materials using various manufacturing methods, they can have diverse shapes, sizes, and distinct properties.

2.2.1 Liposomes and lipid based nano-carriers

Liposomes are bilayer vesicles made of phospholipids. Phospholipids have a polar phosphate group as its head, and a long hydrocarbon chain as its tail. In the presence of water, the heads are attracted to it, and line up to form a hydrophilic surface facing the water. The long non-polar tails are repelled by the water,
and form a hydrophobic surface away from it. As there is water on the outside and inside the liposome, there are two layers of phospholipids, with two surfaces of heads facing the aqueous environment, as shown in Figure 2.2.1. Since liposome encapsulates a region of aqueous solution inside a hydrophobic membrane, the dissolved hydrophilic drugs cannot pass through the lipid bilayer [31].

![Figure 2.2.1 Structure of liposome](image)

Liposomes can non-selectively deliver the molecules to the site of action by fusing with the bilayers of the cell membrane [31]. They can be targeted to tumors by conjugating their surface with ligands or antibodies, allowing them to enter the cell via receptor-mediated endocytosis [31]. Liposomes can also deliver drugs through pH mediated release [32].

### 2.2.2 Polymeric nanoparticles

To prevent nanoparticles from entering reticulo endothelial system (RES) in the body, they should be coated with hydrophilic polymers. This increased hydration also helps nanoparticles to be more soluble and less sensitive to enzymatic degradation [33]. This has led to the development of polymer-based drug delivery systems. Polymeric materials exhibit several desirable properties including biocompatibility, biodegradability, surface modification, and ease of functionalization of polymers. Polymeric systems also allow for greater control of pharmacokinetic behavior of the loaded drug, leading to more appropriate steady levels of drugs [34]. These nanoparticle systems can be made either from non-biodegradable or biodegradable polymers, with drugs either dissolved or encapsulated or covalently attached to the polymer matrix with different structures and unique functionalities (Fig. 2.2.2) [35].

![Figure 2.2.2 Various techniques for the preparation of polymer nanoparticles](image)

SCF: supercritical fluid technology, C/LR: controlled/living radical [36].
2.2.3 Quantum Dots

Quantum dots (QDs) are colloidal, inorganic nanoparticles with unique chemical and physical properties [37]. Their high photoluminescent quantum efficiency, photostability, tunability, narrow emission spectral band and prolonged fluorescence lifetime, make them excellent alternatives to fluorescent proteins. QDs have been extensively used in various cellular imaging applications. They have also been used as probes to image and track multiple tumors simultaneously. Their light emission is size tunable (from 400-2000nm) and have a stable fluorescence signal with a broad absorption spectra and narrow emission profile [30, 38]. This allows for the excitation of multiple species of QDs using a single light source without much signal cross-talk [38]. Quantum Dots made from CdSe are hydrophilic and have a surface that is adaptable to many biological applications [30]. These QDs tend to be over-coated with ZnS, an inorganic material that increases luminescent yields. Recently amphiphilic di- and tri-block copolymers typically containing polyacrylic acids have been developed to encapsulate QDs [39]. This coating not only maintains the phospholuminescent properties of the QDs, but also provides carboxylic acid functionalities to the QDs which provide solubility in water and chemical functional groups for conjugation to primary amines in proteins [39].

2.2.4 Magnetic Nanoparticles

Magnetic nanoparticles (MNPs), commonly composed of magnetic elements, such as iron, nickel, cobalt and their oxides, offer controlled size, and ability to be manipulated externally (Figure 2.2.3). Non-toxic super paramagnetic nanoparticles with functionalized surface coating can conjugate chemotherapeutic drugs or be used to target ligands/proteins, making them useful for drug delivery, targeted therapy, magnetic resonance imaging, transfection, and cell/protein/DNA separation. Magnetic particles may be used to selectively attach and manipulate or transport targeted species to a desired location under the influence of an external magnetic field.

![Figure 2.2.3 Biomedical applications of magnetic nanoparticles](image-url)
2.3 Biological barriers to nanoparticles

One of the greatest challenges that limit the successful applications of nanoparticles is their ability to reach the therapeutic sites at necessary doses while minimizing accumulation at undesired sites. The biodistribution of nanoparticles is determined by the human body's biological barriers. The success of nanoparticles for biomedical applications relies upon their ability to overcome these biological barriers that are manifested by body in several distinct ways. For intravascular delivery, the first barrier arises as the immune clearance of nanoparticles in the liver and spleen by the reticuloendothelial system (RES) [41]. Many approaches have been developed in order to overcome this barrier. These include PEGylation, modification with CD47, modulation of mechanical properties, engineering particle morphology and hitchhiking on red blood cells [41].

Another barrier is the permeation across the endothelium into target tissues. The tightest endothelium in the body is seen in the central nervous system (CNS) in the form of blood-brain barrier (BBB). Nanoparticles have also been designed to cross such barrier via receptor mediated endocytosis [42–44]. TfR and IR receptor targeting ligands and some peptides have been used for the transport of nanoparticles into the brain. However, the design of a highly efficient BBB shuttle is the most challenging issue in drug development. Another barrier is posed by the tissue interstitium which is composed of collagen and elastic fiber network of proteins and glycosaminoglycans that form the ECM. Transport of nanoparticles in tumor interstitium has been enhanced by weakening the dense matrix of ECM.

The next set of barriers for nanoparticles is the plasma membrane and intracellular localization in the target cells. Nanoparticles cannot simply enter the cells via diffusion [45]. Therefore, for specific targeting and internalization, nanoparticle surface is usually conjugated with surface targeting moieties such as antibodies, proteins, aptamers, peptides, folate and other small molecules. Nanoparticles can be internalized by phagocytosis, pinocytosis, specific binding of ligands to cell surface receptors and subsequent receptor-mediated endocytosis, and diffusion of nanoparticles through the lipid double layer forming the cell membrane, including transporter/channel proteins [45]. Also, gaining access to the cell is not sufficient to maximize the therapeutic outcomes of the drugs. Drug molecules must be delivered to the right target by avoiding their lysosomal degradation. After endocytosis, drug molecules can be localized at four major intracellular organelles: the cytoplasm, mitochondria, nucleus and lysosome. The internalization and localization mechanisms highly depend on nanoparticle size, surface properties and types of cells involved [46].

It is crucial to understand the structure function relationships between nanoparticle physicochemical properties and pharmacological behavior to overcome technical bottlenecks. There is a need of more sophisticated material and bioconjugate chemistry methods to fabricate nano sized objects with more fine-tuned properties and in a high throughput manner.
2.4 Targeting of nanomedicines

2.4.1 Passive targeting

Passive targeting has been an important strategy for targeting nanoparticle systems to tumors [47]. Nanoparticles can have many advantages even when untargeted due to their small size and the unique properties of tumors such as the enhanced permeability and retention (EPR) effect and the tumor microenvironment [48], which allow for long-circulating nanomedicines to accumulate in tumors over time. This strategy is generally referred to as passive targeting since this way of guiding nanomedicines to tumors only rely on the pathophysiological state of cancerous tissue. A schematic view of the EPR effect is shown below in Figure 2.4.1.

These effects can lead to a 10-fold or greater increase in drug accumulation simply by delivering a drug using nanoparticles rather than the free drug [50]. The rate and amount of accumulation of the nanoparticles in tumors depends on factors such as the size, surface characteristics, and the circulation half-life of the nanoparticles as well as the degree of angiogenesis.

2.4.2 Active targeting

Active targeting can be achieved by conjugating ligands to the surface of a nano-carrier that promotes binding to specific cell surface receptors expressed at the target site as shown in Figure 2.4.2. The targeting ligands increase the specificity of the nanoparticle system. Interactions between the ligand-attached nano carrier and receptors at the cell membrane will cause the nanoparticles to be internalized through the same endocytosis pathway as the ligand alone. A large variety of substances can be used as targeting ligands, including antibodies and other proteins, lipoproteins, hormones, charged molecules, and saccharides [51]. It is important that the ligand binds selectively to the desired receptor, and the target tissue should overexpress the surface marker in order to maximize specificity of the interaction, so that the nanoparticles are internalized by the desired cells, leading to high intracellular concentration of the drug.
2.4.3 Targeting by triggered drug release

Another means of targeting the drug to the desired site is the design of nano carriers that selectively release their contents upon exposure of an external stimulus. When certain stimulus is applied to the pathological area from outside of the body, the properties of the nano-formulation is changed, allowing for enhanced or controlled drug release, as shown in Figure 2.4.3. Examples of externally provided stimuli include heat, light, ultrasound and magnetic fields. They are designed to release the therapeutic agent upon application of spatially confined triggers only, thereby maximizing drug release in tumor tissue and at the same time protecting healthy tissues against damage and minimizing side effects [53].

![Diagram of temperature-responsive hybrid magnetic nanoparticles for remotely triggered drug release upon ac magnetic field application][1]

2.4.4 Targeting by electroporation

To bypass the slow and inefficient endocytosis process, researchers have been using electroporation to directly deliver nanoparticles into cell cytosol. Electroporation is a physical delivery approach in which short electrical pulses are applied to cells to create transient and reversible pores on the cell membrane. With these electro-pores, the cell membrane becomes highly permeable to exogenous substances in the surrounding media thus facilitating cellular uptake [55]. Electroporation efficiency is influenced by many factors, such as voltage, pulse width, cell condition, operation temperature, serum concentration in electrical buffer [56, 57].
2.5 Cellular uptake / endocytosis

One of the best features of nano carriers is the ability to cross cellular barriers in order to facilitate the delivery of therapeutic agents. There are several mechanisms by which cells can uptake exogenous material. These include phagocytosis, macro-pinocytosis, receptor-mediated endocytosis, clathrin mediated endocytosis, caveolin-mediated endocytosis, and clathrin and caveolin independent endocytosis [58].

![Cellular uptake / endocytosis diagram](image)

**Figure 2.5.1 Principal internalization pathways in mammalian cells** [59].

Clathrin-mediated endocytosis (CME) pathway involves uptake of high-affinity receptors and their bound ligands into clathrin-coated pits on the plasma membrane. The pits are formed by assembly of cytosolic coat proteins, mainly clathrin and the adaptor protein complex 2 (AP2) [59]. The other component of the machinery driving CME is dynamin. It is a multi-domain GTPase that is recruited to the necks of coated pits, where it assembles into a spiral collar that upon GTP hydrolysis, mediating release of clathrin-coated vesicles [60]. Once inside the cell, the pits form part of the classic endosome network, beginning with early endosomes and ending with lysosomes or recycling endosomes, acquiring and losing cellular factors along the way [61].

Caveolae-dependent pathway involves flask-shaped invaginations called caveolae that are present in cholesterol and sphingolipid-rich domains of the plasma membrane. The shape and structure of caveolae are given by a dimeric protein called caveolin-1 that binds to cholesterol. It inserts into the inner leaflet of the plasma membrane, and self-associates to form a caveolin coat on the surface of the membrane invaginations [59], [62]. Dynamin recruitment has been implicated in this pathway as well [63], [64] and the actin cytoskeleton is believed to play a significant role in the initial uptake and inward transport [65]. Endocytosis via caveolae leads to formation of cytoplasmic organelles known as caveosomes. These organelles are stable, cholesterol-enriched structures that are devoid of markers of the classical endocytic and biosynthetic organelles, including the known endocytic Rab GTPases [66].

Macropinocytosis is a signal-triggered endocytic mechanism used by cells to internalize larger amounts of fluid and membrane into large vacuoles called macropinosomes. These organelles are formed by closure of characteristic membrane ruffles triggered by activation of receptor tyrosine kinases and subsequent downstream changes in actin dynamics [67, 68]. Once inside the cell, macropinosomes either recycle
back to the cell surface or acquire early endosome proteins required for homotypic fusion [69–71]. While Na+/H+ exchangers are important for this endocytic process, dynamin does not appear to be [72, 73].

2.5.1 Cell penetrating peptide (CPP) mediated endocytosis

Cell-penetrating peptides (CPPs) are also called protein transduction domains (PTDs) or arginine-rich intracellular delivery peptides. They are a group of short peptides that have been considered as candidates for mediating drug delivery since they possess the ability to penetrate cell membrane [74]. Zorko and Langel categorized CPPs into three classes: protein-derived CPPs, model peptides and designed peptides [75]. Transactivator of transcription (TAT) and penetratin, two peptides derived from specific protein domains, are typical examples of protein-derived CPPs [75]. Model CPPs include sequences that contain repeat motifs or poly-residues, such as MAP (KLAL) peptide and polyarginine [75]. Designed CPPs are chimeric peptides comprised of various functional domains of target proteins.

CPPs are powerful penetrating biomaterials that are able to deliver cargoes/ bioactive macromolecules such as proteins, nucleic acids, peptide nucleic acids, inorganic particles such as quantum dots, and liposomes into cells of various species [76, 77]. Two major routes for CPPs to enter cells have been documented: the endocytosis-mediated pathway and direct membrane translocation (also called the pore-opening mechanism) [78]. Molecules entering cells via the direct membrane translocation pathway does not incur endosomal entrapment and thus, this route is usually preferred in drug delivery [79]. Several studies have found that arginine-rich CPPs, such as nona-arginine (R9) peptide, destabilized the plasma membrane creating transient pores for cellular penetration [80, 81].

2.5.2 Epidermal growth factor receptor (EGFR) mediated endocytosis

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that constitutes one of four members of the erbB family of tyrosine kinase receptors [82]. Binding of a ligand such as EGF (epidermal growth factor) or TGFα (transforming growth factor α) to the receptor’s extracellular domain initiates signal transduction pathways that regulate cell proliferation and differentiation. A promising target structure for cancer therapy is the epidermal growth factor receptor (EGFR) that is overexpressed on a wide variety of different tumors [83]. To increase the selectivity and efficiency of drug or gene delivery to diseased cells, nanocarriers can be equipped with targeting moiety (such as antibody developed against EGFRs). During the past few years, in vitro and in vivo studies have demonstrated that recombinant full-length EGF can be coupled to gene and drug delivery systems providing their efficient and fast receptor-mediated endocytosis [84, 85].

2.5.3 Size dependent cellular uptake of nanoparticles

A few studies have been conducted which suggested a correlation between the size of the nanoparticles and their uptake [9–11]. A study involving endocytosis of fluorescent microspheres of sizes 50, 100, 200, 500 and 1000 nm in diameter by the murine melanoma cell line, revealed the relevance of particle size itself, i.e. devoid of ligands, as an important factor in governing the cellular pathway of entry and
processing [9]. In another study involving polystyrene beads of sizes 22 nm, 100 nm or 1000 nm, it was observed that a large number of the 22 nm-fluorescent particles was taken up by the PC12 cells than the 100 nm particles. However, the 100 nm-particles were taken up more frequently than the 22 nm-particles. Conversely, the 1000 nm-particles were rarely taken up by the cells [10]. Another study involving cellular uptake of silica nanoparticles by HeLa cells showed that cellular uptake efficiency is size-dependent in the order of 55 nm > 167 nm > 300 nm [11].
3 MATERIALS AND METHODS

3.1 Nanoparticles

Five types of nanoparticles were used for the project. Four of these were red fluorescent polystyrene beads (PSBs, purchased from Micromod, Germany) with excitation and emission wavelengths of 552nm and 580nm respectively, of sizes 25nm, 50nm, 70nm and 100nm, each with carboxylic acid groups (–COOH) on their surface. The 100nm magnetic nanoparticles (MNPs) with streptavidin conjugated surface were purchased from Chemicell, with excitation and emission wavelengths of 547nm and 581nm respectively.

3.1.1 Nanoparticle conjugation with streptavidin

The nanoparticles were conjugated with streptavidin molecules so as to further conjugate the nanoparticle surface with the peptides such as cell penetrating peptides and epidermal growth factor, using the biotin-streptavidin high bonding affinity. The carboxylic group on the surface of polystyrene nanoparticles was used to conjugate the beads with streptavidin. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, also called EDC, is a carbodiimide crosslinker which reacts with carboxylic acid groups to form an active O-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. The primary amine of the streptavidin forms an amide bond with the original carboxyl group on the nanoparticle, and an EDC by-product is released as a soluble urea derivative, as shown in Figure 3.1.1.

![Figure 3.1.1 Schematic of chemistry behind the conjugation of carboxylic surface nanoparticles, represented by 1, with streptavidin molecule, represented by 2.](image)

The procedure for conjugation involved spinning down the stock solution of nanoparticles, originally in water, using bench top ultracentrifuge at 45,000 g for 30 minutes. The particles were then diluted using 10mM borate buffer (pH 7.4). After uniform suspension, streptavidin was added to the solution followed by EDC. After constant stirring for 2 hours, the conjugate solution was filtered through a 0.2 µm PES
membrane unit and was transferred to a clean centrifugal ultrafiltration unit. The solution was then spun for 5 buffer exchanges with 50mM borate buffer, pH 8.3, in order to remove the free streptavidin from the solution. Finally the conjugated material was passed through a 0.2 µm syringe filter and stored in 4°C.

3.1.2 Surface modification with CPP and EGF

Biotin-TAT peptide was purchased from AnaSpec (Fremont, CA). CPP conjugated nanoparticles were prepared by incubating streptavidin-coated polystyrene beads/magnetic nanoparticles with biotinylated TAT at a ratio of 1 to 100 for 1 hour at room temperature. Biotin-XX EGF was purchased from Invitrogen (Carlsbad, CA). EGF conjugated nanoparticles were prepared by incubating streptavidin-coated polystyrene beads/magnetic nanoparticles with biotinylated EGF at a ratio of 1 to 10 for 1 hour at room temperature.

3.1.3 Particle size characterization

The particle size for each nanoparticle sample, before and after conjugation with the peptides, was characterized using dynamic light scattering instrument for quantitative analysis, and transmission electron microscopy for qualitative analysis.

3.1.3.1 Dynamic light scattering

Malvern Zetasizer Nano ZS was used for size characterization for nanoparticles with and without surface modification. 2ml of samples were prepared by diluting each nanoparticle solution to 1nM concentration in milli q water and passing the solution through 0.2µm filter. The samples were sonicated in an ultrasonic sonication bath for a minute to avoid cluster formation, and then transferred to a glass cuvette to put in the instrument to get the data. For 25nm and 50nm nanoparticles samples, both with and without conjugation, number size distribution was considered. For larger particles, intensity size distribution was considered.

3.1.3.2 Transmission electron microscopy

Hitachi H-7100 TEM was used. 100µl of samples were prepared by diluting each nanoparticle solution to 25nM in milli q water and sonicating them for a minute. Samples were then prepared for imaging by drying nanoparticles on a copper grid that was coated with a thin layer of carbon. The particles with surface modification were stained with heavy metals. A small drop of solution was placed onto a grid and allowed to evaporate. The sample was then imaged at 150k × and the images were collected.

3.1.4 Estimation of surface coating

The streptavidin-conjugated nanoparticles were incubated with biotinylated carboxy fluorescein (cF dye) in a ratio of 1:100 for 1 hour at room temperature. To remove the free dye, the solution was then transferred to a clean centrifugal ultrafiltration unit (Amicon Ultra-4, Millipore Corp) and centrifuged at
4000g for 10 min. It was repeated for 3 buffer exchanges with 50 mM borate buffer, pH 8.3. The filtrand was then applied to a glass slide and sealed with a cover slip. It was then visualized under the Nikon Eclipse Ti-E inverted microscope with a Photometric CoolSnap HQ2 camera (Roper Scientific) and a 20×/1.41NA objective lens. The samples were excited using the TRITC filter (for nanoparticles) as well as FITC filter (for cF dye), and images were captured to see if there was an overlap.

### 3.2 Fluorescence characterization using plate reader

To check the relative fluorescence intensity of all the polystyrene nanoparticles, TECAN Safire 2 fluorescence plate reader was used. 1nM of each sample was prepared in a 96 well plate with 200 µl in each well. For each sample 5 serial dilutions with a dilution factor of 20 was done using borate buffer of pH 8.3. Blanks were also prepared using borate buffer of pH 8.3. The samples were excited at 550nm and the emission spectra were recorded.

### 3.3 Cell cultivation

BSC-1 cells, epithelial cell line of African green monkey kidney origin, was grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). HEK 293, Human Embryonic Kidney cell line, was grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH 3T3, mouse embryonic fibroblast cell line, was also grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BSC-1 cells, 3T3 cells and HEK cells were cultivated in T-25 flasks. Mouse hippocampus neurons were grown in Neurobasal medium supplemented with 2% B27 and 2 mM GlutaMAX. They were plated in glass-bottom cell culture MatTek dishes which were pre-treated with poly-L-lysine and laminin solutions.

### 3.4 Incubation of cells with nanoparticles

BSC-1 cells, 3T3 cells and HEK cells were trypsinized from the T-25 flasks using Trypsin-EDTA (0.25%) and transferred to the center of the glass-bottom cell culture MatTek dishes, with a cell density of approximately 200,000 cells per dish. The cells were allowed to stick to the glass bottom by incubating them for 3-4 hours in a humidified 37°C/5% CO₂ incubator. Each type of nanoparticles was diluted to 0.1 nM in the respective medium. For the conjugated nanoparticles sample, biotin-TAT and biotin-EGF was added to the nanoparticles in a ratio of 1:100 and 1:10 respectively, and incubated for 1 hour at room temperature with gentle shaking. The nanoparticle-medium solution was then added to the center of the MatTek dishes, and were kept for incubation for 1-4 hours, depending on the different cell type and experimental design, in a humidified 37°C/5% CO₂ incubator.

### 3.5 Cell imaging

After incubating the cells in MatTek dishes with nanoparticles, the cells were washed using fresh culture medium. Cells were then counterstained with Hoechst 33342 (purchased from Invitrogen (Carlsbad,
CA)) to label the nucleus. After incubation for 10 minutes, cells were washed using fresh culture medium three times and then imaged using a Nikon Eclipse Ti-E inverted microscope with a Photometric CoolSnap HQ2 camera (Roper Scientific), and a 60× and 100×/1.41NA oil objective lens.

3.6 Estimation of nanoparticle uptake using flow cytometry

3.6.1 Incubation of cells with nanoparticles

BSC-1 cells, 3T3 cells and HEK cells were plated in 6 well plates with a cell density of approximately 400,000 cells and 3ml respective media in each cell. The cells were incubated for 24 hours in the humidified 37°C/5% CO₂ incubator. Each type of nanoparticles was diluted to 1 nM in the respective medium. For the conjugated nanoparticles sample, biotin-TAT and biotin-EGF were added to the nanoparticles in a ratio of 1:100 and 1:10 respectively, and incubated for 1 hour at room temperature with gentle shaking. The nanoparticle-medium solution was then added to the wells with the cells, and was incubated for 2-3 hours. The cells were then washed 3 times using 1× PBS buffer to remove free nanoparticles. They were then incubated with Trypsin-EDTA (0.25%) for 5 minutes. To stop the trypsinization process the PBS buffer was added and the cells were transferred to 15ml centrifuge tubes and spun down at 1000g for 5 minutes. The cells were then suspended in 500 µl of 1× PBS buffer and transferred to 1ml Eppendorf tubes. Samples with no nanoparticles added were also analyzed as controls.

3.6.2 Flow cytometry setup

Analyses of samples were performed in a BD FacsVantage flow cytometer. Samples were transferred to facs tubes for analysis. A total of 5,000 cells were counted from each sample, or the data collection was aborted after analyzing the sample for 180 seconds. Samples were excited with a green laser of wavelength 561 nm and detected through a 582 nm filter with a 15 nm band pass. By plotting the forward scatter signal against the side scatter signal in a dot plot, a collection gate was established to exclude cell debris and clusters. From these events, a single parameter histogram of counts versus fluorescence intensity was obtained. Control samples were analyzed to exclude the auto-fluorescence contribution. Once all the data was collected, FlowJo software was used for data analysis, and to acquire overlays of histograms.
4 RESULTS

4.1 Particle size characterization

The size of the polystyrene beads (PSBs) and magnetic nanoparticles (MNPs) before and after the conjugation of the nanoparticles with streptavidin-biotin-TAT (cell penetrating peptide), were characterized using the dynamic light scattering (DLS) instrument and transmission electron microscope (TEM).

4.1.1 Dynamic light scattering (DLS)

Using the DLS instrument the Intensity size distribution histograms of all the nanoparticles were collected, as shown in Figures 4.1.3, 4.1.6, 4.1.7 and 4.1.8. Using the raw data, the mean and standard deviation for each type of nanoparticles was calculated and the bar graph was plotted to compare their sizes, as shown in Figure 4.1.1.

![Intensity Size distribution using DLS](image)

*Figure 4.1.1 Diameters of unconjugated and conjugated nanoparticles obtained from “Intensity size distribution” for polystyrene beads by DLS instrument. Errors bars show standard deviation, as determined by DLS.*

It was observed that the diameters obtained from the “Intensity size distribution” for the unconjugated 25nm and 50nm nanoparticles were not consistent with the specifications provided by the company (Micromod, Germany). This could be explained by the fact that the intensity size distribution is based upon the intensity of light scattered by particles which makes it very sensitive to the presence of even a very small number of large particles/aggregates in the sample. The nanoparticle cluster formation or the streptavidin aggregation might contribute to this deviation from true results.

However, the number size distributions for the 25nm and 50nm nanoparticles provided results in a more acceptable range. The plot involving the diameters obtained from “Number size distribution” for 25 nm
and 50nm polystyrene beads, and from “Intensity size distribution” from 70nm and 100nm polystyrene beads, is shown in Figure 4.1.2

![Size distribution using DLS](image)

**Figure 4.1.2** Diameters of unconjugated and conjugated nanoparticles; obtained from “Number size distribution” for 25 nm and 50nm polystyrene beads, and from “Intensity size distribution” for 70nm and 100nm polystyrene beads. Errors bars show standard deviation, as determined by DLS.

The following figures are the histograms obtained after analyzing the unconjugated and conjugated nanoparticle samples using the Zetasizer DLS instrument. Figure 4.1.3 represents the histogram of the “Intensity size distribution” for 25nm polystyrene beads. It shows a bimodal distribution which could be due to the cluster formation or presence of some free streptavidin clumps in the solution. And since Intensity size distribution is very sensitive to aggregates and larger particles it produces a wide range of sizes from 20nm to 100nm with a maximum peak at 69.55nm in the case of unconjugated 25nm PSB sample and a range of 50nm to 1000nm with a maximum peak at 190nm in the case of conjugated 25nm PSB sample.

![Intensity size distribution of unconjugated 25nm PSB](image)
Figure 4.1.3 Intensity size distribution of 25nm polystyrene beads generated by Malvern Zetasizer Nano ZS. 
(A) Beads without conjugation, with a maximum peak at 69.55nm, and (B) Beads with conjugation of streptavidin-biotin-TAT, with maximum peak at 190.1 nm

On the other hand, the number size distribution, which is a transformation derived using Mie theory and is highly dependent on the optical properties of the particle such as refractive index and particle absorption, gives relatively more accurate size distribution for 25nm and 50nm PSBs with and without conjugation, as shown in Figure 4.1.4 and 4.1.5. The size for the conjugated particle comes out to be approximately double to that of the unconjugated particle because of the hydrodynamic radius formed due to the peptides on the nanoparticle surface. The unconjugated beads have –COOH groups on their surface which might contribute to a small increase in the size of the nanoparticles obtained from the DLS as compared to its actual core diameter.

Figure 4.1.4 Number size distribution of 25nm polystyrene beads generated by Malvern Zetasizer Nano ZS. 
(A) Beads without conjugation, with a maximum peak at 32 nm, and (B) Beads with conjugation of streptavidin-biotin-TAT, with maximum peak at 61.06 nm

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Similarly for 50nm polystyrene beads, number size distribution reflected more accurate results as shown in Figure 4.15.

![Number size distribution of unconjugated 50nm PSB](image)

Figure 4.15 Number size distribution of 50nm polystyrene beads generated by Malvern Zetasizer Nano ZS. (A) Beads without conjugation with a maximum peak at 43.82 nm, and (B) Beads with conjugation of streptavidin-biotin-TAT, with maximum peak at 68.06 nm

For 70nm and 100nm polystyrene beads and 100nm magnetic nanoparticles, the Intensity size distributions were obtained as shown in Figures 4.1.6, 4.1.7 and 4.1.8, respectively.

![Intensity size distribution of unconjugated 70nm PSB](image)
Figure 4.1.6 Intensity size distribution of 70nm polystyrene beads generated by Malvern Zetasizer Nano ZS. (A) Beads without conjugation with a maximum peak at 68.08 nm, and (B) Beads with conjugation of streptavidin-biotin-TAT, with maximum peak at 79.80 nm.

Figure 4.1.7 Intensity size distribution of 100nm polystyrene beads generated by Malvern Zetasizer Nano ZS. (A) Beads without conjugation with a maximum peak at 105.70 nm, and (B) Beads with conjugation of streptavidin-biotin-TAT, with maximum peak at 122.40 nm.
The unconjugated magnetic nanoparticles when bought from the company already had streptavidin coated on their surfaces, because of which the size obtained for the MNPs using the DLS was 135nm. The conjugated MNPs had Biotin-TAT in addition unlike the polystyrene beads which had streptavidin-biotin-TAT on their surfaces, resulting in a very small difference in the sizes of conjugated and unconjugated MNPs.

![Intensity size distribution of unconjugated 100nm MNP](image1)

![Intensity size distribution of conjugated 100nm MNP](image2)

**Figure 4.1.8 Intensity size distribution of 100nm Magnetic nanoparticles generated by Malvern Zetasizer Nano ZS.** (A) Beads with streptavidin with a maximum peak at 135.90 nm, and (B) Beads 100nm Magnetic nanoparticles with conjugation of streptavidin-biotin-TAT, with maximum peak at 145.10 nm

The diameters of the nanoparticles obtained from the DLS instrument is summarized in Figure 4.1.9.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Unconjugated (nm)</th>
<th>Conjugated (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25nm PSB</td>
<td>32</td>
<td>61.06</td>
</tr>
<tr>
<td>50nm PSB</td>
<td>43.83</td>
<td>68.06</td>
</tr>
<tr>
<td>70nm PSB</td>
<td>68.08</td>
<td>79.80</td>
</tr>
<tr>
<td>100nm PSB</td>
<td>105.70</td>
<td>122.40</td>
</tr>
<tr>
<td>100nm MNP</td>
<td>135.90</td>
<td>145.10</td>
</tr>
</tbody>
</table>

**Figure 4.1.9 Comparison of sizes of unconjugated and conjugated nanoparticles; obtained from “Number size distribution” for 25 nm and 50nm nanoparticles, and from “Intensity size distribution” for 70nm and 100nm nanoparticles**
4.1.2 Transmission electron microscopy (TEM)

The size and geometry of the nanoparticles was further characterized using TEM. Under the TEM microscope, no difference was observed in the sizes between nanoparticles with and without conjugation. This might be because of the low density of the surface coating which was not sufficient enough to hold the heavy metal stain applied to the samples in order to observe the coating. Also, from the images of each sample of nanoparticles, shown in Figure 4.1.10, it could be seen that the nanoparticle size is not strictly uniform; this is consistent with the histograms obtained from the DLS analysis of the nanoparticles.

Figure 4.1.10 Images of unconjugated nanoparticles obtained from transmission electron microscope. (A) 25nm polystyrene beads, (B) 50nm polystyrene beads, (C) 70nm polystyrene beads, (D) 100nm polystyrene beads, and (E) 100nm magnetic nanoparticles. Scale bar: 100nm
4.2 Assessment of surface coating using fluorescence microscopy

To check the success of conjugation of the 25nm, 50nm, 70nm and 100nm polystyrene beads (PSB) with streptavidin, nanoparticles were incubated with biotinylated carboxy fluorescein (cF) dye. The purpose of using the different color dye was to be able to distinguish the streptavidin coating from the nanoparticle core. The samples were visualized under the microscope by first exciting the beads using TRITC filter and taking the images as shown in figures 4.2.1 (A) for 25nm PSB, 4.2.2 (A) for 50nm PSB, 4.2.3 (A) for 70nm PSB and 4.2.4 (A) for 100nm PSB, and then exciting the cF dye on the beads using FITC filter and taking the images as shown in figures 4.2.1 (B), 4.2.2 (B), 4.2.3 (B) and 4.2.4 (B). The perfect overlap of the images obtained from the TRITC filter with the images obtained from FITC filter, as shown in figures 4.2.1 (C), 4.2.2 (C), 4.2.3 (C) and 4.2.4 (C), showed that the beads were successfully conjugated.

Figure 4.2.1 Estimation of surface coating of 25nm PSB. (A) conjugated 25nm PSB using TRITC filter, (B) conjugated 25nm PSB + biotin-cF dye using FITC filter, at 20× magnification. (C) Overlay of the images (A) and (B).

Figure 4.2.2 Estimation of surface coating of 50nm PSB. (A) conjugated 50nm PSB using TRITC filter, (B) conjugated 50nm PSB + biotin-cF dye using FITC filter, at 20× magnification. (C) Overlay of the images (A) and (B).

Figure 4.2.3 Estimation of surface coating of 70nm PSB. (A) conjugated 70nm PSB using TRITC filter, (B) conjugated 70nm PSB + biotin-cF dye using FITC filter, at 20× magnification. (C) Overlay of the images (A) and (B).
4.3 Fluorescence characterization of nanoparticles

The purpose of fluorescence characterization of the nanoparticles was to find the relative fluorescence intensities of the nanoparticles’ cores so as to be able to normalize the data obtained from the flow cytometry analysis to measure the relative uptake of the nanoparticles of different sizes by the cell lines.

In order to find the relative fluorescence intensities of the polystyrene beads, the samples were excited at 550nm and the emission wavelengths were obtained for each sample, using a TECAN Saphire plate reader, with different dilutions. A linear correlation was observed between the fluorescence intensity and dilution rate. The emission spectra for samples at 1nM are shown in figure 4.3.1. Fluorescence intensities at $\lambda_{\text{max}}$ of 485 a.u. for 25nm PSB, 977 a.u. for 50nm PSB, 639 a.u. for 70nm PSB, and 2255 a.u. for 100nm PSB were obtained. This showed that 100nm PSB is 4.65 times more fluorescent than 25nm PSB, 2.31 times more fluorescent than 50nm PSB and 3.53 times more fluorescent than 70nm PSB.

**Fluorescence characterization**

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**Figure 4.3.1 Fluorescence characterization of polystyrene beads.** Emission spectra obtained with a max at 585nm as a result of excitation at 550 nm. Fluorescence intensity at $\lambda_{\text{max}}$ of 485 a.u. for 25nm PSB, 977 a.u. for 50nm PSB, 639 a.u. for 70nm PSB, and 2255 a.u. for 100nm PSB.
Figure 4.3.2 Relative fluorescence intensity of polystyrene beads. 100nm PSB is 4.65 times more fluorescent than 25nm PSB, 2.31 times more fluorescent than 50nm PSB and 3.53 times more fluorescent than 70nm PSB.

4.4 Fluorescence live cell imaging of nanoparticle uptake

To check for internalization of nanoparticles by the BSC-1 cells, the cells were incubated with conjugated nanoparticles and were visualized under the microscope. Uniform endocytosis was observed when 25nm, 50nm and 70nm polystyrene beads, conjugated with cell penetrating peptide TAT, were added to the BSC-1 cells attached to the MatTek dishes and incubated for an hour, as shown in Figures 4.4.1, 4.4.2, and 4.4.3.

Figure 4.4.1 Uniform endocytosis of TAT-25nm PSB by BSC 1 cells. Addition of beads to BSC1 cells attached to the dish and incubation for 1 hour, (A) DIC image at 100x, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing uniform endocytosis, and (D) Overlay. Scale bars: 10µm.
**Figure 4.4.2 Uniform endocytosis of TAT-50nm PSB by BSC 1 cells.** Addition of beads to the BSC1 cells attached to the dish and incubation for 1 hour, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing uniform endocytosis, and (D) Overlay. Scale bars: 10µm.

**Figure 4.4.3 Uniform endocytosis of TAT-70nm PSB by BSC 1 cells.** Addition of beads to the BSC1 cells attached to the dish and incubation for 1 hour, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing uniform endocytosis, and (D) Overlay. Scale bars: 10µm.
In case of 100nm MNP conjugated with TAT, there was no uniform endocytosis and cluster formation could be seen near the membrane, when the MNPs were added to the cells attached to the MatTek dishes before incubation, as shown in Figure 4.4.4. But when the MNPs were added to the suspended cells and incubated for 3 hours, a more uniform endocytosis was observed, as shown in Figure 4.4.5.

**Figure 4.4.4 Non-uniform endocytosis of TAT-100nm MNPs by BSC 1 cells.** Addition of MNP to the BSC1 cells attached to the dish and incubation for 3 hours, (A) DIC image at 60x, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing cluster formation on membrane, and (D) Overlay. Scale bars: 10µm.

**Figure 4.4.5 Uniform endocytosis of TAT-100nm MNPs by BSC 1 cells.** Addition of MNP to the suspended BSC1 cells and incubation for 3 hours, (A) DIC image at 60x, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing uniform endocytosis, and (D) Overlay. Scale bars: 10µm.
When the TAT conjugated-100nm MNPs were incubated with mouse hippocampus neurons, there was no endocytosis as shown in Figure 4.4.6. But incubating the neurons with 70nm PSBs showed the nanoparticles attaching to the surface of the cell body and the neurites unlike 100nm nanoparticles, as shown in figure 4.4.7. The endocytosis drastically improved on conjugating the 70nm PSB with TAT as well as EGF, as shown in Figures 4.4.8 and 4.4.9.

![Figure 4.4.6 No uptake of TAT-100 nm MNP by the mouse hippocampus neurons.](image1)

Addition of MNPs to neurons and incubation for 3 hours, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing no endocytosis, and (D) Overlay. Scale bars: 10µm.

![Figure 4.4.7 Uptake of 70 nm PSB by the mouse hippocampus neurons.](image2)

Addition of beads to the neurons and incubation for 3 hours, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing some endocytosis, and (D) Overlay. Scale bars: 10µm.
Figure 4.4.8 Uptake of TAT-70 nm PSB by the mouse hippocampus neurons. Addition of beads to the neurons and incubation for 3 hours, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing some endocytosis, and (D) Overlay. Scale bars: 10µm.

Figure 4.4.9 Uptake of EGF-70 nm PSB by mouse hippocampus neurons. Addition of beads to the neurons and incubation for 3 hours, (A) DIC image at 60×, (B) Image using DAPI filter to capture nucleus, (C) Image using TRITC filter to capture nanoparticles showing some endocytosis, and (D) Overlay. Scale bars: 10µm.
4.5 Quantification of nanoparticle uptake using FCM

4.5.1 Dependence of uptake on particle size

Overlay histograms were obtained by doing flow cytometry (FCM) analysis on the BSC1, HEK and 3T3 cell lines which were incubated with same concentration of 25nm, 50nm, 70nm and 100nm polystyrene nanoparticles. Since the fluorescence intensity for each size of nanoparticle at a given concentration was different so the data obtained from FCM was normalized using the relative fluorescent values of the particles obtained using TECAN plate reader. The bar graph was then plotted using the normalized data.

For the first experiment the BSC-1 cells were incubated with the unconjugated beads, and the FCM analysis was performed to obtain the histogram as shown in Figure 4.5.1 (A). The corresponding normalized data showing the relative uptake of the nanoparticles by the cells is represented using the bar graph, shown in Figure 4.5.1 (B). The results show that the 70nm nanoparticles are taken up by the cell line most efficiently, followed by 50nm and then 100nm and 20nm.

![Figure 4.5.1 Dependence of the unconjugated PSBs uptake by the BSC 1 Cells on the nanoparticle size. (A) Overlay histogram obtained in FCM analysis of BSC-1 cells incubated with unconjugated polystyrene beads of sizes 25nm, 50nm, 70nm and 100nm, for 3 hours. (B) Normalized data showing the relative uptake of polystyrene beads by BSC-1 cells.](image)
The other experiments were performed to check the dependence of the cellular uptake of the nanoparticles by the BSC-1 cells, HEK cells and 3T3 cells when the nanoparticles were conjugated with TAT peptides. The histograms obtained from the FCM for each experiment are shown in Figures 4.5.2 (A), 4.5.3 (A) and 4.5.4 (A), respectively. The normalized bar graphs representing the relative uptake of the nanoparticles for each set of experiment is shown in Figures 4.5.2 (B), 4.5.3 (B) and 4.5.4 (B), respectively.

The results show that 70nm nanoparticles were uptaken by the cells most efficiently, followed by 50nm, 20nm and 100nm.

Figure 4.5.2 Dependence of the conjugated PSBs uptake by the BSC 1 Cells on the nanoparticle size. (A) Overlay histogram obtained in FCM analysis of BSC-1 cells incubated with TAT conjugated polystyrene beads of sizes 25nm, 50nm, 70nm and 100nm, for 3 hours. (B) Normalized data showing the relative uptake of conjugated polystyrene beads by BSC-1 cells.
Figure 4.5.3 Dependence of the conjugated PSBs uptake by the HEK Cells on the nanoparticle size. (A) Overlay histogram obtained in FCM analysis of HEK cells incubated with TAT conjugated polystyrene beads of sizes 25nm, 50nm, 70nm and 100nm, for 3 hours. (B) Normalized data showing the relative uptake of conjugated polystyrene beads by HEK cells.

Figure 4.5.4 Dependence of the conjugated PSBs uptake by the 3T3 Cells on the nanoparticle size. (A) Overlay histogram obtained in FCM analysis of 3T3 cells incubated with TAT conjugated polystyrene beads of sizes 25nm, 50nm, 70nm and 100nm, for 3 hours. (B) Normalized data showing the relative uptake of conjugated polystyrene beads by 3T3 cells.
4.5.2 Effect of CPP coating

To visualize and analyze the effect of TAT conjugation to beads, the histograms obtained from cells incubated with unconjugated beads, were plotted against the histograms obtained from cells incubated with TAT-conjugated beads. This was done for each size of nanoparticles, as shown in Figure 4.5.5.

The results show that there is approximately a 10 fold increase in the intake of 25nm, 50nm and 70nm PSB by the cells when the PSBs are conjugated with TAT peptides. However, there is no significant change in uptake of the TAT conjugated 100nm PSB when compared to the unconjugated 100nm PSB.

Figure 4.5.5 Effect of the cell penetrating peptide (TAT) coating on the cellular uptake of nanoparticles. Overlay histograms obtained in FCM analysis of BSC-1 cells, red representing cells with unconjugated beads, and blue representing cells with TAT-conjugated beads, of sizes (A) 25nm, (B) 50nm, (C) 70nm and (D) 100nm.
4.5.3 Effect of EGF coating

To visualize and analyze the effect of EGF conjugation to beads, the histograms obtained from cells incubated with unconjugated beads, were plotted against the histograms obtained from cells incubated with EGF-conjugated beads. This was done for the 50nm and 70nm nanoparticles with the different cell lines, as shown in Figure 4.5.6.

The results show that there was no significant change in the uptake of the EGF conjugated PSB when compared to the unconjugated PSB, by the non-cancer cell lines.

![Figure 4.5.6](Image)

**Figure 4.5.6 Effect of the epidermal growth factor (EGF) coating on the cellular uptake of nanoparticles.**

Overlay histograms obtained in FCM analysis, red representing cells with unconjugated beads, and blue representing cells with EGF-conjugated beads, of (A) 50nm with 3T3 cells, (B) 70nm with 3T3 cells, (C) 50nm with BSC1 cells and (D) 70nm with BSC1 cells
5 DISCUSSION AND CONCLUSION

5.1 Size characterization

5.1.1 Size difference with and without conjugation

The conjugated nanoparticles showed to have a larger diameter than the unconjugated particles. The reason behind this could be explained by hydrodynamic radius which is formed as a result of the peptide chains on the surface of the nanoparticles, as shown in Figure 5.1.1. In Dynamic Light Scattering (DLS) light intensity fluctuations taking place at microsecond or millisecond scales are measured. Those fluctuations are a measure of the diffusion constant (Brownian motion) of the molecules and are related to the hydrodynamic radius of a molecule.

![Figure 5.1.1 Hydrodynamic diameter $d_H$, formed as a result of the peptides present on nanoparticle's surface.](image)

The other observation from the size characterization using DLS method was that the difference of size after and before conjugation in case of 25nm beads was the most when compared to other particles. The reason behind this could be the larger surface area of the 50nm, 70nm and 100nm particles as compared to 25nm ones. Smaller the surface area might mean that the peptide chains are present on the surface more compactly, leading to repulsion and thus extended/longer lengths of peptides above the surface of the nanoparticle as shown in Figure 5.1.2.

![Figure 5.1.2 An illustration showing the effect of surface area on the length of the peptide on the surface of the nanoparticle. If ‘X’ is the vertical length of peptide on the larger NP and ‘Y’ is the vertical length of peptide on the smaller NP, then due to steric hindrance, ‘Y’ may be greater than ‘X’.](image)

5.1.2 Difference in size characterization between TEM & DLS

Dynamic light scattering (DLS) method gives the hydrodynamic radius of the particle whereas the transmission electron microscope (TEM) gives an estimation of the projected area diameter. So as far as DLS is concerned, the theory states that when a dispersed particle moves through a liquid medium, a thin
electric dipole layer of the solvent adheres to its surface due to presence of some molecules such as carboxylic groups. This layer influences the movement of the particle in the medium. Thus the hydrodynamic diameter gives us information of the inorganic core along with any coating material and the solvent layer attached to the particle as it moves under the influence of Brownian motion.

While estimating size by TEM, this hydration layer is not present hence, we get information only about the inorganic core. The projected area diameter estimated by TEM is theoretically defined as the area of a sphere having the same area as the projected area of the particle resting in a stable position. Sometimes due to poor contrast in TEM the size measurement of the coating layer if present could be underestimated or missed. Hence, the diameter obtained by DLS generally was greater than the size estimated by TEM.

5.2 Increase in uptake efficacy due to surface modification

From the results obtained from flow cytometry analysis it is evident that the conjugation of nanoparticles with TAT improves the uptake efficiency. But this improvement is dependent on the particle size as well since 25nm, 50nm and 70nm particles showed 10 fold more intake when conjugated with TAT whereas there was not much improvement in case of 100nm particles. From the previous studies it had been shown that modification of nanoparticle surface with EGF or EGFR antibody improved the uptake of the nanoparticles by the cancer cell lines. This study shows that there is an improvement in the uptake of EGF coated nanoparticles by the mouse hippocampus neurons, but there is no increase in the uptake efficiency of nanoparticles conjugated with EGF by the non-cancer mammalian cell lines (BSC-1, HEK and 3T3). This might be explained by the low expression of EGF receptors on the non-cancer cells as compared to the cancer cell lines, and high expression of EGF receptors on the neurons.

5.3 Influence of size on uptake efficacy

The flow cytometry analysis shows that the uptake efficiency of 70nm particles is the maximum irrespective of the surface modification with the cell penetrating peptide. This was followed by 50nm, 25nm and 100nm particles. Live cell imaging showed that the 25nm, 50nm and 70nm beads were uniformly distributed inside the cell after 2 hours of incubation of cells with the beads. The 100nm particles tend to form clusters near the periphery of the cell.

5.4 Effect of incubation on uptake efficacy

Two sets of MatTek dishes were prepared with same cell density at the center of the dish. Same concentration of 100 nm particles was added to both the dishes. In one dish the particles were added when the cells had attached to the dish whereas in the other dish particles were added when the cells were in the suspended form. After incubating the dishes for around 3 hours, the cell imaging was done to study the endocytosis. The results showed a uniform endocytosis in the 2nd dish (the one with suspended cells) whereas cluster formations in the first dish (the one with pre-attached cells). This might be explained by the surface area of the cell exposed to the nanoparticles which is more when the nanoparticles are exposed to suspended cells. Another factor could be the change in the morphology which the cell undergoes while attaching to a surface which might lead to a temporary increase in pores in the cell membrane.
In summary, the study has investigated the effect of particle size and surface modification on the cellular uptake of the nanoparticles by the mammalian cells. The cell imaging analysis shows that there is a uniform endocytosis of 25nm, 50nm and 70nm nanoparticles in the non-cancer cell lines, whereas there are cluster formations on the cell periphery in the case of 100nm nanoparticles. Addition of the 100nm nanoparticles to the cells in the suspended form, leads to a much more uniform endocytosis. The FCM analysis reveals that the 70nm size range is taken up by the cells most efficiently as compared to the 25nm, 50nm and 100nm nanoparticles, irrespective of the surface modification with cell penetrating peptides. The study also shows that conjugating the cell penetrating peptides to the surface of nanoparticles in the size range of 25nm to 70nm, significantly improves the cellular uptake rate. Furthermore, the study reveals that the conjugation of the nanoparticles with epidermal growth factor does not improve the cellular uptake by the non-cancer cell lines. However, the conjugation of nanoparticles with either cell penetrating peptides or epidermal growth factor increases the uptake of nanoparticles by the mouse hippocampus neurons.
REFERENCES


