Particle Size Influences Fibronectin Internalization and Degradation by Fibroblasts

by

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Abstract

Particle size is a crucial factor that influences the fate and biological impact of particles and their surface proteins upon internalization. Here, using fibronectin-coated polystyrene nanoparticles and microparticles we examined the effect of particle size on degradation of fibronectin.

Microparticle uptake depended primarily on $\beta 1$ integrins and actin filaments, while nanoparticle uptake relied mainly on lipid rafts and specifically on clathrin-mediated endocytosis. Further, biotinylated fibronectin when coated on microparticles underwent more intracellular processing than fibronectin coated on to nanoparticles. Thus, particle size affects actin and clathrin-dependent internalization, which in turn regulates intracellular fibronectin degradation.

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Publications Arising From Thesis

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List of Abbreviations

ATP	Adenosine 5-triphosphate
BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FITC	Fluorescein isothiocyanate
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine-5-triphosphate
LDL	Low density lipoproteins
mAb	Monoclonal antibody
MP	Microparticle
NP	Nanoparticle
pAb	Polyclonal antibody
PEG	Polyethylene glycol
PLA	Polylactic acid
PLL	Poly-L-lysine
PBS	Phosphate buffered saline
TBST	Tris-buffered-saline with 0.1% Tween-20

Literature Review

I. Description of Nanoparticles

A. Discovery

Over the past thirty years a new branch of technology has been established and developed that implements the design, and applies structures, devices and systems at the nanoscale level ¹. This technology started with the notion that small molecular carriers can be used to deliver a wide variety of biologically active molecules to the cell interior. Earlier, in the 1970s, it was discovered that synthetic polymer nanocapsules can cross the plasma membrane and become concentrated inside cells. The molecules incorporated into these capsules were not transported into cells in the absence of the nanocapsules ². Arising from this discovery, a new strategy was developed and optimized to deliver specific molecules into cells for diagnosis and treatment of a wide spectrum of pathological conditions, including tumors, metabolic disorders, inflammatory syndromes and infectious diseases, as well as the transport of nucleic acids for gene therapy.

B. Definition and General Characteristics

Nanoparticles are <100 nm in their greatest diameter ³. Their physical and chemical characteristics vary widely between different types of nanoparticles. Nanoparticles are synthesized as spheres, tubes or as irregular shapes and may be fused, aggregated or agglomerated.

C. Types of Nanoparticles

1. Structural types

Nanoparticles can be formed from a wide range of materials including metals, oxides, ceramics, semiconductors and organic materials. The particles may be of mixed composition and may

comprise, for example, a combination of a metal core with an oxide shell, or alloys that include several different types of metals. In general, nanoparticles can be classified into several major categories based on molecular composition and morphological characteristics.

a) Carbon-based nanoparticles

Fullerenes are large, complex molecules that contain only carbon atoms and are arranged in the shape of a sphere, ellipsoid or tube. Fullerenes were initially discovered in the 1980s by ablation of graphite with a laser. Subsequently, fullerenes were characterized as one of four types of naturally-occurring forms of carbon ⁴. The general structure of the fullerene molecule is similar to that of graphite; however, in addition to hexagonal carbon rings it contains also pentagonal or heptagonal rings that enable the formation of more complex, three-dimensional structures.

Carbon nanotubes belong to the structural family of fullerene nanoparticles, which are characterized by an elongated tubular structure, 1–2 nm in diameter ³. Nanotubes are formed from a one atom-thick layer of carbon atoms arranged in a hollow cylinder ⁵; multi-wall carbon nanotubes have also been described with diameters up to 20 nm ⁶. Carbon nanotubes exhibit high tensile strength and demonstrate large elastic moduli, which account for nanotubes being one of the strongest and stiffest materials that have been described. Other notable features of nanotubes include high levels of thermal conductivity, surface area to volume ratio, hardness, molecular adsorption capacity as well as demonstration of unique electronic properties ⁷.

b) Noble metal-based nanoparticles

Noble metal-based nanoparticles are synthesized by the colloidal method in which salt precursors are reduced by agents such as citrate, which is followed by exposure to stabilizers for surface protection. Gold nanoparticles manufactured by this method are 9-120 nm in diameter ⁸. To

generate nanoparticles with different morphologies or sizes, the synthesis can be modified by varying the concentration of metal precursors and/or the pH of the reaction ⁹. In a separate synthetic approach, the seed-growth method was developed to provide improved control of size and shape of the nanoparticles ¹⁰. Small, spherical, seed-mediated particles are produced first, which is followed by separate nucleation and growth steps. With this method several different shapes of nanoparticles can be synthesized including those with rod-like shapes ¹¹, stellate shapes ¹² or sea urchin-like shapes ¹³.

c) Quantum dots

Quantum dots are nanometer-sized (2–10 nm) crystalline cores of metal, metal oxide or semiconductor materials ³. Arising from their unique structure, quantum dots exhibit novel electronic, optical, magnetic and catalytic properties that allow them to be used in variety of cell and molecular biology applications that include single particle tracking, intracellular drug delivery, fluorescence resonance energy transfer imaging and multi-modal molecular/tissue imaging ^{14, 15}. Quantum dots are very versatile and can be readily modified to suit the requirements of particular applications. For example, their optical properties can be changed by altering the particle composition and morphology through careful control of the growth steps ¹⁶. Due to their large surface area to volume ratios, and the ease of surface functionalization, a wide variety of target recognition and active biosensing elements can be attached to quantum dots, thereby endowing them with multifunctional properties ¹⁷.

d) Biopolymeric nanoparticles

Compared to carbon based or metal nanoparticles, polymeric nanoparticles have several advantages as they can be manufactured in a wide range of sizes and with distinct surface

characteristics¹⁸. Naturally-derived polymeric nanoparticles are particularly important in biotechnology applications because of their biocompatibility, biodegradability and low immunogenicity¹⁹. For example, protein-based nanoparticles offer several advantages over synthetic polymers as they are readily metabolized by digestive enzymes into biocompatible peptides without the production of toxic degradation products, which is frequently seen with synthetic polymers²⁰. The specific amino acids and the sequences of amino acids dictate the three dimensional structure of proteins, which include for example the formation of α -helix or β -sheet structures. Arising from the ability of proteins to form three dimensional structures, protein-based nanoparticles can be synthesized to form complex shapes, such as fibers and sheets¹⁹. Further, the surface characteristics of protein- and carbohydrate-based nanoparticles can be readily modified due to the presence of multiple functional groups in their chemical structures, which enable specific targeting²¹. A variety of proteins (silk, albumin, collagen and elastin) and polysaccharides (chitosan, alginate, heparin) have been utilized for the preparation of therapeutic nanoparticles²²⁻²⁴.

2. Functional types of nanoparticles

a) First Generation

Nanoparticle design evolved through several stages as nanotechnology was being developed. The first generation of nanoparticles was developed to assess the properties of these new materials and consider possible applications for biomedical research. Nano-particulates such as quantum dots²⁵ and iron oxide nanoparticles²⁶ were modified to achieve stability for application in biological systems. The dynamics of cellular uptake and biocompatibility of several novel nano-carriers was investigated. Various parameters of nanoparticles such as surface charge, size,

morphology and surface functionalization were studied to characterize more specifically, the interactions of nanomaterials with cells.

b) Second Generation

When stable nanoparticles were developed that could predictably interact and be internalized by cultured cells, nanoparticle development was pursued to optimize surface characteristics and to allow targeting to specific organs or tissues¹. In order to increase the exposure time and to enhance the uptake of nanoparticles by specific cell types, the surface of nanoparticles was modified, for example by adding polyethylene glycol (PEG)²⁷. The aim of this particular step was to mask the ligands for various cell surface receptors that would rapidly clear nanoparticles from the blood circulation and potentially decrease the biological half-life of the nanoparticles. When nanoparticles remain in the circulation for prolonged time periods, there is increased probability that the nanoparticles will be internalized and accumulate in therapeutic targets, such as cancer cells²⁸. In this context, several features of polyethylene glycol influence the effectiveness of the nanoparticle protective layer including polymer length²⁹, the mechanism of attachment to the surface of nanoparticles³⁰ and the density of polyethylene glycol on the nanoparticle surface³¹.

The targeting of nanoparticles to specific tumor tissues and their accumulation at these lesions largely depend on the enhanced permeability of blood vessels that perfuse tumors and the increased retention of particles that is exhibited by the tumor vasculature³². Newly-formed tumor vessels often exhibit poorly organized and dysfunctional endothelium with wide fenestrations, features that enhance the retention of nanoparticles circulating in the bloodstream compared with healthy tissues³³. For optimizing nanoparticle targeting in tumor therapy, nanoparticles are modified by coating with ligands that enable recognition by endothelial cells, which facilitates

extravasation into the intra-tumor space. The limitation with this approach is that there is also increased clearance of nanoparticles by cells of the mononuclear phagocytic system in spleen and liver, which recognize ligands on the particle surface that are no longer protected by polyethylene glycol. As a result of this modification, specific targeting of nanoparticles does not consistently lead to increased accumulation in tumor tissues³⁴. Another concern with specific receptor targeting by second generation nanoparticles is their limited penetration through the tumor, which may be caused by nanoparticle retention in the outer layer of the tumor by cell surface receptors that bind to the surface ligand.

c) Third Generation

The most recent generation of nanoparticles was designed specifically to overcome the problems described above. Briefly, modifications of the surface properties of nanoparticles are created so that specific properties become manifest only in the local tissue environment of interest. Based on *a priori* knowledge of local environmental conditions, such as reduction of tissue pH, removal of the protective polyethylene glycol layer exposes the positively charged surface of nanoparticles, which then promotes non-specific internalization by cells³⁵. Variations of tissue pH in discrete microenvironments can also trigger loss of molecules from the nanoparticle surface or can promote degradation of the nanoparticle itself, with subsequent release of active molecules located within the nanoparticle³⁶. This design strategy is particularly effective in chemotherapy since many tumors are characterized by a relatively more acidic pH than normal tissues. Other environmental factors that are used for activation of nanoparticles include oxygen tension, redox levels and enzymatic activity, which are specific to the pathological condition of interest. External triggers can also be used to activate nano-carriers and release bioactive molecules including radiation, electromagnetic fields or ultrasound³⁷⁻³⁹.

II. Intracellular Location and Translocation of Nanoparticles

A. Endocytosis

For normal growth and metabolic function, both prokaryotic and eukaryotic cells must be able to internalize extracellular materials. For eukaryotic cells, endocytosis is a highly regulated process that allows cells to uptake solutes and particles from the extracellular space through interactions with proteins embedded in the plasma membrane. As a result of binding to cell surface receptors, a cascade of signal transduction pathways is often initiated that is critical for a variety of biological pathways and processes inside the cell. For example, antigen processing, antigen presentation by cells of the immune system, receptor down-regulation, cholesterol trafficking, mitosis and apoptosis all require endocytosis⁴⁰.

In general, endocytosis can be divided into two main categories based on the size of the internalized material⁴⁰. Phagocytosis (or cell eating) refers to the internalization of large particles (>200 nm). Pinocytosis (literally, cell drinking) refers to non-phagocytic uptake of fluids, solutes and very small particles. Endocytosis can occur through four discrete mechanisms: clathrin-dependent endocytosis, caveolin-mediated endocytosis, macropinocytosis, and dynamin- and clathrin-independent endocytosis⁴¹.

B. Internalization by phagocytosis and pinocytosis

1. Phagocytosis

Phagocytosis is an actin-based internalization mechanism that unlike pinocytosis occurs primarily in specialized cells known as professional phagocytes, which include macrophages and neutrophils⁴². Other cell types including epithelial cells and fibroblasts also exhibit phagocytic activity but at slower rates of particle internalization than professional phagocytes⁴³. In general,

phagocytosis involves internalization of much larger particles than pinocytosis, although the size of particles internalized for each process, within limits, may vary considerably. For example, polystyrene particles with diameters of ~250 nm to 3 μ m are more rapidly internalized than particles of <250 nm in diameter ⁴⁴.

For phagocytosis by macrophages or neutrophils, the particles are first opsonized by serum proteins, which enable detection and engulfment. The major opsonins of serum include immunoglobulins, complement factors, the cell attachment protein fibronectin and the clotting factor, fibrinogen ^{27, 45}. The surface characteristics of the nanoparticle strongly influence which proteins will be absorbed, largely as a result of ionic or hydrophobic interactions. As increased surface hydrophobicity is a key determinant of opsonization, nanoparticles with hydrophilic surfaces typically absorb less protein ⁴⁶. Opsonized nanoparticles can interact with specific receptors on the surface of phagocytes, such as Fc receptors, non-complement receptor integrins (e.g. $\alpha 5\beta 1$ and $\alpha v\beta 3$, which mediate uptake of particles coated with fibronectin ⁴⁷), lectins such as the mannose receptor ⁴⁸, the lipopolysaccharide receptor CD14 ⁴⁹ and the diverse scavenger receptor group ⁵⁰.

Receptor ligation by ligands activates signaling cascades that include Rho-family guanosine 5-triphosphatases (GTPases) ⁵¹, which promote actin assembly and the formation of cell membrane extensions around the nanoparticle, which is then engulfed. The resulting nanoparticle-containing phagosome traffics through the cytoplasm, undergoing maturation as a result of a specific sequence of fusion events with other vacuolar compartments. As a result of phagosome maturation and fusion with late endosomes, the contents of the phagosome undergo marked changes in protein content. Ultimately, lysosomes fuse with phagosomes to form phagolysosomes ⁵². These organelles become acidified as a result of the activity of vacuolar proton pumps, which are adenosine 5-triphosphatase (ATPases) located in the vacuolar

membrane. Phagolysosomes also acquire various enzymes, including esterases and cathepsins, which enable efficient protein digestion in the vacuolar compartment⁵³.

2. Pinocytosis

Pinocytosis is a process used primarily by cells for the absorption of extracellular fluids but small particles are also internalized by pinocytosis. As indicated above, the size of particles taken up by pinocytosis is much smaller than in phagocytosis. Cells can form vesicles by wrapping plasma membrane around small particles, which are then internalized. Subsequently these vesicles fuse with lysosomes, where digestion of internalized material occurs. An alternative pathway involves recycling of a fraction of the endocytic vesicles back to the plasma membrane for exocytosis.

Unlike phagocytosis and receptor-mediated endocytosis, pinocytosis is non-specific as the majority of substances are internalized by non-specific interactions with the plasma membrane. Another difference between pinocytosis and phagocytosis is that in pinocytosis, cells internalize materials that are already dissolved or digested whereas in phagocytosis, cells engulf large particles that must be processed and degraded by digestive enzymes prior to redistribution throughout the cell.

a) Receptor-mediated endocytosis

Receptor-mediated endocytosis is a specific endocytic pathway that allows cells to internalize various ligands including hormones, growth factors, enzymes and plasma proteins. Receptor-mediated endocytosis exhibits saturation kinetics of ligand uptake when the number of receptors on the cell surface is limiting⁵⁴. A classical example of receptor-mediated endocytosis is the uptake of low density lipoproteins (LDL), the major class of cholesterol-carrying lipoproteins in human plasma. LDL regulates the activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase

activity and cholesterol synthesis ⁵⁵. Through the use of LDL coupled to electron-dense ferritin, the clustering of LDL receptors in clathrin-coated pits can be observed by electron microscopy, which shows that LDL is internalized by LDL-receptor specific endocytosis ⁵⁶. Studies of the LDL receptor also highlight another important characteristic of receptor-mediated endocytosis, specifically the recycling of receptors ⁵⁷. After internalization, vesicular contents are exposed to the decreased pH environment of endosomes, which results in receptor dissociation. Once free from ligand, receptors are recycled back to the cell surface, thereby providing an efficient mechanism for delivery of cholesterol to cells ⁵⁸. The endocytic pathway is also important in regulating signal transduction and a large number of biochemical processes. For example, cells stimulated with epidermal growth factor (EGF) can form clusters of EGF-receptor complexes in clathrin-coated pits ⁵⁹. Similar, ligand-induced, clathrin-mediated endocytosis of G-protein coupled receptors has also been reported ⁶⁰. The endocytic pathway can regulate the relative abundance of G-protein coupled receptors on the cell membrane by cycling receptors to lysosomes where they are degraded ⁶¹. This process can dampen cell signalling processes after prolonged or repeated stimulation by free ligand.

b) Trafficking and fate of internalized proteins

Endosomes are a complex system of membrane-enclosed tubular vacuoles that direct the intracellular distribution of internalized material ⁶². In endocytosis, endosomes undergo stages of maturation, ranging from early endosomes to mature late endosomes. Endosomal maturation is characterized by a decrease in luminal pH, movement to the perinuclear space and the formation of intraluminal vesicles by budding off from the membrane of late endosome ⁶³. The composition of endosomal membranes changes in its protein and lipid composition at different stages of this transition, although it is difficult to identify unique markers of each stage ⁶⁴.

The internalized extracellular material associated with receptors can be transferred from endosomes through several distinct pathways. The first pathway is characterized by the situation when material and receptors are not degraded but instead recycled back to the plasma membrane⁶⁵. This recycling can occur directly from early endosomes or in a separate endosomal vesicle termed a recycling endosome, which traverses a nominal “long recycling loop”⁶⁶. The second pathway involves the situation in which internalized material is retained in a maturing endosome until it is delivered to the lysosome for degradation⁶⁷.

Another important pathway in endocytosis is the system in which cargo is delivered to the Golgi complex for re-packaging and re-distribution throughout the cell. This retrograde transport pathway can direct material from the recycling endosomes, the early endosomes, or the late endosomes to the trans-Golgi complex⁶⁸. Each pathway is characterized by distinct sets of proteins and is used to transport many different types of molecules, such as sorting receptors (e.g. mannose 6-phosphate receptors), integral membrane proteases (e.g. β -secretase and furin) and nutrient and ion transporters^{69,70}. Proteins and other molecules in the Golgi complex can be then distributed to other intracellular and extracellular destinations such as the extracellular space, the plasma membrane, secretory vesicles or other endosomes⁷⁰.

III. Cellular Interactions and Uptake of Nanoscale Matter

A. Interactions between Cells and Nanoparticles

The plasma membrane forms the first barrier with which nanomaterials must interact in order to be internalized. A phospholipid bilayer comprises the primary structural element of the plasma membrane, which exhibits specific physical characteristics that maintain and delineate the functional and structural integrity of the intracellular environment from the extracellular environment. The plasma membrane plays fundamental roles in cell physiology by contributing

to the modulation of signaling and protein function that are critical for cellular interactions with the extracellular environment.

Nanomaterials can influence cell membrane structure as a result of modifying surface charge ⁷¹. When negatively-charged nanoparticles interact with the plasma membrane they can induce gelation of the membrane; in contrast, positively charged nanoparticles can convert stable micro-domains into a more fluid state, facilitating penetration by the particles. As a result, cationic particles have a tendency to be more cytotoxic than anionic or neutrally-charged particles, an effect which is independent of size ⁷². Further, polymeric nanomaterials can directly cause the formation of “holes” in the cell membrane, which correspond to areas of reduced lipid or protein content ⁷³. The resulting permeabilization of cell membranes is associated with structural changes that can enable leakage of cytosolic proteins and contribute to cell death. In comparison to particles with neutral charge, charged particles exhibit preferential uptake, which occurs primarily at sites of high charge density on the cell surface that are able to efficiently mediate endocytosis of positively-charged particles ⁷⁴. Negatively-charged particles can utilize surface receptors for entry into cells that arise through non-specific electrostatic interactions ⁷⁵.

In addition to interactions with phospholipid bilayers, nanomaterials can interact with many proteins embedded in the plasma membrane, some of which are responsible for molecular transport across the plasma membrane and signal transduction. In this context, nanoparticles can readily interact and block ion channels that mediate ion transport. For example, potassium ion channels can be physically blocked by spherical fullerenes (diameter= 0.72 nm) and by carbon nanotubes (diameter= 1-15 nm) ⁷⁶. The structure and function of ion channels can be also be perturbed by semiconductor nanomaterials that do not physically block ion channels but instead induce oxidative damage ⁷⁷.

B. Pathways for Cellular Uptake of Nanoparticles

The outcome of interactions between cells and nanoparticles largely depends on the physical and chemical characteristics of the particle as well as the physiological status of the cell. The major pathway for uptake of nanoparticles is by endocytosis. As reviewed above, unlike the phagocytic behavior that is exhibited by specialized phagocytic cells, endocytosis in most cell types is mediated by one of four main mechanisms: clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis or clathrin and caveolae-independent endocytic mechanisms.

1. Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis is an important pathway for maintenance of normal physiological cell function, including the uptake of nutrients and for signal transduction. Depending on whether specific receptors are used for internalization, endocytosis is divided into receptor-mediated or receptor-independent pathways. Receptor-mediated, clathrin-dependent endocytosis is a common pathway for the cellular uptake of many ligand-receptor complexes. Examples of such ligands include low density lipoprotein, transferrin and epidermal growth factor⁷⁸. When receptors on the cell membrane are ligated in regions rich in polymerized clathrin, a polygonal lattice structure is formed that promotes invagination of the plasma membrane into a clathrin-coated pit⁷⁹. As this process continues, the GTPase dynamin is recruited to the neck of the pit to initiate membrane fission and formation of clathrin-coated vesicles. Clathrin proteins are later removed from the vesicles and re-cycled back to the plasma membrane⁸⁰. The vesicles fuse with early endosomes, which are acidified by ATP-dependent proton pumps; these endosomes later mature into more acidic, late endosomes. The final step involves fusion with pre-lysosomal vesicles and further degradation of internalized material by acid hydrolases.

Receptor-independent clathrin endocytosis follows similar mechanisms with the important difference that internalized particles do not interact with specific receptors on the plasma membrane; instead, non-specifically charged residues and hydrophobic interactions initiate the binding process. As a result of non-specific interactions, materials that are recognized by this pathway are internalized at a slower rate than receptor-mediated endocytosis ⁸¹.

2. Macropinocytosis

Nanoparticles (diameter < 0.1 μm) in the extracellular environment can be internalized non-specifically by the macropinocytosis pathway. When nanoparticles contact the plasma membrane, activation of discrete signaling pathways promotes the formation of actin-mediated membrane protrusions that collapse and fuse with the plasma membrane ⁸⁰, a process that is functionally separate from phagocytosis. This process in turn produces uncoated, irregularly-shaped macropinosomes (average diameter > 1 μm). Depending on the cell type, the intracellular fate of these vesicles varies widely, but in the majority of cases, vesicles acidify and condense as they traverse the endosomal/lysosomal pathway ⁸². Macropinocytosis contributes to the non-specific internalization of larger size nanoparticles, which often is concurrent with other entry mechanisms ⁸³. Nanoparticle uptake by the macropinocytosis pathway is efficient and is employed for pharmaceutical delivery of drugs ⁸⁴.

3. Caveolin-Mediated Endocytosis

Caveolin-mediated endocytosis is the most prominent alternative to clathrin-dependent uptake. The caveolin-mediated endocytic pathway is prominent in basolateral endothelial cells and has also been assessed in smooth muscle cells and fibroblasts ⁸⁵. Caveolae are small, flask-shaped membrane invaginations with a diameter of 50–80 nm ⁸² that are coated with caveolin, a dimeric protein. Invagination is facilitated by dynamin at hydrophobic domains of the plasma membrane

that are enriched with cholesterol and glycosphingolipids⁸⁶. Caveolae are involved in endocytosis of various ligands including folic acid, albumin and cholesterol⁷⁸ but they also provide an entry point for viruses such as SV40 virus⁸⁷ and bacterial toxins such as cholera toxin subunit B, or Shiga toxin⁸⁸. Particle size significantly influences the efficiency of transport by the caveolin-mediated pathway as small particles are transported more efficiently than large particles (small nanoparticles of 20-40 nm in diameter are internalized 5–10 times more quickly than larger nanoparticles of 100 nm diameter)⁸⁹. Larger particles (>500 nm diameter) are very rarely internalized by caveolin-mediated endocytosis⁸³.

The internalization of exogenous material by caveolin-mediated endocytosis is much slower than clathrin-mediated endocytosis⁷⁸. Currently, the fate of internalized material through the caveolin-mediated pathway and its associated intracellular trafficking routes are not well-defined. While non-acidic, non-digestive degradation pathways are prominent and well-established, there may also be connections with lysosomal degradation pathways^{88,90}. As a result of entry into non-degradative pathways, pathogens internalized by caveolin-dependent endocytosis may remain viable and are then transported to the Golgi and endoplasmic reticulum⁹¹. Caveolin-mediated endocytosis can be advantageous for delivery of therapeutics such as peptides or nucleic acids that are sensitive to digestive enzymes in the lysosomal degradation pathway.

4. Endocytosis-Independent Pathways

There is some evidence to suggest that endocytosis-independent pathways can be used by nanoparticles to gain entry into cells. Hong and co-workers showed that positively-charged dendrimeric nanoparticles can actively destabilize supported lipid bilayers and could either form holes (15–40 nm in diameter) or expand holes at pre-existing sites. The authors observed that

these holes led to dendrimer internalization into cells, diffusion of dye molecules into the cells as well as leakage of cytosolic proteins out of cells ⁹². The ability of cationic peptides to disrupt the structure and integrity of membranes has been confirmed in other studies ⁹³⁻⁹⁵.

C. Size- and Shape-Dependent Cellular Uptake of Nanoparticles

1. Size

Nanoparticle size strongly affects the nature of the endocytic pathway and the kinetics of particle internalization. Theoretically, interactions between nanoparticles and receptors are optimal when nanoparticles are 30-50 nm in diameter, and when the concentration of ligand on the nanoparticle surface and receptor abundance on the cell surface are not limiting factors ⁹⁶. However, because different cell types can express varying levels of the target receptor and can utilize different internalization pathways, the optimal size of nanoparticles may depend on the cell type being assayed ¹. For example, gastrointestinal epithelial cells preferentially uptake 100 nm diameter polylactic polyglycolic acid-based particles compared with 500 nm–10 μ m particles, as measured by particle numbers and total mass ^{97, 98}. Similar trends of uptake were found for conjunctival epithelial cells internalizing polylactic polyglycolic particles *in vivo* ⁹⁹ and for poly(ϵ -caprolactone) particles ¹⁰⁰. In murine melanoma B16 cells, the endocytosis pathway used for nanoparticle internalization is size-dependent: nanoparticles < 200 nm are internalized by clathrin-mediated endocytosis while particles >500 nm are internalized by the caveolin-mediated pathway ⁸³. In a separate study of HeLa cells, nanoparticles of 40 nm in diameter were internalized by clathrin-mediated endocytosis while smaller particles (< 25 nm) were internalized by a novel, non-clathrin and non-caveolae-mediated pathway, which was also cholesterol-independent ¹⁰¹. As the macropinocytosis pathway involves uptake of particles with a wide range

of sizes and occurs in complement-dependent and other endocytic pathways, it is difficult to estimate the kinetics and type of particle uptake by macropinocytosis ¹⁰².

2. Shape

Nanoparticle shape can strongly influence cellular uptake. Rod-shaped particles exhibit the fastest uptake followed in order by spheres, cylinders and cubes for nanoparticles >100 nm ⁸⁹. In contrast, for nanoparticles <100 nm, spheres exhibit faster uptake rates than rods ^{40, 103}. Multiple endocytic pathways may be involved simultaneously in these processes, which could explain variations in outcomes from different experimental approaches.

Only a few studies have investigated non-spherical nanoparticles: interactions of cells with these types of particles may be complex since the particle can be presented to the cell in at least two different orientations. Compared with the short side of the particle, the longer side is able to interact with more cell surface receptors ¹⁰⁴. For other shapes of nanoparticles such as “spiky” gold “nano-urchins”, interactions depend on whether the ligand is located on or between the spikes ¹⁰⁵. The increased complexity of interactions observed with complex nanoparticle shapes may enable the use of asymmetrical nanoparticles in biological applications to improve control and versatility of drug delivery.

Particle shape also affects subsequent trafficking. For example, hexagonal or rod-shaped nanoparticles, although internalized through the same endocytic pathway, behaved differently thereafter: hexagonal particles remained in the cytoplasm whereas rod-shaped particles were directed to the nucleus, probably through a microtubule-dependent active transport mechanism ¹⁰⁶. The use of hexagonal or rod-shaped nanoparticles could be exploited for improved control of gene delivery.

D. Surface-Charge-Dependent Cellular Uptake of Nanoparticles

When comparing the effect of nanoparticle charge on uptake by non-phagocytic cells, several studies have shown that charged polystyrene and iron oxide particles are taken up more efficiently than uncharged particles ¹⁰⁷. Positively charged particles generally display higher rates of cell association and internalization than negatively charged particles, possibly due to the negatively charge on the plasma membrane ¹⁰⁸. The influence of surface charge on polystyrene particles or quantum dots on cell uptake is not well-defined. Carboxylate-modified polystyrene particles (1 μm and 50 nm in diameter) were internalized faster by alveolar type I cells than uncharged particles ¹⁰⁹ whereas cationic polystyrene nanoparticles were taken up more rapidly by MDCK cells than uncharged particles ¹¹⁰. There are also conflicting data on the internalization of quantum dots: some studies showed preferential uptake of anionic quantum dots ¹¹¹ while other studies showed faster uptake with positively charged dots ¹¹². Collectively, the data indicate that the uptake of nanoparticles coated with charged polymers largely depends on the charge of the particle. For example, polylactic acid (PLA)-PEG nanoparticles coated with the cationic lipid stearylamine were internalized by clathrin-mediated endocytosis and showed faster uptake by HeLa cells compared with negatively-charged PLA-PEG nanoparticles that were internalized by a different endocytic pathway ¹⁰².

E. Impact of Surface Ligand Coating on Nanoparticle Uptake

Nanoparticles can be modified by surface coating with targeting ligands (i.e. molecules that can be recognized by specific receptors expressed by cells). This method can be successfully employed to deliver a drug to a specific cell population or to control the intracellular trafficking routes of the nanoparticles ¹¹³. One aim of this approach is to direct the nanoparticle coated with specific ligands to internalization through the same pathway as the ligand alone. One possible

advantage of this approach is that the density of ligands on the nanoparticle surface may enable stronger interactions with the cell by a process known as avidity, or molecular clustering of surface adhesion receptors ¹¹³. For example, when folic acid is used to coat nanoparticles for anti-cancer therapy ¹¹⁴ the folic acid binds with high affinity to glycosylphosphatidylinositol-linked folate receptors, which are often over-expressed on the surface of cancer cells compared with normal cells ¹¹⁵. Once the folate receptor is activated, it can facilitate internalization of folic acid by receptor-mediated endocytosis. The folic acid escapes from endosomes into the cytosol ¹¹⁶ and does not undergo lysosomal degradation. Folic acid has been successfully coated onto PEG-ylated polymeric nanoparticles by conjugation of activated N-hydroxysuccinimide folic acid with the aminated methoxy-polyethyleneglycol cyanoacrylate-co-n-hexadecyl cyanoacrylate ¹¹⁷. Surface plasmon surface resonance showed that the folic acid bound to these particles has a 10-fold higher affinity for folate receptor compared with free folic acid. In a different study, liposomes were coated with folic acid by incorporating phospholipid-anchored folic acid ¹¹⁸. These liposomes exhibited enhanced uptake by folate receptor-expressing cells. Taken together, these experiments demonstrate the versatility of nanocarriers to enable targeting of folic acid as a treatment for cancer.

IV. Cytotoxicity of Nanoparticles

Recent advances in the engineering and design of nanoparticles combined with their use in biomedical applications have led to the concern about potentially toxic and hazardous effects on cell function and viability, and human health in general. As discussed above, nanoparticles are readily internalized through various endocytic pathways where they then translocate into different organelles and cell compartments. During these processes nanoparticles interact with various structural components of the cell including proteins, lipids, or nucleic acids and potentially

damage these molecules. One major concern of the use of nanoparticles is the increased production of reactive oxygen species by cells ⁹. High levels of reactive oxygen species can interfere with a wide variety of physiological processes including signal transduction and gene expression, or by direct damage to cells by peroxidation of lipids, which induces inflammation, denatures proteins and damages deoxyribonucleic acid (DNA) structure.

A. Cytotoxicity of Noble Metal Nanomaterials

1. Gold Nanoparticles

Several studies have shown that gold nanoparticles are biocompatible and exhibit relatively low cellular toxicity ^{119, 120}. Gold nanoparticles (60 nm diameter) internalized by murine macrophages were assessed by 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide and lactate dehydrogenase toxicity assays and these data showed low cytotoxicity. Similarly, gold nanoparticles were not found to be pro-inflammatory, as measured by low levels of cytokines and pro-inflammatory mediators in the medium. It should be noted that the toxicity of gold nanoparticles can be influenced by surface molecules that are used to coat the nanoparticles ¹²⁰. Studies of the cytotoxicity of gold nanoparticles with different surface modifications (including cetyltrimethylammonium bromide, polystyrene sulfonate and poly(diallyldimethylammonium chloride) showed that cetyltrimethylammonium bromide -coated particles were much more toxic than the polystyrene sulfonate or poly(diallyldimethylammonium chloride)-coated particles. The proposed mechanism of cell toxicity is that cetyltrimethylammonium bromide can damage the integrity of endosomes and mitochondria, which leads to increased production of reactive oxygen species and cell death ¹²¹. Several recent studies showed that although spherical gold nanoparticles inherently are not toxic, gold nano-rods exhibit very high toxicity. These data are

possibly a reflection of the presence of toxic surfactants that coat the particles and which are used for nanoparticle synthesis through the seed-mediated, surfactant-assisted growth method^{122, 123}.

2. Silver Nanoparticles

Silver nanoparticles have been introduced commercially, primarily as a result of their broad spectrum of antimicrobial activity¹²⁴. Silver-impregnated catheters¹²⁵ and wound dressings¹²⁶ are now under development for therapeutic purposes. However, there are concerns about their biocompatibility and potentially adverse effects on human health. Several studies have indicated that silver nanoparticles can induce changes in normal cell morphology, reduce viability, and disrupt metabolic activity through oxidative stress, which results in cell cycle arrest, inflammatory reactions and DNA damage^{127, 128}. Several mechanisms have been proposed to explain the toxicity of silver nanoparticles. The antibacterial properties of silver and its toxic effects on human cells may be related to the release of silver ions, which bind and modify thiol groups in proteins in the respiratory chain of enzymes, thus causing the collapse of proton motive force and blockade of oxidative phosphorylation¹²⁹. Another mechanism of toxicity may be related to the deposition of silver nanoparticles in mitochondria, which alters their normal function by disrupting electron transport. As a result, highly damaging reactive oxygen species are produced and released inside the cell, which leads to oxidative damage to proteins and DNA (127). Notably, the toxicity of silver nanoparticles cannot be explained solely by the presence of free silver ions as their concentration is very low, suggesting that nanoparticles and silver ions collectively contribute to the overall toxicity^{128, 130}.

B. Cytotoxicity of Carbon-Containing Nanoparticles

Individual fullerene molecules (diameter = 0.7 nm) can enter cells by passive diffusion through plasma membrane pores. As a result, the toxicity of fullerenes is of particular concern and biological importance⁹. Fullerenes can reduce the growth and viability of several cell lines even at small doses when exposed for longer time periods¹³¹. In several cell lines, cytotoxicity after 48 hours exposure was related to lipid peroxidation and the resultant damage to the plasma membrane¹³². Fiber-shaped nanoparticles are generally more reactive and toxic than are spherical particles. For example, carbon nanotubes are even more toxic than fullerenes¹³³. Significant cytotoxicity involving oxidative stress and induction of apoptosis has been observed with carbon nanotubes and graphene¹³⁴.

V. Comparisons of Nanoparticle and Microparticle-Loading of Proteins into Cells

A. Cell Receptor Utilization

Due to their larger size, microparticles (>1 μm diameter) are preferentially internalized by the phagocytic pathway of endocytosis. As described above, before particles interact with the plasma membrane, particles first undergo opsonization. In this process, the microparticle surface is coated with opsonins, which enable interactions with receptors on phagocytic cells. Opsonization rapidly occurs when microparticles are injected into the circulation of a living organism. Opsonins are avidly adsorbed on hydrophobic, highly charged microparticles, a process that depends on the specificity of the molecular interactions¹³⁵. Major opsonins that are important for phagocytosis include soluble components of the immune system such as immunoglobulins and components of the complement system. Other proteins such as laminin, fibronectin, C-reactive

protein and type I collagen are also phagocytosis by non-professional phagocytic cells ²⁷. The corresponding receptors that are involved in recognition of these proteins include Fc receptors and non-complement-receptor integrins, such as $\alpha 5\beta 1$ and $\alpha v\beta 3$. These proteins mediate the uptake of particles coated with fibronectin ⁴⁷, lectins, such as the mannose receptor ⁴⁸ and the lipopolysaccharide receptor, CD14 ⁴⁹.

In contrast to microparticles, nanoparticles are mainly internalized by discrete pinocytic pathways, which include non-receptor mediated clathrin-dependent endocytosis and micropinocytosis, a pathway which relies on non-specific charge and hydrophobic interactions with the cell membrane but without the involvement of specific receptors. Conversely, the uptake of low density lipoprotein, transferrin and epidermal growth factor occurs via specific interactions with membrane-bound receptors, which is followed by internalization by clathrin-mediated endocytosis ⁷⁸. As a result of its efficiency and specificity, clathrin-mediated endocytosis is important for drug-loading into cells using nanocarriers; in this instance, the nanoparticles are coated with targeting ligands on their surfaces.

B. Cell Compartments Containing Internalized Micro and Nanoparticles

After internalization by different pathways of endocytosis, both microparticles and nanoparticles are thought to enter into endosomal tubular-vacuolar systems within the cell. In these pathways, endosomes undergo stages of transition and maturation from early endosomes to mature late endosomes, which then fuse with lysosomes for degradation of their contents. During this process some of the contents can be transferred back through the loop mechanism to the plasma membrane and into the extracellular space or delivered to the Golgi complex for processing and re-distribution in other cell compartments. Due to their large size, microparticles exhibit slow rates of trafficking inside the cell and tend to remain within vesicular compartments while in

contrast, smaller size particles are more efficiently transported to lysosomal compartments ¹³⁶.

The dynamics of intracellular trafficking depends on the dimensions of the intracellular vesicular machinery within each particular cell type: professional phagocytic cells exhibit faster delivery of larger size microparticles than is observed in non-professional phagocytic cells ¹³⁷.

Intracellular trafficking of nanoparticles utilizes pathways that depend on the shape of the particle. Rod-shaped nanoparticles are delivered to the nucleus, while hexagonal, sheet-like nanoparticles remain in the cytoplasm. Nuclear translocation of rod-shaped nanoparticles is mediated by the microtubule network inside the cytoplasm. Currently, the reasons for the involvement of preferential transport systems for particular shapes of nanoparticles are not well-defined ¹⁰⁶.

C. Impact of Nanoparticle Loading on Cell Function and Differentiation

Several parameters of nanoparticles including size, shape, composition, surface charge, and surface hydrophobicity affect cytotoxicity. The effect of nanoparticle size on cytotoxicity depends on the type of cell that is incubated with the nanoparticles. For non-specialized phagocytic cells, small nanoparticles are associated with increased cytotoxicity compared with larger nanoparticles. Indeed, several in vitro studies showed higher cytotoxicity for well-dispersed small mesoporous silica, dolomite, and polystyrene nanoparticles compared with larger microparticles ^{138, 139}. In contrast, compared with nanoparticles, microparticles are more cytotoxic to phagocytic cells such as macrophages and monocytes. More cell damage was observed with silica microparticles versus nanoparticles in T lymphocytes ¹⁴⁰. Further, there was less cytotoxicity in response to 30–70 nm silica nanoparticles compared with 1000 nm particles in macrophage-like THP-1 cells ¹⁴¹.

D. Potential Therapeutic Applications

The application of microparticles and nanoparticles as drug delivery systems to more effectively transport active molecules to target sites has attracted considerable interest in the last decade. The use of nanoparticle delivery can be valuable for therapeutic purposes since it allows modification of the properties of the delivered drug, such as enhancing the solubility or improving the biological distribution. Concurrently, it is possible to achieve more efficient delivery of the drug through improved control of drug release. By establishing continuous release instead of a burst, as well as specific targeting of diseased tissues ¹⁴², microparticles (0.8 μm diameter) were used to deliver a synthetic peptide carrying the major T-cell epitope of Ole e 1, the main allergen of olive pollen. The particles were delivered intranasally to prevent mice from developing allergic sensitivity to the whole protein ¹⁴³. An initial “burst” release of peptide was followed by a slow and sustained release over several weeks. Three consecutive administrations of peptide containing microparticles were sufficient to prevent subsequent sensitization to the whole Ole e 1 allergen. Substantial reductions of IgE and IgG1 antibodies levels were observed in animals treated with this approach ¹⁴³. In another study, microparticles were used to deliver sustained release of the osteogenic growth factor, bone morphogenetic protein-2, which is particularly useful for facilitating bone healing ¹⁴⁴. Further, more effective oral insulin delivery using insulin-loaded microparticles can significantly reduce initially high blood glucose levels in diabetic rabbits ¹⁴⁵.

Due to their small size, nanoparticles can be directed to enter cells by different pathways, which may be advantageous in many clinical situations. For example, the blood–brain barrier, which is comprised of tight junctions between brain microvessel endothelial cells, is a major obstacle for the delivery of drugs that target the central nervous system. In a recent study, neurotoxin-I encapsulated in PLA nanoparticles after intranasal administration in rats was more

effectively delivered across the blood-brain barrier than controls, and this resulted in higher levels of this analgesic peptide in the brain ¹⁴⁶. Similar improvements in delivery and prevention of seizure were observed when nanoparticles loaded with thyrotropin-releasing hormone (an anticonvulsant) were administered intranasally in rats ¹⁴⁷.

E. Applications in Dentistry

1. Periodontal Tissues

There is a considerable interest in application of localized drug delivery systems for the treatment of periodontal diseases. These systems can establish an effective concentration of a therapeutic agent (such as an antibiotic) in the periodontal pocket with minimal systemic side-effects ¹⁴⁸.

Chitosan microspheres containing tetracycline have been developed for these applications; in vitro studies showed sustained tetracycline release and enhanced antimicrobial activity ¹⁴⁹.

Microparticle carriers have been also investigated for their potential use in periodontal regenerative procedures. In a recent study the impact of hydroxyapatite-containing chitosan microspheres on the differentiation of human periodontal ligament fibroblasts were examined in three dimensional cultures. When encapsulated in microspheres, cells differentiated into osteogenic cells more efficiently in the osteoinductive medium than cells that were not encapsulated ¹⁵⁰. In another study, chitosan nanoparticles were incorporated into a porous collagen composite scaffold. These scaffolds were used to deliver expression systems that encoded platelet derived growth factor. In response to this growth factor, the attachment, spreading and growth of periodontal ligament fibroblasts within the pores of the composite scaffold were enhanced ¹⁵¹.

2. Endodontics

Nanoparticles can be used to modify the properties of various obturation materials used in endodontic treatment. Mineral trioxide aggregate modified with silver nanoparticles can improve the efficacy of antimicrobials ¹⁵². Similarly, incorporation of nanoparticles enhances the long-term antibacterial activity of commonly used endodontic sealers ¹⁵³. Various types of nanoparticles are used as a critical component of antimicrobial photodynamic therapy. After activation by light, photoactive drugs encapsulated in nanoparticles generate singlet oxygen species and free radicals that may be a promising adjunct in antimicrobial endodontic treatment ^{154, 155}. Despite these advances, it is currently not clear how the physical properties of nanoparticles or their coatings can be optimized to enhance treatment outcomes.

Statement of the Problem

There is growing interest in the study of interactions between nanoparticles and biological systems. Nanoparticles are used as delivery agents for a wide variety of biologically active molecules that in turn can potentially modify intracellular biochemical processes. However, in the context of dental and periodontal tissues where there are often high levels of inflammation and microbial contamination, the mechanism of uptake of these nanoparticles is not well-defined and the potential toxicity of nanoparticles is a therapeutic concern. Various physical and chemical properties of nanoparticles such as size, shape, surface charge, morphology and surface functionalization influence how nanomaterials interact with cells and ultimately determine their fate. These parameters need to be defined to optimize therapeutic loading of target molecules and to achieve the best clinical outcomes.

Based on the literature reviewed above, my general hypothesis is that in fibroblasts, particle size influences the uptake and internalization of particles coated with the connective tissue matrix protein fibronectin and as a result determines the processing of internalized protein. We consider here that differences in the amount of fibronectin that are available for interaction between the particle and cell surface fibronectin receptors can determine the mechanism of internalization and ultimately the fate of internalized material (depicted pictorially in the Figure 8 in the Figures section).

Specific Aims

- 1) To quantify the binding of fibronectin to polystyrene nanoparticles and microparticles.
- 2) To compare uptake of fibronectin-coated nanoparticles and microparticles by fibroblasts.
- 3) To study the internalization mechanisms of these particles.
- 4) To evaluate intracellular processing of internalized nanoparticles and microparticles
- 5) To assess the viability of cultured cells after incubation with microparticles or nanoparticles.

The following material is derived from a submitted manuscript:

Title: Particle Size Influences Fibronectin Internalization and Degradation by Fibroblasts.

Authors: Peter Bozavikov, Dhaarmini Rajshankar, Wilson Lee, Christopher A. McCulloch.

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Abstract

The application of nanotechnology for drug targeting underlines the importance of controlling the kinetics and cellular sites of delivery for optimal therapeutic outcomes. Here we examined the effect of particle size on internalization and degradation of surface-bound fibronectin by fibroblasts using polystyrene nanoparticles (NPs; 51 nm) and microparticles (MPs; 1 μ m). Fibronectin was strongly bound by NPs and MPs as assessed by immuno-dot blot analysis ($5.1 \pm 0.4 \times 10^{-5}$ pg fibronectin per μm^2 of NP surface; $4.2 \pm 0.3 \times 10^{-5}$ pg fibronectin per μm^2 of MP surface; $p > 0.2$). We estimated that ~193 fibronectin molecules bound to a MP compared with 0.6 fibronectin molecules per NP, indicating that ~40% of nanoparticles were not bound by fibronectin. One hour after incubation, fibronectin-coated NPs and MPs were rapidly internalized by Rat-2 fibroblasts. MPs and NPs were engulfed partly by receptor-mediated endocytosis as indicated by decreased uptake when incubated at 4°C, or by depletion of ATP with sodium azide. Pulse-chase experiments showed minimal exocytosis of NPs and MPs. Internalization of NPs and MPs was inhibited by jasplakinolide, whereas internalization of MPs but not NPs, was inhibited by latrunculin B and by integrin-blocking antibodies. Extraction of plasma membrane cholesterol with methyl β -cyclodextrin inhibited internalization of fibronectin-coated NPs but not MPs. Biotinylated fibronectin internalized by cells was extensively degraded on MPs but not NPs. Particle size affects actin and clathrin-dependent internalization mechanisms leading to fibronectin degradation on MPs but not NPs. Thus either prolonged, controlled release or an immediate delivery of drugs can be achieved by adjusting the particle size along with matrix proteins such as FN.

Key Words: actin, clathrin, flow cytometry, receptor mediated endocytosis, toxicity

Introduction

In macrophage or neutrophil phagocytosis, particles are first opsonized by serum proteins, which enable cellular detection and engulfment. In addition to the major opsonins of serum such as immunoglobulins and complement factors, blood also contains cell attachment proteins including fibronectin (FN; ^{27, 45}). The uptake and degradation of proteins by phagocytosis is not restricted to professional phagocytic cells; other cell types exhibit phagocytosis as part of normal tissue homeostatic mechanisms ¹⁵⁶. Notably, fibroblast-mediated remodeling of the extracellular matrix involves intracellular degradation of extracellular matrix proteins like collagen and FN by the phagocytic pathway ¹⁵⁷.

FN is a major cell adhesive glycoprotein of extracellular matrix that mediates adhesive interactions with cell surface adhesion receptors such as integrins, but also enables binding to other matrix proteins like collagen ¹⁵⁸. FN may provide important regulatory signals that augment macrophage phagocytic responses and can influence resolution of inflammation in connective tissues ¹⁵⁹. As FN participates in cell adhesion and can be readily phagocytosed by fibroblasts ¹⁶⁰, ¹⁶¹ it can facilitate internalization of other matrix proteins like collagen and, when bound to particles, could be used to facilitate gene or drug delivery into connective tissue cells ¹⁶²⁻¹⁶⁴.

Various physical and chemical properties of particles such as size ^{83, 97, 165, 166}, shape ^{89, 103}, surface charge ^{107, 109} and surface functionalization ¹¹⁷ influence how synthetic materials interact with cells and ultimately determine their fate. Particle size is considered to be one of the critical parameters that govern the nature of the endocytic pathway and the kinetics of particle internalization ^{99, 165}. In theory, interactions between particles and cell surface receptors are optimal when particles are 30-50 nm in diameter, and when the concentration of ligand on the particle surface and receptor abundance on the cell surface are not limiting factors ⁹⁶. However,

because different cell types can express varying levels of target receptor and can utilize different internalization pathways, the optimal size of particles for internalization may depend on the cell type being assayed¹. For example, as measured by particle number and total mass, gastrointestinal epithelial cells preferentially uptake 100 nm diameter polylactic polyglycolic acid-based particles compared with 0.5–10 μm particles^{97, 98}. In murine melanoma B16 cells, the endocytic pathway used for particle internalization is size-dependent: particles <200 nm are internalized by clathrin-mediated endocytosis while particles >500 nm are internalized by the caveolae-mediated pathway⁸³, which suggested to us an approach by which the utilization of discrete particle sizes for protein delivery may enable specific engagement of different endocytic pathways. Larger sized particles (>1 μm) are preferentially internalized by the phagocytic pathway of endocytosis¹¹³. Although the optimum size may vary considerably, larger particles (~250 nm to 3 μm) are more rapidly internalized than smaller diameter particles (<250 nm in diameter)⁴⁴. In view of our current lack of definitive understanding of the uptake and processing of nanoparticles and their surface proteins by fibroblasts, we examined the dynamics, mechanisms of FN uptake and FN degradation patterns exhibited by Rat-2 fibroblasts when FN was coated on nanoparticles (NPs; ~50 nm diameter) or on much larger microparticles (MPs; 1 μm diameter).

MATERIALS AND METHODS

Reagents

Carboxylate-coated fluorescent polystyrene MPs (1 μm diameter; yellow-green, FITC) and crimson-red (CRM) MPs were purchased from Polysciences (Warrington, PA) and Molecular Probes (Eugene, OR), respectively. Fluorescent polystyrene NPs (51 nm diameter; FITC or CRM) were purchased from Bangs Laboratories (Fishers, IN). Bovine serum albumin (BSA) was from Miles Diagnostics (Kankakee, IL). Bovine plasma FN, tetramethyl rhodamine isothiocyanate phalloidin, Genistein, Chlorpromazine hydrochloride as well as mouse mAb and rabbit pAb to fibronectin were purchased from Sigma-Aldrich (Oakville, ON). Blocking antibody to mouse fibronectin receptor ($\alpha 5\beta 1$) and mouse mAb to GAPDH were from Millipore (Billerica, MA). Latrunculin B and jasplakinolide were obtained from Calbiochem (La Jolla, CA).

Cells

Rat-2 fibroblasts and NIH 3T3 fibroblasts were cultured at 37°C in complete Dulbecco's Modified Eagle's medium containing 5% fetal bovine serum and antibiotics (0.17% w/v penicillin V, 0.1% gentamycin sulfate, and 0.01% $\mu\text{g/ml}$ amphotericin). Cells were maintained in a humidified incubator gassed with 95% air and 5% CO_2 , and were passaged with 0.05% trypsin with 0.53 mM EDTA (Invitrogen, Burlington, ON).

Fibronectin and BSA particle coating

BSA-coated particles were used to study non-specific uptake of the particles as a control for the FN-coated particles. NPs and MPs were coated with BSA or FN for 1 hour at 37°C with shaking as described previously¹⁶⁷. Briefly, following thorough dispersion by vortexing, 10-100 μl aliquots of MPs or NPs were incubated with 1 ml of FN in PBS (10 $\mu\text{g/ml}$) or with 1% (w/v)

BSA. FN or BSA-coated particles were then sedimented by centrifugation (8160 xg for 3 minutes or 110,000 xg for 15 minutes for MPs and NPs, respectively), re-suspended in PBS and sonicated to ensure even distribution of the particles in solution prior to use for incubation with the cells.

Biotinylated fibronectin preparation

Aliquots of Sulfo-NHS-LC-Biotin (50-100 μ L of 2 mg/mL stock dissolved fresh in DMSO) were mixed by magnetic stirring with FN that was diluted in sodium phosphate buffer (pH 8.2) every hour for five hours, at 4°C. Following addition of the final aliquot, the solution was left stirring at 4°C overnight. The volume of buffer was adjusted so that the final biotinylated-FN concentration was 100 μ g/mL. NPs and MPs were coated with biotinylated-FN solution as described above.

Particle characterization

Dynamic light scattering (DLS) and zeta potential (ζ) analyses of FN-coated NPs and MPs were performed using a ZetaSizer Nano ZS (Malvern Instruments) in the laboratory of Dr. W. Chan (IBBME, University of Toronto) using his previously described methods¹⁶⁸. Particle diameters were also assessed by negative staining with uranyl acetate and electron microscopy of particles incubated on formvar grids.

Dot Blot Analysis

We estimated the amount of FN binding to particles by first eluting FN from particles by boiling for five minutes in Laemmli sample buffer. Aliquots (2 μ L) of the eluates were dotted onto nitrocellulose membranes along with known amounts of FN as separate dots. After air-drying, the membranes were blocked with 5% BSA in TBST and were probed overnight at 4°C with mouse anti-FN antibody. FN dots were then detected by chemiluminescence using a goat anti-mouse antibody conjugated with horseradish peroxidase (BioRad, Mississauga, ON) and Amersham

ECL western blotting reagent (GE Healthcare, Buckinghamshire, UK), imaged with LiCor Odyssey-Fc system (Mandel, Toronto, ON) and quantified using Image J.

Immunofluorescence and Confocal Microscopy

Cells plated on particles were allowed to spread and bind to FN-coated NPs or MPs. The cells were trypsinized with 0.05% trypsin (Invitrogen, Burlington, ON), fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, stained with rhodamine phalloidin and attached to glass slides with a cyto-centrifuge. The spatial distributions of NPs and MPs were examined by fluorescence microscopy.

Electron Microscopy

Cultured cells were fixed in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.3) at 4°C for four hours, washed three times in 0.1 M sodium cacodylate buffer, post-fixed in 2% OsO₄ in 0.1 M sodium cacodylate for 90 minutes at room temperature (21°C), and washed three times in 0.1 M sodium cacodylate buffer. Samples were embedded in Epon 812 resin. Thin sections were placed on nickel grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi) to assess the localization of MPs and NPs in the cytoplasm.

Flow cytometry and fluorimetry

Experiments with FN or BSA-coated NPs and MPs were conducted with Rat-2 fibroblasts grown in 6-well plates (Costar Corp., Cambridge, MA). After sonicating coated particles in PBS to ensure uniform distribution of the particles in suspension, particles were incubated with cells at an estimated MP:cell ratio of 1:10 or with discrete volumes of sedimented NPs. Polystyrene

FITC-MPs or FITC-NPs were used to analyze particle uptake by flow cytometry as described elsewhere^{165, 167} and by fluorimetry (PTI, London, ON).

Impact of actin polymerization and caveolae- or clathrin-dependent pathways

Separate experiments were conducted to study uptake of NPs and MPs after treatment with an inhibitor of actin filament assembly, latrunculin B (an actin monomer sequestering agent) or the actin filament stabilizer jasplakinolide. Cells were pre-incubated with latrunculin B (1 μ M) or jasplakinolide (1 μ M) for 30 and 15 minutes respectively. For assessing the relative importance of raft-dependent endocytosis for the two types of particles, we pre-incubated cells for one hour with methyl β cyclodextrin (M β CD; 10 mM)¹⁶⁹ to extract cholesterol from the plasma membrane prior to adding the particles. Pre-treatment of the cells for an hour at 37°C with genistein (200 μ M) or chlorpromazine hydrochloride (10 μ g/mL) was used perturb caveolae- or clathrin mediated endocytotic pathways, respectively⁸³. NPs or MPs were added to cells following the various pre-treatments for an additional three hours and analyzed by flow cytometry.

Effect of particles on cell membrane integrity

Cells were stained with propidium iodide (Molecular Probes; 20 μ g/ml for 30 minutes) to identify cells with permeable cell membranes, an indicator of dying cells. As a positive control for cell death, cells were fixed with 70% ethanol for one hour prior to propidium iodide staining.

Immunoblotting

Rat-2 cells were incubated with coated or uncoated particles for 24 hours. Cells were trypsinized to ensure the removal of loosely bound particles on the cell surface, pelleted at 370 $\times$ g (<20x speeds used for sedimenting particles), and re-suspended in lysis buffer (PBS containing 0.5% Na-deoxycholate, 1% Igepal CA-630, 0.1% SDS, 2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl

fluoride and Sigma Protease inhibitor cocktail at a ratio of 1:50). After determining protein concentrations with bicinchoninic acid (BCA kit; Thermo Scientific; Waltham, MA), equal amounts of proteins reduced with Laemmli buffer containing 2-mercaptoethanol were separated on polyacrylamide gels and immunoblotted for biotin with streptavidin-rhodamine (Jackson ImmunoResearch Laboratories, West Grove, PA), total FN (using anti-rabbit-IRDye®800CW as the secondary antibody) and GAPDH (using anti-mouse-IRDye®680LT as the secondary antibody). Immunoblots were visualized using the 600 nm, 700 nm and 800 nm channels on LiCor Odyssey Fc system (Mandel, Toronto, ON).

Statistical Analyses

All experiments were repeated at least three times on separate days and with cells at different passage numbers. For continuous variables, means and standard errors of means were computed. Differences between groups were evaluated by Student's unpaired t-test or analysis of variance for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Fibronectin-coated particles characterization

Electron microscopy images obtained at various magnifications of negatively stained FN-coated NPs (Fig. 1A) and MPs incubated on formvar grids were used to estimate the mean diameters for NPs and for MPs with the surface bound FN as 51 ± 3 nm and 1.1 ± 0.1 μ m, respectively (Table 1). We measured the electrostatic potential at the electrical double layer surrounding FN-coated particles in solution, which is referred to as the zeta potential¹⁷⁰. The zeta potential for NPs was not statistically different than that of MPs (Table 1; $p > 0.05$).

The concentration of FN (10 μ g/ml in incubation buffer) used in these experiments was analyzed in pilot experiments based on the lowest concentration of FN in the incubation buffer that was required for optimal bead adhesion. Linear regression was used to examine the relationship between known amounts of FN in the incubation buffer and the density of the immuno-dots (Supporting information Fig. S1A). These data were used in turn to estimate the amounts of FN eluted from varying volumes of particles. From information provided by the manufacturers on the percent volume of solid particles in the particle suspensions and the mean diameter of the particles, and by interpolating the density of dots measured with particles and the density of dots of known amounts of FN (Table 2), we estimated that at the optimum coating concentration of FN, for NPs there was $5.1 \pm 0.4 \times 10^{-5}$ pg FN per μ m² of particle surface area, and for MPs there was $4.2 \pm 0.3 \times 10^{-5}$ pg FN per μ m² of particle surface area, which indicated no statistically significant difference ($p > 0.2$) between particle types.

Sensitivity of assay for quantification of particle internalization

We then determined whether there was a relationship between increased volume (and therefore the numbers) of particles and the fluorescence intensity of these particles, as measured in stirred cuvettes by fluorimetry. These data showed that for both dispersed NPs and MPs, as the packed volume of the particles was increased, there was a corresponding increase of fluorescence photon counts with no evidence of fluorescence quenching at increasing particle concentrations (Supporting information Fig. S1B, C).

We assessed whether there was a relationship between the volumes of NPs or MPs incubated with cells and the fluorescence intensity of internalized particles in single cells, as measured by flow cytometry. Cells were incubated with particles for three hours in 6-well plates (total volume of 1 ml); particles that were not internalized or bound during this time period were removed by trypsinization during preparation of the cell suspensions. The fluorescence channel number of cells containing NPs increased in samples with larger volumes of NPs (up to 2.5 μ l per well) but did not increase further with higher volumes of NPs (Supporting information Fig. S1D). In contrast, the fluorescence channel numbers of cells incubated with MPs increased as the volume of MPs was increased (0.5 - 15 μ l) and did not exhibit a plateau at the highest volume studied (Supporting information Fig. S1E).

Fibronectin-coated particle trafficking

We examined the spatial distribution of NPs and MPs with cells by fluorescence microscopy. Cells were incubated with NPs or MPs for three hours, sedimented on to slides and examined. These images showed that NPs were distributed throughout the cytoplasm as aggregates while some MPs were apparently within the cell or were associated with the plasma membrane (Fig. 1B; left panels). Analysis by transmission microscopy of cell suspensions following three hours

or one day incubation with the particles showed that MPs were aggregated in membrane-coated vesicles, presumably after trafficking through the endocytic vacuolar system (Fig. 1B, middle and right panels). In contrast, NPs were dispersed throughout the cytoplasm. Higher numbers of NPs and MPs were observed after one day of continuous incubation compared to cells harvested after three hours of particle incubation.

Dynamics of nanoparticle and microparticle uptake

We investigated the dynamics of uptake of FN-coated NPs and MPs after incubation for different time periods; uptake was first measured by fluorimetry of cell suspensions in cuvettes with continuous stirring (Supporting information Fig. S2A). These data showed that NPs and MPs were rapidly internalized by Rat-2 fibroblasts over the first three hours of incubation. The abundance of internalized particles increased over six hours and there was no evidence of fluorescence saturation, even after 48 hours of continuous particle incubation.

We conducted experiments in which cells were incubated with very small volumes of green fluorescent NPs or, in separate wells, with green fluorescent MPs (0.25 μ l per well for each type of particle). Cells that internalized NPs or MPs were measured by flow cytometry. These data indicated that the percentage of cells that internalized FN-coated NPs or MPs increased in parallel over the time of incubation (Supporting information Fig. S2B; left panel). However, the mean fluorescence of cells incubated with NPs remained relatively constant over 48 hours of continuous incubation with NPs whereas the fluorescence of cells incubated with MPs remained relatively same for up to six hours and then increased progressively at 24 and 48 hrs ($p < 0.0001$; Supporting information Fig. S2B; right panel).

Localization of fibronectin-coated nanoparticles and microparticles

We examined by fluorescence microscopy the intracellular localization of NPs and MPs that had been incubated simultaneously with cells. Cells were incubated with both NPs and MPs together for three hours, and then were sedimented, stained with DAPI and imaged. These images showed that NPs were distributed in the cytoplasm and that the MPs did not co-localize with the NPs (Fig. 2A). Two colour flow cytometry analysis of cells incubated with the particles of different color and size showed one well-defined population, which presumably represents cells that had internalized both MPs and NPs (Fig. 2B).

We quantified uptake of NPs and MPs in cells that were incubated with both particles simultaneously for 48 hours (Fig. 2C). Within the first three hours of incubation, most of the cells internalized both MPs and NPs, as shown by the progressive increase of the percentage of cells that were labeled with both types of particles over time. NPs were internalized rapidly within one hour, but the retention gradually decreased over time. Conversely, the uptake of MPs slowly increased over six hours before declining. After 24 hours almost all cells had internalized both types of particles.

Processing and exocytosis of fibronectin-coated particles

To investigate processing and exocytosis of FN-coated NPs and MPs, we used pulse-chase experiments. FN-coated fluorescent NPs and MPs were incubated with cells for three hours and the cells were analyzed. In the second related set of samples, particles were incubated with cells for three hours; the cells were separated from the particles by sedimentation and the cells were replated overnight in particle-free medium containing 5% fetal bovine serum to allow cell growth and particle processing. The fluorescence channel number and the percentage of cells that internalized NPs (Fig. 2D) and MPs (Fig. 2E) were analyzed by flow cytometry. Fluorescence

channel numbers were analyzed for only those cells that exhibited supra-threshold labeling. As indicated by the small (<10%) reduction of median fluorescence channel number between cells that were incubated with particles for three hours or that were chased with particle-free medium, there was minimal exocytosis of NPs or MPs after over-night incubation in particle-free medium. Notably, after the over-night incubation, there were ~45% lower percentages of cells with internalized NPs or MPs ($p<0.001$ and $p<0.01$ for NPs and MPs respectively). Taken together with the small reduction of cell fluorescence after the pulse-chase, these data are consistent with the notion that dividing cells did not partition NPs and MPs equally at mitosis. Instead, one of the daughter cells was more likely to receive a much larger volume of NPs or MPs after cell division.

Effect of nanoparticles and microparticles on cell viability

We investigated whether FN-coated NPs or MPs affected cell viability and plasma membrane integrity since endocytosis of exogenous materials is only mediated by cells with intact plasma membranes. Total cell counts were performed over 48 hours in cells that were incubated continuously with FN-coated particles in medium containing 1% serum to prevent cell growth; these data were compared to control cells plated in the same medium but without particles (Fig. S3A). The total number of cells did not change significantly ($p>0.2$) after 48 hours incubation with MPs or NPs. To examine the integrity of the plasma membrane, cells were incubated with FN-coated NPs or MPs and then stained with propidium iodide (30 minutes). The percentage of propidium iodide positive cells was very low for cells exposed to NPs or MPs; this percentage was comparable to untreated controls ($p>0.2$; Fig. S3B) indicating the lack of toxicity of these particles for biomedical applications.

Specificity of nanoparticle and microparticle uptake

Fibronectin is a critical cell adhesion molecule for remodeling of the ECM¹⁷¹ and, similar to collagen, is readily internalized by fibroblasts^{172, 173}. We capitalized on these features of fibronectin and asked if fibronectin can facilitate transport of MPs and NPs into connective tissue cells. In order to determine if the internalization of the particles was dependent on coating, we compared internalization of NPs or MPs coated with FN or bovine serum albumin (BSA). The extent of internalization was estimated from the fluorescence of cell suspensions measured with a fluorimeter (Fig. 3A). These data indicated that there was more internalization of NPs and MPs coated with FN compared with BSA ($p < 0.01$) and that this effect was enhanced after one day of incubation compared with three hours.

We also investigated the effect of FN compared to BSA coating of NPs or MPs on internalization by measuring fluorescence per cell by flow cytometry (Fig. 3B). There was greater internalization ($p < 0.0001$) of FN-coated MPs after three hours or one day of incubation compared with BSA-coated MPs. In contrast, the internalization of FN-coated NPs compared to BSA-coated NPs was slightly increased at three hours, but was decreased after one day of incubation, indicating that the NPs were taken up non-specifically. The non-specific internalization of FN coated onto NPs versus the more specific internalization by FN coated onto MPs was also supported by FN receptor blocking experiments, which showed that $\alpha 5\beta 1$ integrin inhibiting antibody reduced MP internalization compared to control and slightly increased NP internalization (Table 3). Thus, unlike MPs, NPs were internalized equally well with fibronectin or BSA coating or with integrin blocking antibody. Taken together with the earlier observations that almost half of the NPs do not bind FN, and that the binding and internalization of particles is dependent on ligand-induced integrin receptor clustering¹⁶⁷, we conclude that particle

internalization by receptor-ligand interactions is more likely to occur with single MPs compared to single NPs.

Mechanisms of nanoparticle and microparticle uptake

We investigated possible mechanisms by which NPs and MPs are internalized. Eukaryotic cells can internalize extracellular materials through energy-dependent and energy-independent uptake pathways. As low temperature can inhibit energy-dependent internalization processes, we compared particle internalization in cells incubated with FN-coated NPs or MPs at 37°C and 4°C (Fig. 4A). Internalization of FN-coated NPs and MPs was markedly reduced by low temperature ($p < 0.0001$). Similarly, median fluorescence per cell was decreased following ATP depletion by the addition of sodium azide (NaN_3) to the medium ($p < 0.0001$; Fig. 4B). As the internalization of NPs and MPs was inhibited by low temperature and by the depletion of cellular ATP, internalization mechanisms for NPs and MPs appeared to be largely energy-dependent. Our data are in agreement with earlier studies indicating that the efficient internalization of FN-coated NPs or MPs is an energy-dependent process^{83, 174}.

Effect of actin polymerization and lipid rafts on particle uptake

As actin filaments as well as caveolae- or clathrin-mediated endocytic pathways are involved in particle uptake^{83, 167, 175}, we next examined their impact on NP and MP internalization in our model. Flow cytometry analysis was conducted on cells exposed to FN-coated fluorescent NPs or MPs for three hours after pre-incubation with two inhibitors of actin filaments assembly (jasplakinolide or latrunculin B). The actin filament stabilizer jasplakinolide, inhibited the internalization of NPs ($p < 0.001$; Fig. 5A) and MPs ($p < 0.0001$; Fig. 5A). The actin monomer sequestering toxin latrunculin B strongly inhibited the internalization of MPs (Fig. 5A; $p < 0.0001$) but slightly enhanced NP internalization ($p < 0.05$).

We determined whether the clathrin-dependent endocytic pathways were involved in the internalization of NPs and MPs. Methyl β -cyclodextrin (M β CD) was used to selectively extract cholesterol from the plasma membrane, which is required for these endocytic pathways^{83, 90, 169}. M β CD strongly inhibited NP internalization (Fig. 5B; $p < 0.0001$). A three hour wash-out of M β CD prior to incubation with NPs restored internalization of NPs (Fig. 5B; $p < 0.0001$). Conversely, M β CD treatment increased microparticle internalization without or with the removal of the inhibitor prior to particle binding, with corresponding p-values of $p < 0.001$ and $p < 0.0001$ respectively (Fig. 5B).

Mechanism of nanoparticle internalization by endocytosis

In order to elucidate if clathrin- and caveolae- dependent endocytosis were involved in the particle internalization, we used chlorpromazine-HCl that disrupts the processing of clathrin and genistein that inhibits tyrosine kinases required for the activation of Caveolin-1 to block clathrin- and caveolae- mediated pathways, respectively⁸³. Pretreatment with chlorpromazine reduced the internalization of the FN coated NPs (Fig. 6; $p < 0.05$), while internalization of MPs was not affected. In contrast internalization of neither particle was affected by genistein (Fig. 6). Taken together these results support the notion that MPs rely on actin-dependent phagocytosis, which is disrupted when actin assembly is blocked by the action of latrunculin B or jasplakinolide. In contrast, NPs are preferentially internalized by the clathrin-dependent endocytosis, which is disrupted when cholesterol is extracted from plasma membrane and when clathrin processing was interrupted. Notably, for all experiments using inhibitors, only partial inhibition of particle uptake was observed, possibly because of the action of multiple internalization pathways that are acting independently of particle size.

Fate of fibronectin internalized by microparticles and nanoparticles

In order to examine the fate of FN on the MPs and NPs, cells were incubated with biotinylated-FN coated or uncoated NPs or MPs for 24 hours in regular growth media. Confocal microscopy analysis was used to confirm the presence of yellow-green fluorescent MPs, stained with streptavidin rhodamine in cells with coated, but not in the uncoated MPs (Fig. 7A). Further, immunoblots of whole cell lysates that were analyzed for internalized biotinylated FN showed more degraded FN in cells incubated with MPs compared to cells incubated with NPs (arrow; Fig. 7B; Lanes 4 and 6, respectively). These data are consistent with the notion that MPs are trafficked slowly through lysosomal pathways, which subject surface-bound proteins to increased degradation by lysosomal cathepsins.

DISCUSSION

In this study we examined the dynamics, mechanisms of uptake and intracellular degradation patterns of FN exhibited by Rat-2 fibroblasts when FN was coated on nanoparticles (NPs; ~50 nm diameter) or on much larger microparticles (MPs; 1 μ m diameter). The validity of the experimental models used in these experiments relies in part on the relative uniformity of particle composition and ligand abundance with different particle sizes. As the composition of the particle itself as well as its surface can play an important role in determining the nature of interactions between particles and cells^{113, 176}, we used polystyrene particles coated with a known concentration of FN, an important and ubiquitous ECM protein¹⁷⁷.

The relative abundance of FN per μ m² on the surface of the NPs and MPs is an important experimental consideration as ligand surface density is a central determinant of the dynamics of particle binding and uptake¹⁷⁸. Productive binding relies on supra-threshold abundance of ligand on the particle surface¹⁷⁹. As a result of this requirement, increasing the density of ligand molecules on the particle surfaces often enhances cellular uptake¹⁸⁰. Indeed, based on our dot blot data, we estimated that ~193 molecules of FN are attached to the surface of a single, 1 μ m diameter microparticle while there are only 0.6 molecules of FN attached to the surface of a single, 50 nm NP. These data indicate that the local concentration of FN molecules that are available for specific interactions with cell surface receptors will much more likely favor binding and internalization of MPs compared with NPs because on average, ~40% of the NPs will not be coated with FN. This problem may be overcome by increasing the concentration of FN and/or using higher bead:FN ratios. Notably, the specific physical and chemical characteristics of the particle as well as the chemistry of the coating protein determine the density of the absorbed protein. In comparison to our data on polystyrene, recent estimates indicate that ~69 molecules of BSA molecules are bound by 30 nm poly(ethylene glycol)-b-poly(ϵ -caprolactone) particles¹⁸¹.

Transmission electron microscopy showed that after incubation, microparticles and nanoparticles were located inside cells and presumably entered endosomal tubular-vacuolar systems. In these pathways, endosomes undergo stages of transition and maturation from early endosomes to mature late endosomes⁸², which then fuse with lysosomes to enable degradation of their contents¹⁸². Our results showed that microparticles were aggregated together in membrane-coated vesicles, presumably as a consequence of trafficking through the endocytic system. In contrast, nanoparticles were dispersed throughout the cytoplasm. These results are in agreement with earlier studies showing that MPs, possibly due to their larger size, exhibit slower rates of trafficking through the cell and tended to remain within vesicular compartments¹³⁶. In contrast, the smaller sized NPs are more rapidly transported through lysosomal compartments¹³⁶. Another possible consideration here is that the dimension of the intracellular vesicular machinery in Rat-2 fibroblasts is not optimized for rapid delivery of larger size MPs, which is frequently observed in professional phagocytic cells like macrophages¹³⁷. Our data from biotinylated FN degradation experiments are consistent with the notion that MPs are trafficked slowly through lysosomal pathways, which subject surface-bound proteins to degradation by lysosomal cathepsins.

Our data are in agreement with earlier studies indicating that the efficient internalization of fibronectin-coated nanoparticles or microparticles is an energy-dependent process^{83, 174}. We found limited uptake of nanoparticles or microparticles at low incubation temperatures or in the presence of sodium azide, suggesting that uptake of nanoparticles or microparticles by energy-independent pathways can occur only to a small extent.

We investigated the impact of actin filament and clathrin-dependent endocytic pathways, which are thought to be involved in microparticle and nanoparticle^{83, 167, 175}. We used the actin filament stabilizer jasplakinolide, which as expected, inhibited microparticle internalization but unexpectedly had much less effect on the internalization of nanoparticles. Consistent with these

data, the actin monomer sequestering toxin latrunculin B strongly inhibited the internalization of microparticles but slightly enhanced nanoparticle internalization. Previous work has shown that latrunculin B, can, under specific experimental conditions, enhance phagocytosis by increasing integrin adhesion receptor mobility¹⁸³. Thus NP uptake may be facilitated by latrunculin B. Whereas binding of FN-coated MPs strongly enhances actin filament assembly at adhesion sites, which in turn enhances internalization¹⁸⁴, the relative paucity of FN that is available on NPs for engagement of FN receptors is evidently insufficient to involve the actin filament machinery in the endocytic process. We believe that these data relate to the relative lack of NP engagement with integrin receptors, as described above.

In contrast to the data on actin filament-dependent pathways, we found that methyl β -cyclodextrin, an agent that selectively extracts cholesterol from the plasma membrane¹⁶⁹, strongly inhibited nanoparticle internalization but did not affect microparticle internalization. These results support the notion that microparticles rely on actin-dependent phagocytosis, which is disrupted when actin assembly is blocked by the action of the actin toxins. Conversely, nanoparticles are preferentially internalized by the clathrin-dependent endocytosis, which is strongly disrupted when cholesterol is extracted from plasma membrane, followed by disruption of lipid rafts and inhibition of internalization. Notably, for all experiments using inhibitors, only partial inhibition of particle uptake was observed, possibly because of the action of multiple internalization pathways that act independently of particle size.

Nanomaterials are gaining rapid recognition for their role in targeted, controlled release of drugs and genes in medicine¹⁸⁵. Surface functionalization with adhesion molecules, such as ECM proteins can further facilitate this process^{162, 163}. As the architects of tissue remodeling fibroblasts are the ideal targets of such interventions¹⁸⁶. However much of the work have been done on transformed cell lines¹. In this study we show that fibronectin can be used to facilitate

endocytic loading of microparticles into cells of connective tissue while nanoparticles showed no significant enhancement compared to BSA. The notion of optimizing drug delivery by particle vehicles is of considerable interest for localized drug delivery and for the treatment of various inflammatory lesions, including periodontitis. Bead-loading systems can establish an effective concentration of a therapeutic agent (such as an antibiotic) in the periodontal pocket with minimal systemic side-effects¹⁴⁸. Chitosan microspheres containing tetracycline have been developed for these applications and in vitro studies showed sustained tetracycline release and enhanced antimicrobial activity¹⁴⁹.

Thus, as summarized in Fig. 8, particle size affects actin and clathrin-dependent mechanisms that mediate internalization and degradation of FN when coated on to particles. FN-coated MPs were internalized primarily through actin-dependent pathways, indicating that FN can facilitate transport of MPs into connective tissue cells like fibroblasts. In contrast, FN-coated NPs were more dependent on clathrin-dependent internalization and were internalized equally well with FN or BSA coating. We also found that MPs were aggregated together in membrane-coated vesicles, presumably as a consequence of trafficking through the endocytic system and, as a result, FN that was coated on to MPs was much more extensively degraded than FN on NPs, which is consistent with our fluorescence microscopy data that NPs were dispersed throughout the cytoplasm. Collectively our data indicate that the size of particles that are used to present FN to cells impacts the internalization pathways and the degradation of FN.

Conclusions and Future Directions

Our main finding is that internalization and degradation of FN is affected by the particle size that is used for FN delivery. Actin-dependent pathways are primarily involved in uptake of larger FN-coated MPs. Internalization by this mechanism is very efficient since specific cellular receptors for FN such as the $\alpha 5 \beta 1$ integrin are utilized. FN can thus facilitate transport of MPs into connective tissue cells like fibroblasts. Once MPs are inside the cell, the MPs are trafficked through the endocytic system in membrane-coated vesicles, which enable extensive degradation of FN. In contrast, FN-coated NPs were more dependent on clathrin-dependent internalization, which showed less specificity as NPs coated with FN or BSA were internalized equally well. Through this pathway, FN was less subject to degradation by lysosomal cathepsins.

Our data show that when 1 μ m microparticles or 50 nm nanoparticles are coated with fibronectin, they are efficiently internalized by fibroblasts. This system or a modification thereof could be used to deliver biologically active molecules locally into cells of the marginal periodontium and possibly other accessible oral connective tissues that are affected by disease. A potential advantage of NP-mediated endocytosis is that it bypasses entry of internalized materials into degradation-associated vacuolar pathways, potentially offering promise for intracellular delivery of molecules and avoidance of digestion by lysosomal enzymes that can degrade biologically active agents.

Arising from the work presented here, one potential next step for further investigation would be to use and optimize this system for localized drug delivery into periodontal tissues and for the treatment of various inflammatory lesions, including experimental periodontitis in a mouse model as has been used previously for assessment of the role of adhesion receptor complexes for disease progression¹⁸⁷.

FIGURE LEGENDS

Figure 1: Characterization of MPs and NPs coated with FN and visualization of the internalized NPs and MPs by confocal and transmission electron microscopy. (A) Electron microscopy images of negative stained NPs coated with FN at different magnifications. (B) Images of Rat-2 fibroblasts after three hours or 1 day incubation with NPs or MPs coated with FN. The images indicate that NPs (red arrow) were distributed throughout the cytoplasm (yellow asterisk) whereas MPs (black arrowheads) were more aggregated.

Figure 2: Localization and exocytosis of FN-coated NPs and MPs. (A) Localization of FN coated NPs and MPs in Rat-2 fibroblasts by fluorescent microscopy. Green NPs and crimson-red MPs did not co-localize when incubated with cells simultaneously. (B) Flow cytometric bivariate plots of cell populations incubated simultaneously with NPs (green; FITC) and MPs (crimson-red; CRM). (C) Analysis by flow cytometry of cells continuously incubated with green NPs and crimson-red MPs simultaneously for the indicated time periods. (D, E) Pulse-chase experiments to study processing of NPs (D) and MPs (E) by Rat-2 fibroblasts. FN-coated fluorescent NPs or MPs were incubated with cells for three hrs and then analyzed or were incubated with cells for three hrs, washed and incubated overnight in 5% FBS-DMEM. Median fluorescence and the percentage of cells that internalized NPs or MPs were analyzed by flow cytometry. #; $p < 0.05$, *; $p < 0.01$ and **; $p < 0.001$ at three hours pulse-chase compared to corresponding controls. Mean + SEM. $n=3$ for all experiments.

Figure 3: Effect of ligand coating on internalization of NPs and MPs. (A) NPs and MPs were coated with FN or BSA and incubated with Rat-2 fibroblasts. Binding and uptake were measured by fluorimetry. (B) Effect of FN versus BSA-coating of NPs or MPs on binding and uptake as

measured by flow cytometry. *, $p < 0.01$ and ***, $p < 0.0001$ for BSA coated particles compared to the corresponding FN coated particle. Mean + SEM. $n=3$ for all experiments.

Figure 4: Effect of temperature and ATP depletion on internalization of FN-coated NPs and MPs. Uptake of NPs or MPs by cells was quantified by measuring median fluorescence per cell for NPs (A) and MPs (B) as well as effect of ATP depletion by sodium azide (NaN_3) on internalization of NPs (C) or MPs (D) were analysed by flow cytometry. ***, $p < 0.0001$ with temperature or sodium azide treatment compared to corresponding control for the indicated particle. Mean + SEM. $n=3$ for all experiments.

Figure 5: Effects of disruption of actin filament assembly and lipid raft stability on uptake of FN-coated NPs and MPs. (A) Flow cytometry analysis of cells incubated with FN-coated fluorescent NPs after pre-incubation with latrunculin B or jasplakinolide for three hours. Jasplakinolide decreased uptake of both MPs and NPs whereas Latrunculin B affected only internalization of MPs. (B) Pre-incubation of cells with methyl- β -cyclodextrin ($\text{M}\beta\text{CD}$) decreased NP uptake while internalization of MPs was not affected. Removal of $\text{M}\beta\text{CD}$ prior to incubation with particles strongly re-established NP uptake. #, $p < 0.05$, **, $p < 0.001$ and ***, $p < 0.0001$ in the presence of inhibitor compared to corresponding control for the indicated particle. Mean + SEM. $n=3$ for all experiments.

Figure 6: Effects of disruption of clathrin processing and cavolin-1 activation on uptake of FN-coated NPs and MPs. Flow cytometry analysis of cells incubated with FN-coated fluorescent NPs after pre-treatment with chlorpromazine or genistein for one hour. Chlorpromazine decreased uptake of NPs, but not MPs whereas genistein affected neither. #, $p < 0.05$ for chlorpromazine treatment compared to corresponding control for the indicated particle. Mean + SEM. $n=3$ for all experiments.

Figure 7: Localization and processing of biotinylated FN-coated MPs. (A) Rat-2 fibroblasts incubated for 24 hours with biotinylated FN coated or uncoated YG-MPs were immunostained with Streptavidin-Rhodamine and imaged using confocal microscopy. (B) Whole cell lysates of Rat-2 cells incubated for 24 hours with biotinylated FN coated or uncoated MPs or NPs, were immunoblotted for biotin, FN and GAPDH, as described in Material and Methods. Lane designations: M: Protein markers; 1: Biotinylated FN coated MPs only; 2: Rat-2 cells only; 3: Rat-2 cells with uncoated MPs; 4: Rat-2 cells with biotinylated FN coated MPs; 5: Rat-2 cells with uncoated NPs; 6: Rat-2 cells with biotinylated FN coated NPs.

Figure 8: Schematic overview of the particle uptake mechanisms and processing of surface bound FN on NPs and MPs.

TABLE 1 FN-coated particle characterization:

Summary of particle size and surface electrostatic potential of FN coated particles.

Data are mean $\pm$ s.e.m. from three independent trials.

Sample	Particle diameter (nm)	Zeta Potential (mV)
FN-coated nanoparticles	51 ± 3	-33.2 ± 2.3
FN-coated microparticles	1100 ± 100	-26.4 ± 1.2

TABLE 2 Estimation of FN eluted from particles by dot blot analysis

The amount of FN eluted from beads was estimated from interpolation of linear plots of known FN standards and blot density (presented in Fig. S1A).

Dot # NPs	Volume of bead (μ L)	Concentration of FN (μ g/mL)	Density	Amount FN/dot (μ g/ μ L)
1	5	5	2.32	12.7
2	5	10	3.32	18.1
3	5	100	8.38	45.5
4	10	5	2.78	15.1
5	10	10	4.14	22.5
6	10	100	7.72	41.9
7	20	5	3.15	17.2
8	20	10	1.77	9.7
9	20	100	9.65	52.4

Dot # MPs	Volume of beads (μ L)	Concentration of FN (μ g/mL)	Density	Amount FN/dot (μ g/ μ L)
1	5	5	16.9	44.0
2	5	10	58.1	152.7
3	5	100	8.14	20.9
4	10	5	1.44	3.2
5	10	10	43.1	113.1
6	10	100	14	36.3
7	20	5	0.322	0.2
8	20	10	22.5	58.8
9	20	100	56.6	148.8

TABLE 3 Fluorescence Intensities of Internalized Particles per Cell

Single cell suspensions analyzed by flow cytometry

Data are mean $\pm$ s.e.m. of fluorescence intensity units from three independent trials.

	Microparticles	Nanoparticles
Control Antibody	80.1$\pm$0.4	2.9$\pm$0.4
FN Receptor Inhibiting Antibody	49.2$\pm$4.6	4.4$\pm$0.7

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8