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Nanoparticles in Medicine: Selected Observations and Experimental Caveats

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Abstract

Medically useful nanoparticles measure 1-100 nm in at least one dimension and are engineered and manufactured for specific diagnostic and treatment applications. Most nanoparticles used currently used in medicine are engineered and manufactured for specific purposes. Medically significant nanoparticles are composed of a 1) central core that is usually the medically active component, 2) one or more layers of organic or inorganic materials that forms a capsule (corona) covering the core and 3) an outer surface layer that interacts with the environment and/or targeted cells and tissues. Effective nanoparticle function in the living, intact animal or human requires electrochemical stability necessary to bypass the reticuloendothelial system (RES) and avoid filtration through the renal glomerulus into the urine. Nanoparticles are present in “natural” as well as the manufacturing and clinical environments thus could pose as significant toxins because of their small sizes, their chemical and drug content and potential effect of causing long term disease including allergies, chronic inflammation and cancer. Currently published studies have focused on the effects of nanoparticles on cells in the extremely artificial environments of cell cultures. More clinical and preclinical studies documenting the short term and long term effects nanoparticle in the intact experimental animal and human are needed.

Keywords

nanoparticles; endocytosis; phagocytosis; pinocytosis; autophagocytosis; toxicity

2.0 Physical and Chemical Properties

2.1 Definition and Size

Nanoparticles, by definition, measure 1-100 nm in at least one dimension. Medically effective nanoparticles range from 10 – 500 nm in size. When injected into the blood circulation, nanoparticles smaller than 10 nm seem to pass readily through the fenestrations of the capillaries of the renal glomeruli in the kidneys of normal people thus the particles can leave the body via the urine soon after injection. Nanoparticles measuring larger than 500 nm and the larger microparticles measuring 1000 to 5000 nm (1-5 μm) when injected in blood vessels are cleared by the reticuloendothelial system (RES) with the spleen and liver being the largest participants in the cleanup. It has been reported that manufactured particles greater than 5 μm (5000 nm) will not be internalized by single cells in cell cultures. But, *in vivo*, these larger particles can be surrounded by phagocytic cells of the RES and, if the particle is too large for a single cell, the phagocytic cells will fuse to form larger multinucleated giant cells (foreign body giant cells) that completely sequester the particle.¹⁻²

2.2. Composition and Shape

Most nanoparticles used currently used in medicine are engineered and manufactured for specific purposes. Medically significant nanoparticles are composed of a) central core that is usually the medically active component, 2) one or more layers of organic or inorganic materials that forms a capsule (corona) covering the core and 3) an outer surface layer that interacts with the environment and/or targeted cells and tissues.^{1, 2, 3-5} [Figure 1]

The core can be drugs, proteins, viruses or portions of viruses or RNA and DNA segments that need to be delivered into cells for maximum treatment effect. Other cores are composed of metals or metallic oxides that interact with applied magnetic fields to generate heat within cells and tissues. Cores composed of optically active crystals that fluoresce (“quantum dots”) are useful for diagnostic imaging of specific molecules of cell surfaces.⁶⁻¹² The cores of other nanoparticles may not contain the medically active component but act as support for the medically active capsule or nanoparticle surface (See below)

The capsules of most medically important nanoparticles are composed of one or more layers of organic polymers and films that can 1) protect the active components of the core, 2) sequester different drugs between capsule layers to allow controlled and timed release of the drugs and/or 3) stabilize the particles in relatively hostile environments for efficient delivery of the medically active agent(s) over time. Many capsular components are bipolar polymers that can interact with aqueous and lipid rich environments as well participate in stable chemical bonds that allow 1) attachment to the core, 2) sequestration of small and large, frequently polar and charged drug molecules within the capsule layers 3) fend off chemical predation by the numerous enzymes and proteins in the delivery environment and 4) resist physical dissociation due to hostile ionic, pH, temperature, and solvent changes (aqueous vs lipid) until they are safely in their target cells and tissues.

On the other hand, the capsular material of other types of nanoparticles can be the medically active component(s) of the nanoparticle. The core merely functions to determine the size and shape of the nanoparticle and to support the effective treatment component of the particle.

An example is the nanosphere and nanorod- shaped cores of nanoshells that are made of silica and covered by a thin capsule of metallic gold that interacts with applied light to generate heat in the target tissues.⁸

The shape, size, chemistry and electrical charge of the surface of the nanoparticle determine the interaction of the particle with the 1) delivery environment and 2) its targeted cells and tissues.^{1, 9, 13-18} The intracellular and extracellular environments of all living things are water-based solutions and gels separated by lipoprotein membranes into subcellular organelles and cells. Effective delivery of the particles depends on their remaining stable in an aqueous (blood and tissue fluids) or gaseous (inspired or body surface air) environment until they can be selectively and, hopefully, specifically attached to their target.

Nanoparticles with no surface modifications tend to aggregate into larger clumps measuring as much as a few microns particularly when in the environment of biological fluids such as blood plasma. These relatively large clumps will be readily be taken up by the very efficient phagocytic cells of the RES and rarely will survive in the blood for more than a few passes of the blood in circulation. Therefore, numerous chemical, electrical, biological and physical modifications of the particle surfaces have been used to fabricate nanoparticles that will survive in the blood for longer periods. In the past these nanoparticles were called “stealth” nanoparticles because of their ability to remain in the blood circulation. Currently, modified nanoparticles have been reported to have circulation half-lives of several hours to a few days.¹

Addition of organic molecules to the nanoparticle surface such as antibodies, particular metabolites or electrically charged or neutral lipoproteins will increase particle biological compatibility with cell surfaces and allow specific stable attachment to cell surface antigens, receptors or specifically defined membrane regions. [Fig. 1]

2.3 Fabrication

In general, medically useful nanoparticles need to be specifically engineered 1) to withstand the complex environments presented by the patient and preserve the stability of the particle and 2) to home toward and attach to the specifically targeted cell or tissue. Currently, the fabrication of nanoparticles is based on 1) self-assembly of atoms, molecules, membranes, films, micelles etc. under defined chemical and physical conditions, 2) directed and guided construction from prepared materials or 3) combinations of both manufacturing approaches.¹

Multilayered liposomes are the most common nanoparticles manufactured by self-assembly of hydrophobic and hydrophilic organic polymers (micelles) useful for protected delivery of drugs to targeted cells and tissues. Carbon-based nanoparticles such as fullerenes (“bucky balls” and nanotubes) have been touted as medically-useful nanoparticles but concerns about their biological compatibility and toxicity have precluded their total acceptance in the medical field.^{4, 14-16} Dendrimers, branched carbon-based polymers arranged in 2 or 3-dimensional arrays around a central carbon core are being explored as useful as carriers of drugs.^{11, 17}

Nanoparticles manufactured by directed construction from pre-formed materials are composed of specific materials that are then subjected to fabrication techniques such as milling and grinding, molding by templates, microfluidic techniques and lithography to get to their final size and shape.¹ Although these directly constructed particles tend to have more uniform sizes and shapes than those fabricated under self-assembly, the constructed particles tend to be larger (few microns to hundreds of nanometers) because of the constraints of the manufacturing techniques. In addition, these particles have to be further modified to make them biocompatible if they are to be used in medicine.

2.4 Nanoparticles in the environment: potential toxicity problems

Nanoparticles are found in the “natural” environment include complex organic structures such as diesel exhaust products, oil and gas combustion particles and certain types of asbestos fibers. However, the greatest concern should be the nanoparticle manufacturing environment where these tiny particles can easily escape into the air and water and settle on solid surfaces including clothes worn by the workers. For example, several types of micro and nanoparticles such as zinc oxides and titanium oxides are routinely added to cosmetics, toothpastes and sunscreens. Talc that comes from mines are ground into fine powders that are used to control skin moisture for babies and adults.^{5, 19} Some of these small particles have been associated with granulomatous lung diseases (silicosis) and cancers (asbestos with mesothelioma and talc with ovarian and lung cancers. (120. Robbins Pathology)

Most published toxicity studies for the use of nanoparticles are based on relatively short-term, cell culture studies. Except for the historical and experimental studies linking carbon exhausts and certain minerals with lung disease, there is very little literature exists concerning the toxic effects of nanoparticle (and microparticles) on the health of living humans. The toxic and pathological effects of nanoparticle exposure to cells in culture cannot and do not predict the response of cells and tissues in the intact organism over time.

3. Anatomic distribution of medically-effective nanoparticles

The anatomic distribution of nanoparticles depends on their mode of entrance that include 1) applications on the skin, 2) ingestion by mouth, 3) inhalation into the respiratory system, 4) injection into the circulatory system, 5) injection into body cavities and 6) direct injection into the diseased tissues.^{5, 20}

3.1 Skin

In general, aqueous-based nanoparticles cannot pass by the barrier formed by the tight junctions of epidermal cells or through the waxy, kerato-hyaline extracellular substance of the stratum corneum layer of the skin. However, some nanoparticles embedded in lipid-rich carriers can penetrate the epidermis to be exposed to the dendritic cells that are phagocytic cells of the immune system in the epidermis and the phagocytic cells of the dermis, the macrophages. Thus, potential acute and chronic cutaneous allergic responses could result from exposure to nanoparticles on the skin.

3.2 Ingestion into gastro-intestinal track

Ingested nanoparticles are also taken up by phagocytic cells and immune cells present in the entire mucosal lining of the gastrointestinal track from the mouth to the anus. Absorptive cells found in the intestines could also transport nanoparticles across the epithelial lining using pinocytosis (See below). However, very few specific studies have been done that actually demonstrate this mechanism in the intact, living organism using currently available medically used nanoparticles.

3.3 Inhalation into respiratory track

Inhaled nanoparticles, if they remain airborne, can be transported into the alveoli, the terminal air sacs of the lungs, where they can be taken up by the alveolar macrophages perhaps to enter into various pathways of host inflammatory and immune responses. If the nanoparticles have become clumped into larger particles, chronic granulomatous inflammation such as seen in silicosis can occur. Phagocytosis of biologically non-degradable particulate material into macrophages will always lead to chronic exposure of the cell to the materials forming the particle. Even if the material is “non-degradable” small amounts of the foreign material can be expected to leach off the surface that could produce cellular and subcellular toxic effects and possibly initiate allergies for years after the initial exposure. Inhaled nanoparticles can also land on the olfactory nerve region of the nose and could be transported into the brain via the olfactory nerves.

3.4 Injection into the circulatory system

Nanoparticles injected directly into the blood stream will be quickly taken up by endothelial cells (via pinocytosis) and the phagocytic cells that line the blood vessels of the liver, spleen and bone marrow. A substantial amount of the injected particles, if they are not coated by biologically compatible materials, can form large clumps in the blood plasma and be taken up by the phagocytic cells within a few passes of blood never to be available to the targeted cells and tissues. However, some chemotherapies exploit these mechanisms to specifically encase certain anticancer drugs to treat certain cancers of the reticulo-endothelial system such as lymphomas and leukemias that frequently involve the liver, spleen and bone marrow. In addition, certain anti-fungal antibiotics against fungi that infect the liver, spleen and bone marrow are effectively delivered via liposomes injected into the blood circulation.

3.5. Injection into body cavities

Solutions of nanoparticles can be injected into body spaces for treatment of diseased tissues that line these spaces. Injection of colloidal gold into joint spaces has been a recognized treatment of arthritis for years. Some cancers such as ovarian carcinomas and malignant mesothelioma characteristically grow on the peritoneal surfaces of abdominal organs and cavities. Nanoparticles can be constructed with surface modifications to bind specifically with the targeted cancer cells and capsules to sequester strong chemotherapeutic drugs that are released only when the nanoparticle is attached and/or ingested into the tumor cells. The advantage of this method is that the very large peritoneal surface area that can harbor the cancer cells can be treated with minimal damage to the rest of the abdominal organs. However, distribution of the particles into every nook and cranny of the peritoneal cavity is

not easy particularly when fibrous adhesions and peritoneal thickening is associated characteristically with the growth of these cancers.

3.6 Direct injection into diseased tissues

Injection of nanoparticles directly into the diseased tissues allows deposition of a large concentration of particles into a relatively defined volume such as a tumor. The advantages of this delivery system include greater control of dosage in the targeted tissue and decreasing the possibility of toxic side effects in distant tissues. The greatest problem with local injection is the possibility that the injected material does not reach all the diseased tissues such as the invasive margins of cancers. Also, the concentration of the nanoparticles could be so great that massive kill off of the abnormal as well as the normal tissue could produce large defects that would persist over time.

4. Endocytosis: Transport of nanoparticles into the targeted cells and tissues

The transport of nanoparticles from outside to inside the cell involves the three major mechanisms of endocytosis (“inside the cell”), 1) phagocytosis, 2) macropinocytosis and 3) pinocytosis.^{1, 21-23} These mechanisms are influenced by the particle size and shape and the chemical composition and electrical charge of the particle surface.⁹ [Figure 2]

4.1 Phagocytosis

Phagocytosis (“eating by the cell”) involves the movement of the cellular membrane and underlying cytoplasm over larger particles (≈ 500 nm to $\approx 5\mu\text{m}$ particle) to engulf it to form an intracellular vacuole bound by a double membrane called a phagosome whose original size is determined by the size of the engulfed particle. Phagocytosis in the intact organism usually is performed by specifically differentiated cells of the body including 1) tissue macrophages, 2) circulating blood monocytes and 3) dendritic lymphatic cells of the immune system and 4) neutrophils, white blood cells that engulf bacteria and necrotic debris during the acute inflammatory response of infection and tissue necrosis.^{20 & 21}

Occasionally, some carcinoma cells can demonstrate phagocytosis *in vitro* or *in vivo* but this is not frequent and the mechanism(s) and circumstances of their phagocytosis are not well known. Phagocytosis of apoptotic bodies may be due to resident intratumor or tissue macrophages and/or adjacent parenchymal or cancer cells.

Phagocytosis can be initiated by extracellular chemical and physical signals from nearby “foreign materials” and/or binding of the particle to the cell surface via specific ligands/receptors. Once inside the cell the phagosome fuses with lysosomes to form secondary lysosomes membrane covered vacuoles contained fragmented and digested materials engulfed by the cell. Lysosomes are intracellular vacuoles covered by a single-layered membrane and containing enzymes in an acidic environment. Lysosomes are found in most cells but are most abundant in cells whose job is to process ingested foreign material contained in endosomes. Secondary lysosomes are the hallmarks of endocytosis.

Larger foreign bodies injected into the body cavities and circulation systems of higher invertebrates and all vertebrates will be engulfed by a few or several phagocytic cells (macrophages) that will be stimulated to fuse and proliferate to form large, multinucleated foreign body cells that function as part of the intrinsic immune response of the host.²⁰

4.2 Macropinocytosis

Macropinocytosis is a non-phagocytic endocytotic mechanism (not illustrated in Figure 1) that target particles that measure around 1µm and have specific ligands that bind with receptors on the host cell surface. Upon binding, the regional actin of the cell surface will be activated to produce “ruffling” of the surface cell membrane. The flaps of the ruffles then bend towards the cell surface to capture the small particles and adjacent materials within intracytoplasmic endosomes (micropinosomes) that are covered by the double layered cell membrane. The micropinosomes enlarge and combine with acidic, enzyme filled lysosomes to form secondary lysosomes that can be indistinguishable from any other secondary lysosomes.²¹⁻²³

4.3 Pinocytosis

Pinocytosis (“cell drinking”) is the general mechanism utilized by many cells to transport very small particles (such as single nanoparticles), large and small molecules and ambient extracellular water and solutes into and through cells. Pinocytosis is a characteristic morphological and physiological feature of muscle cells, absorptive cells found in the intestines and kidneys, red blood cells and certain nerve cells.

Pinocytosis frequently involves specific attachment of the particle or molecule to specialized regions of the cell surface that activates invagination of the cell membrane. Pinocytotic process are usually are characterized by one of two specialized submembranous protein coats that mediate the formation of the endosome from the invaginated cell membrane. One, clatherin-mediated pinocytosis (endocytosis) is associated with the formation of a geometric mesh of the protein, clatherin, that surrounds the endosome as it forms and travels into the cytoplasm. As it travels, the clatherin coat dissociates to reform at the cell surface and the “bare” endosome fuses with a lysosome to form a secondary lysosome that again is morphologically indistinguishable from any other secondary lysosomes.

The other protein specific pinocytotic endosome formation mechanism is the caveolin-mediated. In this case, the protein coat is caveolin, a granular, dimeric protein located and aggregated just below the cytoplasmic surface of the cell membrane. It covers the endosome during invagination, engulfment and intracytoplasmic travel of the endosome. However, caveolin-mediated endosomes do not fuse with lysosomes but travel to be incorporated into the golgi apparatus or the endoplasmic reticulum where they slough their calveolin and deposit their contents (amino acids, metabolites, etc.). The sloughed calveolin is recycled back to the surface cell membrane.

Non-clatherin and non-caveolin mediated pinocytosis. Pinocytosis has been described to can occur without the mediation of either of these specific submembranous cytoplasmic proteins but they are not well described or understood.

5. Autophagocytosis

Autophagocytosis is normal intracellular process in which the “garbage” of the cell is gathered into a membrane bound vacuole that fuses with lysosomes to form an autophagosome. The “garbage” can be fragments of damaged membranes and organelles produced overtime due to exuberant or poor metabolism, cell damage due to cytotoxins, ageing processes and poor protein assembly. In general, autophagosomes act to sequester these materials away in “garbage cans” to protect the rest of the cell. ²²⁻²⁸

Autophagocytosis is initiated by the formation of a double-membrane forming a curved structure called a phagophore. A specific protein, light chain 3 (LC-3) is synthesized in the cytoplasm and attaches to both surfaces of the phagophore. As the cellular “garbage” is surrounded completely by the membrane, the autophagosome is formed and soon fuses with lysosomes. As the “garbage” is processed by the lysosomal enzymes, the undigestible parts are compacted and the digested material escapes into the cytoplasm to be recycled as synthetic and metabolic components. The LC-3 proteins then dissociate from the autophagosome into the cytoplasm perhaps to be used again by the same cell. The mature autophagosome will survive in the cell as long as it lives.

Whether autophagocytosis is a causative mechanism of cell death has not been proven but has been implicated in several neurodegenerative diseases particularly Parkinson’s and Alzheimer’s disease. Autophagocytosis is a frequent morphological and chemical change of cells in cell cultures exposed to many kinds of nanoparticles and other toxins and drugs. ^{5, 7, 14, 22, 24-28} Cells do die in these *in vitro* situations but the accumulation of autophagosomes in the living, dying and dead cells may be a reflection of the cells’ response to the injury not the direct cause of death. The cascade of distinctive biochemical and genetic steps that have been described for apoptosis and necrosis has not been demonstrated for a separate mechanism of death by autophagy. It is possible that the insult to the cell that produces autophagy can also trigger death by the well-described mechanisms of apoptotic or necrotic cell death. In addition, the finding of dead cells filled with autophagosomes in autopsied tissues of humans is seen rarely. The lipofuscin material found in living liver, cardiac muscle and brain cells of some people with chronic neurological diseases or chronic debilitating illnesses may represent a special autophagocytic effect but has not been shown to cause cell death.

6.) Conclusion: final thoughts and caveats

Nearly all published studies of toxic effects of nanoparticles are of cells in cell cultures, not intact animals or in patients. The results of these studies cannot be translated to the clinical situation since a single cell grown in a highly artificial environment cannot predict the reactions of cells and tissues in intact, living human patients. Since the side effects and toxicity of these very chemically and physically complicated structures have not been widely addressed for intact organisms, the current status of clinical applications is limited. At this time, the clinical studies involve evaluation of the usefulness of nanoparticles (mostly liposomes) for controlled drug delivery or for localization and diagnosis and their usefulness as generators of low-temperature heat treatments using applied light or magnetic

fields in cancer therapies. Some of the nanoparticles composed of non-biodegradable materials may produce long term health concerns by being sequestered in long living phagocytic cells where they can leak of toxic materials from the central cores over time, producing chronic inflammations and allergic immune reactions that could result in long term degenerative diseases. These reactions cannot be predicted from cell culture experiments or computer models.

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Figure 1.

Schematic Image of Nanoparticle (Liposome). This image illustrates the three layers of a medically effective, specifically constructed nanoparticle in the form of a liposome. The surface is composed of specific polar, biocompatible polymers such as polyethylene glycol (PEG) or dextran that can resist uptake by the RES. Interspersed among the polymers are molecules that function as specific ligands such as antibodies that will bind only with cells that have the compatible, specific receptor. The capsule is a multilayered membranous construct of lipid-polar polymers that further enhance the stability of the liposome in the numerous lipid-aqueous environments that it can encounter and allow the sequestration of additional drugs within its layers. The usually aqueous core contains the various materials that forms the therapeutic “payload” that has to be delivered to the targeted cells (tissues).

Figure 2.

Schematic Image of Endocytosis and Autophagocytosis. Endocytosis is the general term describing the various mechanisms by which particles and large molecules are transported from the outside to the inside of cells. Phagocytosis is the process of certain differentiated cells of the tissues and blood which ingest larger particles ($\sim 500\text{nm}$ to $\sim 5\mu\text{m}$) that are perceived to be “foreign” such as bacteria, fungi, metallic and chemical crystals, dirt, etc. These are enclosed in the cytoplasm in a phagosome, an organelle formed by a double membrane derived by the protrusion and later invagination of the cell membrane to surround the foreign material. Primary lysosomes containing proteases and lipases in an acid environment fuse with the phagosome, discharge their enzymes into the phagosome to form a secondary lysosome in which the biodegradable materials are digested and sometimes discharged into the cytoplasm to be recycled. Those materials that resist digestion are compacted into complex structures that remain in the secondary lysosome for the remainder of the cell’s life. Pinocytosis is the ingestion of smaller particles ($\sim 150\text{ nm}$) and large molecules whose specific attachment to the cell membrane activates certain submembranous proteins to assemble and form small endosomes that fuse to form larger endosomes. Some of these endosomes fuse to lysosomes that form secondary lysosomes and digest the ingested material. Some endosomes seem to deliver their specific contents (usually metabolites) to the endoplasmic reticulum or Golgi apparatus by fusing with the membranes. These membranes are recycled to the cell’s membranes therefore conserving them. Autophagocytosis is an entirely intracellular event in which organelles such as damaged mitochondria, membrane fragments and malformed proteins are engulfed by a C-shaped double membrane structure called a phagophore. The phagophore is coated inside and out by a special protein, light chain-3 (LC3), that is a marker for autophagocytosis. The phagophore closes up to form the autophagosome that sequesters the damaged goods from the rest of the cell cytoplasm. When the autophagosome fuses with a lysosome, its contents are digested and compacted to form larger, membrane bound complexes within the mature autophagosome which remains within the cell until it dies. Some of the digestion products are recycled into the cytoplasm to provide precursors for protein synthesis. The mature autophagosome is usually distinguishable from other secondary lysosomes by the presence

of recognizable organelle fragments and its large size, complicated mixture of components and irregular shape.

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