

## NANOVEHICLES: AN EFFICIENT CARRIER FOR ACTIVE MOLECULES FOR ENTRY INTO THE CELL

Gurudatta Pattnaik \*, K Siva Rama Raju, B Heeralal, Md. Sajid Ali.

National Institute of Pharmaceutical Education and Research (NIPER), ITI compound, Raebareilly, Uttar Pradesh, India.

\*Email: [gurudutta.pattnaik@rediffmail.com](mailto:gurudutta.pattnaik@rediffmail.com)

### ABSTRACT

Nanovehicle as a carriers offer exclusive possibilities to overwhelmed cellular obstruction in order to improve the delivery of active substances, including the promising therapeutic biomacromolecules (i.e., nucleic acids, proteins). There are number of mechanisms lead to nanocarrier cellular internalization that is desperately affected by nanoparticles' physicochemical properties. The pharmacological actions of nanocarriers may be depends on the different paths cellular uptake and intracellular trafficking. In this article we are trying to foccus on several opportunities, starting with the phagocytosis pathway, which has followed in the treatment of certain cancer and various infectious diseases. On the other side, the non-phagocytic pathways accomplished various complex mechanisms, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis, which are more challenging to control for pharmaceutical drug delivery scientists.

**Keywords:** Nanoparticles, Endocytosis, Phagocytosis, Clathrin, Caveolae, Macropinocytosis.

### INTRODUCTION

Nanomaterials because of their very smaller size offer potential benefits with application in biomedical and industrial applications for human health and environment as reported in several annals<sup>1, 2</sup>. A new era of nanotechnology that uses devices of nanoscale size to address urgent needs for improved diagnosis and therapy of diseases is being etched in the 21st century. Different types of nanoscale formulations such as liposomes, polymer-drug conjugates, polymeric micelles, quantum dots, biodegradable nano-particles, dendrimers, gold nanoparticles, silica nanoparticles, etc. are extensively undergoing research and preclinical development, or already used in the clinic<sup>3,4</sup>. Due to the limitations of current drug delivery systems, which have been hampered by their inefficiencies in traversing the cell membrane, there is a pressing need to develop methods for increasing intracellular delivery of protein-based cargoes. The use of polymeric nanoparticles has been shown to be promising in cancer chemotherapy, intracellular viral and bacterial infections and many other pathological states due to their high internalisation into cells compared to larger micron size particles<sup>5</sup>. Infact, nanoparticles can significantly affect the cellular pharmacokinetic profiles of the drugs by altering the cellular uptake and residence time of the drug. These nanomaterials, collectively called "nanomedicines", can deliver most of the drugs, proteins, DNA (genes) etc. to the focal areas in the body for targeted delivery with decreasing the number of untoward effects to maximize clinical benefit. These nanomedicines are also improved for cellular imaging and diagnosis because of their selective uptake by the tumor cells. At the cellular level some important insights have come from studies on cellular response to carbon nanotubes<sup>6</sup>, calcium selenide nanoparticles<sup>7</sup>, and gold nanoparticles (GNPs)<sup>8</sup>. Most of the recent reports concentrate on the effect of various factors such as size, shape, surface properties on the

cellular uptake of the nanoparticles, bioavailability of the drug entrapped and tissue distribution of the particles. Hence, a new paradigm for drug delivery and nanomedicine requires nanomaterials to differentially interact with the surface of their target cells and undergo intracellular trafficking that would lead to determined locations inside cells<sup>9</sup>. Thus the study of the cell internalisation and the intercellular transport of the nanoparticles become important to know the specificity of the nanoparticulate drug delivery systems.

### TOOLS TO STUDY INTRACELLULAR TRAFFICKING

The intracellular trafficking of the nanomaterials can be studied any one of the following approaches. A) colocalisation of the nanoparticles along with the suitable endocytosis markers. B) use of the inhibitors of the specific pathway of uptake of the particles by the cells. In case of colocalization approach specific probes will be attached to the surface of the particle or included in its composition. Then the movement of these probes can be studied by using various methods. One of the mechanism reported for this approach is "pulse- chase" design, in which the marker is given simultaneously or before giving the nanomaterial and inclusion of the nanomaterials in the same vesicle can be tracked at different time points. Some of such markers are fluorescent dyes, rhodamine and Texas Red etc. Then the uptake of particles can be seen by using confocal microscopy or fluorescence microscopy. The most commonly used method is the use of fluorescent probes and analysing the spread of the compound or fluorescence resonance energy transfer (FRET) across the cell lines using confocal microscopy. Hearan et. al. demonstrated the use of flourescein thiocyanate for the study of the uptake of the biodegradable nanoparticles in vascular smooth muscle cells. But the main disadvantage of these approaches is the less selectivity of the markers for specific type of receptor mediated uptake of the particles. We can study



only the rate of uptake of the particles. So there is the need for transfecting specific probes for each type of mechanism to get the exact mode of uptake such as Green Fluorescent Protein (GFP). Recently many specific probes for specific cell organelles have been developed some of them are listed in the table 1.

**Table 1:** Different probes used and their function in imaging.

Probe	Function
MitoTracker™	Tracking of the mitochondria
Fluorescently labelled Glibenclamide	Sulphonylurea receptors of ATP-sensitive K <sup>+</sup> channels in ER
Lysotracker™	accumulate in lysosomes

Apart from confocal microscopy, the electron microscopy is also highly useful as it allows visualizing nanomaterials coupled with electron dense labels in different vesicular structures under very high resolution<sup>10</sup>. Atomic Force Microscopy (AFM) has also been used recently to demonstrate the interactions of nanomaterials with the cell membrane<sup>11</sup>.

Use of the inhibitors of the specific pathways is the most reliable method for the study of uptake of the materials by specific receptors. Some of the commonly used inhibitors from the each pathway are given in the table. After the use of the inhibitors the cellular uptake can be studied by using the confocal microscopy. The uptake of nanomaterials can be also quantified by flow cytometry, fluorescent microscopy or simple radioactivity sampling<sup>12</sup>. Some of the inhibitors used for study are given in table 2.

Imaging studies are always a problem for membrane-trafficking events as such it is often difficult to resolve different structures. Many kinds of actin structures are present in mammalian cells, that are used at various locations in a cell for structural support i.e. motility and trafficking. As well as, clathrin located in the plasma membrane, internal organelles such as endosomes and the Golgi complex. The studies of endocytosis can be performed by total internal reflection fluorescence (TIRF) microscopy which excites fluorescent molecules at the boundary between the active substances and the cover glass. In TIRF microscopy, the fluorescent molecules are excited with a beam of light (usually from a laser) which is directed at the sample at an angle that is greater than the critical angle such that all of the light is reflected at the coverglass-sample interface. Although no light gets into the sample, an evanescent wave is created on the sample side. This wave dissipates rapidly as the distance from the surface of the coverglass increases. The evanescent wave excites fluorophores that are within ~200 nm of the coverglass surface. TIRF has been used to reveal the actin polymerization bursts at endocytic sites in mammalian cells and the localization of clathrin at endocytic sites<sup>13,14</sup>.

**Table 2:** Different inhibitors used and their specific functioning.

Name.	Function
CPM	Inhibitor of Rho GTPase (clathrin)
Genistein	Inhibit F-actin recruitment to clathrin pits (clathrin)
MbCD	Cholesterol-depletion reagent (caveolae/ lipid rafts)
Lovastatin	Cholesterol depletion (caveolae/lipid rafts)
CytD Inhibit	F-actin polymerization (cytoskeleton)
Nocodazole	Disrupts microtubule (cytoskeleton)
BMA1	Inhibits endosome acidification (endosome)
CRQ	Inhibits endosome acidification (endosome)
BFA	Interferes with Golgi, endosome and lysosome
NaN3	ATP inhibitor (ATP)
DMA	Na <sup>+</sup> /H <sup>+</sup> exchanger inhibitor (macropinocytosis)
WMN	PI3K inhibitor (macropinocytosis)
PTX	Inhibitor of Gi a subunit (GPCR)
CTX	Activator of Gs a subunit (GPCR)
U-73122	PLC inhibitor (downstream of GPCR)
SRP	PKC inhibitor (downstream of GPCR)
NCM	Melanosome inhibition (melanosome)
TrpI	Inhibitor of PAR-2 pathway (melanosome)
AcLDL	Ligands for scavenger receptor
PolyI	Scavenger receptor inhibitor
FCD	Scavenger receptor inhibitor
LDL	Ligands for LDL receptor

## MECHANISMS OF THE CELLULAR UPTAKE OF THE NANOPARTICLES:

When the nanoparticles are placed in the milieu of the cells they will be taken up by the cells. As partitioning across membranes is not possible for macromolecules, entry into cells is largely governed by biological mechanisms of endocytosis<sup>15</sup>. Endocytosis occurs in several steps mainly includes three steps. In the first step the molecule will be engulfed by the cell membrane invaginations to form vesicles known as endosomes in the cells which are variant in number in the cells. The second step involves movement of the engulfed molecule to different sites in the cell based on their nature. Then in the third step they will reach the required sites in the cell or get released from the cell after destruction or get transferred to other cells by a mechanism known as transcytosis.

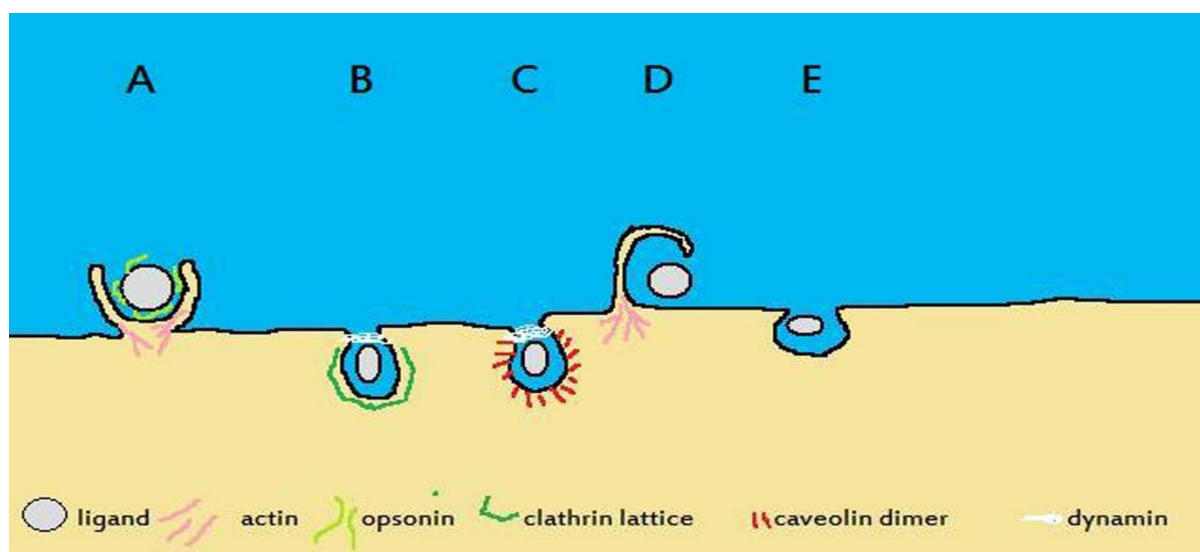


Endocytosis include the uptake by phagocytosis, pinocytosis and receptor mediated endocytosis. These processes are given in brief in table 3. These processes are integral to key physiological functions such as intracellular digestion and cellular immunity. Endocytotic pathways into cells can either lead to the endosomal and lysosomal compartments (conventional endocytosis) or else via cell-surface lipid raft associated domains known as caveolae which avoids the degradative fate of material entering the endosomal/lysosomal system. Phagocytosis of large particles (0.25–10  $\mu\text{m}$ ) will be performed by specialized cells such as macrophages and neutrophils, and a variety of other endocytic processes at a smaller scale. Pinocytosis involves the processes such as clathrin mediated endocytosis (CME), clathrin independent endocytosis. Clathrin independent again includes potocytosis or caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis, which further includes Arf6-dependent, flotillin-dependent, IL2R $\beta$ -dependent, CLIC/GEEC type and RhoA-dependent endocytosis. The routes of endocytosis most prominently described in the literature in recent

years are the receptor-mediated routes. These routes require recognition of some ligand (surface molecule or epitope) by a specific biological receptor. However, the receptor-mediated routes of uptake do not account for all uptake of material into cells, and other mechanisms which could come under the term pinocytosis described in earlier literature and which can account for modes of uptake not involving receptors must also be operative. These routes of uptake, which include macropinocytosis, can potentially allow uptake of materials up to 300 nm in diameter. All these endocytic routes of uptake involve delivery of material into a subcellular compartment, the endosome, which is still separated from the cytoplasm of the cell by a membrane. Most of these endocytic routes also end up in a degradative compartment of the cell, the lysosome, where materials are exposed to high concentrations of a wide variety of hydrolytic enzymes active on proteins, polysaccharides and nucleic acids. Figure 1 shows various mechanisms involved in the endocytosis process. Different forces that are effecting the cellular uptake are give in table 4.

**Table 3:** Different uptake mechanisms and their description<sup>16-18</sup>.

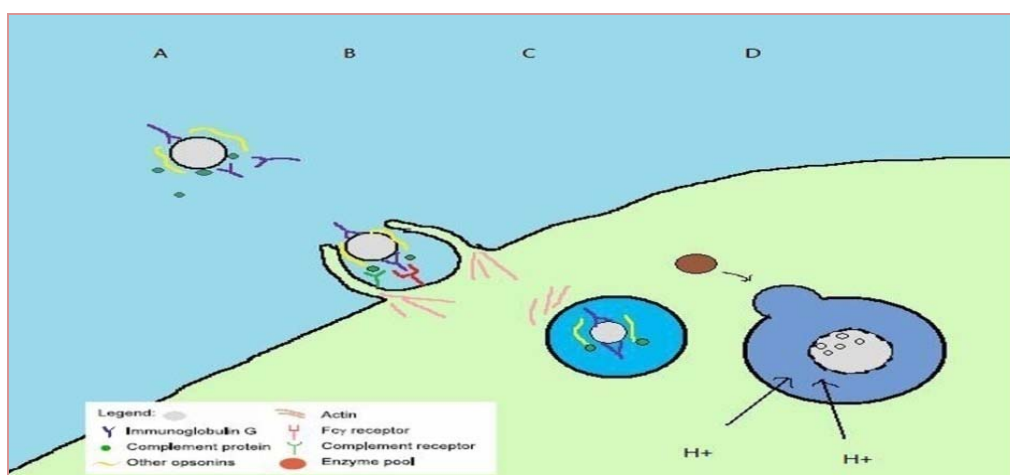
Mechanism	Description in brief
Endocytosis	A complex and highly regulated process of macromolecule and particle internalization by cells; includes two subcategories: phagocytosis and pinocytosis.
Phagocytosis	'Actin-dependent endocytic mechanism restricted to professional' phagocytes: macrophages, dendritic cells and neutrophils; also defined as 'cell eating', uptake of large particles; subcategories include Fc $\gamma$ receptor-, complement receptor- and mannose receptor-mediated phagocytosis
Pinocytosis	Endocytic mechanism for the cellular uptake of fluids and solutes, which is further subcategorized into macropinocytosis (endocytic vesicle (EV) size >1 $\mu\text{m}$ ); clathrin-mediated endocytosis (EV size ~120 nm); caveolin-mediated endocytosis (EV size ~60 nm) and clathrin- and caveolin-independent endocytosis (EV size ~90 nm); only macropinocytosis is actin-dependent, the three other pathways are actin-independent.



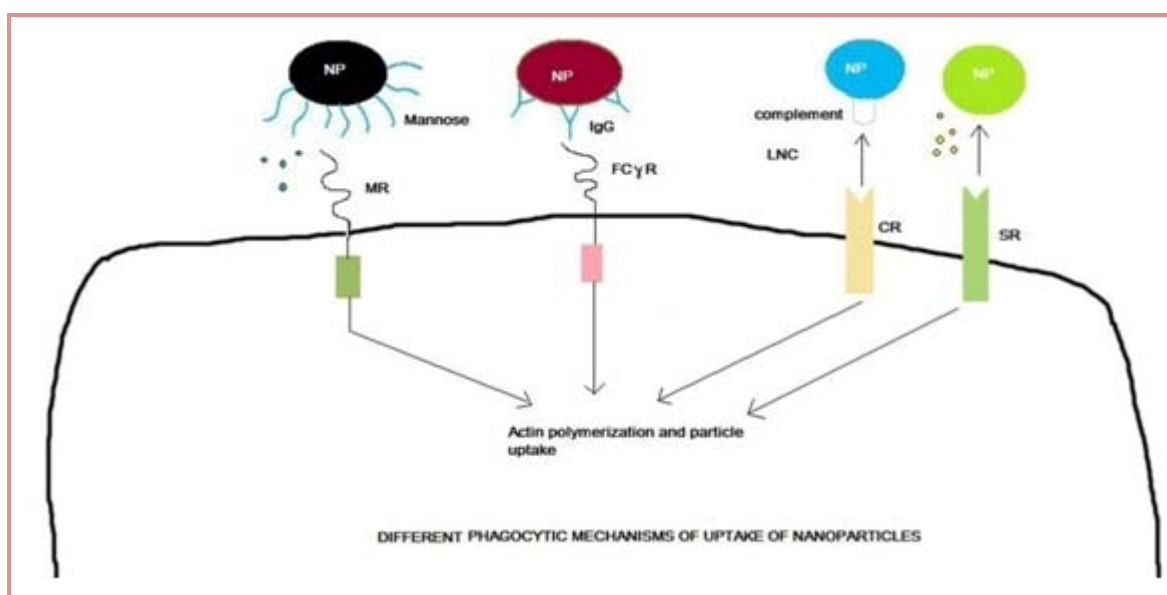
**Figure 1:** Endocytosis of the nanoparticles by different mechanisms. A) phagocytosis, B) clathrin mediated endocytosis (CME), C) caveolae mediated endocytosis ( CvME), D) macropinocytosis, E) other clathrin independent mechanisms.

**Table 4:** Forces that resist and promote nanoparticle wrapping at the surface membrane.

	Resistive forces
<ul style="list-style-type: none"> <li>➤ <u>Specific binding</u>: ligand–receptor interactions</li> <li>➤ <u>Nonspecific binding</u>: particle surface characteristics</li> <li>➤ Free energy release at contact site</li> <li>➤ Optimal particle size and shape</li> <li>➤ Energy-dependent membrane and cytoskeletal components, motile forces (for example in the formation of a clathrin cage that binds to cytoskeletal proteins)</li> </ul>	<ul style="list-style-type: none"> <li>➤ Stretching and elasticity of cell membrane</li> <li>➤ Thermal fluctuations of cell membrane</li> <li>➤ Receptor diffusion to adhesive front</li> <li>➤ Hydrophobic exclusion of polar surface by surface membrane</li> <li>➤ Stretching of receptor–ligand bonds, bond elasticity factor</li> </ul>



**Figure 2:** Schematic representation of typical process of phagocytosis.



**Figure 3:** Schematic representation of various receptors involved in the uptake of the nanoparticles by phagocytic cells. MR (mannose receptor) receptors causes the uptake of nanoparticles coated by mannose units. FC $\gamma$ - receptors mediates the the binding of particles opsonised by immunoglobulins (IgG & M). CR (complement receptor) receptors mediates the uptake of the nanoparticles having surface attached proteins complementary the the receptor. SR (scavenger receptor) receptors mediates the scavenging of some nanoparticles by noninflammatory pathways.

### a. Phagocytosis

Phagocytosis occurs mainly by the phagocytic cells as monocytes, macrophages, neutrophils and dendritic cells and also to a lesser extent by the fibroblasts and endothelial cells<sup>18</sup>. The phagocytic process is facilitated by factors called opsinins located naturally in blood plasma that coat the foreign particles and contain the receptors that then allow leucocytes or other phagocytes to attach to the element. Most commonly observed opsinogens are immunoglobulins (IgG, IgM) and some complements (C3, C4, C5) and some other blood proteins. Some of the receptors involved in phagocytosis are Fcγ Receptor, Complement Receptor (CR), Mannose Receptor mediated and scavenger receptor mediated phagocytosis. Fcγ receptor receptors binds to the surface immunoglobulins present on the surface of the particle. Complement receptors bind to the complement proteins adsorbed on the particle surface. Mannose receptors bind to the mannose units present on the surface<sup>19</sup>.

On the interaction of the ligand molecules with the receptors actin molecules rearrange leading to the formation of phagosomes which engulf the particles. Through a series of fusion and fission events, the vacuolar membrane and its contents will mature, fusing with late endosomes and ultimately lysosomes to form a phagolysosome. If the material is too large for the cell to phagocytose, this situation is called frustrated phagocytosis during which phagocytes release proteases, free radicals, and lysosomal enzymes that attempt to slowly degrade the material. The rate of these events depends on the surface properties of the ingested particle, typically from half to several hours [19]. The phagolysosomes become acidified due to the vacuolar proton pump ATPase located in the membrane and acquire many enzymes, including esterases and cathepsins<sup>20</sup>. The enzymatic content of these intracellular vesicles is a key issue for synthetic polymeric nanoparticles, since polymer biodegradability is required in pharmaceutical applications, both to ensure drug release and to avoid accumulation of the ingested material, which can lead to further toxicities. The typical process of phagocytosis is shown in the figure 2.

The size and shape dependence of nanoparticle uptake was given in several articles<sup>18</sup>. Different nanoparticles taken up by this mechanism are Polystyrene based nanoparticles<sup>21</sup>, poly(alkylcyanoacrylate) based nanoparticles<sup>22</sup>, Liposomes<sup>23</sup> etc.

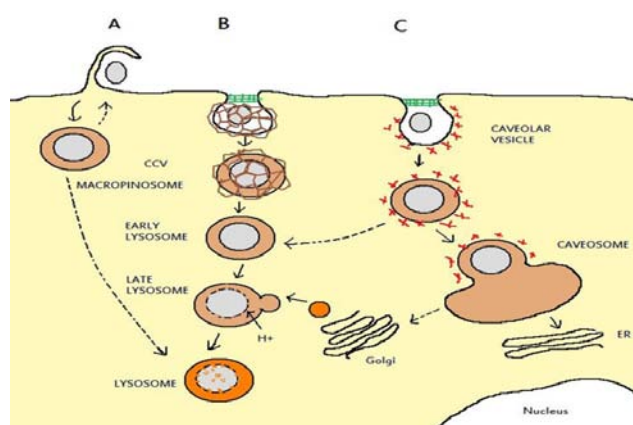
### a. Clathrin-Mediated Endocytosis

Endocytosis via clathrin-coated pits, or clathrin-mediated endocytosis (CME), occurs constitutively in all mammalian cells, and fulfills crucial physiological roles, including nutrient uptake and intracellular communication. For most cell types, CME serves as the main mechanism of internalization for macromolecules and plasma membrane constituents. This mechanism involves the binding of the ligand molecules to the receptor followed by assembly proteins such as AP-2 and AP180 mediation

for assembly of the clathrin on the membrane. Formation of the endocytosis vacuole is driven by assembly of a basket-like structure<sup>24</sup> formed by polymerization of clathrin units. Clathrin is a three-leg structure called triskelion. These triskelia assemble in polyhedral lattice just on the cytosolic surface of the cell membrane, which helps to deform the membrane into a coated pit of approximately 150 nm. Slowly clathrin polymerises to form polygonal lattice which results in formation of a coated pit. Then the dynamin (GTPase protein) comes to the neck of the pit and forms spiral collar. Then the GTPase undergoes hydrolysis which promotes the scission of the membrane and the formation of clathrin coated vesicles (CCV)<sup>25</sup>. These slowly get converted to endosomes by the removal of the clathrin coat and further to lysosomes. The resulting endocytic vesicle may have an average size of 100<sup>26</sup> or 120 nm. The clathrin molecules will be recycled by going back to the membrane.

Various accessory proteins which aid in the scaffold formation amphiphysin, Eps15 and intersectin<sup>27</sup>. This mechanism is responsible for the uptake of essential nutrients like cholesterol carried into cells by low density lipoprotein (LDL) via the LDL receptor, or iron carried by transferrin (Tf) via the Tf receptor. These proteins are now commonly used as markers of CME. CCV are major carriers for protein and lipid cargo at the plasma membrane, the transGolgi network.

Different nanoformulations taken up by this mechanism are Polystyrene based nanoparticles<sup>28</sup>, PLA or PLGA nanoparticles<sup>29</sup>, PLA-PEG nanoparticles<sup>30</sup>, Chitosan-based/coated nanoparticles<sup>31</sup>, Silica-based nanomaterials (SNTs)<sup>32</sup> etc.



**Figure 4:** Different methods of pinocytotic cellular uptake. A) macropinocytosis, B) Clathrin mediated endocytosis, C) Caveolae mediated endocytosis.

### b. Caveolae-Mediated Endocytosis

It is generally clathrin mediated endocytosis is dominating mechanism for cellular uptake by pinocytosis. Other mechanisms are also identified for cellular uptake of which caveolae mediated endocytosis (CvME) is of importance. Unlike CME, CvME is a highly regulated process involving complex signalling, which may be driven

by the cargo itself. Caveolae are characteristic flask-shaped membrane invaginations, having a size generally reported in the lower end of the 50–100 nm range<sup>33,27</sup>. They are lined by caveolin, a dimeric protein, and enriched with cholesterol and sphingolipids. Caveolae are particularly abundant in endothelial cells, where they can constitute 10–20% of the cell surface<sup>17</sup>, but also smooth muscle cells and fibroblasts. After binding on to the surface of the cell, the particle slowly moves into the invaginations on the surface and form the sack like structure which will undergo fission from the membrane, mediated by the GTPase dynamin, then generates the cytosolic caveolar vesicle. The vesicle formed is not having enzymatic degradation by lysozyme as in the CME. This is the mechanism followed by several microorganisms to escape the cellular lysis. This mechanism can be targeted to decrease the degradation of the particles in the cell by changing the surface properties of the particles carrying drugs (e.g., peptides, proteins, nucleic acids, etc.) highly sensitive to enzymes. CvMEs are involved in endocytosis as well as transcytosis of various proteins; they also constitute a port of entry for viruses (typically the SV40 virus)<sup>34</sup> and receive increasing attention for drug delivery applications using nanocarriers. Other endocytic proteins involved in CvME are cavin, Src, actin, PKC. On the whole, the uptake kinetics of CvME is known to occur at a much slower rate than that of CME. Ligands known to be internalized by CvME include folic acid, albumin and cholesterol. Another example is eNOS binds to the caveolin-1 scaffolding domain and remains inactive when bound. The ligands, which disrupt this interaction, enable eNOS activation, which leads to production of nitric-oxide (NO) and increases vascular permeability.

Different nanoformulations following this mechanism are Polymeric micelles with cross-linked anionic core<sup>35</sup>, DOXIL<sup>®326</sup> Polysiloxane nanoparticles<sup>37</sup>, Quantum Dot nanoparticle<sup>38</sup>.

### c. Macropinocytosis

It is a type of clathrin and caveolae independent endocytosis, occurring in many cells, including macrophages [39]. This also similar to that of phagocytosis by the protusions in the membrane driven by actin. But the difference is that the protusions do not zipper up along the ligand-coated particle, instead, they simply melt from the membrane to form vesicles termed as macropinosomes, which sample the extracellular milieu and having the size around 0.5-10  $\mu\text{m}$ . The intracellular fate of macropinosomes varies depending on the cell type, but in most cases, they acidify and shrink. This endocytic pathway does not seem to display any selectivity, but is involved, among others, in the uptake of drug nanocarriers.

### d. Other Endocytosis Pathways

Other clathrin- and caveolae- independent endocytosis pathways have also been described. In particular, pathways similar to CvME involving cholesterol-rich micro

domains called 'rafts,' having a 40–50 nm diameter, have received increasing attention<sup>17</sup>. However, the understanding of their implications in the interactions with drug delivery nanosystems is still in a nascent stage. Based on the effectors the caveolae- and clathrin-independent pathways are presently classified as Arf6-dependent, flotillin-dependent, Cdc42-dependent and RhoA-dependent<sup>40</sup>. All these pathways appear to require specific lipid compositions and are dependent on cholesterol.

Some of the factors that are causing the alterations in the cellular uptake are given in the table 6 below.

**Table 6:** Main bio-physicochemical influences on the interface between nanomaterials and biological systems.

Nanoparticle	Nano-Bio interface
<ul style="list-style-type: none"> <li>• Size, shape and surface area</li> <li>• Surface charge, energy, roughness and porosity</li> <li>• Valence and conductance states</li> <li>• Functional groups</li> <li>• Ligands</li> <li>• Crystallinity and defects</li> <li>• Hydrophobicity and hydrophilicity</li> </ul>	<ul style="list-style-type: none"> <li>• Membrane interactions: specific and nonspecific forces</li> <li>• Receptor-ligand binding interactions</li> <li>• Membrane wrapping: resistive and promotive forces</li> <li>• Biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects</li> <li>• Free energy transfer to biomolecules</li> <li>• Conformational change in biomolecules</li> <li>• Oxidant injury to biomolecules</li> <li>• Mitochondrial and lysosomal damage, decrease in ATP</li> </ul>

## CONCLUSION

Because of the advancement in the nanotechnology, nanoparticles are finding much more application in delivering drugs to various target sites in the body. It becomes necessary to understand the internalisation of the particles in the cells and targeting to the specific cells. Nanoparticles enter into the cells by the mechanism of endocytosis of which the most common is phagocytosis. The particle surfaces of the nanoparticles can be tailored to make it able to be uptake by specific mechanism by specific cells. Now-a-days nanoparticles are finding more application in the diagnosis because of their ability to enter cells. Thus it is very necessary to understand the specific uptake mechanism of these nanoparticles. There are very limited blockers available for receptors involved in uptake. So it is necessary to develop more specific blockers for efficient study of the uptake of nanoparticles.



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