Cellular Trafficking and Subcellular Interactions of Cationic Gene Delivery Nanomaterials

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Abstract: Various cationic nanobiomaterials have been widely used as gene delivery nanosystems (GDNSs) *in vitro* and *in vivo.* Various cellular machineries are involved in trafficking of GDNSs, whose surface functional moieties and architectural properties confer great potential to interact with cell membranes and subcellular biomolecules. It appears such intrinsic inadvertent biological functionalities may impact the outcome of the biomedical applications of these nanobiomaterials. Various advanced materials used as GDNSs may display selective phenotypic effects in target cells/tissues as a result of initiation of various signaling pathways perhaps due to its cellular interactions with plasma cell membranes and/or intracellular compartments including genetic materials. Thus, better understanding about cellular/molecular impacts of GDNSs may maximize their clinical outcomes and accordingly minimize their inevitable undesired consequences. The main focus of this review is based on the cellular trafficking and interactions of cationic gene delivery nanobiomaterials with target cells or subcellular compartments.

Key Words: Cellular trafficking, gene delivery systems, cationic gene therapy, nanobiomaterials.

INTRODUCTION

The development of gene-based nanomedicines (e.g., antisense, siRNA) is being established as the creation of a new pharmacology, where the receptor for a designated genomedicine is a specific sequence of nucleotides in a target RNA [1]. A key step in the development of such platform appears to be the understanding of the structure, functions, and eventually metabolism/degradation of RNA. Of different kinds of gene-based medicines, antisense and short interfering RNA (siRNA) are the most studied gene therapies. For designing of the antisense-based pharmaceuticals, apart from the relatively less successful utilization of computer programs (e.g., MFOLD8) to predict RNA folding, a strategy of 'gene-walking' has been recruited, whereby a series of oligodeoxynucleotides (ODNs) are generated against the target mRNA to identify active sequences despite being strenuous [1]. However, two new strategies (i.e., RNaseH mapping and scanning combinatorial oligonucleotide arrays) have also been exploited with somewhat successes [2-4]. The prim step for successful development of the gene therapy (e.g., antisense technology) is detailed understanding of the mechanism of action. Still, it is not exclusively obvious where and how antisense reaches its target(s), in particular in case of *in vivo* implementations [5 Basically, on the basis of mechanism of action, it is deemed that two classes of antisense oligonucleotide can be discerned, including: a) the RNaseH dependent oligonucleotides that induce the degradation of mRNA; and b) the steric-blocker oligonucleotides that physically prevent or inhibit the progression of splicing or the translational machinery [6].

Most of the antisense drugs investigated in the clinic appear to function through an RNaseH dependent mechanism. The RNaseH is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA/DNA duplex. RNaseH dependent oligonucleotides can result in 80-95% down-regulation of protein and mRNA expression. They can also inhibit protein expression when targeted to virtually any region of designated mRNA. Most of the steric-blocker oligonucleotides seem to be efficient when targeted to the 5' or AUG initiation codon region [7, 8]. Although it has been demonstrated that a 5-bp region of homology is sufficient to induce RNaseH activity [9], the precise mechanism by which RNaseH recognizes duplexes is not fully understood [6]. Antisense molecules steric interferences on distinct RNA regions appear to inhibit pre-mRNA splicing or polyadenylation editing and/or initiate mutations within the encoding gene [10]. For detailed mechanism of action of antisense, reader is directed to see the following citations [11, 12].

For delivery of gene-based therapies to target cells/tissues, both viral and nonviral vectors have been so far exploited. However, use of the viral vectors (e.g., retroviruses and adenoviruses) have been somewhat limited due to their immunogenic impacts when used in

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clinical gene therapy protocols [13]. Despite their high transfection efficiency, the preparation and purification of the viral vectors were shown to be laborious, cost-prohibitive and not amenable to industrial-scale manufacture [14]. Cationic lipids (Figures **1** and **2**) or polymers (Figure **3**) such as Lipofectin™, Oligofectamine™, starburst polyamidoamine dendrimers, linear or branched polyethylenimine are advanced nanomaterials that are used as potentially relatively safer non-immunogenic alternatives, whose development has incorporated diverse technologies in attempts to mimic the efficient gene delivery capacity of viruses.

Their low immunogenicity, lack of pathogenicity, and ease of pharmacologic production continue to make them as attractive gene delivery systems [15], nevertheless our recent investigations resulted in inadvertent occurrence of toxicogenomics by theses cationic lipids and polymers [16, 17]. They also continue to suffer from relatively low levels of gene transfer compared to

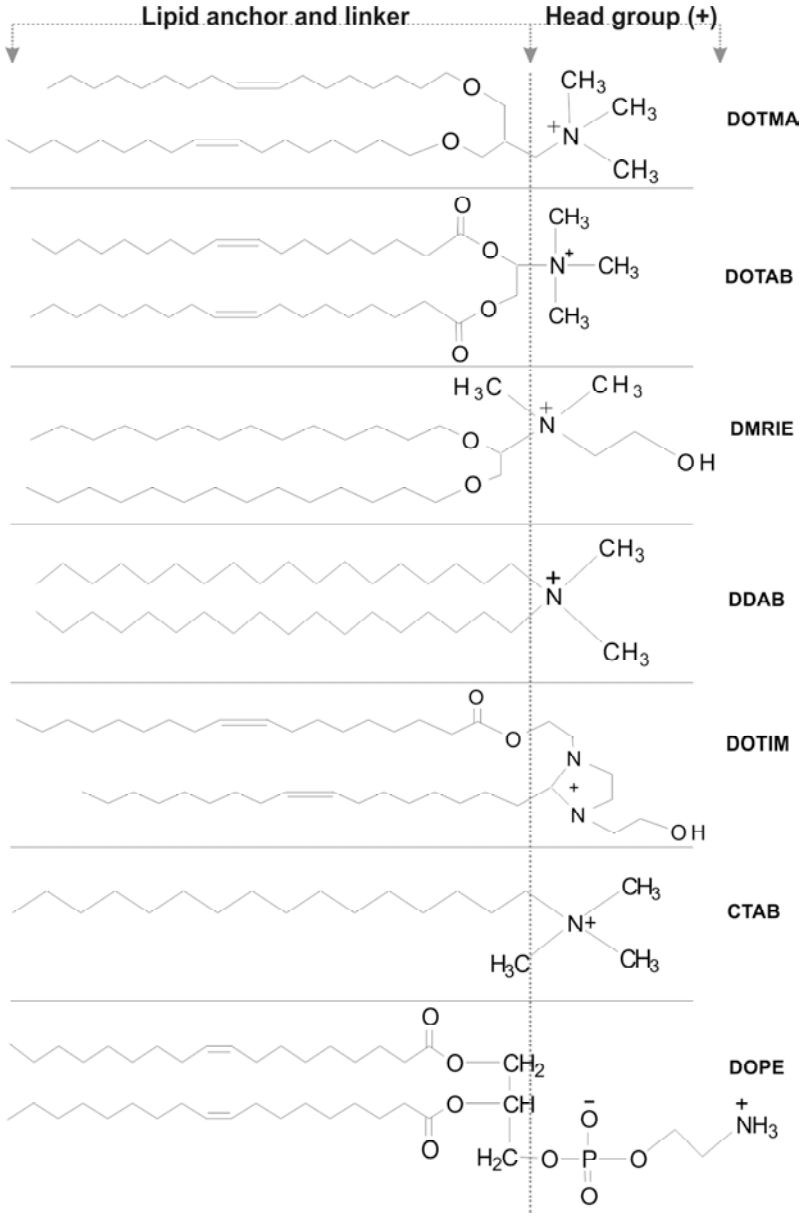

Figure 1: Selected examples of commonly used monovalent cationic lipids in the formation of cationic liposomes. DOTMA: N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride/bromide; DOTAP: N-1(-(2,3-dioleoyloxy)propyl)-N,N,N-trimethyl-ammonium methylsulphate; DMRIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyl ethyl ammonium bromide: DOPE: 1,2-dioleoyl-sn-glycero-3-L-a-phosphatidylethanolamine; DOTIM: 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride; CTAB: cetyl-trimethyl-ammonium bromide; DDAB: dimethyldioctadecylammonium bromide.

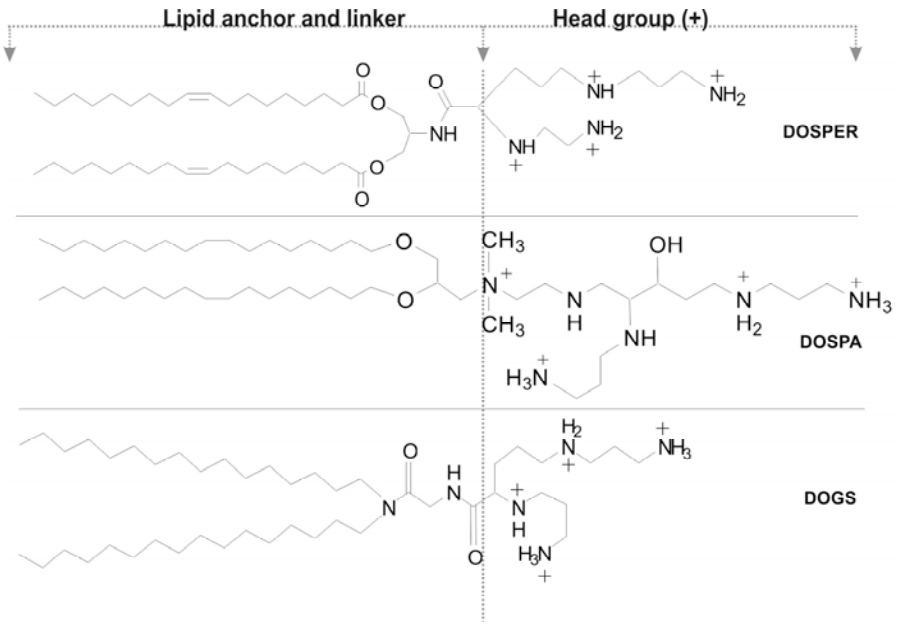

Figure 2: Selected examples of commonly used polyvalent monovalent cationic lipids in the formation of cationic liposomes. DOSPER: 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid; DOSPA: 2'-(1",2"-dioleoyloxypropyldimethyl ammonium bromide)-N-ethyl-6-amidospermine tetratrifluoroacetic acid salt (LipofectAMINE™);. DOGS: spermine-5-carboxy-glycinedioctadecylamide.

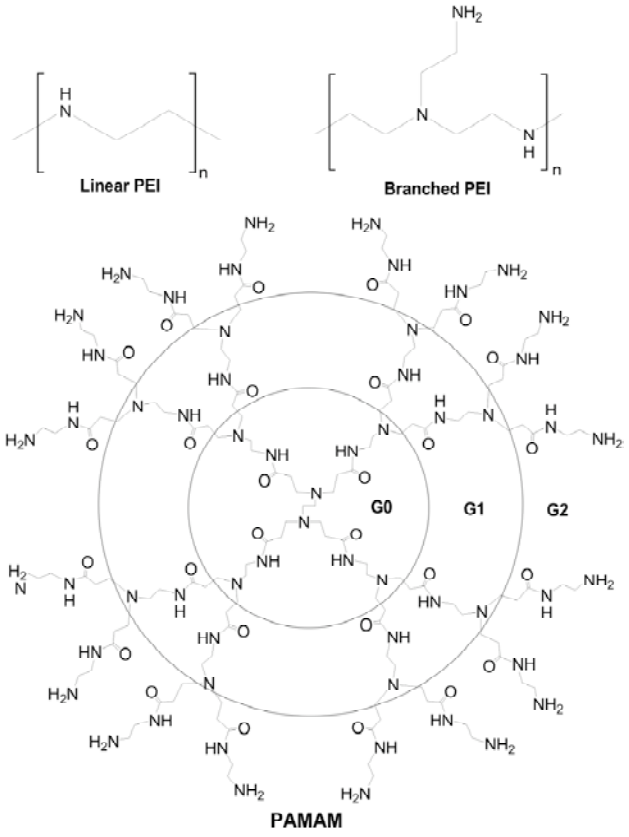

Figure 3: Selected examples of commonly used polycationic polymers. PEI: Poly(ethylenimine); PLL: Poly(L-lysine); PAMAM: Polyamidoamine dendrimer. G0-G2 represent generation 1-2 of PAMAM dendrimer.

viruses, thus there has been considerable progress in increasing the levels of expression using nonviral vectors [18-20].

Numerous studies on vector-cell interactions have reported that nonviral vectors are capable of efficient binding and entering the target cells, however they

yield low transfection. Indeed, critical membranes and barriers exist from the assembly of the vector particle to its disassembly inside the cell, resulting in not premeditated biological impacts. The main focus of this review has thus been devoted to the cellular and/or intracellular interactions of these cationic lipids/polymers for better understanding of their genomic influences.

CELLULAR TRAFFICKING OF CATIONIC GENE DELIVERY NANOBIOMATERIALS

Ideally, corresponding properties for a designated nonviral vector should include: a) protecting DNA against degradation by nucleases, b) facilitating transport of DNA across biological barriers and membranes, c) transporting DNA to the target cells (normally via receptor-mediated endocytosis pathway), and d) promoting the import of DNA into the nucleus. Once the selected nucleic acids inside the target cells, they must overcome the subcellular and/or biomolecular impacts [15]. In fact, the amphipathic sheet like lipid bilayer architecture of the cell membranes along with the integrated proteins separate cells from their environment and form the boundaries of different organelles inside the cells, as a result of which exchange of materials among the different parts of a cell is selectively controlled [21]. Physicochemical properties (in particular chemical architecture, size and surface chage) of GDNSs appear to be cornerstone of its interactions with cellular biomolecules. Accordingly, positively charged cationic polymers are able to bind to the negatively charged surface of target cells mainly through electrostatic interactions [22]. These delivery systems, after binding to cell surface, may internalize by means of one or both of two types of cell membrane transport machineries, i.e. receptor and/or non-receptor mechanisms [15]. Figure **4** represents schematic vesi-

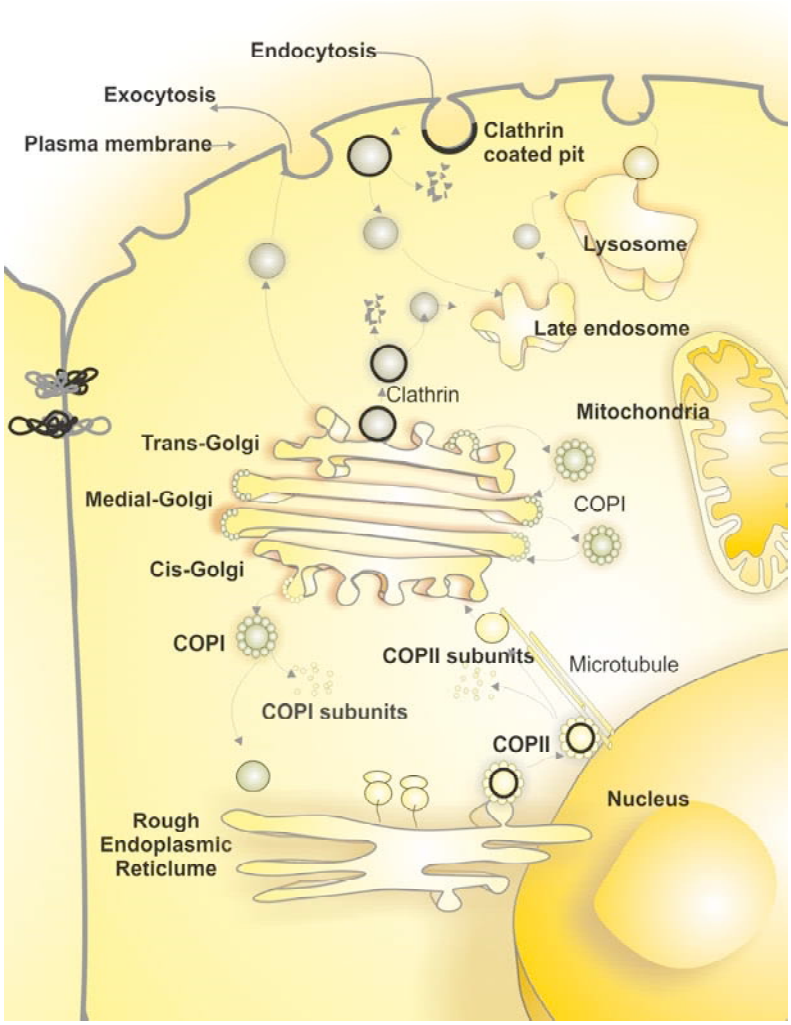

Figure 4: Schematic representation of vesicular trafficking in cell used for delivery of macromolecules.

cular trafficking of macromolecules (e.g., cationic polymer/lipid based genomedicines) which are basically performed through vesicular transport machineries (e.g., clathrin coated pits or caveolea membranes).

In the vesicular transportation pathways, gene-based nanomedicines may engineer its own escape from demise in the lysosome. Lysosomes contain approximately 40 different hydrolytic enzymes that mediate controlled intracellular degradation of macromolecules such peptides and proteins. Because of uniqueness of these organelles in terms of composition and pH, they are of particular interest for the design and delivery of pH-dependent nanomedicines and prodrugs. Genetic deficiency in lysosomal hydrolases or proteins involved in the efflux of metabolites causes lysosomal storage diseases such as ocular manifestation of the mucopolysaccharidosis, where accumulation of undigested metabolites often results in ocular and neurological consequences [23]. These diseases can also occur as a result of some mutations leading to defective localization or trafficking of lysosomal hydrolases to lysosomes from the endoplasmic reticulum or Golgi complex. The gene-based nanomedicines still must be uncoated and release its DNA cargo prior to completing its mission to enter the nucleus [15]. Within the cytosol, the naked DNA and nonviral vector are subjected to a group of binding proteins and enzymes that may result in inadvertent undesired consequences. The delivery system should be cleared from target cells, while the nucleic acids ought to cross through very small and selective nuclear pores. Figure **5** shows the main transport machinery of the cell.

All these processes, one way or another, reveal known/unknown interactions of these gene delivery nanosystems with cellular components as seen for lipoplex-cell interactions [24].

Figure **6** represents the fluorescence images of the internalized antisense labeled with cyanine 3 (panel A) or FITC (panel B) to inhibit the expression of epidermal growth factor receptor (EGFR) in the human breast cancer MCF7 cells (our unpublished data) and lung cancer A549 cells [25].

The intracellular uptake of antisense ODNs linked to transferrin and folic acid was more effective than addition of unmodified antisense [26-28]. Similarly, antisense ODNs targeted to cancer cells via the epidermal growth factor receptor [29] or to dendritic cells through the mannose receptor [30] are taken up more efficiently than naked ODNs. However, once the plasma membrane barrier is overcome by exploiting the endocytic entry path, the next intracellular barrier constitutes the endosomal membrane, which can be considered the crucial limiting step in the overall pathway that eventually elicits the antisense effect [31].

CELLULAR TRANSPORT MACHINERIES

Developing gene and drug delivery tools and more efficient transport is directly related to understanding of

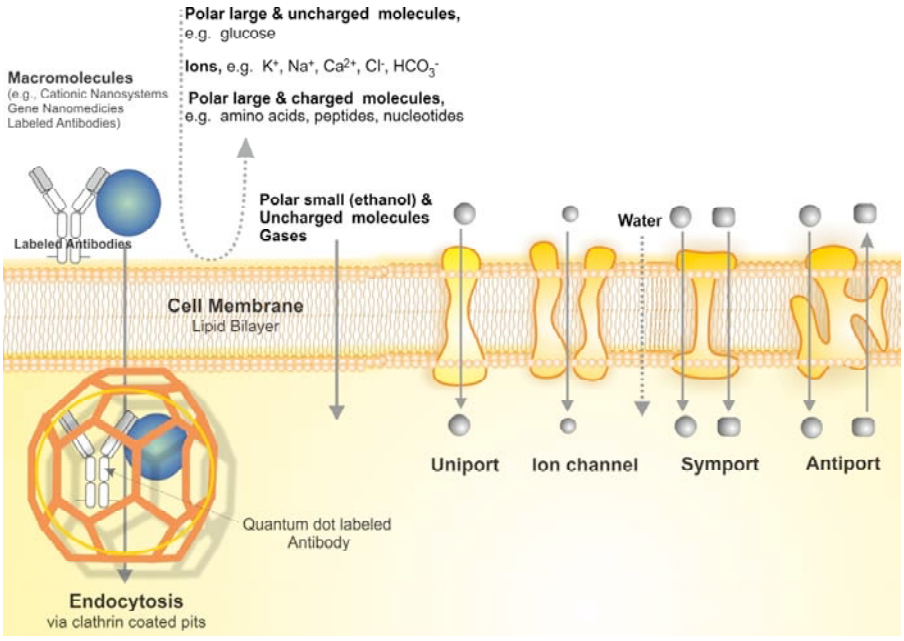

Figure 5: Schematic illustration of cellular transport machineries.

how cellular transport machineries works, in particular endocytosis pathway mechanisms. Hence comprehension of this concept as well as intracellular trafficking leads to effective nano- carries design and as well

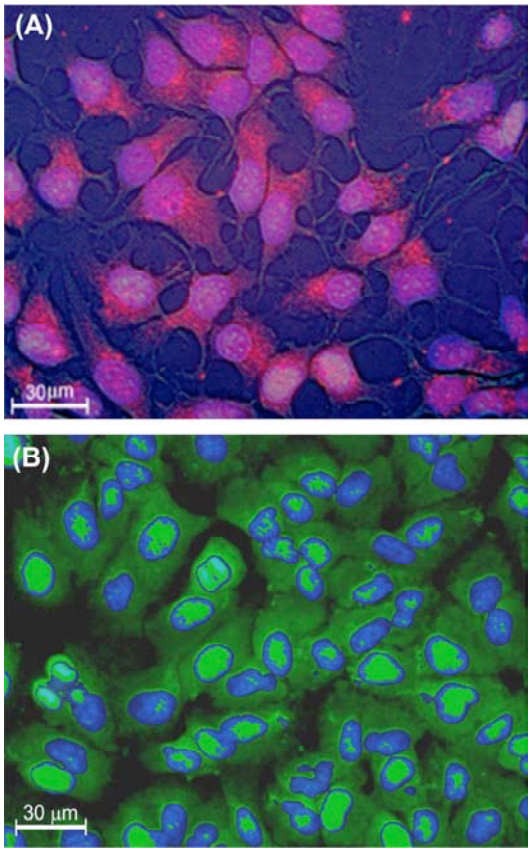

Figure 6: Fluorescence microscopy images of delivered fluorescent-labeled As-ODN in MCF7 and A549 cells. Panel A is a superimposed image of MCF7 cells, representing Cy3-ODN transfected cells (red), nucleus stained with DAPI (blue) and cells itself as phase contrast (unpublished data). Panel B is a superimposed image of A549 cells, representing FITC-ODN transfected cells (green), nucleus stained with DAPI (blue) [25]. As-ODN: antisense oligonucleotides; Cy3:Cyanine 3; FITC: fluorescein isothiocyanate.

entry into cells and target therapy. Thus, in the following subsections the main pathways for traverse of macromolecules into the cells are discussed. Receptor-mediated transport machinery Nanomedicines, as macromolecular cargos, are internalized through endocytosis process which could be a receptor-mediated, adsorptive and fluid phase phenomena. As shown in Figure **4**, during endocytosis, the folding cell membrane around macromolecules are generated leading to creation of protein-coated or noncoated vesicles (Lundmark and Carlsson). At a time when the object is completely enclosed, the vesicle budded and released into the cytoplasm (Figure **1**). It is then often intended for early endosome compartment, upon which it can be: 1) shuttled between membranes (original membrane or opposing membrane), 2) transported to late endosome and ultimately destined to the lysosome for degradation (Lundmark and Carlsson). Endocytosis is accomplished by different processes: 1) receptor mediated endocytosis, 2) adsorptive endocytosis and 3) fluid-phase endocytosis (Lundmark and Carlsson). As demonstrated in Figs. (**4** and **5**), the receptor mediated endocytosis internalize plasma membrane domains at specialized regions or domains of the plasma membrane including clathrin coated pits [32, 33], non-coated lipid rafts [34] and caveolae [35] thereby engulf any associated macromolecules into cells.

Clathrin Coated Pits

Clathrin mediated endocytosis as the main pathway provides specific delivery of various macromolecular ligands (e.g., nanomedicines) into the cells (Figure **7A**), so that clathrin coated pits as major coat proteins at the inner surface of membrane occupies 2% of total plasma membrane. The main scaffold clathrin protein consist of three heavy (180 kDa) and three light (40 kDa) chains whose assembly form a three-legged structure entitled a triskelion (Figure **7B**). These triskelion structures is favorable to stabilize the vesicle budding from the membrane [36]. The major budding process, converting a pit to a vesicle, is relied on correct assembly adaptors such as adaptins and clathrin assisted proteins, such as dynamin which is a cytosolic GTPase mediated biomolecule (Figure **7C**) and can be seen as a highly dense vesicle in micrograph of transmission electron microscopy (Figure **7D**).

Nanostructures are deemed to internalize through these kinds of vesicles which are first sorted to early endosomes and characterized by the presence of the Rab GTPase RAB5. The RAB5 participates in the fusion of early endosomes and the switch between RAB5 and RAB7 mediates the conversion of these endosomes to late endosomes. Adaptins are multisubunit proteins complex that mediate formation of clathrin-coated pits, through interaction with membrane-bound receptors such as epidermal growth factor receptor. Some specific amino acid motifs within the cytoplasmic domain of the receptor and globular domain at the end of each clathrin heavy chain (tyrosine-based and di-leucine motifs) are responsible for such interaction. Among three types of adaptins (AP-1, AP-2, AP-3), AP-2 is crucially involved in the formation of clathrin coated

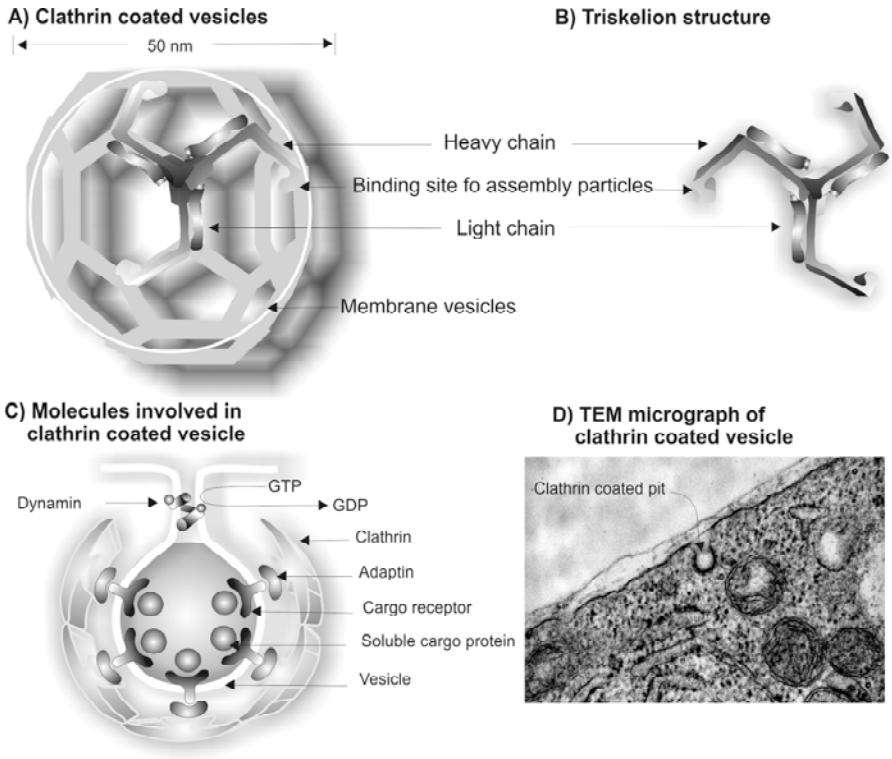

Figure 7: Clathrin mediated endocytosis as the main pathway for specific delivery of macromolecules. **A)** Schematic representation of a clathrin coated pit with **B)** its main scaffold (three-legged structure entitled as triskelion) consist of three heavy (180 kDa) and three light (40 kDa) chains. **C)** The major budding process via assembly adaptors such as adaptins and clathrin assisted proteins (e.g., cytosolic GTPase dynamin). **D)** A micrograph of transmission electron microscopy showing a highly dense vesicle.

vesicles at the cell surface [37]. Dynamin as a molecular motor is a phosphoprotein GTPase (96 kDa) that regulates budding or fission process at last stage through serving as a molecular switch or a mech-anochemical enzyme with right-handed twisting activity. All types of Dynamins (dynamin I, II and III) undergo oligomerization step via protein-protein intractions which leads to accumulation of other proteins (i.e. amphiphysin and endophilin) resulting in vesical forma-tion [37]. Dynamin is the only known to have a twisting activity. Additionally dynamin has been introduced to associate with intracellular events mediated by growth factor receptors, insulin receptors and the beta-adrenergic receptor [38].

It is deemed that an intermediate organelle called the multivesicular body (MVB) mediates the cargo transfer. These multivesicular structures are known as the endosomal carrier vesicle, for biogenesis of which ceramide is involved. Nanostructures destined for degradationare sorted from late endosomes to lysosomes, whose low pH facilitates the activation of enzymes that are responsible for cargo degradation. In fact, this is a key consideration for drug design and delivery of pH-sensitive molecules. Such cargos can also be directly recycled back from early endosomes to the plasma membrane through RAB4 GTPase, while cargo transfer from endosomes to the Golgi complex is carried out by RAB9 in the case of late endosomes.

Vesicles interaction

Clathrin coated vesicles, after being pinched by dynamin, enter into the cytoplasm. They are then subjected to uncoating ATPase phenomenon, mainly through heat shock protein Hsp70 [39], upon which the clathrin coat can be striped and the vesicle is directed to early endosomes around the plasma membrane. Depending on the cargo nature, vesicles can either fuse in the late lysosomes which are involved in the breakdown of the internalized material or retrieve to the membrane, perhaps towards recycling receptors. Two classes of proteins which act as the organizers of targeting and fusion of an endocytic vesicle with other vesicles are SNAREs and targeting GTPases, Rabs. Of these, the soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) is divided to vesicle- and target- SNAREs (v-SNARE and t-SNARE, respectively). Among 40 different Rab proteins distributed on cell membranes, Rab4 and Rab5C

associate with early endosomes and Rab7 and Rab9 with late endosomes, while Rab5A associate with plasma membrane and clathrin-coated vesicles. Rab proteins involve in the specific stages of vesicular transport in a quanine- nucleotide dependent manner. Once a cargo delivered into the cell GDP-bound Rab proteins (inactive form) activated through substitution of GTP for GDP. Activated Rab then mediate v-SNARE with the corresponding t-SNARE, thereby resulted helical domains interact to form a stable SNARE complex. Thereafter Rab proteins recycles to GDP-bound inactive form via intrinsic GTPase activity [40]. In addition to the clathrin coated pits, the endocytosis of macromolecules occurs through various cellular pathways, including caveolae membranes and lipid rafts [41, 42]. The latter process depends on extracellular fluid/matrix components as well as cell type [22].

Caveolae mediated vesicles are sphingolipid and cholesterol rich flask-shaped invaginations in the cell membrane that are smaller than clathrin coated pits and regulate cellular uptake as well as activity of various ion channels [15, 43]. Caveolae cluster some important receptors (e.g., epidermal growth factor, insulin and endothelin), cellular signal transduction components (e.g., PKC, MAPK, eNOS and calmodulin) and transporters (e.g. IP3 receptor and Porin), which enables caveolae membranes to act as a key cellular transport machinery [15. 44]. Additionally, membrane microdomains lipid rafts, enriched in cholesterol, glycosphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins and some membrane proteins, affect the membrane fluidity which is deemed to influence critical cellular processes such as endocytosis, ligand–receptor interactions and functional coupling of occupied receptor through G-proteins to effector enzymes [45]. Also it has been evidenced that there is close relationship between raft microdomains and non-clathrin uptake pathways since they are present in the lipid rafts while clathrin-mediated endocytosis markers are away from these lipid rafts; reader is directed to see [21].

Exosomes

It is deemed that, in many cases, exosomes as an intermediate organelle called the multivesicular bodies (MVBs; also known as the endosomal carrier vesicle) are 30-100-nm in diameter and mediates the cargo transfer [46, 47]. Recent work has shown that these nanostructures are important bioparticles from various viewpoints: 1) as a novel platform for immunotherapy, i.e. cancer vaccines [48, 49], 2) as vectors of mRNAs, 3) lipid mediators acting on target cells, 4) cell signaling and communications [50], and tumor biology and immune regulation [51]. Structurally, ceramide is involved in the biogenesis of these MVBs, which differentiates these vesicular bodies from late endosomes. Lipids and proteins sorted to the intraluminal vesicles of MVBs can be released into the extracellular space by fusion of the MVBs with the plasma membrane as exosomes. Exosomes are involved in different processes such as signalling and release of pathogenic peptides as well as antigen presentation [51]. These pathogenic processes could be inhibited by specifically directing the inhibitors to the exosome-specific MVBs. Proteins destined for degradation, such as the epidermal growth factor receptor (EGFR), are sorted from late endosomes to lysosomes, although the exact sorting mechanism is yet to be fully understood. The low pH of lysosomes facilitates the activation of enzymes that are responsible for cargo degradation; this is a key consideration for drug design and delivery of pH-sensitive molecules [47].

Transcytosis

Trancytosis, first introduced in capillary permeability studies, is a well known transport process through which the cargo moves from one side of the cells to the other side. It is a shuffling process and several trancytosis pathways are capable of avoiding lysosyme degradation depending on the cargo nature [52]. Nanomedicines, in general, enter cells exploiting the endocytic machineries presumably mainly involving clathrin-mediated endocytic pathway [22]. For example, it has been evidenced that DOTAP lipoplexes are internalized by cells solely via clathrin-mediated Endocytosis. However polyplexes prepared with polyethylenimine (PEI), a commercially available cationic polyamine first introduced by *Boussif et al*. [53], are internalized through both clathrin- and caveolae-mediated endocytic pathways [54]. It is believed that these paths have somewhat potential to initiate and stabilize membrane curvature formation, in which the adaptor proteins bind to clathrin pits and augment the inward pull of the membrane towards the cytoplasm leading to vesicle formation [55]. Because of different pattern of the DNA release from lipoplexes and polyplexes, escape of DNA from the endosome at an early stage of endocytosis or degradation of DNA in lysosomes may be occurred.Furthermore, negative charge of serum proteins may play an inhibitory role for nanomedicines entry into cells which could be overcome by various strategies during complexes formations such as providing slight alkaline pH and the presence of NaCl which leads to more efficient transfection [22].This

highlights increasing needs for rational design of novel strategies to overcome such intracellular impediments, likewise recently new strategy presented aiming to enhance non-viral delivery efficiency on the basis of receptor mediated endocytosis via surface modifications. For example, conjugated nanomedicine with the peptides derived from ligands internalize into cells through its cognate receptor mediated endocytosis pathway [56].

CELLULAR INTERACTIONS

Condensation and engulfment of DNA by nonviral cationic polymers or lipids are in favor with the internalization of the gene-based nanomedicines. The cationic nanostructures also interact with the plasma membrane mainly with the gel-like layer of proteoglycans that can serve as a selective molecular sieve to regulate the traffic of migrating cells and signaling molecules. The positive charges of the polycations were shown to mediate vector binding to proteoglycans [57], whose transfection potentials in the presence of glycosaminoglycan inhibitors (i.e., sodium chlorate) appear to be inhibited in a dose-dependent manner reducing gene expression up to 70% compared to untreated cells [58]. Intriguingly, sulfated proteoglycans were reported to act as cellular receptors for the cationic ligands, rather than only passive binding sites [59], and transfection is failed in the proteoglycan-deficient cells. Once inside the cytoplasm, DNA is released from vesicular compartment upon physicochemical properties of the gene based medicine. Such process may be affected by the flip-flop movement of the endosomal membrane cytoplasmic facing monolayer phospholipids, where negative charges of the phopholipides neutralize ion pair of positively charged head group of the cationic lipid. This phenomenon yields DNA release into the cell even though it is less reliable considering the large surface occupied by the cationic lipid bilayer over the endosome membrane surface [22]. The endosomal escape of DNA at an early stage of endocytosis is deemed to be critical for cytosolic DNA delivery and determination of overall transfection efficiency. Among cationic lipids and polymers, DOPE as a helper lipid for liposome-based DNA delivery were reported to induce membrane fusion between the endosome and the liposome and result in membrane destabilization and release of DNA into the cytoplasm [60]. Such destabilization of the vesicular membrane further highlights the interaction impacts of cationic lipids with cellular compartments. Utilization of the cell-specific ligands or antibodies was reported to lower the cytotoxicity, while facilitating tissue targeting [61]. The ligand choice is largely dictated by behavior of the target receptor that may undergo vesicular trafficking and accordingly the endocytic pathway used by the vector is dependent upon the targeting ligand as well as cell type. It is also likely that ionic and amphiphilic synthetic polymers interact with different membrane domains and alter membrane function(s). For example, we have previously reported that polypropylenimine diaminobutane (DAB) dendrimeric nanostructures can induce upregulation of epidermal growth factor receptor (EGFR) and its downstream signalling biomolecule Akt kinase in A431 and A549 cells [25]. Polyanions and polycations can respectively interact with positively and negatively charged groups of the membrane proteins, resulting in the formation of protein clusters within the membrane. Interaction of the polycations with negatively charged lipids may also result in neutralization of the lipid charges, at which lipid-polymer domains is formed due to lateral segregation of the lipids within the membranes [62]. Such interactions are believed to be largely dependent upon the polyion charge density and the hydrophobicity of the backbone itself as well as the side groups of polyions. It is deemed that the surface modification of polymeric vectors can alter its interaction potential with cells. For instance, surface modification of the cationic starburst polyamidoamine (PAMAM) dendrimers with either lauroyl chains or polyethylene glycol 2000 was reported to reduce the cytotoxicity these dendrimers in the Caco-2 cells because of reduction and/or shielding of the dendrimers surface positive charge [63]. Furthermore, the structural architecture of nonviral gene delivery nanosystems may cause inevitable changes in gene expression pattern in human epithelial cells [64], which is mainly dependent upon cell type, in particular the membrane lipid composition and membrane phase state [62]. Adsorption of polycations such as poly(N -ethyl-4-vinylpyridinium) salts (PEVP) in liposomic biomembranes was shown to induce flip-flop of negatively charged lipids (e.g., cardiolipin, phosphatidylserine, and phosphatidic acid) from the inner to the outer leaflet of the liquid liposomal membrane, but not in solid membranes [65, 66]. Further, it was reported that polycations such as poly-L-lysine (PLL), diethylaminoethyl-dextran (DEAE-DEX), poly-amidoamine (PAMAM), poly ethyleneimine (PEI) were shown to elicit the most dramatic increase in membrane permeability by interacting the membranous biomolecules and forming holes in lipid membranes [67-69]. Besides, intrinsic endosomolytic activity of some polycations may facilitate endosomal escape, whereas PLL polymers assist DNA release within the

cell via degrading by intracellular compartments thus result in significantly higher enhancement in gene expression [22]. Such structures could function as gates, through which the lipid molecules can be transported across the biomembranes [62]. Interestingly, the involvement of microtubule-associated motor proteins in the active transport of PEI:DNA nanocomplexes has also been reported [70]. These researchers reported that actively transported complexes in endosomes may undergo motor protein-driven movement guided by microtubules, or they may be physically associated with the motor proteins themselves. These nanocomplexes are deemed to use the same efficient mechanism for transport to the cell nucleus as several viruses (e.g., adenoviruses and adeno-associated viruses). The PEI:DNA nanostructures are thought to reach the perinuclear region within endosomes (that are actively transported on microtubules) and then break free of the vesicles before entering the nucleus. These complexes inside quasi-stationary endosomes may exhibit sub-diffusive behavior which can be decreased upon PEI:DNA nanocomplexes escape from their endosomal cage into the cytosol [70]. Based on differences in cell types, the gene delivery polyions can bind to the cellular compartments and thus may induce compartmentalization within certain areas of the membranes and inadvertently trigger various signaling paths. For example, PAMAM dendrimers were shown to induce nano-scale defects in cells through removing lipid from the fluid domains at a significantly greater rate than for the gel domains [71]. This reinforces compartmentalization effects of synthetic polymers within different membrane domains as well as a differential influence of polymers on functional systems in the membranes that consecutively provoke inadvertent cytoplasmic/nucleic consequences directly and indirectly via secondary messengers such as G proteins. Accordingly, we have shown that the cationic lipids and polymers induce wide-ranging gene changes in cells including molecules involved in cytokine and apoptosis signaling pathways [16, 17, 64, 72-74].

NUCLEAR EVENTS

The cytosolic release of ODNs is a prerequisite need for its nuclear translocation. Of released ODNs in cytoplasm, only a small fraction of internalized nucleic acids penetrates the nucleus. Delivery of nucleic acids may encounter the diffusional and metabolic barriers of various cellular compartments which can result in reduced number of intact molecules reaching the nuclear pore complex (NPC). In fact, nuclear translocation of DNA requires either the disassembly of the nuclear envelope or active nuclear transport via the NPC [75]. The nuclear envelope of a typical mammalian cell consists of inner and outer membrane layers perforated by 3000 to 4000 nuclear pores that are aqueous channels (100 different proteins with an external diameter of 120 nm) surrounded by large protein granules arranged in an octagonal array. Small DNA fragments in the cytoplasm can readily accumulate in the nucleus, presumably by diffusion through the nuclear pore and the passage of molecules with a MW of ~40 kDa appears to be via NPC [21]. Intriguingly, some researchers propose that by modulating the surface properties of the gene delivery systems, the kinetics of such transportation may be controlled, whereupon possibilities for programmable release of the carrier contents can be provided [31]. Diffusion rate of ODNs has been reported to be largely dependent upon its length [76]. These researchers reported that a fragment consisting of 100 bp appeared to be fully mobile in the cytoplasm, displaying a diffusion rate compatible to that of similarly sized fluorescein isothiocyanate (FITC) dextran. Small ODNs, up to several hundreds of base pairs, readily acquire access into the nucleus, where DNA fragments of all sizes were nearly immobile on a distance scale of ~1 micron and a time scale of several minutes. In contrast, similar sized FITC dextrans up to 580 kDa diffused (diffuse) freely in (through) the nucleus. The immobilization of DNA by the nucleus is probably because of extensive DNA binding to nuclear components, including the positively charged histones. These findings clearly highlight that diffusion of DNAs appear to be a significant rate-limiting barrier in the cellular processing of plasmids and large DNA fragments, particularly when diffusion and nuclear uptake compete with degradation by cytosolic nucleases [77]. Further, intranuclear ODNs may extensively bind to the nuclear RNA matrix [5]. When cationic lipids/polymers are used to deliver ODNs, the overall distribution pattern of the ODNs appears to be similar to that obtained upon microinjection of ODNs in the cytosol. The cationic lipids remain associated with endocytic compartments, but not the cationic polymers [78]. The cationic lipids have rarely been observed in association with the nuclear membrane [79]; however some cationic polymers were shown to mediate directly the delivery of ODNs at the nuclear membrane. A typical example is PEI [78] and PAMAM (our unpublished data) capable of mediating a cell cycle-independent nuclear entry of plasmid DNA and anti-EGFR ODNs, respectively. The transcriptional machinery responds to a multitude of endogenous and/or exogenous signals,

which trigger time-ordered expression of genes that control the cellular proliferation and differentiation, the cell cycle, and, eventually, the death of a cell. Controlled gene expression determines the spatiotemporal developmental pattern of cells/tissue. Complex transcriptional machinery sorts and integrates the vast amount of converging and diverging signals. It manages to increase/decrease, at the right moment, pre-messenger RNA production from a particular gene. It has been reported that PEI can prompt the nuclear delivery and DNA release in the nucleus, but not cationic lipids [80]. It is deemed that anionic lipids found on the cytoplasmic face of the plasma membrane may displace DNA from cationic liposomes and help DNA release in the cytoplasm. This could be different in the case of cationic polymers, as they may interact with nuclear targeted proteins and facilitate nuclear transport of plasmid DNA.

Once released into the cytoplasm, DNA should be addressed into the nucleus in order to be transcribed. For the nuclear translocation of ODNs, its cytosolic release as a prerequisite is important. Further, entrapment and degradation of the transferred ODNs within the endolysosomes form a major hindrance to efficient gene transfer, as result of which only a small fraction of internalized nucleic acids penetrates the nucleus. For instance, delivery of the plasmid DNA may encounter the diffusional and metabolic barriers of the cellular compartments that result in reduced number of intact plasmid molecules reaching the nuclear pore complex (NPC). Hence several strategies has been reported to induce endosomal escape such as employing vectors with intrinsic endosomolytic activity (e.g. PAMAM and PEI) or by vector modifications through conjugation of peptides with endosomal disrupting characteristics (e.g. KALA and GALA peptides**)** [22].

While, the diffusion of larger fragments was remarkably slower, in which little or no diffusion was observed for nucleotides with a length beyond 2000 bp.

TRANSCRIPTIONAL MACHINERY

Now, it is well documented that the positively charged cationic nanostructures can vigorously interact with cell surface biomolecules. And once inside of cell, they can also interact with intracellular components and as a result the transcriptional machinery can eventually respond to a multitude of signals created directly or indirectly by these exogenous invaders. Thus, ranges of time-ordered expression of genes are triggered that are involved in various cellular biofunctions such as cell proliferation and differentiation, the cell cycle, and, eventually, the death of a cell. In fact, as reported previously, the cationic gene delivery nanomaterials, depends on the physicochemical characteristics, are able to induce intrinsic early and/or late gene expression changes which determines the cellular responses to the exogenous polycations [16, 17, 73, 74, 81]. Although it appears that the release of DNA in the nucleus is largely dependent on the type of vector used as shown for PEI, the mechanism of such function is unknown – we do not know the direct and/or indirect impacts of such interaction. In contrast, behavior of the cationic and anionic lipids differ as the anionic lipids found on the cytoplasmic face of the plasma membrane are able to displace DNA from cationic liposomes that supports a mechanism of DNA release in the cytoplasm. The nuclear targeted proteins facilitate nuclear transport of plasmid DNA, however it is also unclear how efficiently these proteins release DNA once the complex arrives in the nucleus. As many of these proteins are rich in basic amino acids, they are both ideal and necessary for DNA compaction and protection outside the cell, inside the cytoplasm, and for nuclear delivery. Yet, ironically, their tight DNA binding may actually impede the function of the payload they were meant to protect. In the following section, we will discuss the potential mechanisms by which antisense activity is acquired.

CONCLUDING REMARKS

Implementation of nanbiomaterials for targeted delivery is deemed to allow therapeutic agents to preferentially locate at desired biological sites. This confers an attractive treatment modality with a greater therapeutic index than the conventional formulations of the same agents. So far, many nanomedicines have been clinically used including: liposomal encapsulated doxorubicin and albumin conjugated paclitaxel (approval by FDA in 2005 for treatment of breast cancer). And hundreds of clinical trials are currently underway for the use of these nanoformulations, in combination with established individual drugs (see: http://www.cancer.org/ClinicalTrials.gov) in novel applications beyond the current approved indications of breast, ovarian, head-and-neck cancers, and Kaposi's sarcoma. Given the fact that polycationic nanomaterials can interact with the intricate cellular moieties (cell surface receptors and subcellular transcriptional machineries), they are able to sort and integrates the vast amount of converging and diverging signaling pathways based upon intracellular digitations. This is an important issue since the mechanism(s) by which a

polycationic can interact with subcellular moieties and deliver the genomic entity into the nucleus should be fully investigated is still unknown. Understanding of such mechanism(s) can significantly improve the design of targeted therapy, in particular for gene-based therapies. To pursue such objectives, recruitment and integration of different techniques (from global gene expression to multiple sensing and imaging) appear to be essential.

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