

Macropinocytosis-like endocytic mechanism contributes to the overall internalization of polyethyleneimine polyplexes used in nucleic acid delivery

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Abstract

Successful transport of nucleic acids by nonviral vectors relies, among others, on their cellular uptake route and subsequent intracellular trafficking. Here, we show that Linear Polyethyleneimine (LPEI) polyplexes are taken up by HeLa cells via ATP-dependent mechanisms featuring several overlapping endocytic pathways. Pharmacological blocking of clathrin-, caveolae-, and macropinocytosis-mediated routes demonstrated limited or no declines in uptake of nanocarriers, suggesting effective overlapping or compensatory correspondence across internalization mechanisms. Remarkably, ultrastructural examination by transmission electron microscopy (TEM) alongside scanning electron microscopy (SEM) frequently indicated polyplex internalization through significant membrane ruffling opposed to traditional coated pits, showing a predominant contribution of macropinocytosis or macropinocytosis like mechanism(s). Nonetheless, no effectivity of specific macropinocytosis pathway inhibitors coupled with no or lesser colocalization with traditional macropinocytosis molecular markers confirms the presence of a non-canonical macropinocytosis-like process. Interestingly, dynasore dramatically decreased internalization efficiency, suggesting that dynamin contributes an essential part to fulfill vesicular scission for functional delivery. Our results highlight the significance of ruffle-mediated, dynamin-regulated macropinocytosis-like endocytic mechanism in internalization of LPEI-mediated gene transport and offer new perspectives for improving non-viral carrier constructs through strategic targeting of endocytic routes.

1. Introduction

Nanoparticle-based delivery structures are currently an appealing tool for directed drug and gene transfer owing to their potential to allow for specific transfer of therapeutic agents into cells [1]. Polyethyleneimine (PEI), arguably the most viable option of the polycation-based carriers, is currently demonstrated to effectively transfer DNA both in vitro and in vivo [2]. The efficacy of medication of these nanocarriers depends extensively on their potential to be precisely taken up by cells. Although endocytosis is the primary means of cellular entrance [3], certainly exists emerging evidence that certain nanocarriers may take advantage of other intake approaches, featuring membrane fusion along with passive diffusion, specifically for lipid-based carriers or ultra-small firm particles [4, 5]. Despite progress, current understanding of the molecular and mechanistic particulars regulating the internalization of PEI-based polyplexes remain poorly characterized [6].

Within the realm of endocytosis, multiple different routes have recently been tracked down, notably clathrin-mediated endocytosis (CME), caveolae-mediated internalization, and macropinocytosis [7, 8]. In CME, internalization happens by the emergence of clathrin-coated pits forming clathrin-coated vesicles (CCVs), following dynamin-mediated dissociation [9]. The vesicles rapidly uncoat upon internalization and fuse with early endosomes to undergo acidification to a pH of 5–6 that follows progression to lysosomes for additional acidification to a pH of around 4.5 [9]. Identical to CCVs, caveolin-dependent endocytosis commences with caveolae, characterized by cup-shaped invaginations in the cell surface that are sliced from the membrane by dynamin [10]. Since they have limited acidity, caveosomes are believed

to be immune to lysosomal destruction, unlike clathrin-coated vesicles [11]. Actin-mediated protrusions of the surface membrane trigger macropinocytosis, a particular form of endocytosis resulting in the non-selective capture of external fluid into huge, loose vesicles of macropinosomes [12]. The Arf6-induced ruffle patterns proliferate into vesicles, which can exceed a size of 5 μm [8, 13]. Macropinosomes are prone to avoid lysosomal merging across several cell types, however, potentially could be transported to lysosomes in macrophages [14]. Each of these endocytic routes contributes to the internalization of nanoparticles, functions as an interlinked chain of pathways instead of as independent routes [15]. Current research demonstrates noteworthy interaction and functional overlap among routes, especially within situations during which certain pathways are pharmacologically hindered [16]. Nevertheless, quantitative recognizing of their respective roles and compensatory capability stays limited, particularly in varied polyplex structures where particle size and morphology might significantly impact pathway utilization [17].

Among multiple endocytic routes, macropinocytosis is a prevalent intake pathway marked by the nonspecific transport of extracellular substances and fluids into large capsules [13]. Since there is noticeable membrane ruffling with the mechanism, it is frequently believed these ruffles are an outward indication of macropinocytic activation. However, the conclusion requires specificity, as recent research indicates that only certain surface ruffling events are functionally related to traditional macropinocytosis whereas certain nanoparticles enter cells by ruffle-mediated mechanism akin to macropinocytosis but differ in molecular regulation and signaling requirements [18]. The inability to consistently associate certain internalization pathways with membrane ruffling points to a considerable knowledge gap regarding nanoparticle internalization [19]. In addition, the molecular identities along with functional mechanism regulating such additional routes remain poorly characterized [20].

In the current study, we illustrate the cellular internalization of polyethyleneimine (PEI)/DNA polyplexes in HeLa cells, with a primary focus on an energy dependent non-canonical, macropinocytosis-like route. Following that, we determined the relative significance of endocytic pathways employing ATP-depletion studies along with high-throughput quantitative examination, displaying energy-dependent intake dynamics. To investigate the relative involvement along with interaction of various endocytic paths we utilized a comprehensive panel of pathway-specific inhibitors, revealed significant effective plasticity, wherein compensatory pathways maintain polyplex internalization while traditional pathways are hindered. Advanced electron microscopy revealed an intriguing spatial binding where large polyplexes notably accumulated at prominent membrane extensions ahead of being taken up via ruffle-mediated capture. Of greatest importance, through colocalization analysis along with macropinocytic route blocking, we reveal that these nanocarriers are incorporated by cells employing a non-canonical macropinocytosis-like mechanism having distinct ultrastructural characteristics with traditional macropinocytosis and operating via different cellular machinery.

2. Results

2.1. Polyplex internalization occurs primarily via energy-dependent endocytosis

In order to investigate if uptake of polyplexes by cells involved either an active or passive approach, cells were incubated with polyplexes both at 37°C (standard cell culture conditions) and at 4°C. Lower temperatures impede endocytic internalization since it is evident that ATP as well as other proteins and enzymes are temperature-sensitive [21]. Polyplexes were robustly internalized by HeLa cells maintained at 37°C (16.4 ± 3.2 particles/cell), which is indicative of active, ATP-dependent endocytosis (Fig. 1a). Contrarily, cells cultured at 4°C, condition known to inhibit all ATP-dependent physiological activities, HeLa cells showed significant reduction of cellular fluorescence, with internalization efficiency decline to 0.2 ± 0.8 particles per cell. Quantitative analysis demonstrated a robust temperature dependence of polyplex intake, with a significant decrease in nanocarrier internalization at lower temperatures in comparison to 37°C (Fig. 1b).

To further establish active ATP facilitated endocytic internalization, we subsequently examine into applying sodium azide to chemically suppress oxidative phosphorylation. Azide-treated cells showed a substantial decrease in ONTs internalization and subsequent release in cytoplasm, as evident from FITC-positive cells, in comparison with untreated controls (Fig. 1c). Control cells demonstrated efficient uptake of polyplexes with 15.3 ± 2.5 particles/cell whereas pharmacological suppression of ATP in chemical-treated cells revealed dramatic reduction in particle internalization with 2.6 ± 1.9 particles/cell. Importantly, decline observed in polyplex intake was indicated by particular metabolic suppression instead of generalized cytotoxicity, as verified by viability analyses indicating > 90% cell longevity throughout experimental conditions (Fig. 1c). Quantification examination of nanoparticle numbers per cell confirms the statistically substantial decrease (Fig. 1d).

2.2. Overlapping endocytic routes compensate for LPEI-Mediated ONTs internalization even with a particular pathway suppression

Thus far, the findings imply that the cellular uptake of polyplexes is primarily mediated by ATP-driven endocytosis, while pharmacological blocking or low-temperature inhibition substantially decreases overall transfection efficiency. The intracellular fate of polyplexes is significantly determined by primary cellular entry route(s) [22]. To gain insight into the cellular internalization mechanism of LPEI complexes, several pathway specific chemical inhibitors were applied to inhibiting various endocytic pathways.

Clathrin-mediated endocytosis was inhibited by chlorpromazine and cytochalasin D, caveolae entrance was inhibited by filipin III, and macropinocytosis was inhibited by LY294002. Furthermore, blebbistatin was employed as a myosin II blocker, jasplakinolide was used to obstruct actin-driven endocytosis, and nocodazole was utilized to hinder with microtubule-dependent trafficking. To accomplish transport blocking, all the inhibitors were employed in accordance with the manufacturer's instructions. Within

such conditions, no toxic impact of the inhibitors was observed, as performed by MTT analysis (data not shown), and the cellular internalization of polyplexes was evaluated.

As illustrated in Fig. 2a, neither of the pharmacological inhibitors against particular endocytic routes prompted a significant decline in the overall entry of LPEI polyplexes in HeLa cells. It is possible that no specific endocytic mechanism contributed majorly for polyplex internalization since pharmacologically blocking individual pathways did not significantly hinder overall internalization of polyplexes.

Remarkably, pharmacological suppression of primary endocytic routes involving clathrin-mediated cellular entry (chlorpromazine), caveolae-dependent intake (filipin III), and macropinocytosis (LY294002), were unable to reduce LPEI polyplex internalization in HeLa cells. Collectively, these findings contributed to support the notion that while effective polyplex uptake occurs in HeLa cells through conventional endocytic pathways, additional alternative routes also facilitate internalization. Alternatively, the continuing success of LPEI polyplex internalization regardless of pharmacological blockade of traditional endocytic routes reveals the activation of compensatory function among various endocytic pathways that retain overall polyplexes entry (Fig. 2b).

2.3. Polyplexes are internalized predominantly in the form of large complexes through specialized membrane ruffles

Oligonucleotides internalization data suggested that PEI can use multiple endocytic pathways to enter HeLa cells. To further confirm this and to see the initial binding and internalization of PEI polyplexes into HeLa cells we used Transmission and Scanning Electron Microscopy (TEM and SEM) approaches.

SEM examination was initially employed to analyze the initial interaction of polyplexes to the cellular membrane. As demonstrated in Fig. 3b–3e, only a minimal number of small polyplexes were seen at the cellular surface. Alternatively, larger clustered polyplexes were frequently noticed in conjunction via prominent membrane ruffles (arrowheads in Fig. 3b-3e). At various sites, these cellular extensions were observed to essentially attach to the polyplex aggregates, showing active membrane activation during the initial phases of particle interactions. SEM image analysis further confirmed that the cell-surface extensions frequently interact with polyplex clumps. The extensions were seen to surround or transport particles toward membrane regions appropriate for intake, indicating a coordinated approach of invading particle at the cell membrane. Particularly, partial membrane ruffling along with protrusive activity corresponded with an uptake route similar to macropinocytosis.

TEM evaluation was carried out to investigate that these surface-attached polyplex aggregations had been internalized as intact structures. While pharmacological inhibition studies had indicated the contribution of several conventional pathways, TEM analysis of different membrane profiles showed a remarkable lack of caveolin-1-positive incursions (< 1%) and clathrin-coated pits (< 2% incidence) associated with internalizing polyplexes (arrow in Fig. 3g, h). Alternatively, a significant fraction of internalization occurrences featured larger polyplexes, which were mostly spotted at a location of entrance into cells usually possessed a diameter of more than 300 nm (arrowhead in Fig. 3h, 3j).

The TEM data as illustrated in Fig. 3f revealed healthy cellular structure, showing that the cells retained intact morphology under control conditions. It is evident from Fig. 3g-3j clumps of polyplexes were frequently located near or shortly next to the plasma membrane. Such positioning indicates active interaction of membrane with polyplexes at initial phase of uptake. Additionally, TEM examination of the surface topology in Fig. 3h and 3j displayed particular membrane reshuffling and partial ruffling surrounding polyplexes, demonstrating that the cell membrane experiences structural remodeling during the initial phases of particle interaction.

TEM examination further demonstrates that the incorporated polyplexes were wrapped in compact vesicles of relatively similar diameter (> 400 nm; Fig. 3g-j). These vesicles remained consistent in appearance and lacked larger vacuoles that are formed during conventional macropinocytosis. Individual vesicle frequently carried several polyplexes, suggesting that the cells were packing the nanoparticles via a more targeted vesicular approach as alternative to large-scale fluid encapsulation. The appearances of these smaller sacs, opposed to the lack of large macropinosome-like organelles, highlight an alternate uptake pathways. While membrane ruffling was evidently triggered at the cell surface, subsequent events were unable to form standard macropinosomes. Rather, the internalization mechanism is more correlated with a sophisticated macropinocytosis-like mechanism, whereby polyplexes precedes membrane remodeling and advancing into a regulated, alternative vesicular trafficking pathway.

2.4. Specific macropinocytosis inhibitors and colocalization analysis reveals polyplexes trafficking via non-classical macropinocytosis like compartments

EM data hint towards the possible involvement of macropinocytosis like mechanism. Therefore, to either confirm or rule out potential role of macropinocytic like mechanism, fluorescently labeled ONTs were complexed with PEI and were subsequently added to HeLa cells to evaluate intracellular trafficking, utilizing specific chemical inhibitors of macropinocytosis mediated uptake for 30 min pre-incubation. Investigations showed a non-canonical endocytic route for LPEI polyplexes which continued to be operationally intact regardless of pharmacological suppression of macropinocytosis. The entry of nanoparticles was not significantly altered by treatment with well-characterized inhibitors aimed at different nodes of the macropinocytic route, such as wortmannin (PI3K/mTOR suppression), bafilomycin A1 (vacuolar ATPase blocking), and LY294002 (PI3K inhibition), as shown in Fig. 4a. According to quantitative fluorescence intensity observations, there was no substantial difference in intake rates for control and macropinocytosis-inhibited cells, indicating significant polyplex internalization in both cultures. Statistical analysis confirmed this finding, showing that the number of particles per cell was about the same in all experimental situations (Fig. 4b).

To further rule out the major contribution of macropinosome-associated compartment that regulates polyplex accumulation, we utilized a specific colocalization approach employing established molecular markers of macropinosomes (Rabankyrin-5). During 2h incubating point, related to the maximum cellular

trafficking, polyplexes were found within endosomal membranes (Fig. 4c). However, nanocarriers showed no detectable colocalization with rabankyrin-5 molecular marker. Interestingly, regardless of no colocalization of nanocarriers with traditional endocytic marker, an elevated intracellular accumulation of LPEI-based nanocarriers was noticed (Fig. 4c). To further evaluate the colocalization among these signals, fluorescence intensity line profile examination was carried out (Fig. 4d). The subsequent line graph showed two different fluorescence intensity peaks that exhibited distinct patterns throughout the selected area. The fluorescent lines of both signals was not coincided, demonstrating the absence of discernible colocalization across the polyplexes and Rabankyrin 5 positively tagged regions.

2.5. Overall internalization of polyplexes through macropinocytosis-like pathways is regulated by dynamin

Several inhibitors were previously employed in an attempt to provide a mechanistic analysis and possible cellular variables; however, none of these inhibitors hindered the cellular entry of polyplexes. Although the cytoskeleton facilitates macropinocytosis likewise in the present instance, internalization was not significantly inhibited by cytochalasin D or similar cytoskeleton blockers such as jasplakinolide and blebbistatin. Consequently, the ultimate objective turned out to experimentally ascertain if biological components are potentially engaged in the macropinocytosis-like route that allows LPEI polyplexes to enter HeLa cells. Dynamin, a significant GTPase governs various endocytic routes and is particularly hampered by dynasore. Therefore if LPEI polyplex cellular entry is accomplished through dynamin-dependent macropinocytosis-like mechanism, dynasore treatment ought to substantially hinder polyplexes uptake.

After 30 minutes of dynasore preincubation, LPEI polyplexes were added to the HeLa cells. As a control, cells were exposed to polyplexes with no inhibitor. A substantial amount of nanocarrier absorption was noted as shown in Fig. 5a, which is in accordance with LPEI polyplexes internalizing through a variety of endocytic routes in control cells. On the contrary, drug-induced suppression of dynamin in dynasore-treated cells resulted to a pronounced decrease in cellular internalization regardless of particle sizes, with intake decreased by 68–84% compared to untreated controls. Interestingly, internalization was not entirely eliminated, indicating that residual uptake gets facilitated by dynamin-independent channels (Fig. 5b). Taken together, our findings strongly suggest that dynasore majorly inhibited the internalization of polyplexes through macropinocytosis-like mechanism. Since ruffles-mediated internalization was not considerably impaired by treatment with specific macropinocytosis blockers, we are certain that the suppression of macropinocytosis-like pathway is precisely because of dynasore, contribute to the significant inhibition in the internalization.

3. Discussion

The current results indicate that the internalization of polyplexes in HeLa cells is carried out primarily via an energy driven endocytic pathway. Endocytic events, notably membrane reconfiguration, endocytic vesicle formation and the recruiting of trafficking proteins, are primarily ATP-dependent. It has been

demonstrated that HeLa cells showed strong intracellular polyplex intake at the appropriate temperature (37°C), showing efficient internalization in accordance to energy-dependent endocytosis (Fig. 1a, 1b). However, suppression of cellular internalization at low temperatures implies that active cellular machinery is necessary for polyplex entrance, as opposed to passive diffusion (Fig. 1c, 1d). These observations correlates with previously reported results showing that nanocarrier structures display substantially reduced entrance while cellular ATP were hampered [23]. It has been extensively studied that lower temperature inhibits ATP formation and is associated with membrane processes, notably cellular ruffles, membrane scission, and the function of GTPases along with ATPases required for endocytosis, consequently reducing internalization efficiency [24].

The inhibitory impact of sodium azide provides additional confirmation for polyplexes undergo ATP-triggered endocytosis. Sodium azide had been proven to severely hamper clathrin-, caveolae-and macropinocytosis-associated uptake pathways via blocking oxidative phosphorylation promptly depleting intracellular ATP accumulates [25]. Similar findings have been reported in recent research, showing the considerable decrease in polyplex internalization after azide therapy (Fig. 4c) implies that ATP depletion considerably decreased the effectiveness of cellular internalization. Crucially, cell viability stayed over 90% during the experiment, demonstrating that declines in polyplexes internalization were the result of metabolic inhibition instead of cytotoxic consequences (Fig. 4c). The pronounced decline of polyplex uptake at 4°C, coupled with the substantial reduction noticed upon sodium Azide-induced ATP suppression, provides strong evidence that internalization in HeLa cells proceeds mainly via an energy-induced endocytic pathway rather than passive or diffusion-driven event.

The data thus far confirmed that LPEI complex internalization involved an energy-driven mechanism, however a specific endocytic pathway mainly contributed to the effective internalization of polyplexes has remained unclear. Polyplexes uptake into inhibitor-treated HeLa cells was mostly unaffected by blocking of clathrin-induced endocytosis (chlorpromazine), caveolae-dependent accumulation (filipin III), macropinocytosis (LY294002), actin polymerization (jasplakinolide), or myosin II-mediated activity (blebbistatin), demonstrating relatively slight or insignificant declines in intracellular fluorescence (Fig. 2a, b). The overall susceptibility to a particular pathway inhibitor showed that polyplex entrance was not primarily relying on an individual route but instead was facilitated by an overlapping and compensatory network of endocytic pathways.

The adaptability of polyplex uptake subject to different inhibitory situations may be related, partially, to their strong cationic composition, facilitating robust electrostatic interactions with negatively charged cell surface constituents including heparan sulfate proteoglycans (HSPGs) [26]. These interactions frequently initiate localized signaling events which trigger substantial surface remodeling and uptake, irrespective of conventional receptor-driven routes [27]. The little disruption of inhibitors targeting actin patterning (jasplakinolide), myosin II pathway (blebbistatin), and microtubule-mediated transportation (nocodazole) more thoroughly highlights the durability of LPEI polyplex entrance. Actin reorganizing was usually accepted as vital to macropinocytosis along with multiple clathrin- unrelated pathways, nevertheless persistent accumulation in actin-perturbed cells implies that cellular compensatory

membrane interactions could evade regional cytoskeletal disruption [28]. Cationic nanocomplexes have been shown to trigger localized membranes deformation and ruffling, facilitating entrance regardless of whether normal actin-mediated pathways were blocked [29]. Additionally, the notion of endocytic crosstalk along with compensatory uptake provided a convincing interpretation for recent findings that cells probably redistributed the integration of traffic to other paths in order to retain homeostasis once conventional pathways were impeded [30].

When taken as a whole, thus far the results support the notion that the endocytic framework whereby LPEI polyplexes interact with cells was intrinsically adaptable, permitting the cell to actively redistribute uptake burden across multiple pathways. Such overlapping pathways potentially allows nanocarriers to sustain efficient delivery even with inhibition of particular routes, thereby leading to the minimal decrease in uptake throughout all examined pharmacological situations. This mechanistic analysis provides the basis for further ultrastructural examinations, aiming at clearly illustrating the surface ruffling, vesicular formation, and non-traditional uptake processes that result in successful polyplex internalization. The ultrastructural findings acquired in the current examination reveal significant structural proof that clarifies the internalizing procedure proposed by the pharmacological analysis. Although the inhibitor-based analysis in Fig. 2 referred to a divergent route, the TEM and SEM examinations currently demonstrate specific morphological patterns of specific macropinocytosis-like mechanism that exhibits noticeable cellular ruffling along with the formation of distinctive, nanocarrier-packed vesicles. However, the alternate internalization pathways lack prominent cylindrical ruffles alongside large macropinosomes that characterize the traditional macropinocytic route.

SEM imaging revealed the large complexes were mostly attached to protruding structures and noticeable membrane ruffles (Fig. 3b–e). This finding implies that the polyplexes ultimately serve as a preliminary uptake signal, inducing polymerizing of actin and membrane modification on interaction site [31]. The minimal number of complexes found in clathrin-covered or caveolin-1-positive invaginations (< 2% occurrence), as evidenced by TEM imaging (Fig. 3g, h), provides clear structural confirmation to the pharmacological insensitivity shown in Fig. 2.

Interestingly, the subsequent intracellular fate of the complexes diverged from the conventional macropinocytic path. TEM analysis demonstrated that the polyplexes were wrapped inside small, circular vesicles of a reasonably constant size (Fig. 3g–3j) instead of being enveloped within enormous, uneven macropinosomes. The observed pattern indicates that polyplexes internalization initiated a highly controlled procedure whereby the initial extensive surface ruffling advanced into a precisely regulated splitting or cutting-off step, as opposed to the spontaneous vesicular assembly regularly observed in fluid-phase macropinocytosis. Such regulation certainly depends on membrane-scission proteins notably dynamin II that regulate and maintain membrane curvature to generate vesicles of specified shape [32]. This macropinocytosis-like method, depicts a refined cellular approach, permitted the cell to target particulate cargo whereas inhibiting additional fluid accumulation, offering a more targeted and highly efficient pathway for nanoparticle uptake.

The trafficking events noticed in the present analysis further strengthened the findings that LPEI complexes move into HeLa cells via a specialized, macropinocytosis-like route that operates irrespective of conventional regulatory junctions. Although previous ultrastructural findings suggested that polyplexes provoked cellular ruffling analogous of macropinocytic intake, current inhibitor experiments showed that the mechanism was contrary to conventional macropinocytosis. Notably, nanoparticle entry is mostly unaffected in chemical treated cells (Fig. 4a) despite suppression of essential regulatory molecules of macropinocytic signaling, which includes PI3K activation (LY294002), PI3K/mTOR (wortmannin), and vacuolar ATPase-mediated vascular acidification (bafilomycin A1). The recurrence of sufficient complex intake under all inhibitor-regulated situations showed that whereas surface remodeling patterns were apparent during entrance, subsequent trafficking lacked reliance on the primary signaling axes, usually the standard PI3K-associated macropinocytic pathway essential to effective internalization [33].

To further locate the endocytic compartment liable for polyplex distribution, colocalization examination was carried out via Rabankyrin-5, an essential mediator of macropinosomes. At their maximum integration phase (2 hours), no or very less polyplexes were confined to Rabankyrin-expressing endosomal compartments, indicating the existence of a macropinocytosis-like endocytic internalization. Nevertheless, the overlap between signals was continuously absent, as no colocalization was detected by line profile analysis along with fluorescent microscopy. Unsurprisingly, the intracellular accumulation of complexes persisted high, demonstrating that a large number of nanocarriers trafficked by means of Rabankyrin-independent endocytic compartments. This pattern correlates with recent research revealing cellular redundancy and channel adaptability in nanoparticle entrance, during which cells utilize hybrid or alternative endocytic strategies to sustain internalization proficiency even though primary pathways are interrupted [34].

Potentially current results imply that polyplex intake occurs through alternate, macropinocytosis-like endocytic pathways, with noticeable operational redundancy that maintains productive cellular internalization. Morphological signs of membrane protrusions, frequently linked with macropinocytosis, were prominent during the phase of internalization, contributing a significance role in polyplexes uptake. Yet pharmacologically inhibiting macropinocytosis did not result in a significant decrease of nanocarrier uptake, suggesting that macropinocytosis solely is not responsible for the observed cellular entry of polyplexes. Furthermore, a considerable portion of polyplexes internalized, without colocalization with rabankyrin-5 suggests that nanocarriers accumulated in the Rabankyrin-independent endosomal compartment, thereby illustrates a non-classical macropinocytic-like pathway. Additionally, the current study established the basis to investigate the intracellular molecular regulators involved in polyplex trafficking following internalization by macropinocytosis-like mechanism.

After figuring out the structural and pharmacological characteristics of a divergent entrance route, the current investigation concentrated to identify associated molecular machinery governing the macropinocytosis-like internalization of LPEI complexes. Despite previous inhibition investigation showed that both conventional macropinocytosis and also actin-mediated membrane remodeling was

not entirely responsible for the effective uptake of polyplexes, the pathway continued to be active regardless of whether numerous cytoskeletal or contractility-associated processes were impaired. Since the uptake method was visually similar to macropinocytic ruffling, the observed pattern suggested that the process required different molecular regulators that were not associated with traditional macropinocytosis.

Although dynamin had been generally associated to the scission of clathrin-capped vesicles, a growing number of investigations highlight dynamin contribution in multiple clathrin-independent routes, particularly the introduction of large particulates and some pathogens [35]. In accordance with these analyses, dynamin activity was believed to be essential for the scission of vesicles generated after cellular remodeling. In conformity with the previous evidence, dynasore administration displayed a significant and measurable inhibition of polyplex intake, decreasing internalization nearly by 68–84% for each sized particles (Fig. 5a, 5b). Such dramatic decrease shows that dynamin is essential for facilitating the vesicle dissection phase essential for LPEI complexes to enter productively. Furthermore, the suppression was not confined to a particular particle size, suggesting that dynamin-induced scission was an essential prerequisite for both average-sized and larger complex aggregates, associated with their intake via ruffle-mediated pathways. The remaining, dynamin-independent uptake (~ 16–32%) provided more evidence that cells had alternative membrane splitting machinery. Moreover, compensatory endocytosis showed that following dynamin suppression, alternative strategies including actin-mediated contraction along with others BAR domain proteins may partially regulate vesicle release, however with reduced efficacy [36].

In current study, we focused primarily on internalization of LPEI-constituted polyplexes, without expanding the investigation to subsequent transfection or gene expression. Although cellular internalization reflects the initial crucial step in the delivery of nucleic acid, a successful transfection involves further cellular steps, notably endosomal release, which is impacted by both the characteristics of the nanocarrier and a particular endocytic route. In addition, transfection is also dependent on nuclear transfer for plasmid DNA. Such following stages further complicate the pathway, making it challenging to specifically associate internalization with overall gene expression. Through emphasizing internalization, this research offers a detailed mechanistic basis for polyplex entrance and cellular regulation, providing a substantial basis for further studies focusing on endosomal release and nuclear delivery.

4. Materials and methods

4.1. Reagents and antibodies

FITC-labeled Oligonucleotides (ONTs) acquired via Invitrogen, while linear polyethyleneimine (LPEI; MW 22 kDa) was acquired from Polyplus-transfection. Primary antibody comprised rabbit anti-Rabankyrin-5 were obtained from Sigma and corresponding secondary antibody included Alexa Fluor 555 were purchased from Invitrogen. All the additional materials were bought from Sigma-Merck (Netherlands).

4.2. Cell lines

HeLa cell lines were obtained from ATCC and cultured in DMEM supplied with 10% FBS and 1% penicillin–streptomycin at 37°C with 5% CO₂. Subculturing was carried out via trypsinization, and cells were grown at a density of 1×10^5 cells/cm³. Cells from passages 3 through 10 were used for each study. Before the experiments, cells were seeded on sterilized glass coverslips in 6 well plates and permitted to remain attached for one day according to standard growth conditions achieving > 70% confluency.

4.3. Polyplexes formation and ATP-mediated internalization

Following 24 hour of initial culturing, cells were washed once with HBSS and sustained in incomplete medium during the experiment. Polyplexes were synthesized by complexing fluorescently tagged ONTs using linear polyethyleneimine (LPEI) at a N/P ratio of 5 and left at room temperature for 20 minutes, according to manufacturer protocol.

Cells were pre-incubated subjected to different circumstances in order to evaluate energy dependence: either incubation at 4°C or subjection to 10 mM Sodium Azide at 37°C for 30 minutes, followed by polyplex administration (0.1 nmol of ONTs per well) for two hours for each condition. As a positive control, polyplexes were introduced to cells at 37°C with no inhibitors and incubated for 2–3 hours in 5% CO₂. Following a 20-minute fixation with 4% PFA, the cells were washed three times with PBS and mounted on glass slides using mounting media. Cells were examined using fluorescence microscopy using (Olympus fluorescence microscope). The proportion of PEI positive cells relative to all nuclei in five randomly selected areas per coverslip were used to measure internalization.

4.4. Pharmacological inhibitors of endocytic route

HeLa cells were pretreated with a variety of pharmacological inhibitors for 30 minutes before the introduction of polyplexes, unless specified differently. Clathrin-mediated endocytosis was inhibited by chlorpromazine (CPM; 6 µg/mL), and cytochalasin D, whereas caveolae-mediated internalization was blocked by filipin III (5 µg/mL). LY294002 (10 µM), bafilomycin A1 (100 nM), and wortmannin (100 nM) were used to hinder macropinocytosis. Dynasore (80 µM) was used to block multiple endocytic pathways [37]. Furthermore, blebbistatin (50 µM) was employed as a myosin II blocker, jasplakinolide (1 µM) was used to obstruct actin-driven endocytosis, and nocodazole (10 µM) was utilized to impair microtubule-dependent delivery. All inhibitors were acquired from Sigma-Merck, while absence of cytotoxic consequences at specific levels was verified utilizing the MTT cell viability tests.

4.5. Polyplex Facilitated ONTs internalizing Assay

HeLa cells were plated in 6-well plates at density of 1.5×10^5 cells/well one day before the experiment. Once being pre-treated with the appropriate inhibitors for 30 minutes, cells were subjected to LPEI polyplexes complexed with FITC-labeled ONTs for two hours with inhibitors still present. For setting up

FACS examination, the cells were trypsinized for 5 minutes at 37°C followed by resuspending in complete medium with 0.2% trypan blue reagent to extinguish extracellular fluorescence. Fluorescence magnitude was determined utilizing a CytoFLEX Flow Cytometer (Beckman Coulter, Woerden, the Netherlands) fitted with a 488 nm excitation and a 530 nm emission filter. Each sample with no less than 10,000 events collected, and FlowJo software (FlowJo LLC) was used for data analysis.

4.6. Electron microscopy examination

For electron microscopy investigation, overnight seeded HeLa cells were exposed to polyplexes for ninety minutes, thereafter, rinsed with PBS, and fixed overnight at 4°C in 2% glutaraldehyde plus 4% PFA diluted in 0.1M sodium cacodylate buffer (pH 7.4). Following one hour at room temperature, post-fixation was carried out using 1% osmium tetroxide. Through progressively increasing ethanol concentrations (30–100%), the samples were dehydrated. In order to prepare for TEM analysis of intracellular ultrastructure, the samples were sectioned into 70–90 nm segments, which were subsequently gathered on grids of copper and contrasted using uranyl acetate along with lead citrate to enhance visibility. The examination of stained grids were carried out utilizing a JEOL JEM-1400Plus TEM that was powered by an 80 kV Gatan Orius SC1000 CCD camera.

For SEM examination of surface topology and polyplex interactions, cells cultured on coverslips were then incubated with polyplexes and followed through the same initial fixing and dehydration procedure as outlined for TEM. Next to dehydration, the cells were carefully point-dehydrated in order to maintain sensitive cellular frameworks. After being dried, the samples were placed on aluminum stubs and coated with a 5 nm covering of palladium/gold. Imaging was carried out employing a JEOL 6301F field-emission scanning electron microscope with an exponential voltage of 3 kV.

4.7. Colocalization analysis

HeLa cells were seeded on microscope coverslips in 6-well plates for 24 hours before treatment, as mentioned earlier. Endocytic inhibitors were added 30 minutes prior to the addition polyplex. FITC-labelled ONTs were complexed with LPEI to produce polyplexes, which were subsequently added to cells and incubated for further two hours. Subsequently, cells were rinsed thrice with PBS and mounted using 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature, followed by quenching with 0.1 M glycine in PBS. Cells were made permeable using 0.2% Triton X-100 for 2 min while nonspecific interaction was prevented by employing PBS comprising 1% BSA and 0.05% Tween-20 for an hour. After one hour incubation with primary antibodies diluted in PBS-T at 37°C, the cells underwent four PBS rinses with a single PBS-T wash. Secondary recognition was carried out via Alexa Fluor-coupled secondary antibodies (1:1000 in PBS-T) duration of 30 min at 37°C. Follow-up PBS washes were followed by fixing of the coverslips utilizing fluorescent mounting media (Dako, Carpinteria, USA). Fluorescence analysis was performed using a Leica TCS SP2 confocal microscope (Leica Microsystems, Germany) containing a 60× oil-immersion objective lens. Colocalization analysis was performed by means of ImageJ software (NIH), with Pearson's coefficients to determine the converge within fluorescent signals.

Declarations

Funding declaration:

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Author Contribution

F. Q.: Investigation, Data curation, Formal analysis, Writing—original draft. M. G.: Investigation, Data curation. S. K.: Investigation, Methodology. M. D. K.: Investigation, Sample preparation. N. M.: Investigation, Data curation. N. W.: Writing—review & finalization of the manuscript. Z. U. R.: Conceptualization, Funding acquisition, Supervision, Methodology, Writing—review & editing. All authors have reviewed the manuscript and agree to submit to the Scientific Reports.

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Data Availability

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. The raw data, including experimental results and related materials, are securely stored on a password protected system at Kohat University of Science and Technology, Kohat.

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Figures

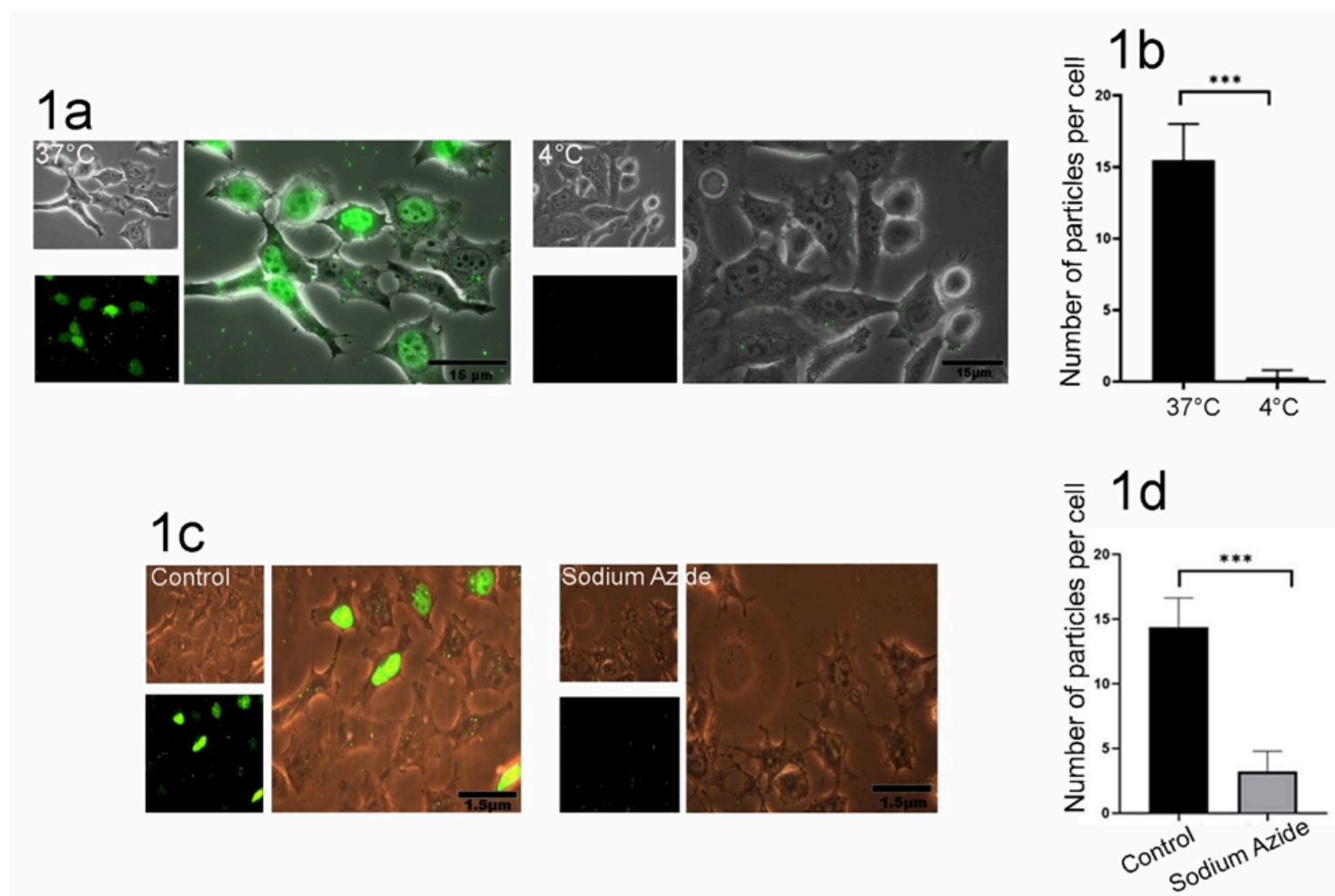


Figure 1

Polyplex uptake predominantly proceeds through energy-dependent endocytosis. 1a-1b. ATP is inhibited in response to temperature, whereas 1c-1d. Shows pharmacological blocking. (1a) Upper brightfield panels showed cell morphology while lower images displayed fluorescently tagged polyplexes in the same area. Merged images shows the overlay of both the images. (1c) Representative brightfield and fluorescence images of control and ATP-depleted (sodium azide treated) cells were shown, with merged images revealing the distribution pattern of fluorescently-tagged polyplexes within cells. (1b, 1d) Corresponding graphs quantified the number of polyplexes per cell across experimental groups and

control. Statistical study verified these findings where ANOVA via Duncan’s post hoc test along with t-tests demonstrated strongly significant variations among conditions ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$; Fig. 1b, 1d). The scale bars in figures a and c indicate 15 μm and 1.5 μm , respectively.

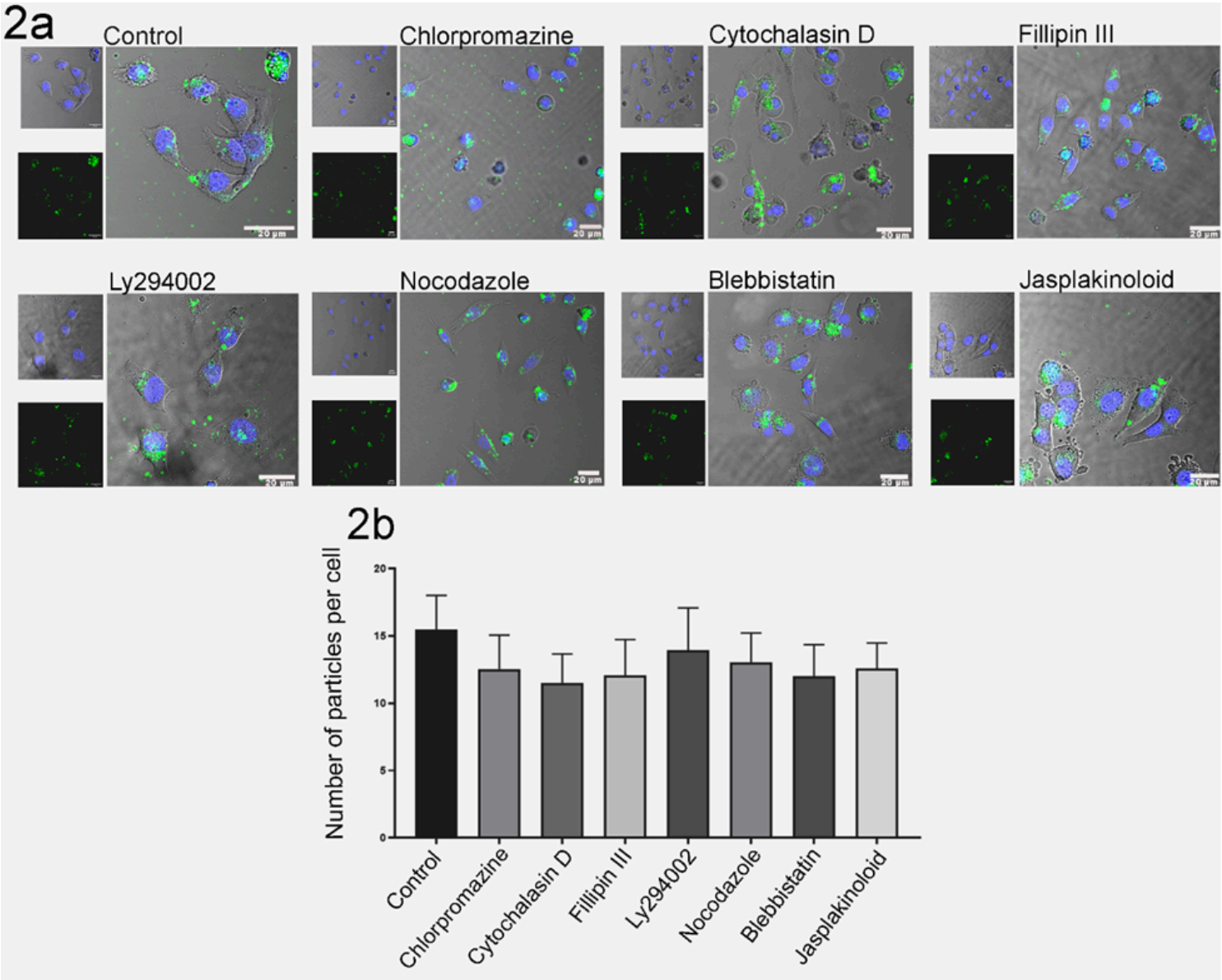


Figure 2

Endocytic inhibitors possess minimal or no impact on the overall internalization of polyplexes. (2a) FITC-labeled ONTs (green) complexed with unlabelled LPEI were added to cell and incubated for two hours before fixing the cells. Upper left corner frames in each condition displayed brightfield images of cells and lower images represented fluorescently tagged polyplexes within similar areas. Right panels exhibited merged images of brightfield with fluorescence. Nuclei of the cells are labelled with DAPI (blue) (2b) Quantitative analysis of the number of polyplex particles per cell under inhibitor-treated and control conditions allowed for statistical analysis of the internalization of nanocarriers across different treatments (Elkin et al. 2015). Data comprised three separate studies. Scale bars = 20 μm .

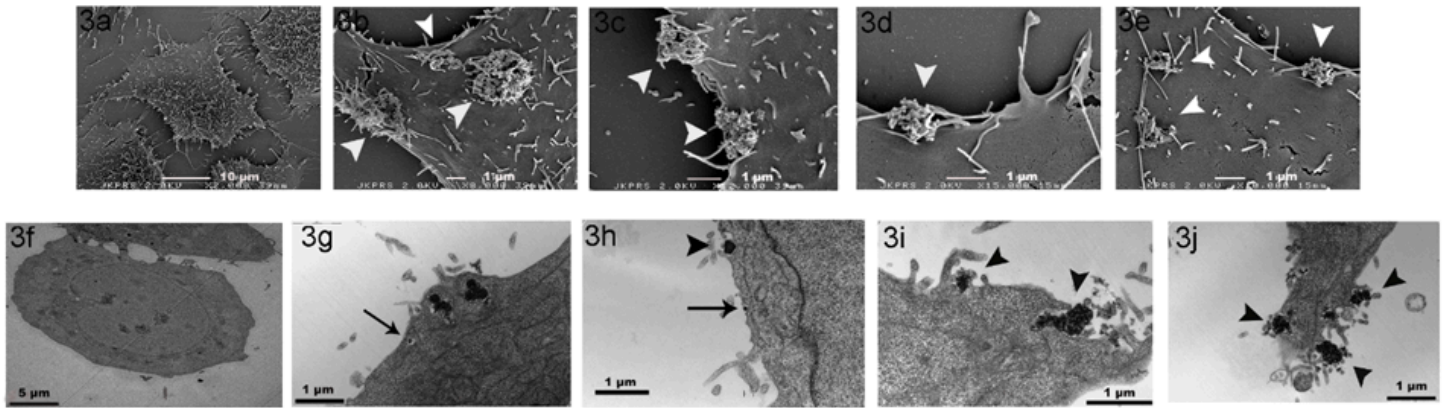


Figure 3

Substantial portions of polyplexes are primarily internalized into the cells in the form of large complexes via a macropinocytosis-like mechanism. (3a, 3f) Lower magnification SEM image of untreated control cell, showing regular shape surrounded by cellular protrusions (3b-3e) SEM images displaying the interaction of polyplexes with a cellular membrane. Larger polyplex particles (arrowheads) are discernible across membrane ruffles. (3g-3i) TEM images illustrate the ultrastructural particulars showing either Clath/Cav mediated internalization of polyplex (show with arrow) or clusters of polyplexes internalizing via macropinocytosis like (ruffle mediated) endocytosis (arrowhead). Images are representative of three individual independent experiments. Scale bars = 10 μm for (a), 5 μm for (f) and 1 μm for (b-e and g-j) respectively.

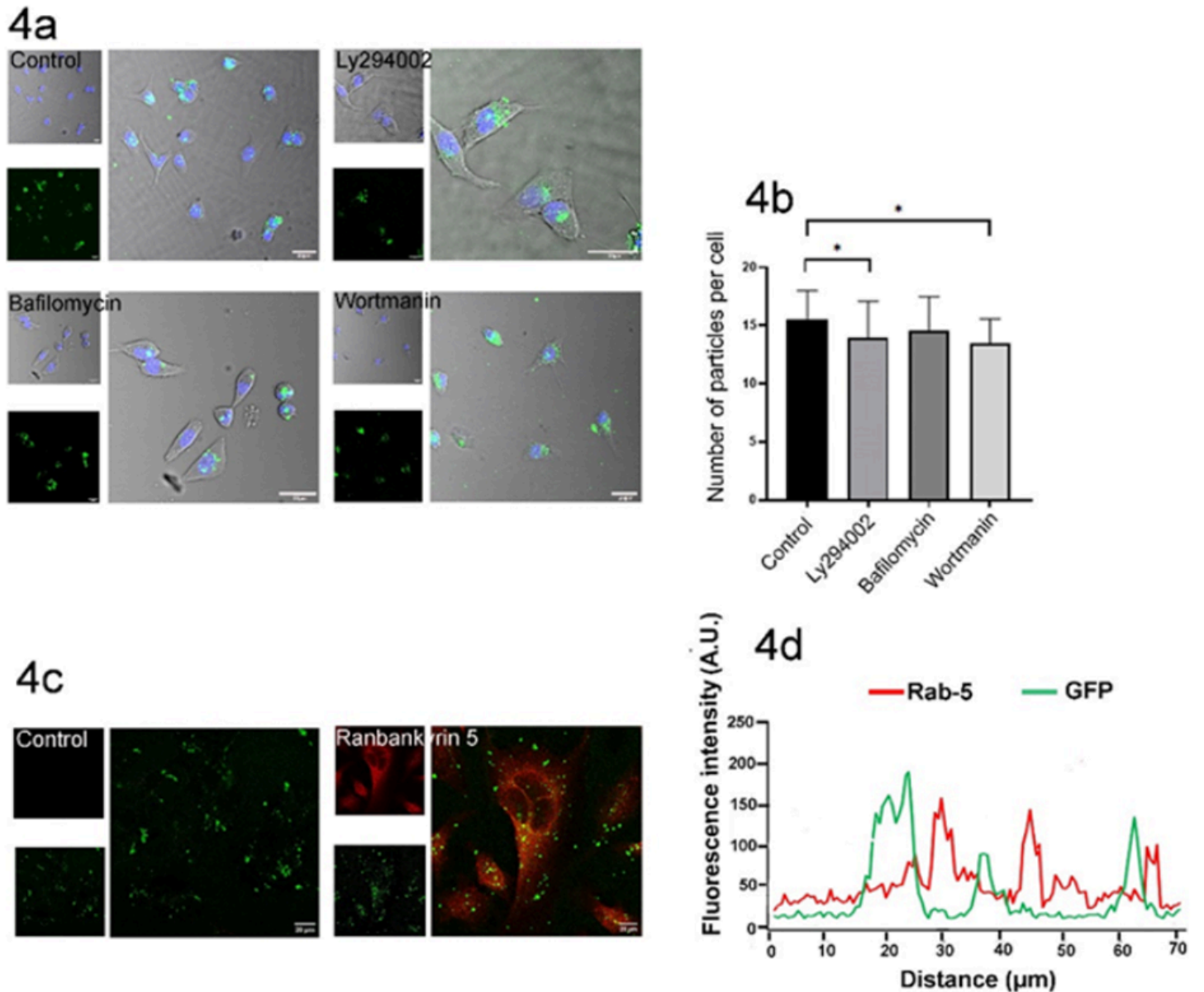


Figure 4

Specific macropinocytosis inhibitors and colocalization analysis reveals polyplexes trafficking via non-classical macropinocytosis like compartments:(4a) Representative merged brightfield–fluorescence frames and appropriate green-channel micrographs shown cells subjected to fluorescently tagged polyplexes (green) under control conditions or following treatment with LY294002, bafilomycin, or wortmannin for 30 minutes. Cell nuclei are labelled with DAPI shown in blue (4b) The quantitative measurement of particle numbers per cells indicates that inhibitor-treated cells expressed internalization intensity corresponding to the control group. (4c) Colocalization analysis of LPEI (green) with macropinocytosis maker Rabankyrin 5 (pseudocolored red). In control only secondary antibody was added The panels represent individual channels and overlays pictures, enabling for evaluating the spatial relationship across internalized polyplexes and Rabankyrin-5–labeled endosomes. Scale bars are shown in overlay images. Data is representative of two independent experiments. (4d) Line graph at the right

bottom represents a fluorescence intensity profile associated with the respective merged image, with red indicating molecular marker (Rabankyrin 5) and green showing a tagged polyplexes throughout the area of interest.

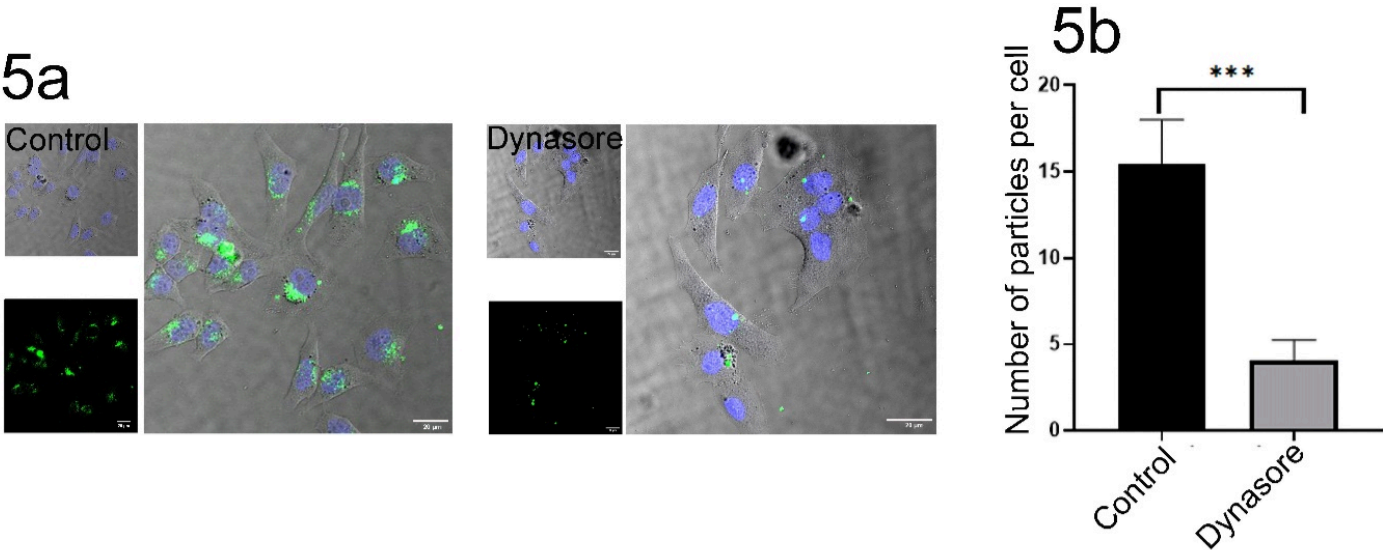


Figure 5

Overall internalization of polyplexes via macropinocytosis-like pathways is impeded through dynasore. (5a) Untreated control and dynasore-treated cells incubated with LPEI (green) and incubated for 2 hours before processing for imaging. Brightfield (upper frames) and corresponding fluorescent pictures (bottom frames) and overlay images are shown. Large number of punctate fluorescent signals were widely dispersed intracellularly throughout control cells. Dynasore-treated cells, on the other hand, exhibit noticeably lower fluorescence intensity inside cells. Nonetheless, overall internalization was not entirely halted as considerable fluorescent signal was still apparent. (5b) Quantitative presentation of polyplexes distribution inside cells revealed a considerably decreased number of particles per cell in dynasore-treated populations in contrast to the control, a finding consistent with the observations made in Fig. 5a. Scale bar = 20 μm .