Super-Resolution Imaging of Proton Sponge- Triggered Rupture of Endosomes and Cytosolic Release of siRNA

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ABSTRACT: The intracellular delivery of nucleic acids and proteins remains a key challenge in the development of biologic therapeutics. In gene therapy, the inefficient delivery of small interfering RNA (siRNA) to the cytosol by lipoplexes or polyplexes is often ascribed to the entrapment and degradation of siRNA payload in the endosomal compartments. A possible mechanism by which polyplexes rupture the endosomal membrane and release their nucleic acid cargo is commonly defined as the “proton sponge effect”. This is an osmosis-driven process triggered by the proton buffering capacities of polyplexes. Herein, we investigate the molecular basis of the “proton sponge effect” through direct visualization of the siRNA trafficking process, including analysis of individual polyplexes and endosomes, using stochastic optical reconstruction microscopy. We probed the sequential siRNA trafficking steps through single molecule super-resolution analysis of subcellular structures, polyplexes, and silencing RNA molecules. Specifically, individual intact polyplexes released in the cytosol upon rupture of the endosomes, the damaged endosomal vesicles, and the disassembly of the polyplexes in the cytosol were examined. We found that the architecture of the polyplex and the rigidity of the cationic polymer chains are crucial parameters that control the mechanism of endosomal escape driven by the proton sponge effect. We provide evidence that in highly branched and rigid cationic polymers, such as glycogen or polyethylenimine, immobilized on silica nanoparticles, the proton sponge effect is effective in inducing osmotic swelling and rupture of endosomes.

KEYWORDS: endosomal escape, polyplexes, proton sponge effect, STORM, glycogen

The complexation of nucleic acids with cationic lipid and polymers to form lipoplexes and polyplexes, respectively, is widely used for intracellular transfection of DNA, siRNA, microRNAs, and lately CRISPR-Cas9.^{1,2} To efficiently deliver their cargo and promote alteration

of gene expression and transcription, these vectors must overcome multiple biological barriers, including substantial cellular uptake, endosomal escape, and intracellular targeting devoid of off-target effects. The endolysosomal escape³⁻⁷ and endocytic recycling⁸ processes are reported to be the major bottlenecks encountered in the intracellular release of nucleic acid payloads. In particular, the translocation of intact and functional RNA double strands from late endosomes to the cytosol prior to their degradation in the lysosome compartments is usually considered the rate-limiting step in siRNA-mediated knockdown of protein expression (RNAi).^{3,6} It has been estimated that the escape of siRNAs from endosomes to the cytosol occurs at a very low efficiency (0.01–2%).^{5,7} The delivery of siRNAs, mediated by lipoplexes and polyplexes, has been investigated by time-lapse confocal and electron microscopy.³⁻⁶ This qualitative and quantitative image-based analysis of lipid and polymer-based nanocarriers, in fixed and live cells, have revealed different mechanisms of release of RNA molecules from the maturing endosomal compartments.

In the case of lipid-mediated delivery of siRNAs, real-time imaging studies⁴ have shown that siRNAs molecules are gradually transferred to the cytosol without rupture of the endosomal membrane. This finding suggests that free siRNAs cargoes, rather than intact lipid carriers, escape into the cytosol presumably *via* the formation of multiple transient pores in the endosomal membrane. The transfection of nucleic acids by polyplexes primarily relies on the use of polyethyleneimine (PEI)^{9,10} or polyamidoamine dendrimers.¹¹ After cellular uptake of polyplexes by endocytosis, the inner endosomal compartment is acidified owing to the activity of the adenosine triphosphate (ATP)-dependent proton pump. This leads to a change in pH inside the vesicles from around ~7.2 to ~6.3 in the early endosome and to 5.5 in the late endosomes.¹² Polyplexes are designed with pK_a values in the range of the estimated endosomal “pH change window” so that they can trigger a large inflow of protons (H^+) and chloride (Cl^-) counterions on

account of their buffering capacity.¹³⁻¹⁵ The resulting osmotic imbalance causes a major water intake into the endosomes, resulting in vesicle swelling, rupture, and eventually release of the cargo into the cytosol. This mechanism is defined as the “proton sponge effect”. However, the molecular basis of endosomal escape of polymer–nucleic acid complexes is not well understood and is still under investigation.

Image-based analyses of the intracellular trafficking of PEI polyplexes have recently raised questions about the proton sponge effect mechanism.^{3,6} This is largely because neither complete rupture of endosomes nor release of intact polyplexes into the cytosol has been observed by direct confocal microscopy imaging of either live or fixed cells. These studies suggested that dissociation of the siRNA might occur in the endosomal compartments. At the time of endosomal escape, the PEI component and the nucleic acid payload are separately ejected into the cytoplasm, presumably *via* transient pore(s) within the endosomal membrane.⁶ Part of the positively charged polymer remains temporarily associated with the endosomal compartment, whereas the nucleic acids are released into the cytosol. In another recent study,³ it was pointed out that endosomal bursting, caused by the proton sponge effect triggered by PEI polyplexes, was dependent on endosome size and cell type. Smaller vesicles were more prone to be ruptured by the osmotic pressure effect than larger vesicles³ and leaky and compromised cell membranes were ineffective in building an osmotic pressure, regardless of endosomal size. Overall, these recent findings provide evidence that the proton sponge effect mediated by polycations may not be as destructive to the integrity of the endosomal membrane as initially thought. In addition, there is general consensus that the endosomal escape of siRNA is the key step that governs the kinetics and efficiency of RNA interference.

Herein, we employed stochastic optical reconstruction microscopy (STORM), a single molecule super-resolution technique, to elucidate the endosomal escape route and intracellular fate of polyplexes. We could probe the different trafficking processes with 20 nm lateral resolution and visualize single intact polymer–siRNA constructs released in the cytosol upon rupture of endosomes, damaged endosomal vesicles, and the disassembly of the polyplexes in the cytosol. By monitoring individual intracellular events, we gain insight into an additional mechanistic factor that influences proton sponge-based endosomal escape of polyplexes. We show that the architecture of the polymer–siRNA construct and the rigidity of the cationic polymer chains are key parameters to controlling the mechanism of endosomal escape driven by the sponge effect. We have performed a detailed analysis of the intracellular trafficking of cationic glycogen-based polyplexes and PEI-coated silica nanoparticles. Our findings suggest that in the case of highly branched and rigid cationic polymers, such as glycogen or PEI, immobilized on silica nanoparticles, the proton sponge effect is effective in inducing osmotic swelling and complete rupture of endosomes. Furthermore, we provide evidence that the rate-limiting step for intracellular delivery of siRNA mediated by cationic glycogen is the cytosolic disassembly of the complex. The sustained release of siRNA payload is dictated by the slow equilibrium dissociation process of the construct.

RESULTS AND DISCUSSION

We have recently reported the engineering of glycogen–siRNA constructs, hereafter referred to as glycoplexes, which can efficiently target PC3 prostate cancer cells, penetrate into three-dimensional multicellular spheroids of PC3 human prostate cancer cells, and knock down the expression (~60%) of different upregulated proteins.¹⁶ Glycogen is a randomly branched,

dendrimeric nanosaccharide composed of repeating units of glucose connected by linear α -D-(1–4) glycosidic linkages with α -D-(1–6) branching, with a highly branched outer surface and a dense core.^{16,17} Controlled assembly of siRNA with 20 nm, positively charged ethylenediamine (EDA)-modified bovine glycogen (BG) (BG-EDA, and the PEGylated derivative BG-EDA-PEG) led to the formation of glycoplexes with a mean diameter of approximately 120 nm and a slightly negative surface charge (Figure 1 and Table S1). Each glycoplex was estimated to accommodate between 1200 and 3300 molecules of siRNA.¹⁶ EDA was selected for glycogen modification because of its potential to trigger the proton sponge effect *via* the secondary amine group (pK_{a1} 6.4) and to complex siRNA *via* the primary amine groups (pK_{a2} 10).¹⁶

However, the use of glycogen as a versatile carrier of nucleic acids has raised several questions. Which endocytic mechanisms are responsible for glycoplex uptake? To which endocytic compartments are glycoplexes transported? Once transported by the endosomes, what is the mechanism and the kinetics of release of siRNAs escaped from endosomes? We sought to answer these questions by performing STORM imaging of subcellular processes at high resolution in space.

STORM Characterization of Glycoplexes. STORM images of BG-EDA (Figure S1A) and BG-EDA-PEG (Figure S1B) glycoplexes labeled with the photoswitchable Alexa Fluor 647 dye (AF647) in aqueous buffer (*e.g.*, phosphate-buffered saline (PBS) buffer, pH 7.4, in the presence of 50 mM 2-mercaptoethanol (MEA) and oxygen scavenging system) were acquired.¹⁸ Imaging and analysis of the particles adsorbed on glass coverslips showed monodisperse particle populations for both samples, with particle sizes centered at ~115 nm (BG-EDA glycoplexes, Figure S1A) and ~118 nm (BG-EDA-PEG glycoplexes, Figure S1B). These results agreed with those obtained by dynamic light scattering (DLS) data shown in Table S1.

The stability and integrity of the glycoconjugates in cell culture medium containing serum proteins was also confirmed by STORM imaging of the glycoconjugates tagged with activator–reporter pair Alexa Fluor 488–Alexa Fluor 647 (AF488/AF647)¹⁹ (Figure 2A,B). There was no evidence of aggregation, but rather a slight increase in size was observed for BG-EDA and BG-EDA-PEG glycoconjugates to 140 ± 30 nm and 190 ± 40 nm, respectively. These results indicated limited interaction of glycoconjugates with serum proteins, likely driven by hydrogen bonding or weak electrostatic forces.

Figure 1. Schematic illustration of glycoconjugate formation. Glycogen from bovine liver (BG) is modified with ethylenediamine (EDA) and polyethylene glycol (PEG) by reductive amination reaction. Modified glycogen (BG-EDA or BG-EDA-PEG) is then mixed with siRNA to form glycoconjugate. Orange circles in the glycogen structure indicate ethylene amine functionalities, whereas black strands represent flexible PEG chains (BG-EDA-PEG). To perform STORM

imaging, BG-EDA and BG-EDA-PEG were fluorescently-labeled with an activator–reporter pair of dyes, AF488 (green circle) and AF647 (red circle) respectively. *Values are reproduced from ref. 16.

Figure 2. Characterization of glycoplexes in biological and cellular environment. Size distribution analysis ($n = 110$) of (A) BG-EDA and (B) BG-EDA-PEG glycoplexes incubated with cell culture medium, imaged, and analyzed by STORM. STORM images were acquired on glycoplexes adsorbed on glass slides after incubation in complete cell culture medium for 5 h. (C) Fluorescence intensity histograms of PC3 cells treated with BG-EDA and BG-EDA-PEG glycoplexes and endocytic inhibitors. (D) Effect of endocytic inhibitors on the uptake of BG-EDA and BG-EDA-PEG glycoplexes by PC3 cells, after 2 h of incubation with the particles.

Mechanism of Internalization of the Glycoplexes. To gain insight into the mechanism of intracellular trafficking of glycoplexes, the endocytic pathway through which the constructs enter

the cells was examined, as it is known that this pathway determines the subsequent intracellular processing and strongly influences gene delivery efficiency. Typically, lipid- and polymer-based vectors enter cells by endocytosis after cell association.²⁰⁻²² Several internalization pathways have been identified including macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis. Importantly, these pathways are not necessarily mutually exclusive. For instance, PEI-based polyplexes are internalized *via* the clathrin- and caveolae-mediated pathways.²³ It is important to determine whether glycoplexes are trafficked *via* a pathway that relies on compartmental acidification. Inhibitors of endocytosis can be used to block and investigate the specific endocytic pathway used by the construct to enter cells. To be consistent with our previous studies,¹⁶ prostate cancer PC3 cells were used as a model system.

We first assessed the effect and specificity of the metabolic inhibitors pitstop 2, filipin from *Streptomyces filipinensis*, and 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on PC3 cells using markers for the different endocytic pathways. EIPA, pitstop 2, and filipin are inhibitors of micropinocytosis-, clathrin-, and caveolae-dependent endocytosis pathways, respectively. Transferrin, cholera toxin B subunit, and 10 kDa dextran were used as endocytic markers for clathrin-, caveolae-mediated endocytosis, and macropinocytosis, respectively (Figure S2). We confirmed that none of the studied inhibitors significantly inhibit pathways other than those targeted in the PC3 cell line. For instance, filipin did not reduce the uptake of transferrin or dextran, whereas the uptake of cholera toxin B was reduced up to 60%. The treatment of cells with EIPA inhibited the internalization of dextran, whereas no marked effect was observed on the uptake of transferrin and cholera toxin B. Pitstop 2 reduced the uptake of transferrin to approximately 50%. These results demonstrate the activity and specificity of the studied inhibitors on PC3 cells.

Next, PC3 cells were treated with the same endocytosis inhibitors for 15 min (Figure 2C,D). In addition, control experiments were set up at a low temperature (4°C) to rule out nonendocytic pathways such as membrane fusion pathway. Subsequently, cells were incubated with Alexa Fluor 555 (AF555)-labeled glycoplexes for 1 h, and the uptake was measured by flow cytometry.

In Figure 2C, to compare the results obtained for the PC3 control cells and cells treated with BG-EDA and BG-EDA-PEG glycoplexes and endocytic inhibitors, the fluorescence intensity histograms were not normalized for the spectroscopic fluorescence values of the samples. However, it is worth noting that in our previous work,¹⁶ we demonstrated that the uptake efficiency of BG-EDA-PEG is lower than BG-EDA when normalized values are used.

Figure 2D shows that >98% of internalization of both BG-EDA and BG-EDA-PEG glycoplexes is inhibited by treatment with NaN₃ (ATPase inhibitor) and incubation at low temperature. This indicates that BG-EDA and BG-EDA-PEG glycoplexes are transported inside the cells by one or a combination of the endocytic pathways rather than *via* the membrane fusion pathway. No inhibition in the uptake of BG-EDA-PEG glycoplexes and limited inhibition in the uptake of BG-EDA glycoplex (<18%) were observed upon incubation with pitstop 2, indicating that clathrin-dependent endocytosis is not the dominant mechanism of internalization. Both filipin and EIPA caused a significant reduction in the uptake of BG-EDA glycoplexes (~60% and ~85% of inhibition, respectively), suggesting that a combination of caveolae-mediated endocytosis and macropinocytosis is involved in the uptake of these glycoplexes. Conversely, EIPA did not influence the endocytosis of BG-EDA-PEG glycoplexes, whereas filipin caused a ~20% inhibition in their internalization process. These results indicate that BG-EDA-PEG glycoplexes are internalized by the caveolae-dependent pathway. These results also showed that even a small

degree of PEGylation (5% degree of substitution) of glycogen nanoparticles can significantly influence the endocytic routes of the nucleic acid cargo.

Super-Resolution Imaging of the Intracellular Trafficking of Glycoplexes: Endosomal Escape and Disassembling. To unravel the mechanisms underlying glycogen-mediated delivery of siRNAs, the intracellular trafficking of glycoplexes internalized in PC3 cells was first examined by confocal microscopy. The direct visualization of intracellular compartments and glycoplexes would help to confirm the colocalization studies of constructs with endocytosis markers.

By using marker-specific antibodies, it is possible to distinguish between different stages of endosome maturation. Endosome staining was performed with antibodies against Early Endosome Antigen 1 (EEA1) and Rab7. EEA1 is a protein exclusively localized on early endosomes. Acting as an effector of Rab5 receptor, EEA1 is responsible for docking of newly formed endocytic vesicles and for inducing fusion with early endosome. Rab7 is a terminal marker used for identifying late endosome-to-lysosome maturation and trafficking, and it is localized in both these compartments.²⁴

Figure 3 shows representative confocal microscope images of PC3 cells incubated for 4 h with AF555-labeled BG-EDA and BG-EDA-PEG glycoplexes after immunostaining with anti-EEA1 and anti-Rab7. Confocal microscopy images show limited colocalization (yellow dots) of BG-EDA and BG-EDA-PEG glycoplexes within both early and late endosomes or lysosomes (red dots). The confocal microscopy images were analyzed and Pearson's correlation coefficient (PCC) values were calculated to determine the extent of colocalization of glycoplexes with early and late endosomes. Considerably low PCC values (0.19 and 0.08 values for early and late endosomes, respectively) indicate limited colocalization of BG-EDA and BG-EDA-PEG glycoplexes with

both early and late endosomes or lysosomes. This result is the first indication of endosomal escape and cytosolic localization of the constructs after cell incubation for 4 h.

Figure 3. Colocalization studies of glycoplexes in PC3 cells using confocal laser scanning microscopy. Images were taken 4 h after transfection of PC3 cells with AF555-labeled BG-EDA and BG-EDA-PEG glycoplexes (green). Endosomes (red) were stained using mouse anti-EEA1 monoclonal antibody (early endosome) or rabbit anti-Rab7 monoclonal antibody (late endosome), followed by a secondary AF647 conjugated antibody.

However, confocal microscopy imaging is restricted to the diffraction resolution (~250 nm), thus restricting the observation of individual nanoparticles and vesicles. To advance the understanding of the intracellular route of glycoplexes and the mechanism of release of siRNA payload, a super-resolution microscopy technique, STORM, was used. Unlike conventional optical microscopy, STORM allows detailed inspection of the cellular and nanocarrier architecture with 20-nm lateral resolution by reconstructing images on the basis of the accurate localization of single, stochastically blinking fluorescent molecules. Recently, super-resolution microscopy has been exploited to image cellular structures²⁵ and nanomaterials.²⁶⁻³⁰ However, to our knowledge, the use of STORM to probe nanomaterial–cell interactions³¹ and track the intracellular events associated with siRNA delivery remains unexplored to date.

The transfection of PC3 cells with glycoplexes was conducted for 2 h. After the initial incubation, the transfection medium was discarded and the cells were cultured for the following 48 h in fresh culture medium. The intracellular trafficking of the constructs was analyzed immediately after medium replacement (2 h incubation) and after 4 and 7 h incubation on fixed cells. A glycogen sample labeled with AF488/AF647 activator–reporter pair and a tandem-labeled secondary antibody with AF555/AF647 pair was prepared. This allows for multicolor acquisition on an STORM instrument, as different activators are concomitantly used to identify nanoparticles and cellular vesicles.³² To visualize the initial and later phases of trafficking, immunostaining for early (anti-EEA1 antibody) and late endosomes (Rab-7 antibody) was performed, and the colocalization of these vesicles with glycoplexes was examined.

Figure 4 and Figure S3 show representative STORM images acquired for PC3 specimens after 2 h and 4 h incubation with BG-EDA (Figure 4A,C) and BG-EDA-PEG (Figure 4B,D) glycoplexes, and early endosomes staining.

Figure 4. Multicolor STORM super-resolution images of glycoplexes and early endosome compartments. Intracellular trafficking of (A and C) BG-EDA and (B and D) BG-EDA-PEG glycoplexes and early endosome compartments after 2 h (A and B) and 4 h incubation (C and D) with PC3 cells. Image 1: large view of a representative PC3 cell; Image 2: bright-field image of the analyzed cell; Images 3-6: high-magnification images showing details of intracellular imaged nano-objects; The green signal stems from dual-labeled BG-EDA and BG-EDA-PEG glycoplexes

(AF488/AF647 pair), whereas the red signal denotes early endosomes (anti-EEA1 dual-labeled AF555/AF647 immunostaining).

Figure 5 shows representative STORM images acquired for PC3 specimens after 7 h incubation with BG-EDA (Figure 5A) and BG-EDA-PEG (Figure 5B) glycoplexes and early endosomes staining. Figure S4-S6 show representative STORM images acquired for PC3 specimens after 2h, 4 h and 7 h incubation respectively with BG-EDA (Figure S4-6A) and BG-EDA-PEG (Figure S4-6B) glycoplexes and late endosomes staining.

Figure 5. Multicolor STORM super-resolution images of glycoplexes and early endosome compartments. Intracellular trafficking of (A) BG-EDA and (B) BG-EDA-PEG glycoplexes and

early endosome compartments after 7 h incubation with PC3 cells. Image 1: large view of a representative PC3 cell; Image 2: bright-field image of analyzed PC3 cell; Images 3-6: high-magnification images showing details of intracellular imaged nano-objects; The green signal indicates dual-labeled BG-EDA glycoplex (AF488/AF647 pair), whereas the red signal denotes early endosomes (anti-EEA1 dual-labeled AF555/AF647 immunostaining).

First, the imaged nano-objects and signals in Figure 6 were categorized as follows: (i) glycoplexes colocalized with endosomes (“colocalized perfect”); (ii) glycoplex nanoparticles approaching ruptured endosomes (“colocalized bursting endosomes”); (iii) non-colocalized glycoplex nanoparticles (“non-colocalized glycoplex”); (iv) non-colocalized disassembled glycoplex molecules (“non-colocalized disassembled glycoplex”); and (v) non-colocalized endosomes. To quantify the extent of colocalization of the glycoplexes with early and late endosomal compartments, a statistical analysis of the acquired STORM images as a function of incubation time was performed (Figure 7). The objects in (i) and (ii), categorized as colocalized signal, were grouped.

Figure 7 shows that after incubation for 2 h, approximately 60–50% and 40–30% of BG-EDA and BG-EDA-PEG glycoplexes are colocalized with early and late endosomes, respectively. These data suggest that most glycoplexes are confined within endosomal compartments after incubation for 2 h. However, for longer incubation times (4 and 7 h), a significant decrease in colocalization of glycoplexes with both early (15–10% at 7 h) and late endosomes (5–2% at 7 h) was observed.

The lack of colocalization with both early and late endosomes or lysosomes indicated that cytosolic distribution of glycoplexes occurred in the first 7 h of intracellular trafficking. The fraction of glycoplexes (20–40 %) trafficked from early to late endosomes in the first 2 h (Figure 7C) can represent glycoplexes either released from endosomes or degraded in the late

endosomes/lysosomes. In addition, widespread diffusion of glycogen molecules in the cell cytoplasm (Figure 5B, Images 5 and 6) was more pronounced in cells transfected with BG-EDA-PEG glycoplexes than in cells transfected with BG-EDA.

		Colocalized				Non-colocalized glycoplex		Non-colocalized endosomes	
		Perfect		Bursting					
Early Endosome	2 h								
Early Endosome		Colocalized (bursting endosomes)				Non-colocalized glycoplex		Non-colocalized endosomes	
	4 h								
	7h								
Late Endosome		Colocalized			Non-colocalized glycoplex			Non-colocalized endosomes	
	2 h								
Late Endosome		Colocalized		Non-colocalized glycoplex				Non-colocalized endosomes	
	4 h								
Late Endosome		Non-colocalized glycoplex (only)							
	7 h								

Figure 6. Gallery of intracellular nano-objects and signals acquired by STORM at different incubation times, showing glycoplexes colocalized with endosomes, glycoplexes approaching bursting endosomes, non- colocalized glycoplexes and non-colocalized endosomes.

Figure 7. Statistical analysis of STORM images describing the extent of colocalization of glycoplexes with endosomal compartment at different incubation timepoints. (A) Fractions of BG-EDA glycoplexes colocalized with the early endosomes as a function of incubation time. (B) Fractions of BG-EDA-PEG glycoplexes colocalized with the early endosomes as a function of incubation time. (C) Fractions of BG-EDA glycoplexes colocalized with late endosomes as a

function of incubation time. (D) Fractions of BG-EDA-PEG colocalized with late endosomes as a function of incubation time.

Interestingly, images showing 300-500 nm sized objects with a patchy-like spherical structure (Figure 8) were acquired for PC3 specimens after incubation for 2, 4, and 7 h with glycoplexes and early endosomes staining. Approximately 30 nano-objects per cell of this type were observed in each of the 20 cells examined. Of note, by using the 2D STORM-TIRF approach to maximize signal-to-noise ratio and improve the quality of images, we probed a sample thickness of approximately 200 nm. On these spherical nano-objects, the green signal arising from glycoplexes is not colocalized with the red signal arising from the early endosome membrane. Hence, we speculate that the glycoplexes observed on these images are not located inside the endosomes but rather on the surface or in close proximity. This observation suggests that the endosomal membrane has been subjected to a stress that compromised its integrity, likely causing the formation of large pores. Literature defines the critical area strain for membranes to be 5%,³³ which suggests a fixed burst criterion. Taken together, these sub-diffraction STORM/TIRF microscopy images show that the early endosome membranes are ruptured and the glycoplexes can protrude throughout the lipid membrane.

Figure 8. Gallery of STORM images showing ruptured early endosomes and glycoplexes protruding throughout the lipid membrane. For comparison purposes, images representing colocalized and non-colocalized endosomes are shown (first row). The green signal indicates dual-labeled BG-EDA glycoplex (AF488/AF647 pair), whereas the red signal denotes early endosomes (anti-EEA1 dual-labeled AF555/AF647 immunostaining). See also Figure 6 for classification of the different nano-objects. Scale bar: 100 nm.

To support our speculations that endosomal escape of glycoplexes is driven by the proton sponge effect, we performed a control experiment in the presence of bafilomycin A1 (Figure 9). Bafilomycin A1 is a macrolide antibiotic that prevents acidification of endosomal vesicles. Cells

were treated with bafilomycin A1 for 15 min prior to incubation with glycoplexes. STORM analysis of the treated cells was performed after incubation for 2 and 4 h, as previously described. We observed over 90% of perfect colocalization (Images 2 and 3 in Figure 9A and 9C and corresponding statistical analysis in Figure 9E) of glycoplexes with early endosomes after incubation for 2 h (Figure 9A) and 4 h (Figure 9C). We also observed colocalization (over 90%) of BG-EDA-PEG glycoplexes with early endosomes after incubation for 2 h of (Images 2 and 3 in Figure 9B and Figure 9F). After incubation for 4 h, we could also observe colocalization of both BG-EDA and BG-EDA-PEG glycoplexes (approximately 80–90%) (Image 2 in Figure 9D), whereas the number of non-colocalized objects slightly increased to ~20%. When comparing the data shown in Figure 7A and B obtained without bafilomycin pre-treatment and Figure 9E and F obtained with bafilomycin pre-treatment, we can infer that by preventing acidification of endosomal vesicles, a large number of glycoplexes remain confined in early endosomes. Overall, these results strongly suggest that endosomal escape of glycoplexes is driven by the buffering capacity of glycoplexes resulting in the proton sponge effect.

Figure 9. Multicolor STORM super-resolution images of glycoproteins and early endosome compartments after treatment of PC3 cells with bafilomycin A1. Intracellular trafficking of (A and C) BG-EDA and (B and D) BG-EDA-PEG glycoproteins and early endosome compartments after 2 h (A and B) and 4 h (C and D) incubation with PC3 cells. Image 1: large view of a representative

PC3 cell; Image 2-3: high-magnification images showing localization of glycoplexes and early endosomes. The green signal indicates dual-labeled BG-EDA glycoplex (AF488/AF647 pair), whereas the red signal denotes early endosomes (anti-EEA1 dual-labeled AF555/AF647 immunostaining). Statistical analysis of STORM images describing extent of colocalization of (E) BG-EDA and (F) BG-EDA-PEG glycoplexes with endosomal compartments at different timepoints after treatment with bafilomycin A1.

Super-Resolution Imaging of the Intracellular disassembling and release of siRNA Release.

To gain insight into the kinetics of disassembly of glycoplexes in the cytosol and associated influence on the target gene knockdown activity in PC3 cells, a combination of STORM and gene silencing studies were performed. The knockdown efficacy of glycoplexes was evaluated in PC3 cell lines using NF- κ B as a model target. NF- κ B is a ubiquitous transcription factor that regulates fundamental metabolic processes, and it is upregulated and constitutively activated in prostate cancer PC3 cells.³⁴ Cells were incubated for 48 and 72 h with glycoplexes loaded with an siRNA formulation targeting NF- κ B and analyzed at the different time points. Knockdown efficacy was evaluated using protein-specific immunostaining by flow cytometry and western immunoblotting. The efficacy of BG-EDA and BG-EDA-PEG glycoplexes in siRNA transfection *in vitro* was compared to that of the commercially available cationic lipid-based transfection agent lipofectamine RNAiMax. The data in Figure 10A,B show the downregulation of protein expression after treatment with glycoplexes loaded with either protein-specific or eGFP siRNA, which was used as a negative control for NF- κ B siRNA. A time-dependent silencing effect of the target protein was observed. No significant downregulation was observed in cells treated with glycogen loaded with eGFP siRNA (negative control), indicating that the observed knockdown was specific and that no off-target effects were caused by the transfection itself. These results

suggested that a prolonged treatment of cells with glycoplexes was necessary to detect significant downregulation of NF- κ B expression. This observation agrees with previous studies performed with glycoplexes to downregulate luciferase and survivin in 293T, PC3 monolayer cell cultures, and 3D multicellular spheroids.¹⁶

Figure 10. Silencing activity of glycoplexes upon disassembly in the cytosol. (A) NF- κ B p105/p50 protein knockdown in PC3 cells after 48 and 72 h post-transfection measured by flow cytometry. EGFP siRNA was used as a negative control. (B) Representative Western Blot image of NF- κ B knockdown in PC3 cells. (C) STORM images of AF647-labeled siRNA (red) after 5 h incubation with PC3 cells. Image 1: large view of analyzed cell; Image 2: large view of siRNA localized within glycoplex structure in the cytosol; Image 3: image of glycoplex likely disassembling and releasing siRNA in the cytosol; Image 4: bright-field image of analyzed cell. (D) STORM images

of AF647-labeled siRNA (red) after 48 h incubation with PC3 cells. Image 1: large view of analyzed cell; Image 2 and 3: image of siRNA molecules diffusing from glycoplexes in the cytosol and Image 4: bright-field image of analyzed cell.

STORM imaging was then used to obtain insight into the kinetics of glycoplexes dissociation and siRNA cargo release in the cytosol. PC3 cells were analyzed after transfection with glycoplexes at two time points: 5 h (Figure 10C and Figure S7A) and 48 h (Figure 10D and Figure S7B). In this study, AF647-labeled siRNA and unlabeled glycogen were used. At the early and late stage of trafficking, nanometer-sized objects (Figure 10C, image 2; Figure 10D, image 2) enclosing siRNA were observed together with spread single siRNA molecules (Figure 10C, image 3; Figure 10D, images 2, 3). On the basis of previous discussion on endosomal escape of glycoplexes, we can assume that after 48 h, siRNA assemblies or single molecules either are localized in the cytosol. These results indicate that siRNA is tightly immobilized into glycogen nanoparticles and gradually transferred in the cytosol where it ultimately binds to the RNA-induced silencing complex

It is necessary to point out that STORM and other microscopy approaches based on a single fluorescent probe do not allow for discriminating intact and functional siRNA molecules from inactive siRNA degraded by cellular nucleases.

Overall, our results suggest that the mechanism of glycogen-mediated delivery of siRNA is different from that of siRNA delivery from PEI-based polyplexes reported in the literature.^{3,6} The direct and single molecule visualization of the individual components of glycoplexes by STORM indicates that the disassembly of glycoplexes, rather than endosomal escape, is the rate-limiting step for their intracellular delivery. Microscopy images acquired and processed by using STORM and tandem dye labeling technique allowed us to capture all events involved in the trafficking of

glycoplexes with sub-diffraction resolution. To summarize our findings, a schematic representation of the intracellular events observed by STORM imaging is shown in Figure 11.

Figure 11. Schematic representation of intracellular trafficking of BG-EDA and BG-EDA-PEG glycoplexes. Glycoplexes are internalized by a combination of macropinocytosis and caveolae-mediated endocytosis. After endocytosis, caveolae vesicles and macropinosomes fuse with EEA1 positive vesicles, leading to localization of glycoplexes within early endosomes. As a result of adenosine triphosphatase (ATPase) pump activity, early endosome undergoes acidification causing a decrease in pH from ~6.3 to ~5.5. The maturation of endocytic vesicles leads to loss of EEA1 and recruitment of Rab7 marker, characteristic for late endosome. Glycoplexes have a pK_a of ~6.4 and can buffer early and late endosomes and induce proton sponge effect. As a result, intact glycoplexes are released into cytosol. A sustained disassembly of glycoplexes in cytosol enables siRNA molecules to bind to RISC.

Importantly, unlike previous aforementioned studies,^{3,6} the present study provides evidence that the membrane of the released endosome ruptures to enable the release of nanometer-sized constructs into the cytosol. The observed mechanism of endosomal escape suggests bursting of early endosomes triggered by the proton sponge effect, followed by fast release of intact glycoplexes into the cytosol, and slow detachment of siRNA molecules. The latter process is deemed to be the major bottleneck.

Although endosomal membrane rupture can be the outcome of either the osmotic swelling mechanism or polycation-induced permanent destabilization of the endosomal membrane,³⁵ the rigid structure of glycogen would suggest their inability to engage with the bilayer lipids components.

We speculate that the architecture of the cationic glycogen, which is dendritic but also highly compact and rigid, prevents dynamic association with the hydrophobic endosomal membranes. Amine groups, embedded into the glycogen scaffold, may exert the proton sponge effect without

directly interacting with the lipid membrane. The intrinsic rigidity of glycogen nanoparticles is due to the highly branched dendritic structure of glycogen. High-molecular weight dendrimer polymers, such as glycogen, display a rigid surface scaffolding owing to enhanced surface congestion. Thus, these polymers behave as rigid globular spheres.³⁶ The small angle neutron scattering studies performed on glycogen suggest that glycogen nanoparticles behave similarly to hard sphere colloids.³⁷ The rigidity of dendrimer polycations has been directly correlated to their mild cytotoxicity and inability to disrupt lipid membranes.³⁸ In our previous work,¹⁶ the cytotoxicity of PEI and glycoplexes was compared in different cell lines, including A549 and PC3 cells. These results show that PEI becomes toxic even at low concentrations (*e.g.*, 0.06 mg mL⁻¹). Unlike PEI, glycogen–siRNA constructs, and particularly BG-EDA and BG-EDA-PEG, show a desirable toxicity profile even at high concentrations (*e.g.*, 0.5 mg mL⁻¹).¹⁶ This can be explained by the dendritic, however, compact and rigid architecture of cationic glycogen, which is less prone to entanglement with the membrane lipids than PEI.

As a result, the high buffering capacity of cationic glycogen nanoparticles likely makes endosome swelling and bursting the predominant route of releasing intact glycoplexes into the cytosol. To obtain supporting evidence for this mechanism and justify the different findings reported herein when compared to those reported in several previous studies^{3,6} that have shown that PEI-based polyplexes can induce endosomal leakiness without membrane rupture through the PEI-lipid bilayer interaction, a positive control experiment was performed. PEI chains were grafted onto the surface of rigid silica nanoparticle scaffolds (PEI-SiNPs) and their endosomal escape was examined.

We expect that PEI chains immobilized on rigid templates to display rigidity features and buffering capacity that are similar to cationic glycoplexes.

The buffering capacity of glycoplexes and PEI-coated silica was compared, as shown in Figure S8. From the results, PEI and BG-EDA each possess two pK_a values at 6.1 and 9.4 for PEI and 6.4 and 10.3 for BG-EDA. The pK_a values at 6.4 and 6.1 are within the range of endosome acidification. This indicates that the two polymers have a similar buffering capacity within early endosomes. The rigidity of polymeric PEI chains is considerably enhanced upon chemical conjugation to the silica template, as evidenced by the NMR spectra (Figure S9). Sharp peaks were observed in the spectrum of free PEI. In contrast, very broad and featureless NMR signals were obtained upon attachment of the PEI chains to the surface of the template, indicating that the PEI chains have limited rotational mobility.

Therefore, PEI chains were grafted onto the surface of rigid silica nanoparticle scaffolds and their endosomal escape route was investigated by STORM. Silica nanoparticles (120 nm diameter, Figure S10) were functionalized with PEI and labeled with AF488/AF647 fluorophores pair, as described in the *Methods* section.

To track the intracellular fate of the nanoparticles and monitor the progressive stages of the endocytic route, cells were incubated with PEI-SiNPs and the same immunostaining protocol based on marker-specific antibodies against EEA1 and Rab7 was used. The colocalization of PEI-SiNPs and early endosomal compartments in cells cultured in the presence of the particles for 2 h (Figure 12A) were first examined. The acquired images showed a perfect overlap between the PEI-SiNPs (green signal) and early endosomes vesicles (red signal), visualized as round yellow objects distributed in the cell cytoplasm (Figure 12A, Images 2 and 3). These results suggested that most of the PEI-SiNPs were internalized and confined within round-shaped endosomal compartments,

retaining their structural features. However, non-colocalized PEI-SiNPs (Figure 12A, Image 4) were also observed. The following stage of endocytic trafficking was next monitored. Cells were incubated for 2 h with the PEI-SiNPs and subsequently cultured for additional 4 h in fresh culture medium. Labeling of late endosome-lysosome compartments was performed by immunostaining of Rab7, as previously described for the glycoplexes. Interestingly, very limited colocalization of the PEI-SiNPs (green signal) and stained vesicles (red signal) was observed, and most of the signals visualized in the cytosol were either green or red nano objects (Figure 12B, Images 2-4). Focusing onto a single STORM object enabled direct visualization of endosomal escape. A single PEI-SiNP could be observed to likely free itself from the enveloping vesicle upon rupture (Figure 12B, Image 3). Taken together, these data suggest that endosomal escape of single and intact PEI-SiNPs occurs through breach of the internalizing endosomal compartments.

Our results also point out that PEI chains immobilized on the rigid silica nanoparticle scaffolds display a behavior similar to that of glycogen nanoparticles.

This suggests that the stiffness of the PEI-nanoparticle construct can lead to the endosomal escape mediated by osmotic swelling over other mechanisms hinging on membrane leakiness induced by direct interaction of polycations with the vesicle lipid membrane.

Figure 12. Multicolor STORM super-resolution images of PEI-SiNPs and early and late endosome compartments in the cells. The red signal is associated with the analyzed vesicles, whereas the green signal originates from the PEI-SiNPs. (A) Intracellular trafficking of PEI-SiNPs and early endosome compartments after 2 h incubation with cells. Image 1: large view of a representative cell showing cytosol distribution of early endosomal compartments and PEI-SiNPs; Images 2–4: high-magnification images of cytosol showing colocalization of PEI-SiNPs with early endosomes (yellow), non-colocalization, and escape of single PEI-SiNPs (green). (B) Intracellular trafficking of PEI-SiNPs and late endosome compartments after 6 h incubation with cells. Image 1: large view of a single representative cell showing predominant cytosol distribution of late endosome compartments and PEI-SiNPs; Image 2: high-magnification image showing intact PEI-SiNPs (green dots) with no overlap with lysosome membranes (red spots); and Image 3: super-resolved image of a single PEI-SiNP escaping from the enveloping late endosome–lysosome compartment; Image 4: single late endosome compartments without PEI-SiNPs.

CONCLUSIONS

In the present study, the molecular basis of the proton sponge effect for highly branched and rigid cationic polymers was examined using STORM. Snapshots of individual intact polyplexes released in the cytosol upon rupture of endosomes, the damaged endosomal vesicles, and the disassembly of the polyplexes in the cytosol were acquired. The present findings provide direct evidence that in highly branched and rigid cationic polymers, such as glycogen or PEI, immobilized on silica nanoparticles, the sponge effect is effective in inducing osmotic swelling and endosome rupture. Noteworthy, the architecture of the polyplex and the rigidity of the cationic polymer chains are important parameters that control the mechanism of endosomal escape driven by the sponge effect. The direct and single molecule visualization of the individual components of glycoplexes by STORM suggests that the rate-limiting step for intracellular delivery of siRNA mediated by cationic glycogen is the cytosolic disassembly of the complex. Furthermore, the present study highlights the versatility of STORM to directly visualize the intracellular trafficking of therapeutic biomolecules such as siRNA and warrants further studies on the intracellular dynamics of therapeutic proteins.

METHODS

Materials. Glycogen from bovine liver, sodium cyanoborohydride, sodium metaperiodate, EDA, SiNPs, (3-aminopropyl)triethoxysilane (APTES), triethylamine (TEA), succinic anhydride, dimethyl sulfoxide (DMSO), *N,N'*-diisopropylcarbodiimide (DIC), *N*-hydroxysuccinimide (NHS), PEI, dimethyl formamide (DMF), NaN₃, filipin from *S. filipinensis*, transferrin-FITC, choler toxin B subunit-FITC, 10kDa Dextran-FITC, bafilomycin A1, EIPA, Dulbecco's phosphate-buffered saline (DPBS), radioimmunoprecipitation assay (RIPA) buffer, and goat anti-rabbit peroxidase antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pitstop 2 was purchased

from Abcam (Cambridge, UK). Methoxypolyethylene glycol amine (methoxy-PEG-amine; 2 kDa) was obtained from JenKem Technology (USA). Triton X-100 and Tween-20 were purchased from Chem-Supply (SA, Australia). Dialysis tubing (10 kDa molecular weight cutoff) and Protease Inhibitor Cocktail were purchased from Thermo Fisher Scientific (VIC, Australia). Alexa Fluor NHS dyes (488, 555, and 647), lipofectamine RNAiMAX, trypsin, and rabbit anti-NF- κ B p105/p50 polyclonal antibody were purchased from Life Technologies (Australia). Dulbecco's modified Eagle medium (DMEM) was purchased from Lonza (Allendale, NJ, USA). Fetal bovine serum (FBS) was purchased from Bovogen (Australia). Illustra NAP-10 columns were purchased from GE Healthcare and Life Sciences (NSW, Australia). Polyvinylidene fluoride (PVDF) membrane was obtained from Pall Corporation (VIC, Australia). Illustra NAP-5 and NAP-10 columns were purchased from GE Healthcare and Life Sciences (Silverwater, NSW, Australia). PC3 human prostate epithelial cancer cells (CRL-1435) and HeLa (CCL-2) were obtained from ATCC. Paraformaldehyde solution (4%) was obtained from ProSciTech (QLD, Australia). SiRNA NF- κ B sequence (sense: 5'-GGG UAU AGC UUC CCA CAC U[dTdT]-3'; anti-sense: 5'-AGU GUG GGA AGC UAU ACC C[dTdT]-3') was purchased from Sigma-Aldrich (St. Louis, MO, USA). SiRNAscr sequence: eGFP Silencer (sense: 5'-GCA CCA UCU UCU UCA AGG A[dTdT]-3'; anti-sense: 5'-UCC UUG AAG AAG AUG GUG C[dTdT]-3') was purchased from Thermo Fisher Scientific (VIC, Australia). Clarity ECL substrate was obtained from Bio-Rad Laboratories (CA, USA). Rabbit anti- β -Actin and rabbit anti-Rab7 monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-EEA-1 monoclonal antibody was purchased from BD Biosciences (San Diego, CA, USA). Goat anti-mouse IgG secondary antibody and goat anti-rabbit IgG secondary antibody, and Alexa Fluor conjugates were supplied by Invitrogen (Carlsbad, CA, USA). All chemicals were used as received without further purification.

High-purity water with a resistivity greater than 18.2 M Ω cm was obtained from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation, Billerica, MA, USA).

Synthesis of Glycogen-Ethylenediamine and Glycogen-Ethylenediamine-Polyethylene Glycol. Bovine glycogen-ethylenediamine (BG-EDA) and bovine glycogen-ethylenediamine-polyethylene glycol (BG-EDA-PEG) nanoparticles were prepared by the method previously described.¹⁶ Briefly, 100 mg BG was dissolved in 5 mL of 0.6 M acetic buffer (pH 5.0) followed by addition of sodium periodate (26 mg, 0.12 mmol). The reaction was kept stirring for 2 h in the dark. Then, 36 mg (0.6 mmol) of EDA or 70 mg (0.035 mmol) of methoxy-PEG-amine (2 kDa) was added, followed by addition of 76 mg sodium cyanoborohydride (1.2 mmol). The reaction was stirred overnight. For BG-EDA-PEG, the product was isolated by precipitation from cold ethanol, followed by reaction with EDA. BG-EDA and BG-EDA-PEG were purified by dialysis against Milli-Q water for 3 days and freeze dried.

Synthesis of Dual-Labeled PEI-Coated Silica Nanoparticles. Commercially available spherical SiNPs (200 mg) were dispersed in 10 mL ethanol and mixed with 100 μ L APTES and a catalytic amount of TEA. The mixture was agitated overnight at room temperature. Then, the amino-modified SiNPs were centrifuged (3000 g, 1 min) and washed thrice with ethanol. The reaction produced a shift in ζ -potential of SiNPs from -15 ± 2 to $+19 \pm 2$ mV. Next, 100 mg of the amino-modified SiNPs was dispersed in 10 mL of a 100 mM solution of succinic anhydride in DMSO. The mixture was agitated overnight at room temperature. The resulting carboxylate-modified SiNPs were centrifuged (8000 g, 1 min) and washed thrice with DMSO. The success of the reaction was confirmed by a change in the ζ -potential of the modified SiNPs from $+19 \pm 2$ to -31 ± 1 mV. To obtain PEI-coated nanoparticles, the above carboxylate groups were first transformed into reactive succinimidyl esters. The carboxylate-modified SiNPs (50 mg) were

dispersed in 10 mL dry DMSO in the presence of DIC (250 mM) and NHS (250 mM). The mixture was agitated overnight at room temperature under inert nitrogen atmosphere. Then, the NHS-modified SiNPs were centrifuged (8000 g, 1 min) and washed thrice with DMSO. To graft the PEI chains, 40 mg NHS-modified particles was dispersed in 5 mL DMF and mixed with 1 g branched, high molecular weight PEI (average $M_w \sim 25,000$). The mixture was stirred overnight at room temperature. The PEI-coated SiNPs were then centrifuged (8000 g, 1 min) and washed thrice with DMF and ethanol. PEI coating also induced a ζ -potential shift from -31 ± 1 mV to $+17 \pm 1$ mV. ^1H -NMR spectra of PEI and PEI-coated SiNPs at concentration of 5 mg mL^{-1} were obtained using a 400 MHz Varian INOVA system in D_2O at 21°C .

For dual labeling with dyes suitable for performing STORM analysis, 5 mg of the above PEI-coated SiNPs was dispersed in 2 mL DMF containing $10 \mu\text{L}$ of 1 mg mL^{-1} AF647 NHS solution and $30 \mu\text{L}$ of 1 mg mL^{-1} AF488 NHS solution. The mixture was agitated for 3 h at room temperature. The dual-labeled PEI-coated SiNPs were then centrifuged (8000 g, 1 min) and washed thrice with ethanol.

Preparation of Glycoplexes. BG-EDA and BG-EDA-PEG glycoplexes were prepared at a predetermined glycogen-to-siRNA weight ratio.¹⁶ Briefly, siRNA was diluted in 30 mM PBS solution to achieve a concentration of $1 \mu\text{M}$. BG-EDA was prepared in 30 mM PBS to obtain a final concentration of 0.054 mg mL^{-1} (w/w ratio 4) and BG-EDA-PEG was prepared to obtain a final concentration of 0.081 mg mL^{-1} (w/w ratio 6). The glycogen and siRNA solutions were then mixed together and incubated for 15 min at 20°C to form glycoplex.

Mechanism of Internalization of Glycoplexes. PC3 cells were seeded at 80,000 cells per well in a 12-well plate (Corning, USA) and incubated at 37°C , 5% CO_2 , overnight. Endocytosis inhibitors (pitstop 2, filipin from *S. filipinensis*, EIPA, and NaN_3) were added to the cells to achieve

final concentrations of 12, 5, and 15 $\mu\text{g mL}^{-1}$, and 120 mM, respectively. After 15 min incubation with endocytosis inhibitors, BG-EDA and BG-EDA-PEG glycoplexes labeled with AF555 were added to achieve a final concentration of 5 $\mu\text{g mL}^{-1}$ and incubated with the cells for 2 h, after which, the culture medium was removed and cells were washed twice with PBS and detached with trypsin. The detached cells were suspended in 1% bovine serum albumin (BSA) in PBS solution, centrifuged for 5 min at 400 g and 4 °C, and washed twice with fresh portion of PBS. The cells were analyzed with BD Accuri™ C6 flow cytometer, registering 10,000 events per sample. For control experiment using endocytic markers: transferrin (final concentration 5 $\mu\text{g mL}^{-1}$), dextran (final concentration 100 $\mu\text{g mL}^{-1}$) cholera toxin B subunit (final concentration 10 $\mu\text{g mL}^{-1}$) were added and incubated for 20 min, 1 h and 1.5 h, respectively. Data were analyzed using FlowJo.

Intracellular Trafficking by Confocal Microscopy. PC3 cells were seeded at 40,000 cells per well in Labtek 8-well chamber slides and incubated overnight. The medium was replaced with fresh standard media. BG-EDA and BG-EDA-PEG glycoplexes labeled with AF555 were added to achieve a final concentration of 5 $\mu\text{g mL}^{-1}$. After 4 h of incubation, the medium was removed. The cells were washed thrice with pre-warmed PBS-BSA (1% BSA in PBS) to remove unbound particles from cells and to prevent nonspecific binding on the cell surface. Cells were fixed with 4% paraformaldehyde for 15 min, washed, permeabilized with 0.1% Triton X-100 solution in PBS for 5 min, and washed again thrice with 1% BSA in PBS. Samples were then incubated for 1.5 h with mouse anti-EEA1 monoclonal antibody or rabbit anti-Rab7 monoclonal antibody at 2.5 $\mu\text{g mL}^{-1}$. The cells were washed thrice and incubated for 1 h with goat anti-mouse or goat anti-rabbit AF647 conjugate antibody (2 $\mu\text{g mL}^{-1}$). The cells were imaged with a Nikon A1R confocal microscope with a 60× 1.4NA oil immersion objective.

Stochastic Optical Reconstruction Microscopy. STORM images were acquired using a Nikon N-STORM system configured to achieve total internal reflection fluorescence imaging (TIRF). The focus and TIRF angle were adjusted and tuned to maximize the signal-to-noise ratio. Fluorophores were excited by 488, 561, and 647 nm laser lines. No activation by ultraviolet light was used. Fluorescence was collected through a Nikon 100× 1.4NA oil immersion objective and passed through a quad-band pass dichroic filter. All time-lapses were recorded onto a 256 pixels × 256 pixels region by an EMCCD camera. For each channel, 3000–5000 frames were sequentially acquired. STORM movies were processed and analysis of the population of nano-objects were performed with the STORM module of the NIS Elements Nikon software.

STORM Size Distribution Analysis. For STORM analysis, BG-EDA and BG-EDA-PEG glycoplexes were prepared using with AF647-conjugated siRNA. Glycogen–siRNA constructs were moved to Labtek 8-well chamber slides at the final concentration of 5 $\mu\text{g mL}^{-1}$ and allowed to adsorb on the slide. To analyze the stability of the constructs in serum, the glycoplexes (5 $\mu\text{g mL}^{-1}$) were added to DMEM supplemented with 10% FBS and incubated in 37 °C for 5 h.

Intracellular Trafficking Analysis by STORM. For dual-fluorescent labeling, BG-EDA (10 mg) was dissolved in 2 mL of 0.1 M sodium bicarbonate buffer (pH 8.0) and mixed with 100 μL AF488 NHS (1 mg mL^{-1}) and 50 μL AF647 NHS (1 mg mL^{-1}) and kept stirring overnight in the dark. Excess dye was removed by purification on a NAP-10 filter column and freeze dried.

Dual-labeled secondary antibodies were prepared as follows: 50 μL secondary antibody AF555 NHS (2 mg mL^{-1} in PBS) was mixed with 50 μL of 0.1 M NaHCO_3 and 10 μL AF647 NHS (activator dye/reporter dye 2:1). The reaction proceeded for 30 min at 25° C, protected from light and on a shaking platform. The reaction volume was adjusted to 0.5 mL. Then, the antibody was purified using NAP-5 gel filtration column, after equilibration in PBS.

To perform intracellular trafficking studies, PC3 cells (approximately 30,000) were seeded on 8-well Lab-Tek chamber slides, followed by addition of 0.5 mL culture medium and incubation overnight. The transfection of PC3 cells with dual-labeled glycoplexes and PEI-SiNPs at a final concentration of $5 \mu\text{g mL}^{-1}$ was conducted for 2 h at 37°C in 5% CO_2 . After the initial incubation, the transfection medium was discarded and the cells were cultured for the following 48 h in fresh culture medium. The intracellular trafficking of construct was analyzed immediately after medium replacement (2 h incubation) and after 4, 7, and 48 h incubation. At the different time points, the cells were fixed and permeabilized. Immunostaining for early endosomes and late endosome was performed as follows. The cells were incubated with mouse anti-EEA1 primary antibody ($2.5 \mu\text{g mL}^{-1}$, 1 h, 25°C) for early endosome or rabbit anti-Rab7 primary antibody ($2.5 \mu\text{g mL}^{-1}$, 1 h, 25°C) for late endosome, washed with PBS (thrice), subsequently incubated with AF555/AF647 dual-labeled goat anti-mouse secondary antibody ($2 \mu\text{g mL}^{-1}$, 1 h, 25°C), and washed again with PBS (thrice). To image the intracellular disassembly of glycoplexes, PC3 cells were incubated with AF647 glycoplexes (AF647 tagged siRNA loaded) using the same protocol and analyzed after 5 and 58 h incubation. To inhibit acidification of endosomes, cells were treated with bafilomycin A1 at final concentration of 100 nM for 30 min prior addition of glycoplexes.

NF- κ B p105/p50 Downregulation. PC3 cells were plated on 12-well plates (Costar, Corning, MA, USA) at a seeding density of 8.0×10^4 cells per well. Transfection was done at equivalent of 100 nM concentration of siRNA. Gene knockdown was evaluated using flow cytometry. Briefly, after 48 or 72 h, cells were washed with DPBS, detached, fixed with 4% paraformaldehyde, and permeabilized using 0.1% Triton X-100 and 1% BSA in PBS solution. NF- κ B p105/p50 knockdown was investigated using rabbit anti-NF- κ B p105/p50 polyclonal antibody. A 1:200 dilution in 200 μL was used for both antibodies with 2 h of incubation time. Goat anti-rabbit

AF647-conjugated antibody ($2\ \mu\text{g mL}^{-1}$) was used for secondary staining. Cells were always washed twice with 1% BSA in DPBS and centrifuged (400 g, 5 min) between each step. Cells were analyzed using a BD Accuri™ C6 flow cytometer, registering 10,000 events per sample. Data were analyzed using FlowJo.

Western Immunoblotting

PC3 cells were transfected with BG-EDA or BG-EDA-PEG glycoplexes at concentration equivalent to 100 nM siRNA and incubated with for 72 h. The whole cell extract was prepared by lysing cells for 30 min on ice in RIPA buffer containing a protease inhibitor cocktail and separated from cell debris by centrifugation (16,000 g, 15 min, 4 °C). Proteins in the extract were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane using standard protocols. The membrane was washed twice with PBS, followed by incubation with blocking buffer (5% w/v skim milk powder in PBS-Tween20 (PBS-T)). The membrane was incubated with anti-NF- κ B p105/p50 (1:2000 in 5% BSA/PBS-T buffer) and anti- β -Actin primary antibodies (1:5000 in 5% BSA/PBS-T buffer) overnight. The membrane was then incubated with anti-rabbit peroxidase-conjugated antibody (1:10000 in PBS-T). Bound antibodies were detected using Clarity Western ECL substrate and imaged with a ChemiDoc XRS+ imaging system (BioRad, USA).

Potentiometric Titration

The pKa of BG-EDA and PEI were measured by potentiometric titration. For the measurement, 10 mg of BG-EDA and PEI was dissolved in 5 mL of 200 mM NaCl solution, and pH was adjusted to 11 using 0.1 N NaOH. The suspension was titrated with 0.01 N HCl to pH of ~2.5. The pKa was determined using the first derivative method.

ASSOCIATED CONTENT

Supporting Information. STORM images, DLS data, TEM image of PEI-coated SiNPs, inhibition of endocytic markers, ¹H-NMR of PEI and PEI-coated siNPs, potentiometric titration

The following files are available free of charge.

ACKNOWLEDGMENT

This work was funded by Future Fellowship Scheme (F. Cavalieri FT140100873) and a National Health and Medical Research Council Senior Principal Research Fellowship Scheme (F. Caruso APP1135806), respectively. This work was also supported by a University of Melbourne Establishment Grant (F. Cavalieri) and Endeavour Research Fellowship (A. Bertucci #5574) granted by the Australian Government, Department of Education and Training. This work was performed at the Australian Research Council (ARC) Centre of Excellence in Convergent Bio-Nano Science and Technology (project number CE140100036). This work was performed in part at the Materials Characterization and Fabrication Platform (MCFP) at The University of Melbourne and the Victorian Node of the Australian National Fabrication Facility (ANFF).

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