ChemComm

Guanylurea-functionalized conjugated polymer enables RNA interference in ex vivo human airway epithelium

Journal:	*ChemComm*
Manuscript ID	CC-COM-04-2019-002856.R1
Article Type:	Communication

Authors

Md. Salauddin Ahmed,[a+] Rajib Kumar Dutta,[b+] Prakash Manandhar,[a] Xuerong Li,[a] Hooman Torabi,[a] Alfonso Barrios,[a] Ping Wang,[c] Srinivasan Chinnapaiyan,[b] Hoshang Jehangir Unwalla*[b] and Joong Ho Moon*[a]

Affiliations

[a] Department of Chemistry and Biochemistry, Biomolecular Sciences Institute, Florida International University, 11200 SW 8th St., Miami, FL 33199, USA

E-mail: jmoon@fiu.edu

[b] Department of Immunology and Nanomedicine, Florida International University, 11200 SW 8th St., Miami, FL 33199

[c] Department of Electric Engineering, Florida International University, 11200 SW 8th St., Miami, FL 33199

Acknowledgements

JHM acknowledges generous financial support from NSF (DMR 1352317). HU acknowledges support from Flight Attendant Medical Research Institute (CIA150086).

+MSA and RKD contribute equally

COMMUNICATION

Guanylurea-functionalized conjugated polymer enables RNA interference in ex vivo human airway epithelium†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

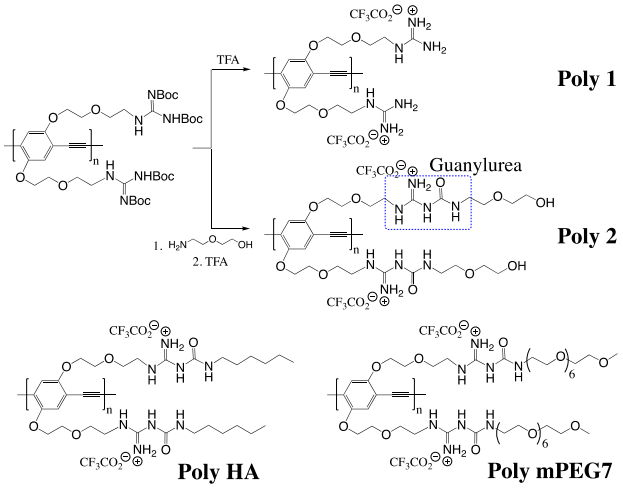
Here, we demonstrate a successful target gene knockdown in ex vivo normal human bronchial epithelium (NHBE) cells covered with mucus layers using the guanylurea functionalization technique modulating the chemical environment at the positive charge of a gene carrier. While conventional lipid-based and positively charged carriers exhibit no or very poor transfection efficiency, guanylurea-functioned positively charged conjugated polymer (CP) exhibits good knockdown efficiency in primary epithelium cells owing to enhanced short interfering RNA (siRNA) complexation, diffusion through the mucus layer, and RNA delivery.

RNA interference (RNAi) induced by small RNA molecules has been used for studying the loss-of-function phenotypes or developing therapeutics by knockdown of disease causing or related genes.[1] Efficient delivery of negatively charged RNA molecules to target cells is pivotal for a successful application of RNAi technology.[2] RNA molecules are highly susceptible for enzymatic degradation and too big to penetrate the cellular membranes. Although various types of delivery materials have been developed and used at the in vitro tissue culture level, gene regulation at the ex vivo or in vivo level has been largely unsuccessful due to poor intracellular siRNA availability.[3]

In human airway epithelium possessing additional extracellular barriers such as mucus layers, transfection using conventional lipid-based or positively charged carriers is extremely limited.[4] As the critical physical barrier interfacing environmental stimuli, the mucosal surfaces of epithelium tightly regulate various physiological and immunological processes. In the mucus layer, dense mucin fibers and negatively charged proteoglycans provide the adhesive and viscous protective layer that often trap and remove positively charged carriers, resulting in poor delivery of payloads to the underlying epithelial cells.[5] Because understanding on the genetic and molecular changes upon external stimuli or viral infections is pivotal to develop proper preventive or therapeutic tools by controlling the gene expressions,[6] developing a novel delivery material that overcomes the biological barrier is highly needed. Very few options are currently available for delivering nucleic acids to the airway epithelium. Mucus-altering or mucolytic agents can be used as adjuvants of gene carriers, although high millimolar concentrations are often needed to disrupt or disturb the mucus layers.[7] Alternatively, block copolymers of polyethyleneimine (PEI) and polyethyleneglycol (PEG) have been developed to deliver plasmid DNA (pDNA) to the lung airways.[8] The positively charged PEI and negatively charged pDNA form ionic complexes, while the PEG block shields the positively charged block from the negatively charged mucus layers and provides diffusion through the nanometer sized mucus meshes. However, the PEG block often causes poor gene complexation and reduced cellular entry. In addition, while pDNA can form smaller ionic complex with PEI-PEG copolymers due to the molecular topography of pDNA enabling compaction to nanoparticles, inefficient complexation of stiff and short RNA molecules possibly induces larger complex sizes.[9] Despite optimization opportunity to balance the ratios between the charged and PEG segments, block copolymer architectures in biological fluids containing ions and proteins will likely complicate the surface properties and thus influence the biological functions.[10]

Conjugated polymers (CPs) have been used for labeling of intracellular organelles and delivery of siRNA.[11, 12] CPs are synthetic polymers with aromatic π-electrons delocalized in rigid hydrophobic

Scheme 1. Synthetic scheme of guanylurea-functionalized conjugated polymers.



† Electronic Supplementary Information (ESI) available: Synthesis, characterization, cellular study, and theoretical simulations. See DOI: 10.1039/x0xx00000x

backbones.[13] In addition to the intrinsic fluorescent properties that are highly advantageous for labeling and tracking, the charged CPs are structurally similar to short arginine-rich peptides or synthetic mimics of protein transduction domains exhibiting efficient cellular entry, especially in charge density and backbone rigidity.[14] To address the issues associated with the PEG copolymers for siRNA delivery, we have sought a straightforward positive charge-modulating technique that enhances mucus penetration and cellular entry efficiencies while maintaining ionic complexation ability.

Here, we report a direct chemical modulation technique that introduces hydrophilic group at the guanidine group via guanylurea bond formation. A guanylurea side chain-containing CP exhibits an efficient knockdown of a target gene in well-differentiated primary normal human bronchial epithelium (NHBE) cells, while conventional lipid-based and positively charged materials exhibit no or very poor transfection efficiency. The guanylurea formation chemistry allows coupling of various amine-containing molecules to guanidine via urea bond formation[15] and both natural and synthetic guanylurea containing molecules have been reported as antibiotics.[16] Unlike to previous attempts to functionalize guanidine group that substantially decrease the cellular entry efficiency,[17] the added functional group at guanidine via urea bond maintains or enhances cellular entry, presumably due to the urea extension not hindering the ionic and hydrogen bonding with the membranes.

A poly(phenyleneethynylene) containing t-butyloxycarbonyl (Boc)-protected guanidine side chains was synthesized by the Sonogashira cross-coupling reaction in a mixed solution of THF and diisopropylamine (DIPA) at room temperature. After repeated precipitation in methanol, the resulting polymer was divided into two portions. The first portion was used for direct deprotection of the Boc group to make **Poly 1** (a conventional positively charged CP). By reacting Boc-protected guanidine with 2-aminoethoxyethanol followed by Boc deprotection, a positively charged hydrophilic guanylurea group-functionalized **Poly 2** was successfully synthesized in high yields (Scheme 1). Hexylamine and a short ethylene glycol (n=7)-containing polymers (i.e., Poly HA and Poly mPEG7) were similarly synthesized as a control for studying the effects of the guanylrea group on ionic complexation and mucus penetration abilities. A characteristic amide proton at ~12.3 ppm was used to confirm the formation of the guanylurea group. All polymers exhibit good solubility in common organic solvents (e.g., DMSO) and characteristic absorption/emission profiles of PPEs. **Poly 2** shows ~three-fold increased fluorescent quantum yield than **Poly 1**, due to the protective and solvating functional group introduced at the charge site (see Electronic Supporting Information, ESI).

The hydrodynamic diameters (HDs) of the polymers and polymer/siRNA complexes in water were analyzed by dynamic light scattering technique. The HD determination of both **Poly 1** and **2** was unsuccessful due to very weak light scattering signals in water even at near millimolar concentrations, implying that both polymers are relatively well-solvated by not forming dense aggregations in water. Spectroscopic analysis also suggests that both polymers have very weak π-π interaction among the polymer backbones in water. While absorption spectra of both polymers in either a good (e.g., DMSO) or a poor (e.g., water) solvent exhibit similar profiles (i.e., similar λ_{max} and spectral shape), emission spectra of polymers in water show slightly increased the peak intensity accounting for the π-π interaction of conjugated backbones compared to those in DMSO (ESI). When the positive charges of polymers were screened by negatively charged siRNA upon ionic complexation, substantially increased light scattering intensities were observed from both polyplexes due to increased hydrophobic interaction among the

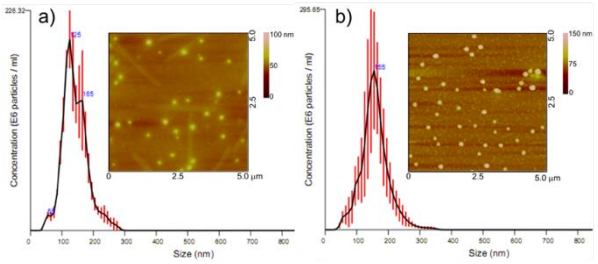


Fig. 1. The HDs and AFM height image (inset) of **Poly 1**/siRNA (a) and **Poly 2**/siRNA (b).

polymer chains. The HDs of polyplexes formed at the nitrogen (N)-to-phosphate (P) (N/P) ratio of 4 were determined as 137 ± 40 and 152 ± 44 nm for **Poly 1** and **2**, respectively, by nanoparticle tracking analysis. Addition of a hydrophilic group at the guanidine slightly increased the HD (Fig. 1). Atomic force microscopy images (Fig. 1 insets) show that both polyplexes are spherical particles. By analyzing three independent images containing more than 40 particles, the average sizes of **Poly 1**/siRNA and **Poly 2**/siRNA were determined as 130 ± 50 and 127 ± 23 nm, respectively, which are in the similar size range of the HDs. During the imaging, we noticed that **Poly 2**/siRNA complexes showed better particle contrast with clear boundary (Fig. 1b, inset), while **Poly 1**/siRNA complexes exhibited somewhat diffused boundary. This morphological difference indicates that the guanylurea modification influences the complexation behaviors of CPs. Zeta potentials of both polyplexes were slightly positively charged (~ +13 mV). In order to examine how the guanylurea formation at the positive charge of CPs influences siRNA complexation, an agarose-based gel retardation assay was conducted. The guanylurea-functionalized **Poly 2** shows better siRNA complexation than the guanidine-containing **Poly 1**. While entire siRNA was complexed by **Poly 2** at the N/P ratio of ~2, **Poly 1** showed only ~50% complexation at the ratio. The balanced hydrophilicity introduced at the guanidine through the urea extension was critical for the ionic complexation. The hydrophobic hexylamine and longer mPEG7-containing polymers exhibit poor complexation efficiency than **Poly 2** (ESI). As mentioned earlier, the longer hydrophilic chain (I.e., PEG or mPEG7) introduced at the positive charge exhibits interference in siRNA complexation.

Both polymers exhibit no noticeable viability inhibition of immortalized human bronchial epithelial cell line (BEAS-2B) up to 10 μM, indicating that modulation at the guanidine group causes no toxic effects to BEAS-2B cells (ESI). Using a fluorescently labeled control siRNA (i.e., siGLO Red), CP-mediated siRNA delivery was confirmed by confocal microscopy (Fig. 2). After an hour of incubation at the N/P ratio of ~8 (i.e., all siRNA were complexed by the polymer), both CPs (green) and siGLO (red) were observed inside of BEAS-2B cells, while relatively high amount of siGLO was observed from cells incubated with **Poly 2** (Fig. 2d-f), indicating that guanylurea modification increased the siRNA delivery efficiency. In addition to better siGLO complexation by **Poly 2** (ESI), the higher amounts of intracellular siGLO can be understood by enhanced cellular entry of the complex due to the balanced hydrophilicity and charge density. When the positive charge of guanidine in cell penetrating peptides is balanced with hydrophobic moieties, many guanidine-containing carriers are known to exhibit better ionic, hydrogen bonding, and hydrophobic interaction with the membrane, resulting in high intracellular entry.[18] Guanylurea formation did not inhibit the basic guanidine function, instead the introduced hydrophilic group at the charged guanidine via the urea bond increased the cellular entry of polyplexes. While the combined images of Fig. 2(c) indicate that **Poly 1** and siGLO are mainly overlapped, somewhat diffused orange color

was observed from Fig. 2(f), implying that some portions of siGLO were released from **Poly 2**. From the result, we believe that hydrophilic modification at the positive charge of CPs allows better siRNA complexation, efficient cellular entry, and subsequent intracellular release of siRNA.

To evaluate the mucus penetration effects of the guanylurea

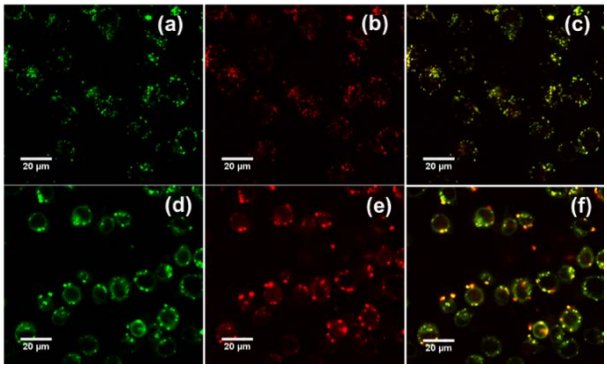
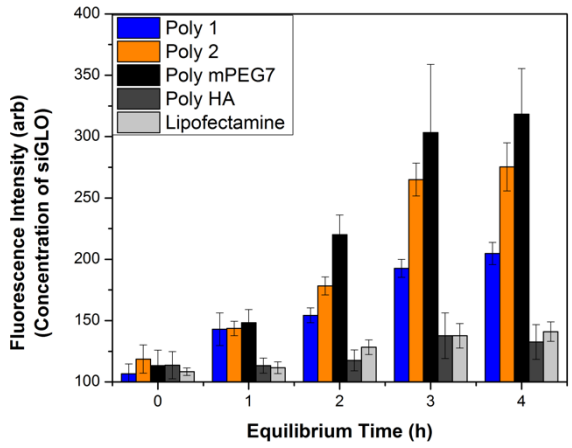


Fig. 2. Confocal microscopic images of BEAS-2B cells incubated with CP/siGLO polyplexes for 1 h visualize CP-mediated siRNA delivery. Green dots were observed from CPs located in the cytosol (a and d). Red signals from siGLO were observed from cells treated with CPs (b and e), while the intensity of siGLO was much stronger from cells treated with **Poly 2**/siGLO (e). While the combined image of Poly 1 shows that CP and siGLO are mainly overlapped (c), some portions of siGLO were released from Poly 2 (f). Scale bar: 20 μm.

functional groups, various CP/siGLO complexes were loaded to the top of the Transwell inserts filled with mucin type III (10 mg/ml),[19]



and the amounts of siGLO in the basolateral Transwell were

Fig. 3. Penetration of CP/siGLO complexes and Lipofectamine/siGLO through the model mucus layer in a Transwell assay setup. The fluorescence intensity of siGLO in the basolateral side of Transwell was measured at 580 nm. Data represents mean ± SD (n=3). Both Poly 2 and Poly mPEG7 with hydrophilic guanylurea groups exhibited good mucus penetration, while Poly HA (Hexylamine) and Lipofectamine showed little penetration through the mucus layer.

monitored as a function of the equilibrium time by measuring the fluorescent intensity at 580 nm. As shown in Fig. 3, **Poly 2**/siGLO complexes exhibit ~40% better diffusion than **Poly 1**/siGLO complexes during the four hours of equilibrium period, indicating that the positively charged carriers tend to be trapped in the complex mucus network by electrostatic and hydrophobic interactions. Meanwhile, the introduced hydrophilic aminoethoxyethanol group through the guanylurea extension (i.e., **Poly 2**/siGLO complex) allowed better diffusion of siRNA through the mucus layer. Poly mPEG7 containing longer hydrophilic ethylene glycol groups exhibited a slightly enhanced penetration efficiency, while siRNA complexation efficiency was substantially lower than **Poly 2** (ESI). Both Poly HA and Lipofectamine showed almost no penetration through the mucus layer. This observation supports that the guanylurea chemistry can effectively change the chemical environments at the positive charge (guanidine) of carriers through the urea extension, allowing efficient diffusion through the hydrophilic and negatively charged mucus layers. We conclude that balanced hydrophilicity at the positive charge is critical to enhance the mucus penetration efficiency while maintaining the strength of ionic complexation.

To evaluate the guanylurea-functionalized CP for delivery of siRNA in a physiological setting, ex vivo primary bronchial epithelial cells were incubated with **Poly 2**/siGLO polyplex. Primary bronchial epithelial cells obtained from nasal turbinates or cadaver lungs can be grown either on plastic dishes or on porous supports at the air liquid interface (ALI).[20] While the cells grown on plastic dishes present a poorly differentiated squamous phenotype, the cells grown on porous supports at the ALI closely recapitulate their normal in vivo morphology including the cell-matrix and cell-cell interactions, differentiation of mucus, goblet and ciliary cells, polarized epithelial ion transport, and regenerating the native bronchial epithelium ex vivo.[20] Therefore, ex vivo primary human bronchial epithelial cells are considered as the best models of the airway epithelial constitute and used for ex vivo drug delivery studies before extrapolating to large animal models or human clinical studies. However, the transfection efficiency in the airway epithelium is very poor as their primary function is to block pathogens or particulates from the environment using combined functions of secretory and ciliated cells.

The re-differentiated NHBE cells were tested for ciliation by staining acetylated tubulin (ESI). In order to confirm the polarity and integrity of the epithelium, the transepithelial electrical resistance (TEER) was measured after 21 days of differentiation at the ALI. TEER is a well-accepted method to quantitatively evaluate the integrity of the tight junction of epithelium and the values are strongly correlated to the integrity of the cellular barrier.[21] The primary NHBE cells exhibit a mean TEER value of 731 ohms/cm^2, indicating efficient barrier formation. The apical chloride ion flux was also monitored by treating albuterol, which activates the cystic fibrosis transmembrane conductance regulator (CFTR) protein to stimulate chloride ion flux. A sharp current increase was monitored right after addition of albuterol (ESI). The specificity of CFTR-mediated efflux was confirmed by the decreased current after addition of a CFTR inhibitor. From the assay, we have confirmed that the cultured NHBE cells exhibit the characteristic membrane properties of the epithelium layer.

Fluorescence microscopic images clearly indicate that **Poly 2** delivers siGLO to NHBE cells, while cells treated with **Poly 1**/siGLO and Lipofectamine/siGLO, respectively, exhibited only background signals (ESI). Uniform and strong green (**Poly 2**) and red (siGLO red) color was clearly observed from the characteristic packed cuboidal morphology of NHBE cells. We believe that the added hydrophilic groups at the positive charges allow better diffusion of the ionic complex through the mucus layer followed by efficient intracellular entry (see Fig. 3). Despite the hydrophilic group at the positive charge, which could possibly lower the complexation and cellular entry efficiency due to the steric effects added at the positive charge, **Poly 2** exhibits better siRNA complexation efficiency (ESI), better mucus diffusion (Fig. 3), and better epithelium entry (ESI).

The knockdown efficiency of **Poly 1** and **2** was initially evaluated at the in vitro level using siRNA against histone deacetylase (HDAC) (siHDAC) in BEAS-2B cells. The mRNA expression levels were

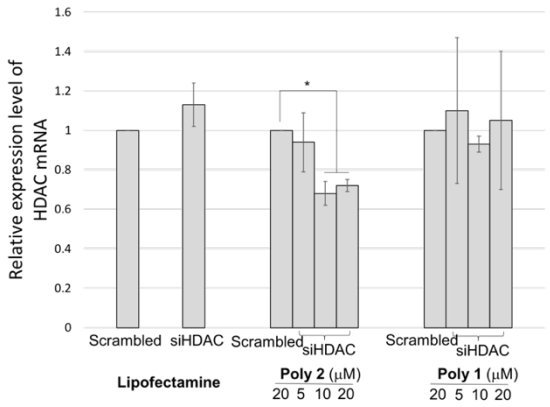


Fig. 4. Relative HDAC mRNA expression levels of NHBE cells treated with Lipofectamine 2000 (a lipid-based), **Poly 1** (cationic), and **Poly 2** (modulated chemical environment at positive charge). *p < 0.008

quantified by real time quantitative polymerase chain reaction (RT-qPCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control gene. Inhibition of HDACs have been shown to suppress proliferation of non-small cell lung cancer (NSCLC) and restore the drug sensitivity to NSCLC.[22] The guanidine-modified **Poly 1** exhibits relatively poor knockdown efficiency even in immortalized BEAS-2B cell lines. **Poly 2** and Lipofectamine exhibit a dose-dependent gene knockdown, supporting that the guanylurea modification enhances RNAi efficiency (ESI).

As we expected from the microscopic images (ESI) and mucus penetration assay (Fig. 3), both lipid-based (e.g., Lipofectamine) and purely positively charged carriers (e.g., **Poly 1**) exhibit no or very poor HDAC knockdown efficiency in well-differentiated NHBE cells (Fig. 4) due to poor cellular siRNA delivery to the epithelium cells. Meanwhile, **Poly 2** consistently exhibits the average of ~30% knockdown efficiency over eleven independent lung samples at the N/P ratio ~4 or higher. Due to the negatively charged hydrophobic mucus layers, positively charged **Poly 1** and lipid-based carriers experience difficulty in diffusion through the mucus layer (Fig. 3). The hydrophilic environment introduced at the positive charge of guanidine allows efficient ionic complexation, diffusion through the mucus layer, entry to epithelial cells, and release of siRNA, resulting in high knockdown efficiency in well-differentiated NHBE cells. Considering the difficulty to deliver siRNA to primary epithelium covered with mucus, the consistent knockdown efficiency shown in Fig. 4 supports the feasibility of the guanylurea modulation chemistry for tuning the chemical environments at the positive charge of carriers to achieve high knockdown efficiency in primary cells. A cellular entry mechanism study indicates that **Poly 1**/siRNA complex uses various energy-dependent and non-energy dependent entry pathways in BEAS-2B cells (ESI).

In conclusion, we have demonstrated that a facile modification technique introducing nonionic functional group at the positive charge of guanidine-containing gene carriers enhanced ionic complexation, diffusion through the mucus layer, cellular entry, and gene knockdown efficiency in primary cells having additional biological barriers. Because the primary culture of NHBE cells reproduces the in vivo morphology and key physiologic processes to regenerate the native human bronchial epithelium,[23] the transfection efficacy demonstrated in this report can be reasonably correlated to real in vivo environment. The guanylurea formation chemistry can be applicable for many existing therapeutic carriers including lipids and synthetic/natural polymers to modulate and enhance the biophysical properties, contributing to enhanced therapeutic efficacy in the future.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1. D. Castanotto and J. J. Rossi, *Nature*, 2009, **457**, 426.
2. K. A. Whitehead, R. Langer and D. G. Anderson, *Nature Rev. Drug Discov.*, 2009, **8**, 129.
3. R. Kanasty, J. R. Dorkin, A. Vegas and D. Anderson, *Nature Mater.*, 2013, **12**, 967.
4. J. K. W. Lam, W. L. Liang and H. K. Chan, *Adv. Drug Deliv. Rev.*, 2012, **64**, 1.
5. S. K. Lai, Y. Y. Wang and J. Hanes, *Adv. Drug Deliv. Rev.*, 2009, **61**, 158.
6. F. Schembri, S. Sridhar, C. Perdomo, A. M. Gustafson, X. L. Zhang, A. Ergun, J. N. Lu, G. Liu, X. H. Zhang, J. Bowers, C. Vaziri, K. Ott, K. Sensinger, J. J. Collins, J. S. Brody, R. Getts, M. E. Lenburg and A. Spira, *Proc. Nat. Acad. Sci. USA*, 2009, **106**, 2319.
7. S. Ferrari, C. Kitson, R. Farley, R. Steel, C. Marriott, D. A. Parkins, M. Scarpa, B. Wainwright, M. J. Evans, W. H. Colledge, D. M. Geddes and E. Alton, *Gene Ther.*, 2001, **8**, 1380.
8. J. S. Suk, A. J. Kim, K. Trehan, C. S. Schneider, L. Cebotaru, O. M. Woodward, N. J. Boylan, M. P. Boyle, S. K. Lai, W. B. Guggino and J. Hanes, *J. Control. Rel.*, 2014, **178**, 8.
9. D. J. Gary, N. Puri and Y. Y. Won, *J. Control. Rel.*, 2007, **121**, 64.
10. A. Malek, O. Merkel, L. Fink, F. Czubayko, T. Kissel and A. Aigner, *Toxicol. Appl. Pharm.*, 2009, **236**, 97.
11. J. H. Moon, E. Mendez, Y. Kim and A. Kaur, *Chem. Comm.*, 2011, **47**, 8370.
12. M. Twomey, E. Mendez, R. K. Manian, S. Lee and J. H. Moon, *Chem. Comm.*, 2016, **52**, 4910.
13. D. Tuncel and H. V. Demir, *Nanoscale*, 2010, **2**, 484.
14. E. G. Stanzl, B. M. Trantow, J. R. Vargas and P. A. Wender, *Acc. Chem. Res.*, 2013, **46**, 2944.
15. M. S. Ahmed, T. Annamalai, X. R. Li, A. Seddek, P. Teng, Y. C. Tse-Dinh and J. H. Moon, *Bioconjugate Chem.*, 2018, **29**, 1006.
16. C. G. Yuan and R. M. Williams, *J. Am. Chem. Soc.*, 1997, **119**, 11777.
17. J. B. Rothbard, T. C. Jessop and P. A. Wender, *Adv. Drug Deliv. Rev.*, 2005, **57**, 495.
18. J. P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M. J. Gait, L. V. Chernomordik and B. Lebleu, *J. Biol. Chem.*, 2003, **278**, 585.
19. B. Poinard, S. Kamaluddin, A. Q. Q. Tan, K. G. Neoh and J. C. Y. Kah, *ACS Appl. Mater.Int.*, 2019, **11**, 4777.
20. M. L. Fulcher and S. H. Randell, *Met. Mol. Biol.*, 2013, **945**, 109.
21. B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler and J. J. Hickman, *Jala*, 2015, **20**, 107.
22. C. Damaskos, I. Tomos, N. Garmpis, A. Karakatsani, D. Dimitroulis, A. Garmpi, E. Spartalis, C. F. Kampolis, E. Tsagkari, A. A. Loukeri, G. A. Margonis, M. Spartalis, N. Andreatos, D. Schizas, S. Kokkineli, E. A. Antoniou, A. Nonni, G. Tsourouflis, K. Markatos, K. Kontzoglou, A. Kostakis and P. Tomos, *Anticancer Res.*, 2018, **38**, 37.
23. A. Dvorak, A. E. Tilley, R. Shaykhiev, R. Wang and R. G. Crystal, *Am. J. Resp. Cell Mol. Biol*, 2011, **44**, 465-473.

For Table of Contents use only

A positive charge modulation chemistry via the guanylurea group allows an efficient target gene knockdown in primary normal human bronchial epithelial cells covered with mucus.