

# Mimetic Proteins: A Non-Toxic Cytosolic Delivery Strategy

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## Introduction

- Our collective understanding of the molecular regulation of cellular homeostasis and processes such as endocytosis have increased rapidly over the last 20 years (Fig 1).<sup>1 & 2</sup>
- This has allowed membrane trafficking systems to be exploited for cytosolic drug delivery.<sup>1 & 2</sup>
- In order to assimilate cytosolic targets, many organisms have evolved virulence factors that can manipulate mammalian endomembrane trafficking and associated cargo sorting systems (Fig 1).
- Protein virulence factor toxicity may be ablated using site-specific mutagenesis whilst retaining the ability to access the cytosol without causing toxicity.
- We have exploited such a system to facilitate the cytosolic delivery of pharmacologically active antisense oligonucleotides (ASO) *in vitro*.

## Results

- Gene knockdown *in vitro*:** Syntaxin-5 knockdown was demonstrated both morphologically (Fig 2) and by Western blotting, after normalizing syntaxin-5 expression levels to those of a housekeeper (Derlin1) (Fig 3).
- After 48h only  $43 \pm 4.1\%$  (SEM) of the protein labeled using an antibody to syntaxin-5 was observed relative to a non-ASO containing control (*i.e.* HeLa cells exposed to only the delivery system).
- In vitro* toxicity:** We have shown  $88.6 \pm 4.6\%$  viability (SD; n=4) in Vero at 100  $\mu\text{g/ml}$  (DDS protein concentration) after 3 days. HeLa cells were more sensitive showing  $54.5 \pm 3.9\%$  viability at 100  $\mu\text{g/ml}$  (DDS protein concentration) after 3 days (SD; n=4) (Fig 4).
- Under similar experimental conditions the  $\text{IC}_{50}$  values obtained for poly(ethyleneimine) (PEI) were between 2.4 and 7.4  $\mu\text{g/ml}$  (Table 1).
- The propensity of a commercial lipid based transfection system to induce transfection artifacts was also documented in relation to the protein based system described herein (Fig 5). This was performed by monitoring the distribution of lysosomal associated membrane protein (LAMP)1 after transfection.

Fig 1: Membrane trafficking in mammalian cells; non-disruptive routes to the cytosol. Of the many routes that exist, two examples are shown

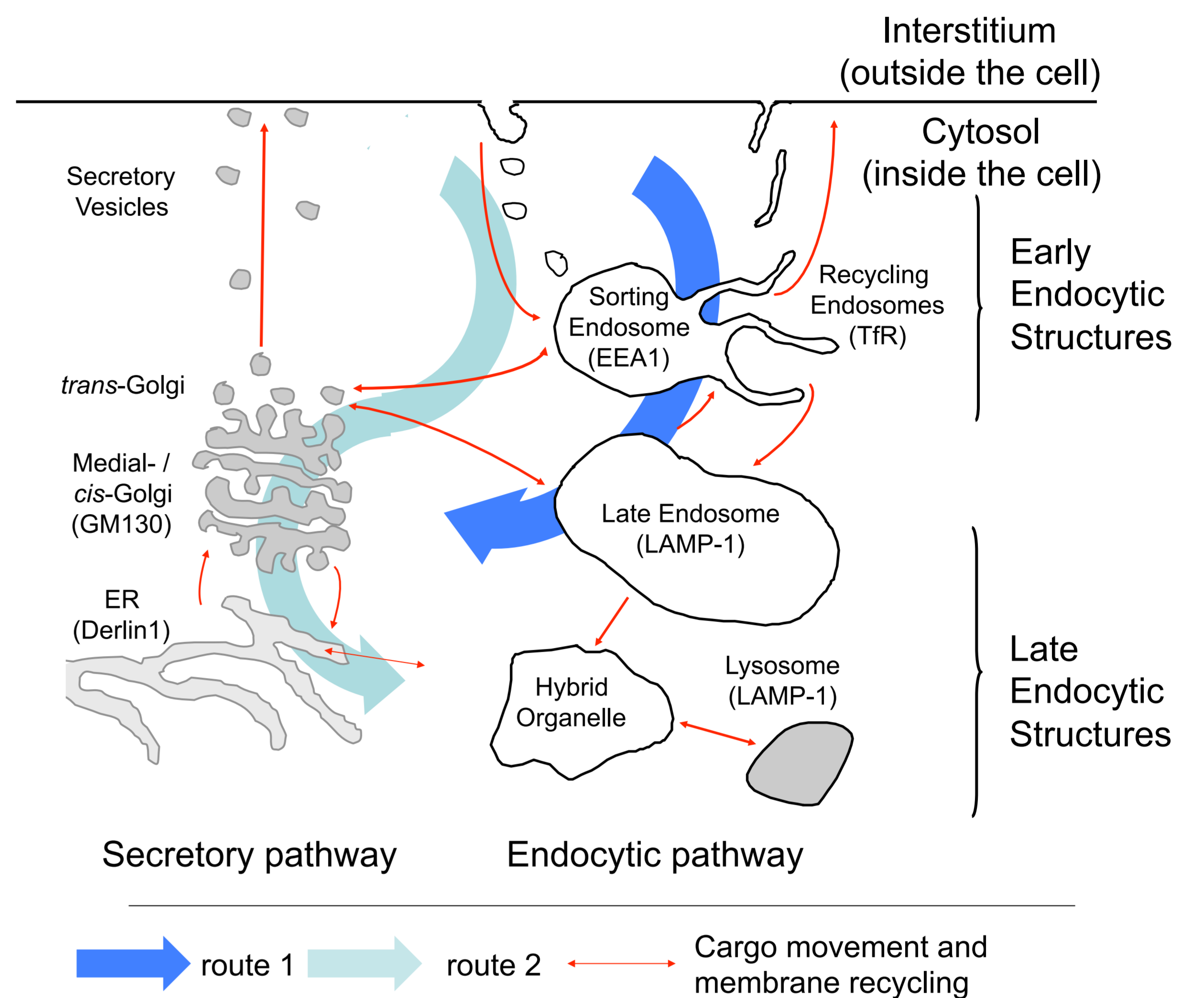
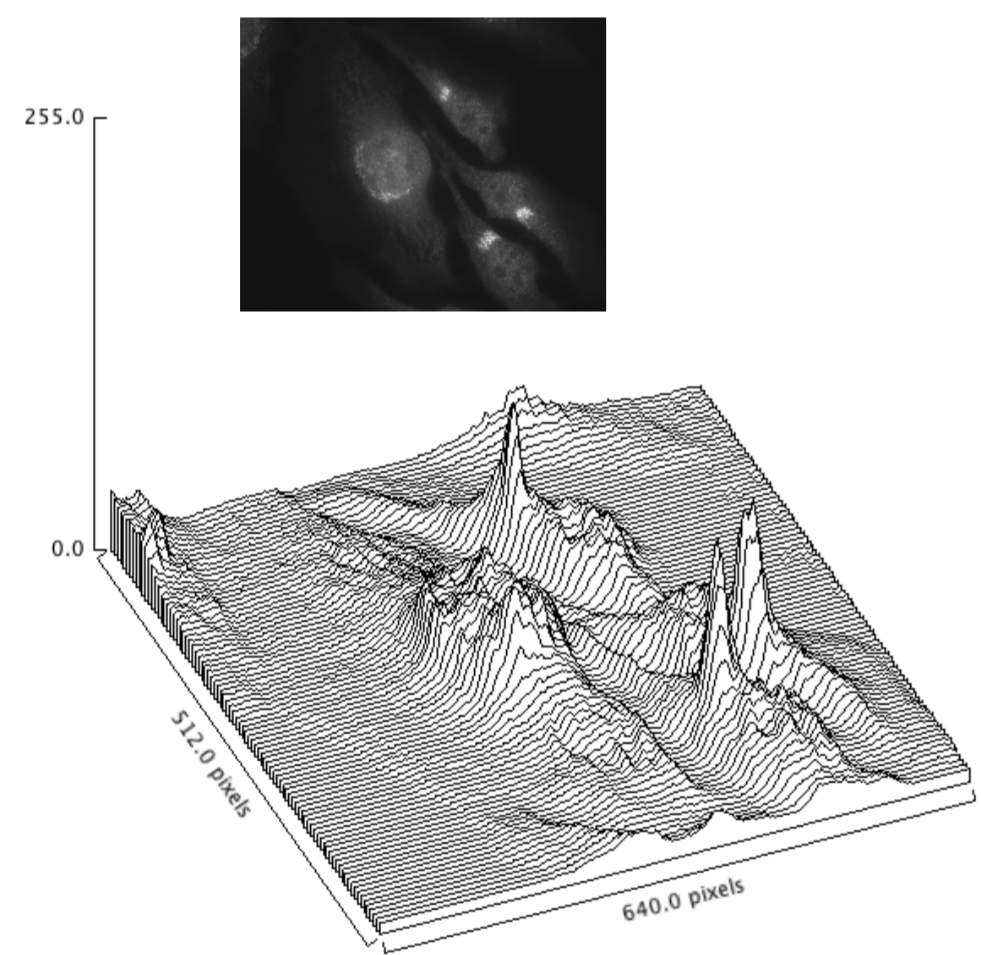
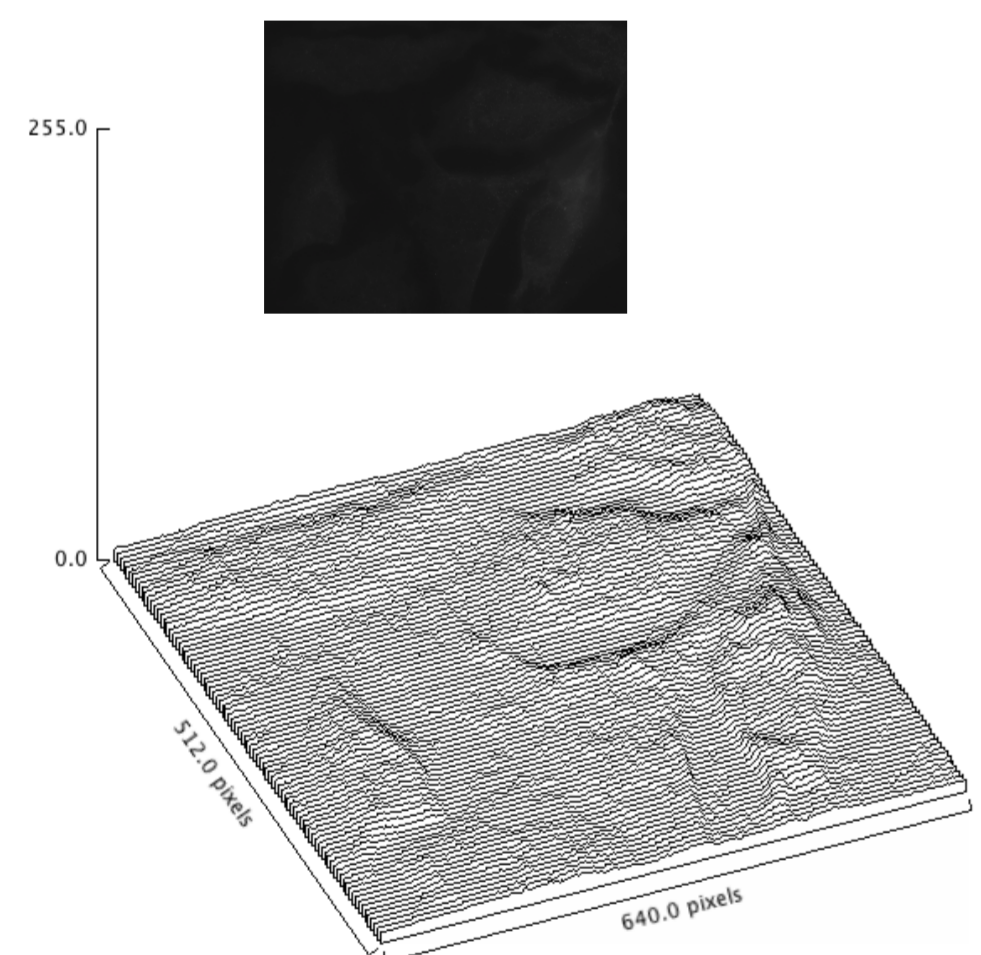


Fig 2: Syntaxin-5 protein levels at 24 Hours (by microscopy)

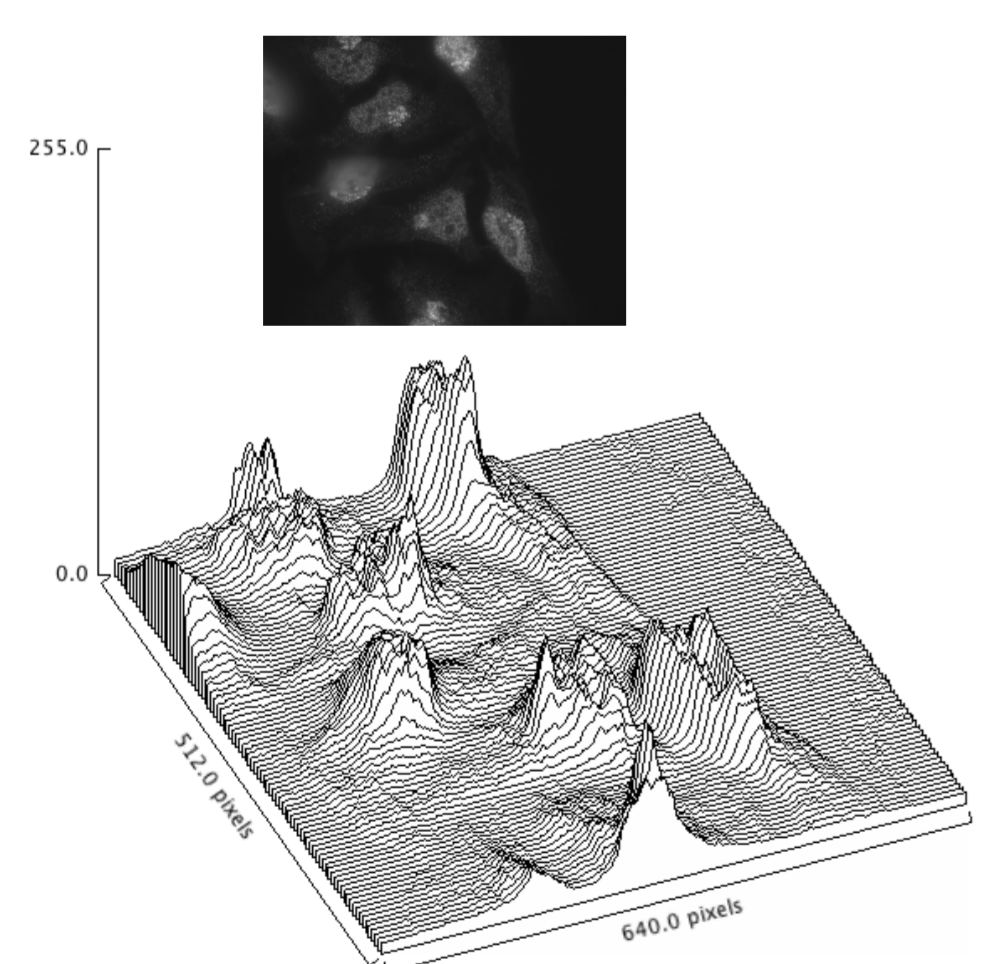
DDS-No ASO



DDS+ASO (STX5)



DDS+ASO (GFP)



Syntaxin 5 Expression at 24h (n=4  $\pm$  SEM)

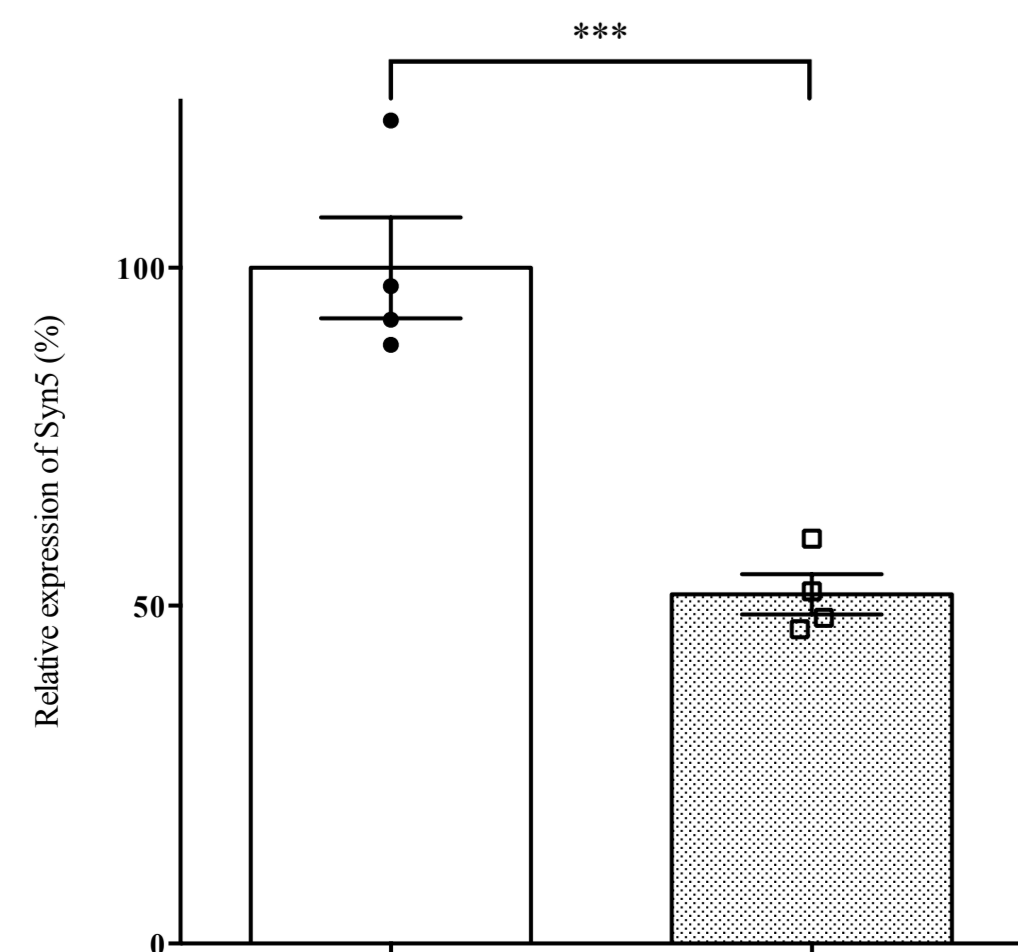
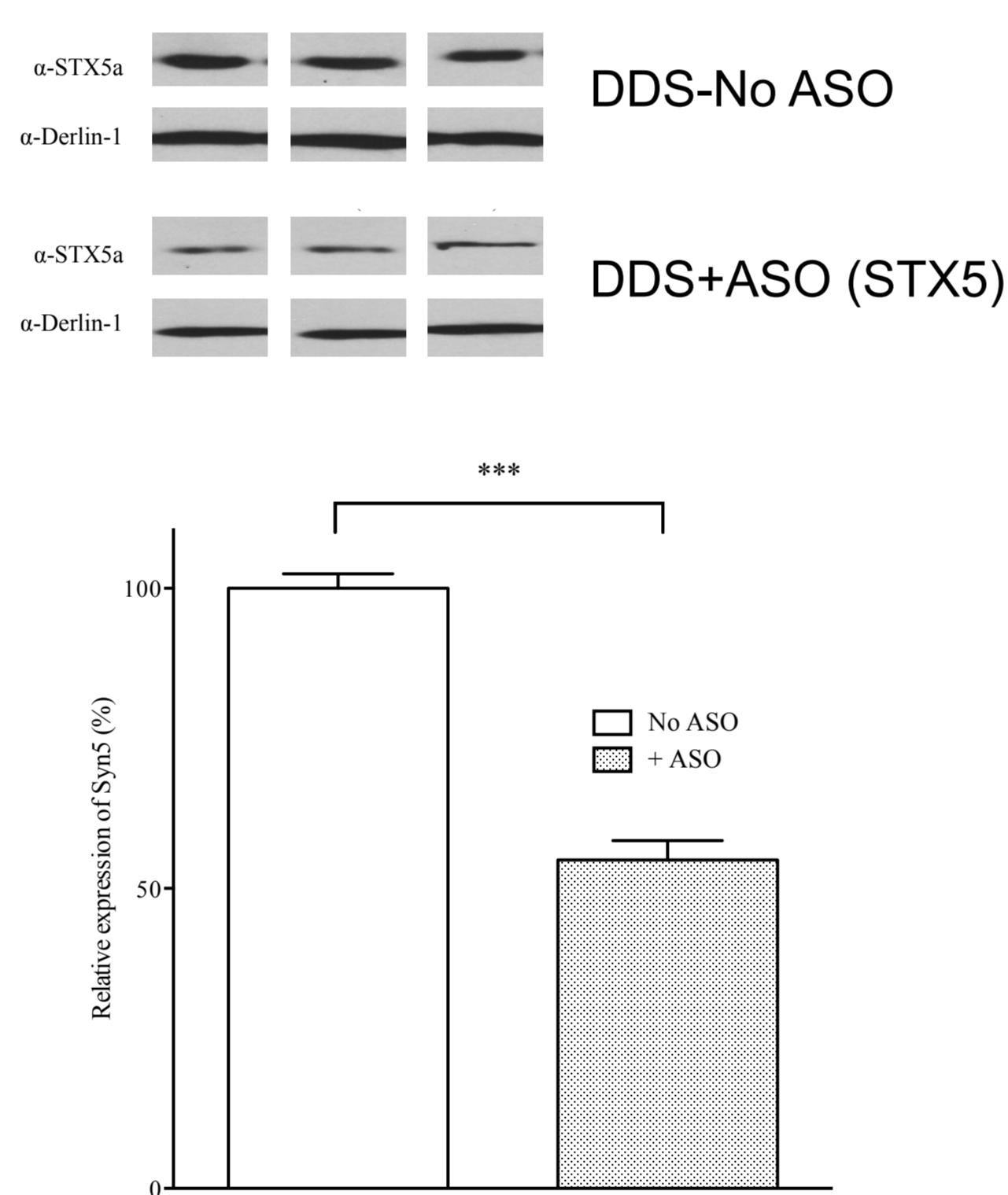


Fig 3: Syntaxin-5 protein levels at 48 Hours (by Western blotting)



## Methods

Cell culture, immunofluorescence experiments and Western blotting were performed as described in the cited literature<sup>3, 4, 5, 6, 7</sup> under the conditions stated. PEI and general reagents were from Sigma (Dorset UK), recombinant proteins were produced using sequences obtained from GenBank, sub-cloned into the pET151 expression system (Invitrogen, Paisley, UK) using standard protocols and characterized by SDS PAGE and Coomassie staining as well as Western blotting and immunodetection after affinity chromatography using appropriate antibodies.<sup>4</sup> Electroporation was carried out using a Nucleofector 2b (Lonza Biologics plc Cambridge, UK) following the manufacturer's instructions.

## Discussion

This departure from well-characterized, non-viral ASO delivery technology, (*i.e.* utilizing either cationic lipids or cationic polymers), has demonstrated activity equivalent to that of nucleofection (electroporation) over a 48h time course. Further, this recombinant DDS was tested for toxicity *in vitro* against epithelial cells (Vero and HeLa) using MTT.

Many of the pharmacokinetic and toxicity problems associated with current non-viral intracellular delivery technologies are directly attributable to the cationic nature of the delivery vector. As this charge is also required for membrane destabilization and payload delivery, we are very keen to continue investigating the possibilities afforded by this predominantly uncharged system, designed around known protein architecture.

Fig 4: *In vitro* toxicity of DDS

Toxicity in Vero and HeLa cells by MTT over 72h (n=8)

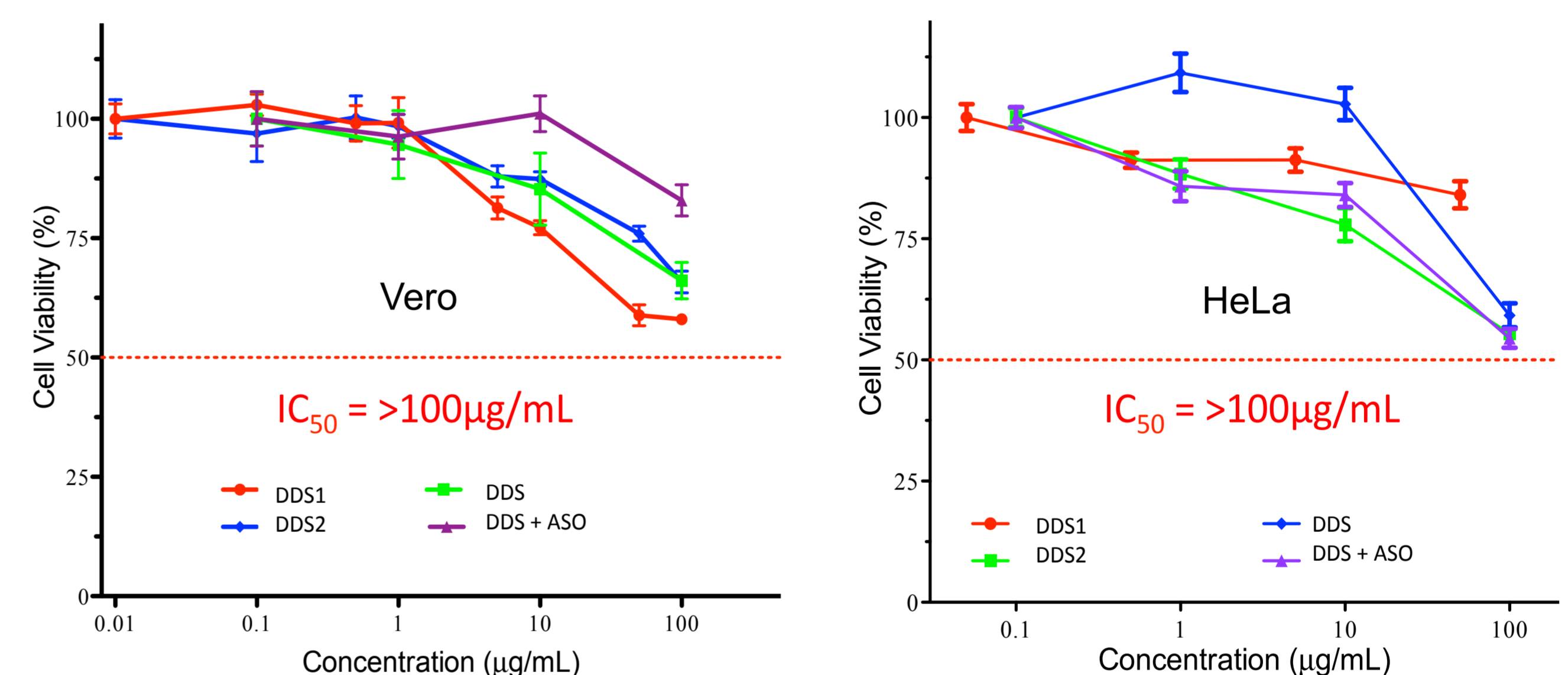
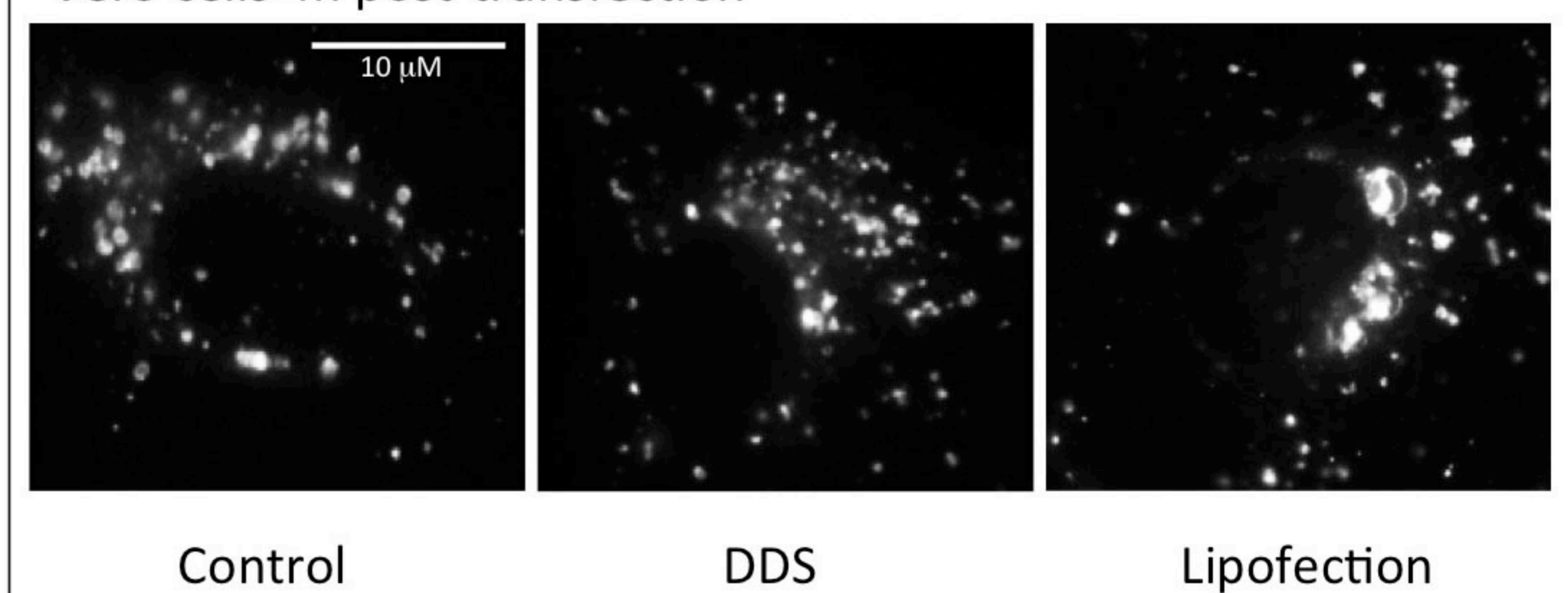


Table 1 : Poly(ethyleneimine) *in vitro* toxicity over 72h using MTT (n=8)

	HeLa ( $\mu\text{g/ml}$ )	Vero ( $\mu\text{g/ml}$ )
25 KDa Branched PEI	$2.9 \pm 0.6$	$7.3 \pm 0.1$
0.8 KDa Branched PEI	$2.4 \pm 0.2$	$7.4 \pm 0.3$
20 KDa Linear PEI	$3.0 \pm 0.1$	$6.9 \pm 0.5$
DDS+ASO	>100	>100

Fig 5: LAMP1 distribution in response to transfection

Vero cells 4h post-transfection



## References

- [1] Dyer P. D. & Richardson S.C. (2011) *Expert Opin. Drug Deliv.* **8**(4): 403-7; [2] Duncan R. & Richardson S.C.W. (2012) *Mol Pharm.* **9**(9): 2380; [3] Richardson S. C. *et al.*, (2008) *J. Controlled Release* **127**(1):1; [4] Richardson S.C. *et al.*, (2004) *Mol. Biol. Cell*, **15**(3):1197-210; [5] Richardson S.C. (2010) In: *Organelle-Specific Pharmaceutical Nanotechnology*, Ed., Weissig V & D'Souza G. John Wiley and Sons Inc. pp177-192; [6] Richardson S. C. *et al.*, (2010) *J. Controlled Release* **142**: 78-88; [7] Dyer P. D. *et al.*, (2013) *Methods in Mol. Biol.* **911**: 195.