# Cationic cell penetrating peptides with fatty acyl groups improve siRNA-delivery by chitosan for gene therapy

*Review Article*

**Gene therapy is rapidly becoming a mainstream approach for the treatment of a variety of inherited and acquired human diseases, but viral delivery systems can have issues of immunogenicity and mutagenesis. This has stimulated the development of non-viral delivery systems. El-Sayed and collaborators based in Egypt and the USA have shown that chitosan complexed with fatty-acyl derivatives of a cationic cell-penetrating peptide, CGKRK, shows promise as a method for delivering intact short interfering RNA (siRNA) to breast cancer cells *in vitro*.**

## Gene therapy and siRNA

After thirty years of promise and setbacks, a number of gene therapies for inherited immune disorders, like hemophilia, eye and neurodegenerative disorders, and lymphoid cancers have recently been approved in the United States and Europe, or are anticipated to receive approval in the near future (1). One approach to gene therapy is to suppress the expression of specific proteins by gene silencing using siRNA. A number of siRNA-based drugs are now in clinical trials and one, ONPATTRO™ (patisiran) lipid complex injection, a first-of-its-kind RNA interference (RNAi) therapeutic, has been recently approved for the treatment of the nerve damage caused by the rare disease hereditary transthyretin-mediated amyloidosis (hATTR) in adults (http://www.alnylam.com).

## Aims and limitations of delivery systems

With their high transfection efficiency, viruses have been viewed as promising vectors for gene therapy but it also emerged that they can induce immunogenicity and cause insertional mutagenesis. A large range of non-viral delivery systems have therefore been developed to (a) stabilize complexes, (b) escape phagocytosis, and (c) protect the drug payload from degradation by serum nucleases. Examples include polycationic liposomes, cationic polymers, and cationic cell-penetrating peptides (CPPs). These non-viral delivery systems also have disadvantages, stimulating efforts to improve cost-effectiveness, transfection efficiency, safety, biocompatibility, biodegradability, and selectivity.

With this in mind, the authors (2) used several building blocks to construct a delivery system for siRNAs that would protect them from serum nucleases and dissociation by other competing species such as heparin:

**Chitosan:** A linear polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan has several valuable properties including a dense cationic nature, excellent biocompatibility, and almost negligible cytotoxicity and immune response (https://en.wikipedia.org/wiki/Chitosan).

**Hydrophobic moiety:** Previous studies have highlighted the importance of including a hydrophobic moiety into the siRNA polymeric carrier to enhance uptake of the siRNA, promote its endosomal release into the cytoplasm, protect the siRNA from serum nuclease digestion, and improve the transfection efficacy.

**CPPs:** These peptides promote efficient active and passive transport of payloads into the target cell. Conjugation with CPPs has been shown to improve the transfection capacity of chitosan. The CGKRK peptide, for example, promotes cellular internalization mediated by heparin sulfate receptors, which are overexpressed in cancer cells. The authors had also developed a library of fatty acyl CGKRK peptides that efficiently bind to siRNA, protecting them from enzymatic degradation and also targeting specific cancer cells.

## Peptide synthesis and construction of the delivery system

The CGKRK peptide was synthesized by solid-phase synthesis using N-(9-fluorenyl) methoxy carbonyl (Fmoc)-based peptide chemistry on a Tribute® automated peptide synthesizer. The fatty acylation was performed on a solid support using palmitic acid, stearic acid, and oleic acid, with HBTU and DIPEA as coupling reagents.

The three fatty acyl derivatives of CGKRK peptide were then coupled to chitosan oligosaccharides (COS; MW = 4000–5000 Da, with 90% degree of deacetylation) using sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate sodium salt (sulfo-SMCC).

The polymer/siRNA polyplexes were then prepared by mixing scrambled siRNA with the polymer solutions at different ratios.

## Characterization of the conjugates

The hydrodynamic size of the polymer/siRNA polyplexes varied depending on the polymer ratio and the hydrophobic moiety conjugated to the CGKRK peptide. In addition, conjugating COS to the fatty acyl-CGKRK peptide improved complex formation with the siRNA and reduced the hydrodynamic size. The zeta potential, which indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion, depended on the polymer:siRNA ratio and the acyl group used. The fatty-acyl CGKRK-COS polyplexes had higher zeta potential compared to unmodified COS at the same ratio of polymer:siRNA. Unmodified COS had a higher affinity for siRNA than the fatty acyl peptide-COS conjugates.

## Improved serum stability with low cytotoxicity

Previous studies had shown that attaching a fatty acyl moiety to the CGKRK peptide improved its ability to protect siRNA from degradation by serum RNases. The results presented here revealed that protection from serum degradation depended on the polymer ratio and its degree of hydrophobicity. Generally, increasing the ratio of peptide:siRNA increased the protection of siRNA from serum degradation (Figure 1).

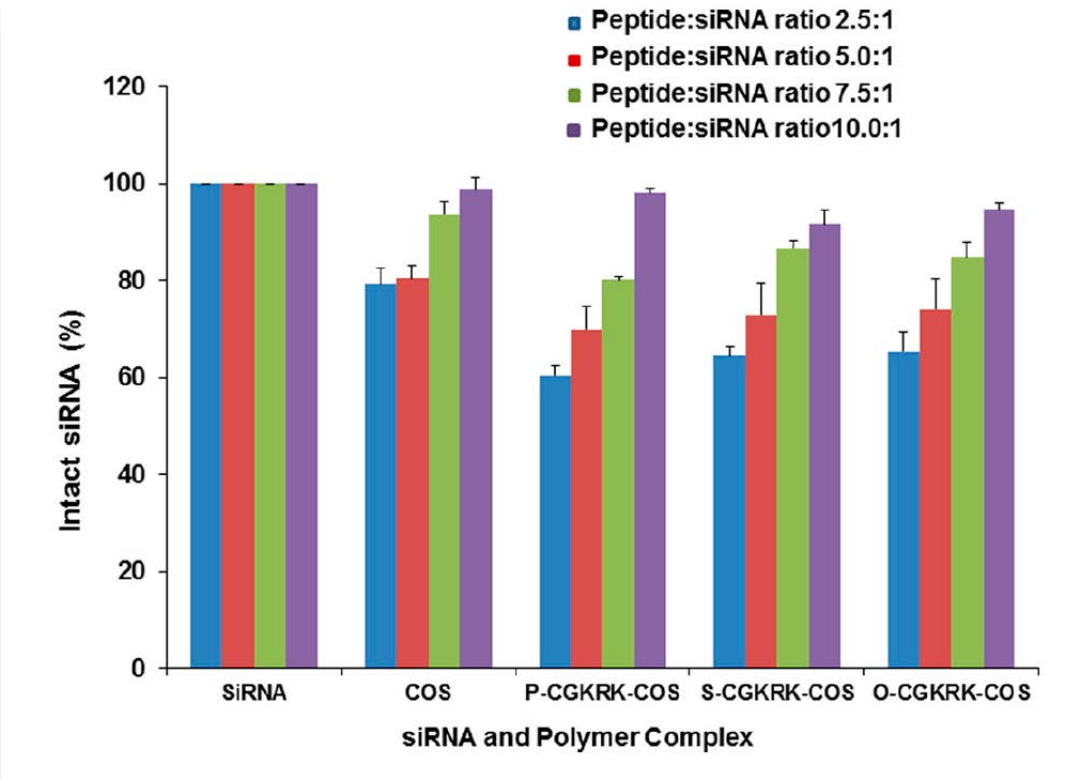


*Figure 1. The serum stability of the polymer/siRNA polyplex depends on the peptide:siRNA ratio and also the nature of the acyl group on the modified CGKRK peptide. High ratios of peptide:siRNA afford almost complete protection. Figure 6. From El-Sayed et al, 2018.a*

Cytotoxicity tests using a MTT (yellow 3-(4,5-dimethythiazol- 2-yl)-2,5- diphenyl tetrazolium bromide) assay on a human breast cancer cell line indicated that COS, fatty acyl-CGKRK-COS alone, and fatty acyl-CGKRK-COS/siRNA polyplexes all had either negligible or mild cytotoxicity even at high polymer concentrations.

## A promising siRNA carrier

The researchers concluded that fatty acyl-CGKRK-COS appears to be a promising carrier for siRNA and that including a hydrophobic moiety into chitosan improves its ability to complex with siRNA and protect it from degradation. They plan to further investigate the use of O-CGKRK-COS for siRNA delivery into a multi-drug resistant breast cancer cell line.

## References

1. Gene therapy comes of age. Dunbar CE *et al*. Science. 2018 Jan 12;359(6372). pii: eaan4672. doi: 10.1126/science.aan4672. https://www.ncbi.nlm.nih.gov/pubmed/29326244

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