

# Arginine-rich cross-linking peptides with different SV40 nuclear localization signal content as vectors for intranuclear DNA delivery

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## Abstract

The major barriers for intracellular DNA transportation by cationic polymers are their toxicity, poor endosomal escape and inefficient nuclear uptake. Therefore, we designed novel modular peptide-based carriers modified with SV40 nuclear localization signal (NLS). Core peptide consists of arginine, histidine and cysteine residues for DNA condensation, endosomal escape promotion and interpeptide cross-linking, respectively. We investigated three polyplexes with different NLS content (10 mol%, 50 mol% and 90 mol% of SV40 NLS) as vectors for intranuclear DNA delivery. All carriers tested were able to condense DNA, to protect it from DNAase I and were not toxic to the cells. We observed that cell cycle arrest by hydroxyurea did not affect transfection efficacy of NLS-modified carriers which we confirmed using quantitative confocal microscopy analysis. Overall, peptide carrier modified with 90 mol% of SV40 NLS provided efficient transfection and nuclear uptake in non-dividing cells. Thus, incorporation of NLS into arginine-rich cross-linking peptides is an adequate approach to the development of efficient intranuclear gene delivery vehicles.

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**Keywords:** SV40; Nuclear localization signal; Cross-linking peptides; DNA; Transfection; Intranuclear delivery; Non-viral vectors

Gene therapy is a highly promising approach to cure various inherited and acquired diseases.<sup>1,2</sup> The outcome of gene therapy greatly depends on the way of gene delivery. Because of high transfection efficacy the majority of gene therapy clinical trials known so far used viral-mediated delivery. However non-viral carriers being less efficient lack immunogenic and cytotoxic issues are more safe and versatile for nucleic acids (NA) compared to viral delivery.

The main goal of non-viral gene delivery studies concerns development of highly efficient vectors, which are able to overcome all cellular barriers of NA transport.<sup>3</sup> One of the most formidable intracellular barriers for DNA transport along with cellular and endosomal membranes is a nuclear membrane, which controls the exchange of macromolecules between nucleus and cytoplasm and it must be overcome to reach expression of the therapeutic gene. Direct injection of plasmid DNA into cell nuclei provide up to 50% cells expressed transgenes, while their amount was approximately 1% if plasmid was injected into cytoplasm.<sup>4</sup> There are two feasible ways to penetrate nuclear membrane: (I) degraded nuclear envelope during mitosis and (II) penetration of nuclear membrane through the pores with a diameter of approximately 40 nm.<sup>5</sup> The latter represents an only available way for nuclear internalization in non-dividing, slowly dividing and post-mitotic cells. Import of large molecules (>45 kDa) occurs by means of special receptors - importins - transport proteins of nuclear pore complex which recognize the specific sequence - nuclear localization signal (NLS).<sup>6</sup> Viral NLS of SV40 large T-antigen (<sup>126</sup>PKKKRKV<sup>132</sup>) attaches to importin- $\alpha$  on the nuclear envelope surface and form complex with importin- $\beta$  providing intranuclear transport.<sup>7</sup> Direct conjugation of NLS to DNA is inefficient, but nuclear delivery can be increased by NLS-modified liposomes and polymers.<sup>8-10</sup>

Recently we developed arginine-rich, cysteine-flanked peptide R6 which can be modified with certain ligand to achieve receptor-mediated gene and siRNA delivery.<sup>11-13</sup> Oligoarginine stretch in R6 sequence promotes effective electrostatic binding of DNA, while cysteine residues can oxidize to disulfide bonds during template polymerization, allowing cross-linking between peptides and formation of stable DNA/carrier complexes. Histidine residues provide endosomal escape due to their buffering capacity.<sup>14,15</sup>

We developed multifunctional peptide-based non-viral carrier modified with NLS of SV40 large T-antigen through the two molecules of  $\epsilon$ -aminohexanoic acid spacer (PKKKRKVG-Ahx-Ahx-CHRRRRRRHC) and designated it as NL peptide module. Combination of NLS-modified module (NL) and unmodified peptide CHRRRRRRHC (R6) by means of polymerization on DNA template resulted in three carriers with different NLS content: NL1 with 10 mol%, NL2 with 50 mol% and NL3 with 90 mol% of NLS-modified peptides respectively. The aim of current research was to study arginine-rich cross-linking peptides with different SV40 NLS content, as vectors for intranuclear DNA delivery.

R6 and NL peptides were synthesized using a solid phase Boc-chemistry by NPF Verta, LLC (Saint-Petersburg, Russia) (Table 1). They were dissolved in 0.1% trifluoroacetic acid at 2 mg/ml and stored at  $-20^{\circ}\text{C}$ . The purity of the peptides determined by high-performance liquid chromatography was 90-95%. Polyethyleneimine (branched PEI 25 kDa; Sigma) was used as at 0.9 mg/ml (pH 7.5). We used pCMV-lacZ plasmid (pDNA) with  $\beta$ -galactosidase gene under cytomegalovirus promoter control (gift from Prof. B. Sholte, Erasmus University Rotterdam, the Netherlands), diluted to 1 mg/ml and stored at  $-20^{\circ}\text{C}$ . DNA/peptide complexes were prepared at various N/P ratios (peptide nitrogen/DNA phosphorus ratio) in the range 0.1-8 and PEI to DNA 8:1. pDNA was diluted in 20  $\mu\text{g}/\text{ml}$  in Hepes-buffered mannitol (HBM) (5% w/v mannitol, 5 mM Hepes, pH 7.5). Then peptides at 2 mg/ml were added to the DNA solution and vortexed. Complexes were left at room temperature for 2 h for template polymerization. Template polymerized carriers NL1, NL2 and NL3 were obtained by mixing solutions of NL and R6 peptides before the addition of pDNA (10 mol% and 90 mol%; 50 mol% and 50 mol%; 90 mol% and 10 mol%, respectively). Peptide-DNA binding was monitored by agarose gel retardation and the ethidium bromide (EtBr) fluorescence quenching assay. Peptides' ability to protect DNA from endonuclease degradation was checked by reaction of complexes with 0.5 units of DNase I (Promega, Madison, WI, USA) for 30 min at  $37^{\circ}\text{C}$ . Transfection experiments were performed in human cervical carcinoma (HeLa) cell line (Institute of Cytology RAS, Saint-Petersburg, Russia). Cell cycle was blocked with 2.5 mM hydroxyurea (HU) (Sigma). Before transfection culture medium was replaced with serum-free medium. Complexes were incubated with cells for 4 h. The  $\beta$ -galactosidase activity in cell extracts was measured with MUG reaction solution (Sigma) using a Wallac 1420D scanning multilabel counter (355 nm/460 nm).  $\beta$ -galactosidase activity (mU) was determined by means of calibration curve using diluted  $\beta$ -galactosidase with known activity (Promega). The reporter gene expression was normalized by the total protein concentration of the cell extracts, measured with Bradford reagent (Helicon, Russia). For intranuclear delivery visualization pDNA was mixed with intercalating dye YOYO-1 iodide (491 nm/509 nm) - 1 mM solution in DMSO (Invitrogen) in ratio 1 molecule of dye per 50 base pairs. Then transfections with or without HU were carried out and cell nuclei were stained with the Hoechst 33258 (Sigma-Aldrich, USA) (0.5 mkg/ml) in Dulbecco buffer (pH 7.2 - 7.4). DNA was visualized in 24 h after transfection by means of the Olympus FluoView™ FV1000 confocal laser scanning microscope. Quantitative data are presented as the percentages of transfected nuclei containing fluorescent signal of YOYO-1. Mean values were presented and standard deviations of triplicate samples were counted for 600 nuclei per sample. To confirm the intranuclear localization in the 3D images, 4-sectioned images were overlapped into a single image. The nuclei were considered positive if YOYO-1-generated fluorescent signal was present inside nucleus after 3D reconstruction procedure. The cytotoxicity of complexes was evaluated using Alamar blue assay (BioSource International, USA). Statistically significant differences were analyzed by Mann-Whitney *U* test and by Student's *t*-test, using InStat (GraphPad Software Inc., USA).  $p < 0.05$  was considered statistically significant.

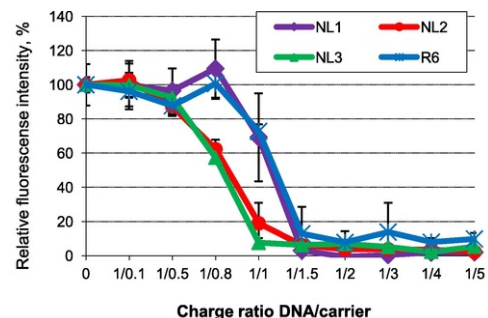
**Table 1** Composition of modular peptide carriers.

Name		Formula
Control peptide R6		CHRRRRRRHC
Large SV40 Tag - modified peptide module NL		PKKKRKVG-Ahx-Ahx-CHRRRRRRHC
NL peptide module-consisting carriers	NL1	10 mol% NL + 90 mol% R6
	NL2	50 mol% NL + 50 mol% R6
	NL3	90 mol% NL + 10 mol% R6
Control carrier PEI		Branched PEI 25 kDa

Table 1 demonstrates the list of carriers, which we used in the present study. Our group previously developed R6 peptide which we used as a control.<sup>11</sup> NL1 carrier comprises the PKKKRKVG motif and the DNA-binding sequence of R6 (CHRRRRRRHC), separated from each other with two  $\epsilon$ -aminocaproic acid molecules (Ahx). NL1 is a combination of 10 mol% of NL and 90 mol% of R6 peptide, NL2 carrier includes

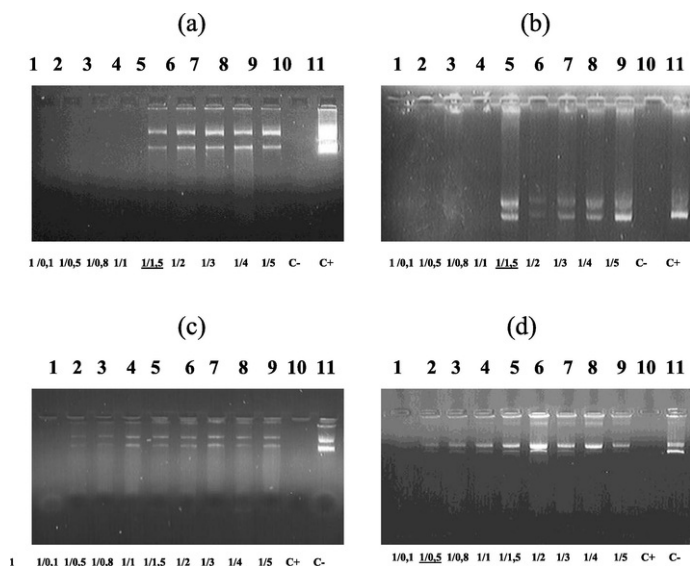
50 mol% of NL peptide and 50 mol% of R6, NL3 peptide is a combination of 90 mol% of NL and 10 mol% of R6 peptide.

We studied DNA-binding capacity of the carriers by means of ethidium bromide fluorescence quenching assay. EtBr releases out of DNA during complex formation which causes fluorescence intensity decrease. Free DNA was used as a control with maximal fluorescence taken as 100%. We observed clear difference in DNA condensation by peptides with different NLS content. Complete complex formation by unmodified R6 peptide and NL1 carrier started at 1.5:1 peptide/DNA charge ratio, whereas for NL2 and NL3 carriers it began at 1:1 (Fig. 1). The results of this assay generally agreed with gel retardation test (data not shown). Thus it was concluded that modification of R6 peptide with NLS module improves its DNA-condensation properties, due to the increasing of the carrier's positive charge. Previously Sipsrashvili with co-authors clearly showed that increase of cationic residues number in peptide carriers leads to augmentation of their DNA-condensing properties.<sup>16</sup>



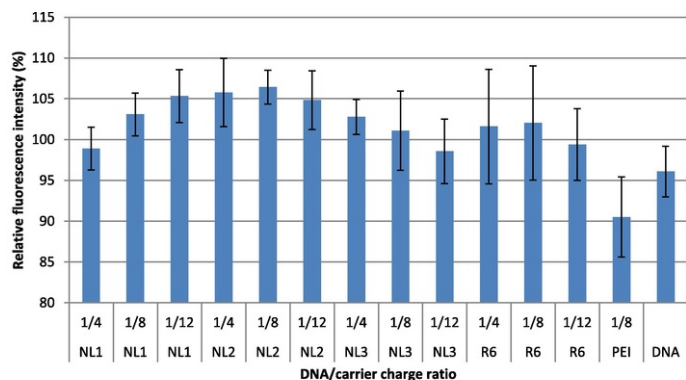
**Fig. 1** DNA binding studies by ethidium bromide exclusion from complexes of DNA and NL1, NL2, NL3 and R6 carriers. Values are means  $\pm$  standard deviation of the mean of triplicates.

An important property of synthetic DNA-carriers is ability to protect DNA from endonuclease degradation.<sup>17</sup> Degree of DNA condensation by cationic peptides directly correlates with its susceptibility to nuclease degradation.<sup>18</sup> Protective properties of the carriers were estimated by DNase I protection assay. The DNA integrity was compared with this one of native DNA and DNase I treated DNA. We demonstrated that NL2 and NL3 protected DNA from degradation at N/P ratio 0.5:1, while NL1 partly protected it from DNase I at 1.5:1 N/P ratio similarly to unmodified carrier R6 (Fig. 2). Thereby NL2 and NL3 carriers possess the most effective DNA-protection properties, suggesting that at least 50 mol% content of NLS-containing module in the carrier is required for optimal DNA protection. Improved DNA protection by means of carriers with higher NLS-content could be explained by steric hindrances caused due to presence of NLS and Ahx-linker.



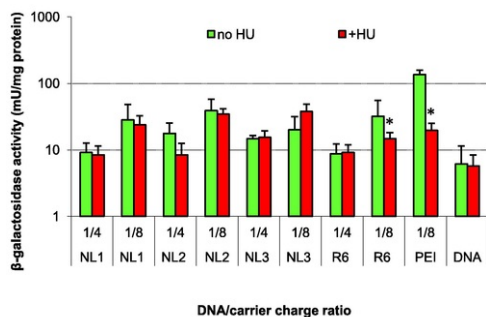
**Fig. 2** DNase I protective ability of DNA-complexes formed with R6 (a), NL1 (b), NL2 (c) and NL3 (d) carriers. Underlined charge ratio indicates beginning of DNA protection. C-“naked” plasmid DNA treated with DNase I; C+ untreated plasmid DNA.

Cytotoxicity of peptide/DNA complexes after transfection *in vitro* was evaluated at three charge ratios (4:1, 8:1 and 12:1) using the Alamar blue assay. We observed significantly higher degree of cell viability after transfection with DNA/peptide complexes at 4:1 and 8:1 N/P ratios compared to the PEI/DNA in HeLa cells (Fig. 3). These results demonstrated that studied peptides possessed less cytotoxicity compared to PEI. Also there was no statistically significant difference in cytotoxicity between native DNA and complexes of NL1, NL2, NL3 and DNA at 4:1 and 8:1 N/P ratios in HeLa cells. These results match previous data on role of disulfide bonds reduction in decrease of the carrier toxicity.<sup>11,19</sup>



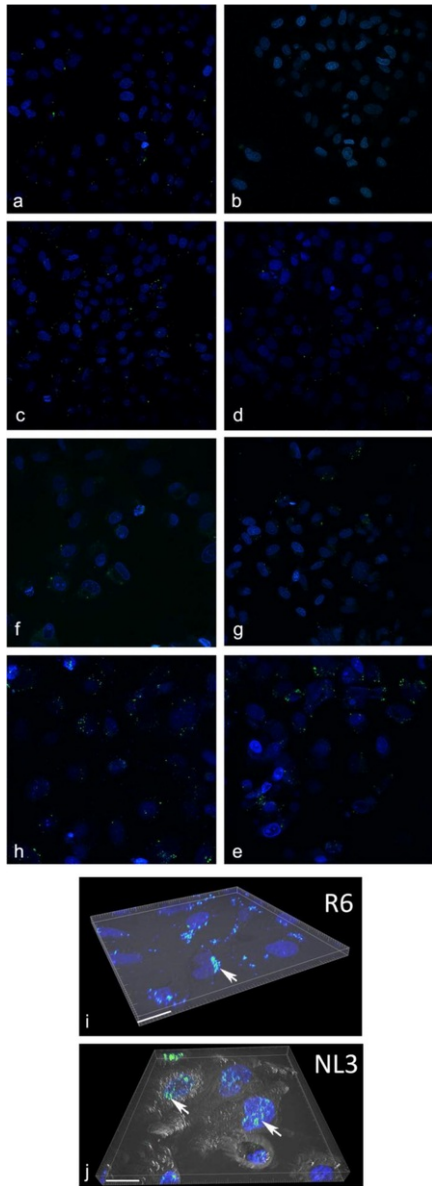
**Fig. 3** Cytotoxicity evaluation of peptide/DNA complexes by means of Alamar Blue assay. Values are means  $\pm$  standard deviation of the mean of triplicates.

In the present study, a non-dividing cell model was made by synchronization of the HeLa cell cycle at G1 phase via treatment with 2.5 mM of hydroxyurea (HU) which affects over 90% of cells.<sup>20</sup> No differences in transfection efficacy in HeLa cells between dividing cells and arrested cells were found, whereas efficacy of PEI/DNA and R6/DNA complexes was significantly decreased after HU treatment (Fig. 4). Thereby, increasing of the complexes' transfection efficacy in non-dividing cells can be associated with the presence of specific NLS, which gives them an opportunity to overcome nuclear envelope barrier and provide the same level of gene delivery, which is typical for dividing cells. It is known, that the presence of an NLS peptide increases number of transfected cells in dividing and non-dividing cells.<sup>21</sup>

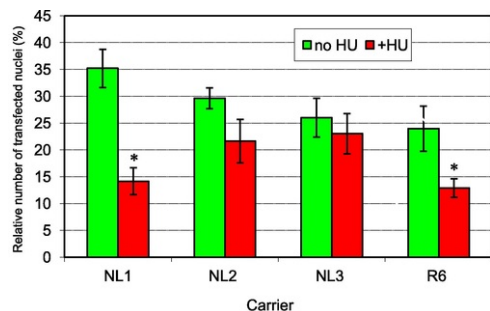


**Fig. 4** Transfection efficacy evaluation. The HeLa cell line was transfected with NL1/DNA, NL2/DNA, NL3/DNA, R6/DNA complexes at charge ratios 4:1 and 8:1, PEI/DNA complexes at charge ratio 8:1 and pCMV lacZ plasmid alone. Reporter *lacZ* gene expression is given as milliunits (mU) per milligram of total protein. Values are means  $\pm$  standard deviation of the mean of triplicates. Asterisk indicates  $p < 0.05$  compared with cells without hydroxyurea (HU) treatment.

With a confocal microscopy we demonstrated localization of plasmid DNA in HeLa cells after transfection and estimated efficacy of intranuclear delivery. YOYO-1 - labeled pDNA in complexes with the NL1, NL2 and NL3 at N/P ratio of 8:1 was studied. R6/DNA complexes at the same N/P ratio and naked DNA served as a control. The cells treated with naked DNA had negligible green fluorescence (data not shown), whereas those treated with peptide/DNA complexes displayed obvious intracellular green fluorescence. DNA delivered by R6 and NL1 peptides localized mainly in cytoplasm and perinuclear region, indicating low transfection efficacy (Fig. 5a, b, c, d). However NL2 and NL3 peptides provided more efficient pDNA delivery and promoted its preferable localization inside the nuclei (Fig. 5f, g, h, e). 3D reconstruction of the pDNA-positive cells, transfected with pDNA/peptide complexes allowed us to identify YOYO-1-positive nuclei and to distinguish the fluorescence from complexes located in the perinuclear and the intranuclear areas (Fig. 5i, j). The nuclear uptake rate of pDNA was assessed by confocal microscopy image analysis followed by quantification of YOYO-1-positive nuclei (Fig. 6).



**Fig. 5** Intracellular uptake evaluation. Intracellular localization of YOYO-1-labeled pCMV lacZ plasmid in HU-treated (b, d, g, e) and untreated (a, c, f, h) HeLa cells after delivery by R6/DNA (a, b), NL1/DNA (c, d), NL2/DNA (f, g) and NL3/DNA (h, e) complexes at charge ratio 8:1; perinuclear and intranuclear localization (arrows) of YOYO-1-labeled pCMV lacZ plasmid after delivery by R6/DNA (i) and NL3/DNA (j) complexes at charge ratio 8:1 in the 3D images of HeLa cells; bar scale represents 10  $\mu$ m.



**Fig. 6** Relative number of YOYO-1-positive nuclei in HeLa cells after delivery by NL1/DNA, NL2/DNA, NL3/DNA and R6/DNA complexes at charge ratio 8:1. Values are means  $\pm$  standard deviation of the mean of triplicates. Asterisk indicates  $p < 0.05$  compared with cells without hydroxyurea (HU) treatment.

We found that cells treated with NL1 and R6 polyplexes demonstrated 35.2% and 24% of YOYO-1-positive nuclei, whereas the amounts of transfected nuclei dropped down to 14.1% and 12.4%, respectively, after HU-mediated cell cycle blockage (Fig. 6). Meanwhile, according to quantitative analysis of positively transfected nuclei the efficacy of DNA delivery with NL2 and NL3 carriers remained the same irrespective of HU treatment. It has been found that NL2 and NL3 polyplexes provided intranuclear DNA delivery into 29.6% and 26% of nuclei without HU treatment. In cycle arrested cells efficacy of intranuclear DNA delivery mediated by NL2 and NL3 carriers dropped insignificantly (21.6% and 23% of nuclei, respectively) (Fig. 6). Thus, confocal microscopy analysis confirmed improved transfection activity due to the increased pDNA intranuclear delivery by means of SV40 NLS-modified carriers.

In the present study we designed novel transfection enhancing cross-linked peptide-based carriers by conjugation of NLS to arginine-rich module, which increase intranuclear DNA delivery without obvious cytotoxicity. We assume that pDNA is not fully released from the polyplexes before reaching nucleus. Actually, the disassembly of the polyplexes is the final requirement for the transcription machinery to access pDNA for efficient transfection. Although the interaction of polyamine gene vectors with intracellular RNA leads to the dissociation of pDNA-carrier complexes, it is generally accepted that at least part of the polyplexes localize intact to the nucleus, where they presumably undergo dissociation.<sup>22,23</sup> Interaction of R6/DNA polyplexes with the polyanions has been studied previously and it has been shown that R6 peptide-based polyplexes are not susceptible to relaxation by polyanions.<sup>11</sup> Thus, it can be suggested that NL peptide-based polyplexes will also be stable in an intracellular environment and will not be completely destroyed when interacting with RNA. Also it should be noted that in confocal images the polyplexes were found to be accumulated in cytoplasm and in cell nuclei as bright green dots (fluorescence from YOYO-1 iodide-labeled pDNA) and they have particle shapes, meaning that pDNA was still condensed with the peptide carriers (Fig. 5). Thus, it can be assumed that pDNA should be at least partly bound by cationic NLS-modified peptides used in our study as carriers for intranuclear delivery.

Also we showed that an increase in content of NLS-conjugated modules improves DNA condensation and protection properties of the carriers. Although the difference between NL and R6 polyplexes can be seen only at N/P ratio range 1-2 this finding may provide insights into transfection and nuclear translocation abilities of the polyplexes. Previously it has been shown that at N/P ratio of about 2, cationic polymer molecules completely neutralize DNA, but addition of more polymer to this neutral core leads to the formation of a shell of extra carrier molecules around the core that results in formation of positively charged polyplexes with their highest gene transfer activity.<sup>24</sup> In our study we used for transfection N/P ratios  $\geq 4$  and it can be suggested that NL2 and NL3 polyplexes may differ from NL1 and R6 polyplexes in transfection properties because their neutral cores are formed at different N/P ratios (Figs. 1, 2).

According to obtained results modular carrier NL3 with 90 mol% of SV40 NLS-modified peptide provided the most efficient transfection and nuclear uptake by non-dividing cells. Our study proves the efficiency of cross-linking peptide modification with nuclear localization signal for the gene delivery into non-dividing cells.

## Acknowledgements

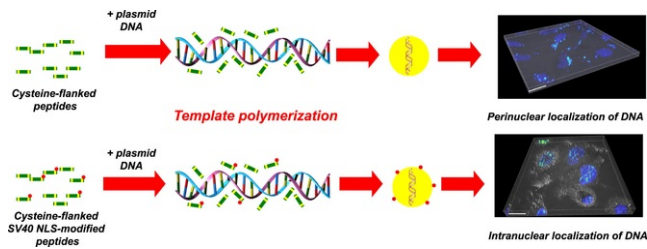
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