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Co-liposomes comprising a Lipidated Multivalent RGD-Peptide and a Cationic Gemini Cholesterol Induce Selective Gene Transfection in $\alpha\beta3$ and $\alpha\beta5$ Integrin Receptor-rich Cancer CellsSantosh K. Misra,^a Paturu Kondaiah,^b Santanu Bhattacharya,^{a,c,*} Didier Boturyn,^{d,e,*} and Pascal Dumy^{e,f,*}

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The $\alpha\beta3$ and $\alpha\beta5$ integrin, transmembrane glycoprotein receptors, are over-expressed in numerous tumors and in endothelial cells that constitute tumor blood vessels. As this protein selectively binds to Arg-Gly-Asp (RGD) sequence containing peptides, it is an attractive way to target tumor. Herein we have developed novel formulations for integrin mediated selective gene delivery. These formulations are composed of a novel palmitoylated tetrameric RGD containing scaffold (named RAFT-RGD), cationic gemini cholesterol (GL5) and a natural helper lipid 1, 2-dioleoyl-L- α -glycero-3-phosphatidylethanolamine (DOPE). We have optimized a co-liposomal formulation to introduce the multivalent RGD-containing macromolecule in GL5: DOPE (GL5D) mixture to produce GL5D-RGD. We have unambiguously shown the selectivity of these formulations towards cancer cells that over express $\alpha\beta3$ and $\alpha\beta5$ integrins. Two reporter plasmids, pEGFP-C3 and PGL-3 were employed for the transfection experiments and it was shown that GL5D-RGD liposomes increased exclusively the transfection in $\alpha\beta3$ and $\alpha\beta5$ -overexpressing HeLa cells.

Introduction

Selective cell targeting has been considered as a major objective in gene therapy to combat different diseases.¹ The gene carrier species should have specific ligands which can help it to recognize the victim cell, so that it can deliver the cargo to the desired cells only. The concept of cell targeting introduces an effort to recognize natural targets on tumor cell surfaces. Integrin receptors have been recognized as natural targets for such selective transfection.² Integrins belong to a major class of $\alpha\beta$ heterodimeric transmembrane glycoprotein receptors.³⁻⁵ It is known that $\alpha\beta3$ and $\alpha\beta5$ integrin are expressed on various cell types such as endothelial cells,⁶ osteoclasts,⁷ macrophages,⁸ platelets,⁹ and melanomas.¹⁰ They play a significant role in angiogenesis, vascular intimal thickening and proliferation of malignant tumors.¹¹ Some important cancer cell lines expressing high $\alpha\beta3$ integrin are A375 (melanoma), M21 (melanoma), SK-MEL-28 (melanoma), M19-MEL (melanoma) while HeLa cells (carcinoma) over-expresses $\alpha\beta3$ as well as $\alpha\beta5$.¹² However, some cell lines do not express $\alpha\beta3$ and $\alpha\beta5$ integrins. These include HEK293¹³ 293T¹⁴ and NIH3T3¹⁵ cells, which express another integrin receptor as $\alpha5\beta1$ in abundance.

Cells expressing $\alpha\beta3$ or $\alpha\beta5$ integrins bind with a broad range of ligands such as fibronectin and vitronectin mainly through the recognition of the ubiquitous triad sequence RGD (Arg-Gly-Asp).¹⁶ The latter has served as a basis for the

development and discovery of high and selective integrin-peptide ligands such as c[RGDfV-],¹⁷ that was exploited for a wide range of applications.¹⁸⁻²³ Additionally, the principle of multivalency has then been recognized as an important approach for the design of synthetic ligands.²⁴ In this context, enhancements of biological activity are obtained from multivalent RGD peptide ligands used to target cell surface receptors such as $\alpha\beta3$ integrin.²⁵⁻²⁷ For instance, we have shown that tetrameric RGD-containing scaffolds²⁸ exhibit advantageous biological properties for imaging²⁹⁻³¹ and for targeted drug delivery.^{32,33}

Two strategies may be conceived for achieving cell selective targeting using carrier liposomes. Firstly the covalent conjugation of RGD based molecules to the lipid carrier³⁴ and secondly the non-covalent loading of RGD based molecules to lipid carriers by physical mixing in optimized molar ratios.³⁵ Very recently, we prepared RGD-containing lipopeptide to construct fluid supported lipid bilayers (SLB).³⁶ We then studied RGD-mediated cell adhesion to an SLB surface providing the average ligand spacing to trigger cell adhesion and spreading on a fluid substrate. Herein we present the results of non-covalent RAFT-RGD inclusion in cationic liposomes³⁷ for cancer cell targeting *in vitro* (Figure 1).

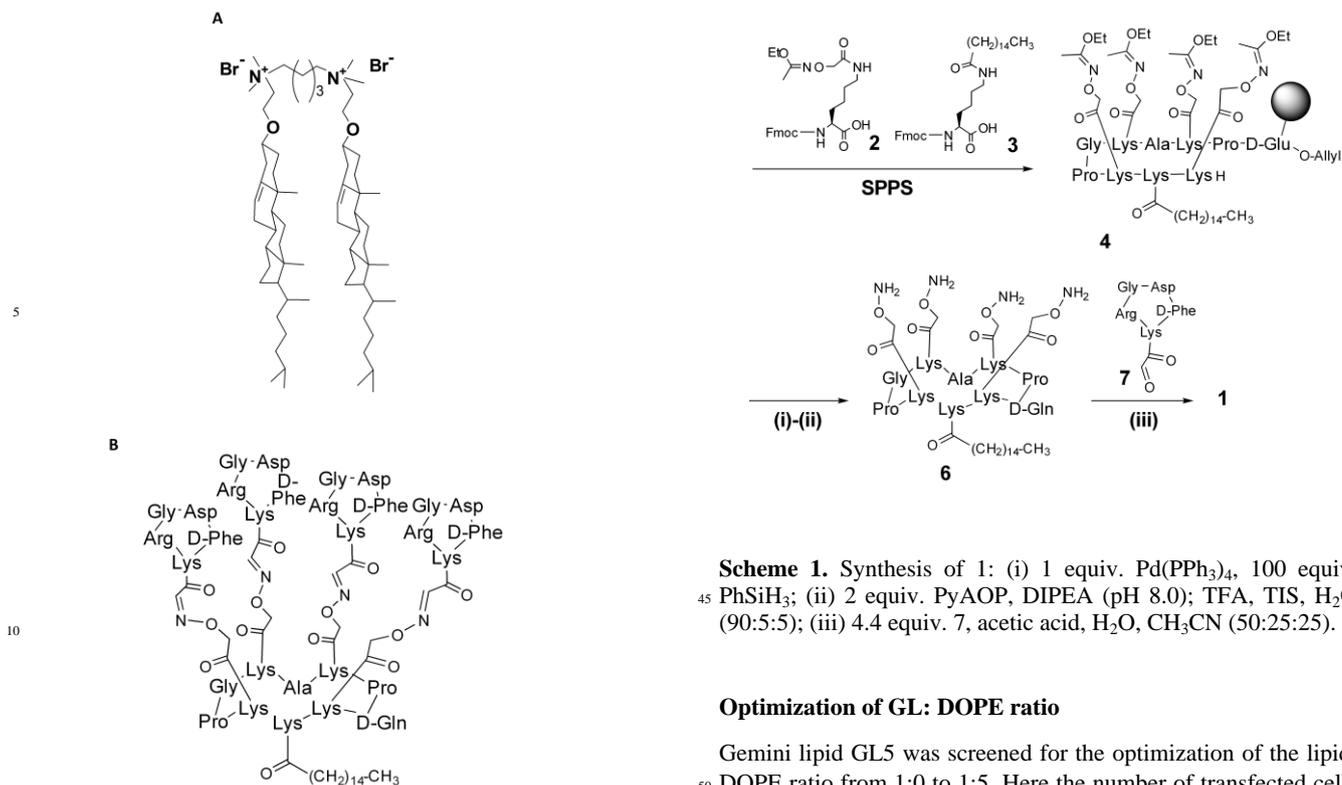


Figure 1. Molecular structures of the gemini lipid (A) GL5 and (B) a Palmitoyl-RAFT-RGD₄ (**1**) ligand used in the present investigation.

Results

The concept of cell targeting introduces an effort to recognize natural targets *viz.* integrin receptors for selective transfection. The multivalency has been established as an important approach for the design of ligands to increase the targeting efficiency. We applied the non-covalent palmitoylated RAFT-RGD inclusion strategy to develop a co-liposomal formulation of GL, DOPE and a palmitoylated RAFT-RGD macromolecule **1** to transfect pEGFP-C3 and PGL-3 in $\alpha\beta 3$ and $\alpha\beta 5$ positive HeLa and negative HEK293 and NIH3T3 cells. This methodology of using lipopeptide **1** was found highly beneficial for introducing high selectivity in gene therapy.

Synthesis of Palmitoyl-RAFT-RGD₄

The synthesis of lipopeptide **1** was produced *via* solid-phase peptide syntheses (SPPS) according to the methods already developed by our group (Scheme 1). Stable oxime bonds were chosen to append aldehyde-bearing RGD motifs to a cyclodecapeptide scaffold. Building blocks **2** and **3** including respectively the protected amino-oxo function and the palmitoyl group were directly introduced during the SPPS. This strategy decreases the number of steps and the combination of protecting groups required so far for the production of lipopeptide **4**. The following head-to-tail cyclization on resin and the subsequent deprotection under acidic conditions furnished the lipopeptide **6**. The grafting of glyoxylyl aldehyde containing-RGD targeting elements **7** provided the expected palmitoyl-RAFT-RGD₄ **1** in satisfying 15% overall yield.

Scheme 1. Synthesis of **1**: (i) 1 equiv. Pd(PPh₃)₄, 100 equiv. PhSiH₃; (ii) 2 equiv. PyAOP, DIPEA (pH 8.0); TFA, TIS, H₂O (90:5:5); (iii) 4.4 equiv. **7**, acetic acid, H₂O, CH₃CN (50:25:25).

Optimization of GL: DOPE ratio

Gemini lipid GL5 was screened for the optimization of the lipid: DOPE ratio from 1:0 to 1:5. Here the number of transfected cells (% GFP cells) was higher for the GL: DOPE at ratios 1:3 and 1:5 compared to that in 1:4. However, the amount of DNA transfected to each cell (MFI) was very high when a molar ratio of 1:4 was used. A gemini lipid to DNA ratio of 1:4 was used as the optimal ratio for further studies (Figure S1).

Optimization of Palmitoyl-RAFT-RGD₄ inclusion in liposomes

Incorporation of the Palmitoyl-RAFT-RGD₄ **1** into cationic liposomes was achieved either by the preparation of co-liposomes or *via* post-injection method. In the first method, an optimized GL5D was mixed with the lipidated RGD in molar ratios of 2000:1 and 1000:1, respectively. Liposomes were prepared from these mixtures as mentioned earlier and were termed as co-liposomal formulations, i.e., Co2000 and Co1000, respectively. A second method was used by injecting an ethanolic solution of the lipidated RGD in preformed GLD suspensions. These formulations have been termed as post-injection formulations, i.e., Po2000 and Po1000, respectively. FACS analysis of the pEGFP-C3 reporter gene transfection showed a distinct advantage of the co-liposomal preparation over the one prepared by the post-injection protocol. Liposome GL5D-RGD was prepared by co-liposomal method showed at least 50% increase in the transfection efficiency compared to the corresponding gemini lipid-DOPE-RGD formulations prepared by the post injection method (Figure S2A).

Optimization of GLD: RAFT-RGD molar ratio

Molar ratios of GL5D: RGD were varied from 1:0 to 50:1 for the pEGFP-C3 transfection in HeLa cells. A molar ratio of 100:1 was found to be the best ratio for achieving high and cell-specific transfection. Beyond this ratio, the transfection efficiency decreased considerably (Figure S2B). This is probably because of

the alteration in the liposomal organization and/or aggregation leading to the formation of significantly larger aggregates of the GL5D-RGD formulations at GL5: RGD molar ratio of 50:1 (Figure S2C)

5 Palmitoyl-RAFT-RGD₄ mediated cell targeting

Optimized GLD and GLD-RGD formulations were used for transfecting three different cell lines, HeLa, HEK293 and NIH3T3 using 0.8 μ g pEGFP-C3 per well using the GL/DNA charge ratio (N/P) 0.5 (Figure 2). In HeLa cells, GL5D induced ~50% GFP cell transfection with a MFI of ~25 only while GL5D-RGD furnished ~75% GFP cells with a MFI of ~75 at the N/P charge ratio of 0.5. In HEK293 cells, GL5D-RGD gave ~40% GFP cells with a MFI of ~20 at the N/P charge ratio of 0.5. Lipofectamine2000® in presence of 10% FBS (Lipo2000*) was used as positive control in all the cell lines. GL5D-RGD was found to be better than Lipo2000* at least in HeLa and NIH3T3 cells.

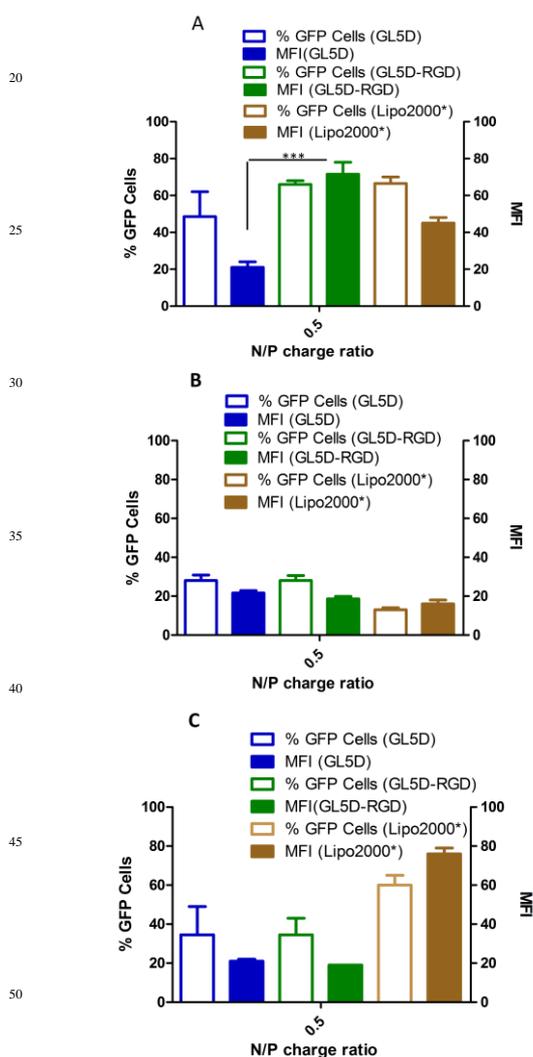


Figure 2. Transfection efficiency of GL5D gemini lipid with and without incorporation of Palmitoyl-RAFT-RGD₄ (100:1) and Lipo2000* in presence of serum (-FBS+FBS) at N/P charge ratio 0.5. Concentration of the plasmid DNA (pEGFP-C3) = 0.8 μ g/well, was used for the experiment, which was performed on

HeLa (Figure 3A), NIH3T3 (Figure 3B) and HEK293 (Figure 3C) cells. Statistical difference in MFI from GL5D-RGD and GL5D are labelled as *** indicating $P < 0.001$.

We further measured the luciferase activity with respect to N/P charge ratios and the relevant data are shown as RLU/mg of protein. In HeLa cells, GL5D-RGD gave around 5-fold higher transfection efficiency compared to that of GL5D at the N/P of 0.5. On the other hand in NIH3T3 cells, the overall transfection was found to be strikingly low and GL5D-RGD showed no improvements in poorer transfection efficiency compared to GL5D. Thus GL5D-RGD was found significantly better transfecting agent in HeLa cells compare to GL5D with high biostatistical significance with $p < 0.001$ (Figure 3).

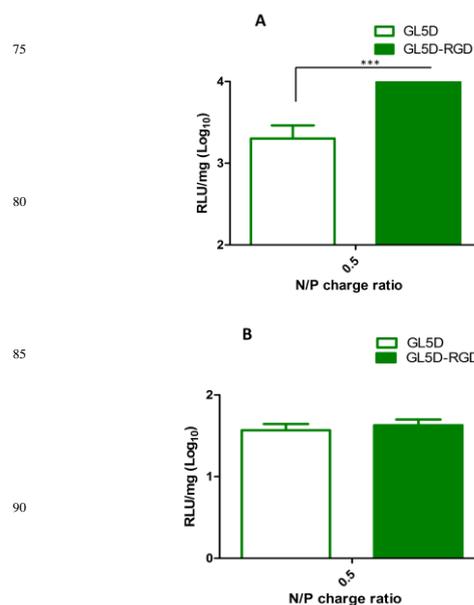


Figure 3. Transfection efficiency of GL5D gemini lipid with and without RGD-RAFT (100:1) in presence of serum (-FBS+FBS) at N/P (lipid/DNA) charge ratio of 0.5. Plasmid DNA (PGL-3) [0.8 μ g/well] was used for the experiment. Data are expressed as relative light unit/mg of protein as obtained from single luciferase assay. Experiment was performed on HeLa (Figure 3A) and NIH3T3 (Figure 3B) cells. Statistical difference in transfection efficiency of GL5D-RGD from the control GL5D are labelled as *** indicating $P < 0.001$.

105 Gel electrophoresis

DNA complexation efficiency of the GLD and GLD-RGD formulations were examined at different N/P charge ratios 0.125, 0.25, 0.75 and 1. The GL5D and GL5D-RGD gave around 100% complexation at N/P charge ratio of 0.5. Inclusion of Palmitoyl-RAFT-RGD₄ (1) did not however affect the DNA binding efficiency of the GLDs (Figure 4). Micellar SDS mediated decomplexation of DNA from the lipoplexes was analyzed using lipoplexes of N/P ratio 3 followed by incubation with SDS at SDS/GL ratio varying from 1 to 4 (Figure 5). At SDS/GL ratio of ~1, GL5D-RGD/DNA ~60% release of DNA was recorded while

GL5D/DNA could release only 40% even at SDS/GL of 5.

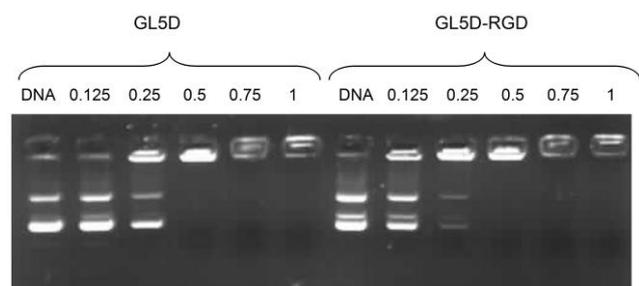


Figure 4. DNA binding efficiency of GLD and GLD-RGD liposomes. Experiment was performed using 0.2 μ g pEGFP-C3 plasmid DNA per well. Numbers above the lanes indicate N/P charge ratio used in the formulation.



Figure 5. SDS mediated release of the DNA from GLD and GLD-RGD based lipoplexes. Experiment was performed using 0.2 μ g plasmid DNA per well. Lipoplexes were prepared at N/P ratio 3. Numbers above the lanes indicates SDS/GL charge ratios while DNA and D/N indicates only DNA and GL/DNA charge ratio respectively.

DNA protection ability of the cationic liposomes was also examined by incubating DNase I (1 unit) for 30 min at 37 $^{\circ}$ C. Whereas 1 unit DNase I could digest/degrade whole DNA in this condition, all lipoplexes at N/P ratio 3 could protect DNA completely from any degradation (Figure 6).

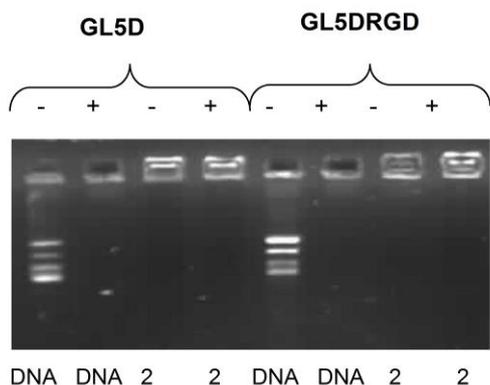


Figure 6. DNA protection ability of various liposomes against DNase I. Experiment was performed using 0.2 μ g plasmid DNA per well. Lipoplexes with and without RAFT-RGD (N/P ratio 2) were treated with 1 unit of DNase I possessing 1x DNase buffer

for 30 min at 37 $^{\circ}$ C. The + and - sign indicate the presence and absence of DNase I in formulations respectively. Numbers below the lanes indicates N/P charge ratio in the formulation while DNA indicates only DNA in lane.

Stability of the lipoplexes in presence of FBS was measured both using a fixed N/P ratio while varying the % of FBS (v/v) (Figure S3A) and by keeping the % of FBS fixed while altering the N/P charge ratio (Figure S3B). It was found that even 50% of FBS could not dissociate the lipoplexes at N/P charge ratios for the liposomes with or without RGD whereas in presence of 70% FBS, an N/P charge ratio of 2 was enough to protect the DNA from being released.

Effect of RGD on the lipoplex delivery efficiency

Overall the transfection efficiency of liposomes was found to be related with the DNA delivery efficiency. The UV absorbance of the undelivered DNA was found to be higher in case of HeLa cells when they were transfected with GL5D compared to GL5D-RGD. In NIH3T3 and HEK293, however, it was found to be the opposite (Figure S4).

Cell surface binding

A flow cytometry experiment was performed on both the HeLa and NIH3T3 cells to find out the selective cell surface binding efficiency of the lipoplexes with the Palmitoyl-RAFT-RGD₄ (Figure 7).

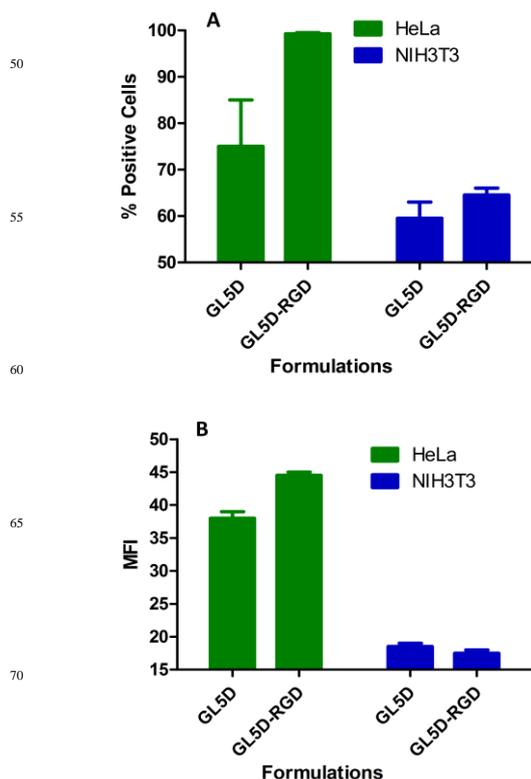


Figure 7. Surface binding efficiency of GLD and GLD-RGD formulations in HeLa and NIH3T3 cells. Bar diagrams shows high surface binding of formulations with RAFT-RGD in HeLa cells compared to NIH3T3 cells.

HeLa cells treated with GL5D-RGD: pyrene, selectively expressed ~95% pyrene positive cells compared to ~70% in case of GL5D: pyrene. In NIH3T3, GL5D-RGD: pyrene as well as GL5D: pyrene both expressed ~65% pyrene positive cells without any selectivity. Presumably the presence of the RAFT-RGD in GL5D-RGD: pyrene assisted selective and highly efficient surface binding to cells containing the integrins receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$ (HeLa cells). In contrast, in NIH3T3 cells (which are devoid of $\alpha v\beta 3$ and $\alpha v\beta 5$) the RAFT-RGD could not influence the surface binding percentage to any significant extent.

Confocal Fluorescence Microscopy

Cells were observed under a confocal microscope to visualize the expression of green fluorescent protein (GFP) in the cell cytoplasm. Green fluorescence was clearly visible in the cell cytoplasm, which was obtained *via* cell transfection using the cationic gemini cholesterol formulations with or without the RAFT-RGD in both HeLa and NIH3T3 cell lines (Figure 8) similar to the cells transfected with the commercially available agents Lipofectamine2000® and Effectene.

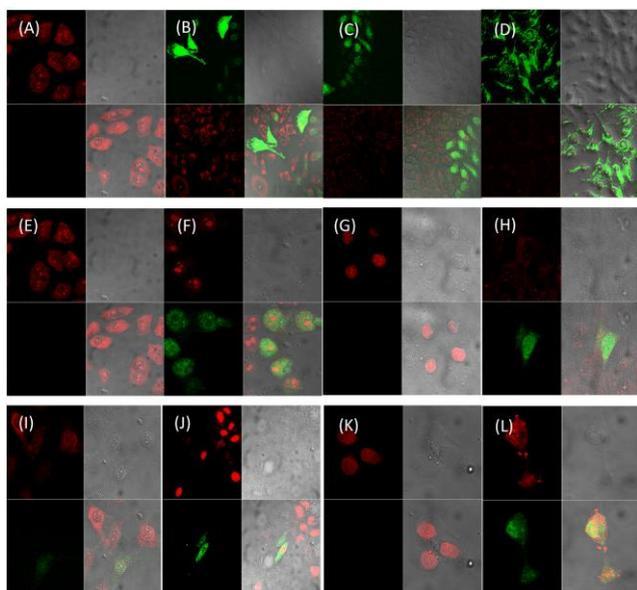


Figure 8. Confocal images showing GFP expression of pEGFP-C3 gene transfected to HeLa (A-F) and NIH3T3 (G-L) cells. Liposomal formulations were used at N/P charge ratio 0.5 using 0.8 μg DNA per well. (A) HeLa; (B) + Effectene; (C) + GL5D; (D) + GL5D-RGD; (E) + only pEGFP-C3; (F) + Lipo2000*; (G) NIH3T3; (H) + Effectene; (I) + GL5D; (J) + GL5D-RGD; (K) + only pEGFP-C3; (L) + Lipo2000*.

Cytotoxicity

The MTT assays of optimized GLDs and GLD-RGDs, with and without DNA were obtained in HeLa, NIH3T3 and HEK293 cell lines (Figure 9). All formulations without DNA were screened for concentrations 0.75 μM to 6 μM while formulations with DNA were screened for N/P charge ratios varying from 0.125 to 1. In the case of the final optimized formulations, the % cell viability was found to be ~80% which dropped to ~60% at higher concentrations and at higher N/P charge ratios, specifically in the

case of other non-optimized formulations across each cell line. Importantly an inclusion of addition of RAFT-RGD in the liposomal formulation did not reduce it at all, in any cells, e.g., HeLa, NIH3T3 and HEK293 examined.

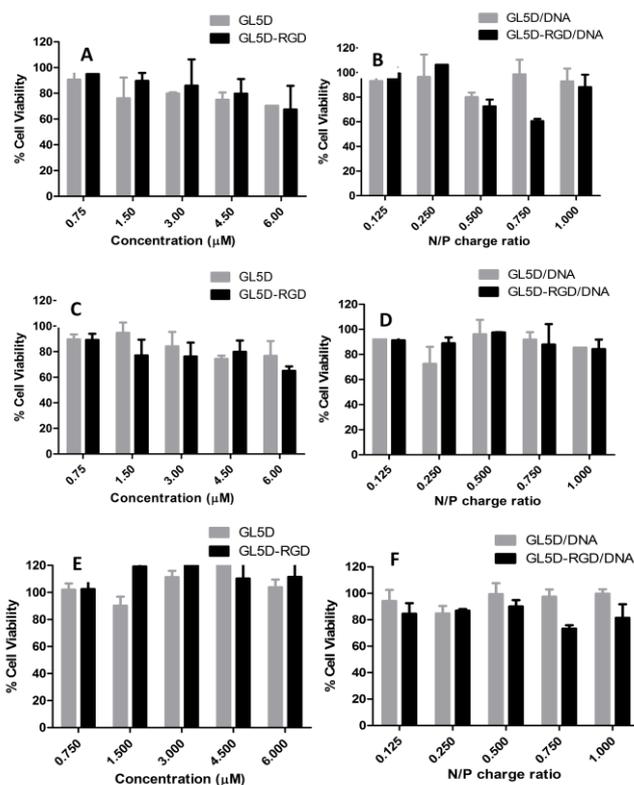


Figure 9. Cytotoxicity assay (MTT) performed on HeLa (A and B), NIH3T3 (C and D) AND HEK293 (E and F) cells. Experiment was performed by using 0.2 μg DNA per well in 96 well plates. Experiment was performed in triplicate.

Discussion

There are many barriers that impede effective transfection using non-viral gene vectors. Stability of vector/DNA complexes in the bloodstream and specific cellular uptake by the target tissues/organs are some of the main obstacles for *in vivo* gene delivery.^{38, 39} Liposomal formulations of the cationic cholesterol derivatives are known to achieve DNA transfection in cells with greater efficiency than a number of commercial transfection reagents. These cationic cholesterol based formulations are more efficient than the cationic lipid suspensions based on pseudoglycerol skeleton in term of gene transfection.^{40,41} Moreover gemini lipids have been shown to be more efficient than their monomeric counterparts in terms of their ability to transfect eukaryotic cells.³⁷ Earlier we have reported serum compatible cholesterol based GLs as efficient transfecting agents.^{42,43} But to establish a precisely targeted gene delivery, there was a need to design and develop newer formulations with efficient cell targeting moiety also capable of efficient gene transfection. Accordingly we have utilized the cationic cholesterol derivatives as cytofectins for our studies. The spacer

lengths between the two monomeric units in such gemini lipids play important role in determining the transfection efficacies of the cationic lipids.^{38, 41}

Recognition of specific integrin receptors on cell surfaces by the particular tripeptidic sequence (RGD), allows the binding of RGD loaded liposomal formulations with cell surfaces having abundance of compatible integrin receptors. One can employ different ways of using RGD templates in gene delivery formulations for achieving integrin receptor specific targeting; firstly *via* a covalent modification of the cationic lipid molecules with RGD based tripeptides³⁴ or secondly *via* a non-covalent incorporation of an optimized amount of RGD based lipid soluble molecules. To avoid the complexity of molecular geometry and in order to achieve convenient variation of ratios of RGD to GLs, we decided to proceed with the non-covalent strategy.

For the present work, cationic gemini cholesterol (GL5) was used with DOPE at defined proportions to form liposome GL5D, which also contained specified amount of palmitoylated multivalent RGD to prepare GL5D-RGD *via* co-liposomal and post-injection methods. During the co-liposome formation, presumably the long fatty acid chain in the palmitoylated multivalent RGD ligand has full freedom to be anchored into the hydrophobic region of the vesicular aggregates. Also in case of the palmitoyl-RAFT-RGD₄, the presence of the sterically demanding cyclic peptide residues might ensure preferential partitioning of these molecules on the outer periphery of the co-liposomes. It is certainly the reason for the increase of the transfection efficiency by using the co-liposomal methodology.

Co-liposomal formulation was used for the optimization of the GLD: RGD molar ratio for highest transfection with best selectivity. It was found that 100:1 molar ratio was the best transfecting formulation. Incorporation of RGD templates in molar ratios more or less than 100:1 led to decreases in the transfection efficiency (Figure 2B). Probably, at lower ratios (2000:1 or 1000:1), the number of RGD tags onto the co-liposomes was insufficient to achieve maximum while at a higher ratio (50:1) too many RGD tags might have altered the surface properties of the co-liposomes (GLD) in such way that it reduced the transfection efficiency by probably due to the instability and/or aggregation of liposomes with significantly larger hydrodynamic diameter for the GL5D-RGD formulation at GL5: RGD molar ratio 50:1 (Figure S2C)

The overall optimized GL5D-RGD liposomes were then used for transfection of HeLa, HEK293 and NIH3T3 cells using plasmid pEGFP-C3 and PGL-3 vectors. In all the experiments, GL5D, devoid of RGD, were used as negative controls. Transfection of pEGFP-C3 revealed that in HeLa cells GL5D-RGD formulations were significantly better transfectants (Figure 2A) compared to the GL5D ($P < 0.001$) whereas in NIH3T3 and HEK293 cells, inclusion of RGD did not lead to an enhancement in the transfection efficiency (Figure 2B-C). Similar results were obtained in transfection of PGL-3 plasmids in HeLa and NIH3T3 cells as well. In this instance RGD inclusion in GLDs increased the transfection efficiency by ~5 fold in HeLa cells with $P < 0.001$ in bio-statistical analysis (Figure 3A). In contrast in NIH3T3 it did not change to any considerable extent (Figure 3B).

An introduction of RAFT-RGD in the GL5D formulation at

N/P charge ratio of 0.5 causes significant improvement in HeLa cell selectivity as revealed from the FACS analysis. Similarly the luciferase assay shown in Figure 3 indicates that an introduction of RAFT-RGD in GL5D improves the transfection efficiency at N/P charge ratios of 0.5 in HeLa cells only. It may be further noted here that luciferase assay was performed using PGL-3 plasmid while plasmid pEGFP-C3 was used for the FACS analysis.

It is possible that either an improved DNA binding, a better DNA release, an improved serum tolerance or better DNase I sensitivity of the formulations in presence of RGD might have resulted in a high transfection efficiency with specific targeting. But gel electrophoresis experiments performed on formulations with and without RAFT-RGD indicated that the presence of RGD did not enhance the binding of DNA (Figure 4). This also did not enhance the SDS mediated release of DNA (Figure 5) or even afford better DNA protection against DNase I (Figure 6). Even the stability of lipoplexes in presence of serum was not affected by the presence of RGD (Figure S3). These results clearly suggest that there might be another factor, which governs the enhancement of transfection efficiency by the co-liposomes loaded with the RGD. The HeLa cells possess high abundance of $\alpha\beta3$ and $\alpha\beta5$ transmembrane integrin receptors while the HEK293 and NIH3T3 cells are devoid of them. As expected, Palmitoyl-RAFT-RGD₄ present in the co-liposomal formulations improves the transfection efficiency in HeLa cells.

In support of the RGD-mediated targeting of the $\alpha\beta3$ and $\alpha\beta5$ integrins, undelivered lipoplex measurement assay was performed. This indicates that in HeLa cells, GLD-RGD could deliver more lipoplexes in the cytoplasm compared to that of the GLD alone (Figure S4A). In contrast, in NIH3T3 and HEK293 cell lines, the observed pattern was just the opposite. Thus with GLD-RGD, higher amount of lipoplexes were left undelivered. Similarly, GLD-RGD: pyrene was able to tag higher % of cells compared to the GLD: pyrene while NIH3T3 showed no enhancement in surface binding (Figure 7), indicating a selective targeting induced by the RGD peptide (Figure S5).

Confocal fluorescence microscopy was performed to find out the GFP expression of the pEGFP-C3 plasmid in HeLa and NIH3T3 cells (Figure 8). It was found that each formulation, with or without RGD along with commercially available Effectene and Lipofectamine2000® (Lipo2000*) (two positive controls), were able to transfect the cells. Confocal fluorescence microscopy data shown here indicate a qualitative expression of GFP. This cannot be used here for any quantitative comparison of different formulations including positive controls, Effectene and Lipofectamine2000®. The results obtained from FACS and luciferase assays are however, quantitative for this purpose. Finally, the cytotoxicity of each formulation was measured using the MTT assay protocol. It confirmed the considerably low toxicity of the formulations with the palmitoylated-RAFT-RGD₄ (Figure 9). Thus, the co-liposomal methodology of RGD tagging on cationic liposomes has been demonstrated to be an efficient and convenient way of cell targeting in the gene delivery experiments.

Conclusions

A new gene delivery formulation has been developed which is

composed of a cationic gemini cholesterol (GL5), helper lipid (DOPE) and a palmitoyl-RAFT-RGD₄ template to imbibe the selectivity in liposomes towards cells therefore endowed with abundance of $\alpha\text{v}\beta 3$ and/or $\alpha\text{v}\beta 5$ integrins. Stable formulations were developed by a non-covalent co-liposomal mixing of GL, DOPE and palmitoylated RGD-RAFT in different optimized molar ratios. Expression of pEGFP-C3 and PGL-3 in $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ positive cell (HeLa) was found to be considerably high for the GL5D-RGD formulations compared to other $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ negative cells (HEK293 and NIH3T3). Similar gel electrophoretic patterns of liposomes with (GL5D-RGD) or without (GL5D) RGD confirmed that the RGD played no role in enhancing the DNA binding, anionic micelle induced DNA release, serum stability or even DNA protection against DNase I to improve the transfection. Measurement of the extent of undelivered lipoplexes and % GLD-RGD: pyrene labeled positive cells support the role of RGD in $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ mediated cell selectivity by the present gemini lipid based transfectants. The formulations were good transfecting agents in comparison to commercially available Effectene or Lipofectamine2000® (Lipo2000*). As inclusion of RGD did not increase the cytotoxicity of the liposomes and corresponding lipoplexes, it may be highly beneficial to use this formulation for *in vivo* gene delivery processes. We are currently developing this strategy and *in vivo* evaluation will be reported in due course.

Experimental

Materials and Methods

Gemini lipid (GL), GL5 has been synthesized (Supporting Information) as described elsewhere [37]. Palmitoyl-RAFT-RGD₄ was prepared through a combination of solid and solution-phase syntheses according to the methods previously described (Supporting Information) [36]. All reagents, solvents, and chemicals used in this study were of the highest purity available. The solvents were dried prior to use. Column chromatography was performed using 60-120 mesh silica gel.

NMR spectra were recorded using a Jeol JNM-300 (300 MHz for ¹H and 75 Hz for ¹³C) spectrometer. The chemical shifts (δ) are reported in ppm downfield from the internal standard, TMS, for ¹H-NMR and ¹³C-NMR. Mass spectra were recorded on a Kratos PCKompact SEQ V1.2.2 MALDI-TOF spectrometer, a MicroMass ESI-TOF spectrometer or on ESI-MS (HP1100LCMSD) spectrometers. Infra-red (IR) spectra were recorded on a Jasco FT-IR 410 spectrometer using KBr pellets or as neat.

Trans-sonic T 460H Elma bath sonicator (40W) was used for sonic dispersal of lipid suspension. Blood serum used in cell culture experiments was obtained from GIBCO. Cell culture flasks and multi-well plates were obtained from NUNC. Dulbecco's modified phosphate buffer saline (DPBS) and Dulbecco's modified eagles medium (DMEM) was obtained from SIGMA. Public domain software, WinMDI was used for the FACS data analysis.

Preparation of lipopeptide

Palmitoylated Multivalent RGD-Peptide **1** was prepared as described in the literature (see the supporting information).

Preparation of liposomes

Liposomes were prepared using a literature protocol.³⁷ In brief, each of the gemini lipid was mixed with DOPE in an appropriate molar ratio as solution in chloroform. The organic solvent was evaporated under rotary evaporator and finally dried under vacuum. The vacuum dried films of lipid mixtures were subjected to hydration with addition of appropriate amount of buffer, repeated freeze-thaw cycles followed by sonication at 70 °C for 15 min. Each liposomal suspension was found to be transparent and very stable while stored at 4 °C in sterile conditions. Formulations were re-sonicated at room temperature for 5 min before use for each experiment.

Plasmid DNA

Plasmids pEGFP-C3 and PGL-3, which encode for an enhanced green fluorescence protein (GFP) under a CMV promoter and firefly luciferase protein under a SV40 promoter respectively, were amplified in *Escherichia coli* (DH5 α) and purified using Qiagen Midi Prep plasmid purification protocol (Qiagen, Germany). The purity of the plasmid was checked by electrophoresis on 1.0% agarose gel. The concentration of DNA was estimated spectroscopically by measuring the absorption at 260 nm and confirmed by gel electrophoresis. Plasmid preparations showing a value of OD₂₆₀/OD₂₈₀ >1.8 were used.

Cell culture

Cells (HeLa, HEK293 and NIH3T3) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) in T25 culture flasks (Nunc, Denmark) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in PBS (pH 7.4).

Transfection procedure

Transfection experiments were performed using literature protocol.³⁶ In brief, 24 h before transfection, 24-well plates were seeded with 60, 000 cells/well in antibiotic-free media. Transfection experiments were performed in presence of serum (-FBS+FBS) where lipoplex preparation was carried out in absence of serum but the lipoplexes were incubated with the cells in presence of serum. At the time of transfection, cells were at least 70% confluent. Liposomal formulations and plasmid DNA were diluted in DMEM. Lipoplexes were prepared in DMEM by mixing liposome and DNA in required N/P charge ratio and the total volume was made up to 200 μ l. The N/P values represent the ratio of charges on the cationic gemini cholesterol (in mol) to nucleotide base molarity and were calculated by considering the average nucleotide mass of 330. Lipid-DNA complexes were incubated for 30 min at room temperature followed by dilution with 200 μ l of 20% FBS containing DMEM (final DNA concentration 12.12 μ M). Formulations were added to the wells and incubated for 6 h in optimal condition. After 6h of incubation, old medium was replaced with new medium having 10% FBS. Cells were further incubated for a period of 42h before checking the reporter gene expression. The extent of reporter gene expression was examined by fluorescence microscopy and/or luciferase assay technique and expression was quantified

either by flow cytometry analysis or by luminometry. All the experiments were performed in duplicate, and the results presented are the average of at least duplicates of two such independent experiments performed on different days. Lipofectamine2000 was used as positive control using manufacturers protocol in 10% serum, abbreviated as Lipo2000* throughout the manuscript.

Flow cytometry

Transfection efficiency of different liposomal formulations for the reporter gene pEGFP-C3 was examined by performing flow cytometry assay (FACS).⁴⁴ Transfected cells were regularly observed under fluorescent microscope for the intensity of fluorescence due to green fluorescence protein (GFP) expressed by the cells. Finally, 42h post transfection, cells were trypsinized by adding 100 μ l of 0.1% trypsin and cells were collected in 5% FBS containing DPBS. Duplicate cultures were pooled and analyzed by flow cytometry immediately using a Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm. FACS data were analyzed using public domain WinMDI2.8 software.

Luciferase assay

Transfection efficiency of various formulations for the reporter gene PGL-3 expression was quantified further by luciferase assay. Transfected cells were assayed using single-luciferase assay kit, provided by Promega (U.S.A.). In a typical assay, after 48 h of transfection, the old medium was removed from the wells and the cells were washed twice with 200 μ l of PBS and 100 μ l of 1x cell lysis buffer was added to each well. Cells were lysed for 30 min in a horizontal rocker at RT. The cell lysate was transferred to centrifuge tubes and centrifuged (4000 rpm, RT) for 2 min. Supernatant was stored at -70°C until used for assay. For the assay, 10 μ l of supernatant was mixed with 10 μ l of luciferase assay substrate (Promega). The lysate and the substrate were both thawed to RT before performing the assay. The luciferase activity was measured in a luminometer (Turner designs, 20/20, Promega, U.S.A.) in standard single-luminescence mode. The measurement was performed for 10 s. A delay of 2 s was given before each measurement. The protein concentration was estimated in each case using Bradford's method with bovine serum albumin as a standard.⁴⁵ Comparison of the transfection efficiencies of the individual lipids was presented using data for luciferase assay expressed in relative light units (RLU)/mg of protein or luciferase activity/mg of protein.

Statistical analysis

Statistical significance of differences between control and samples were evaluated using two-way ANOVA using GraphPad Prism 5.0 with Bonferroni analysis wherever applicable. Results were considered statistically significant when the p value was less than 0.05 (* represents $p < 0.05$; ** represent $p < 0.005$ and *** represent $p < 0.001$).

Optimization of Lipid: DOPE ratio

DOPE, a natural lipid, is known to enhance gene transfection efficacy of different cationic liposomal formulations,⁴⁶ has been used to prepare high output transfecting agents. Gemini lipid:

DOPE ratio was varied from 1:0 to 1:5 of molar ratio to optimize the efficiency of transfection. Efficiency of each liposomal formulation was checked by performing pEGFP-C3, reporter gene transfection on HeLa cells at N/P charge ratio of 0.5 in 10% FBS condition.

Optimization of Palmitoyl-RAFT-RGD₄ inclusion methodology

Two different methodologies were used for non-covalent incorporation of the palmitoyl-RAFT-RGD₄ in GLD liposomes. In the first method, optimized amount of GLD was mixed with the RGD-containing scaffold in molar ratios of 2000:1 and 1000:1. Liposomes were then prepared from each of these mixtures as mentioned earlier. These formulations were termed as co-liposomal formulations Co-2000 and Co-1000 respectively. A second method was used *via* injection of an ethanolic solution of RGD-containing scaffold in pre-formed GLD suspensions in buffered media. Formulations were termed as post-injection formulation Po-2000 and Po-1000 respectively. Efficiency of formulations was checked by performing pEGFP-C3, reporter gene transfection on HeLa cells at N/P charge ratio of 0.5 in 10% FBS condition.

Optimization of GLD: Palmitoyl-RAFT-RGD₄ molar ratio

Palmitoyl-RAFT-RGD₄ was mixed with the GL5D formulation in ratios of GLD: RGD-peptide 1:0, 2000:1, 1000:1, 100:1 and 50:1 respectively. GL5D was optimized as 100:1 molar ratio as best with respect to the transfection efficiency. This optimized ratio was used for further optimization of the N/P charge ratio.

Gel electrophoresis

Complexation and de-complexation ability of the GLD and GLD-RGD at different GL/DNA ratios,⁴⁷ DNase I stability of lipoplexes derived from GLD/DNA and GLD-RGD/DNA⁴⁸ and the stability of lipoplexes in presence of FBS⁴⁹ were all assayed by gel electrophoresis. Lipoplexes were prepared at different GL/DNA charge ratios. After 30 min of incubation, lipoplexes were run on 1% agarose gel to perform gel retardation assay. Lipoplexes were incubated for 15 min in presence of micellar SDS at different SDS: GL molar ratio and run on the 1% agarose gel to perform de-complexation assay whereas DNase I (1 unit) was incubated along with lipoplexes for 30 min at 37 °C and run on the 1% agarose gel to find out the DNA protection efficiency of lipoplexes against DNase I. Stability of lipoplexes in high serum percentage was performed by incubating the lipoplexes with high FBS percentage and run on the 1% agarose gel. We also performed gel electrophoresis for the lipoplexes that remained un-internalized to the cell after 6h of incubation. Observed band intensity proportional to the amount of lipoplexes could not enter inside the cells even after 6h. High intensity band in the electrophoretic gel indicates low uptake of lipoplexes by cells.

Effect of Palmitoyl-RAFT-RGD₄ on lipoplex delivery efficiency

To measure the effect of palmitoyl-RAFT-RGD₄ inclusion on the DNA delivery efficiency of liposomes in each of HeLa, HEK293 and NIH3T3 cells, we performed UV absorption spectral measurements for the lipoplexes⁵⁰ which could not enter inside

the cells even after 6h of incubation. Experiment performed was similar to general transfection experiment using pEGFP-C3 plasmid complexed with GLD and GLD-RGD. After 6h of incubation, culture medium possessing undelivered lipoplexes was removed. Medium was centrifuged at 13000 rpm for 20 min at 4 °C. Centrifuged lipoplexes were further suspended in water and equally divided into two parts. One part was run on 1% agarose gel and the second part was separated using phenol: chloroform. The absorbance due to DNA was measured using a BioRad UV-spectrometer. Absorbance values (A_{260}) were plotted against individual formulations which indicate the amount of lipoplex left outside the cells. Results were confirmed by gel electrophoresis.

Cell surface binding analysis

Experiment was performed to find out the specificity of palmitoyl-RAFT-RGD₄ loaded liposomes toward the HeLa cell line that over-expresses $\alpha\beta 3$ and $\alpha\beta 5$ integrin receptors. A fluorescent probe, pyrene was included in GLD (GLD: Pyrene molar ratio 100:1) with and without the palmitoyl-RAFT-RGD₄. After 24 h, cells were incubated with formulations possessing either GLD: Pyrene or GLD-RGD: Pyrene for 2h. Cells were then washed with DPBS and collected for FACS analysis. FACS data were analyzed using WinMDI2.8 software.

Confocal fluorescence microscopy

Transfection of pEGFP-C3 reporter plasmid was visualized under a confocal fluorescent microscope and the experiment was performed as described.⁵¹ Cells were cultured in T25 culture flasks, trypsinized and plated in 12-well plates, having autoclaved glass slips in the wells. Then, cells were plated on glass slips in antibiotic free 10% FBS containing DMEM as medium. Cells were grown for 24 h at 99% humidity, 37 °C temperature and 5% CO₂ condition till cell-monolayer gained ~70% confluency. Further, transfection was performed as mentioned above. Plates were then incubated for a period of 42 h before checking under confocal microscopy. Control transfections were performed in each case by using commercially available transfection agent 'Lipofectamine2000' and 'Effectene' using manufacturers protocol in presence (-FBS+FBS) of serum. After 42 h of incubation, all the media were removed from the wells and the cells were washed with DPBS buffer carefully to remove all the cell debris without disturbing the monolayer of cells. Cells were fixed for 10 min with 1 mL of 4% paraformaldehyde in each well. Cells were washed with DPBS for 3x10 min and kept with 1 mL 0.1% Triton-x-100 for 5 min to increase the membrane permeabilization. Cells were washed with DPBS for 3x10 min. Glass slips having fixed and permeabilizable monolayer of cells were taken out from the wells and kept on the glass slides and incubated for 5 min with 1 µg/ml of PI (propidium iodide) to specifically stain the nucleus of the cells. Finally cells were washed with DPBS for 3x10 min to remove extra PI to reduce over-staining. Vector shield was used to mount the glass slips upside down on the glass slides which were observed under a confocal microscope (Zeiss) at objective of Plan Apochromat 63x/1.4 Oil DIC and analyzed using a Zeiss LSM image browser.

Cytotoxicity

Cytotoxicity of GL5D and GL5D-RGD formulations were

determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay in HeLa, HEK293 and NIH3T3 cells.⁵² In brief, 15,000 cells/well were plated in 96 well plates. After 24 h, GL5D, GL5D-RGD, GL5D/DNA and GL5D-RGD/DNA formulations were added to the cells in absence of serum. Liposomal formulations GL5D, GL5D-RGD were complexed with 0.2 µg of the DNA at various N/P ratios for 30 min to give GL5D/DNA and GL5D-RGD/DNA respectively. After 6 h of incubation, lipoplexes were removed and 200 µL of media with 10% FBS was added. After 42 h, 20 µL of MTT solution was added and the cells were incubated further for 4 h. Blue formazan crystals were seen in the wells under a microscope. The entire media were removed and 200 µL of DMSO was added per well. The absorbance was measured using microtiter plate reader. The % viability was then calculated as = $\frac{[A_{590} (\text{treated cells}) - \text{background}]}{[A_{590} (\text{untreated cells}) - \text{background}]} \times 100$.

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Notes and references

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- M.A. Barry, W.J. Dower and S.A. Johnston, *Nat. Med.*, 1996, **2**, 299.
- D. Hallahan, L. Geng, S. Qu, C. Scarfone, T. Giorgio, E. Donnelly, X. Gao and J. Clanton, *Cancer Cell*, 2003, **3**, 63.
- A. Maria, E.W. Croyle, J. Sonia, J.R. Blake and L.A. Gordon, *Human Gene Ther.*, 1998, **9**, 561.
- D. Pramanik, B.K. Majeti, G. Mondal, P.P. Karmali, R. Sistla, O.G. Ramprasad, G. Srinivas, G. Pande and A. Chaudhuri, *J. Med. Chem.*, 2008, **51**, 7298.
- A. Mistry, R. Harbottle, S. Hart and K.M. Hodivala-Dilke, *Curr. Opin. Mol. Ther.*, 2003, **5**, 603.
- P. K. Dubey, D. Singodia, R. K. Verma and S. P. Vyas, *J. Pharm. Pharmacol.*, 2011, **63**, 33-40.
- M.A. Chellaiyah and K.A. Hruska, *Calc. Tissue Inter.*, 2003, **72**, 197.
- D.C. Paik, C. Fu, J. Bhattacharya and M.D. Tilson, *Exp. Mol. Med.*, 2004, **36**, 524.
- K. Chitkara, K. Hogrefe, M. Vasa-Nicotera, N. Swanson and A.H. Gershlick, *J. Invas. Cardiol.*, 2006, **18**, 417.

- 10 O. H. P. Ramos, A. Kauskot, M. R. Cominetti, I. Bechyne, C. L. S. Pontes, F. Chareyre, J. Manent, R. Vassy, M. Giovannini, C. Legrand, H. S. Selistre-de-Araujo, M. Crepin and A. Bonnefoy, *Clin. Exper. Met.*, 2008, **25**, 53.
- 11 P. C. Brooks, R. A. F. Clark, D. A. Cheres, *Science*, 1994, **264**, 569.
- 12 T. J. Wickham, P. Mathias, D. A. Cheres and G. R. Nemerow, *Cell*, 1993, **73**, 309.
- 13 L. Erguang, L. B. Swati, G. S. Dwayne, S. P. Xose, A. C. David and R. N. Glen, *J. Virol.*, 2001, **75**, 5405.
- 14 X. Zhengming, C. Zhen, Z. Xianzhong, P. Manish, C. W. Joseph, S. G. Sanjiv and C. Xiaoyuan, *J. Nucl. Med.*, 2006, **47**, 130.
- 15 S. Klein, A. Bikfalvi, T. M. Birkenmeieri, F. G. Giancotti and D. B. Rifkin, *J. Biol. Chem.*, 1996, **271**, 22583.
- 16 F. M. Fusi and R. A. Bronson, *J. Androl.*, 1992, **13**, 28.
- 17 R. Haubner, R. Gratiias, B. Diefenbach, S. L. Goodman, A. Jonczyk and H. Kessler, *J. Am. Chem. Soc.*, 1996, **118**, 7461.
- 18 J-X. Chen, H-Y. Wang, C-Y. Quan, X-D. Xu, X-Z. Zhang and R-X. Zhuo *Org. Biomol. Chem.*, 2010, **8**, 3142.
- 19 E. Garanger, D. Boturny, J. L. Coll, M. C. Favrot and P. Dumy, *Org. Biomol. Chem.*, 2006, **4**, 1958.
- 20 Y-b. Lim, O-J. Kwon, E. Lee, P-H. Kim, C-O. Yun and M. Lee, *Org. Biomol. Chem.*, 2008, **6**, 1944.
- 21 I. Martin, C. Dohmen, C. Mas-Moruno, C. Troiber, P. Kos, D. Schaffert, U. Lächelt, M. Teixidó, M. Günther, H. Kessler and E. Giralt, *Org. Biomol. Chem.*, 2012, **10**, 3258.
- 22 R. Xu, M. Fisher and R.L. Juliano, *Bioconjugate Chem.*, 2011, **22**, 870.
- 23 M. N. Bongiovanni, D. B. Scanlon and S. L. Gras, *Biomaterials*, 2011, **32**, 6099-6110.
- 24 M. Mammen, S.K. Choi and G.M. Whitesides, *Angew. Chem. Int. Ed.*, 1998, **37**, 2755.
- 25 H. D. Maynard, S. Y. Okada and R. H. Grubbs, *J. Am. Chem. Soc.*, 2001, **123**, 1275.
- 26 R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Asgeirsdottir, M. Everts, D. K. F. Meijer and G. Molema, *Bioconjugate Chem.*, 2002, **13**, 128.
- 27 G. Thumshim, U. Hersel, S. L. Goodman and H. Kessler, *Chem. Eur. J.*, 2003, **9**, 2717.
- 28 D. Boturny, J. L. Coll, E. Garanger, M. C. Favrot and P. Dumy, *J. Am. Chem. Soc.*, 2004, **126**, 5730.
- 29 J. Razkin, V. Jossierand, D. Boturny, Z. Jin, P. Dumy, M. Favrot, J. L. Coll and I. Texier, *ChemMedChem*, 2006, **1**, 1069.
- 30 J. Dimastromatteo, L. M. Riou, M. Ahmadi, G. Pons, E. Pellegrini, A. Broisat, L. Sancey, T. Gavrilina, D. Boturny, P. Dumy, D. Fagret, C. Ghezzi, *J. Nucl. Cardiol.*, 2010, **17**, 435.
- 31 Z.-H. Jin, T. Furukawa, M. Galibert, D. Boturny, J. L. Coll, T. Fukumura, T. Saga, P. Dumy and Y. Fujibayashi, *Nucl. Med. Biol.*, 2011, **38**, 529.
- 32 S. Foillard, Z. Jin, E. Garanger, D. Boturny, M. Favrot, J-L. Coll and P. Dumy, *Chem. Bio. Chem.*, 2008, **9**, 2326.
- 33 S. Foillard, L. Sancey, J-L. Coll, D. Boturny and P. Dumy, *Org. Biomol. Chem.*, 2009, **7**, 221.
- 34 A. J. Schraa, R. J. Kok, A. D. Berendsen, H. E. Moorlag, E. J. Bos, D. K. F. Meijer, L. F. M. H. de Leij and G. Molema, *J. Control. Rel.*, 2002, **83**, 241.
- 35 T. Nishiya and S. Sloan, *Biochem. Biophys. Res. Commun.*, 1996, **224**, 242.
- 36 L. Sandrin, L. Coche-Guerente, H. Basit, A. Bernstein, P. Labbé, P. Dumy and D. Boturny, *Org. Biomol. Chem.*, 2010, **8**, 1531.
- 37 A. Bajaj, P. Kondiah and S. Bhattacharya, *J. Med. Chem.*, 2007, **50**, 2432.
- 38 D. Niculescu-Duvaz, J. Heyes and C. J. Springer, *Curr. Med. Chem.*, 2003, **10**, 1233.
- 39 M. A. Ilies, W. A. Seitz, B. H. Johnson, E. L. Ezell, A. L. Miller, E. B. Thompson and A. T. Balaban, *J. Med. Chem.*, 2006, **49**, 3872.
- 40 Y. K. Ghosh, S. S. Visweswariah and S. Bhattacharya, *FEBS Lett.*, 2000, **473**, 341.
- 41 J. Heyes, D. Niculescu-Duvaz and R. G. Cooper, *J. Med. Chem.*, 2002, **45**, 99.
- 42 P. V. Dileep, A. Antony and S. Bhattacharya, *FEBS Lett.*, 2001, **509**, 327.
- 43 A. Bajaj, P. Kondaiah and S. Bhattacharya, *Bioconjugate Chem.*, 2007, **18**, 1537.
- 44 A. Sandrine, M. Grietje, L. Lou de and H. Dick, *J. Gene Med.*, 2000, **2**, 465.
- 45 A. Noguchi, N. Hirashima and M. Nakanishi, *Pharm. Res.*, 2002, **19**, 933.
- 46 S. Bhattacharya and P. V. Dileep, *Bioconjugate Chem.*, 2004, **15**, 508.
- 47 S. Bhattacharya and S. S. Mandal, *Biochemistry*, 1998, **37**, 7764.
- 48 M. A. Arangoa, N. Duezguenes and C. Tros de Ilarduya, *Gene Ther.*, 2003, **10**, 5.
- 49 S. Fumoto, S. Kawakami, K. Shigeta, Y. Higuchi, F. Yamashita and M. Hashida, *J. Pharm. Exp. Therap.*, 2005, **315**, 484.
- 50 S. F. Ausar, S. B. Joshi and C. R. Middaugh, *Meth. Molecul. Biol.*, 2008, **434**, 55.
- 51 R. Mukthavaram, S. Joyeeta, S. Marepally, M. Koushik, S. Bojja and A. Chaudhuri, *J. Am. Chem. Soc.*, 2007, **129**, 11408.
- 52 D. Fischer, T. Bieber, Y. Li, H. P. Elsasser and T. Kissel, *Pharm. Res.*, 1999, **16**, 1273.