Chapter 9
Development and Evaluation of Targeted Lipoplexes

Don't do anything great if you can't handle the congratulations…

-From "Replacements"

9.1 Development and evaluation of targeted lipoplexes

Targeting to the Bone marrow cells can be achieved through several approaches described earlier (Chapter 2, literature review). Bone matrix proteins such as osteocalcin, sialoprotein and osteopontin which bear specialized characteristic that let them bind effectively to the bone mineral surfaces thorugh their storng binding affinity for the calcium [1-3]. This characteristic is imparted by the negatively charged amino acid residues i.e. aspartic acid and glutamic acid in their structures [4]. To enlist a few, bisphosphonate and acidic oligopeptide based targeting strategies are of particular importance as they ensure the binding to the bone. However, among these two strategies, alendronate (a bisphosphonate) have been shown to be indifferent towards bone-formation or bone-resorption surfaces; while acidic oligopeptide based targeting has been demonstrated to be of specific in terms of its binding affinity towards the bone-forming surfaces and bone resorption surfaces [5] [6]. Structural differences in these oligopeptides are responsible for this difference in the activities. Simple hexapeptide of aspartic acid $(Asp)_6$ and $(Asp)_8$ has been demonstrated to be targeting the bone resorption surfaces more preferentially as compared to the bone formation surfaces, while $(Asp\text{-}Ser\text{-}Ser)_6$ oligopeptide has more preferential binding to the low crystallinity hydroxyappetites at the bone formation surfaces [5]. Two characteristics has been speculated in highly specific interaction of this peptide with bone formation surfaces. One is highly specific interaction between the serine and distinct regions of hydroxyapeptite and secondly, the change in spatial arrangement of acidic aspartate by placing of serine residues in between [5, 7].

The advantage of targeting the bone formation surfaces is that the drug delivery system will be delivered to the osteoblast lineage cells rather than osteoclast lineage cells. Hence, the activity of the therapeutic BMP-9 gene will be concentrated in the preferred region where the activity of BMP-9 will be on the osteoblast precursor cells resulting in more pronounced differentiation of these cells to osteoblasts. Additionally, the liposomes will be saved from the harsh acidic environment at the bone resorption surfaces.

For the development of the bone targeted gene delivery system, based on the above review, acidic oligopeptide $(Asp\text{-}Ser\text{-}Ser)_6$ has been chosen for targeting. Based on the cytotoxicity studies and the cellular uptake studies of different lipoplexes developed, HSA, CSA and CDS lipoplexes were chosen for targeting.

Different liposomes were prepared by replacing the 1 mole% DSPE-mPEG$_{2000}$ with DSPE-PEG$_{2000}$-maleimide (purchased from NanoCS, USA). Lipoplexes were prepared by the incubating pDNA with liposomes at N/P ratio of 2.0. Prepared lipoplexes were incubated with the targeting peptide at DSPE-PEG$_{2000}$-maleimide to peptide molar ratio of 1:2 at room temperature for 2 hr. Complexation efficiency of the lipoplexes were determined by gel electrophoresis to see if the conjugation and/or incubation with the peptide leads to detrimental effect on the lipoplex stability.

It was observed that the complexation efficiency of lipoplexes was not affected by the conjugation with the peptide or the presence of the peptide (**Figure 9.1**and **Table 9.1**). This is due to the presence of the PEG chains which reduce the effect of peptide on the lipoplex. Once complexed to the DSPE-PEG$_{2000}$-maleimide, the peptide will remain on the surface of the lipoplexes.

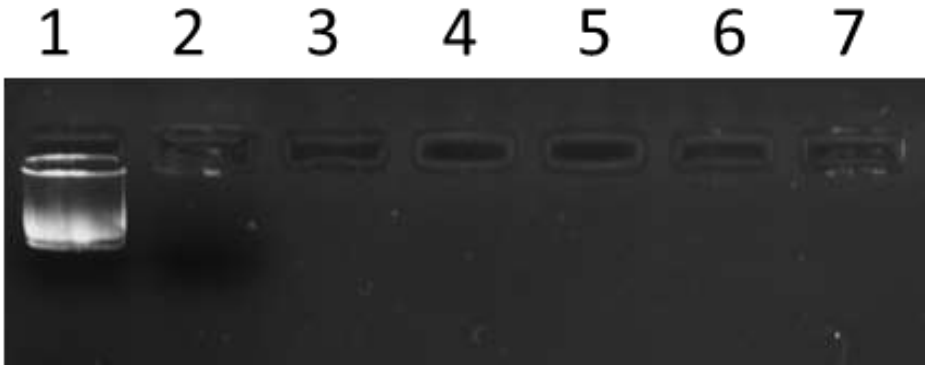

Figure 9.1 Complexation efficiency of lipoplexes before and after peptide conjugation (200 ng pDNA/well, N/P ratio of 2: lane 2-HSA lipoplexes before peptide conjugation, lane 3-HSA lipoplexes after peptide conjugation, lane 4-CSA lipoplexes before peptide conjugation, lane 5-CSA lipoplexes after peptide conjugation, lane 6-HDS lipoplexes before peptide conjugation, lane 7-HDS lipoplexes after peptide conjugation)

Table 9.1 Physicochemical characteristics of the lipoplexes

Physicochemical property	**HSA lipoplexes**		**CSA lipoplexes**		**HDS lipoplexes**	
Before/after conjugation	Before	After	Before	After	Before	After
%Complexation efficiency (Gel electrophoresis)	98.64±1.58	99.55±2.15	99.08±1.81	100.65±2.15	99.14±2.51	98.36±1.74
%Complexation efficiency (UV spectrophotometry)	98.52±2.21	102.15±1.59	100.51±0.93	99.48±2.53	98.93±1.11	100.32±2.56
%Complexation efficiency (QuantiFluor® Assay)	98.75±2.10	101.45±2.15	99.45±1.36	100.36±2.96	99.16±0.97	100.26±2.15
Particle size (nm)	94.6±6.5	92.4±5.9	92.4±9.1	93.1±4.4	128.5±7.8	126.2±4.5
Zeta potential (mV)	29.5±3.1	11.2±0.9	28.9±3.5	13.3±1.4	37.7±5.4	14.59±1.5

The physicochemical properties of the lipoplexes were evaluated for the peptide conjugated lipoplexes. It was observed that the particle size of the lipoplexes did not change after conjugation; however, zeta potential of the lipoplexes was changed significantly for the lipoplexes. This is due to the presence of negatively charged peptide at the surface of the lipoplexes which now governs the potential of the stern layer (bound layer) around the particle. However, due to higher concentration of cationic charge, the lipoplexes still were showing positive zeta potential (~ 11-15 mV). Though zeta potential of the lipoplexes was changed significantly, the complexation efficiency of the lipoplexes was not affected by the complexation. This is due to very low concentration of the peptide used for reaction. Additionally, conjugation of the peptide would not hinder the already complexed pDNA's interaction with the cationic charges; rather this might help prevent loss of the pDNA due to stresses by trapping the pDNA between the attractive forces from the cationic lipids and repelling forces of the negatively charged peptide.

Cytotoxicity studies of the targeted lipoplexes were carried out to compare their cytotoxicity against the non-targeted lipoplexes prepared without targeting peptide. It was observed that there was no significant difference in the cytotoxicity of the lipoplexes before and after conjugation to the targeting peptide (**Figure 9.2**).

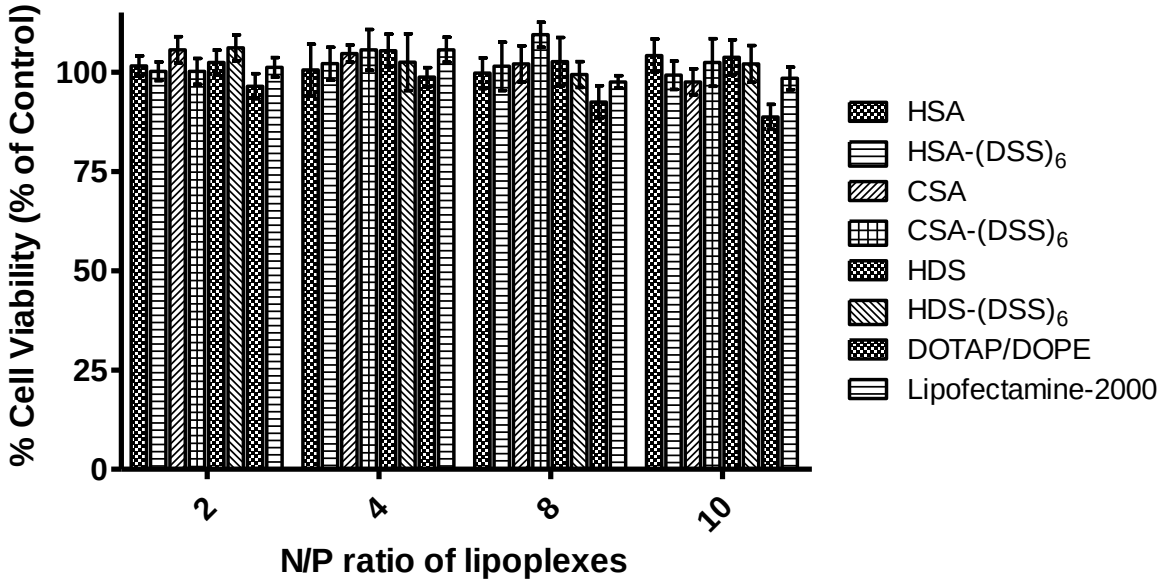

Figure 9.2 Effect of peptide conjugation on the cytotoxicity of the lipoplexes. Cytotoxicity of targeted and non-targeted lipoplexes are shown against DOTAP/DOPE and lipofectamine-2000 lipoplexes. For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.6 µL, 0.8 µL and 1.0 µL.

Quantitative and qualitative cellular gene expression studies were carried out using flow cytometry and confocal microscopy respectively in order to see the effect of the peptide conjugation on the cellular internalization of lipoplexes and gene expression. It was observed that the cellular expression of the lipoplexes did change significantly (**Table 9.2** and **Figure 9.3**). The level of expression of GFP expression in the cells was reduced after peptide conjugation as observed in confocal microscopy (**Figure 9.4**). However, one thing to notice here is that only level of expression in cells was found to be reduced while the number of cells expressing GFP was not found to be reduced significantly. The results were corroborating the results obtained with flow cytometry. This suggests that the peptide conjugation affects the cellular uptake of the formulation but the delivery system still bears the properties suitable for their uptake sufficient enough to show cellular expression of gene in all cells.

Table 9.2 GFP expression after transfection with peptide conjugated and non-conjugated lipoplexes against naked pDNA and Lipofectamine-2000 lipoplexes*

Formulation	% GFP expressing cells
Naked pDNA	5.24±2.15
HSA	94.42±1.85
HSA-$(DSS)_6$	82.15±2.45
CSA	93.67±1.45
CSA-$(DSS)_6$	81.56±1.85
HDS	85.94±1.74
HDS-$(DSS)_6$	70.54±3.21
Lipofectamine-2000	73.48±1.19

*Experiments were performed in triplicate.

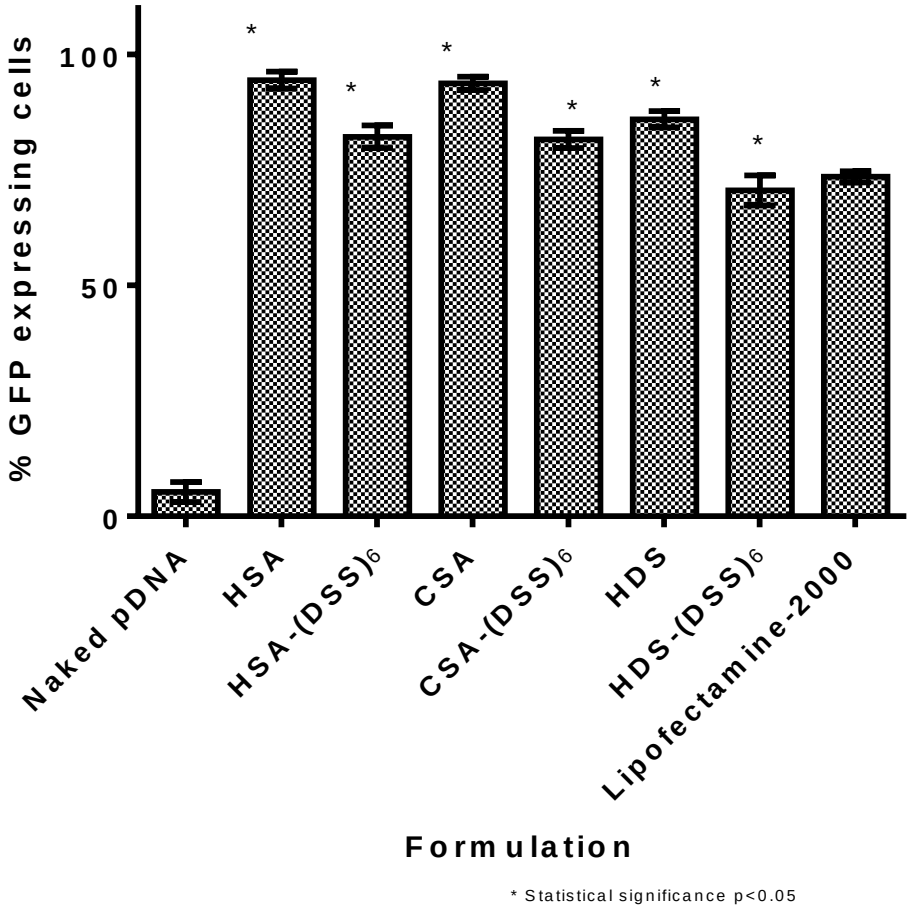

Figure 9.3 %GFP expression observed after transfection with peptide conjugated and non-conjugated lipoplexes. Untreated cells were taken as negative control while cells treated with naked pDNA and lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

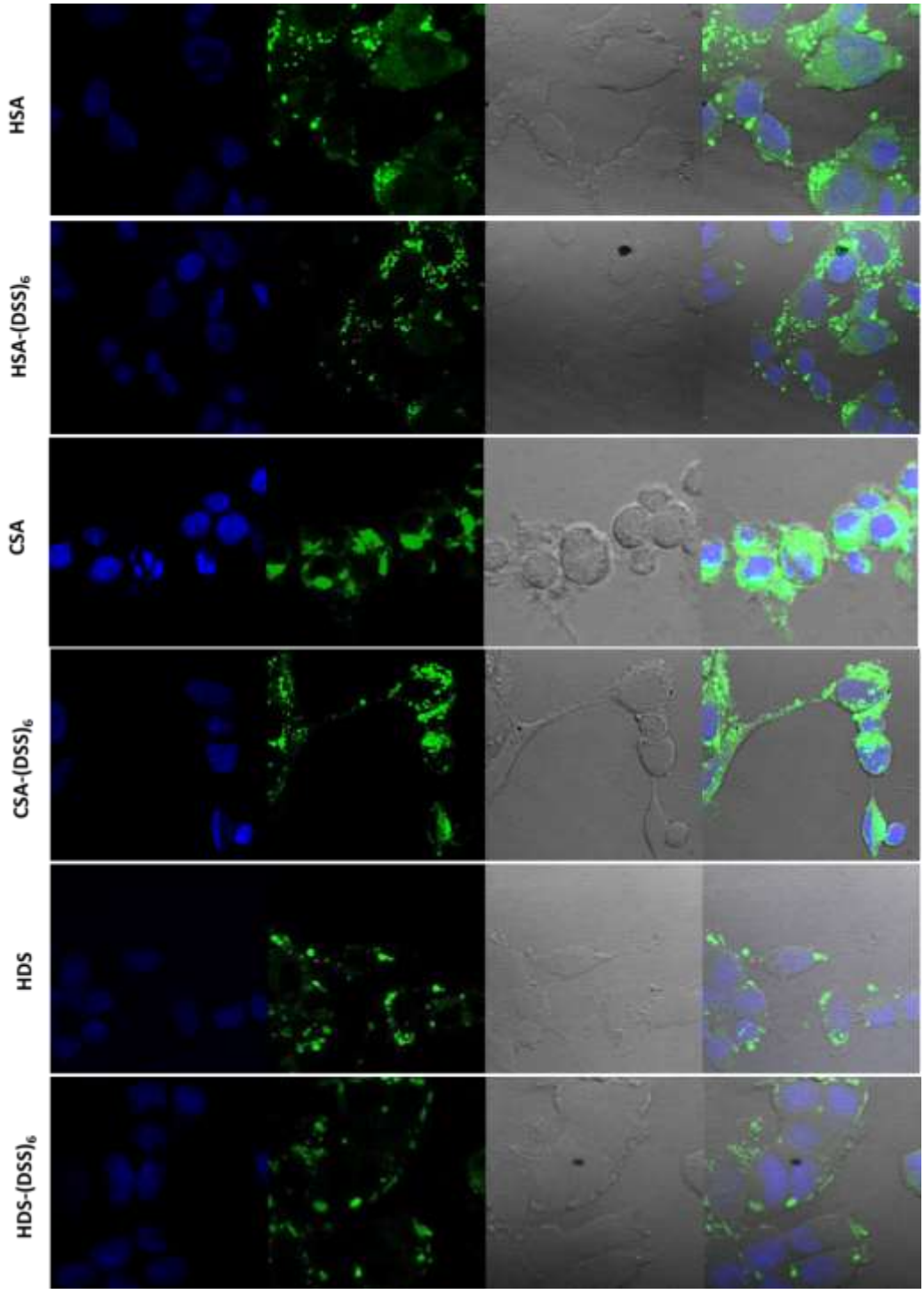

Figure 9.4 Confocal images of GFP expression after transfection with peptide conjugated and non-conjugated lipoplexes.

Further to the cellular GFP expression studies, in vitro calcium deposition studies were carried out in order to evaluate the effect of peptide conjugation on the cellular uptake and subsequent therapeutic activity i.e. calcium deposition. The results of the calcium deposition study are shown for the three targeted lipoplexes in comparison to their non-targeted counterparts (**Figure 9.5**). There was drastic reduction in the calcium

deposition after conjugation of the peptide to the lipoplexes. This corroborates the results of cellular GFP expression studies. i.e. the lesser the uptake, the lower will be the expression of the therapeutic gene and this would lead to reduced trans-differentiation of the cells to osteoblastic lineage ultimately turning out as reduced calcium deposition.

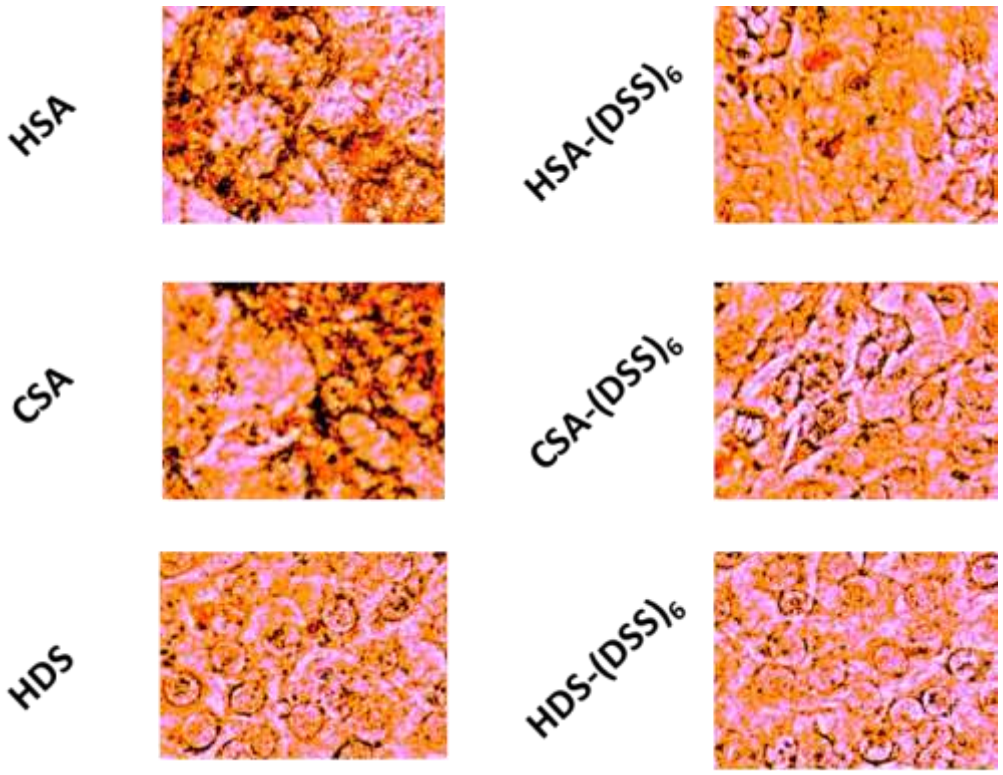

Figure 9.5 Calcium deposition study: Von Kossa staining of cells after transfection with different lipoplex formulations. Von Kossa stained cells 7 days after transfection with peptide conjugated and peptide non-conjugated lipoplexes are shown.

Results show that some characteristics of the lipoplexes were changed for the targeted lipoplexes after peptide conjugation; however, the changes were not detrimental to the performance of the lipoplexes. The results indicated that the therapeutic potential of the lipoplexes was still conserved in the lipoplexes. Targeted lipoplexes were successfully developed which showed promising results for further *in vivo* evaluation.

9.2 References

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