8. STABILITY STUDIES

Stringent requirements have been laid forward by regulatory authorities for stability of nanoparticulate drug delivery systems like liposomes. Hence, it is crucial to determine stability of all nanoparticulate systems designed for delivery of therapeutic genes. Additionally, developed nanoplexes being a complex system stabilized by thermodynamically equilibrated electrostatic interaction between cationic polymers or lipids with negatively charged nucleic acids, there are chances that equilibrium may get shifted and complex may get destabilized over time on exposure to humidity and temperature. Liposome systems have been reported to show various physicochemical changes on storage. Such changes include liposomal aggregation, fusion, loss of drug, etc. (1-4). These parameters will affect the *in vivo* performance of the formulation (8, 9). Similar changes can be expected for polyplex systems, too. Additionally, phospholipids may undergo hydrolysis reaction forming fatty acids and lysophospholipids (5, 6). However, under dried state, there is least possibility for such degradation, but, there are still chances of hydrolysis due to residual water content remaining in lyophilized cakes and also under humid conditions and temperature. Another aspect of stability of liposomes is oxidation of lipids (7). These changes may lead to structural integrity problems in liposomes and this might cause siRNA complexation changes. Thus these effects induce time dependent changes in desired properties of formulation during storage, therefore real time stability studies are potential tools to get an idea of any such possibility.

Both optimized polyplexes and lipoplexes were converted into dry powder form for pulmonary delivery. However, dry powder formulations should retain their original characteristics on stability. Hence, stability studies were performed on dry powder formulations containing polyplexes and lipoplexes by determining siRNA complexation efficiency, particle size and its distribution characteristics and change in water content of the formulation. Stability studies were performed at two conditions i.e. 2-8°C and 25°C as specified by ICH guidelines for products to be stored in refrigerator.

8.1 Method

Dry powder formulations of nanoplexes were evaluated for long term stability for 3 months at accelerated conditions of 25°C ± 2°C, 60% RH ± 5% RH and at 5°C ± 3°C. 20 mg of formulations were filled in hard gelatin capsules and required number of these capsules was

filled in tightly closed HDPE container. At different time points, required number of capsules of each formulation kept at each storage condition was removed. Dry powder was examined visually for any discoloration or shrinkage/collapse of powder. Accurately weighed quantities of dry powder were examined for moisture content using Karl-Fischer titration. Weighed quantity of formulation was reconstituted appropriately with nuclease free water and used for analysis of siRNA integrity using gel electrophoresis, particle size and its distribution (polydispersity index) and zeta potential.

8.2 Results

Results of stability studies of AAP, AHP, ALP and DL performed at two stability conditions are shown in **Table 8.1**, **Table 8.2**, **Table 8.3** and **Table 8.4** respectively.

Table 8.1 Stability of PDPI L1

Sampling time (Month)	Moisture Content (%)	Particle size (nm)	PDI	Zeta potential (mV)
Initial	1.83	140.8±4.5	0.12	22.16±2.75
Accelerated conditions (25°C ± 2°C, 60% RH ± 5% RH)				
1	2.25	145.4±3.7	0.14	23.39±0.88
3	2.79	148.8±2.19	0.14	23.77±1.74
Refrigerated conditions (5°C ± 3°C)				
1	1.95	143.9±2.02	0.13	22.09±2.07
3	2.11	153.3 ±1.98	0.14	22.42±2.58

Table 8.2 Stability of PDPI L2

Sampling time (Month)	Moisture Content (%)	Particle size (nm)	PDI	Zeta potential (mV)
Initial	1.76	159.4±4.5	0.06	14.07±0.32
Accelerated conditions (25°C ± 2°C, 60% RH ± 5% RH)				
1	2.31	165.12±4.09	0.07	14.44±0.48
3	2.88	169.58 ±3.79	0.18	15.25±1.95
Refrigerated conditions (5°C ± 3°C)				
1	1.91	160.86 ±2.02	0.06	14.01±1.19
3	2.07	158.1 ±1.98	0.10	13.96 ±2.75

Table 8.3 Stability of PDPI L3

Sampling time (Month)	Moisture content (%)	Particle size (nm)	PDI	Zeta potential (mV)
Initial	1.73	169.9±2.7	0.11	14.55±1.47
Accelerated conditions (25°C ± 2°C, 60% RH ± 5% RH)				
1	2.33	179.7±7.6	0.13	15.02±0.59
3	2.77	193.4 ±5.6	0.17	16.20±1.19
Refrigerated conditions (5°C ± 3°C)				
1	1.87	168.8 ±3.2	0.12	14.14±3.36
3	2.09	173.5±2.2	0.14	15.29 ±1.26

Table 8.4 Stability study of LDPI

Sampling time (Month)	Moisture content (%)	Particle size (nm)	PDI	Zeta potential (mV)
Initial	1.66	122.3±3.1	0.10	27.43±1.78
Accelerated conditions (25°C ± 2°C, 60% RH ± 5% RH)				
1	2.09	149.6±6.2	0.13	30.29±0.44
3	2.52	201.6 ±4.9	0.23	31.63±2.52
Refrigerated conditions (5°C ± 3°C)				
1	1.83	122.4 ±1.9	0.11	28.91±0.92
3	1.99	136.9 ±2.0	0.12	29.75±1.63

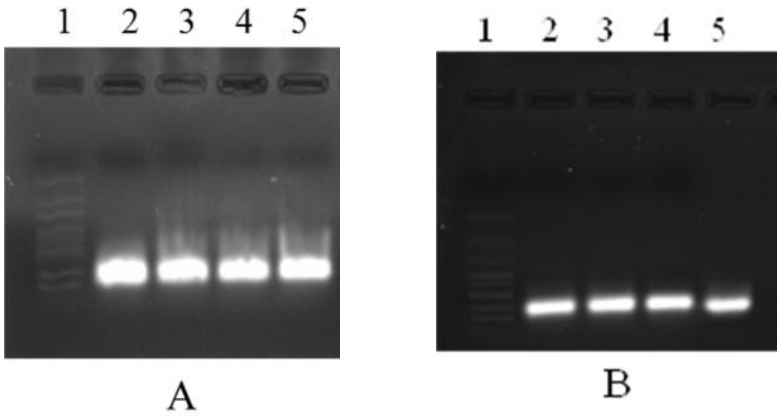

Figure 8.1 siRNA integrity after 3 months stability as compared to 10 bp ladder at 25°C ± 2°C, 60% RH ± 5% RH (A) and 5°C ± 3°C (B).

Lane 1: 10 bp ladder; Lane 2: PDPI L1, Lane 3: PDPI L2, Lane 4: PDPI L3, Lane 5: LDPI.

Visual examination of the dry powder formulations stored at both stability conditions showed no evidence of any physical instability. There was no significant particle size increase on storage either at accelerated condition or at refrigerator condition. However, there was a marginal increase in the polydispersity index of all formulations with that of lipoplexes being higher than that of polyplexes. Our results conform to the results obtained by other authors who have attributed to the retained particle size characteristics with the lyophilization of the

formulations with suitable cryoprotectant (10). Zeta potential change was also recorded to see whether there was change in surface charge properties as well as to have idea about the stability of reconstituted formulations. Studies revealed that, zeta potential was maintained close to initial zeta potential at both storage conditions and this depicts that there was no drastic loss of siRNA from complex.

Moisture content is one of the important parameters that can affect the stability of dry powders. Residual moisture below 1% in lyophilized powders is considered better for storage stability (11). However, higher moisture content can lead to aggregation in lyophilized formulations during storage (12). Such aggregation of particles can lead to increased particle size which can result in suboptimal and inconsistent dose delivery upon aerosolization. It has also been reported that moisture content can lead to destabilization of nanoparticulate systems due to water induced crystallization of carbohydrates when they are held above Tg of the system (13, 14). Water induces shift in the Tg of the system below the temperature of storage augmenting the crystallization processes (14). Hence, moisture content analysis was performed using Karl-Fisher titration. The analysis showed that moisture contents were significantly increased for all formulations at accelerated conditions reaching more than 2% level; however, on storage at refrigerated conditions lyophilized cakes maintained moisture content comparable to initial. Higher moisture contents at accelerated conditions were in concordance with other studies (15, 16). Decrease in complexation efficiency of all formulations at accelerated conditions can be ascribed to loss of siRNA due to water content increase. It has been reported that rise in residual moisture content of lyophilized DPI up to 3% has no detrimental effects on the aerosolization characteristics of powder (17). This ensures consistent in vivo performance of lyophilized DPIs on storage too. Additionally, low moisture content proves the better aerosolization of formulated DPIs ensuring that the DPIs will deliver accurate doses to the lungs *in vivo*. Dry powder formulations were also helped to maintain the integrity of siRNA as shown in **Figure 8.1**. The results of gel are in line with data of particle size and zeta potential.

From the stability studies, all Dry powder formulations were found to be stable under accelerated as well as long term storage condition. However, to ensure better retention of siRNA activity and other characteristics DPIs should be stored in refrigerator.

8.3 References

1. Grit M, Crommelin DJ. The effect of aging on the physical stability of liposome dispersions. Chemistry and physics of lipids. 1992 Sep;62(2):113-22. PubMed PMID: 1423806. Epub 1992/09/01. eng.

2. Cliff RO, Ligler F, Goins B, Hoffmann PM, Spielberg H, Rudolph AS. Liposome encapsulated hemoglobin: long-term storage stability and in vivo characterization. Biomaterials, artificial cells, and immobilization biotechnology : official journal of the International Society for Artificial Cells and Immobilization Biotechnology. 1992;20(2-4):619-26. PubMed PMID: 1391485. Epub 1992/01/01. eng.

3. Fang JY, Lin HH, Hsu LR, Tsai YH. Characterization and stability of various liposome-encapsulated enoxacin formulations. Chemical & pharmaceutical bulletin. 1997 Sep;45(9):1504-9. PubMed PMID: 9332003. Epub 1997/10/23. eng.

4. Slabbert C, Plessis LH, Kotze AF. Evaluation of the physical properties and stability of two lipid drug delivery systems containing mefloquine. International journal of pharmaceutics. 2011 May 16;409(1-2):209-15. PubMed PMID: 21310223. Epub 2011/02/12. eng.

5. Mowri H, Nojima S, Inoue K. Effect of lipid composition of liposomes on their sensitivity to peroxidation. Journal of biochemistry. 1984 Feb;95(2):551-8. PubMed PMID: 6715314. Epub 1984/02/01. eng.

6. Grit M, Zuidam NJ, Underberg WJ, Crommelin DJ. Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation on hydrolysis kinetics. The Journal of pharmacy and pharmacology. 1993 Jun;45(6):490-5. PubMed PMID: 8103093. Epub 1993/06/01. eng.

7. Frokjaer S, Hjorth EL, Worts O. Stability testing of liposomes during storage. In: Gregoriadis G, editor. Liposome Technology: Preparation of Liposomes. Boca Raton, Florida, US: CRC Press; 1984. p. 235-45.

8. Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability—A step closer from promising technology to application. Journal of Controlled Release. 2011 5/10/;151(3):246-55.

9. Cherng JY, Talsma H, Crommelin DJ, Hennink WE. Long term stability of poly((2-dimethylamino)ethyl methacrylate)-based gene delivery systems. Pharmaceutical research. 1999 Sep;16(9):1417-23. PubMed PMID: 10496659. Epub 1999/09/25. eng.

10. Kasper JC, Troiber C, Kuchler S, Wagner E, Friess W. Formulation development of lyophilized, long-term stable siRNA/oligoaminoamide polyplexes. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2013 Oct;85(2):294-305. PubMed PMID: 23747745. Epub 2013/06/12. eng.

11. Tang X, Pikal M. Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice. Pharmaceutical research. 2004 2004/02/01;21(2):191-200. English.

12. Shah SP, Misra A. Liposomal amikacin dry powder inhaler: effect of fines on in vitro performance. AAPS PharmSciTech. 2004;5(4):e65. PubMed PMID: 15760062. Pubmed Central PMCID: PMC2750490. Epub 2005/03/12. eng.

13. Abdelwahed W, Degobert G, Fessi H. Investigation of nanocapsules stabilization by amorphous excipients during freeze-drying and storage. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2006 Jun;63(2):87-94. PubMed PMID: 16621490. Epub 2006/04/20. eng.

14. Chan HK, Clark AR, Feeley JC, Kuo MC, Lehrman SR, Pikal-Cleland K, et al. Physical stability of salmon calcitonin spray-dried powders for inhalation. J Pharm Sci. 2004 Mar;93(3):792-804. PubMed PMID: 14762916. Epub 2004/02/06. eng.

15. Mattern M, Winter G, Kohnert U, Lee G. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems.

Pharmaceutical development and technology. 1999 May;4(2):199-208. PubMed PMID: 10231881. Epub 1999/05/08. eng.

16. Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability--A step closer from promising technology to application. Journal of controlled release : official journal of the Controlled Release Society. 2011 May 10;151(3):246-55. PubMed PMID: 21223985. Epub 2011/01/13. eng.

17. Claus S. Novel dry powder inhalation system based on dispersion of lyophilisates: Ludwig Maximilian University of Munich; 2012.