Chapter 11
Stability Studies

Nothing ends. Nothing ever ends.
-From "Watchmen"

11.1 Stability Studies

Stability study of any formulation on storage is necessary as it reflects whether the desirable properties of the formulation are retained on storage [1, 2]. These desirable properties include integrity of lipid vesicles and size distribution of particles. Upon storage, liposomes are susceptible to many physical changes i.e. lipid particles may undergo fusion and aggregation leading to increase in particle size of liposomes. Also there may occur loss of integrity of liposomes and subsequently leakage of encapsulated drug may take place [3, 4].

Liposomal formulations are not stable enough in an aqueous dispersion form. So to increase their stability the liposomal formulations are freeze-dried (lyophilized). However during lyophilization the liposomal formulation may undergo aforementioned physical changes. To avoid such changes different lyoprotectants like sucrose, mannitol, glycerol, trehelose, povidone, dextran etc. can be used which maintain the product in a good state [5, 6]. The physical testing of such product should be carried out to check whether any changes take place in the liposomal product in terms of its particle size and entrapment efficiency. So after storage period, the liposomal formulation, on rehydration, should retain the same characteristics it possessed before lyophilization.

For liposomal products an attention has been focused on two processes affecting the quality and therefore acceptability of liposomes [7]. Especially, with liposomal product to see the market it should stable during the shelf life (storage or transport). In general, a shelf life of at least one year is a minimum prerequisite criterion for a commercial product. First leakage of entrapped molecules from the vesicles may take place into the extra liposomal compartment. Secondly, there is a possibility of liposomal aggregation and/or fusion, which leads to formation of larger particles [8-11]. These parameters will alter the *in vivo* fate, affecting therapeutic index of the entrapped biomacromolecules. Hydrolysis of phospholipids is one of the parameters like to cause the formation of fatty acids and lysophopholipids [12, 13]. Although under dehydrated storage, there is least possibility of the formulation to encounter hydrolytic degradation. Another aspect to be considered is liposome oxidation [14]. Stability is considered as chemical stability of drug substance in a dosage form. However, the performance of liposomal formulation is not only dependent upon the content of the drug substance, but also dependent on reproducible *in vivo* performance of the formulations.

As per the ICH stability study guideline Q1A (R2), stability studies should be performed on a drug product intended for storage in refrigerator at following storage conditions (**Table 11.1**). Formulations under stability studies were considered chemically stable by evaluating the assay of pDNA using gel electrophoresis. The stability protocol was designed as per ICH guidelines [15] for countries falling under zone III (hot, dry) and zone IV (very hot, humid) [16]; however, only short term studies for 3 months storage period were performed for having the idea of the stability of the product.

Table 11.1 Stability Testing Conditions for Drug Product Intended for Storage in Refrigerator as per ICH Guideline Q1A(R2).

Study	Storage condition	Minimum Time Period for Which Study Should be Carried Out
Long term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C/60% RH ± 5% RH	6 months

Any "significant change" for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation product's exceeding its acceptance criterion;
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;
4. Failure to meet the acceptance criterion for pH;
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

11.1.1 Method

Comparative stability studies were carried out of the potential lipoplex formulations at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for three months and at long-term conditions (2-8°C) up to three months. Lyophilized formulations in Type I tubular glass vials were sealed with chlorobutyl rubber stoppers and sealed with aluminum seals. Sealed vials were stored at above mentioned condition [17-24]. At each

sampling point different vials were used for the stability testing. The lipoplex formulations were examined visually for the evidence of discoloration. The content of the vials were tested for percentage pDNA complexation, assay, particle size, zeta-potential, assay and water content.

11.1.2 Results and discussion

Lipoplex formulations must show physical stability in order to produce a commercially viable product [25]. Preferable stability of formulation up to 1 to 2 years at storage conditions i.e. room temperature condition or at refrigerated condition is required for a pharmaceutically acceptable liposomal product. In order to make the formulation survive these long stability periods on shelf, lyophilization becomes a primary resort for stabilizing the liposomal product. However, this doesn't eliminate the requirements for the real time stability monitoring as the physicochemical properties of the formulations are still prone to change on storage. To evaluate the stability of the pDNA lipoplex formulations, lyophilized liposomal formulations were evaluated for changes in particle size, zeta potential, pDNA assay and complexation efficiency were determined. Results of the study are tabulated in **Table 11.2** and **Table 11.3**. Additionally, lipoplexes that were peptide conjugated and which rerepresented each group of synthesized lipids (amino acid derivative modified stearyl amine and amino acid derivative modified phospholipid) were evaluated for stability studies i.e. HSA-$(DSS)_6$ lipoplexes and HDS-$(DSS)_6$ lipoplexes. Due to similar compositions of the lipoplexes except for the modified lipid, the stability data can be extended to have an idea on the stability of the other lipoplexes as well.

During stability monitoring, no significant differences ($p > 0.05$) were found in all above mentioned parameters at refrigerated condition. Lyophilize formulatins maintained their physical integrity and were observed as white porous cakes. Assay of the formulations stored at both conditions at each time point was between the range of 95-105% of initial levels which was acceptable. There was no significant change ($p>0.05$) in particle size and zeta potential after storage period at both conditions. Water content of the lyophilized cakes was not affected during the storage period ($p< 0.05$). Stability studies at accelerated and refrigerated conditions demonstrate that the product was stable at both conditions for a period of 3 months and suggest that the product will be stable for longer periods at refrigerated conditions.

Table 11.2 Stability Testing Data of HSA-$(DSS)_6$ lipoplexes

Storage condition	Time (Month)	Description	Assay (%)	Complexation efficiency (%)	Water content (%)	Particle size (nm)	Mean Polydispersity index	Zeta potential (mV)
Initial (Before lyophilization)		NA	101.23±2.54	100.52±1.89	NA	91.4±5.7	0.262	12.4±1.2
Initial (After lyophilization)		White lyophilized cake	99.42±2.04	99.25±2.15	1.26±0.30	89.7±4.2	0.271	12.5±1.2
2-8°C	1	White lyophilized cake	100.16±1.58	98.69±1.95	1.34±0.27	94.5±5.1	0.249	11.3±1.4
2-8°C	2	White lyophilized cake	98.56±1.24	99.15±2.10	1.16±0.15	90.4±4.1	0.272	12.9±0.9
2-8°C	3	White lyophilized cake	99.48±1.85	98.88±1.19	1.39±0.21	92.5±3.8	0.266	12.4±1.6
25°C/60% RH	1	White lyophilized cake	98.75±1.48	99.56±2.65	1.35±0.25	89.9±4.9	0.245	13.2±1.1
25°C/60% RH	2	White lyophilized cake	99.71±2.03	99.12±2.36	1.44±0.26	93.5±4.5	0.249	12.4±1.5
25°C/60% RH	3	White lyophilized cake	98.48±1.65	98.78±1.56	1.52±0.34	96.4±2.9	0.254	13.7±1.4

*Experiments were performed in triplicate

Table 11.3 Stability Testing Data of HDS-$(DSS)_6$ lipoplexes

Storage condition	Time (Month)	Description	Assay (%)	Complexation efficiency (%)	Water content (%)	Particle size (nm)	Polydispersity index	Zeta potential (mV)
Initial (Before lyophilization)		NA	99.12±2.29	99.32±1.75	1.21±0.33	124.5±4.9	0.187	13.5±2.9
Initial (After lyophilization)		White lyophilized cake	98.75±3.62	98.69±3.26	1.19±0.41	125.8±5.9	0.185	12.9±2.2
2-8°C	1	White lyophilized cake	98.62±2.42	99.45±2.25	1.16±0.54	128.5±6.6	0.189	14.2±3.4
2-8°C	2	White lyophilized cake	99.05±2.42	98.56±1.26	1.22±0.42	124.6±3.8	0.198	13.6±1.9
2-8°C	3	White lyophilized cake	97.89±1.69	98.03±1.99	1.36±0.21	127.0±5.1	0.192	12.4±2.2
25°C/60% RH	1	White lyophilized cake	99.03±2.06	97.68±1.98	1.36±0.49	129.3±5.4	0.186	13.6±2.5
25°C/60% RH	2	White lyophilized cake	98.69±1.36	98.56±0.87	1.24±0.23	129.5±4.6	0.201	12.8±3.1
25°C/60% RH	3	White lyophilized cake	99.06±2.08	98.26±1.58	1.25±0.26	129.9±5.7	0.195	12.7±1.5

*Experiments were performed in triplicate

11.2 References

1. Guidance for Industry Q1C Stability Testing for New Dosage Forms. U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER),ICH, Federal Register (1996). At <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073374.pdf >.

2. ICH Guidelines, Guidance for Industry of New Drug Substances and Products Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products Substance, Human Services, 32, 1-18 (2003).
3. Cacela C, Hincha DK. Low Amounts of Sucrose Are Sufficient to Depress the Phase Transition Temperature of Dry Phosphatidylcholine, but Not for Lyoprotection of Liposomes. Biophysical Journal 2006; 90(8): 2831-42.
4. Guidance for Industry Liposome Drug Products . U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER),ICH, Federal Register 12 (2002). at <http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm262292.htm>.
5. Fakes MG, Dali MV, Haby TA, Morris KR, Varia SA, Serajuddin AT. Moisture sorption behavior of selected bulking agents used in lyophilized products. PDA J Pharm Sci Technol 2000; 54(2): 144-9.
6. Crowe LM, Crowe JH, Rudolph A, Womersley C, Appel L. Preservation of freeze-dried liposomes by trehalose. Arch Biochem Biophys 1985; 242(1): 240-7.
7. Talsma H, Crommelin DJ. Liposomes as Drug Delivery Systems, Part III: Stabilization. Pharmacetucial Technology 1992; 17: 48-59.
8. Grit M, Crommelin DJ. The effect of aging on the physical stability of liposome dispersions. Chem Phys Lipids 1992; 62(2): 113-22.
9. Cliff RO, Ligler F, Goins B, Hoffmann PM, Spielberg H, Rudolph AS. Liposome encapsulated hemoglobin: long-term storage stability and in vivo characterization. Biomater Artif Cells Immobilization Biotechnol 1992; 20(2-4): 619-26.
10. Fang JY, Lin HH, Hsu LR, Tsai YH. Characterization and stability of various liposome-encapsulated enoxacin formulations. Chem Pharm Bull (Tokyo) 1997; 45(9): 1504-9.
11. Slabbert C, Plessis LH, Kotze AF. Evaluation of the physical properties and stability of two lipid drug delivery systems containing mefloquine. Int J Pharm 2011; 409(1-2): 209-15.
12. Mowri H, Nojima S, Inoue K. Effect of lipid composition of liposomes on their sensitivity to peroxidation. J Biochem 1984; 95(2): 551-8.
13. 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. J Pharm Pharmacol 1993; 45(6): 490-5.

14. 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.
15. Singh S. Drug stability testing and shelf-life determination according to international guidelines. Pharm Technol 1999; 23: 68-88.
16. US FDA (CDER), Draft Guidance for Industry Liposome Drug Products. CMC Documentation.
17. Ugwu S, Zhang A, Parmar M, Miller B, Sardone T, Peikov V, et al. Preparation, characterization, and stability of liposome-based formulations of mitoxantrone. Drug Dev Ind Pharm 2005; 31(2): 223-9.
18. Winterhalter M, Lasic DD. Liposome stability and formation: experimental parameters and theories on the size distribution. Chem Phys Lipids 1993; 64(1-3): 35-43.
19. Changsan N, Chan HK, Separovic F, Srichana T. Physicochemical characterization and stability of rifampicin liposome dry powder formulations for inhalation. J Pharm Sci 2009; 98(2): 628-39.
20. Yang T, Cui FD, Choi MK, Lin H, Chung SJ, Shim CK, et al. Liposome formulation of paclitaxel with enhanced solubility and stability. Drug Deliv 2007; 14(5): 301-8.
21. Anderson M, Omri A. The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. Drug Deliv 2004; 11(1): 33-9.
22. Manosroi A, Podjanasoonthon K, Manosroi J. Stability and release of topical tranexamic acid liposome formulations. J Cosmet Sci 2002; 53(6): 375-86.
23. Manosroi A, Kongkaneramit L, Manosroi J. Characterization of amphotericin B liposome formulations. Drug Dev Ind Pharm 2004; 30(5): 535-43.
24. Bhalerao SS, Raje Harshal A. Preparation, optimization, characterization, and stability studies of salicylic acid liposomes. Drug Dev Ind Pharm 2003; 29(4): 451-67.
25. Fildes F. Liposomes: The Industrial view point. In: Liposomes from Physical Structure to Therapeutic Applications. Knight C, editor. New York, US: Elsevier Biomedical Press; 1998.