Chapter 1
Introduction

Hope is a good thing, may be the best of the things, and no good thing ever dies

-*From "The Shawshank Redemption"*

1.1 Introduction

Osteoporosis is a disease of bones that leads to an increased risk of fracture. It has been denoted a silent disease due to its character of occurring without symptomatic changes in the body. Worldwide estimates show that osteoporosis accounts for over 8.9 million fractures annually which turns out to be an osteoporosis related fracture every 3 seconds [1]. Osteoporosis affects 200 million women worldwide with approximately one-tenth of women aged 60 affected by osteoporosis [2]. It affects the aged people making them bedridden affecting their quality of life. Worldwide, 1 in 3 women above 50 years age and 1 in5 men above 50 years age are affected by osteoporotic fracture [3-5]. This shows that osteoporosis is a global healthcare burden.

1.1.1 Osteoporosis pathophysiology

In osteoporosis, the bone mineral density (BMD) gets reduced, deterioration of bone microarchitecture takes place, and the amount of various transcription factors, growth factors and cytokines etc. in bone are altered. Imbalance between bone resorption and bone formation is the underlying mechanism in all cases of osteoporosis. In normal bone, matrix remodeling of bone is constant. Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells. The three main mechanisms by which osteoporosis develop are (a) an inadequate peak bone mass (the skeleton develops insufficient mass and strength during growth), (b) excessive bone resorption, and (c) inadequate formation of new bone during remodeling.

These occurs due to various hormonal level defects that lead to cascades of processes which cause increased bone resorption by osteoclasts and/or decreased bone generation by osteoblasts. Lack of estrogen (e.g. as a result of menopause) increases bone resorption, as well as decreasing the deposition of new bone that normally takes place in weight-bearing bones. In addition to estrogen, calcium metabolism plays a significant role in bone turnover, and deficiency of calcium and vitamin D leads to impaired bone deposition; in addition, the parathyroid glands react to low calcium levels by secreting parathyroid hormone (parathormone, PTH), which increases bone resorption to ensure sufficient calcium in the blood. Main hormones that regulate bone metabolism are as follows:

Decrease bone resorption: Calcitonin, estrogen

Increase bone resorption: parathyroid hormones (PTH), glucocorticoids, thyroid hormones, high dose vit. D

Increase bone formation: Growth hormone, vit. D metabolites, androgens, insulin, low dose parathyroid hormone

Decrease bone formation: Glucocorticoids

Various growth factors, cytokines and transcription factors are involved in the pathogenesis of osteoporosis. These include RANK (receptor activator of nuclear factor κβ), RunX2 (Runt related factor X2), VEGF (vascular endothelial growth factor), TNF (tumor necrosis factor), TGF (transforming growth factor), BMPs (bone morphogenetic proteins), OPG (osteoprotegerin), OTX (osterix). Many of these factors have been studied for their potential use in osteoporosis.

1.1.2 Treatment and management of osteoporosis:

Osteoporosis risk can be reduced with lifestyle changes and medication; in people with osteoporosis, treatment may involve both. Lifestyle change includes diet and exercise, and preventing falls. Medication includes supplemental calcium, vitamin D, calcitonin, bisphosphonates (zaledronic acid, ibandronate, etc), bone morphogenetic proteins (BMP-2 and -7) and several others. Most of the therapies are long term therapies and require closer monitoring to avoid any adverse effects. Some of the demerits of the current therapeutics of osteoporosis are described in the **Figure 1.1** below.

Effectiveness of oral calcium and vitamin D supplementation has been evaluated extensively. The analyses show that calcium supplementation alone and vitamin D supplementation alone are not effective in preventing fractures in osteoporotic patients as the combination thereof [6, 7]. Effect of intravenous calcium infusion has also been evaluated in osteoporotic women for treating osteoporosis, but it was found to be ineffective in altering bone calcium turnover in osteoporotic women. Loss of total body calcium was similar to that in untreated subjects with osteoporosis [8, 9].

Glucocorticoid-induced osteoporosis and osteoporosis related to aging are mainly outcome of reduced bone formation due to reduced number of osteoblasts. Moreover, calcium and vitamin D combination therapy has been found to be non-effective in preventing fractures in elderly (age >70 years) [10, 11]. An ideal way to prevent bone loss in such cases would be not only to reduce bone resorption, but also to promote bone formation. There is therefore an important need to develop therapeutic strategies capable of promoting bone formation in osteoporotic subjects.

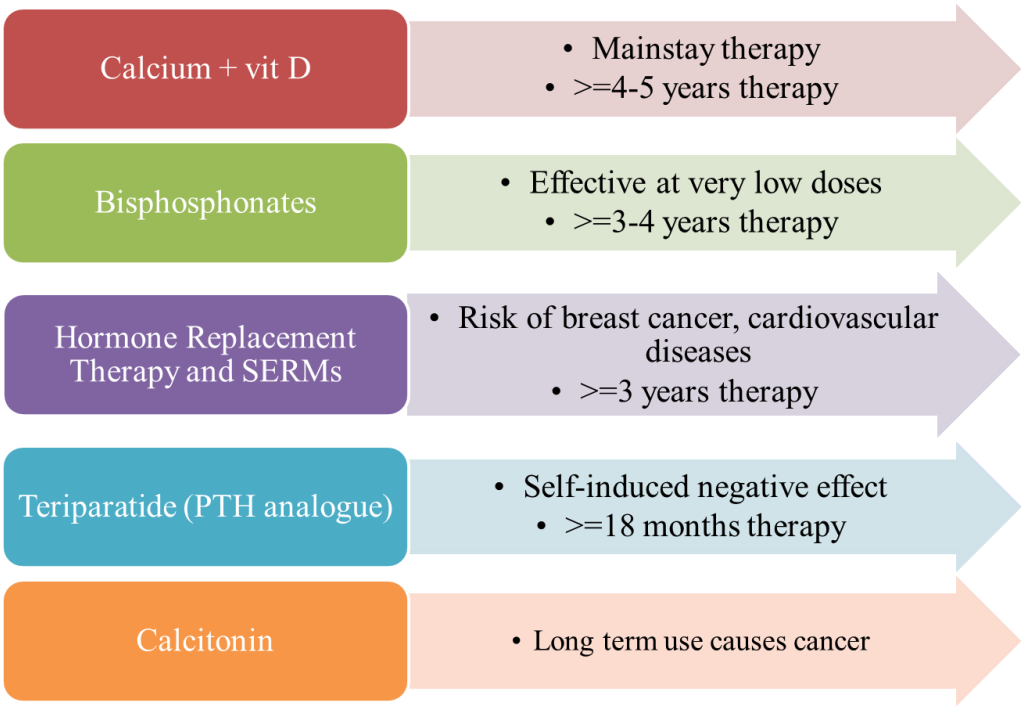

Figure 1.1 Current treatment options of osteoporosis and their drawbacks

1.1.3 Gene Delivery for Osteoporosis: Current Status

In the past decade various gene delivery approaches have been studied for the treatment of osteoporosis. Such gene delivery approaches particularly act either by inducing or one or other growth factors, cytokines, transcription factors, other mediators or their receptors that are implicated in osteoporosis. Advancements made in the treatment of osteoporosis with gene delivery are described below with brief review of various gene delivery systems evaluated for osteoporosis treatment in animals (**Table 1**).

Various cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been strongly implicated in postmenopausal osteoporosis occurring due to estrogen deficiency. Both of these cytokines are powerful inducers of bone resorption. From this information, it follows that inhibiting the biological activities of IL-1 and TNF should reduce bone loss under conditions of estrogen deficiency. Genes encoding for IL-1 receptor antagonist (IL-1Ra) or soluble form of TNF receptors would ameliorate the osteoporotic bone loss by inhibiting osteoclastic activity [12, 13].

Intravenous delivery of human osteoprotegerin (hOPG) gene using viral vectors results in systemic circulation of the OPG which in turn inhibits osteoclastic activity. The

mechanism involves the binding of OPG to RANKL (receptor activator of nuclear factor κβ ligand) which prevents the binding of latter to RANK. This in turn suppresses its ability to increase bone resorption by osteoclasts [14, 15]. LIM mineralization protein (LMP) which induces the bone mineralization and expression of various osteogenic genes, BMP-2, RunX2 (Runt related transcription factor X2), OSX (Osterix) etc., and thereby promotes the osteoblast differentiation. One study has also shown that it induces bone formation more efficiently than even BMP-2 [16].

Among all gene delivery approaches, delivery of genes of bone-morphogenetic proteins has been most extensively evaluated (**Table 1**). Bone morphogenetic factors (BMPs), mainly BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9, are other osteogenic proteins that have been studied for bone regeneration in fractured bone healing, osteoporosis and osteopenia [17]. Recombinant human bone morphogenetic protein-2 and -7 have been recently granted United States Food and Drug Administration approval for select clinical applications in bone repair [13, 17]. These BMPs act primarily as differentiation factors, turning responsive mesenchymal cells into cartilage- and bone-forming cells. [18] While significant progress has been made in the delivery of recombinant osteogenic proteins to promote bone healing, the short half-life and instability of the protein requires the delivery of milligram quantities of factor or multiple dosages [13]. So, delivery of genes encoding for various BMPs have been investigated in various studies (Table 1). Various transcription factors and growth factors such as VEGF, RunX2, TGF etc. have also been found to enhance the effects of various BMPs. Among various BMPs, BMP-9 has been shown to provide most robust and effective osteogenic activity in animal studies.

Table 1 Various gene delivery approaches used in osteoporosis

Gene therapy with	Encoded protein		Vector for transfection	Use of	Route of administration	Remarks	Ref.
pDNA	IL-1Ra(interleukin-1 receptor antagonist		Adenovirus	Recombinant adenovirus	Intramedullary injection		[12]
pDNA	BMP-2		Adenovirus	Recombinant adenovirus	--		[19]
pDNA	Bone morpho-genetic proteins (BMPs)	BMP-2	Adenovirus	Recombinant adenovirus and AdBMP-transduced osteoblast progenitors	Intramuscular injection (in quadriceps)	Activity inhibited by BMP-3	[20]
		BMP-3				Negative regulator of bone formation	
		BMP-6				Most robust and mature ossification, Activity inhibited by BMP-3	
		BMP-7				Activity inhibited by BMP-3	
		BMP-9				Most robust and mature ossification, Activity inhibited by BMP-3	
pDNA	BMP-2		Baculovirus	Recombinant baculovirus-transduced hMSCs (mesenchymal stem cells)	Injection into back subcutis		[21]
pDNA	BMP-2		Adenovirus with RGD tripeptide containing coat	Recombinant adenovirus-transduced hMSCs		Coat with RGD peptide enhances interaction with MSCs' surface integrins and thus enhance transfaction.	[22, 23]
pDNA	BMP-2 and RunX2		Adenovirus	Recombinant adenovirus-transduced pluripotent C3H10T1/2 cell	Subcutaneous implant	Complementary effect of RunX2 and BMP-2 on bone formation	[24]
pDNA	BMP-4 and vascular endothelial growth factor (VGEF)		Retrovirus	Recombinant retrovirus	Implantation into defect	VEGF and BMP-4 appeared to act synergistically to enhance bone healing	[25]
pDNA	BMP-9		Adenovirus	Recombinant adenovirus-transduced hMSCs	Intramuscular injection	-	[26]

pDNA and fusion construct of pDNA with immune-globulin constant domain (pDNA-Fc)	Human osteoprotegerin (hOPG)	Adenovirus	Adenovirus	Intravenous injection	-	[27]
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intravenous injection	-	[14]
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intramuscular injection	-	[15]
pDNA	BMP-2 and VGEF	Nonviral gene transfer	Gene transfer	Intramuscular injection	VGEF synergized the effect of BMP-2 on ossification	[28]
pDNA	BMP-7	Nanostructured calcium phosphate (NanoCaP)	Fibrin gel matrix of pDNA-NanoCaP	Intramuscular implantation	-	[29]
pDNA	BMP-2	Nanostructured calcium phosphate (NanoCaP)	Collagen Gene activated matrix of pDNA or pDNA-NanoCaP	Subcutaneous transplant or injection in bone-marrow	Modification of GAM with CaP effective in tissue regeneration at lower pDNA level	[30, 31]
pDNA	BMP-2	Nonviral gene transfer	BMP-2 gene-modified autologous MSCs or β-tricalcium phosphate	--	-	[32]
pDNA	LMP-3	Adenovirus	Recombinant adenovirus	Intramuscular injection	More efficient ectopic bone formation in-vivo than BMP-2	[16]

1.1.4 Gene Delivery Vectors

Among various vectors researched for gene delivery, those used in osteoporosis include viral vectors mainly adenoviral vector, adenoassociated viral vector, baculoviral vector and retroviral vector. Though providing very efficient transfection ability, viral vectors bear a lot of disadvantages mainly higher oncogenic, inflammatory and immunogenic potential and also virus insert their genome into host genome in random pattern restricting functioning of host genes. This is changing the scenario of gene delivery from viral based delivery to non-viral gene delivery. However, recently a few instances have been reported where non-viral gene delivery have been used in vivo preclinically. These vectors include calcium phosphate nanoconstructs and lipoplexes. This opens a possibility to develop and use liposomal vector (synonymously used terms are lipoplexes, lipid-DNA complex, liposomes etc.) for gene delivery in osteoporosis.

Lipoplexes have become the most used gene delivery vector for in vitro gene delivery to cells and have been successfully evaluated in vivo in animals for treatment of various genetic conditions such as cancer, osteoporosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, viral infections, cardiovascular diseases and many more [33].

Among the non-viral gene delivery systems, naked DNA and lipofection has been used in clinical trials with 5.9% of clinical trials employing lipofection as a gene delivery system [34]. With increasing attention on the nanotechnology based gene delivery systems and advancing understanding of viral and non-viral gene delivery systems, lipoplexes based gene delivery are projected to be used most used gene delivery system.

Liposome mediated gene transfer occurs by endocytosis where liposomes can bind to cell membrane and get engulfed into the cells. Endocytosed liposome-DNA complexes can release DNA into cytosol [35-37]. Cytosolic release is often promoted by the helper lipids which have fusion capabilities i.e. DOPE or the lipids which have capability to destruct endosomal wall. Additionally, enhanced transfection can be rendered by the lipids which provide buffering effect. DNA released can migrate to nucleus. Additionally, transfection can also follow the direct cytosolic uptake through direct fusion to cell membrane of the lipoplexes [35-37]. Lipoplexes offer inherited low toxicity characteristics of biocompatible

bilayer structure. Moreover, lipoplexes can be modified in order to provide advantages such as a) ability to target various organs by modifying liposome surface by attaching appropriate ligands, b) reduced immunogenic response, c) differential release characteristics and d) protection of the complexed gene [35].

A few positively charged lipids can be used for entrapment of negatively charged DNA. Few cationic lipis are

- N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride(DOTMA)
- [1,2-bis(oleoyloxy)-3-(trimethylammonio)popane] (DOTAP)
- 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), and dioctadecylamidoglycylspermine (DOGS)

Few shortcomings of the liposomal gene delivery systems are cellular toxicities, low transfection efficiency, uptake by reticuloendothelial system cells, low target organ delivery, low protection of DNA against in vivo milieu etc. To overcome these shortcomings, novel lipoplexes need to be developed.

1.2 Aim of Work

The aim of the current project is to develop a novel gene delivery approach for treatment of osteoporosis.

1.3 Hypotheses

Gene delivery approach will provide a suitable corrective approach for the treatment of osteoporosis. Synthesized cationic lipids will provide a better and safe delivery alternative for gene delivery. In addition, developed liposomal carrier will provide better stability to therapeutic gene from degradation and also surface modification with a targeting ligand will provide bone targeted delivery of gene delivering it to the progenitor niche in the bone providing higher concentration of gene at the target site. Targeted delivery will ensure higher exposure of therapeutic gene to the target cells and will provide enhanced effectiveness.

1.4 Plan of Work

1. Synthesis of novel cationic lipids and their evaluation
2. Preparation of the cationic liposomes and lipoplexes
3. Characterization of formulated liposome and lipoplexes
    a. Particle size and ζ potential
    b. Complexation efficiency
4. *In vitro* performance studies: Stability in presence of electrolytes, serum, To check *in-vitro* cell-uptake and transfection efficiency of lipoplexes, *in vitro* mineralization assay
5. *In vivo* performance studies: Osteogenic activity in animal model of osteoporosis

1.5 References

1. Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 2006; 17(12): 1726-33.
2. WHO Technical report, WHO Press, Switzerland, 2007.
3. Melton LJ, 3rd, Atkinson EJ, O'Connor MK, O'Fallon WM, Riggs BL. Bone density and fracture risk in men. J Bone Miner Res 1998; 13(12): 1915-23.
4. Melton LJ, 3rd, Chrischilles EA, Cooper C, Lane AW, Riggs BL. Perspective. How many women have osteoporosis? J Bone Miner Res 1992; 7(9): 1005-10.
5. Kanis JA, Johnell O, Oden A, Sembo I, Redlund-Johnell I, Dawson A, et al. Long-term risk of osteoporotic fracture in Malmo. Osteoporos Int 2000; 11(8): 669-74.
6. Garcia Vadillo JA. [Are calcium and vitamin D supplements for everyone?: Pro]. Reumatol Clin 2011; 7 Suppl 2: S34-9.
7. Nowson CA. Prevention of fractures in older people with calcium and vitamin D. Nutrients 2010; 2(9): 975-84.
8. Dudl RJ, Ensinck JW, Baylink D, Chesnut CH, 3rd, Sherrard D, Nelp WB, et al. Evaluation of intravenous calcium as therapy for osteoporosis. Am J Med 1973; 55(5): 631-7.
9. Jensen H, Christiansen C, Munck O, Toft H. Treatment of osteoporosis with calcium infusions. An osteodensitometric study. Scand J Clin Lab Invest 1973; 32(1): 93-6.

10. Grant AM, Avenell A, Campbell MK, McDonald AM, MacLennan GS, McPherson GC, et al. Oral vitamin D3 and calcium for secondary prevention of low-trauma fractures in elderly people (Randomised Evaluation of Calcium Or vitamin D, RECORD): a randomised placebo-controlled trial. Lancet 2005; 365(9471): 1621-8.
11. Porthouse J, Cockayne S, King C, Saxon L, Steele E, Aspray T, et al. Randomised controlled trial of calcium and supplementation with cholecalciferol (vitamin D3) for prevention of fractures in primary care. BMJ 2005; 330(7498): 1003.
12. Baltzer AW, Whalen JD, Wooley P, Latterman C, Truchan LM, Robbins PD, et al. Gene therapy for osteoporosis: evaluation in a murine ovariectomy model. Gene Ther 2001; 8(23): 1770-6.
13. Kofron MD, Laurencin CT. Bone tissue engineering by gene delivery. Adv Drug Deliv Rev 2006; 58(4): 555-76.
14. Kostenuik PJ, Bolon B, Morony S, Daris M, Geng Z, Carter C, et al. Gene therapy with human recombinant osteoprotegerin reverses established osteopenia in ovariectomized mice. Bone 2004; 34(4): 656-64.
15. Ulrich-Vinther M, Carmody EE, Goater JJ, K Sb, O'Keefe RJ, Schwarz EM. Recombinant adeno-associated virus-mediated osteoprotegerin gene therapy inhibits wear debris-induced osteolysis. J Bone Joint Surg Am 2002; 84-A(8): 1405-12.
16. Pola E, Gao W, Zhou Y, Pola R, Lattanzi W, Sfeir C, et al. Efficient bone formation by gene transfer of human LIM mineralization protein-3. Gene Ther 2004; 11(8): 683-93.
17. Gautschi OP, Frey SP, Zellweger R. Bone morphogenetic proteins in clinical applications. ANZ J Surg 2007; 77(8): 626-31.
18. Ebara S, Nakayama K. Mechanism for the action of bone morphogenetic proteins and regulation of their activity. Spine (Phila Pa 1976) 2002; 27(16 Suppl 1): S10-5.
19. Musgrave DS, Bosch P, Ghivizzani S, Robbins PD, Evans CH, Huard J. Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. Bone 1999; 24(6): 541-7.
20. Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, et al. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther 2004; 11(17): 1312-20.

21. Chuang CK, Sung LY, Hwang SM, Lo WH, Chen HC, Hu YC. Baculovirus as a new gene delivery vector for stem cell engineering and bone tissue engineering. Gene Ther 2007; 14(19): 1417-24.
22. Tsuda H, Wada T, Ito Y, Uchida H, Dehari H, Nakamura K, et al. Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. Mol Ther 2003; 7(3): 354-65.
23. Tsuda H, Wada T, Yamashita T, Hamada H. Enhanced osteoinduction by mesenchymal stem cells transfected with a fiber-mutant adenoviral BMP2 gene. J Gene Med 2005; 7(10): 1322-34.
24. Yang S, Wei D, Wang D, Phimphilai M, Krebsbach PH, Franceschi RT. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. J Bone Miner Res 2003; 18(4): 705-15.
25. Peng H, Wright V, Usas A, Gearhart B, Shen HC, Cummins J, et al. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. J Clin Invest 2002; 110(6): 751-9.
26. Dayoub H, Dumont RJ, Li JZ, Dumont AS, Hankins GR, Kallmes DF, et al. Human mesenchymal stem cells transduced with recombinant bone morphogenetic protein-9 adenovirus promote osteogenesis in rodents. Tissue Eng 2003; 9(2): 347-56.
27. Bolon B, Carter C, Daris M, Morony S, Capparelli C, Hsieh A, et al. Adenoviral delivery of osteoprotegerin ameliorates bone resorption in a mouse ovariectomy model of osteoporosis. Mol Ther 2001; 3(2): 197-205.
28. Samee M, Kasugai S, Kondo H, Ohya K, Shimokawa H, Kuroda S. Bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) transfection to human periosteal cells enhances osteoblast differentiation and bone formation. J Pharmacol Sci 2008; 108(1): 18-31.
29. LEE P, OLTON D, KUMTA P, SFEIR C, editors. Non-viral gene delivery of nanostructured-calcium-phosphate (NanoCaP) carriers for bone regeneration. AADR 37th Annual Meeting and Exhibition; 2008 2-5 April 2008; Dallas, Texas, USA.

30. Endo M, Kuroda S, Kondo H, Maruoka Y, Ohya K, Kasugai S. Bone regeneration by modified gene-activated matrix: effectiveness in segmental tibial defects in rats. Tissue Eng 2006; 12(3): 489-97.
31. Kuroda S, Kondo H, Ohya K, Kasugai S. A new technique with calcium phosphate precipitate enhances efficiency of in vivo plasmid DNA gene transfer. J Pharmacol Sci 2005; 97(2): 227-33.
32. Yue B, Lu B, Dai KR, Zhang XL, Yu CF, Lou JR, et al. BMP2 gene therapy on the repair of bone defects of aged rats. Calcif Tissue Int 2005; 77(6): 395-403.
33. Al-Dosari MS, Gao X. Nonviral Gene Delivery: Principle, Limitations, and Recent Progress. The AAPS Journal 2009; 11(4): 671-81.
34. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 – an update. The Journal of Gene Medicine 2013; 15(2): 65-77.
35. Balazs DA, Godbey W. Liposomes for use in gene delivery. J Drug Deliv 2011; 2011: 326497.
36. Liu SQ. Bioregenerative Engineering: Principles and Applications: Wiley; 2007.
37. Simoes S, Filipe A, Faneca H, Mano M, Penacho N, Duzgunes N, et al. Cationic liposomes for gene delivery. Expert Opin Drug Deliv 2005; 2(2): 237-54.