World Journal of Pharmaceutical ReseaRch
SJIF Impact Factor 5.045
Volume 3, Issue 8, 723-734. Research Article ISSN 2277 – 7105

ENCAPSULATION OF Λ DNA IN CHITOSAN MICROSPHERES AS A GENE DELIVERY VEHICLE

[1]Hire Nitin N*, [2]Derle Dilip V, [3]Bhagat Anirudh B.

[1,2]Department of Pharmaceutics, M.V.P. Samaj's College of Pharmacy, Shivaji Nagar Gangapure Road, Nashik-,422002; Maharashtra, India.

[3]Department of Biotecnology, M.V.P. Samaj's K.T.H.M. College, Shivaji Nagar Gangapure Road, Nashik-422002; Maharashtra, India.

Article Received on
08 August 2014,

Revised on 30 August 2014,
Accepted on 24 Sept 2014

***Correspondence for Author**
Dr. Hire Nitin N
Department of Pharmaceutics, M.V.P. Samaj's College of Pharmacy, Shivaji Nagar Gangapure Road, Nashik Maharashtra, India.

ABSTRACT

The biodegradable polymers like Chitosan are considered as the green eco-friendly materials due their biocompatibility and non-toxic properties. Biodegradable microspheres and nanoparticles have proven to be very useful in protein and DNA delivery systems. These are easily taken up by immunocompetent cells, shows prolonged antigen release characteristics and provide a long lasting immunity. Micro and nano-particulate based protein and DNA delivery systems have its importance for various therapeutic and biomedical applications. Microspheres were formulated by complex coacervation method and characterised for their surface morphology, size, loading efficiency, and release profile study. The microsphere morphology was examined by SEM and Zeta sizer. It was found that Chitosan encapsulated with λ DNA showed loading efficiency more than 82% It was also found that The particle size for Chitosan was varying between 162-373 nm. Release of λ DNA from encapsulated Chitosan microspheres were checked spectrophotometrically with optical density 260 nm for λ DNA, by taking samples at different time intervals dissolved in PBS (phosphate saline buffer, at pH 7.4).

KEYWORDS: Chitosan, SEM, microsphere, *in vitro*.

INTRODUCTION

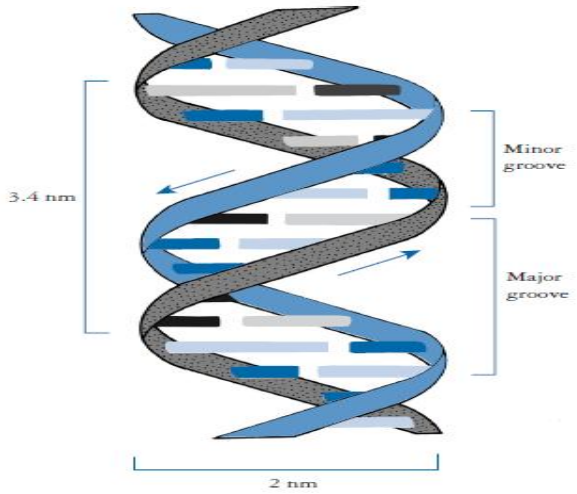
The term genetic engineering is often thought to be rather emotive or even trivial, yet it is probably the label that most people would recognize. However, there are several other terms

that can be used to describe the technology, including gene manipulation, gene cloning, recombinant DNA technology, genetic modification, and the new genetics. There are also legal definitions used in administering regulatory. Mechanisms in countries where genetic engineering is practised. Although there are many diverse and complex techniques involved, the basic principles of genetic manipulation are reasonablysimple. The premise on which the technology is based is that genetic information, encoded by DNA and arranged in the form of genes, is a resource that can be manipulated in various ways to achieve certain goals in both pure and applied science and medicine. many areas in which genetic manipulation is of value, including the following:

1. Basic research on gene structure and function.
2. Production of useful proteins by novel methods.
3. Generation of transgenic plants and animals.
4. Medical diagnosis and treatment.
5. Genome analysis by DNA sequencing.

The discovery of the structure of DNA by James Watson and Francis Crick in 1953 provided the stimulus for the development of geneticsat intense activity and excitement as the main features of the gene and its expression were determined. This work culminated with the establishment of the complete genetic code in 1966 -- the stage was now set for the appearance of the new genetics. In 1967 the enzyme DNA ligase was isolated. This enzyme can join two strands of DNA together, a prerequisite for the construction of recombinan molecules, and can be regarded as a sort of molecular glue. This was followed by the isolation of the first restriction enzyme in 1970, amajor milestone in the development of genetic engineering. Restriction enzymes are essentially molecular scissors that cut DNA at precisely defined sequences. Such enzymes can be used to produce fragments of DNA that are suitable for joining to other fragments. Thus, by 1970, the basic tools required for the construction of recombinant DNA were available. The first recombinant DNA molecules were generated at Stanford University in 1972, utilising the cleavage properties of restriction enzymes (scissors) and the ability of DNA ligase to join DNA strands together (glue). The importance of these first tentative experiments cannot be overestimated. Scientists could now join different DNA molecules together and could link the DNA of one organismto that of a completely different organism.

The DNA molecule *in vivo* usually exists as a right-handed doublehelix called the *B*-form. This is the structure proposed by Watson and Crick in 1953. Alternative forms of DNA include the *A*-form (righthanded helix) and the *Z*-form (left-handed helix). Although DNA structure is a complex topic, particularly when the higher-order arrangements of DNA are considered, a simple representation will suffice here, as shown in Fig. 2.5.



What Are Plasmids

Many types of plasmids are found in nature, in bacteria and some yeast. They are circular DNA molecules, relatively small when compared to the host cell chromosome, that are maintained mostly in an extra chromosomal state. Although plasmids are generally dispensable (*i.e.* not essential for cell growth and division), they often confertraits (such as antibiotic resistance) on the host organism, which can be a selective advantage under certain conditions. The antibiotic resistance genes encoded by plasmid DNA (pDNA) are often used in the construction of vectors for genetic engineering, as they providea convenient means of selecting cells containing the plasmid. When plated on growth medium that contains the appropriate antibiotic, only the plasmid-containing cells will survive. This is a very simple and powerful selection method. Plasmids can be classified into two groups, conjugative and non-conjugative plasmids. Conjugative plasmids can mediate their own transfer between bacteria by the process of conjugation, which requires functions specified by the *tra* (transfer) and *mob* (mobilising) regions carried on the plasmid. Non-conjugative plasmids are not self transmissible but may be mobilised by a conjugation-proficient plasmid.

Diagnosis of Infection

Despite traditional methods being applied in many cases, there may be times when these methods are not appropriate. Infection by in some cases, viral infections the human immunodeficiency virus (HIV) is one case in point. The virus is the causative agent of acquired immune deficiency syndrome (AIDS). The standard test for HIV infection requires immunological detection of anti-HIV antibodies, using techniques such as ELISA (enzyme linked inmmunosorbent assay, sometimes known as the enzyme immunoassay), Western blot, and IFA (indirect immunofluorescence assay). Patterns of inheritance

Since it was rediscovered in 1900, the work of Gregor Mendel has formed the basis for our understanding of how genetic characteristics are passed on from one generation to the next. We have already seen that the human genome is made up of some 3 billion base pairs of information. This is organized as a diploid set of 46 chromosomes, arranged as 22 pairs of autosomes and one pair of sex chromosomes. Prior to reproduction, the haploid male and female gametes (sperm and oocyte, respectively) are formed by the reduction division of meiosis, which reduces the chromosome number to 23. Onfertilisation of the oocyte by the sperm, diploid status is restored, with the zygote receiving one member of each chromosome pair from the father and one from the mother. In males the sex chromosomes areX and Y, in females XX, and thus it is the father that determines the sex of the child.

Genetically Based Disease Conditions

Genetic problems may arise from either chromosomal abnormalities(aberrations) or gene mutations. An abnormal chromosome complement can involve whole chromosome sets (variation in the ploidynumber, such as triploid, tetraploid, *etc.*) or individual chromosomes Although chromosomal abnormalities are a very important type of genetic defect, it is in the characterisation of gene mutations that molecular genetics has had the most impact. Many diseases have now been almost completely characterised, with their mode of transmission and action defined at both the chromosomal and molecular levels. We will consider some of these in more detail to outline how a disease can be characterised in terms of the effects of a mutated gene.C ystic fibrosis (CF) are the most common genetically based disease found in Western Caucasians, appearing with a frequency of around1 in 2000--2500 live births. It is transmitted as an autosomal recessive characteristic and, therefore, the birth of an affected child may be the first sign that there is a problem in the family.

Material and Method

Standard λ DNA was obtained from Genei PVT Bangalore, India.

Chitosan was obtained as a gift sample from Central institute of fisheries technology, Cochin, India. Na_2SO_4 and all other chemicals were obtained from Sigma.

Ultraviolet (Uv) Spectroscopy

UV spectrum of 200 µg/ml solution of the λ DNA in 1.5 ml of water was recorded in the range of wavelength from 200nm to 400nm using Visible Double beam Spectrophotometer (UV-250 1PC, Shimadzu).

Preparation of Dna – Loaded Microspheres

Plasmid loaded chitosan microspheres were prepared by complex co-acervation method as sodium sulphate solution (20% w/v) containing plasmids were dropped into the chitosan solution (0.50% w/v) and stirred at 500 rpm for 1 hour. Formed particles were separated by centrifugation for 10 min at 12000 rpm and stored at 4^0C after freeze-drying

In Vitro Release Studies

Releases of plasmids from chitosan microspheres was determined in phosphate buffered saline (PBS, pH 7.4) at 37 ± 0.50C and at appropriate time intervals samples were taken and supernatants were separated by centrifugation. The release DNA was measured spectrophotometric ally at 260 nm. After each sampling, the microspheres were resuspended in the fresh medium. Corrections due to chitosan were also made during the spectrophotometric measurement therefore empty microspheres were used as a blank. Released samples were checked with agarose gel electrophoresis. For this purpose, released plasmid DNA was precipitated by ethanol and dissolved in TAE buffer prior to electrophoresis

Gel Electrophoresis

Plasmid DNA stability and topology were assessed by 1.0% agarose gel electrophoroesis λ DNA (50 µg equivalent weight of DNA in each lane) was applied using TAE buffer (24.2 gm of tris base 57.1 ml glacial acetic acid and 0.5 M EDTA, pH~8.). DNA was visualized under the UV light.

Zeta Potential

The microparticles were dispersed in deionized water at pH 6.0 and the surface charge (zeta potential) was measured by laser doppler anemometry using a Zetamaster (Malvern, UK).

Scanning Electron Microscopy

Scanning electron photomicrograph of λ DNA loaded Chitosan microspheres were taken. A small amount of microspheres was spread on glass stub. Afterwards the stub containing the sample was placed in the scanning electron microscope chamber. Scanning electron photomicrograph was taken at the acceleration voltage of 20 KV, Chamber pressure of 0.6 mm Hg. At different magnification the photomicrograph of microspheres is depicted in Fig.

Automated Total Reflectance (ATR)

ATR spectra were taken (Alpha, 1005151/06, Bruker) instrument to investigate the possible chemical interactions between the drug and polymer λ dna, Chitosan loaded microspheres were scanned in the range between 4000 and 500 cm^{-1}.

RESULT AND DISCUSSION

Preparation of λ DNA Microspheres

λ DNA Microspheres were prepared by complex coacervation method. Briefly, sodium sulphate solution (20% w/v) containing plasmids were dropped into the chitosan solution (0.50% w/v) and stirred at 500 rpm for 1 hour. Formed particle were separated by centrifugation for 10 min at 12000 rpm and stored at 4^0C after freeze-drying Size of microspheres was determined by using an ocular micrometer in a light microscopy.

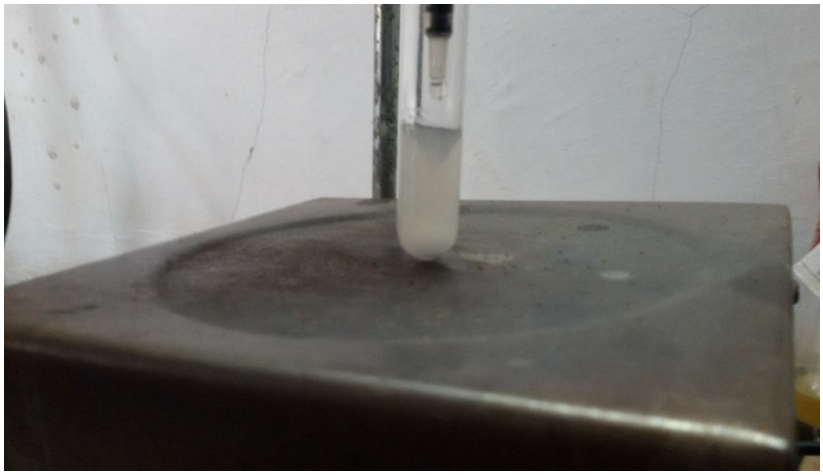


Fig: - 1Preparation of λ DNA Microspheres using magnetic stirrer.

Ultra-Violet (UV) Absorption Spectroscopy

Ultra-Violet (UV) absorption spectroscopy is mostly used for quantitative analysis but this may be used to characterize the drug. The drug sample showed good absorptivity in UV range of the radiation. Wavelength of maximum absorption (λ_{max}) were found to be matching

with the reported values λ DNA showed maximum absorption at shorter wavelength in Distilled water shown by fig:2

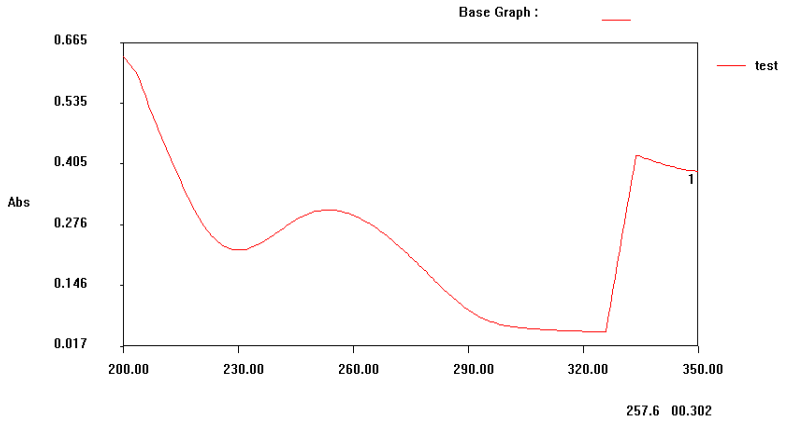


Figure:-2 UV spectra of the obtained λ DNA in distilled water

In Vitro Release Study

Within a period of seven days study, Chitosan microspheres released almost 75% of the encapsulated plasmid DNA. The physico-chemical properties of the stabilizer added seem to affect the release profile significantly. The DNA diffusion was expected due to the porous network like structure formed in particles during lyophilisation process. The viscosity of the solution inside the particles increase due to hydration of the of the polymer chains. Depending upon the types and quantity of stabilizer, drug solubilisation via changes in internal matrix pH, rate and extent of matrix hydration and polymer erosion can be demonstrated (Chambina *et al.*, 2004). The release profile of these particles showed a biphasic pattern of DNA release. Within first hour, the antigen is released as burst release and gradually the rate of release decreases. For smaller particles, a large number of antigen accumulated on the surface resulting in a greater initial burst release (Rin *et al.*, 2005).

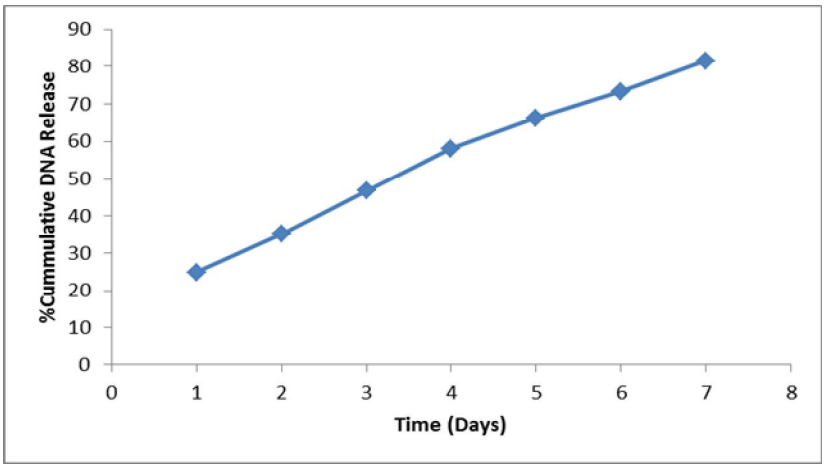


Figure:-3 λ DNA release pattern with chitosan Microspheres.

Surface Characterization by SEM

The morphology of Chitosan microspheres were spherical structures as determined using scanning electron microscope (SEM) as shown in fig.4 The surface of the particles are rough and rounded that possesses pores of varying size.

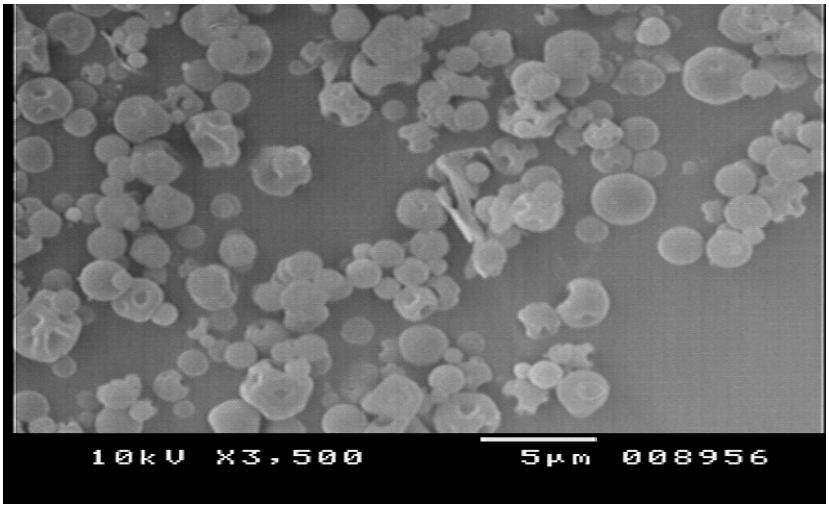


Figure:-4 Scanning electron microscopy of λ DNA- Chitosan microspheres at 5000 magnification.

Zeta Potential

The microspheres were suspended in Phosphate buffer (pH 1.2) for 30 minutes. The suspension (2% w/v) was employed for the determination of zeta potential. The results are presented in figure no.1

Table: 1 Zeta Potential of Selected Microsphere Formulations

Formulation	Zeta potential (mV)
λDNAChitosanMicrosphere	20.24

Zeta potential measurements were used to determine the surface charge on the particles prepared by complex coacervation method The incorporation of chitosan reduced the overall negative surface charge in particles fabricated both with and without DNA The surface charge became positive for blank particles in which half or more chitosan was incorporated, and only in these particles was there a statistical difference ($p < 0.05$) in surface charge with DNA (Fig. 5). Of the formulations evaluated, chitosan resulted in a positively charged surface when fabricated with DNA (Fig. 5).

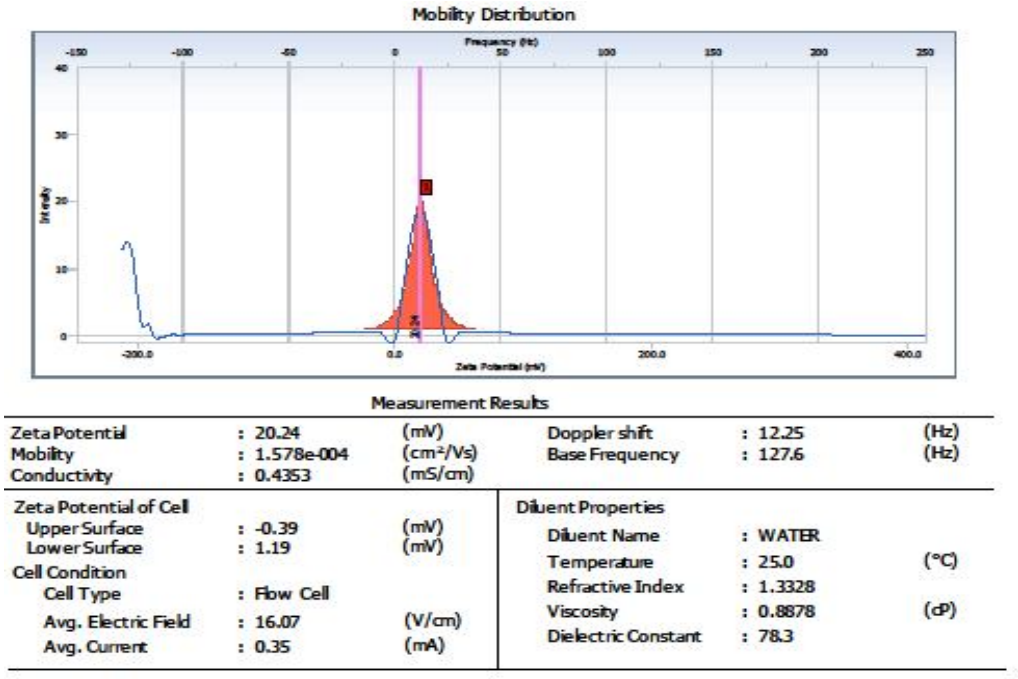


Figure:-5 Zeta potential of λ DNA Chitosan Microspheres

Automated Total Reflectance (ATR)

ATR can be considered as first line analytical technique to study compatibility of drug with excipients. Figure: 6 showed that characteristic ATR absorption peaks of λ DNA and polymer can be observed in ATR spectrum of the mixture of λ DNA and polymer. This indicated that there was no chemical reaction between drug and the polymers used.

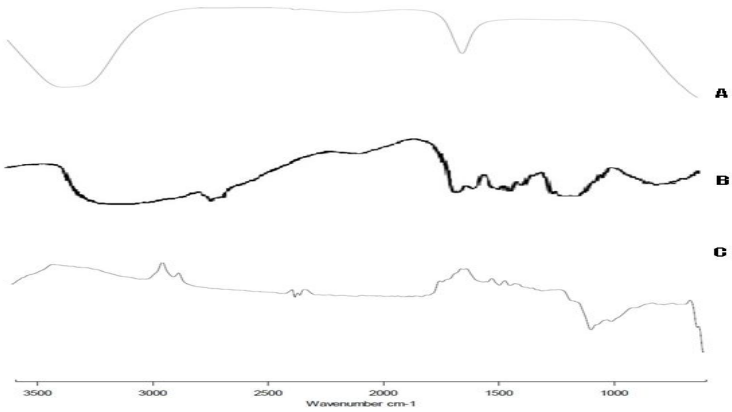


Figure:-6 ATR Spectra of A Λ DNA, B Chitosan, C Λ DNA and Chitosan Microspheres

Agarose Gel Electrophoresis of Λ Dna

After encapsulation process, agarose gel electrophoresis was carried out to assess the integrity of encapsulated λ DNA. After digestion with PBS, microspheres extracts were

separated and supernatants applied on agarose gel and bands were compared with the bands of λ DNA. No change was observed in the electrophoretic mobility of DNA (Figure: 7).As seen in the gel photograph, lane A showed the bands similar to that of the λ DNA

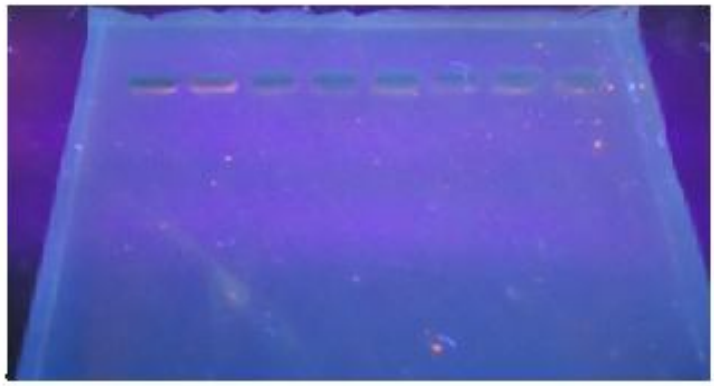


Figure:-7 Agarose gel electrophoresis of released λ DNA from chitosan microspheres.

Lane A Free λ DNA

Lane B λ DNA from chitosan microspheres.

CONCLUSION

The results of this study suggest that a complex coacervation system is an excellent choice for sustained gene delivery and that it can potentially be utilized for delivery of multiple genes. In conclusion, it was shown that microspheres are released for extended period of 7 days from a complex coacervation system; probably on account of bio molecular interaction and these released complexes are capable of transfection both *in vitro* and *in vivo*. Thus, it is Evident that the complex coacervation system provides an enhanced method of extended Transfer of genes.

ACKNOWLWDGEMENT

The author thanks to Prof Anirudha Bhagat for providing λ DNA And agarose gel electrophoresis for studies.

REFERENCES

1. Roemer, K. and Friedmann, T. (Concept and strategies for human gene therapy). Eur. J. Biochem, 1992; 208:211-225.

2. Lasic D.D. and Templeton, N.S., (Liposomes in gene delivery. Adv. Delivery Res), 1996; 20:221-266.
3. Lee, K.Y., Kwon, I.C., Kim, Y.H., Jo, W.H. and Jeong, S.Y. (Preparation of chitosan self-aggregates as a gene delivery system.) J. Cont. rel, 1998; 51:213-220.
4. Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C. J., Tsai, Y.J., Border, R., Ramsey, P., Martini, M. and Felgner, P.L., (Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulation). J. Biol. Chem. 1994; 269: 2550-2561.
5. Tinsley- Bown, A.M., Fretwell, R, Dowsett, A.B., Davis, S.L. and Farrar, G.H., (Formulation of poly(D,L-lactic co-glycolic acid) micro particles for rapid plasmid DNA delivery) .J. Contr. Rel, 2000; 66:229-241.
6. Truong-Le, V.L., August, J.T. and Leong, K.W., (Controlled gene delivery DNA – gelatin nanospheres. Human gene ther), 1998; 9(12):1709-1717.
7. Wang, D., Robinson, D.R., Kwon, G.S. and Samuel, J., (Encapsulation of plasmid DNA in biodegradable poly (D,L-lactic- co-glycolic acid) microspheres as a novel approach for immunogene delivery).J.Contr.Rel, 1999; 57:9-18.
8. Walter, E, Moelling, K, P Pavlovic, J. and Merkle, H.P, (Microencapsulation of DNA using poly (D, L-lactic –co-glycolide) stability issues and release characteristics). *J.Contr. Rel.1999;* 61:361-374.
9. Carrero- Gomez, B. and Duncan, R., (Evaluation of the biological properties of soluble chitosan and chitosan microspheres.) Int. J. Pharm, 1997; 148:131-140.
10. Leong, K.W., Mao, H.Q., Truong-Le, V.L., Roy, K Walsh, S.M. and August, J.T., (DNA-polycation nanospheres as non- viral gene delivery vehicles). J. Cont. Rel, 1998; 53:183-193.
11. Aral, C., Ozbas- Turan, S., Kabasakal, L., Keyer- Uysal, M. and Akbuga, J., (Studies of effective factors of plasmid DNA- loaded chitosan microspheres: I Plasmid size, chitosan concentration and plasmid addition techniques). STP Pharma. Sci, 2000; 10:83-88.
12. Akbuga, J., Kabasakal, L. and Ozbas- Turan, S., (Physical and transfection properties of aged DNA- chitosan microspheres. 10th Annual Meeting of European Society of Gene Therapy, Antibes) - France, 2002.
13. Mao, H.Q., Roy, K., Truong- Le, V., August, J.T. and Leong, K.W., (DNA- chitosan nanospheres: Derivazation and storage stavility). Proceed. Int. Symp. Control. Rel. Bioact. Mater, 1997; 24:671-672.
14. Birnboim, H.C., (A rapid alkaline extraction method for the isolation of plasmid DNA. Meth. Enzymol), 1983; 100:243-255.

15. Berthold, A., Cremer, K. and Kreuter J., (Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs). J. Cont. Rel, 1996; 39:17-25.
16. Mao, H- Q., Roy, K., Troung- Le, V.L., Janes, K.A., Lin, K.Y., Y., August, J.T. and Leong, K.W., (Chitosan DNA nanoparticles as gene carriers: synthesis characterization and transfection efficiency). J. Contr. Rel, 2001; 70:399- 421.
17. Bradford, M.M., (A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein- dye binding. Anal, Biochem), 1976; 72:248-254.
18. 18. Bancroft, J.D.; Cook, H.C. (Manual of histological techniques and their diagnostic application. Churchill Livingstone, London, 1994.