BIODEGRADABLE POLYMERS (PLA AND PLGA) BASED NANOPARTICLES IN PROTEIN AND PLASMID DNA DELIVERY

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Life science

SUBMITTED BY

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CERTIFICATE

This is to certify that the thesis entitled "**BIODEGRADABLE POLYMERS (PLA AND PLGA) BASED NANOPARTICLES IN PROTEIN AND PLASMID DNA DELIVERY**" submitted to National Institute of Technology, Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by Kautilya Kumar Jena under my supervisions and guidance.

Dr.(Miss) B.Nayak

Advisor

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DECLARATION

I hereby declare that the thesis entitled "Biodegradable Polymers (PLA and PLGA) Based Nanoparticles in Protein and Plasmid DNA Delivery", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafied and original research work carried out by me under the guidance and supervision of Dr. (Miss) Bismita Nayak, Assistant Professor, Department of Life Science , National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:
Place: NIT, Rourkela KAUTILYA KUMAR JENA

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ABSTRACT

The biodegradable polymers like poly lactic acid (PLA) and poly (lactide-co-glycolic acid) (PLGA) 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. PLA and PLGA microparticles and nanoparticles were formulated by double solvent emulsion evaporation (w/o/w) method and characterised for their surface morphology, size, loading efficiency and release profile study. The microsphere and nanosphere morphology were examined by SEM and Zeta sizer. It was found that PLA encapsulated with BSA (2.5%) showed loading efficiency more than 82% and that with plasmid DNA (Concentration: 1mg/ml), it was found to be 41%. It was also found that the particle size for PLA was varying between 162-373 nm. Similarly for PLGA particles when encapsulated with BSA, the loading efficiency became 91% whereas for encapsulated plasmid DNA, the loading efficiency was 44%, with their respective particle size between 113-335 nm. *In vitro* release of BSA and plasmid DNA from encapsulated PLA and PLGA nanoparticles were checked spectrophotometrically with optical density 562 nm for protein and 260 nm in case of plasmid DNA, by taking samples at different time intervals dissolved in PBS (phosphate saline buffer, at pH 7.4).

Key Words: PLA, PLGA, BSA, SEM, nanosphere, microsphere, *in vitro*.

INTRODUCTION

Vaccines are very effective means to control or eradicate microbial transmissible diseases, and also effective for immunotherapeutic point of view. There are some difficulties associated with certain vaccines such as: (i) the requirements of multiple injections schedule primary immunization followed by periodic boosters as required maintaining immunity. (ii) A short product shelf life at room temperature requiring refrigeration. (iii) The induction of a biased immune response towards the humoral system. These are not optimal for many applications such as intracellular viral, bacterial and parasitic infections as well as tumour immunotherapy. So, new antigen delivery technologies are essential to fulfil some of these limitations.

Modern vaccinology emphasises on sub unit immunogens or related nucleic acids. These vaccines primarily consist of proteins or polysaccharide antigens and the related DNA, RNA or oligonucleotides from the target pathogen. We need delivery system for the delivery of antigens. A delivery system for antigens and vaccine DNA may be defined as the pharmaceutical formulation that enhances or facilitates the action of antigen or vaccine by delivering, ideally the correct amount of antigen or vaccine to the site of action at the correct rate and timing, in order to maximize the immunological response and minimize the undesired effects. In a more extended definition, antigens and DNA vaccine delivery system may also encompass the controlled release of specific maturation stimuli for the antigen presenting cells. Thus it can be derived from these definitions that a delivery system for antigen and vaccine DNA is a modern and sophisticated form of an adjuvant with engineered immunological properties.

Polymeric micro and nanoparticles, as well as colloidal lipid and surfactant-based particulate delivery system, play an increasing role in vaccine development. Besides exhibiting adjuvant properties, some of these particulate systems are also designed to deliver the antigen or related nucleic acid in a controlled, sustained manner, so that fewer injections are needed to provide a fully protective response. DNA delivery system for vaccines has a strong emphasis on biodegradable micro and nanospheres, in addition to virosomes and immune stimulating complexes, all of which posses high potential as both preventive and therapeutic vaccines for parenteral, nasal and possibly oral administration.

Nanoparticulate delivery systems, such as those based on poly (lactic-co-glycolic acid) (PLGA) and poly (lactic acid) (PLA) polymers, have been studied since many years. For the past three decades, lots of work has been done to utilize biocompatible and biodegradable polymers for drug delivery systems. PLGA and PLA polymers have the advantage of being well characterized and commercially used for microparticulate and nanoparticulate drug delivery systems (Allemann and Leroux, 1999). PLGA and PLA polymers are biocompatible, biodegradable. Polymeric nanoparticles are widely use as pharmaceutical dosage form of proteins and peptides. Many methods are recently applied for the preparation of polymeric nanoparticles, such as emulsification–evaporation method, salting-out procedure and nanoprecipitation method. Nevertheless, several difficulties have been found for adopting these methods. The usage of solvent may cause toxicity, stabilizers such as polyvinyl alcohol (PVA) cannot be accepted for intravascular usage, and the salts are incompatible with bioactive compound.

Poly (lactic acid) (PLA):

Fully biodegradable synthetic polymers have been available since many years, such as poly (lactic acids) (PLA). Among all biopolymers, PLA was extensively studied in medical implants, suture, and drug delivery systems since 1980s due to its biodegradability (fig. 2). The structure of PLA polymer is given in fig. 1.

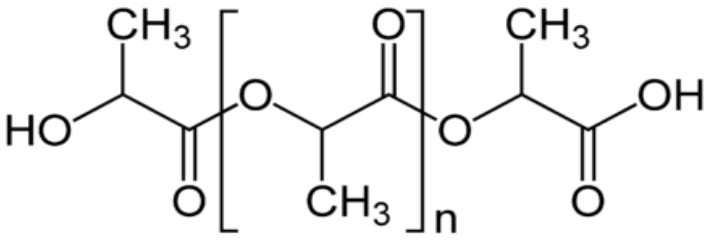


Fig. 1: Structure of Poly lactic-acid; n- no of chains

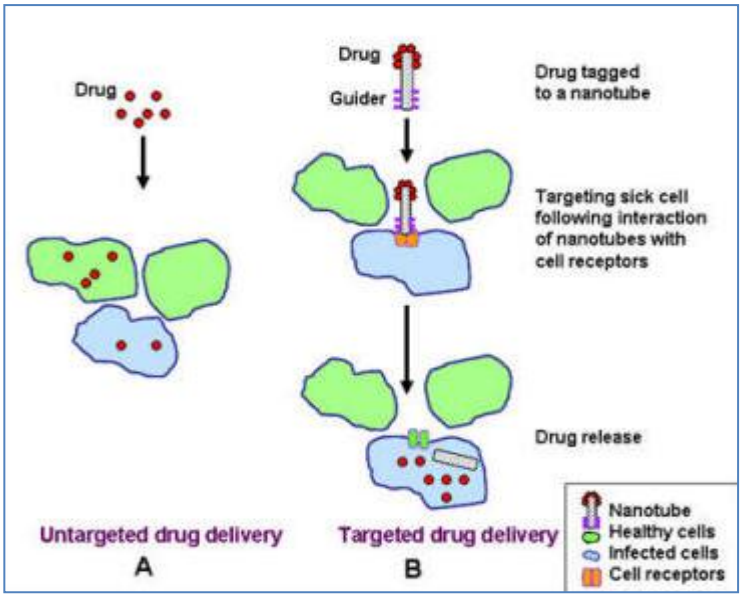


Fig. 2: NTS (Nanotechsystems inc.) utilizes polylactic acid (PLA) based nano particles that have been formulated to encapsulate a drug, allowing for an intracellular site of action. In this case, the drug binds to the cytoplasmic receptors and the subsequent drug-receptor complex is transported to the nucleus resulting in the expression of the drug product.

Poly (lactic-co-glycolic acid) (PLGA):

Over the past few decades, biodegradable polyesters, such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA), have been extensively studied for a wide variety of pharmaceutical and biomedical applications. The biodegradable polyester family has been regarded as one of the few synthetic biodegradable polymers with controllable biodegradability, excellent biocompatibility, and high safety. Among these polyesters PLGA plays an important role in drug delivery system. The structure of PLGA as given in fig. 3.

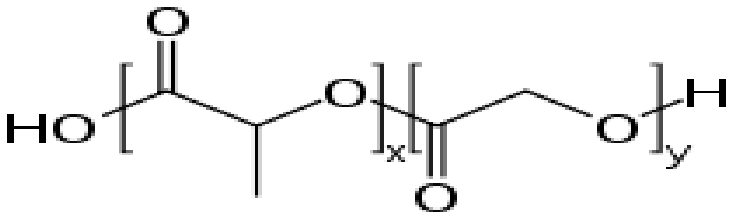


Fig. 3: Structure of poly (lactic-*co*-glycolic acid). *x*= number of units of lactic acid; *y*= number of units of glycolic acid.

Poly (lactic-co-glycolic acid) have also been called poly (lactide-co-glycolide), according to the nomenclature system based on the source of the polymer. Although the name was used in many references in the past, a recent trend is to follow the nomenclature system of the International Union of Pure and Applied Chemistry (IUPAC) that is based on the repeating unit structure. PLGA can be degraded into non-toxic substances and removed from the human body. Accordingly, they have taken centre stages in a variety of research efforts.

Biodegradable poly (lactic-*co*-glycolic acid) (PLGA) and poly (lactic acid) (PLA) polymers show interesting properties for biotechnology through their biocompatibility and their authorization by the Food and Drug Administration (FDA) for drug delivery. Various polymeric drug delivery systems like microparticles or nanoparticles have been developed using these polymers for the delivery of a variety of drugs (Jain, 2000). However, the technology processes often use organic solvents to dissolve the water-insoluble PLGA. Usually, halogenated solvents, such as methylene chloride and di-chloro methane are used in the microencapsulation process.

OBJECTIVES:

1. To prepare microparticles and nanoparticles based drug delivery system using natural polymer (PLA and PLGA) with biocompatible properties.
2. Formulation and evaluation of protein and DNA delivery system using PLA and PLGA nanoparicles and microparticles.
3. Protein (Bovine Serum Albumin) was taken from Sigma chemicals and plasmid DNA (*E. coli*) was purified, encapsulated into these polymer particles.
4. Characterization of prepared nanoparticles and microparticles like measurement of encapsulation efficiency, release study and surface characterization by SEM.
5. To prepare an efficient oral, intranasal and intramuscular drug delivery system using these biodegradable PLA/PLGA nanoparticles.

REVIEW OF LITERATURE

Biodegradable particles (0.1–1.5μm) prepared from poly(lactide-co-glycolide) (PLGA) and poly(lactic acid) (PLA) polymers have generated considerable interest in recent years for their use as a delivery vehicle for various pharmaceutical agents. According to Perrin and English, (1997) these polymers are the most common biodegradable polymer used for the controlled delivery of drugs due to its early use and approval as a compatible biomaterial in humans. Lewis, (1990) reported that, by varying the molecular weight and lactide /glycolide ratio, the degradation time of the PLA and PLGA and the release kinetics of the active agent can be controlled.

The multiple emulsion-solvent evaporation technique being used for preparation of PLGA and PLA nanoparticles is believed to produce heterogeneous size distribution. Various formulation factors and characteristics of the nanoparticles have a key role to play in biological applications like drug delivery systems. The foremost factor that could have an influence on the transfection and cellular uptake is the size of the nanoparticles. Prabha *et al.,* (2002) have studied the size-dependency of nanoparticle-mediated gene (plasmid DNA) transfection with fractionated nanoparticles. Recent reports suggests that a fraction of the stabilizer PVA always remains associated with the nanoparticles despite repeated washings because PVA forms an interconnected network with the polymer at the interface. We came across similar factors while formulating nanoparticles using PVA as a stabilizer. Above all, the stability and biological activity of the plasmid have been major concerns due to the involvement of organic solvents during the preparation process.

Formulation of PLA and PLGA polymeric particles:

Lemoine *et al.,* (1996) reported that, biodegradable colloidal particles have received considerable attention as a possible means of delivering drugs and genes by several routes of administration. Special interest has been focused on the use of particles prepared from polyesters like PLGA, due to their biocompatibility and resorbability through normal bioprocesses of the body. Various methods have been reported for making nanoparticles viz., emulsion-evaporation (Gurny *et al.,* 1981), salting-out technique (Allemann *et al.,* 1992), nanoprecipitation (Fessi *et al.,* 1989), cross-flow filtration (Quintanar-Guerrero *et al.,* 1998) or emulsion-diffusion technique

(Choi *et al.,* 2002 and Niwa *et al.,* 1993). Indeed PLGA particles are extensively investigated for drug (Schachter and Kohn, 2002 and Lamprecht *et al.,* 2001) and gene delivery (Cohen-Sacks *et al.,* 2002 and Prabha *et al.,* 2002), but still improvements in the existing methods are needed to overcome the difficulties in terms of reproducibility, size, and shape. The size and shape of the colloidal particles are influenced by the stabilizer and the solvent used. Most investigated stabilizers for PLGA lead to negatively charged particles and the plasmid incorporation is achieved via double emulsion technique during particle preparation. This could generate problems in the stability and biological activity of the plasmid due to the involvement of organic solvents during the preparation processes. This can be overcome by using cationically modified particles that can bind and condense negatively charged plasmids by simply adhering / encapsulating the plasmid or vice versa. Vandervoort and Ludwig. (2002), suggested that PVA as most popular stabilizer for the production of PLGA nanoparticles leading to negatively charged particles, nevertheless, investigations have been carried out using other stabilizers as well.

PLA and PLGA Particles in Protein Delivery:

According to Bittner *et al.,* (1998), PLGA has a negative effect on protein stability during the preparation and storage, primarily due to the acid-catalyzed nature of its degradation. Its hydrolysis leads to the accumulation of acidic monomers, lactic and glycolic acids within the drug delivery device, thereby resulting in a significant reduction of pH of the microenvironment and denaturation of the encapsulated proteins. In addition, processing conditions used in the manufacturing of PLGA drug delivery vehicles have detrimental effects on certain protein secondary structures (Johansen *et al.,* 1998).

For encapsulating peptide or protein using PLGA NPs/MPs, mainly three methods are used: water–oil–water (w/o/w) emulsion technique, phase separation methods and spray drying (Freitas, 2005). Peptides or proteins are either dispersed in an organic solution of PLGA or preferably processed in an aqueous solution of water-in-oil (w/o) emulsion. The dispersion step is carried out using high speed sonicator.

Raghavendra *et al.,* (2008), reported that, MPs are produced by either extracting organic solvent or by adding a non-solvent i.e., silicone oil, thereby inducing coacervation. The first process is frequently referred as w/o/w method, while the latter

is known as the phase separation technique. In both the cases, particle formation occurs in the liquid phase. In spray drying technique, particle formation is achieved by atomizing the emulsion into a stream of hot air under vigorous solvent evaporation. Different methods are schematically displayed in Fig: 4.

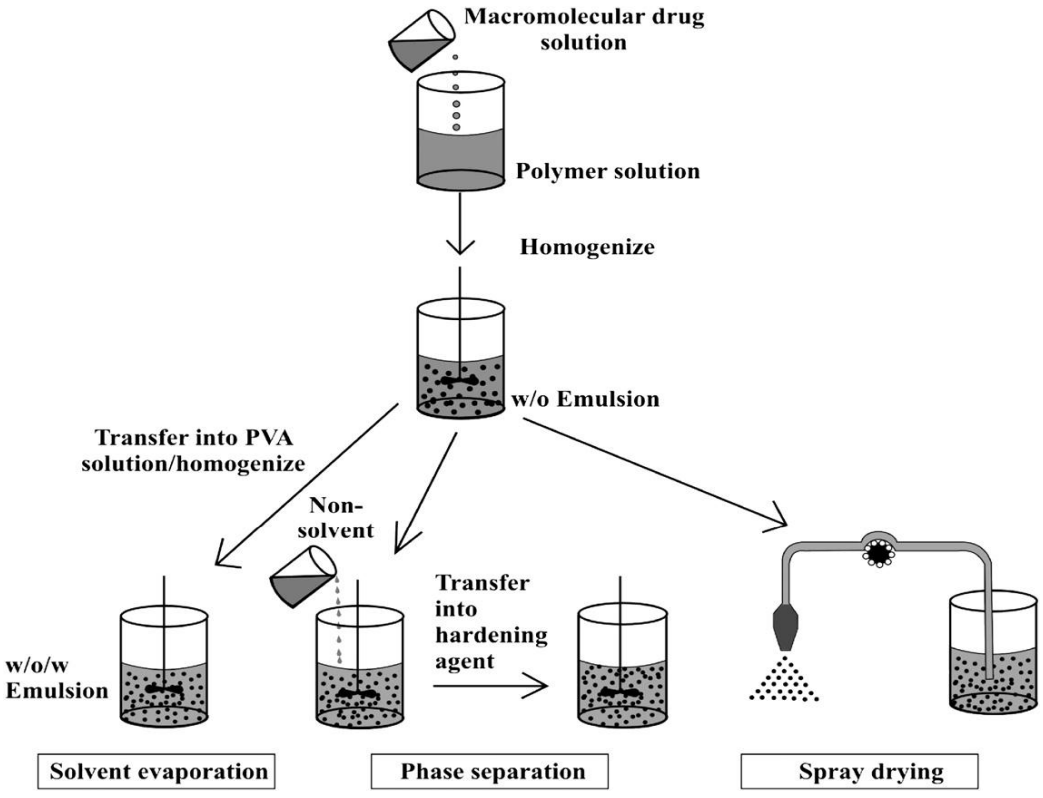


Fig. 4: Comparison of microencapsulation methods: (i) solvent evaporation, (ii) polymer phase separation and (iii) spray drying. Aqueous solution is dispersed in the organic polymer solution by ultrasonication (w/o) emulsion; the w/o emulsion is processed further by specific methods to prepare the drug-loaded microparticles

According to Raghavendra *et al.*, (2008), proteins encapsulated by w/o or w/o/w techniques into NP or MP are susceptible to denaturation, aggregation, oxidation and cleavage, particularly at the aqueous phase-solvent interface. Protein denaturation may also result in a loss of biological activity. Improved protein integrity has been achieved by the addition of stabilizers such as carrier proteins (e.g., albumin), surfactants during the primary emulsion phase or molecules such as trehalose and mannitol to the protein phase. Protein stability may also be enhanced if the protein is encapsulated as a solid rather than in solution. It should be noted that all the nano/micro-encapsulation

techniques create mechanical, thermal and chemical stresses on the system under investigation.

PLA and PLGA Particles for Plasmid DNA Delivery:

Biodegradable poly (lactide) (PLA) or poly (lactide-co-glycolide) (PLGA) microparticles and nanoparticles were demonstrated to represent a potent delivery platform for DNA vaccines (Johansen *et al.,* 2000). When embedded in biodegradable polymeric particles, encapsulated plasmid DNA can be protected from enzymatic degradation and released in a controlled manner, mimicking conventional vaccines (Thomasin *et al.*, 1996).

According to Niidome and Huang (2002), plasmid DNA delivery by physical methods generally results in low but sustained expression *in vivo*, which is limited by poor uptake due to factors such as degradation and clearance.

Herweijer and Wolff (2003), reported that, physical methods (e.g., ultrasound, hydrodynamic injection) are continually being improved to enhance cellular uptake of DNA by altering cell permeability. Intrinsic cellular processes may be involved due to plasmid uptake, but the processes governing intracellular transport remain elusive. Following delivery to the nucleus, expression can typically occur over time scales of days to weeks or months. Extracellular factors that limit delivery include plasmid clearance or degradation, which can be mediated by sequence-specific recognition from the immune system. Immune responses to the plasmid are affected by the methylation pattern of CpG sequences that can affect the duration of transgene expression.

Beginning in the 1980s, various groups could demonstrate that intramuscular injection of plasmid DNA led to its transcription in myocytes resulting in the secretion of the encoded protein (Benevisty and Rashef, 1986; and Wolff *et al.*, 1990). Then Tighe *et al.,* (1998) and Srivastava and Liu (2003) reported that, specific antibodies against the encoded proteins related to the Th1 pathway were found in the serum of the vaccinated animals. Using diverse delivery routes (intramuscular, intradermal, subcutaneous or oral), a variety of animal models and various doses of DNA, it was shown that DNA vaccination can be efficient to concomitantly induce Th1 immune response with antibody production and cytotoxic T lymphocyte (CTL) response (Jilek et al., 2005).

Ledley (1996), reported that, polymeric delivery represents an alternative approach that can increase residence time within the tissue and protect against

degradation. Plasmids (10^3–10^4 bp) have effective hydrodynamic diameters in excess of 100 nm and a highly negative surface charge density. The large size of the DNA limits transport through tissues, resulting in diffusion coefficients on the order of 10^{-9} to 10^{-12} cm^2/s (Zaharoff *et al.,* 2002), and promotes localized delivery when polymers are inserted into a tissue (Bajaj and Andreadis, 2001).

In vitro Release study

Biodegradable microparticles prepared from PLA or PLGA have been studied for their controlled release properties for more than a decade (Johansen *et al.,* 2000). Their main advantages for this purpose are their technical versatility, biocompatibility and biodegradability (Berkland *et al.,* 2002 and Berkland *et al.,* 2003). Indeed, various parameters such as polymer composition, size or surface properties can be customized to achieve a distinct polymer erosion profile in order to control the release of the encapsulated therapeutics (Walter *et al.,* 1999).

Plasmid DNA interacts weakly with many polymers leading to in vitro release from the vehicle with rates modulated by the polymer properties. Many synthetic and natural polymers are negatively charged, and thus the weak interactions likely result from repulsive charge interactions between plasmid and polymer.

According to Ochiya *et al*., (2001) and Shea *et al*., (1999), controlled release systems typically employ polymeric biomaterials that deliver vectors according to two general mechanisms: (i) polymeric release, in which the DNA is released from the polymer, or (ii) substrate-mediated delivery, in which DNA is retained at the surface. For polymeric release, DNA is entrapped within the material and released into the environment, with release typically occurring through a combination of diffusion and polymer degradation.

Cleland *et al.,* 1997 and Thomasin *et al.,* 1996, reported that PLGA MS can provide antigen release over weeks and months following continuous or pulsatile kinetics. It was hoped that the pulsatile antigen release would mimic the booster doses necessary with most other nonlive vaccines (Aguado and Lambert, 1992) by controlling polymer properties (Kissel *et al.,* 1997) and due to the fact that PLGA MS are readily recognised and ingested by macrophages and dendritic cells, an important property for stimulating the immune system (Walter *et al.,* 2001).

A major problem hindering the progression of MS based vaccine formulations for human use is the issue of antigen stability during microencapsulation, storage and release (Hanes *et al.,* 1997 and Uchida *et al.,* 1996). Nonetheless, means to retain and maintain antigen stability and immunogenicity have been proposed (Johansen *et al.,* 1998; Sanchez *et al.,* 1999 and Lee *et al.,* 1997). Consequently, this review will focus on in vitro antigen stability and release issues, with an attempt to elaborate on some of the different approaches and strategies employed to overcome these limiting factors.

OBJECTIVES

- To formulate an efficient drug delivery system using natural polymers (PLA and PLGA) to enhance the release and stability of plasmid and protenaceous drugs.
- To protect the protein and DNA property from degradation with the addition of stabilizer.

PLANS OF WORK .

Preparation of PLA and PLGA microparticles/nanoparticles

↓↓

Loading bovine serum albumin (BSA) and plasmid DNA to PLA and PLGA microparticles/nanoparticles

↓↓

Calculation of LC (Loading efficiency) using BCA protein estimation method

↓↓

Study of in-vitro release of protein and DNA from PLA and PLGA particles

↓↓

Measurement of particle size and zeta potential using Zeta sizer

↓↓

Morphological characterization by using scanning electron microscope (SEM)

MATERIALS AND METHODS

MATERIALS

- Poly(L-lactic)acid (PLA)[Sigma-Aldrich]
- Poly(lactic-*co*-glycolic acid) (PLGA) [Sigma- Aldrich]
- Bovine serum albumin fraction –V(HiMedia)
- Poly vinyl alcohol(PVA) [Sigma-Aldrich]
- BCA™ protein estimation kit
- Ultrapure water from Milli-Q water system.

EQUIPMENTS

- Stratos low-temperature high-speed centrifuge(Thermo, Germany)
- Cooling centrifuge (REMI)
- Freeze Dryer (Lab Tech)
- Magnetic stirrer
- Sonicator
- Zeta sizer (Malvern)
- Scanning electron microscope
- Fourier transform infra-red (FTIR)
- Gel electrophoresis unit (Biorad)
- Gel documentation system (BioRad)

METHODS:

Isolation of plasmid DNA from *E. Coli*

The plasmid was isolated from *E.coli*. Primary culture was done in Maconkey agar medium then the culture was transferred to Luria Bertni broth medium and kept in shaker incubator for 48 hours at 37^{o}C. Then plasmid was isolated from the culture by mini preparation method following steps.

- First 1.5 ml culture was transferred in to 1.5 ml eppendrof tube.
- Then centrifuge was done at 11,500 rpm for 5 mins at 4^{o}C.
- After centrifugation the supernatant was removed by decanting.
- The pellet was resuspended in the little (20-30µl) remaining supernatant by vortex.
- 300µl of TENS (at 37^{0}C) was added and vortex vigorously for 30 seconds.
- 150µl of 3M Sodium acetate (pH- 5.2), was added at RT and vortex for 30 sec.
- Centrifuge was done at 12,000 rpm for 5 mins at 4^{o}C.

- The supernatant was then transferred to a clean 1.5 ml eppendorf tube (300µl).
- 1 ml absolute ethanol (100%) was added and mixed gently by inverting partially (only once).
- Centrifuge was then done at 12,000 rpm for 5 mins at 4^{o}C.
- Absolute (100%) ethanol was removed by aspirator.
- 700µl of 70% ethanol was added.
- Then centrifuge was done with 12,000 rpm for 5 mins at 4^{o}C.
- All the ethanol was removed carefully by an aspirator.
- White smear was seen in the tube and marked it.
- Then the pellet was allowed to dry.
- 25µl of TE was added in the tube.
- Then the culture was stored at -20^{o}C.

Phenol chloroform extraction

- Plasmid DNA (25µl) was diluted with 200µl of TE buffer.
- 200µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and vertex for 20 sec.
- Then centrifuge was done with 12,000 rpm for 6 mins at 4^{o}C.
- Upper aqueous phase (300µl) was taken into another fresh 1.5 ml eppendorf tube.
- 30µl of 3M Sodium acetate and 800µl of absolute (100%) ethanol was added.
- Then the tube was kept in -20^{o}C for 2 hours.
- Centrifuge was done with12, 000 rpm for 25 mins at 4^{o}C.
- Absolute (100%) ethanol was removed by aspirator without touching the DNA.
- 500µl of 70% ethanol was added then centrifuged at 12,000 rpm for 12 mins at 4^{o}C.
- Then ethanol was aspirated carefully, marked the pellet, dried and dissolved in 25µl of TE buffer.

DESCRIPTION OF SAMPLES WITH THEIR RESPECTIVE COMPOSITIONS

Table-1: Description about PLA samples with different compositions.

Sl.No	Sample Name	PLA (mg)	DCM (ml)	IAP		EAP (ml)	Conc^{n}. (OP:IAP)
				BSA + Sucrose (µl)	Plasmid (µl)		
1	101 (Normal)	200	4	_	_	16	_
2	102	200	4	800	_	16	1:5
3	103	200	4	500	_	16	1:8
4	104	200	4	400	_	16	1:10
5	105	125	2.5	_	1000	10	1:2.5
6	106	125	2.5	_	500	10	1:5
7	107	125	2.5	_	250	10	1:10

Table-2: Description about PLGA samples with different compositions.

Sl. No	Sample Name	OP		IAP		EAP (ml)	Conc^{n}. (OP:IAP)
		PLGA (mg)	DCM (ml)	BSA + Sucrose (µl)	Plasmid (µl)		
1	201	200	4	_	_	16	_
2	202	200	4	800	_	16	1:5
3	203	200	4	500	_	16	1:8
4	204	200	4	400	_	16	1:10
5	205	125	2.5	_	1000	10	1:2.5
6	206	125	2.5	_	500	10	1:5
7	207	125	2.5	_	250	10	1:10

[PLGA: Poly (lactide-co-glycolic acid, OP: Organic Phase, DCM: Dichloromethane, BSA: Bovine Serum Albumine (2.5%), Plasmid DNA: Final concentration is 1mg/ml, IAP: Internal Aqueous Phase, EAP: External aqueous phase]

Preparation of Protein and DNA loaded PLA and PLGA Particles

PLA and PLGA particles were prepared using double solvent evaporation method as given in fig. 5. The details description of different phases in particles formulation as given in table- 3.

Table-3: General description of materials in particles formulation.

Condition	IAP	OP	EAP
Protein (BSA)	BSA (2.5%) $NaHCO_3$ (2%) Sucrose (10%)	PLA/PLGA (200mg) DCM (4ml)	PVA (1%)
Plasmid DNA	Plasmid DNA (1mg/ml)	PLA/PLGA (125mg) DCM (2.5 ml)	PVA (1%)

- Polymer was dissolved in organic phase (4ml) then, sonicated with addition of IAP to make primary emulsion.
- For the formation of secondary emulsion 16 ml of EAP was added drop wise manner into the primary emulsion during sonication of primary emulsion.
- Then the secondary emulsion was kept in magnetic stirrer for overnight for excess DCM to evaporate.
- Particles were separated through centrifugation at 15000 rpm for 20 mins.
- Separated particles were washed twice with ice cold MQ water then particles were lyophilized to obtain dry particles and stored in -20^{o}C for further use.

Same methodology was followed in case of plasmid DNA loaded particle formation, but 10ml of EAP was used instead of 16ml and there was no use of sucrose and sodium bicarbonate in IAP. The systematic representation of solvent evaporation method was given in fig. 6.

Preparation of nanoparticles

(Double emulsion-solvent evaporation method)

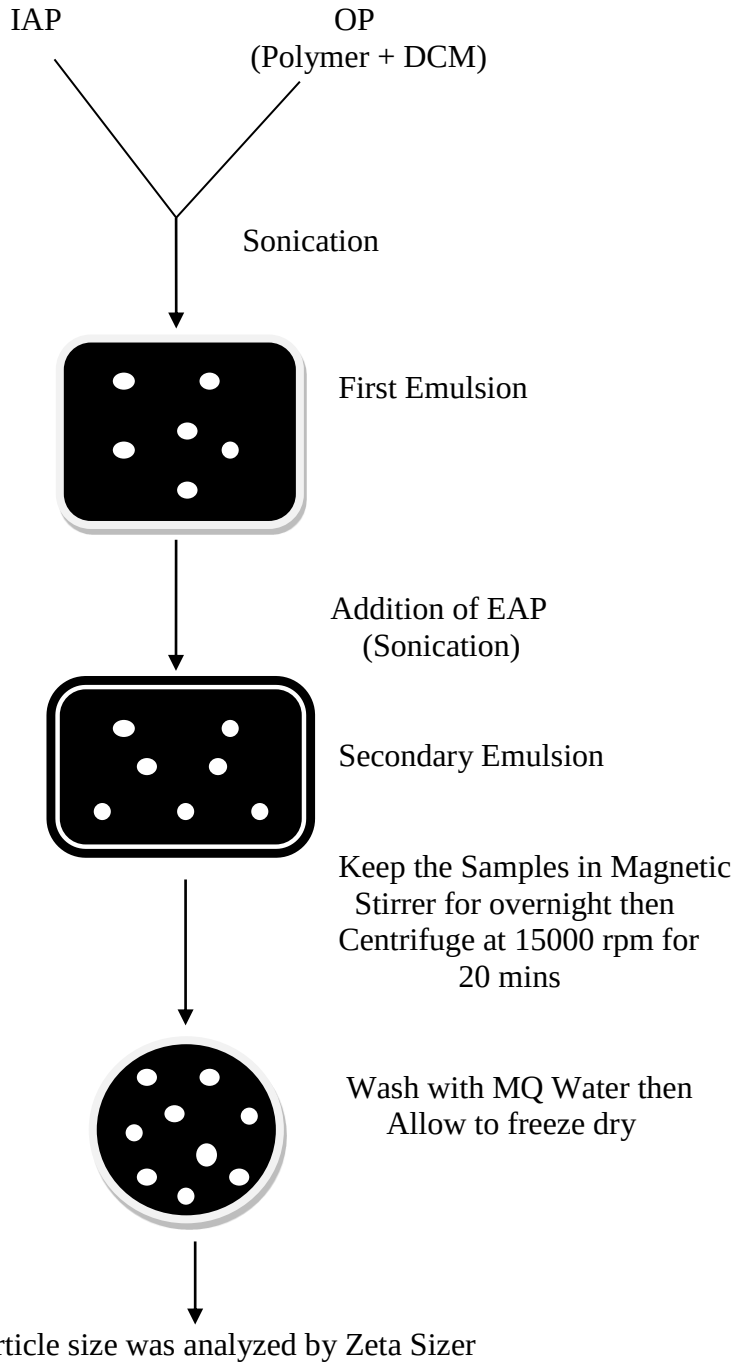


Fig. 5: Solvent evaporation method for the preparation of PLA and PLGA nanoparticles

Emulsion Solvent Evaporation Method

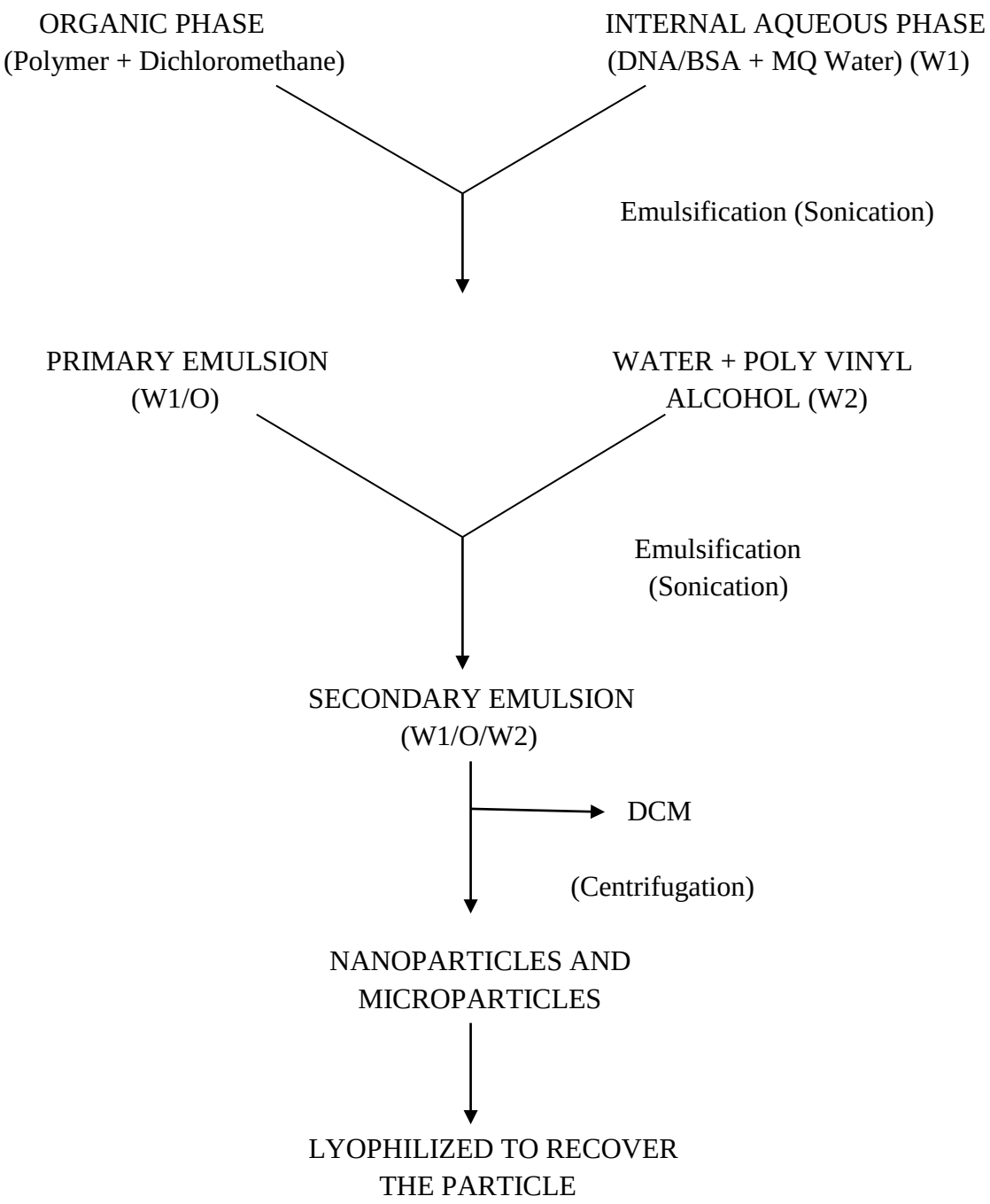


Fig. 6: Preparation of Nano and Microparticles by Emulsion Solvent Evaporation Method

Estimation of protein (BSA) and plasmid DNA encapsulation efficiency

The encapsulation efficiency or loading efficiency of PLA and PLGA particles was calculated by spectrophotometrically (at OD 562nm). While separating the particles by centrifugation the supernatant was collected and analysed by spectrophotometer. The amount of DNA in the supernatant was substracted from the amount used in IAP. This amount was used for the calculation of entrapment efficiency. Then the loading efficiency of protein (BSA) was calculated by BCA method (Biocinchroninic acid protein assay).

$$LE(\%) = \frac{\text{total amount of BSA} - \text{free BSA}}{\text{total amount of BSA}} \times 100$$

Where LE = Loading efficiency

***In vitro* release of encapsulated protein and DNA**

In vitro release studies of prepared nanoparticles were carried out at 37°C. Approximately 10 mg of nanoparticles were suspended in 1 ml of PBS (phosphate saline buffer, pH 7.4) taken in 1.5ml eppendorf placed in incubator shaker for the period of study (200 rpm at 37°C). Then the samples were collected periodically after centrifugation at 13000 rpm for 20mins. Then the supernatant was collected and the amount of protein (BSA) released was estimated by BCA™ kit using spectrophotometer. Similarly for plasmid DNA released was determined by spectrophotometrically. Then the pellet was reconstituted, resuspended in 1ml fresh PBS and kept in shaker for further sampling. The systematic representation of release study for encapsulated particles as shown in fig. 7.

Release study from PLA and PLGA Particles

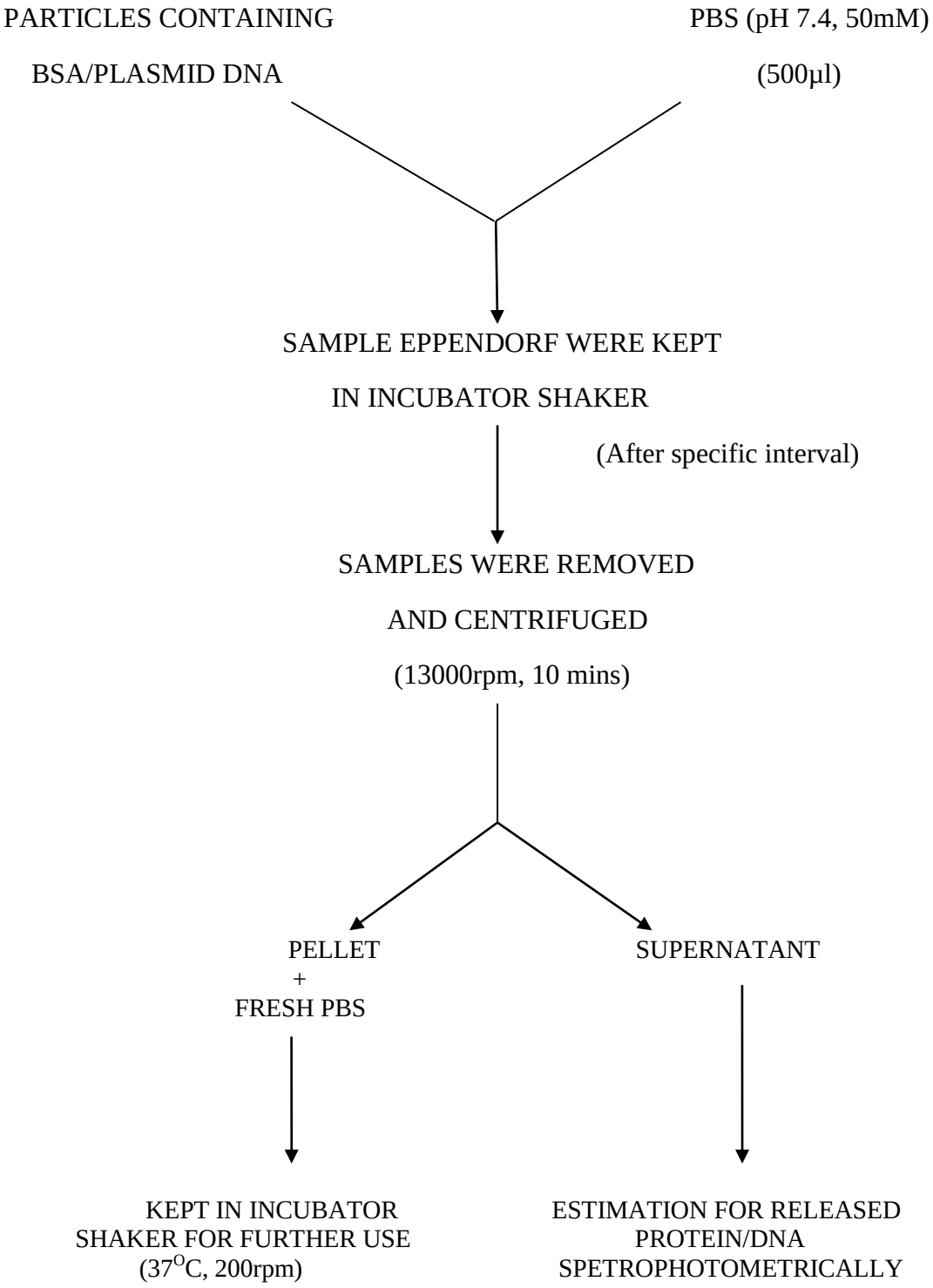


Fig. 7: Release Study of Protein/DNA for PLA and PLGA Nanoparticles

Particle Size and Surface Characterization :

10mg of the lyophilized polymer particles were redispersed in 10ml of MQ water, sonicated in water bath for 15 mins and the size was measured in Zeta sizer (Malvern). For surface morphology characterization, lyophilized particles were examined by scanning electron microscope (SEM). For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum.

RESULTS AND DISCUSSIONS

Isolation of Plasmid DNA

Plasmid DNA was isolated from *E.coli*, using Mini preparation (Mini prep.) method. The isolated plasmid DNA was run on 1% agarose gel electrophoresis to check the integrity of the plasmid. The isolated plasmid was ~1500 base pair in length and was supercoiled in conformation. The plasmid had kanamycin resistance gene to serve as selection marker. Picture of gel with plasmid DNA and marker DNA are shown in fig. 8. Then plasmid DNA was purified through phenol/chloroform purification method. The concentration and purity of plasmid DNA were determined by spectrophotometry as given in table: 4.

Table-4: Details about the plasmid DNA purified from *E.coli.*

Concentration (µg/ml)	Purity (260/280)	Purity (230/260)
1791	1.684	0.824

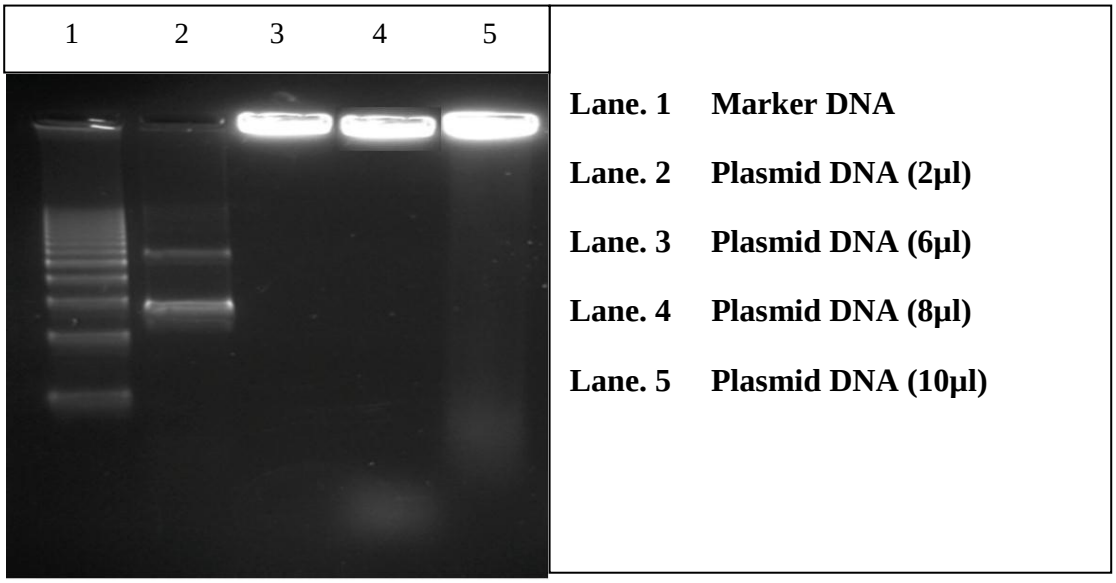


Fig. 8: Agarose gel electrophoresis of plasmid DNA (*E.coli*).

Preparation PLA and PLGA Nanoparticles

PLA and PLGA micro and nanoparticles were prepared by double solvent evaporation method. Then BSA (2.5%) (PI of 4.8) was used as a model protein and plasmid DNA (final concentration 1mg/ml) was used as a DNA carrier for evaluation of the properties of the microparticles and nanoparticles. The particle size was determined by Zeta sizer. Particles which were prepared by sonication for both primary and secondary emulsion were in nanometre range. The particle size was varied from 43 nm to 373nm for PLA polymer and for PLGA polymer the particle size was 113.5 nm to 336 nm. Respective PDI (polydispersity index) values of the formulations were evaluated. Polydispersity index (PDI), a term in polymer chemistry referring to the molecular weight distribution of polymers. PDI is the mass average degree of molecular weight to the number average degree of molecular weight. Zeta potential is a scientific term for electro-kinetic potential (McNaught and Wilkinson, 1997). The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

BSA and Plasmid DNA loaded PLA Particles

Sample 101 was the normal sample in which there was no IAP (BSA/pDNA), the particle size was 43 nm. When BSA was added to the particles (sample 102, 103 and 104), the size were as 258.3 nm, 196.8 nm and 162.7 nm. The loading efficiency was calculated using BCA standard curve for BSA protein by using spectrophotometer as shown in fig. 9. The loading efficiency was 58.7%, 69.6% and 82.4% for the samples 102, 103 and 104 respectively. For sample 104 the loading efficiency was high in this sample the OP: IAP ratio was 1:10. Similarly for plasmid DNA, when plasmid DNA was added (sample 105, 106 and 107), then the particle size were ranged from 194.8nm to 373.2 nm. Then the loading efficiency was calculated by spectrophotometrically as shown in fig. 10. The loading or encapsulation efficiency for these samples were as 42%, 37.6% and 31.12% respectively.

Sample 105 was shown high loading efficiency in which the OP:IAP ratio was 1:2.5. The detail parameter like particle size, zeta potential, PDI and loading efficiency were shown in table 5.

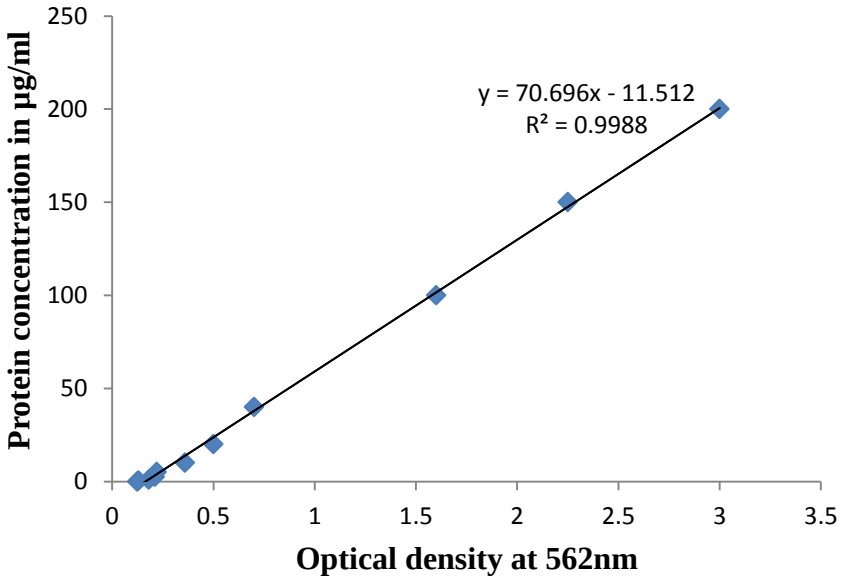
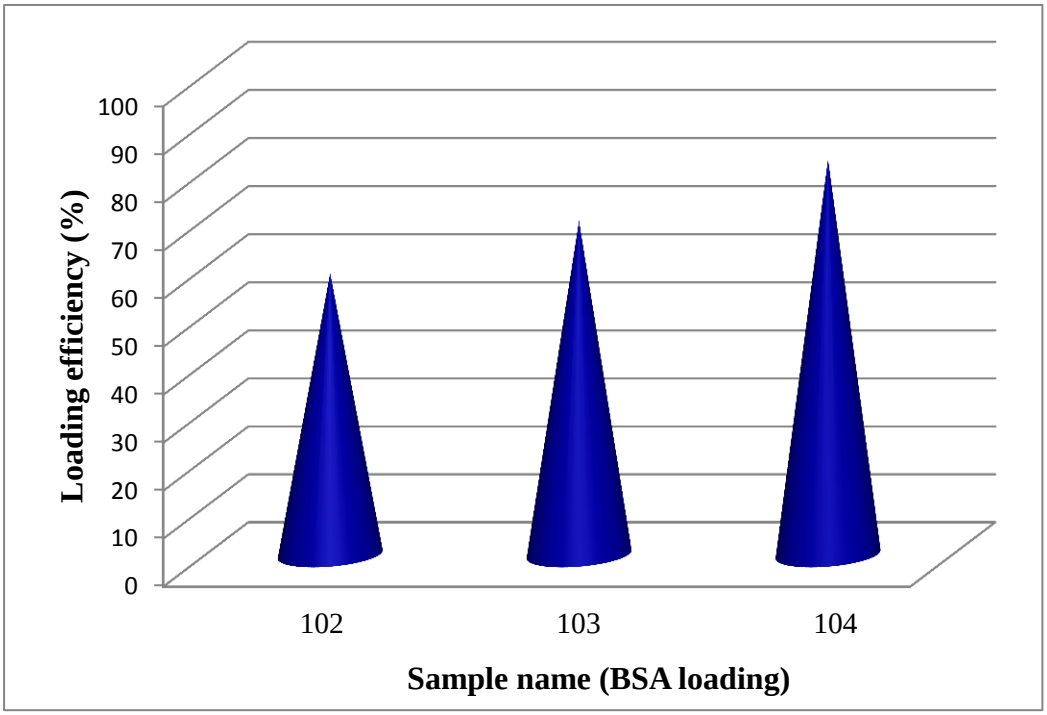


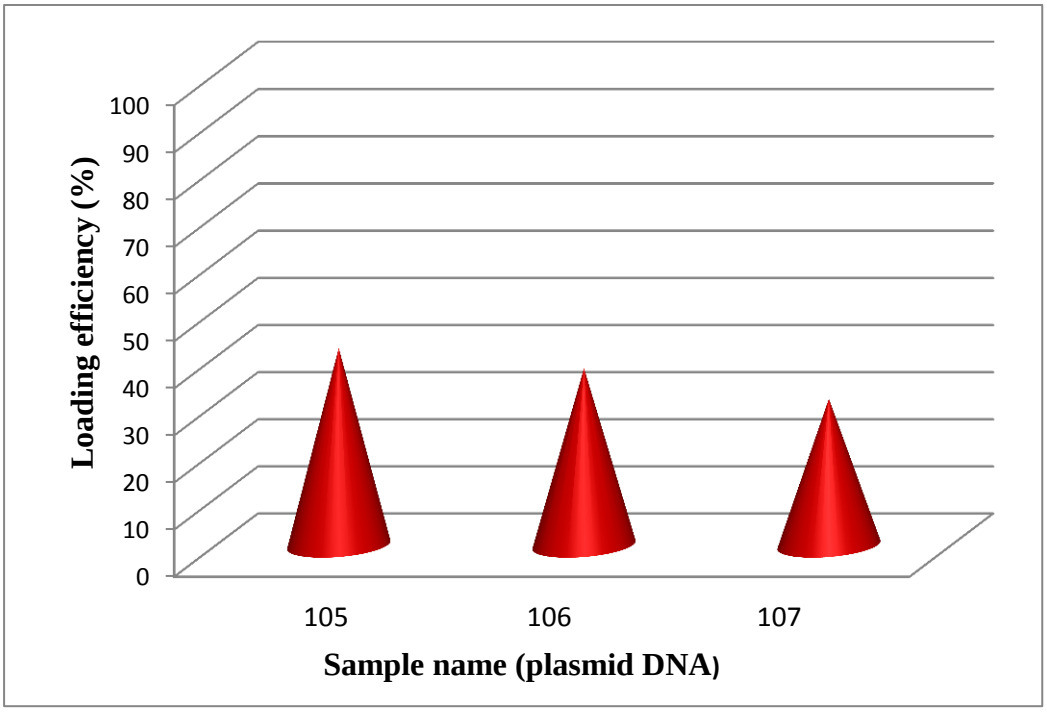
Fig. 9: BCA standard graph using BCA™ kit.

Table-5: Fomulation of PLA nanoparticles with their mean particle size, PDI (poly dispersity index), zeta potential and loading efficiency.

Sl.No	Sample Name	Mean Particle Size	Poly dispersity index (PDI)	Zeta Potential	Loading Efficiency (%)
1	101	42.93	1.000	-24.8	No loading
2	102	258.3	0.337	-32.7	61.6
3	103	196.8	0.403	-20.5	69.6
4	104	162.7	0.465	-24.7	82.4
5	105	194.8	0.359	-19.5	41.95
6	106	227.9	0.987	-30.3	37.6
7	107	373.2	0.241	-19.7	31.12



(A)



(B)

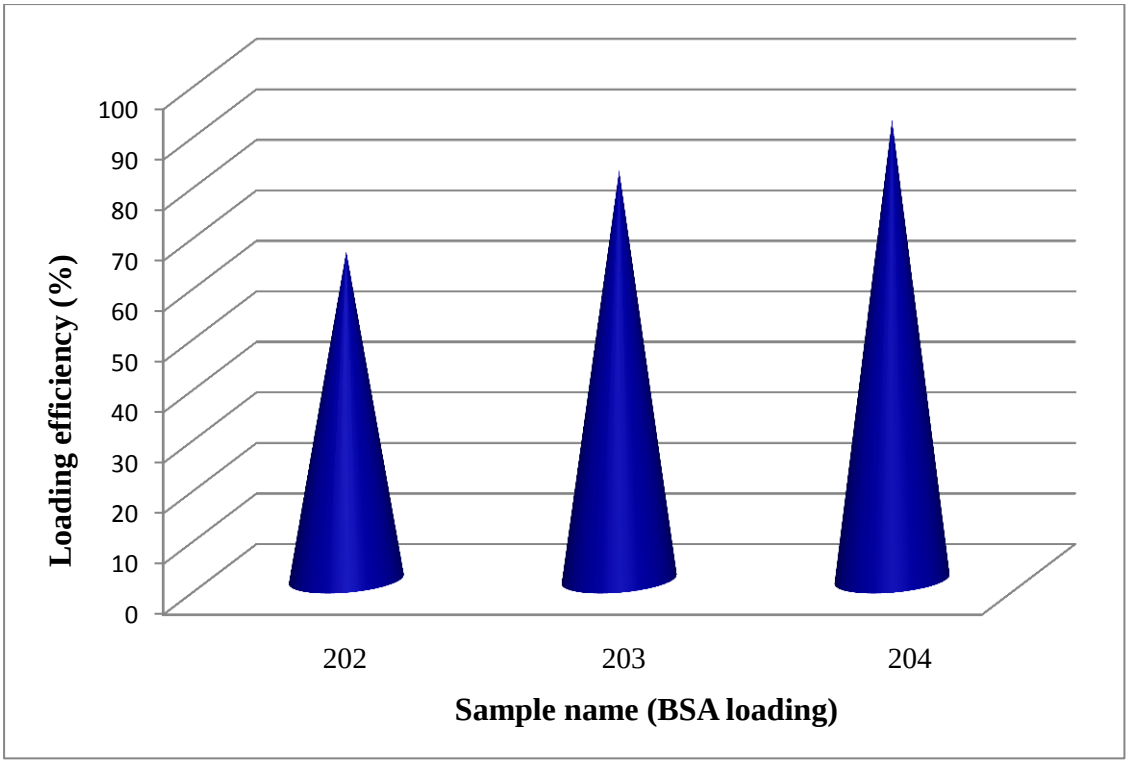
Fig. 10: Loading efficiency of BSA (A) and plasmid DNA (B) in PLA nanoparticles.

BSA and Plasmid DNA loaded PLGA Particles

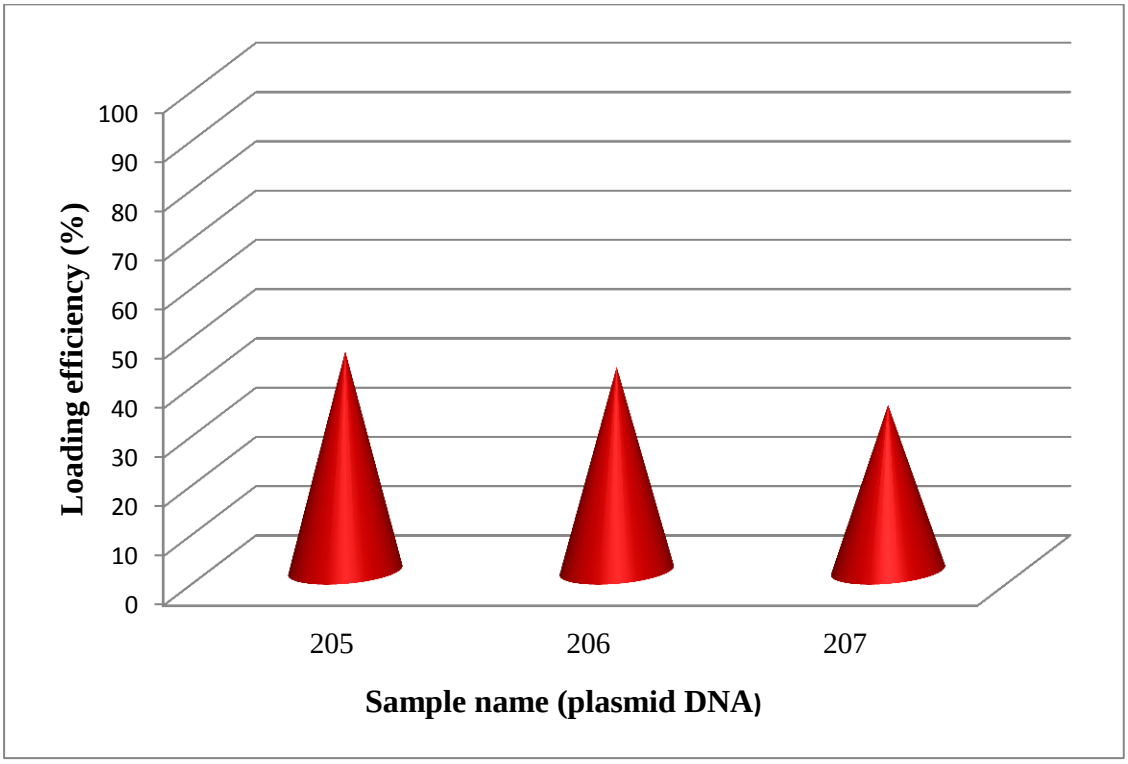
Sample 201 was the normal sample in which there was no IAP (BSA/pDNA), the particle size was 178.6nm. When BSA was added to the particles (sample 202, 203 and 204), the size were as 335.6 nm, 138.5nm and 113.5nm respectively. The loading efficiency was calculated using BCA standard curve for BSA protein by spectrophotometer. The loading efficiency were 65.3%, 81.3% and 91.2% for the samples 202, 203 and 204 respectively as shown in fig. 11 (A). For sample 204 the loading efficiency was high in this sample and the OP: IAP ratio was 1:10. Similarly for plasmid DNA, when plasmid DNA was added (sample 205, 206 and 207), then the particle size were ranged from 276.6nm to 143.9 nm respectively. Then the loading efficiency was calculated by spectrophotometrically as shown in fig. 11(B). The encapsulation efficiency for these samples were 44.6%, 41.4% and 33.7% respectively. It was shown that sample 205 had highest loading efficiency in which the OP: IAP ratio was 1:2.5. The detail parameters like particle size, zeta potential, PDI and loading efficiency were shown as given in table 6.

Table-6: Fomulation of PLGA nanoparticles with their mean particle size, PDI (poly dispersity index), zeta potential and loading efficiency.

Sl.No	Sample Name	Mean Particle Size	Poly dispersity index (PDI)	Zeta Potential	Loading Efficiency (%)
1	201	178.6	0.324	-19.9	No loading
2	202	335.6	0.356	-19.1	65.28
3	203	138.5	0.518	-30.1	81.28
4	204	113.5	0.537	-20.3	91.22
5	205	276.6	0.989	-18.5	44.6
6	206	143.9	0.958	-17.7	41.4
7	207	156.2	0.624	-17.7	33.7



(A)



(B)

Fig. 11: Loading efficiency of BSA (A) and plasmid DNA (B) in PLGA nanoparticles.

Size and Potential Study of Particles

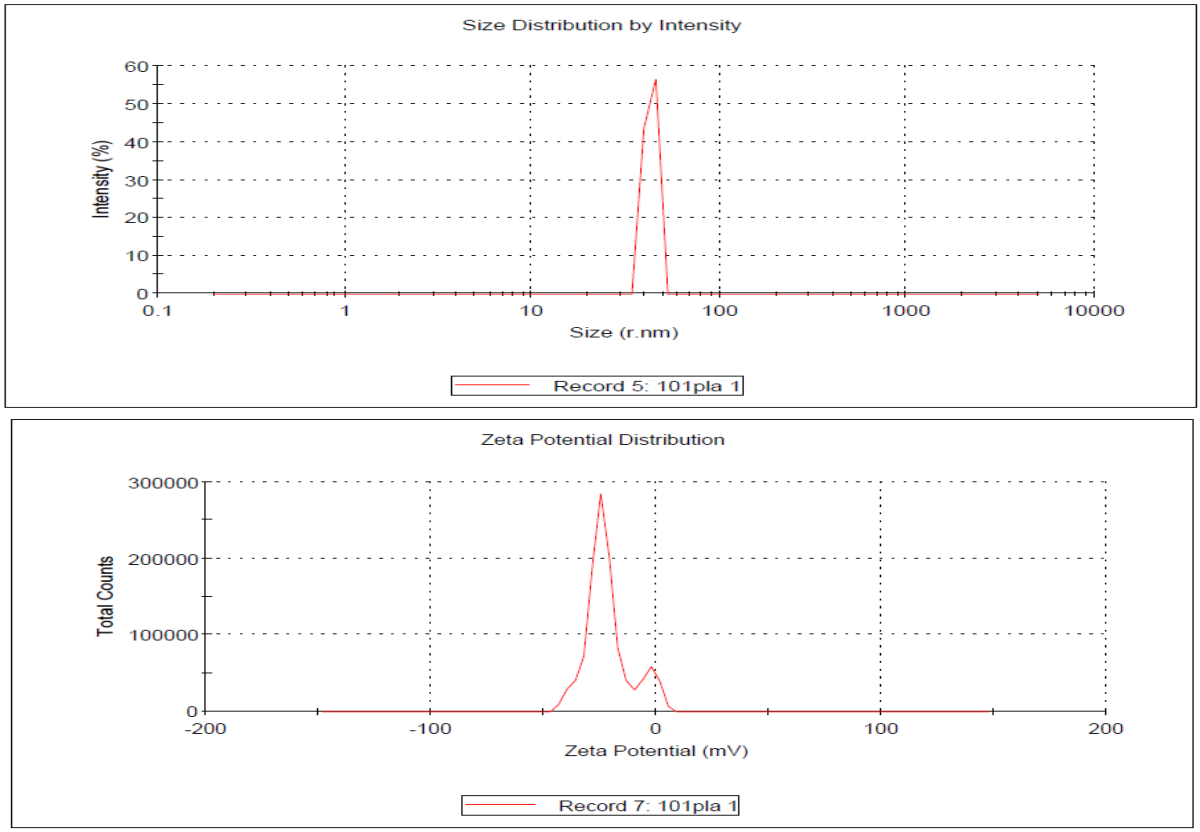


Fig. 12.1: Sample 101 showing the size (42.93nm) and potential (-24.8mv)

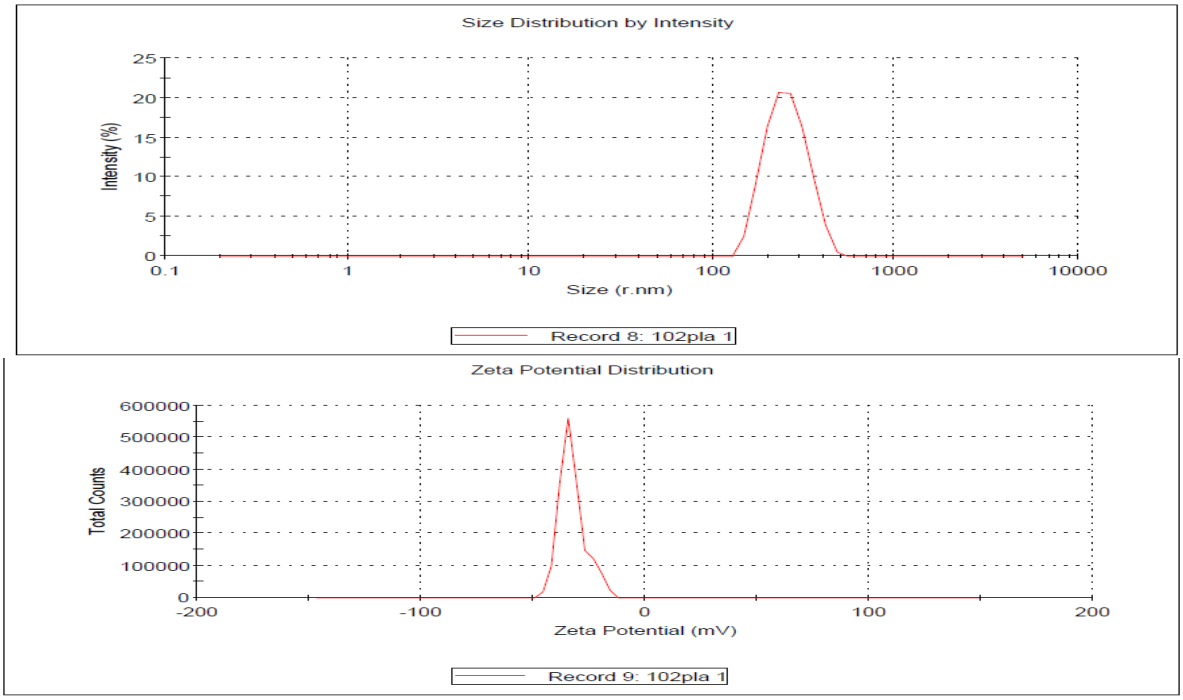


Fig. 12.2: Sample 102 showing the size (258.3nm) and potential (-32.7mv)

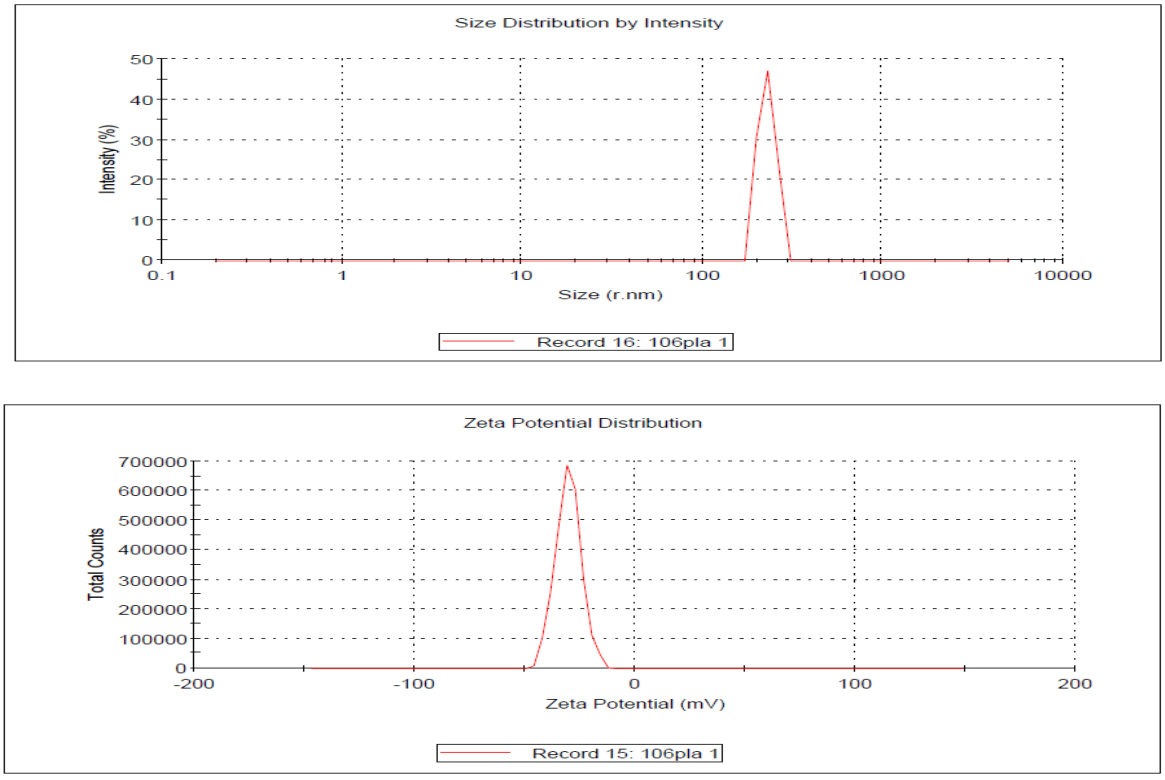


Fig. 12.3: Sample 106 showing the size (227.9nm) and potential (-30.3mv)

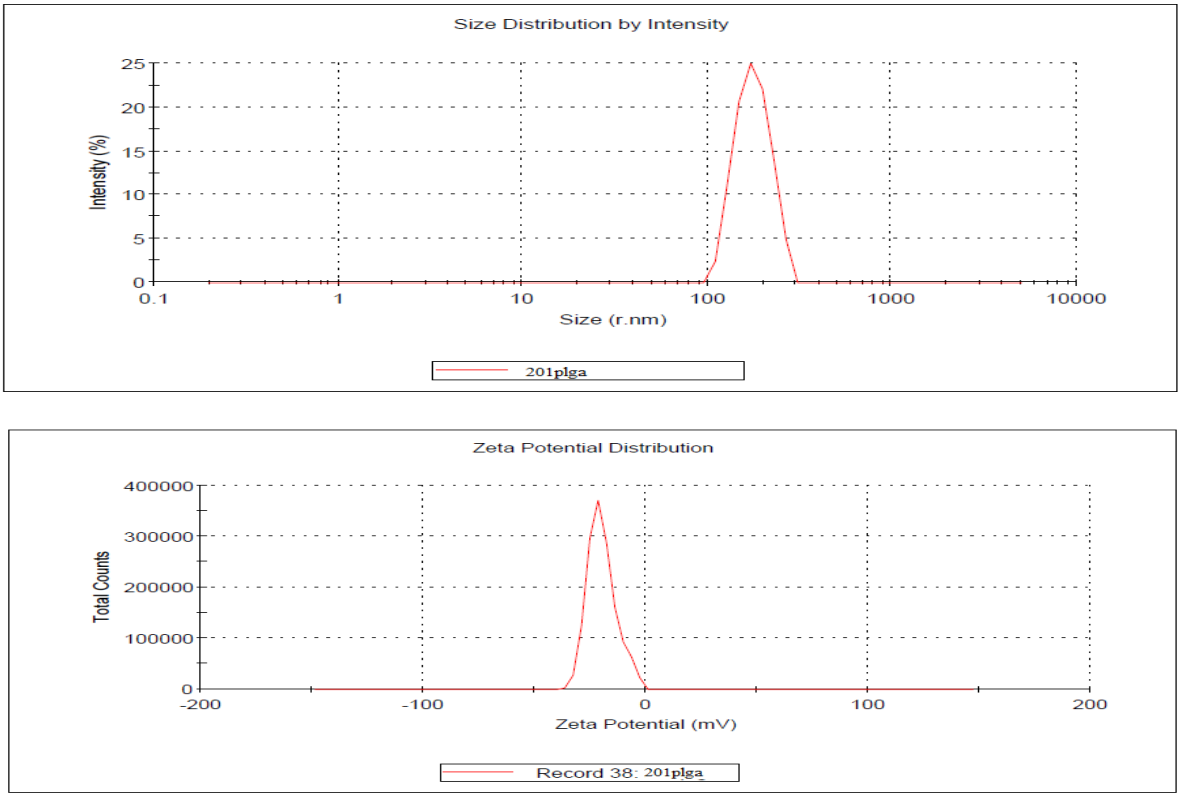
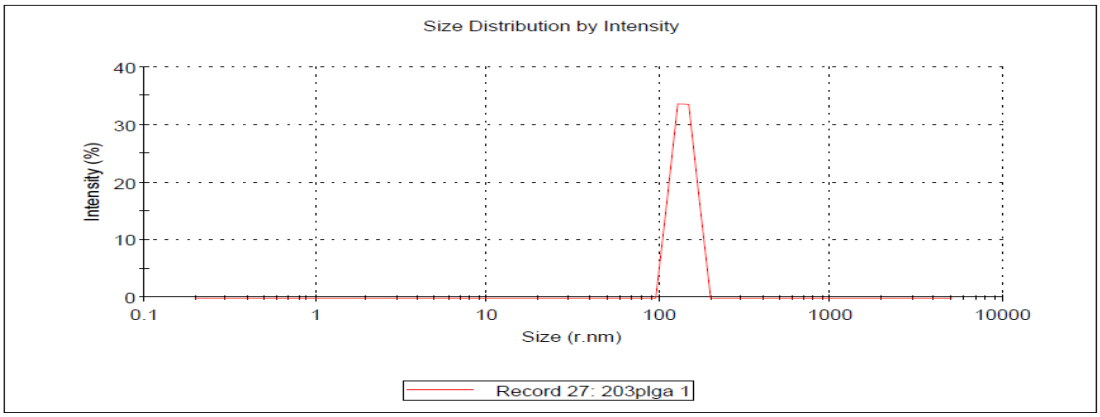


Fig. 12.4: Sample 201 showing the size (178.6nm) and potential (-19.9mv)



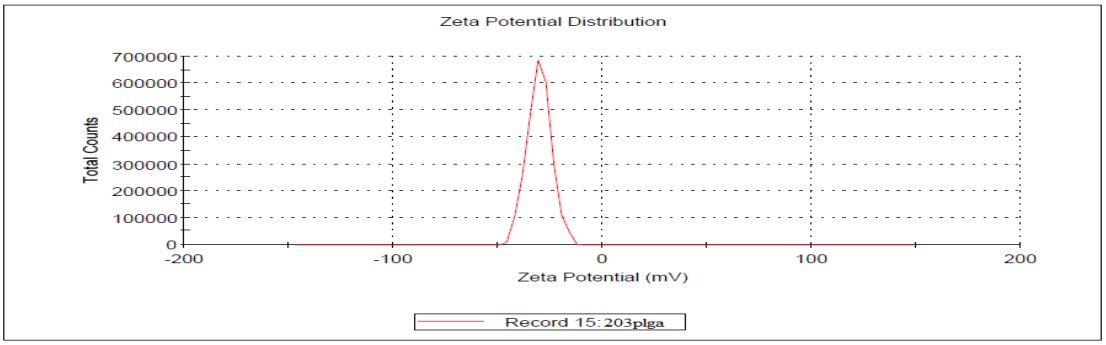
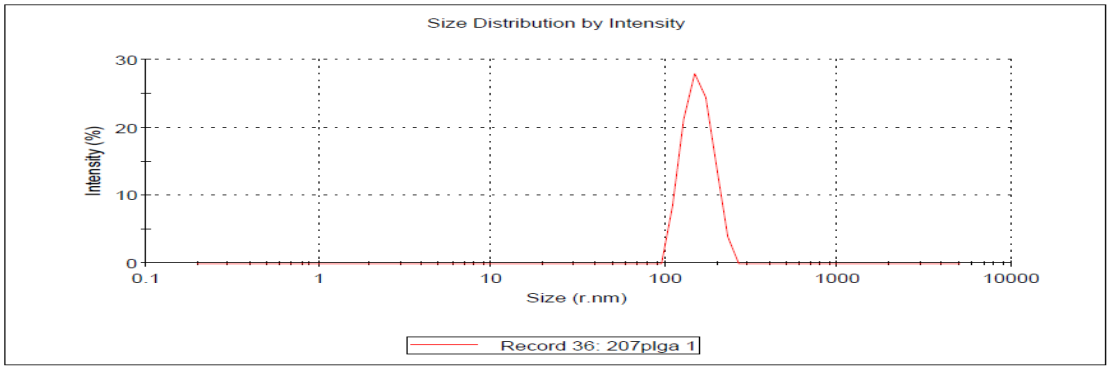


Fig. 12.5: Sample 203 showing the size (138.5nm) and potential (-30.1mv)



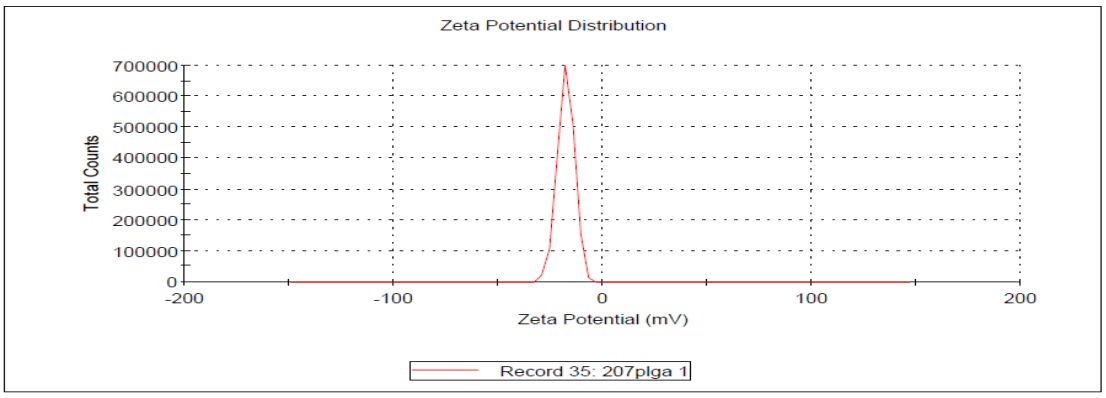


Fig. 12.6: Sample 207 showing the size (156.2nm) and potential (-17.7mv)

In vitro Release Study

The physico-chemical properties of the stabilizer added seem to affect the release profile significantly. The protein and 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. Here sucrose was used as a stabilizer, which reduced the release rate of protein (Tunon *et al.,* 2003). The basic salt like $NaHCO_3$ can reduce the acidic effect produced during the catalyzed degradation of polymer. 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 protein and 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).

In vitro protein release study

The particles with stabilizers have an extended and slow release profile. The serum albumins added along with sucrose as stabilizer provides a hydrophobic layer around the aqueous droplet containing the protein during emulsification process. This provides a hydrophobic barrier which shields the active protein from DCM (Dichloromethane). The conformational change was brought about by the albumin molecules towards the surface of the droplet that forms a hydrophobic layer around the droplet and stabilizes the suspension. It not only protects the protein from denaturation (Duncan *et al.,* 1996), but also leads to the formation of stable primary emulsion. Within a period of one week, PLGA nanoparticles released almost 100% of the encapsulated proteins. There was an extended release of the protein with the adding of stabilizers. In case of PLGA nanoparticles, more than 56% of the encapsulated protein was released as burst release and after 6 hours more than 89% entrapped protein was released. In case of PLA nanoparticles, only 42% of encapsulated protein was released in the first burst release and after 6 hours around 70% of the entrapped protein released *in vitro as* given in Fig.13. Some similar results were obtained by Nayak *et al.,* 2008.

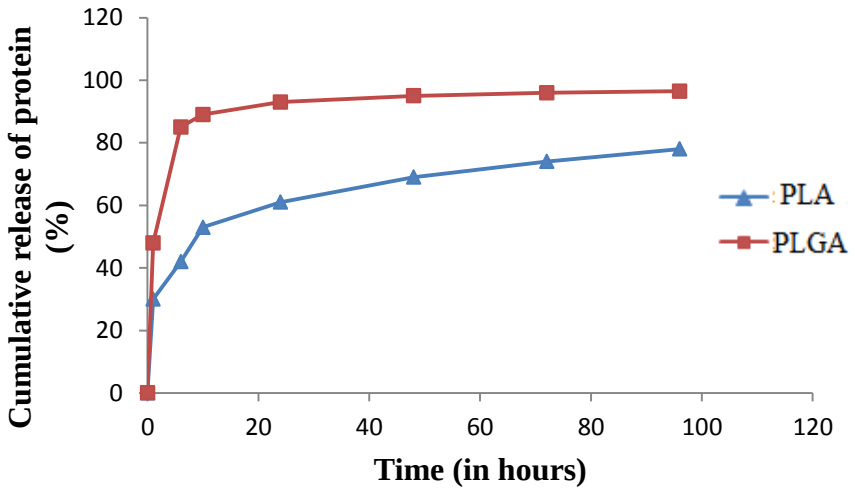


Fig. 13: *In vitro* protein (BSA) release from encapsulated particles.

***In vitro* plasmid DNA release study**

Within a period of four days study, PLGA nanoparticles released almost 75% of the encapsulated plasmid DNA. There was an extended release of the plasmid DNA with the adding of stabilizers. In case of PLGA nanoparticles, more than 27% of the encapsulated DNA was released as burst release and after 6 hours more than 46% entrapped pDNA was released. In case of PLA nanoparticles, only 20% of encapsulated pDNA was released in the first burst release and after 6 hours around 39% of the entrapped protein released *in vitro* as shown in fig. 14. Zou *et al.*, 2009, had described similar results.

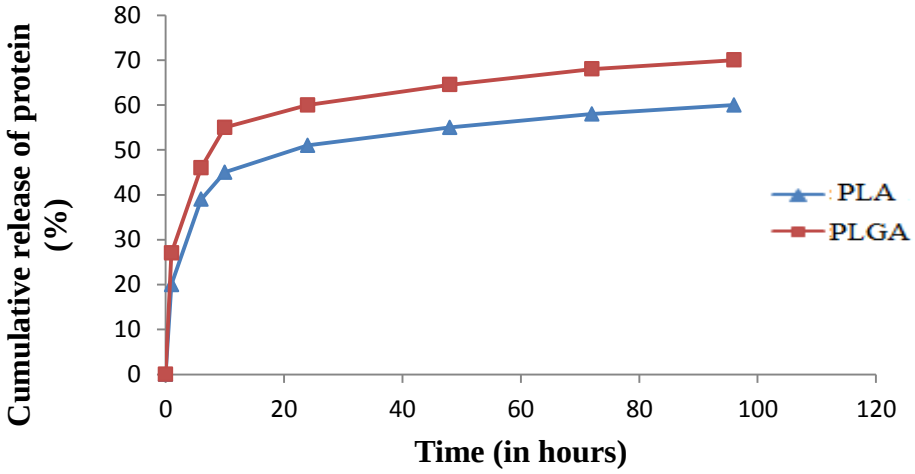


Fig. 14: *In vitro* plasmid DNA release from encapsulated particles

Surface Characterization by SEM

The morphology of these PLA and PLGA particles were spherical structures as determined using scanning electron microscope (SEM) as shown in fig. 15. Fig. 15(A) is the structure of PLA particles whereas fig. 15(B) is the structure of PLGA particles. The surface of the particles are rough and rounded that possesses pores of varying size. It was reported that, when the ratio of the IAP to EAP was increased, the relative size of the pores increased.

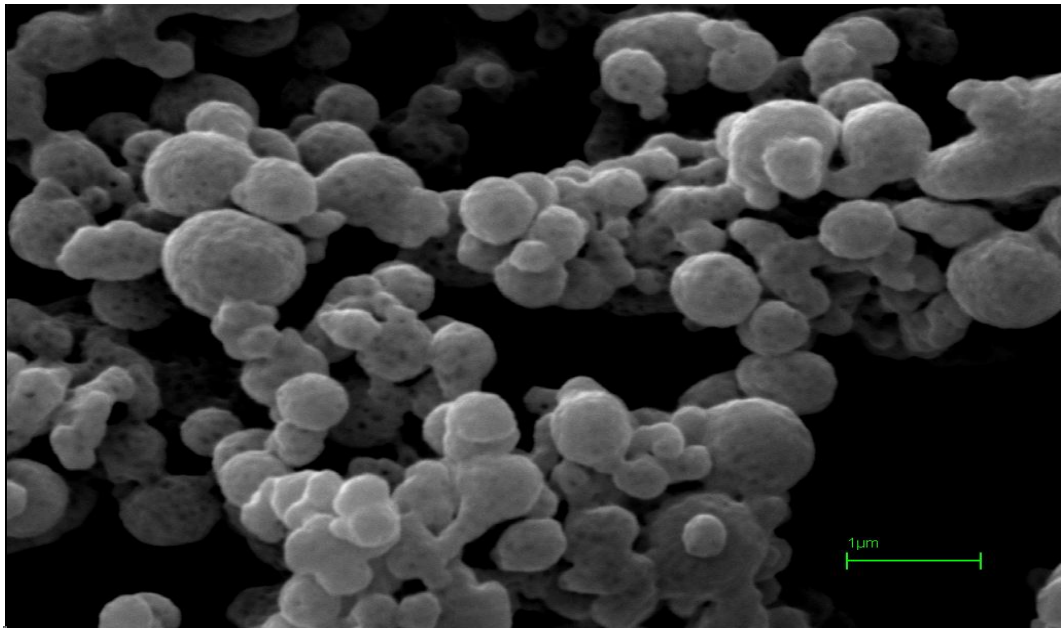


Fig.15(A): SEM structure of PLA nanoparticles.

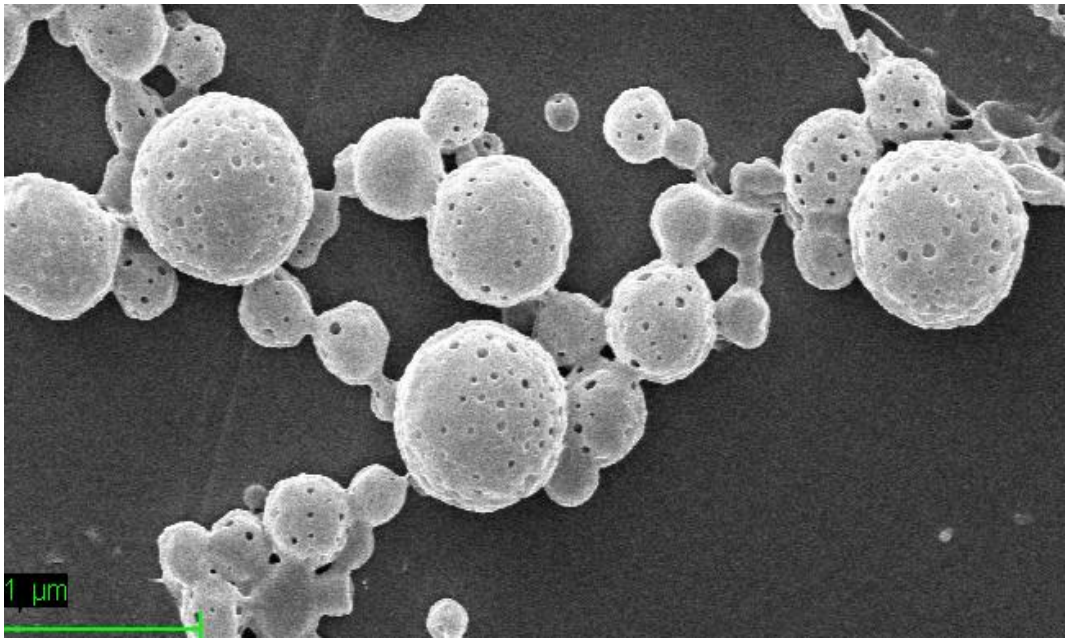


Fig. 15(B): SEM structure of PLGA nanoparticles

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