Nano-Micro hybrid carriers for drug delivery as tuberculosis therapeutics

M.Sc. Thesis

By

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BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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Nano-Micro hybrid carriers for drug delivery as tuberculosis therapeutics

A THESIS

Submitted in partial fulfillment of the

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by

Aniruddha Dan

BIOSCIENCES AND BIOMEDICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**Nano-Micro hybrid carriers for drug delivery as tuberculosis therapeutics**" in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the period from August 2020 to May 2022. Thesis submission under the supervision of Dr. Abhijeet Joshi, Associate professor, BSBE, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Aniruddha Dan

27/05/2022

Signature of the student
(Aniruddha Dan)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

AB Joshi 27th May 2022

Signature of the Supervisor
(Dr. Abhijeet Joshi)

Aniruddha Dan has successfully given his/her M.Sc. Oral Examination held on **May 5, 2022**.

P.V. Kodgire

Signature(s) of Supervisor(s) of MSc thesis
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Convener, DPGC
Date: 31/05/2022

P.V. Kodgire

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Date: 31/05/2022

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Signature of PSPC Member
(Dr. Parimal Kar)
Date: 27-5-2022

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Abstract

Tuberculosis is regarded as one of the most dangerous disease and it's threat is a matter of high concern globally. Yes, there are anti-TB drugs available in the market but occurrence of Multi drug resistant (MDR)/extensively drug resistant (XDR) TB has proved them ineffective and pushed the scientific society to develop a new approach to combat TB. Nanotechnology is a very recent multidisciplinary science and nanoparticle-based drug delivery approaches are in need. These delivery systems target the drug properly to the site of infection leading to high drug concentration at the site and they promote sustainable release of drug for a prolonged time, also have lesser side effects compared to these free drugs. In case of free drugs, patient has to take frequent large dosage of medicine, but these nanoparticle mediated drug delivery approaches have reduced both the frequency and dosage of drugs. Available diagnostic methods to detect the TB are time-taking, inefficient and unreliable. Nanoparticles can be effective in diagnosis also, due to their special physical and optical properties. Nanoparticle based diagnostic assays are quite easy, reliable and will be completed in a short time. The main aim of this project is to develop front-line (Isoniazid, Pyrazinamide, Rifampicin, Ethambutol dihydrochloride) anti-tuberculosis drugs loaded PLGA nanoparticle using the ultrasonic atomizer for the treatment of TB and colorimetric diagnostic method using super-paramagnetic iron-oxide nanoparticle.

LIST OF PUBLICATIONS

"Hybrid carriers combining front-line anti-tuberculosis drugs for their synergistic effect against against Mycobacterium tuberculosis"- (Manuscript under preparation)

"Construction of magnetite-based biosensor for diagnosis of TB"- (Manuscript under preparation)

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Abbreviations

M.Tb	Mycobacterium tuberculosis
PLGA	Poly(lactic-co-galactic) acid
np	nanoparticles
PVA	Poly viny alcohol
Isoniazid	Isonicotinic acid hydrazide
SPIONs	Super paramagnetic iron-oxide nanoparticles
EDX/EDS	Energy-dispersive x-ray spectroscopy
FT-IR	Fourier-transform infrared-spectroscopy

Chapter 1: Introduction

Tuberculosis is the deadliest killer infection, as according to W.H.O, every year more or less 1.5 million people die due to tuberculosis.[1] Also due to emergence of resistance, it is making the available drugs in-effective. It's known that tuberculosis is caused by Mycobacterium tuberculosis, but it's not the only one causing agent, there are others like *M. bovis, M. africanum, M. caprae, M. microti, M. pinnipedii* and these collectively form a group called MTB complex.[2]

1.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis is an anaerobic bacterium, which doesn't form spore and is rod in shape. It transmits from one person to another via droplets released in the air, when an infected person coughs or sneezes.

1.2 Pathogenesis of tuberculosis[1], [2]

The droplets which contain the bacilli, are released into the air, when an infected person sneezes or coughs. The droplets containing the bacilli enters into lungs of healthy person, through inhalation. Alveolar macrophages uptake the bacilli through phagocytosis. Macrophages, B-lymphocytes, T-lymphocytes collectively form shell like structures called **Granuloma**, where infected macrophages remain at the center and other immune cells mostly T-lymphocytes surrounds them. Bacilli are contained within these granulomas and replicate within it. This is called **Latent TB,** because in this case the person remains asymptomatic. After some times the granulomas burst and the mycobacterium bacilli spread throughout the lungs and then to others organs, this condition is referred to as **Active TB.**

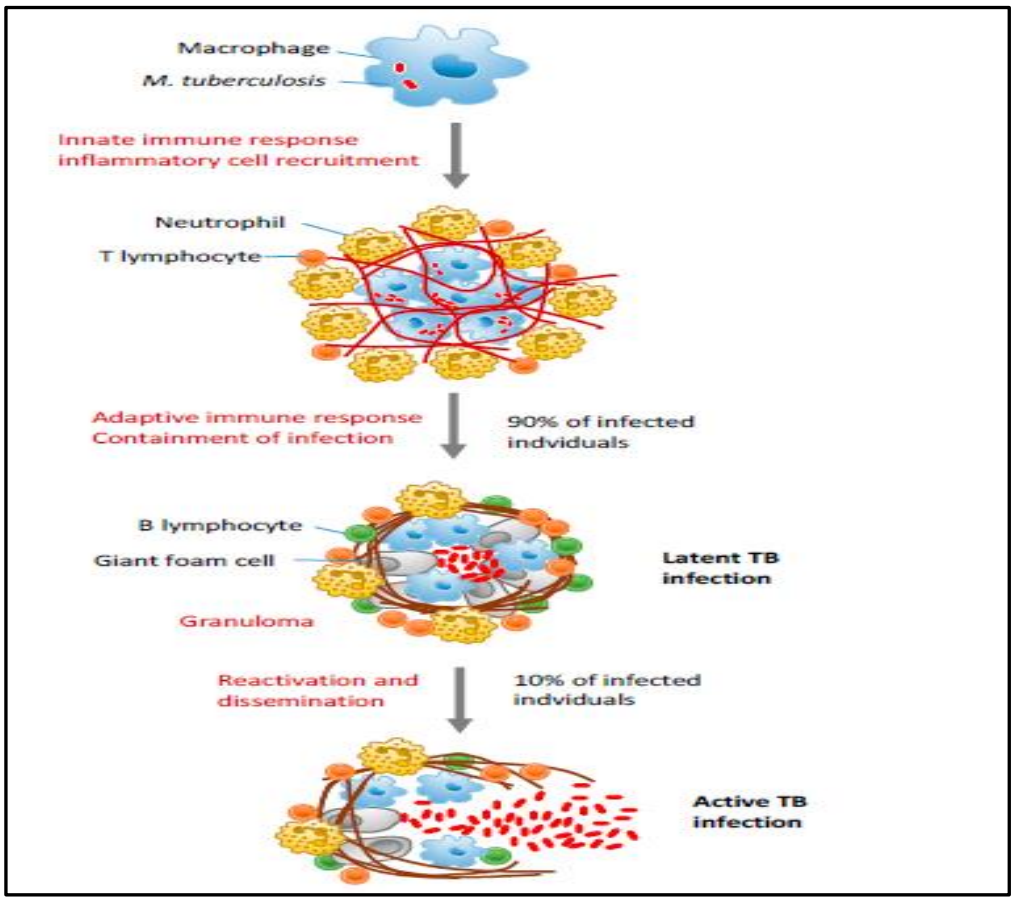

Figure 1-1 Pathogenesis of tuberculosis. Adapted from "Tuberculosis-Associated MicroRNAs: From Pathogenesis to Disease Biomarkers"-Sinigaglia et al. Cells 2020, 9, 2160; doi:10.3390/cells9102160.

From latent TB progress can be either one of two ways-

One, Phago-lysosome forms inside macrophages and the mycobacterium bacilli are killed inside these phago-lysosome.

Two, Mycobacterium blocks the phago-lysosomes formation, evade other immune responses and spread through the organ.

The transition from latent to active TB depends on several parameters, like age of the infected person, diet of that person and most importantly the immunity of that person, immune-compromised individuals are very much more prone to develop active TB disease than immune-competent individuals.

1.3 **Available anti-TB drugs-** There are several anti-TB drugs like Isoniazid, Pyrazinamide, Rifampicin, Kanamycin, Ethambutol, Levofloxacin. They act in various ways like some inhibit bacterial cell-wall synthesis, some of them inhibits steps of central dogma.

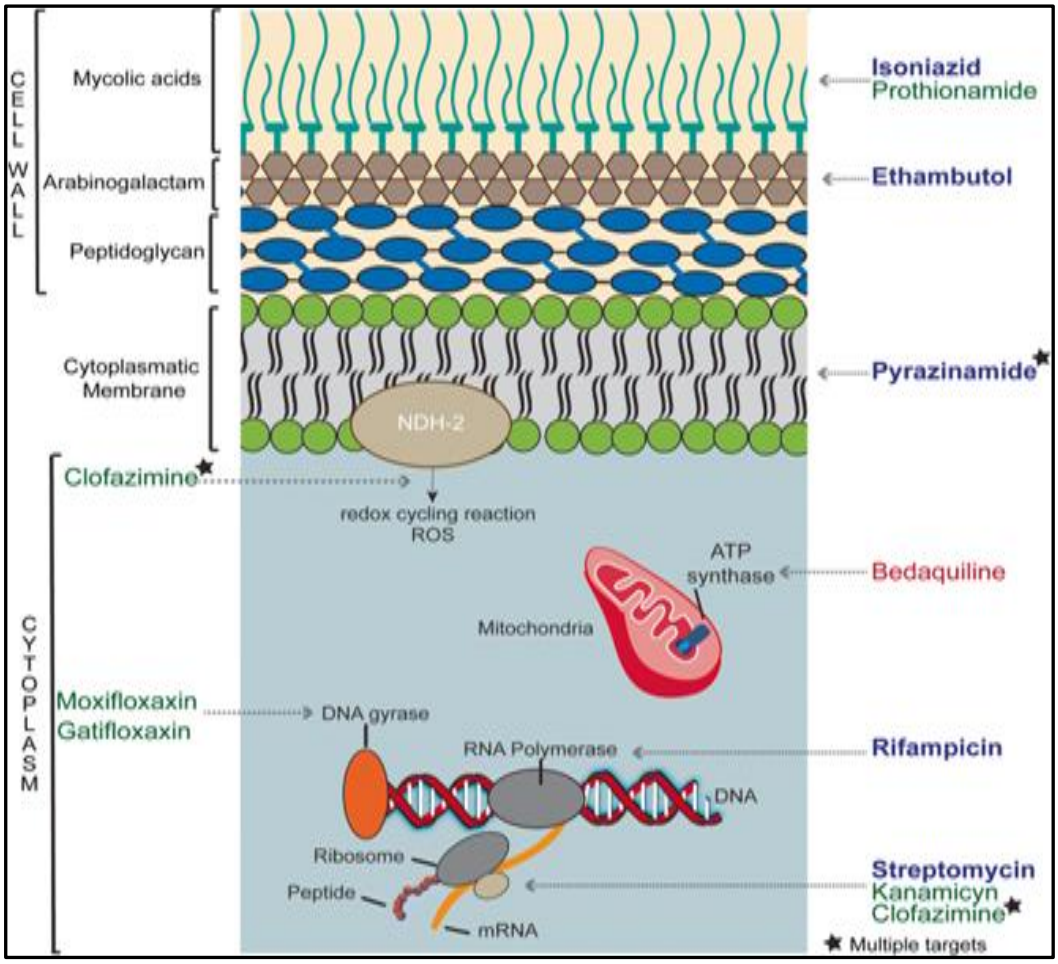

Figure 1-2 Mechanism of action of anti-tuberculosis drugs. Adapted from "Nanotechnology in Tuberculosis: State of the Art and the Challenges Ahead"- Grotz, E.et al. Pharm Res 35, 213 (2018). https://doi.org/10.1007/s11095-018-2497-z

1.4 Mechanism of action & side effects of anti-tuberculosis drugs

Table 1 Mechanism of action & side effects of anti-tuberculosis drugs[3]–[7]

Drug	**Mechanism of action**	**Adverse/side effects**
Isoniazid	Mycolic acid production block	Hepatitis, fever
Rifampicin	Cut off the production of bacterial RNA polymerase	Flu-like symptoms, rashes
Ethambutol	Inhibits bacterium arabinogalactan synthesis, which interrupts production of cell wall.	Visual disturbance can be disturbed.
Pyrazinamide	Inhibit the membrane energy	Joint pain, hepatitis
Streptomycin Kanamycin Capreomycin Amikamycin	Inhibits the translation process permanently	Electrolyte abnormalities, Nephrotoxicity
Amino salicylic acid	PABA gets blocked and folic acid metabolism doesn't proceed	Hypothyroidism, Gastro-intestinal disorders
Cycloserine	Incorporation of D-alanine in the pentapeptide bridge in the cell wall gets inhibited.	Problem in central nervous system

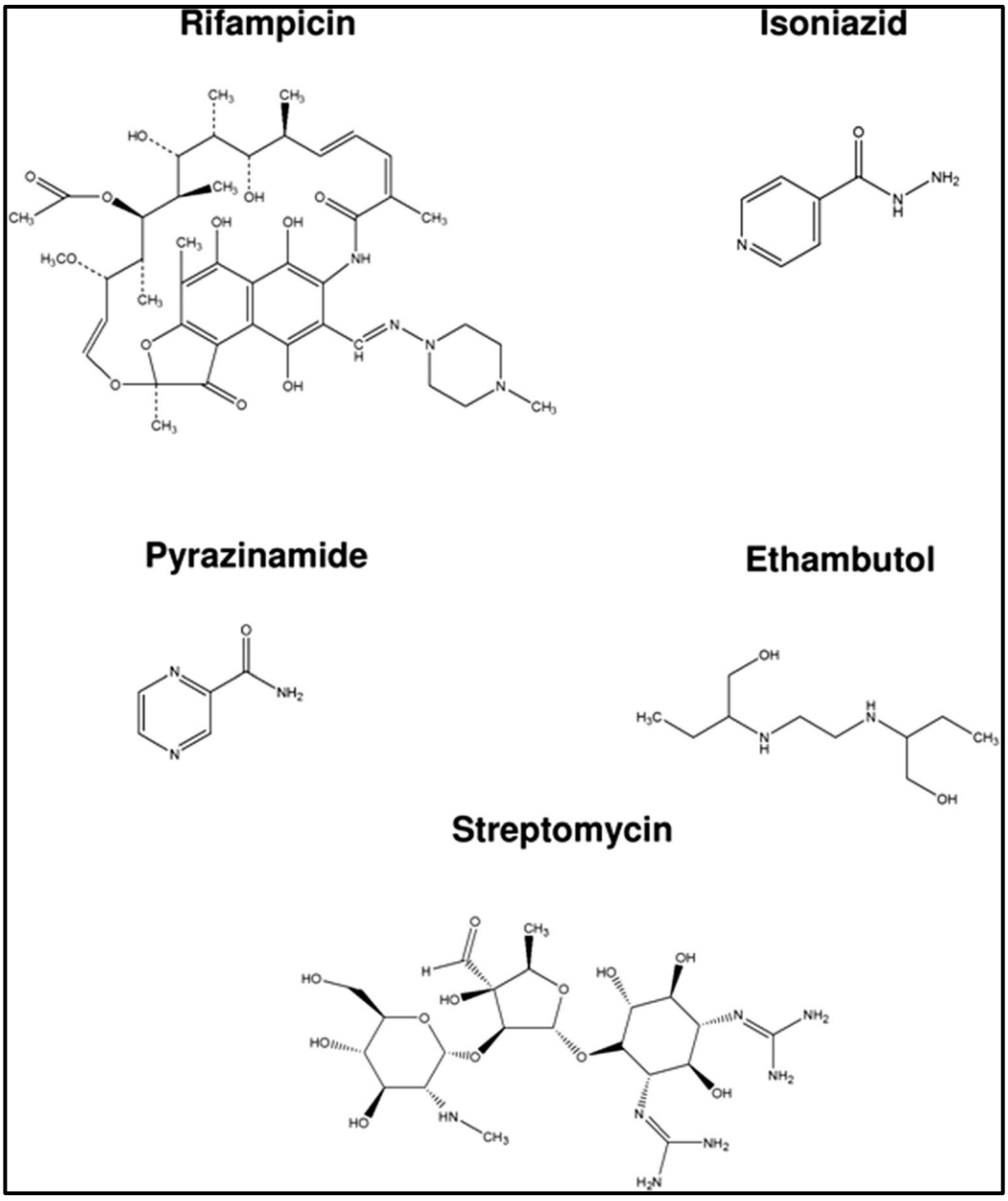

Figure 1-3 Structure of front-line anti-TB drugs. Adopted from “Nanotechnology in Tuberculosis: State of the Art and the Challenges Ahead”- Grotz E et al . Pharm Res (2018) 35:213

1.5 Drug resistivity of Mycobacterium tuberculosis[2]

Although there are several anti-tuberculosis drugs commercially available and also many are lined up in clinical and pre-clinical trial, but the development of drug-resistivity in Mycobacterium tuberculosis is making it more difficult to treat. They have developed certain mechanisms, through which they are becoming resistant to the drugs. These mechanisms are

- They change the membrane permeability, so that drugs can't enter the cell.
- They lower the pH, so the drugs can't act in acidic environment
- They alter the drug targets (enzyme pockets), causing the drugs worthless.
- They produce drug efflux pumps at a very high amount, so that drugs can never reach the needed concentration inside the cells.
- They produce mutations in DNA, as a result DNA binding drugs can't work against it.
- They produce specific drug degrading enzymes, so in case if somehow the drug enters inside, they are degraded before they can act.

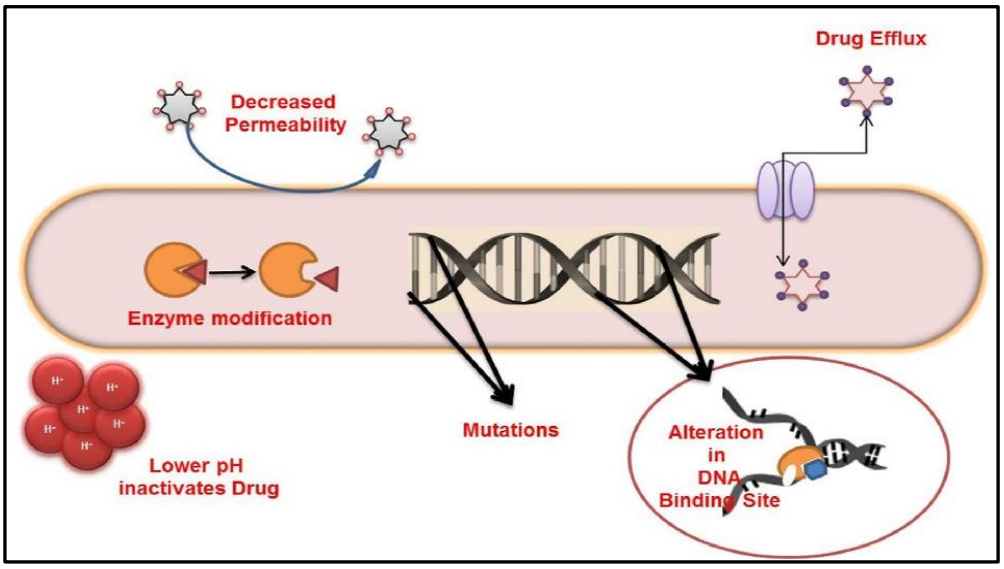

Figure 1-4 Mechanism of drug resistivity of Mycobacterium tuberculosis. Adapted from- "Multidrug resistant tuberculosis – Diagnostic challenges and its conquering by nanotechnology approach – An overview". L. Muthukrishnan et al. Chemico-Biological Inter

1.6 Bad sides of conventional anti-TB drugs

1. Rapid drug degradation
2. Stability problems
3. Low accumulation at site
4. High dosage is used
5. More frequent dosage
6. Non-targeted
7. Side effects
8. Drug resistivity

1.7 Advantages of nanoparticle mediated drug delivery system[1], [8]–[10]

a) They are biocompatible

b) They properly target the drugs to cancer cells, un-affecting the healthy cells. There by toxicity, side effects can be avoided.

c) These nano-carriers decrease the immunogenicity, renal clearance of chemotherapeutic agents.

d) They aid in sustained release of drugs, so that the half -life of drugs are increased and the patient don't have to take the medicine in larger dose or more

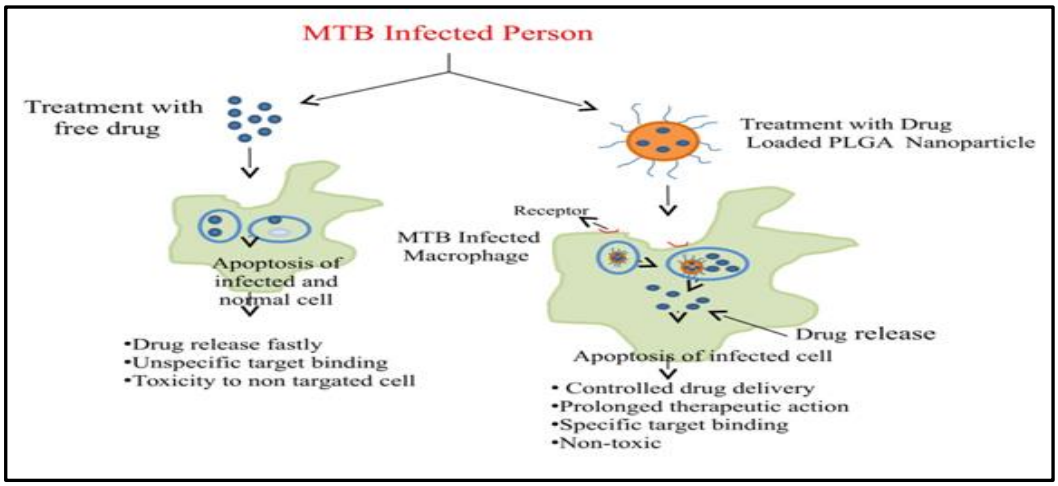

Figure 1-5 Advantages of nanoparticle in case of drug delivery.Adapted from- "Delivery of antituberculosis drugs to Mycobacterium tuberculosis H37Rv infected macrophages via polylactide-co-glycolide (PLGA) nanoparticles". Int J . Verma et al. Mol Biol Open

1.8 Nano-based delivery systems for therapeutic agents in tuberculosis[1], [9], [11]–[17]

Nanoparticle based drugs delivery systems vary in formulations and properties according to their different uses. Several types of drug delivery systems are used like solid lipid nanoparticle, liposomes, niosomes, polymeric micelles, polymeric nanoparticles, nano-capsules, nano-emulsion, dendrimer etc. They are used as carrier of the drugs as they can load a comparatively high amount of variable drugs and they are stable, also they are feasible to administrate through different routes like oral, pulmonary, intravenous, intramuscular. The most special feature about these drug delivery systems is that, there surface can be modified with antibody, ligand, dye etc according to the need.

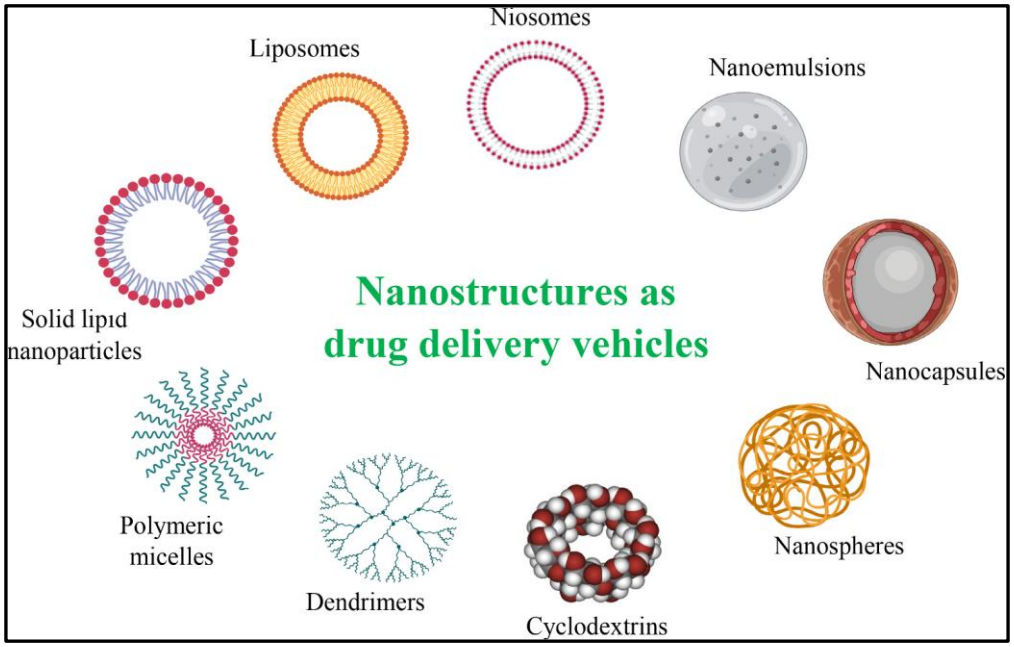

Figure 1-6 Types of nano-sized structures used as carrier of drug in TB therapy. Adapted from "Multidrug resistant tuberculosis – Diagnostic challenges and its conquering by nanotechnology approach – An overview "-L. Muthukrishnan et al. Chemico-Biological

1.9 Polymeric nanoparticle[11], [18], [19]

Drug loaded polymeric nanoparticles have **a outer periphery made of a polymer like chitosan, PLGA, alginate** etc and **a core, which consists the drug/therapeutic chemical**. They are in size of about 50-300 nm. Polymeric nanoparticles are of mainly two types, **one is polymeric nanosphere** and the other one is **polymeric nano-capsule**. In nanosphere the drugs are attached covalently or adsorbed to the surface. In case of nano-capsule, there is a core of oil/water, where the drug is encapsulated and the core is surrounded with an outer shell of polymers.

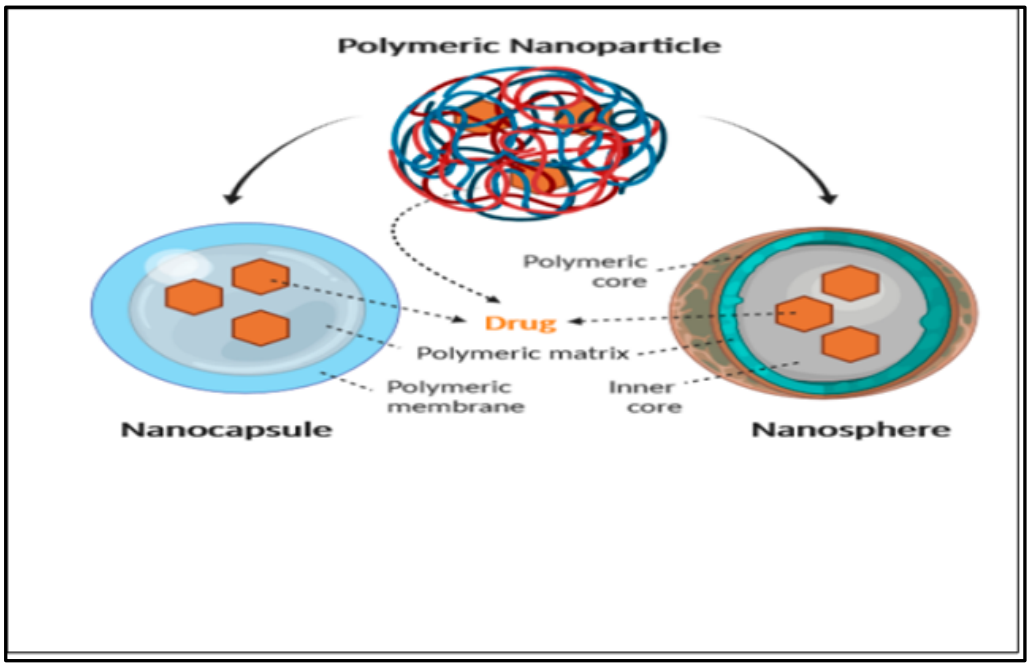

Figure 1-7 Structure of different polymeric nanoparticles; nano capsules and nanospheres. Adapted from "Polymeric nanoparticle: production, characterization, ecotoxicology" - Zielinska Aleksandra et al. Molecules2020, 25,3731.

1.10 Diagnosis of tuberculosis[2], [20]

There are several methods for diagnosis of tuberculosis, but they are not up to the expectations. They are time taking process, also not much reliable. The available diagnostic methods can be divided into two groups,

- Conventional methods- These methods identify the presence of pathogen in patient's sputum sample generally, with the help of microscopes, the results are declared after 24-48 hours.
- Growth based approaches- Here the sample taken from patient is cultured in a selective media for a very longer time, usually 3-4 weeks, to ensure if there is infection due to mycobacterium tuberculosis or not.

1.11 Diagnostic gaps-

- ✓ M.tuberculosis grows very slowly and, therefore we can't depend of it's growth-based detection methods.
- ✓ The TB patients don't have symptoms at the very beginning of infection, resulting in delay the patient care.
- ✓ Even active TB cases sometimes sputum shows low number of bacteria, so smear microscopy is a failure there.
- ✓ The devices are non-portable.

1.12 Nanoparticle mediated diagnostic method-

In case of nanoparticle mediated diagnostic method, gold nanoparticle is preferable because of their unique SPR (surface plasmin resonance) properties.

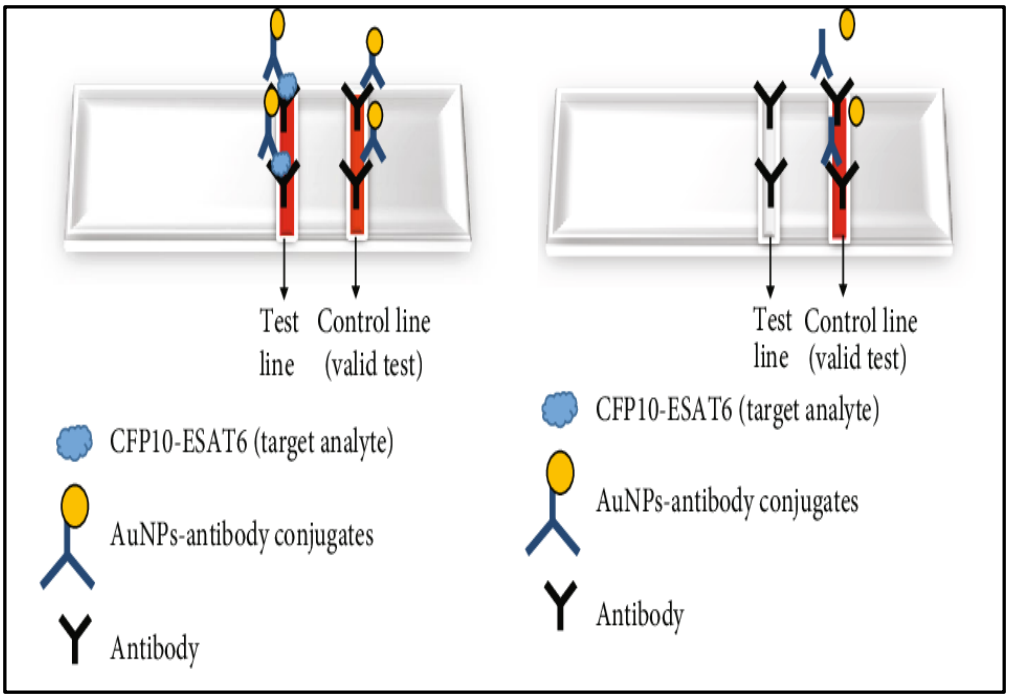

Figure 1-8 Schematic representation of gold nanoparticle mediated naked eye detection of M.Tuberculosis specific antigen. Adapted from- "Lateral Flow Immunoassay for Naked Eye Detection of Mycobacterium tuberculosis"-Ariffin N et al. Hindawi Journal of Sensors https://doi.org/10.1155/2020/1365983.

Gold nanoparticles can be coated with anti-bodies against the mycobacterium tuberculosis specific antigens (ESAT-6, CFP-10). Then after adding the sample having the antigen specific to M.tuberculosis , the colour will change, depending upon colour, we can detect the presence of M.tuberculosis.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Cell lines: RAW 264.7 macrophage cells

2.1.2 Chemicals: Isoniazid, Pyrazinamide, PLGA(Poly(lactic-co-glycolic acid) (50:50)), PVA (Polyvinyl alcohol), Alginate and Dialysis membrane were brought from Sigma-Aldrich India. Rifampicin, Ethambutol, HAuCl4 were acquired from SRL. Acetone, CaCl2 and DMSO were brought FINAR. AgNO3 was supplied by EMPLURA. Sodium citrate, NaOH, FeCl3, FeSO4 were provided by RANKEM. DMEM,96 well plate. 90% methanol was used for preparing the calibration curves and during the process of calculating entrapment efficiency. PBS and citrate buffer were used for conducting drug release studies.

2.2 Methods

2.2.1 Preparation of UV-spectra of Isoniazid, Pyrazinamide, Rifampicin, Ethambutol drugs in 90% methanol solution

10 ml milli Q water was taken and 90 ml pure methanol was added to it to obtain the 90% methanol solution. Each of the drugs were measured with weighing balance and mixed separately with required amount of freshly prepared 90% methanol to obtain concentration of 1mg/ml for those drugs. This was our stock solution. At first standard was set by keeping 90% methanol in both sides of UV-spectrophotometer. Then drug solution was loaded in one side and scanned for each drug at a range of 200 nm to 800 nm to obtain the spectra of these drugs.

2.2.2 Preparation of calibration curve for Isoniazid, Pyrazinamide, Rifampicin, Ethambutol drugs in methanol solution

10 ml milli Q water was taken and 90 ml pure methanol was added to it to obtain the 90% methanol solution. Each of the drugs were measured with weighing balance and mixed separately with required amount of freshly prepared 90% methanol to obtain concentration of 1mg/ml for those drugs.

This was our stock solution. From the stock solution of each drug, dilutions were made. Each dilution was made in triplicate. Absorbance of each dilution of each drug were measured at particular wavelength (Isoniazid-262 nm, Pyrazinamide-269 nm, Rifampicin- 330 nm, Ethambutol- 224 nm). Averages of absorbance values were taken and plotted against concentration(µg/ml) in graph.

2.2.3 Preparation of anti-tuberculosis drug loaded PLGA nanoparticles

Here PLGA nanoparticles were prepared using the ultrasonic atomizer

All the four anti-tuberculosis drugs were taken in different concentrations (5 %, 10 %, 15 %, 20 %, 40 %, 60 %, 80 %) according to the concentration of PLGA is taken. For aqueous phase 1% PVA (500mg in 50 ml H_2O) solution is prepared and kept in magnetic stirrer for 5-10 minutes to be mixed properly.30 mg PLGA is taken and dissolved well in 3 ml acetone solution through vibrator. This is the organic phase. From the stock solution of PLGA polymer, 0.25%(w/v) PLGA is made. PLGA in acetone solution (0.25% w/v) is added to each of the three anti-tuberculosis drugs. Aqueous phase (PVA solution) is kept on magnetic stirrer (1500 rpm) and by the syringe pump (0.3 ml/min), drug sample was pushed to the ultrasonic atomizer(130KHz), which chops the solution into nano-sized particles and sprays into the aqueous phase. After that the nanoparticle solution is kept upon magnetic stirrer for about 10-20 minute(50-55^{0}C), for evaporation of the solvent (acetone).

Later the prepared nanoparticle solutions are ultracentrifuged (60000g-10^{0}C, 30 min) and washed for three times. The nanoparticle solutions are kept in refrigerator for further use. Nanoparticle solution is sonicated for 15 minutes for 2 rounds before any type of further use to prevent aggregation.

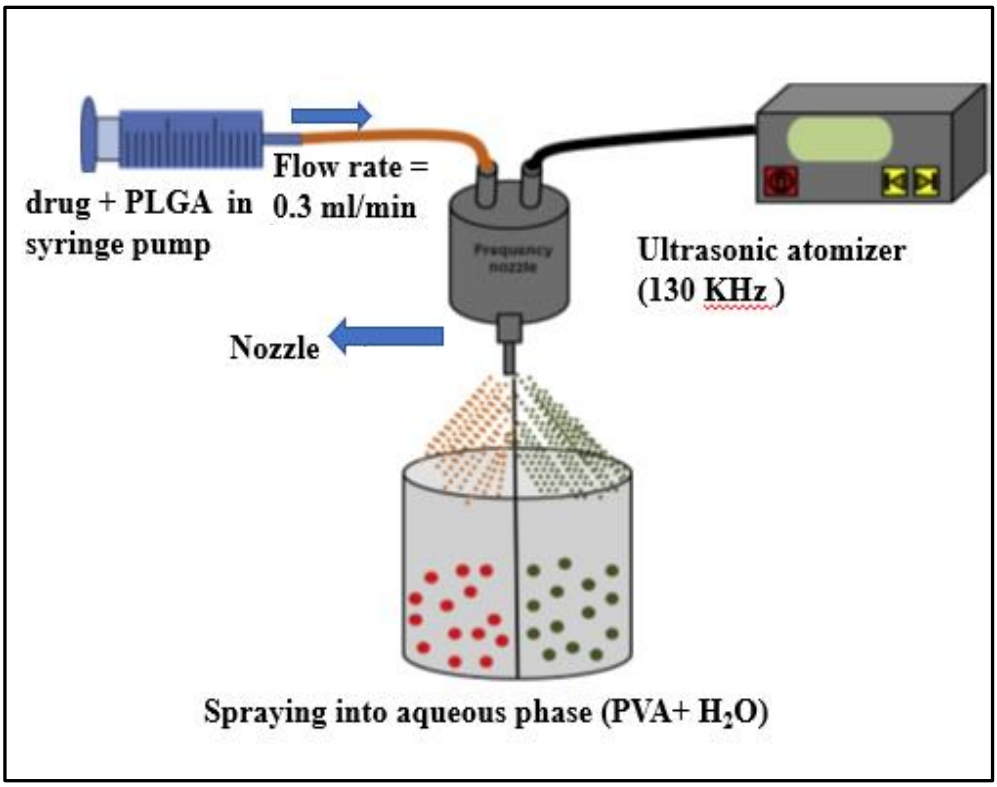

Figure 2-1 Schematic representation of the production of nanoparticles using the ultrasonic atomizer.

Adapted from- "Ultrasonic atomizer driven development of doxorubicin-chitosan nanoparticles as anticancer therapeutics: Evaluation of anionic cross-linkers"-B. Joshi, et al. Journal of Drug Delivery Science and Technology 57 (2020) 101618.

2.2.3.1 Morphological characterization

To detect the particle size and surface morphology of Synthesized NPs SEM was done. NPs were suspended in double distilled water and drop-casted on a glass film allowed to dry and observed under SEM. NP samples were further diluted for dynamic light scattering (DLS) to observe the hydrodynamic radius of the synthesized NPs, also their zeta potential and polydispersity index (PDI) were determined to detect their surface charge and their stability.

2.2.3.2 Entrapment efficiency

To calculate the difference in drug encapsulation efficiency due to vary in drug concentration (5 % - 80 % drugs of 0.25 %(w/v) PLGA) entrapment efficiency is calculated. Entrapment efficiency tells us how much portion of drug is loaded in the NPs, by comparing the concentration of drug loaded to the drug used initially during nanoparticle synthesis.

2.5.1. Direct method

$$Encapsulation\ efficiency\ (\%) = \frac{A1}{A0} * 100$$

2.5.2. Indirect method

$$Encapsulation\ efficiency\ (\%) = \frac{A0 - A2}{A0} * 100$$

Initial drug loaded = A_0, Amount of drug encapsulated in Nps = A_1, Amount of drug left in the supernatant = A_2.

Figure 2-2 Calculation of entrapment efficiency.

Adapted from- "Ultrasonic atomizer driven development of doxorubicin-chitosan nanoparticles as anticancer therapeutics: Evaluation of anionic cross-linkers"-B. Joshi, et al. Journal of Drug Delivery Science and Technology 57 (2020) 101618.

2.2.4 Drug release study of anti-tuberculosis drug loaded PLGA nanoparticle in pH 7.2 & pH 5.5

Here PLGA nanoparticles of al the four drugs were prepared using the ultrasonic atomizer according to the previously mentioned method.

PBS of pH 7.2 and citrate buffer of pH5.5 was prepared. After that only pellets of the nanoparticles were collected and 1ml PBS was added to pellet of each of the four drugs. Dialysis bags containing the sample were prepared. Conical flasks were filled with 120 ml of PBS or citrate buffer for each and dialysis bags

were added into PBS inside the conical flasks. Conical flasks containing the dialysis bags were stirred continuously upon magnetic stirrer at 37°C, 200 rpm for 120 hrs.10 ml of solution was taken from these conical flasks at an interval of 2h until 12^{th} hour, then at an interval of 24h until 120h and replaced with same amount of fresh PBS or citrate buffer. Then absorbance was taken of these solutions at the particular wavelength of particular drug.

2.2.5 Determining the cytotoxicity of anti-tuberculosis drugs loaded PLGA nanoparticles against RAW 264.7 macrophages

RAW macrophage cells were grown in DMEM which contains 10%(v/v) fetal bovine serum, 1% (v/v) penicillin streptomycin. Cells were maintained in a CO_2 incubator (5% CO_2, 37°C). RAW 264.7 macrophage cells were seeded in 96-well plates at a density of 15000 cells/well and cultured for 24 hrs before use. Anti-tuberculosis drugs loaded PLGA nanoparticles were prepared using ultrasonic atomizer and nps were suspended in 0.8 % DMSO. The following day, the culture medium was removed and the np + DMEM solutions were added at different concentrations (1,10,25,50,100 µg/ml). Cells treated with blank PLGA nps and only DMEM were taken as control. After 24 hours of incubation, the culture medium was removed and replaced by MTT+ fresh DMEM media. The plate was incubated for 4 hours at 37°C in the dark. After that, MTT solution was discarded and formazan crystals were solubilized using MTT dissolving solution. The plate was kept at room temperature for 10 minutes and absorbance (570 nm).

2.2.6 Preparation of alginate microspheres of PLGA nanoparticle

Here PLGA nanoparticles were prepared using modified oil in water emulsion method with ultrasonic atomizer. The pellets of the nanoparticles are resuspended in 1 ml milli Q water. 4% $CaCl_2$ -water (w/v) (1.6 gm in 40 ml H_2O) solutions are made.6 mg alginate is added to 2 ml water to make the 0.3 % (w/v) stock. From that stock of alginate 1ml is taken and added to the 1ml of nanoparticle in milli Q solution. The alginate & nanoparticle are our sample for

next round of spraying and for preparing the blank alginate microsphere 1 ml of alginate is added to 1 ml of milli Q. $CaCl_2$ is kept on the magnetic stirrer at 1500 rpm and the sample is sprayed through the ultrasonic atomizer (the flow rate of the syringe pump is 0.3 ml/minute). After that the solution was washed for three times (7000 rpm at 10°c). Then the pellets were collected and 500 µl water was added to it then kept in vortex for 5 minute and sonicated for 15 minutes.

2.2.7 Preparation of silver nanoparticles

2.2.7.1 Regular method-

50 ml sodium citrate and 8.8 ml silver nitrate is prepared. At 170 °c the sodium citrate solution was heated until it starts to boil with constantly stirring. After it has started to boil, the temperature is reduced to 150°c. Then the silver nitrate solution is added dropwise to the sodium citrate solution and stirred for 40 minutes. After letting the resulting solution to cool down, it is centrifuge at 2040 RCF for 30 minutes at 10°c to obtain the silver nanoparticles as pellets.

2.2.7.2 Frens method-

50 ml silver nitrate and 500 µl sodium citrate is prepared. At 170 °c the silver nitrate was heated until it starts to boil with constantly stirring. After it has started to boil, the temperature is reduced to 150°c. Then the sodium citrate solution is added drop-by-drop to the constantly stirring silver nitrate solution and stirred for 20 minutes. After letting the resulting solution to cool down, it is centrifuge at 2040 RCF for 30 minutes at 10°c to obtain the silver nanoparticles as pellets.

2.2.8 Synthesis of Super-paramagnetic iron oxide nanoparticles

6g $FeCl_3$ and 4g $FeSO_4$ were added to 25 ml of distilled water and stirred for proper mixing of these constituents. After these are properly mixed, the temperature was raised up to 80°c and 8.5M NaOH is added under vigorous stirring until the pH of the resulting solution becomes 8-12. Then it is kept for 60 minutes, under vigorous stirring. The solution was washed two times at 7000 rpm, 10°c for 15 minutes. Pellets are collected and dried in hot-air oven for overnight to get fine particles.

2.2.9 Synthesis of gold nanoparticle

The glassware which were used in this process, was first washed with aqua-regia. 0.8mM solution of $HAuCl_4$ was prepared. 1% Sodium Citrate (100 mg in 10 ml) was prepared as the reducing agent. The $HAuCl_4$ was stirred continuously at 750 rpm, 150°c for boiling. After it has started to boil, 2 ml of the sodium citrate solution was added to it and stirring was continued. After 4-5 minutes the solution turned into purple, the solution was stirred continuously until the solution becomes wine red (after 1.30 hr) then after it has come to normal temperature, it was stored in dark.

2.2.10 Synthesis of gold coated spions

SPIONs were produced using the previous mentioned method. 10 ml $HAuCl_4$ was added to 20 mg of dried spions and let the solution in rest for 1 hr, so that the spions are first soaked in gold chloride solution and complexes can form on the surface of spions. Then 20 ml of sodium citrate was mixed with the solution and constantly stirred for 3 hours at 60°c, the colour of the resulting solution changes from brown to burgundy, over the time

Chapter 3: Results and discussion

3.1 Preparation of UV-spectra of Isoniazid, Pyrazinamide, Rifampicin, Ethambutol drugs in 90% methanol solution

The UV-spectra delivers that all the four front line anti-tuberculosis drugs have given significant peaks specific to them.

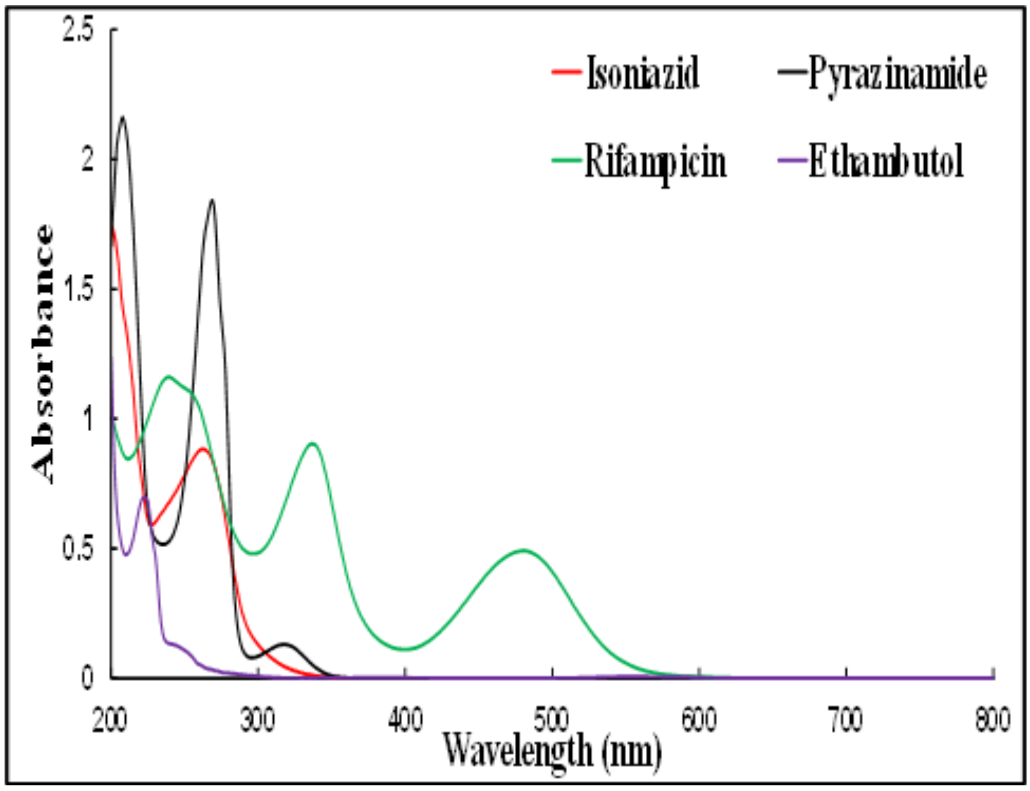

Figure 3-1 UV- spectra of Isoniazid (262 nm), Pyrazinamide (269 nm), Rifampicin (330 nm), Ethambutol (224 nm) in 90 % methanol.

3.2 Preparing calibration curve of four anti-tuberculosis drugs

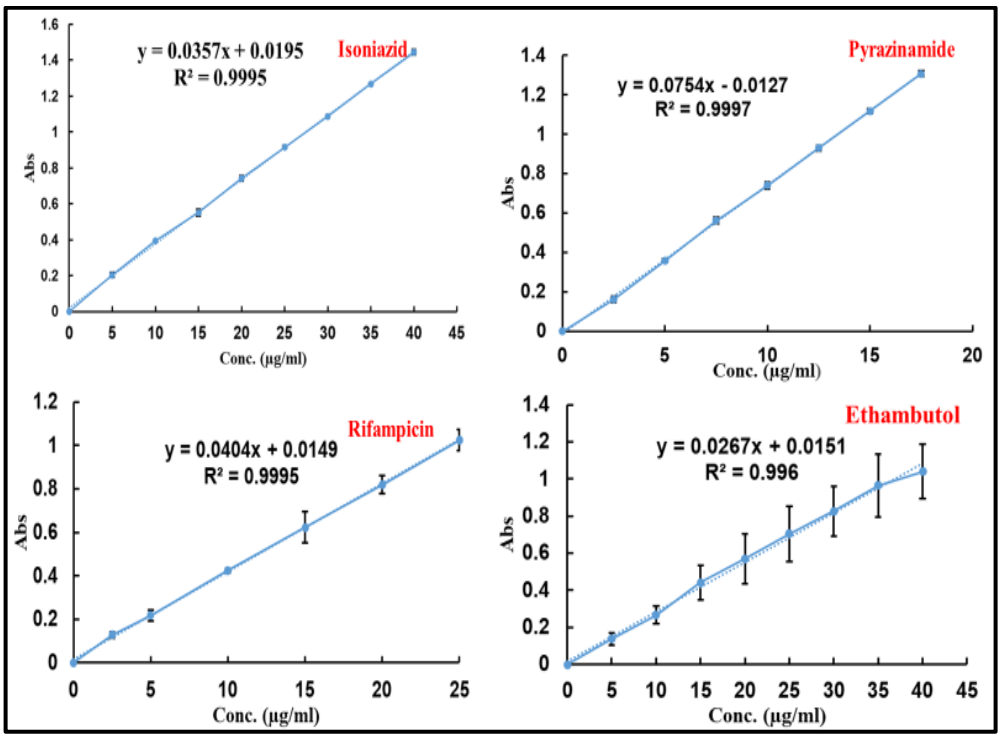

Figure 3-2 Calibration curve of anti-tuberculosis drugs

Calibration curves were prepared from each of the dilutions of each drug taken in triplicate. Calibration curve provides the linearity coefficient and regression equation. These calibration curve shows that, the linearity co-efficient is near to 1 and these drugs obey the Beer-lambert's law. Upon depending these calibration curves, we have calculated the entrapment efficiencies and made the drug release profile.

3.3 Preparation of anti-tuberculosis drug loaded PLGA nanoparticles

Drug loaded NPs are synthesized using ultrasonic atomizer. In ultrasonic atomizer a spray nozzle is connected to a syringe pump and creates vibration of waves at the tip of nozzle, due to which liquid is transformed to nano-sized droplets by the nozzle.

3.3.1 Morphological examination

Morphological properties like size, shape, surface charge etc decide the activity of the nanoparticle. Synthesized NPs were analyzed under SEM to check the size and surface morphology of synthesized particles.

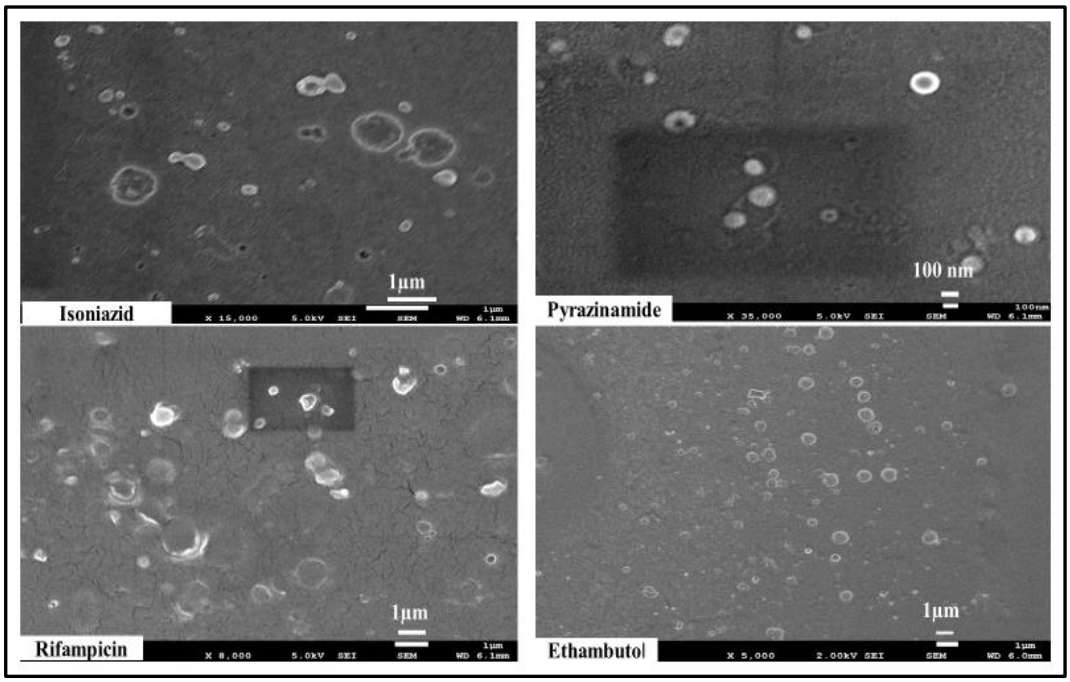

Figure 3-3 SEM image of synthesized anti-tuberculosis drug loaded PLGA NPs.

SEM images of synthesized anti-tuberculosis drug loaded PLGA NPs are spherical in shape and well mono-dispersed.

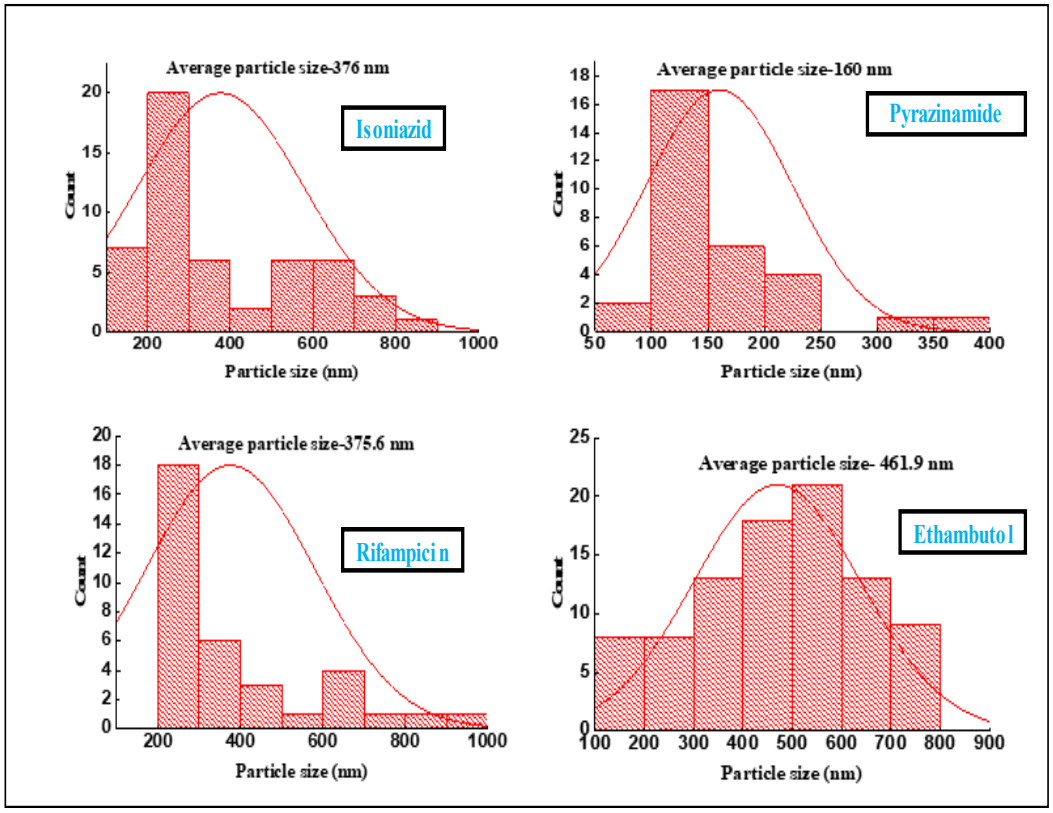

Figure 3-4 Histogram of size of anti-tuberculosis drug incorporated PLGA NPs by SEM analyzed using ImageJ software.

From the histogram, is depicted that, among produced NPs, most of the Isoniazid loaded PLGA NPs have size in the range of 160-350 nm, whereas most of Pyrazinamide loaded, Rifampicin loaded, Ethambutol loaded PLGA NPs are in the size range of 100-170 nm, 200-400 nm, 400-525 nm respectively.

3.3.2 Entrapment efficiency

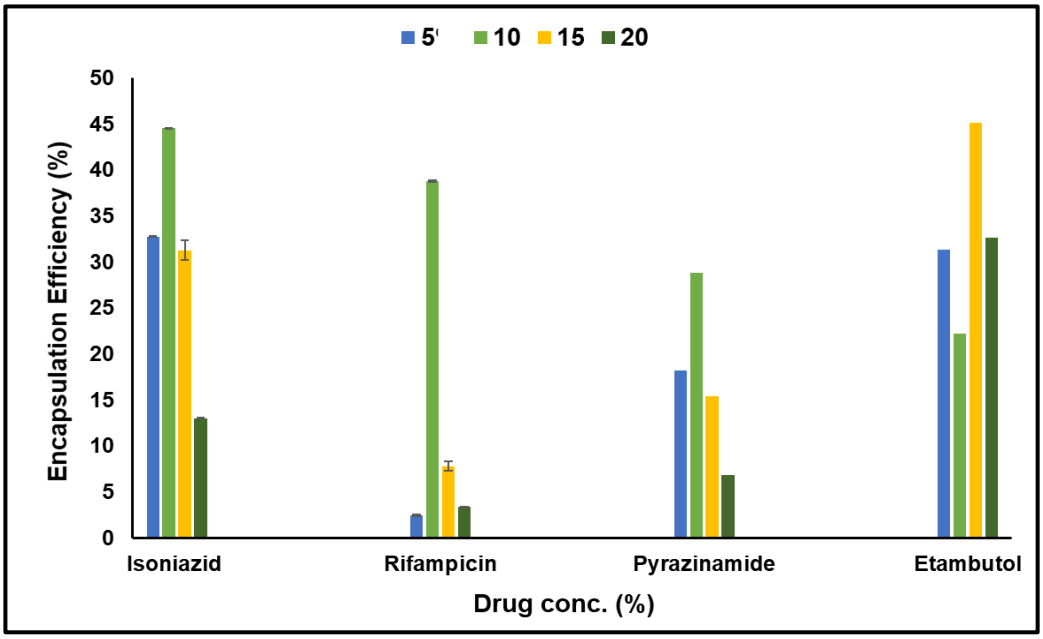

Figure 3-5 The percentage of drug encapsulation in PLGA NPs at different drug concentrations with respect to (0.25%: w/v) PLGA concentration

Entrapment efficiency of four anti-tuberculosis drugs loaded PLGA nanoparticle was determined through direct and indirect method using the standard calibration curve of all the four drugs (Isoniazid, Pyrazinamide, Rifampicin, Ethambutol) developed using UV-Spectrophotometer at specific wavelengths (Isoniazid-262 nm, Pyrazinamide-269 nm, Rifampicin-330nm, Ethambutol-224nm) for each drug. All the four drugs were encapsulated using different concentrations (5%, 10%, 15%, 20%) with respect to the polymer (PLGA) concentration. Isoniazid, Rifampicin, Pyrazinamide has the highest entrapment efficiency at 10 % drug concentration but Ethambutol has highest entrapment efficiency at 15 % drug concentration with respect to the polymer used.

3.4 Drug release study of anti-tuberculosis drug loaded PLGA nanoparticle

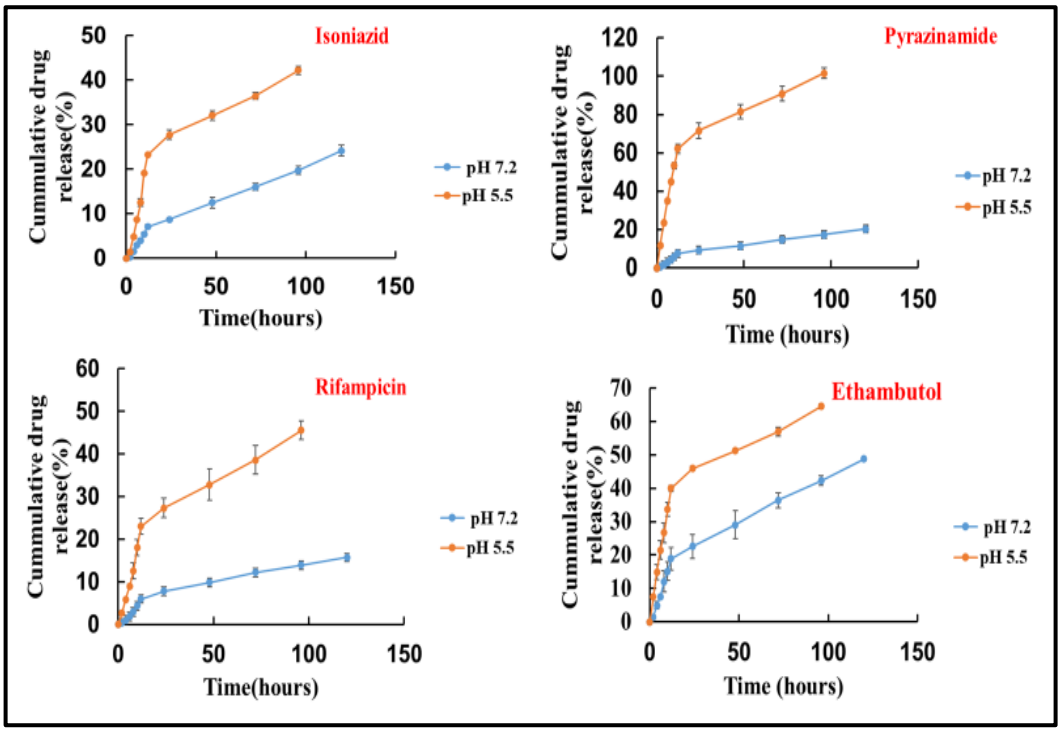

Figure 3-6 Release profile of four frontline anti-tuberculosis drugs in neutral and acidic pH

In-vitro release pattern of anti-tuberculosis drug loaded PLGA nanoparticles were studied through dialysis method at a pH of 7.2 (PBS). The amount of released drug was calculated with respect to how much drug was loaded. All the drugs loaded PLGA NPs released the drug much more sustainably for over 96hours. This depicts that PLGA nanoparticles are releasing the drugs sustainably over a longer period, whereas, the free drugs remain in the therapeutic concentration for only 2-6 hours. The drugs are being released more rapidly in acidic condition than in neutral condition.

3.5 Determining the cytotoxicity of anti-tuberculosis drugs loaded PLGA nanoparticles against RAW 264.7 macrophages

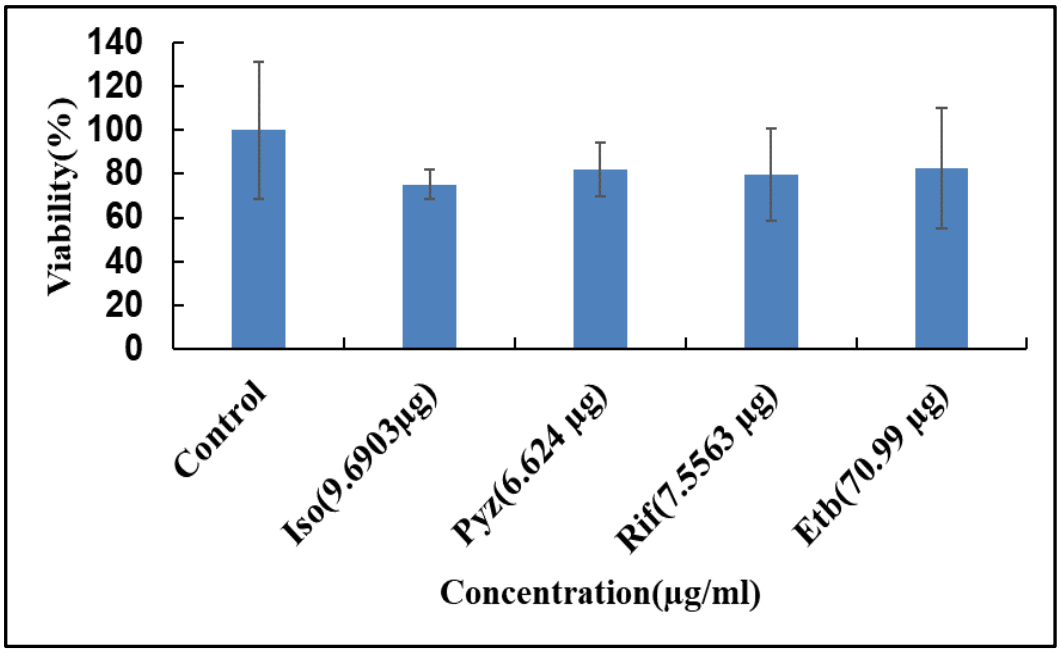

Figure 3-7 Cytotoxicity of PLGA NPs of anti-tuberculosis drugs in RAW macrophages

Table 2 Comparison of the amount of released drug from PLGA nps with respect to MIC of those drugs

Drug	Released in 24 hours (our data)	MIC (MINIMAL INHIBITORY CONCENTRATION) reported
Isoniazid	9.6903µg	0.25 µg/ml
Pyrazinamide	6.624 µg	0.08 µg/ml
Rifampicin	7.5563 µg	1 µg/ml
Ethambutol	70.99 µg	8 µg/ml

From this data it can be depicted that, after 24 hrs. of incubation PLGA NPs have released the drugs in an amount greater than the MIC and cells have shown 70-80% viability at these amount of drugs. So we can say that, PLGA NPs are not killing the normal healthy cells.

3.6 Preparation of alginate microspheres of PLGA nanoparticle

Alginate microspheres of drug loaded NPs are produced through ultrasonic atomizer. In ultrasonic atomizer a spray nozzle remain attached to a syringe pump and the nozzle vibrates, due to which liquid is transformed to very tiny droplets by the nozzle.

3.6.1 Morphological examination

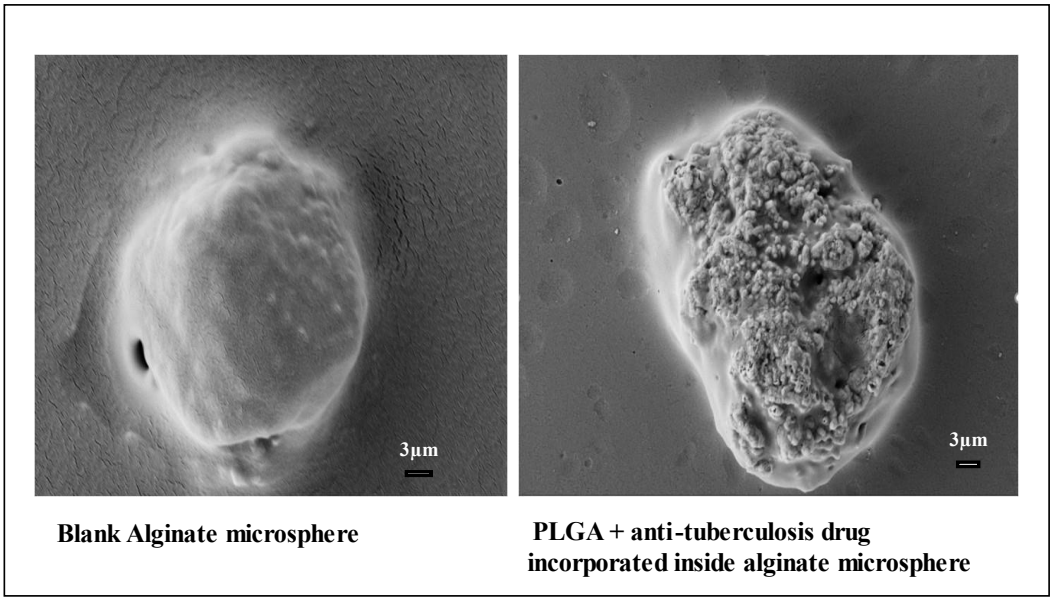

Figure 3-8 SEM images of alginate microspheres. A) Blank microsphere, B) Alginate microsphere of anti-tuberculosis drug loaded PLGA NPs

To check their shape and surface morphology, the synthesized microspheres were analyzed through SEM. SEM images depicts that, microspheres are monodispersed, spherical in shape. They have a size of average 2-3µm.

3.7 Preparation of silver nanoparticles

3.7.1 UV- spectroscopy of silver nanoparticles

Synthesized silver nanoparticles have been characterized through UV-Spectroscopy. They have given peak at wavelength of 417 nm, which is specific for silver nanoparticles. In between these two methods, the frens method is producing more concentrated silver nanoparticles than that of the regular method as the intensity in UV-spectra at 417nm wavelength depicts that.

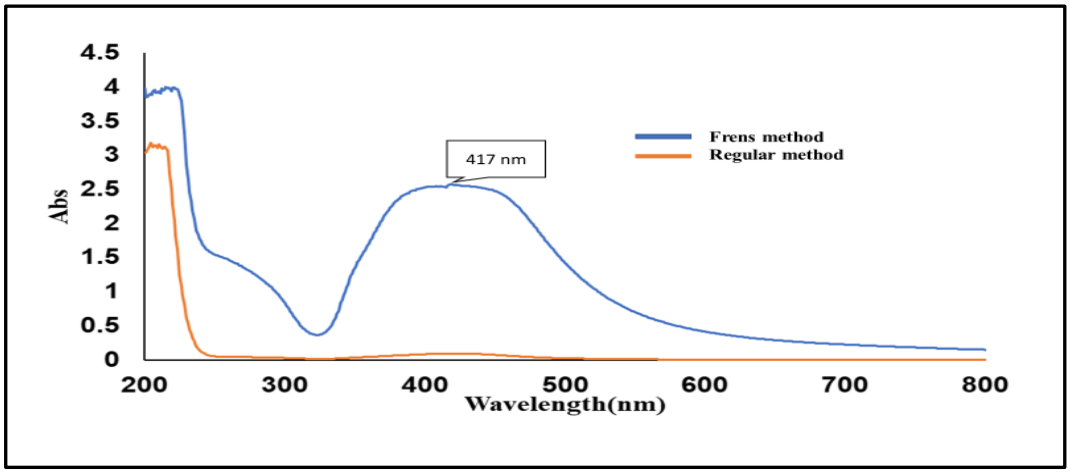

Figure 3-9 UV- spectroscopy of silver nanoparticles

3.7.2 Energy Dispersive x-ray spectroscopy-

To detect the elements, present in those synthesized silver nanoparticles, EDX was done.

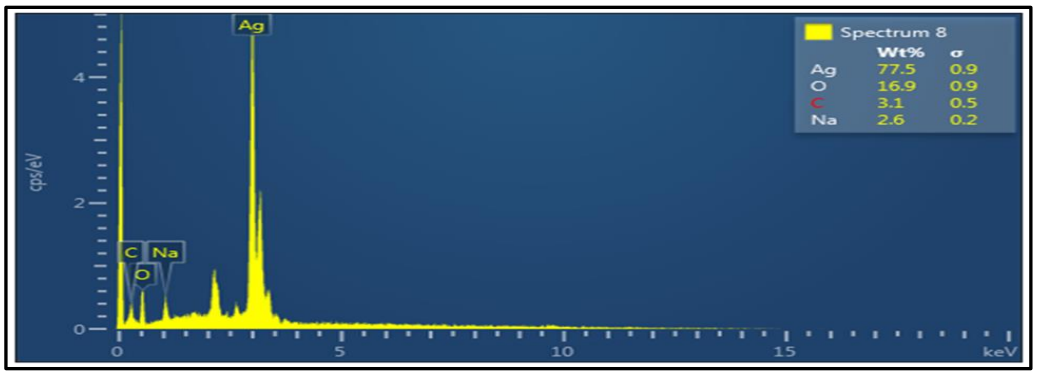

Figure 3-10 EDX of Silver nanoparticles

The presence of Ag depicts that silver nanoparticles have been produced through this method successfully.

3.7.3 Morphological characterization of silver nanoparticles

3.7.3.1 Scanning electron microscopy

The produced silver nanoparticles were observed under SEM to identify their surface morphology along with size.

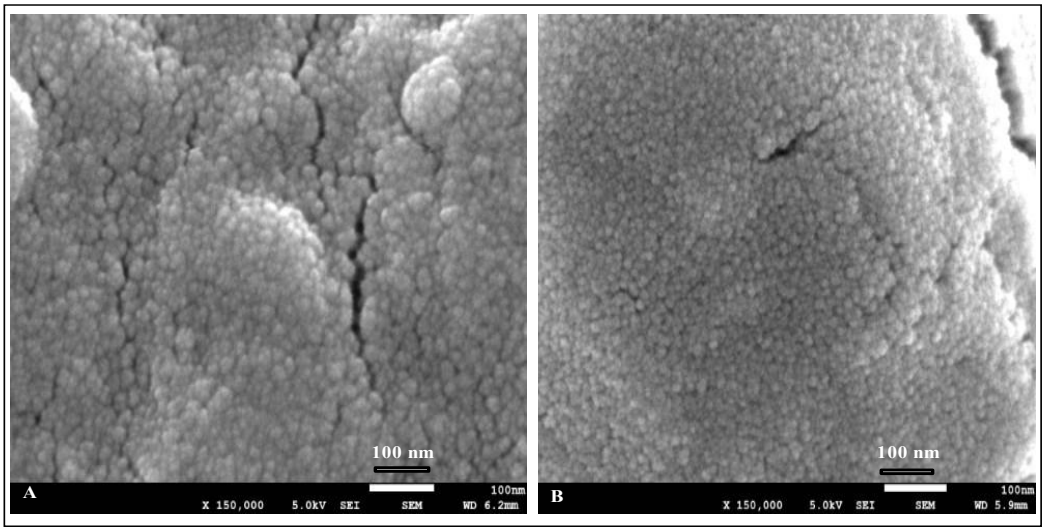

Figure 3-11 SEM images of silver nanoparticles

According to the SEM images the produced silver nanoparticles are spherical in shape and mono-dispersed and they are in the size range of 5-10 nm.

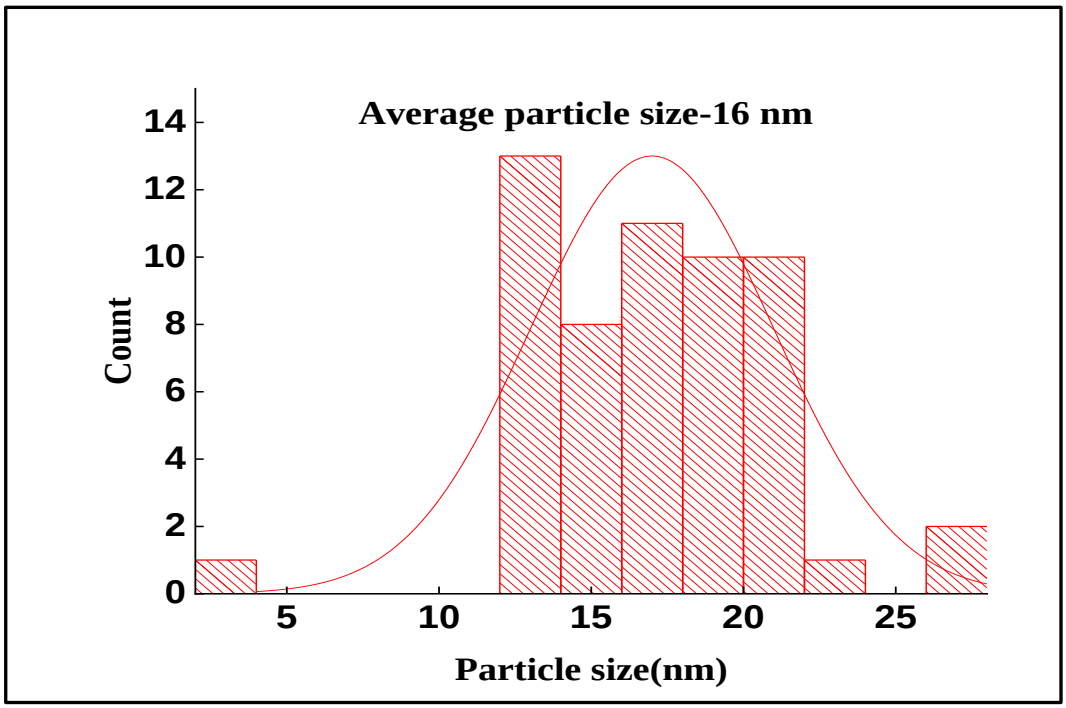

Figure 3-12 Histogram of size distribution of silver nanoparticles

Histogram shows that the produced silver nanoparticles have an average size of 16 nm, which is similar to as previous literatures reported.

3.8 Synthesis of Super-paramagnetic iron oxide nanoparticles

3.8.1 Scanning electron microscopy

The produced SPIONs were observed under SEM to identify their surface morphology, also size.

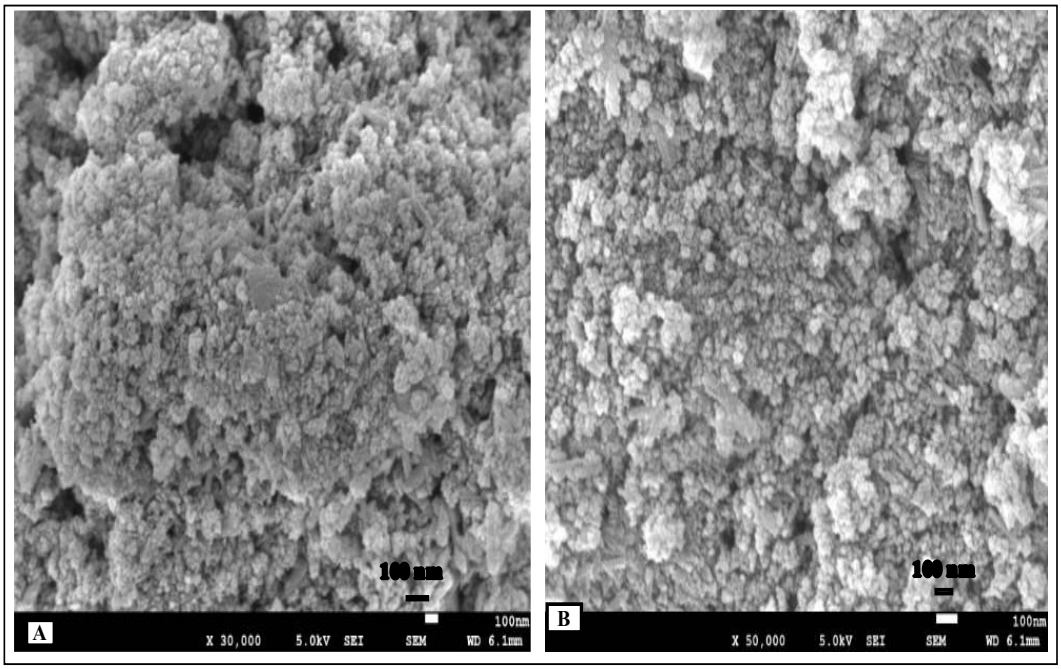

Figure 3-13 SEM images of SPIONs

These SEM images show that, these spions are spherical in shape and well mono-dispersed, they are 5-15 nm in size

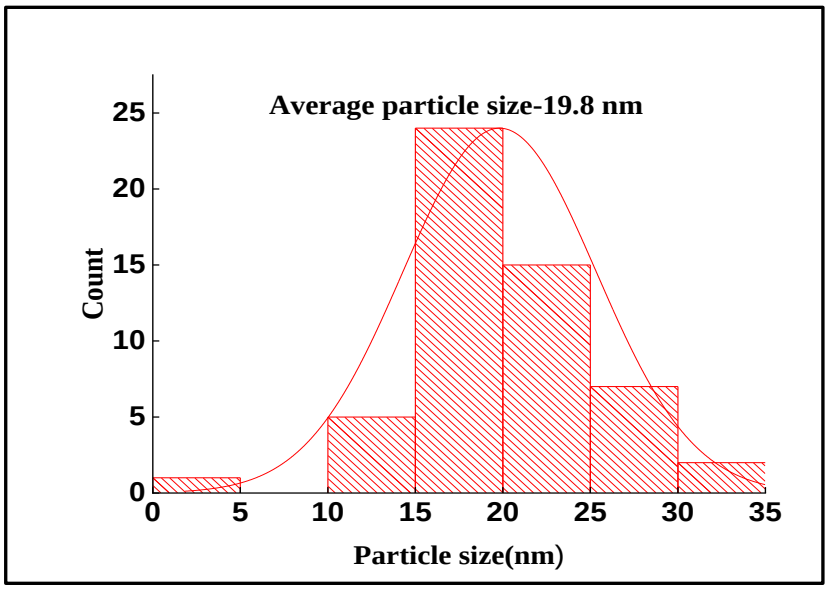

Figure 3-14 Histogram of size distribution of SPIONs

Histogram shows the size distribution of synthesized SPIONs and their average size is near 20 nm.

3.8.2 FT-IR

FT-IR was done to detect the presence of specific bond stretching regarding the SPIONs.

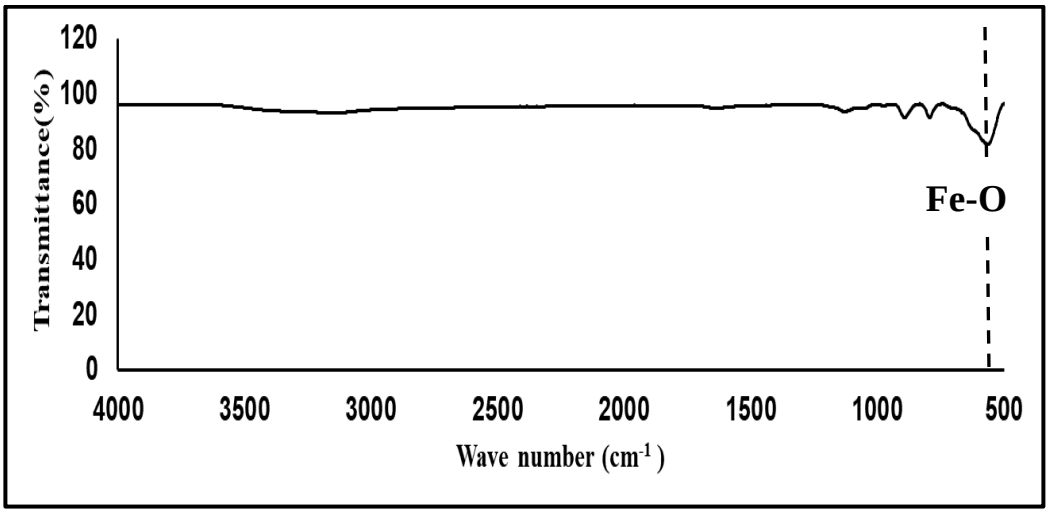

Figure 3-15 FTIR of SPIONs

The specific peak at 500 cm^{-1} is due to the stretching of Fe-O bond, the presence of Fe-O bond depicts that the produced particles are super paramagnetic iron-oxide nanoparticles

3.8.3 Energy Dispersive x-ray spectroscopy-

To detect the elements, present in those synthesized spions, EDS was done.

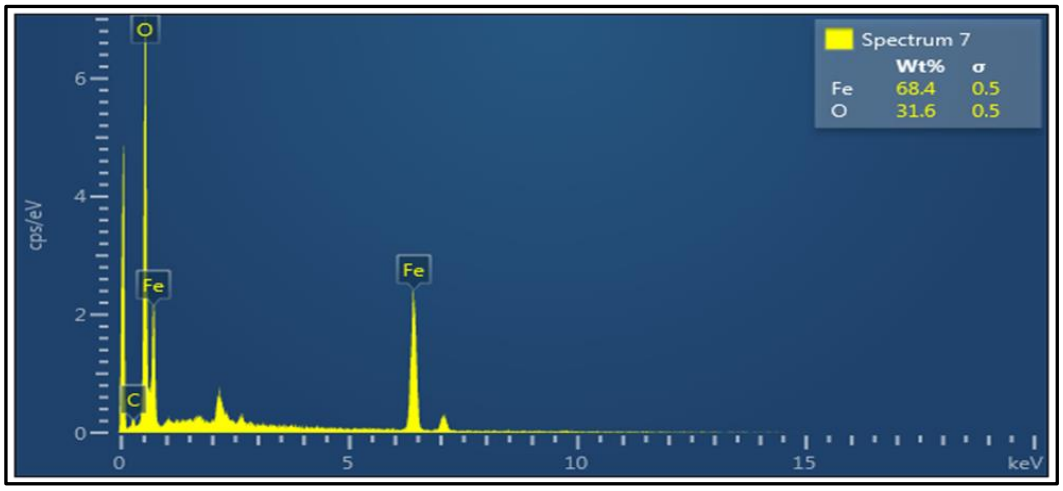

Figure 3-16 EDS of SPIONs

The presence of Fe, O in maximum weight %, depicts that spions have been produced through this method successfully.

3.9 Synthesis of gold nanoparticle

3.9.1 UV-Spectroscopy-To get the spectra of synthesized solution, UV-Spectroscopy was done.

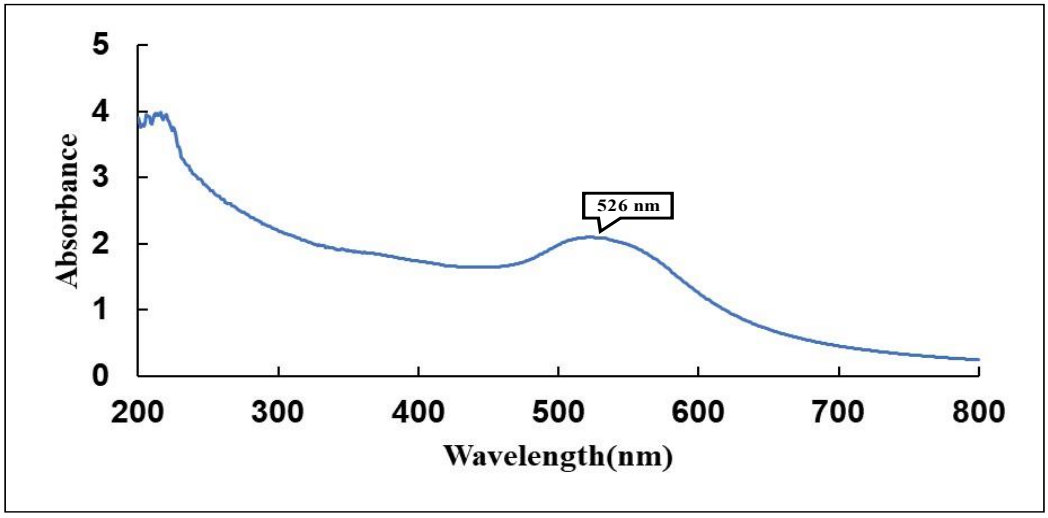

Figure 3-17 UV-Spectroscopy of gold nanoparticles

The synthesized solution has given a peak around 526 nm, this depicts that gold nanoparticle have been produced through this method and they maybe of 5-20nm in size.

3.9.2 Morphological characterization of Gold nanoparticles

3.9.2.1 Scanning electron microscopy

The produced particles were observed under SEM to check their surface morphology, shape, also their size.

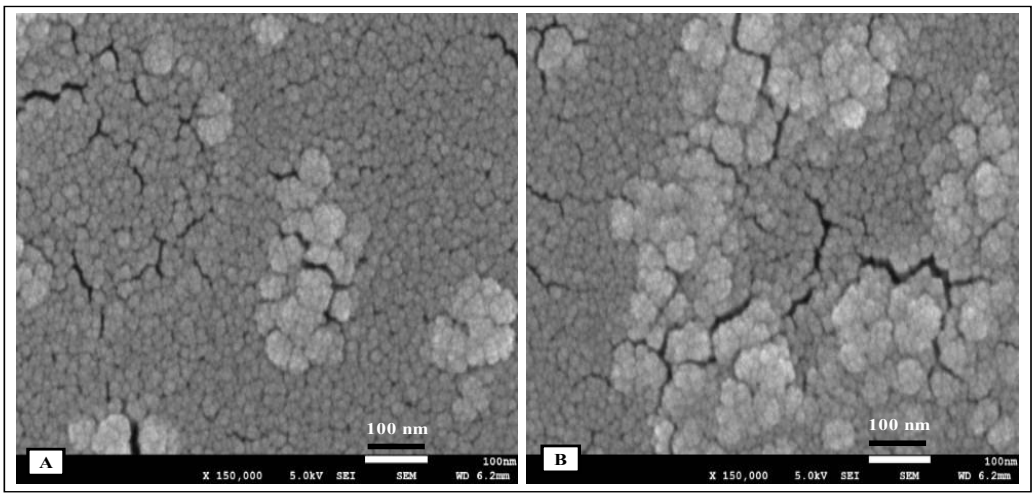

Figure 3-18 SEM images of Gold nanoparticles

SEM images show that synthesized gold nanoparticles are spherical in shape.

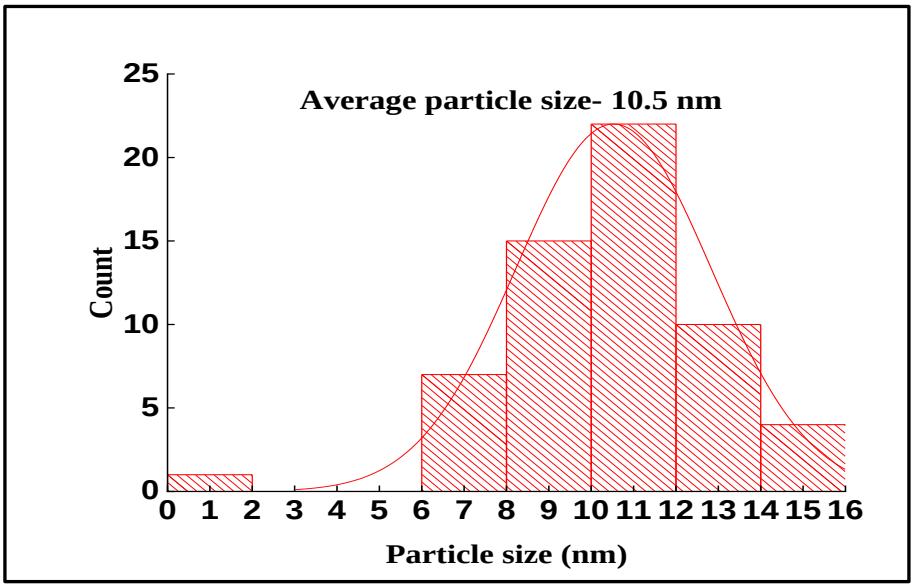

Figure 3-19 Histogram of size distribution of gold nanoparticles

The histogram shows that the gold nanoparticles produced through this method have an average size of 10.5 nm. It can serve our purpose in diagnostic procedure, in case of antibody/antigen binding.

3.9.3 Energy Dispersive x-ray spectroscopy-

To detect the elements, present in those synthesized particles, EDX was done.

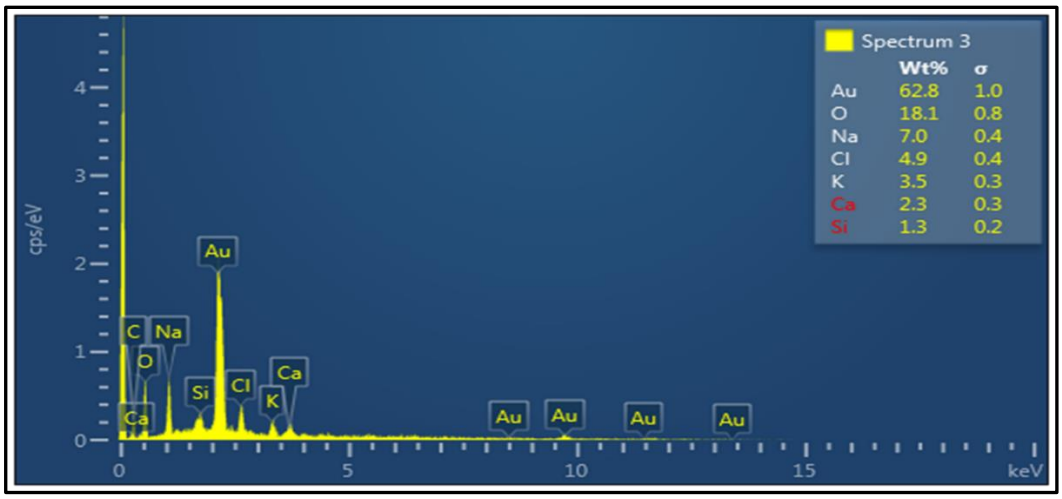

Figure 3-20 EDX of gold nanoparticles

The presence of Au (gold) in maximum wt %, depicts that gold nanoparticles have been produced through this method successfully.

3.10 Synthesis of gold coated spions

3.10.1 UV-Spectroscopy-To get the spectra of synthesized particles, UV-Spectroscopy was done.

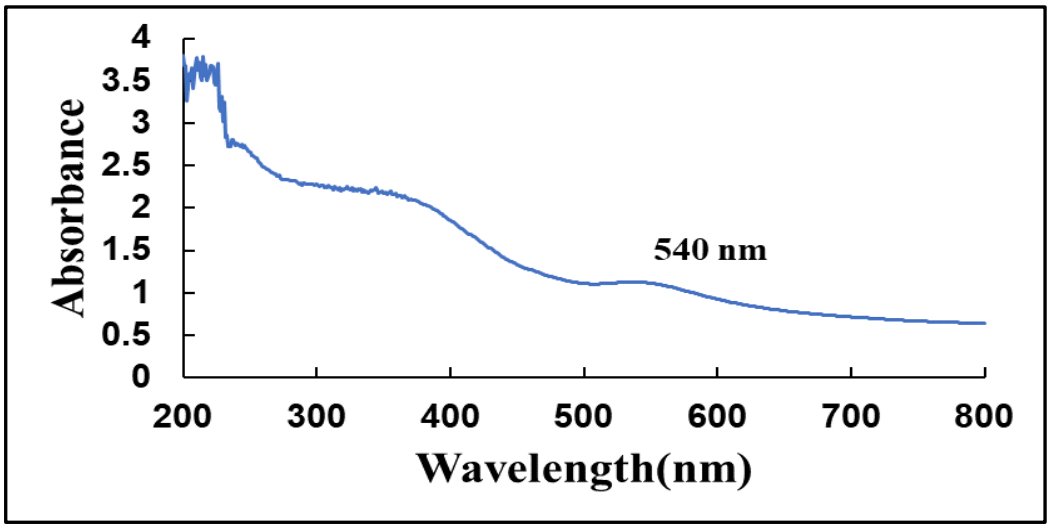

Figure 3-21 UV-Spectroscopy of gold coated SPIONs

The uv-spectra shows that there is a peak at 540 nm. This peak around 540 nm is specific for Au-SPIONs, this ensures that, this method has produced AU-SPIONs.

3.10.2 Scanning electron microscopy-The produced nanoparticles were observed under SEM to identify their size, surface morphology and shape.

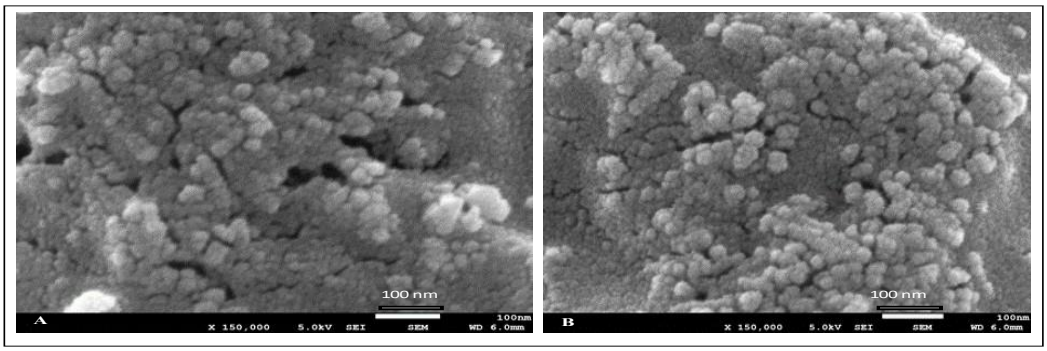

Figure 3-22 SEM images of Gold coated SPIONs

According to the SEM image, produced particles are spherical in shape

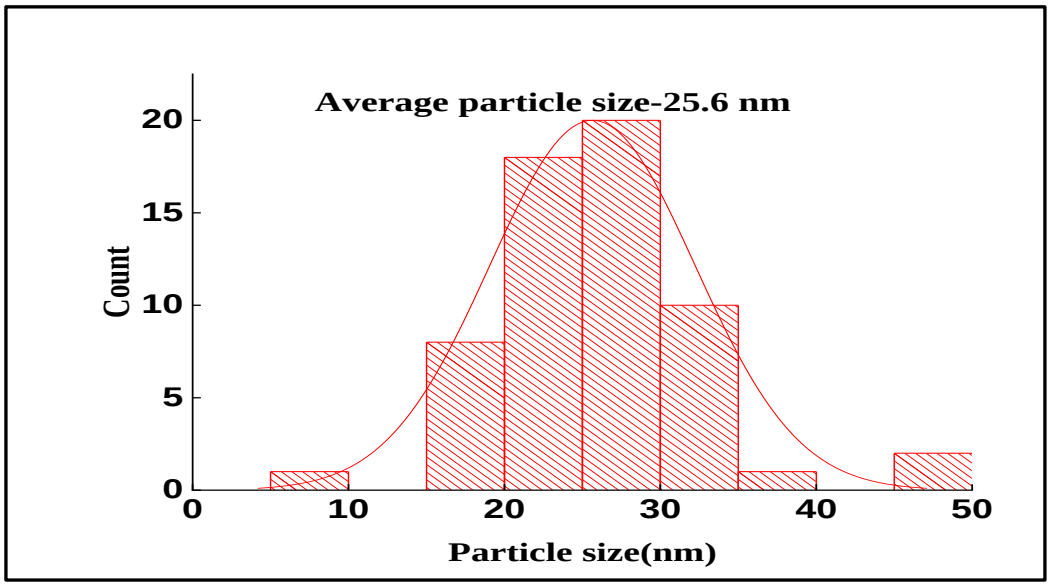

Figure 3-23 Histogram of size distribution of gold coated SPIONs

From this histogram, we can see that, particles have an average size of 25 nm, comparatively greater than the normal SPIONs. From this histogram it can be said that the increase in size is due to the gold coating of the SPIONs.

Chapter 4: Conclusion & future scope-

TB is the most death causing infectious disease, surpassing the AIDS. Also, MDR-TB, XDR-TB has posed a severe threat to the human life. TB remains a neglected disease, but more effective new drugs against TB with lesser side effects and toxicity should be developed. Also, novel reliable drug-delivery systems should be developed to combat TB. Anti-TB drug loaded polymeric nanoparticle has given good results according to many research groups around the world. It's a long run, still a lot to find, a lot to research about the stability, their behavior in human system but drug loaded polymeric nanoparticles can be our hope against TB in near future. In case of fast, reliable, efficient diagnosis of TB, anti-body conjugated to gold nanoparticle or carbon quantum dots can be the possible solution.

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