Synthesis and Characterization of S- PEG for Delivery of Arthritis Drug

A major project report submitted

In partial fulfillment of the requirement for the degree of

Master of Technology

In

Industrial Biotechnology

Submitted by

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DECLARATION

Certified that the project report **"Synthesis and characterization of S-PEG for delivery of Arthritis drug"** submitted by me in partial fulfilment of the requirement for the award of the degree of Master of Technology in Industrial Biotechnology, Delhi Technological University. It is a record of original research work carried out by me under the supervision of **Dr. Jai Gopal Sharma, Associate Professor** Department of Biotechnology, Delhi Technological University, Delhi. The matter embodied in this project report is original and has not been submitted for the award of any Degree/Diploma.

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Certificate

This is to certify that the major project entitled **"Synthesis and Characterization of S-PEG for Delivery of Arthritis drug"** is the bonafied work of Ms. Kriti Shivhare, in the partial fulfilment of the requirements for the award of the degree of Masters of Engineering, Delhi Technological University, Delhi is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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List of Abbreviation

Au	Gold
Abs	Absorbance
BSA	Bovine serum albumin
$CDCl_3$	Deuterated Chloroform
D_2O	Heavy water or Deuterated water
DCC	N,N'-dicyclohexylcarbodiimide
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DLS	Dynamic Light Scattering
DMARDS	Disease modifying antirheumatic drugs
DMAP	4-Dimethyl amino pyridine
EDTA	Ethylene diamine tetraacetic acid
EDC	Ethylene dichloride
ELISA	Enzyme linked immune sorbent assay
FBS	Fetal bovine serum
FTIR	Fourier transform infrared radiation
GFP	Green Fluorescent protein
HEK	Human Embryonic Kidney
IL-1	Interleukin -1
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
KBr	Potassium bromide
MeOH	Methanol
MHz	Mega Hertz
mL	Millilitre
mg	Milligram
µL	Microlitre
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
nm	Nanometre
nDDs	Nano drug delivery system
NaCl	Sodium chloride
Na_2HPO_4	Disodium phosphate
NaN_3	Sodium azide
NMR	Nuclear Magnetic Resonance
ppm	Parts per million
PBS	Phosphate buffer saline
PEG	Polyethyleneglycol monomethyl ether
PLGA	Poly(DL-Lactic-coglycolic acid)
PDI	Polydispersity index
rpm	Revolution per minute
S-PEG	Sulfasalazinepolyethylene glycolmonomethyl

	ether
SDS	Sodium dodecyl sulfate
TLC	Thin Layer Chromatography
TEM	Transmission electron microscope
TNF-α	Tumor necrosis factor-α
UV- VIS	Ultra Violet Visible

Abstract

The Self assembled polymer based nanostructures have been the focus of research in the past decade because of their significant role in various biological system. In this study, a modified sulfasalazine was conjugated with a Polyethylene glycol $_{750}$ monomethylether to construct a multifunctional conjugate, sulfasalazine-PEG in millique into a miscelle type structure and to deliver the arthritis model drug sulfasalazine. The formation of nanostructure was confirmed by TEM and DLS analyses. The mean size of the sulfasalazine PEG was determined i.e. ~212.9 nm and having zeta potential of about -2.49 mV. In this nanocarriers which can be used to encapsulate hydrophobic drug ornidazole and sulfasalzine so there is hydrophobic and hydrophilic interaction between the drug and polymer and release profile of drug was studied at different pH 4.5 and 7. MTT assays showed the non-toxic behaviour of drug and polymer conjugate when performed at different concentration of these conjugate, drug and polymer. So all these results determine that these nanostructures synthesized are used as efficient vector for drug delivery application.

1. INTRODUCTION

Nanotechnology plays a vital role in the field of medicine and drug delivery [1]. Due to its property nanoparticles being able to enhance the efficiency of the drug *invitro* and *in vivo* [2]. Nanoparticles are generally used for delivery purposes due to their exceptional properties such as surface area to mass ratio is much greater than other particle, biocompatibility [3-6], reduce toxicity [7], easily soluble in water [8-9].Due to their proper sizes and surface properties the release of drug occur slowly within the target size. The retention period of drug in blood also increased if the drug is transported in carrier vehicle like a nanoparticles and also results in better accumulation of drug in the tissue due to its enhanced absorptive effect and retention effect [10-11]. The self-assembly of different types of polymers can assemble itself into different types of structures like miscelles [12-13], tubes, fibres, vesicles [14], rods etc. Nanosystems having some different composition and biological properties have been extensively used for drug delivery applications. The most important thing for drug delivery is that there is proper interaction between the nanomaterials and biological environment, target towards the receptors present on the cell surface, release of drug from the nanoparticles, delivery of different types of drugs, stability of therapeutic agents and molecular mechanisms of cell signalling involved [15].The accepted size of nanoparticles for delivery of drug is generally less than < 100 nm. So in this way, nanoparticles can easily adsorb or bind drugs, proteins on their surfaces. The nanoparticles used must be biodegradable so in case of drug delivery the polymer degraded and drug must be reach to their target site. The main aim behind the entrapment of drugs in nanoparticles is to enhance the distribution of drug to their target site and reduce the toxicity of drug to their to non target site [16]. It is very difficult to deliver and transport the very hydrophobic and hydrophilic drug to be released at the site of action so for this amphiphilic nanoparticles are being conjugated with drug [17]. This effective system entails the system being an inactive prodrug in the blood and only taking its active conformation when reach at the target site. This release of drug is totally dependent on pH [18], temperature and enzymatic activity. PEGylation is a technique in which proteins and peptides are generally covalently conjugated with PEG. PEG is an effective carrier because of its properties like lack of immunogenicity, antigenicity, toxicity and protein activity can also decreases [19]. By considering the above points, the main objective behind this work was to prepare a new amphiphilic sulfasalazine conjugated with polyethylene glycol polymer and able to make assembly in water and developed a miscelle type structure which was stable

under cellular environment at different pH conditions and able to encapsulate drug for delivery purposes.

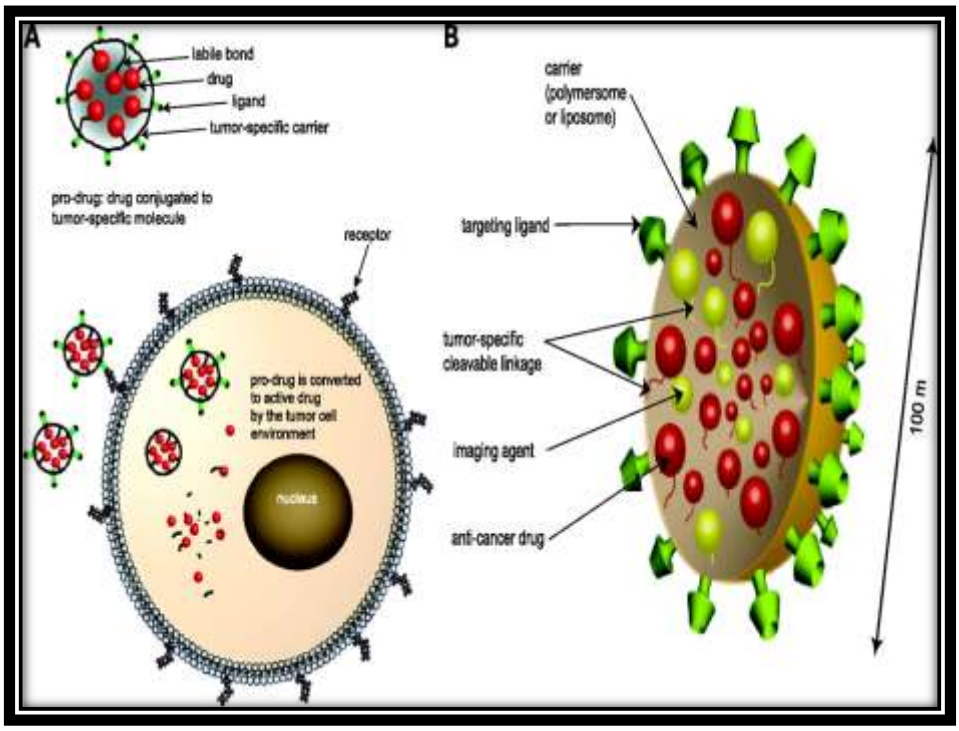

Figure 1: Schematically representation of mechanism of drug delivery to their specific target site

1.1 What is Sulfasalazine?

Sulfasalazine is categorized as non biological DMARDs, disease modifying antirheumatic drugs which is generally used in the medication of arthritis patients to stop the spread of disease along with the relief from the pain. The mechanism of action of DMARDS against inflammation like inhibiton of tumor necrosis factor, suppression of IL-1 and TNF-α, induction of apoptosis of inflammatory cells, by increasing chemotactic factors, inhibit the synthesis of purine and pyrimidine metabolism. Also have important role in diseases like chrohn's disease, psoriatic arthritis, rheumatoid arthritis, juvenile idiopathic arthritis etc [20-22]. Sulfasalazine is basically formed by combination of two compounds i.e aminosalicyclic acid and sulfapyridine and attached through an azo bond. 5-aminosalicyclic acid consists of anti-inflammatory properties and when it administered orally, then upper part of gastrointestinal tract absorbed about 30% of the sulfasalazine and whatever left after absorption passes through the colon, where the azo bond which is present in sulfasalazine is broken by the bacterial enzymes azoreductase, and colon is targeted by 5-aminosalicyclic

acid. The sulfapyridine is completely absorbed and metabolised to N-acetyl sulfapyridine released by hepatic enzymes both sulfasalazine and sulfapyridine have a variety of actions, such as modulates an immune system, antibacterial activity and inhibition of folate dependent enzymes [23]. Sulfasalazine shows delayed release response and also have benefit in use because it have slow radiographic damage [24]. The aim of study was to study to develop arthritis targeted S-PEG_{750} used as carrier encapsulated with sulfasalazine for treatment of rheumatoid arthritis. The quantity of sulfasalazine drug released from the nanoparticles at varies pH was estimated by UV-VIS spectrophotometer at 359 nm. The maximum released percentage of sulfasalazine was 83%.

1.2 Drug used for entrapment

Ornidazole drug is generally used for treatment of amoebiasis. The ornidazole drug is absorbed maximally at gastrointestinal tract and its mechanism of action is long lasting upto 13 h. The objective behind this study was to develop colon targeted in Sulfasalzine-PEG_{750} used as a carrier encapsulated with ornidazole for treatment of amoebiasis. All the formulations were evaluated for the physicochemical parameters and were subjected to *invitro* drug release studies. The amount of ornidazole released from the nanostructures at different pH was estimated by UV spectrophotometer at 317 nm. The maximum released percentage of ornidazole was 99% [25].

In this study, esterification process between the hydroxyl group of polyethylene glycol and the carboxylic acid groups of sulfasalazine is used for synthesis of novel nanoparticles S-PEG_{750}. Subsequently, the S-PEG_{750} loaded with ornidazole and sulfasalazine nanoparticles was prepared and characterized by DLS and TEM. Furthermore, ornidazole release profiles at different pH or lipase activity, *invitro* cytoxicity were systematically investigated.

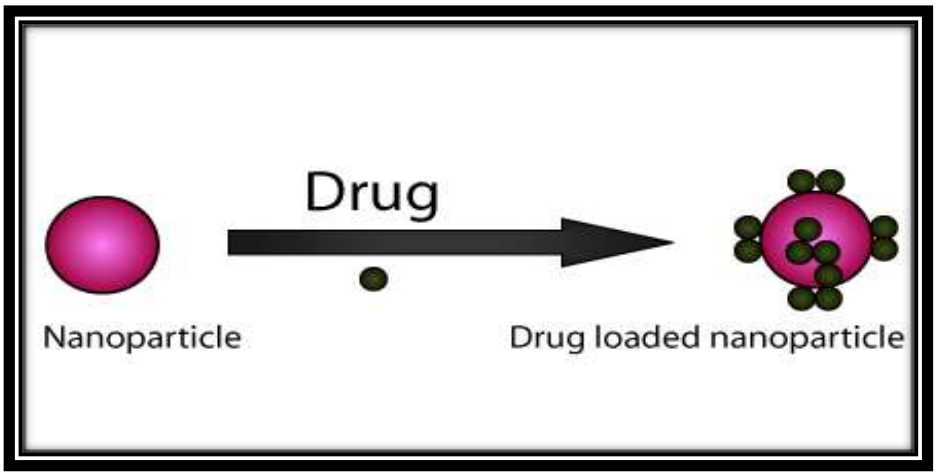

Figure 2: Drug is loaded on the surface of Nanoparticle.

2. OBJECTIVE AND SCOPE OF PRESENT WORK

In the current scenario researchers have mainly focussed on the development of nanocarriers or vehicles that are efficient for drug delivery systems and also have less side effects for treatment of different types of diseases. The biopharmaceutics used are very helpful in treating many diseases but having a poor physical potential and less chemically stable under *invivo* environment due to their molecular properties and also passes slowly to membrane as less permeable. So for delivery purposes of dug efficient vehicle can be used which can improve its properties.

There are a lot of work already has been done for drug delivery application to develop different types of nanoparticles based drug delivery systems such as nanoparticles that are conjugated with polymer, nanoparticles that are conjugated with peptide and nanoparticle in which polymer conjugated with drug. These nano drug delivery system developed due to hydrophobic-hydrophilic interaction which results in self assembled structure of nanoparticles so that drug can easily entrapped inside these nanostructures.

During the past years the main aim of development of small self assembled amphiphillic nanostructures because they are synthesize easily, biocompatible, cheap, easily soluble etc.

These self assembled amphiphilic molecules can arranged themselves into different types of nanostructures as nanovesicles, nanorods, nanofibers, nanospheres etc. These nanocarriers can be designed in such a way that they deliver a drug to particular specific cell-type and also alter their chemical and physical properties upon exposure to external stimuli. The small dipeptides having aromatic amino acid residues, so that they can entrap the drug more efficiently and release of drug occurs in a sustained manner.

With the increasing number of patients suffering from colonic cancer, gastrointestinal tract diseases and arthritis, there is an urgent need to develop strategies to cure these diseases without the side effects of drugs. With the advent of nanobiotechnology, it is possible to have drug delivery by using vehicles to transport the conventional anti –colonic cancer and anti arthritis drug entrapped in between. This helps in prolonging the longevity of drugs in the blood stream and also slows down the release. The polymeric and peptide nature of these vehicles also makes their uptake in cells much earlier, also reducing the toxicity as compared to the anticancer or anti arthritis drug.

The present study make some efforts in the formation of small molecule –based nanoparticles for efficient drug delivery in mammalian cell lines bearing minimal cytotoxicity.

Specific aims

- Design and synthesis of small polymer based self-assembled nanostructures.
- Physico-chemical and bio-physical characterization of these nanostructures.
- Encapsulation and controlled release kinetics of the biomolecules.
- *In vitro* evaluation of the proposed nanostructures.
- *In vitro* cyto toxicity assay of polymer and drug on HEK293 mammalian cell lines.

3. REVIEW OF LITERATURE

Nanobiotechnology is a multidisciplinary area, which is combination of different branches including chemistry, physics, biology and engineering [26-27]. In the current scenario nanobiotechnology mainly focussed in the area of drug delivery and human therapeutics. It might be expected that after using nanomaterials there will be any changes occur in future in pharmaceutical and biotechnology industries [28]. The quantum mechanical behaviour of very small molecules having a molecular dimension helps in changing the magnetic property, electrical conductivity, chemical and biological reactions in different manner at the nano scale. The nanoparticles shows some different properties due to their extraordinary high surface area to mass ratio, so that the chances of interactions with surface increases [29]. The size, electronic property and hydrophobic nature of nanoparticles make them available for different types of applications. [30]. The physical, chemical and biological property of nanocarriers make the conditions most favorable so that they can be easily accepted by cells as compared to micro range particle so can be successfully used for drug delivery applications. These are different nanoparticles which are used as a carrier for delivery of drugs such as liposomes, polymers, dendrimers, carbon and magnetic nanoparticles [31-35].

By combining various disciplines, a multifunctional devices will be designed for biological and chemical analysis characterized by better solutions [36].

Analyses of signalling pathways by nanobiotechnology techniques might provide new introspection into disease processes, thus it is very important to identifying more efficient biomarkers for understanding the mechanism of action of drugs [37]. The binding of nanomaterials with different types of biomolecules like proteins, nucleic acids mainly DNA, toxins, bacteria increases due to some changes in properties of nanomaterials [38].

3.1 DRUG DELIVERY

The polymeric material having some charge plays a very important role in therapeutic applications. nDDS helps in maintaining the concentration of drug in blood for longer period of time due to continuous release of drug from nanocarrier and also lowering the side effects by appropriate binding with the target site by simple diffusion method so the quantity of drug also reduces. [39]. Their release profile depends on one or more release mechanisms (i) release of drug attached to surface, (ii) movement occur from higher concentration to their

lower concentration through matrix, (iii) diffusion through the capsule shell, (iv) matrix erosion and (v) a combined erosion-diffusion process. Among these surface desorption and erosion processes of drugs are the two predominant release mechanisms of drug. The distance of movement of particle from their concentration to lower concentration is smaller in case nDDs when compared to micro particles. [40-41]. The material used if it possesses negative charge so that it can easily form ionic complex with the cationic molecule such as peptide having positive charge , blood proteins and drugs for therapeutic applications. The use of cationic material is mostly preferred because it can easily form electrostatic complexes with the anionic molecules like negatively charged DNA, proteins etc. These positively charged material also possesses antimicrobial activity, some are antioxidants and antitumor property make them more applicable for therapeutic effects. When these nanocarrier encapsulate these molecule like DNA and protein so also helpful in protecting them from degrading enzyme like nucleases or proteases so increases their life span.

To increase the release time of drug, there might be some changes in property of nDDs or providing such conditions so that release time increases. The drug release profile totally depends upon the degradation rate of polymer, by using same polymer but can be differentiate on the basis of molecular weight or by preparing the different ratios of drug and polymer [42-43]. There are also possibilities to encapsulate one or more number of drugs inside the matrix but having different binding ligands or groups and using stimuli-responsive nDDS [44]. These factors which play role in designing and also affect the drug release profile which incudes (i) interaction between nanomaterial and drug, (ii) shape, size and designed structure of the encapsulated drugs, (iii) biodegradability property of the polymer and (iv) elimination time of drug regulation. The interaction between drug and nanomaterial totally decides or we can say it is the prime factor of the release profile of drug. The chemical bonding between the drug and polymeric material mainly include covalent or hydrogen bonding depends upon the chemical structure of the drug. [45].

The material used for encapsulation must be selected in such a way that it will optimum release of drug from it. If the interaction between drug and nanocarriers is less stable then it will induces burst release of the drug which is very common in case of polymeric miscelles. Polymeric miscelles are colloidal particles composed of amphiphilic block polymer. Miscelle formation occurs in such a way that when a nanoparticles self assembled themselves so that hydrophilic head protrudes outwards and hydrophobic tail at the interior of core, proper

complex formation occurs only when the concentration of polymer more than critical miscelle concentration [46-47]. There is inverse relation between the CMC and miscelles formation as more stable the miscelle structure occur when there is lower concentration of CMC. As the polymeric conjugate encapsulated with drug then copolymer concentration decreases in the stable miscelle structure, which results in fast release of drug from polymer. For increasing the stability of miscelles , it is very important to increase the hydrophobic core stability by increasing the crosslinking , hydrophobicity or via electrostatic interactions. [48].

3.2 Polymer

There are different types of polymers used for drug delivery purposes which are either biodegradabale and non biodegradable in nature such as chitosan, poly (lactic acid) (PLA), poly (lactic co-glycolic acid) (PLGA), polyethylene glycol (PEG) etc. PEGylation is the process in which there is a covalent bonding between the alcoholic group of PEG and another group of compounds like peptides, proteins, drugs and bioactive molecules is known so that hydrophobic drug can be easily dissolve in water, its circulation time also increases in blood, binds to their appropriate target site and reach to their tumor specific target site and where they accumulate in tumor cells more in compare to normal cells. The main aim for designing or using nanocarriers are primarily to increase the effectiveness of drug by i) increase the water solubility of drug, only some are relevant to less dissolve in water ii) much more stable against enzymes which digest the DNA and Protein or reduced uptake by reticulo-endothelial system (RES) and iii) Drugs are reached to their specific tumor site. In case of PEG, the ester bond formed is much more stronger so that release of drug occurs only when it reaches to their target site. Extracellular and intracellular enzyme and pH also plays an important role in releasing drug from conjugate. [49].

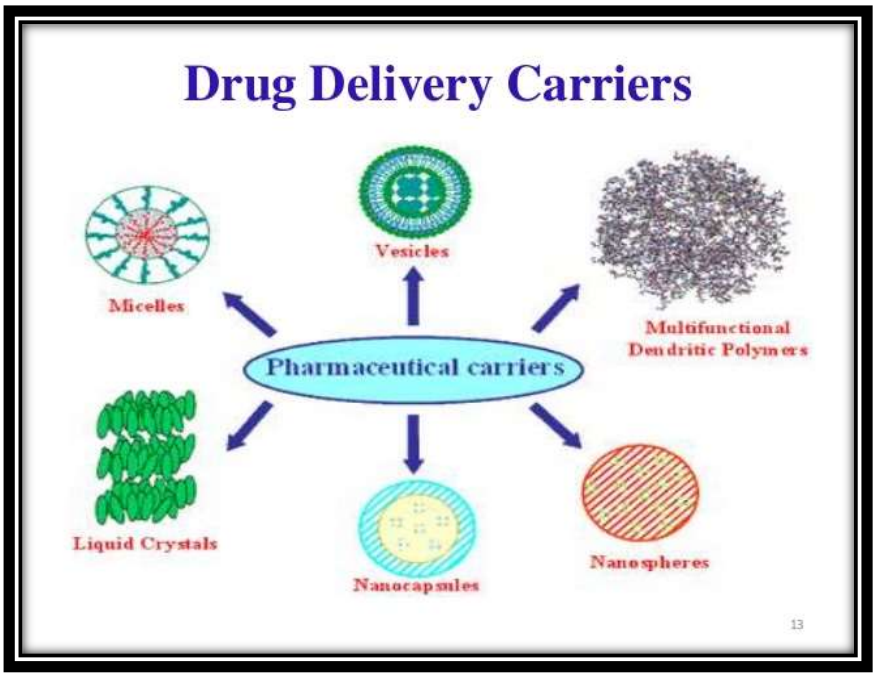

Figure 3: Showing different types of Nanocarriers used for drug delivery

3.3 Applications of Nanobiotechnology

3.3.1Medical applications

Nanobiotechnology mainly focussed on two sectors as health sector and medicine. Nanobiotechnology have many advances in field of pharmaceuticals, medical imaging and diagnosis, treatment of cancer, implantable materials, tissue regeneration etc. [50-52].

3.3.2Diagnosis

The main aim of nanobiotechnology is to design a such methods which can help in diagnosing number of diseases at their initial stage and material used should be cheap and equipment used is much more sophisticated than used in recent technologies [53]. There is lot of research work is going on this area. Nanoparticles such as metal and semiconductor have been used in many biomedical applications. When 25 gm of Au nanoparticles formed a complex with anti-epidermal growth factor receptor then monoclonal antibodies can be efficiently used as in vitro targeting agents for imaging cancer markers [54]. The signal contrast effect of Au nanoparticles is much more intense than antibody-fluorescent dye targeting agents [55]. Diseases like cancer can be easily diagnosed by nanobodies which have ability to be a new generation of antibody-based therapeutics which are highly site

specific and less toxic. The advantage of using conventional antibodies as it is cost effective when produced on a large scale because conventional antibodies are generally small drug molecule.

3.3.3 Gene therapy

Gene therapy is a method in which defective genes can be replaced by repaired genes to prevent genetic diseases. The Mammalian cells generally have diameter in micron range and the diameter of cell organelle are within nanometer range, so that nanodevices can more easily enter the cells as compared to large devices as size is in nano range so they can interact in much better way or we can say in any other way.

Gene therapy uses nanobiotechnology approaches so as to replace the use of viral vectors which are nano size carriers of gene and less active in producing reaction from immune system. The use of nanotechnology, has some advantages in gene delivery; the nucleic acid can be protected by nanoparticles which are degraded by nucleases and the environment and also directs its entry to their specific target site and retension time also increases as compared to other carriers. Poly (DL-lactic-coglycolic acid) (PLGA), polyvinyl alcohol, poly (ethylene-co-vinyl acetate), polyimide, and poly (methylmethacrylate) are the compounds which are used for encapsulating drug, molecules in the recent days [56].

3.3.4 Drug delivery

Controlled delivery systems are those in which drugs are delivered to their target site at their predetermined rate according to the physical environment so that drugs become therapeutic effective, safe for use, less toxic and have minimum side effects[57]. Co-delivery is an another method for delivery of multiple drugs and cannot be used simultaneously with traditional methods. Therefore, nano drug delivery systems can be used to facilitate the delivery of drugs which are not congruous because of differences. They can also be used in thermostics, in which the nanoparticle is used as a device to diagnose and treat the diseases at the simultaneously. They are also being investigated as a tool for the delivery of drugs through the blood–brain barrier [58]. These techniques are also used for the liberation of pharmacological agents against several diseases, such as bacterial infection [59], inflammations [60], and principally cancer, among others. There are different types of factors which plays a major role in drug loading and entrapment efficiency such as hydrophilic and hydrophobic interation between the drug and polymer, chemical structure, molecular weight and charge it bear either positive or negative. Liposomes are one of the

best known example to understand the hydrophobic and hydrophilic interaction between the drug and nanomaterial [61]. Liposomes basically consists of hydrophilic head in the inner zone and hydrophobic tail drugs in lamellae and the release of drug is very low in case of liposomes [62]. Yokoyama *et al.* synthesized a polymeric conjugate in which adriamycin was encapsulated which form covalent bonding with the poly (ethylene glycol)-poly (aspartic acid) block copolymers,[63] so that this results in increasing its stability, easily soluble in water with higher retention time of adriamycin. [64]. Secondly, there is direct effect of molecular weight of polmer and charge on degradation rate of polymeric compound and release of drug from it. The retension time of drug increases in case of physical binding as there is no chemical interactions are present. Different types of polymeric materials have been used for nDDs such as polymers, peptides, lipids, metal nanoparticles, carbon nanotubes, viral capsids etc. The materials should be selected for drug delivery allows optimal release of drug through it and the polymeric material generally shows maximum release percentage of drug.

3.3.5 Pathogen detection

Nanoparticles can also helpful in pathogen detection as the surface area is to volume ratio is very high so that nanoparticles embedded biosensors can allow large number of biomolecules to be immobilised so that large number of sites for reaction are available for binding with a target species. Because of excellent electronic and optical properties nanomaterials can be used in label- free detection, sensitivity of biosensor increases and response time also become improved [65]. Biosensors can be used to identifying the direct molecular target, to find the accuracy of something, assay development, lead optimization and absorption, distribution, metabolism, excretion, and toxicity. The soluble molecules are mainly used for this purpose and over-come most of the limitations that arise with cell-based assays. Biosensors allow the study of receptors which are not required to be removed which is essential in other assay methods.

3.3.6 Food safety

Nanobiotechnology plays the important role in food related research which mainly includes biosensors, antioxidants, antimicrobials and different types of nanomaterials used for packaging. Different types of industries have been using nanoparticles made from food basically to improve the characteristics of products such as cosmetics, medical and pharmaceutical industries. Nanobiotechnology mainly focussed on packaging in case of food

industry as there are some metal nanoparticles such as silver which can be used for packaging due to its antimicrobial property. The bio-based nanocomposite film can be used for packaging because they are easily biodegradable and edible in nature. Due to large area of surface to mass ratio the antimicrobial effect of nanoparticles can be enhanced.

3.3.7Biosurfactants

Biosurfactants are surface active substances that reduce interfacial tension and are produced or excreted at the microbial cell surface. Biosurfactants have been tested in environmental applications, cosmetics, foods, and pharmaceutical industries but also as industrial elements and chemical products for agricultural use. Both natural and synthetic amphiphiles that are able to form self-assembled nanostructures [66]. Biosufactants can also be used as structure-directing agent depending upon their concentration so some of them form nanometres-size miscelles and different types of geometries are obtained like synthesis of various nanostructured silica thin films. Due to high surface area and tunable pore size distribution nanomaterial can be useful for various applications such as photovoltaic electrodes, catalysis, filtration and sensing.

4. MATERIAL AND METHODS

4.1 REAGENTS

Source	Chemicals
Alfa Aesar	Polyethylene glycol $_{750}$ momomethylether
Armar chemicals (Switzerland)	D_2O DMSO
Gibco (Carlsbad,CA)	Fetal Bovine Serum(FBS)
Sigma –Aldrich Chemicals Co. (USA)	Ornidazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 1,1,1,3,3,3- Hexafluoro-2- propanol
Sigma –Aldrich Chemicals Co. (St. Louis, MO)	Trypsin DMEM Bovine serium albumin(BSA)
Sigma Aldrich (India)	Lipase
Spectrum Labs (USA)	Dialysis membrane
Spectrochem, Mumbai	N,N'-dicyclohexylcarbodiimide(DCC) 4-Dimethylaminopyridine(DMAP)
Thermofisher Scientific India pvt.	Methanol
Tokyo Chemical Industry co. Ltd Japan	Sulfasalazine

Table 1: list of reagents and their sources

4.2 BUFFER

4.2.1 Phosphate Buffered Saline (PBS) (10x) (pH 7.2, 1 L)

In 800 ml of Distilled water dissolve-

- 8 g of NaCl
- 0.2 g of KCl
- 1.44 g of Na_2HPO_4
- 0.24 g of KH_2PO_4
- Add water to make 1 Litre.

The constant pH is maintained by using buffer solution. The osmolarity and ion concentrations of the solution usually similar to those of the human body (isotonic), and thus it is non-toxic to cells. Therefore, in tissue culture, cells are washed with PBS.

4.2.2 Cell lysis Buffer (pH 7.2, 50 ml)

- 60.5 mg Tris (for 10 mM concentration)
- 250 mg SDS (for 0.5% SDS)
- 18.6 mg EDTA (for 1mM)
- 30 ml Milli-Q water
- Add water to make 50 ml.

4.2.3 MTT SOLUTION

MTT is reduced to formazan inside living cells, giving purple color. It is made by dissolving 10 mg MTT in 10 ml media i.e. 1 mg/ml solution is prepared.

4.2.4 MTT Lysis Buffer (for two 96 well plates)

- 24 ml isopropanol.
- 136 µl conc. HCl
- 1.25 ml SDS (10%)

4.3 INSTRUMENTS

4.3.1Zetasizer

Size of particles was measured by Dynamic Light Scattering (DLS) principle based Zetasizer Nano-ZS (Malvern Instruments Worcestershire, UK) employing a nominal 5mW He-Ne laser operating at 633nm wavelength. The measurements were generally carried out at following settings: temperature 25℃, viscosity of water is generally 0.89 Cp, refractive index of water 1.33. The size of particle, showed in the present work, is the mean of generally 3 measurements. The particles were dissolved at the concentration of 1mg/mL in filtered millique and measured their particle size including their hydrodynamic diameter in triplicates. The measurement of particle size were carried out as such it takes 20-30 runs thrice in every cycle average of all 3 cycle is the mean particle size from the diffusion of particles moving under the Brownian motion, an intensity auto-correlation graph obtained using the Malvern software package based on strokes-Einstein equation. The nanostructures were formed assembly by dissolving sulfasalazine PEG (1mg) in 1.0 ml of MilliQwater then mixed for 2-3 minutes in vortex. The solution formed was left for 1 h, and Zetasizer Nano-ZS instrument is mainly used for this purpose.

Figure 4: Zetasizer instruments

4.3.2 Fourier Transform Infrared Radiation (FTIR)

IR spectra of the synthesized particles were obtained on a single beam Perkin Elmer (Spectrum BX series), USA with the following scan parameters; scan range, 4400-400cm^{-1}; number of scans, 16; resolution, 4.0cm^{-1}; interval,1.0cm^{-1} ; unit% T. In order to obtain the IR spectra weigh the sample of about 2 mg concentration in microcentrifuge tube sulfalasalazine PEG is a polymer the sample was dissolved in any reasonable volatile solvents and this solution was poured on a rock salt plate KBr and solvent was evaporated by gentle heating.

Figure 5: FTIR instruments

4.3.3 UV-Visible Spectroscopy

In this spectroscopy the absorption or reflection occurs in the UV-VIS region of spectra. The absorption or reflection in the visible range so that colours of the chemicals recognised will be directly affected. The atoms and molecules excited from ground state to higher state when they receive UV-VIS light. If the pi electron or non bonding electrons can absorb the energy in the form of UV or Visible light then these electrons excited to their higher antibonding molecular orbitals. The electron which can excite more easily then it also can absorb the light having longer wavelength.

Figure 6: UV-VIS Spectrophotometer

4.3.4 ^{1}H-NMR (Nuclear Magnetic Resonance)

^{1}H-NMR spectroscopy of compounds was recorded on a 400 MHz spectrometer Bruker Avance, Germany. Methanol, Deuterated water (D_2O) solvents were used to dissolve the compounds.10 mg of Sulfasalazine-PEG_{750} was dissolved in 0.7ml of methanol and D_2O were used to determine the spectra of compound. JEOL Delta software was used for evaluation of spectra, chemical shifts are presented ppm.

Figure 7: NMR instrument

4.3.5 Transmission electron microscope (TEM)

TEM was used to obtain the size and shape of the nanoparticles. In present study, TEM micrographs were acquired using the following procedure. 20µL of the sample was placed, then copper grid that was coated with carbon were kept on for 3 minutes at room temperature further the grids were negatively stained by 1% uranyl acetate resting the grid over solution for additional 3 minute. Then grids were air dried and images were analysed on HR-TEM (Tecnai G2 30-U twin, Tecnai 300KV ultra twin microscope) operating at 200KV.

4.3.6 Lyophiliser

The samples were lyophilized in a Hetovac VR-1. The speed vac comprises a vacuum pump connected to a centrifuged chamber in which the samples were placed. In this process, samples were dried by lowering the pressure so that vacuum was created in the chamber. Samples were dried by the process of sublimation.

Figure 8: Lyophiliser instruments

4.3.7 Centrifuge

During the process of plasmid DNA isolation centrifuge 5430 R was used to pellet out the bacterial cells. Cell suspension was filled in 15 ml or 50 ml falcons, and then into the centrifuge chamber, spun at 5000 rpm for 15 mins at 4°C.

Figure 9: Centrifuge

4.3.8 Fluorescence microscope

NIKON ECLIPSE TE 2000-U inverted microscope, Kanagawa. Japan, fitted with a C-FI epifluorescece filterblock B-24 consisting of excitation filter Ex-450-490 nm/ dichromic mirror DM 505 and barrier filter BA 520 was used to observe GFP protein expression. Sample fluoresced when illuminated with light of particular wavelength. The emitted fluorescence light was at higher wavelength than the illumination, detected through a microscopic objective. The two illuminator used in this technique were an illumination filter and an emmision filter.

Figure 10: Fluorescence microscope

4.3.9 ELISA Plate Reader

μQuant MCX200 microplate reader (Biotek, USA) operated with Gene5 software with high powered xenon flash lamp and tunable monochromator was used to calculate cell viability. The 540 nm and 590 nm wavelength was set to measure the amount of formazan formed after using MTT and for Bradford assay respectively.

Figure 11: Elisa plate Reader

5. PROTOCOLS

5.1 Synthesis of S-PEG

Polyethylene glycol $_{750}$ methyl ether (7.50 gm,10mmol) and DCC (2.472gm,12mmol) were dissolved in ethylene dichloride (EDC) followed by addition of Sulfasalazine (5.97gm,15mmol) and DMAP (0.293gm, 2.4mmol). The mixture was stirred and a white precipitate was obtained. The reaction was monitored by thin layer chromatography (TLC). After overnight stirring, the precipitate was filtered and the filterate was evaporated to dryness. The resulting residue was dissolved in water and the solid was removed by filtration. The filterate was dialysed against double distilled water for 24 hrs with regular change of water (4*6 hr). The dialysed solution was removed from the bag and lyophilised to get monomethyl ether PEG_{750}-Sulfasalazine. The yield obtained was 68%.

5.2Physicochemical characterization

5.2.1Size and surface charge measurements

Size of particles was measured by Dynamic Light Scattering (DLS) principle based Zetasizer Nano-ZS (Malvern Instruments Worcestershire, UK) employing a nominal 5mW He-Ne laser operating at 633nm wavelength. The measurements were generally carried out at following settings: temperature 25℃, viscosity of water is generally 0.89 Cp, refractive index of water 1.33. The particle size, reported in the present work, is the average of generally 3 measurements. The nanoparticles were immersed at the concentration of 1mg/mL in millique and the particle size was measured in terms of hydrodynamic diameter in triplicates. The measurement of particle size were carried out as such it takes 20-30 runs thrice in every cycle average of all 3 cycle is the mean particle size from the diffusion of particles moving under the Brownian motion, an intensity auto-correlation graph obtained using the Malvern software package based on strokes-Einstein equation. The nanostructures were formed assembly by dissolving sulfasalazine PEG (1mg) in 1.0 ml of MilliQwater then mixed for 2-3 minutes in vortex. The solution formed was left for 1 h, and Zetasizer Nano-ZS instrument is mainly used for this purpose.

5.2.2 Fourier Transform Infrared Spectroscopy

IR spectra of the synthesized particles were determined on a single beam Perkin Elmer (Spectrum BX series), USA with the following scan parameters; scan range, 4400-400cm^{-1}; number of scans, 16; resolution, 4.0cm^{-1}; interval,1.0cm^{-1} ; unit% T. In order to obtain the IR spectra weigh the sample of about 2 mg concentration in micro centrifuge tube as each of sulfasalazine- $PEG_{750,}$ Sulfasalazine, $PEG_{750,}$is a solid polymer the sample was dissolved in any reasonable volatile solvents and this solution was poured on a rock salt plate KBr and solvent was evaporated by gentle heating.

5.2.3 ^{1}H NMR(Nuclear Magnetic Resonance)

^{1}H-NMR spectroscopy of compounds was recorded on a 400 MHz spectrometer Bruker Avance, Germany. Methanol , Deuterated water (D_2O) solvents were used to dissolve the compounds.10 mg of Sulfasalazine-PEG_{750} was dissolved in 0.7ml of methanol and D_2O were used to determine the spectra of compound. JEOL Delta software was used for evaluation of spectra . chemical shifts are presented in ppm.

5.2.4 Transmission electron microscope

TEM was used to determine the size and shape of the particles. In present study, TEM micrographs were acquired using the following procedure. 20µL of S-PEG_{750} and S-PEG_{750} entrapped with sulfasalazine drug and S-PEG_{750} degraded by Lipase was placed, carbon coated copper grids were kept on for 3 minutes at room temperature further the grids were negatively stained by 1% uranyl acetate resting the grid over solution for additional 3 minute. Then grids were air dried and images were analysed on HR-TEM (Tecnai G2 30-U twin, Tecnai 300KV ultra twin microscope) operating at 200KV.

5.2.5 Loading studies

Further to study the self assembled ability of peptide amphiphiles and polymeric nanostructures, hydrophobic molecules were encapsulated in the hydrophobic core region of in such ratio as sample (5mg) and drug (1mg and 0.5 mg) and nanostructures were prepared by dissolving these mixture in 100 µl methanol with continous vortexing for 1 min and then 900 µl water was added and again vortexed for 1 min. The solution were left overnight for

formation of polymeric self assembled nanostructures. The resulting mixture then lyophilised to obtain the desired drug loaded nanostructures. The entrapment efficiency and loading efficiency percentage of drug loaded NPs were calculated from the absorption spectra of the drug loaded nanostructures recorded using solutions of concentration 0.1 mg in 1 ml of methanol; in a disassembled state of peptide and polymeric nanostructures. The absorbance of the drug in the solution was measured on a spectrophotometer. Entrapment efficiency was calculated from a curve drawn constructed at particular wavelength using different concentrations of drug. All measurements were conducted thrices and the average values were considered. Two different formulations (sample: drug 5:1 and 5 :0.5) were prepared. The percent encapsulation efficiency (%EE) and drug loading (%DL) were calculated using the formulas given below.

$$Entrappment\ efficiency = \frac{Total\ Drug\ Loaded \times 100\%}{Total\ Drug\ used}$$

$$Loading\ efficiency = \frac{Total\ Drug\ Loaded \times 100\%}{Final\ weight\ of\ Loaded\ Drug}$$

5.2.6 *Invitro* release

In this method drug is entrapped in the hydrophobic core of compound having composition (5:1) and (5:0.5) was performed in a dialysis tube (cut off 100-500 Da, spectra/por, United states) by diffusion technique. Drug released from the compound was studied in a filtered MilliQ and Phosphate buffer saline (PBS) at different pH 4.5 and 7.2. The drug loaded sample (2mg) was placed in a cellulose dialysis bag. The dialysis tube was suspended in 10 mL PBS solution and mixed at 150 rpm under stirring. Aliquots of solution were taken out at definite time intervals and same volume was replaced with fresh buffer solution. The solutions were analysed by UV-VIS spectrophotometer at particular wavelength for sample against the blank reagent. All the experiments were performed in triplicates and the average values are considered. The formulation exhibited continous release of drug from the sample . Nps as a result of diffusion i.e weaking of interactions between drug and polymer. The drug release from compound showed the biphasic release pattern with approximately % release of the total drug in 24 h, followed by a continued release over a period of 31 h.

5.2.7 Enzyme Degradation

Lipase: Lipase extracted from Pseudomonas cepacia was procured from Sigma –Aldrich, Co USA and was used for degradation of S-$PEG_{750.}$ The stock solution of Lipase (10mg/mL) was prepared in phosphate buffer saline. The enzyme degradation was carried out at two different concentrations for comparsion that showed the difference between the two degraded by enzyme. The enzyme degradation was carried out as S-peg_{750} (1mg) was solubilised by adding (100µl and 200 µl) Lipase and (50µl) NaN_3 in two different eppendoff and placed the sample on thermomixer at 37℃ and 350 rpm. The size of sample was measured by DLS after particular time interval and morphology and size of sample was also observed by TEM in dry state**.**

5.2.8 Cell viability assay

Invitro cell viability assay in which mitochondrial succinate dehydrogenase enzyme helps in reduction of MTT in live cells into formazan product which is dark purple in colour. The organic solvents can be used to solubilised the formazan crystals and intensity was measured spectrophotometrically at 540 nm. In present study, HEK 293 cells were seeded in a 96-microtiter well plate and incubation time is about 24-36 hours for attachment on surface after that media was removed and cells were washed once with 1X PBS. After that serum –free DMEM was used to diluted the S-PEG to a final volume of 100 µl and added gently to each well followed by incubation for 24 hours in humidified 5% CO_2 atm. After 24 hrs, MTT (1mg/ml) dissolved in DMEM was added to the cells and incubated for 3-4 hours at 37℃. The supernatant was removed and the formazan crystals formed were suspended in 100µl DMSO. The intensity of colour was measured spectrophotometrically on an ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability. The cell viability (%) was estimated by the given formula:

$$\boldsymbol{Cell\ viability}(\%) = \frac{\boldsymbol{abs[treated]} * \mathbf{100}}{\boldsymbol{abs[control]}}$$

6. RESULTS AND DISCUSSION

6.1 Characterization of sulfasalazine PEG_{750} nanostructures

Polyethylene glycol $_{750}$ methyl ether (7.50 gm,10mmol) and DCC (2.472gm,12mmol) were dissolved in ethylene dichloride (EDC) followed by addition of Sulfasalazine (5.97gm,15mmol) and DMAP (0.293gm, 2.4mmol). The mixture was stirred and a white precipitate was obtained. The reaction was monitored by thin layer chromatography (TLC). After overnight stirring, the precipitate was filtered and the filterate was evaporated to dryness. The resulting residue was dissolved in water and the solid was removed by filtration. The filterate was dialysed against double distilled water for 24 hrs with regular change of water (4*6 hr). The dialysed solution was removed from the bag and lyophilised to get monomethyl ether PEG_{750}-Sulfasalazine. The yield obtained was 68%.

The conjugate formed was characterized by UV-VIS spectrophotometer, FTIR and ^{1}H-NMR techniques. In the NMR spectrum of compound the appearance of peak at δ8.0-6.75ppm due to Ar-H of azobenzene residues confirmed the formation of compound. In D_2O, the potency of aromatic proton was very less in number that could be due to the formation of miscellar structures, which resulted in interment of Ar-H in the core. In methanol, peaks at δ8.0-6.75ppm due to Ar-H of azobenzene while methanol peak at δ4.80, 3.35 and due to some degree of unstability in the miscellar structure, the aromatic protons got exposed and were observed with higher potency. Futher in UV spectra of compound was analysed and maximum absorbance was obtained at wavelength 359 nm and due to interaction between sulfasalazine and PEG_{750} and formation of ester linkage.

The self- assembled nanostructure of S-PEG conjugate formed was studied by DLS. An aqueous solution of S-PEG (1mg ml^{-1}) in 10% methanol and hexafluoro-2-propanol was mixed properly in vortex and left it for 1 h at room temperature to form self assembled nanostructures, after that size was measured by DLS. The results obtained was confirmed that nanostructures which was formed found to be ~212 nm in 10% methanol and ~233 nm in hexafloro-2-propanol (Table 2). The shape and size of these structures was obtained by TEM analysis. Figure (17) showing that spherical shape nanostructures having size in nm range consisting of a hydrophobic shell which is at outside of nanostructure and hydrophobic core on at its inner part. There was difference in the size measured by DLS and TEM may be due to measurements of the hydrodynamic diameter i.e layer formed by water in case of DLS, while in case of TEM size was measured in dry state. The self–assembly of nanostructures

could be confirmed in such a way when a hydrophilic shell with a hydrophobic core, a hydrophobic molecule, ornidazole and sulfasalazine was encapsulated in the hydrophobic core region. DLS showed that there is significant increase in size of particle from ~212 nm to ~ 300 nm (Table 2), when there was encapsulation of ornidazole in the hydrophobic core via hydrophobic interactions, which resulted in the density of nanostructures. Similarly, TEM analysis showed a increase in the size of ornidazole entrapped nanostructures in the range of ~52 nm. These results highlight the potential of these nanostructures to encapsulate hydrophobic molecules. The Zeta potential of S-PEG nanostructures measured which was found to be~ -5.39 mV, whereas the S-PEG encapsulated with sulfasalazine nanostructures showed 0.38 mV.

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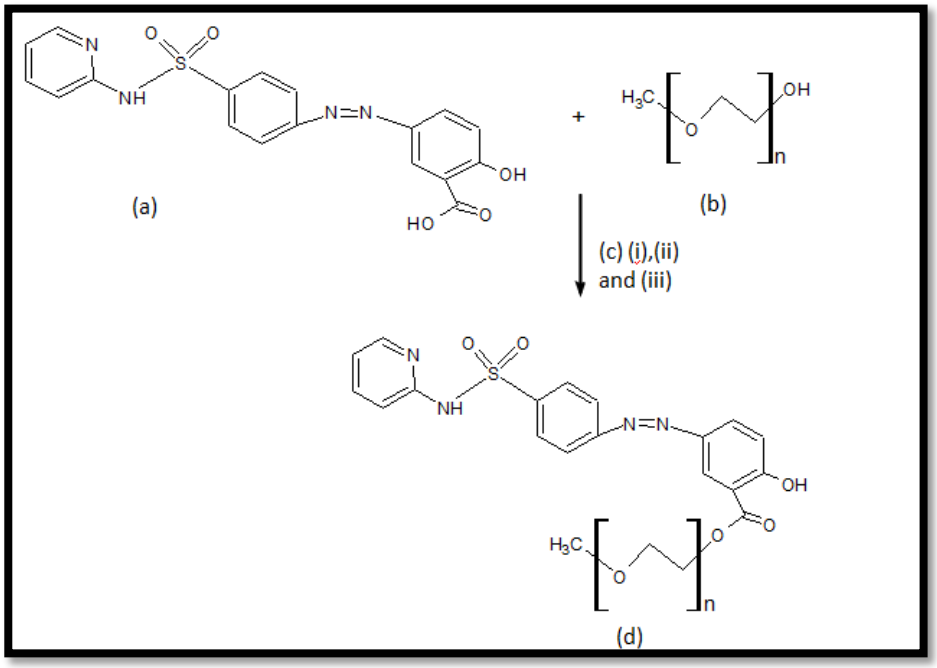


Figure 12 : Schematic representation of synthesis of S-PEG$_{750}$ (a) Sulfasalazine (b) PEG$_{750}$ (c) (i) DCC (ii)EDC (iii)DMAP (d) S-PEG$_{750}$

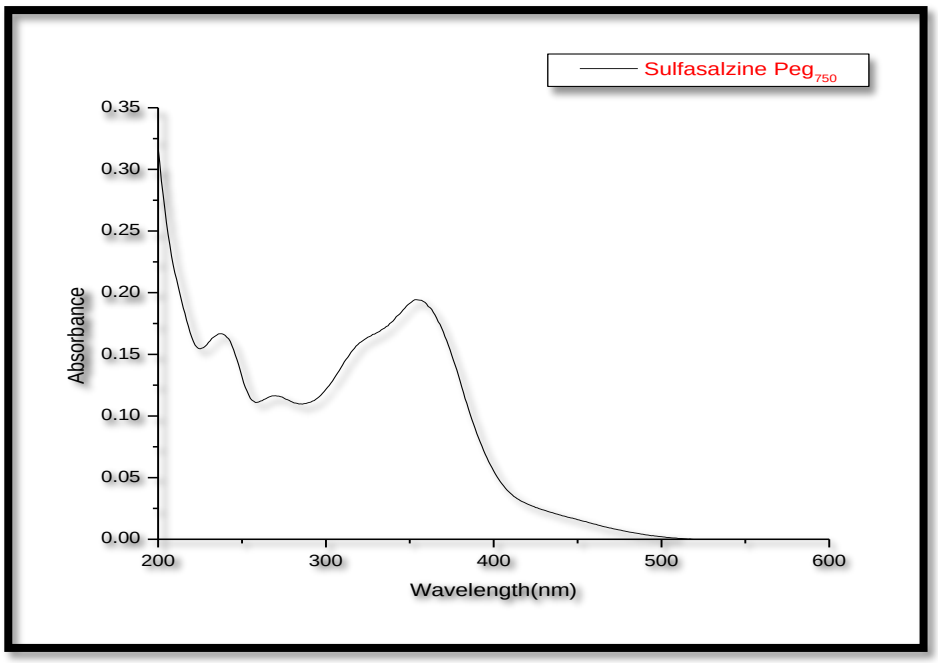


Figure 13: UV-VIS Spectra of Sulfasalazine PEG750.

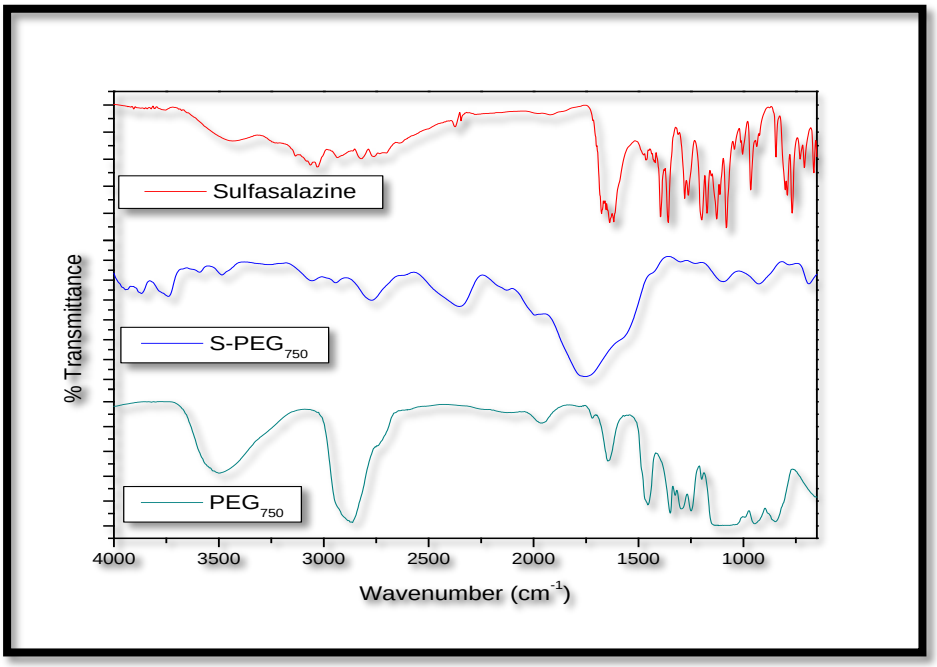


Figure 14 FTIR spectra of sulfasalazine, S-PEG$_{750}$ and PEG$_{750}$.

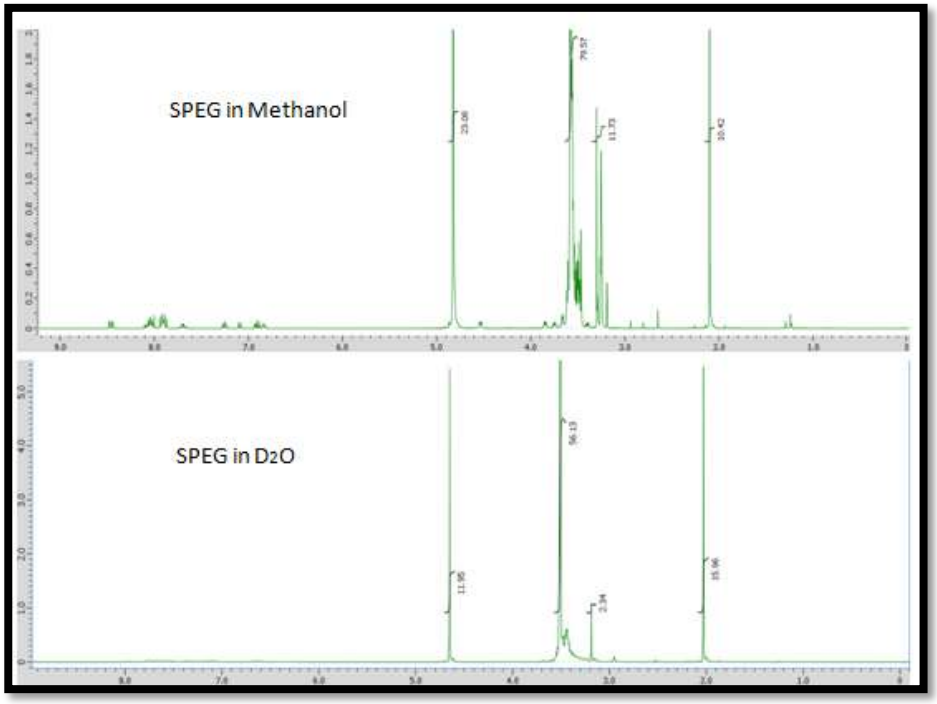
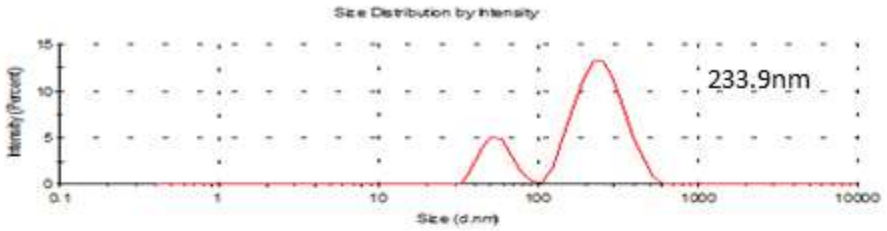


Figure 15: NMR Spectra of S-PEG in methanol and D_2O

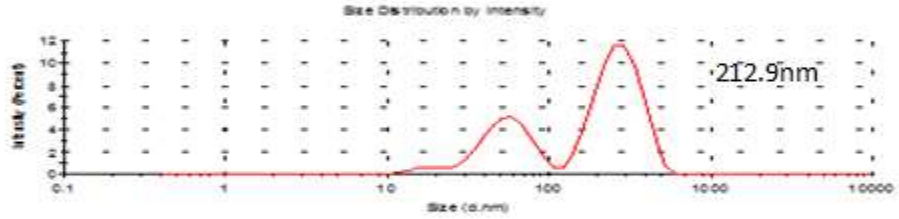
S.no	Sample	Average particle size (d.nm)±S.D.	PDI ± S.D	Zeta potential(in mV) ± S.D.
1.	S-PEG(10% MeOH, in H_2O1 mg ml^{-1})	212.9±321.7	0.336± 0.069	-2.49± 0.34
2.	S-PEG(10% hexafluoro-2-propanol, in H_2O1 mg ml^{-1})	233.9± 94.28	0.464±0.018	-5.39± 10.51
3.	S-PEG (ornidazol 5:1)(in H_2O, 1 mg ml^{-1})	230.3± 18.02	0.514± 0.115	-5.41±1.23
4.	S-PEG (ornidazol 5:0.5)(in H_2O, 1 mg ml^{-1})	300.4± 19.52	0.442± 0.158	-17.4 ± 1.14
5.	S-PEG (sulfasalazine5:1)(in H_2O, 1mg ml^{-1})	265.4± 38.42	0.342± 0.034	-6.89±2.48
6.	S-PEG (sulfasalazine5:0.5)(in H_2O, 1mg ml^{-1})	283.6± 21.56	0.261± 0.061	0.38±0.49
7.	S-PEG degraded by LIPASE(100 µl, 1mg ml^{-1}in PBS)	1036 ± 93.70	0.482± 0.138	-9.70±0.966
8.	S-PEG degraded by LIPASE (200 µl, 1 mg ml^{-1} in PBS)	1192±58.40	0.309 ± 0.125	-11.3±1.31

Table 2: Size and zeta potential measurements of S-PEG750 (10% meoh),(10% hexafluoro-2-propanol) , S-PEG750 (ornidazole and sulfasalazine) and S-PEG degraded by LIPASE(100 and 200 µl, 1mgml^{-1} in PBS)

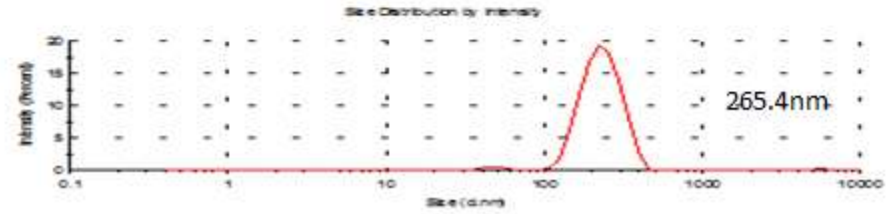
(a)


Size Distribution by Intensity
233.9nm
Size (d.nm)

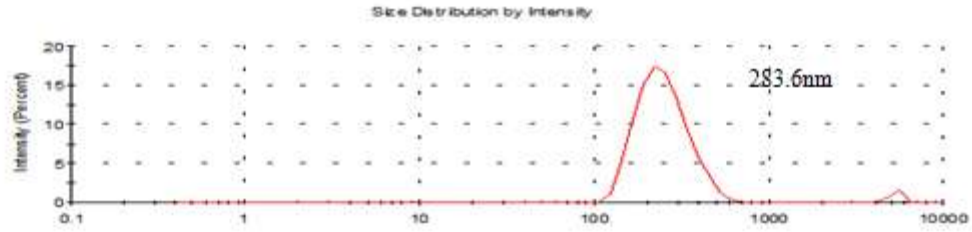
(b)


Size Distribution by Intensity
212.9nm
Size (d.nm)

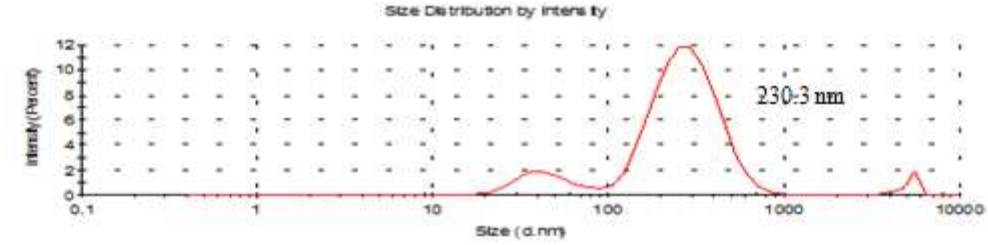
(c)


Size Distribution by Intensity
265.4nm
Size (d.nm)

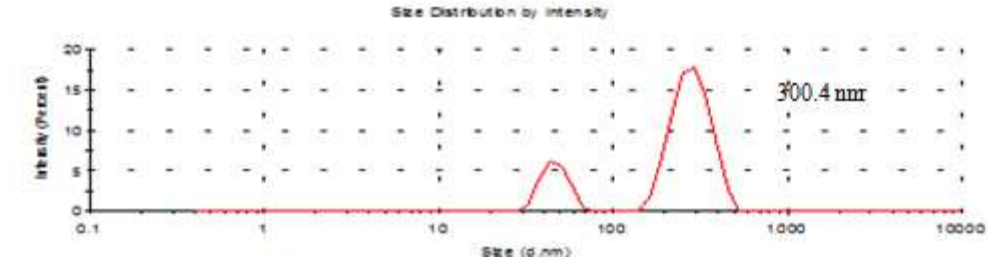
(d)


Size Distribution by Intensity
283.6nm

(e)


Size Distribution by Intensity
230.3 nm
Size (d.nm)

(f)


Size Distribution by Intensity
300.4 nm
Size (d.nm)

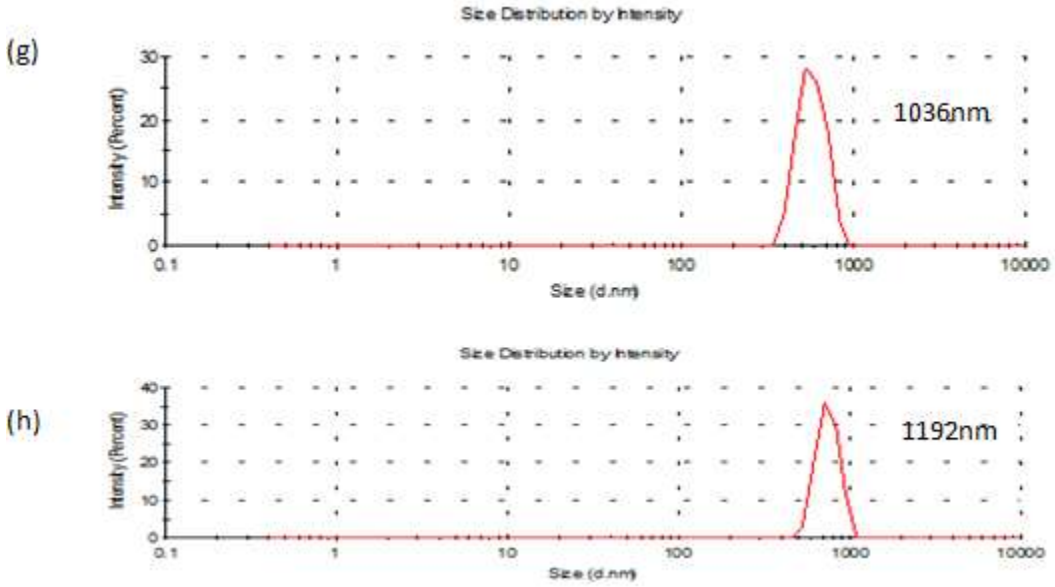
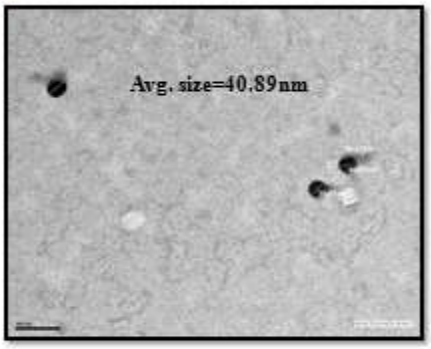
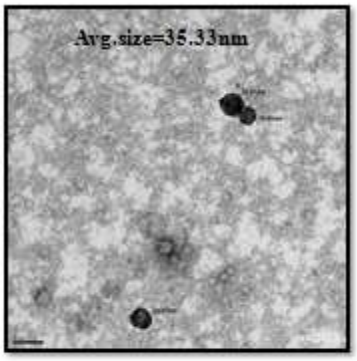


Figure 16 :DLS images of Sulfasalazine PEG_{750}(a) 10% Hexafluoro-2-propanol (b)10% methanol (c)S-PEG entrapped with sulfasalzine 5:1(d) S-PEG entrapped with sulfasalazine 5:0.5 (e) S-PEG entrapped with ornidazole 5:1 (f) S-PEG entrapped with ornidazole 5:0.5 (g) S-PEG degraded by Lipase 100 µl (g) S-PEG degraded by Lipase 200 µl.

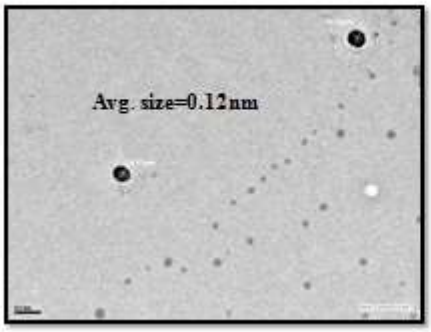
(a)


Avg. size=40.89nm

(b)


Avg.size=35.33nm

(c)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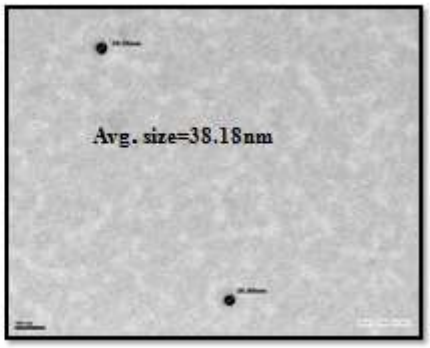
(d)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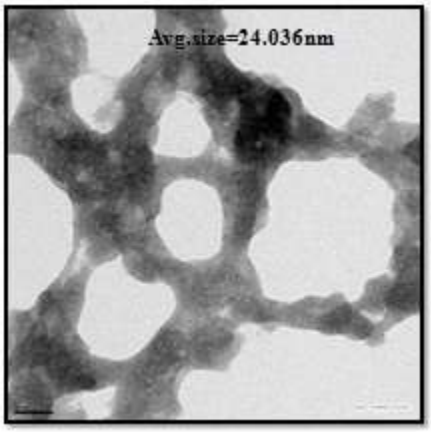
(e)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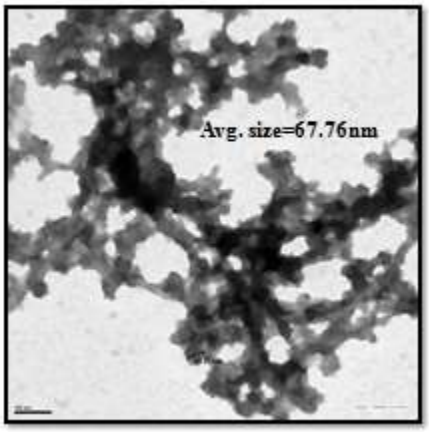
(f)


Avg. size=67.76nm

(g)

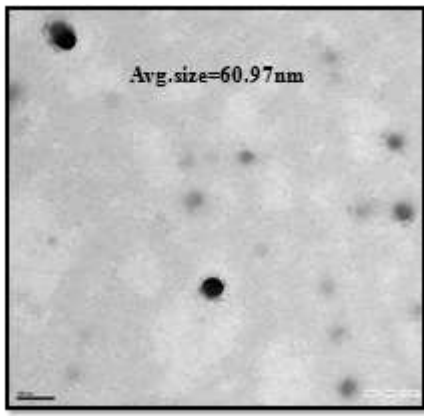


Figure 17 : TEM images of (a) Sulfasalazine PEG (10% meoh, in H_2O1 mg ml^{-1}), scale bar:100nm, (b) Sulfasalazine PEG peptide (10% hexafluoro-2-propanol, in H_2O1 mg ml^{-1}), scale bar: 100nm, (c)S-PEG (sulfasalazine 5:0.5) (in H_2O, 1mg ml^{-1}), scale bar:0.2 µm, (d) S-PEG (sulfasalazine 5:1) (in H_2O , 1 $mgml^{-1}$), scale bar: 100 nm (e) S-PEG degraded by (100µl lipase with remaining PBS to made 1 ml volume), scale bar: 20 nm, (f) S-PEG degraded by(200µl lipase with remaining PBS to made 1 ml volume), scale bar: 100 nm,(g) S-PEG (ornidazole 5:0.5)(in H_2O , 1 $mgml^{-1}$), scale bar:100 nm.

6.2 Effect of pH on the size and zeta potential of the S-PEG nanostructures

Due to change in pH there was direct effect on the size and charge of the nanostructures. The size and zeta potential were measured at three varies pH (4.5, 7.2 and 9) after incubation of 2hr. When the pH of the medium changes then the self assembled nanostructures showed some changes in their size and zeta potential. Table 3 indicates that on changing pH from 4.5 to 7.2 there was decrease in both size and zeta potential of S-PEG were 494 nm and -2.98 mV to 347nm and -17.85mV and on futher increase in pH from 7.2 to 9 there is increase in both size and zeta potential of the S-PEG 426 nm and -10.98mV after 2 hr of incubation. The increase in size was due to protonation of amines which was present in self assembled nanostructures which resulted in repulsion within the ammonium ions, increase in the zeta potential of these nanostructures was due to cationic ammonium ions.

S.no	Sample	Average particle size (d.nm)±S.D.	PDI ± S.D	Zeta potential(in mV) ± S.D.
1.	S-PEG (pH4.5)	493.9± 79.05	0.557± 0.030	-2.98± 1.83
2.	S-PEG (pH7.2)	346.8± 21.67	0.485± 0.082	-17.85±2.96
3.	S-PEG (pH 9)	425.6± 93.99	0.520± 0.052	-10.98± 6.23

Table 3: Size and zeta potential measurements of S-PEG750 at different pH 4.5, 7.2 and 9.

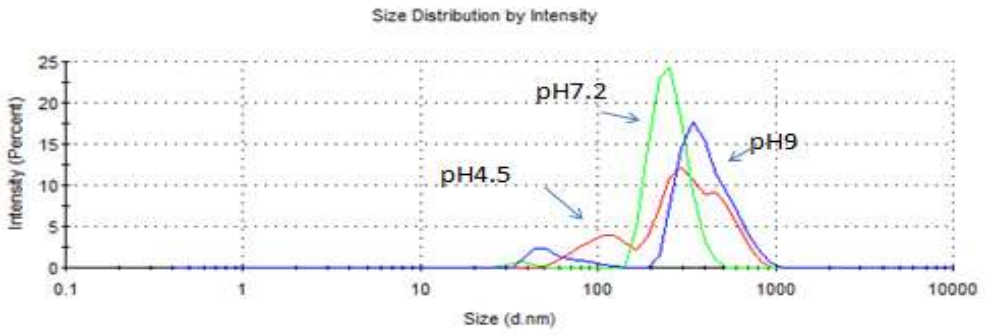


Figure 18: Size of S-PEG$_{750}$ at different pH 4.5, 7.2 and 9.

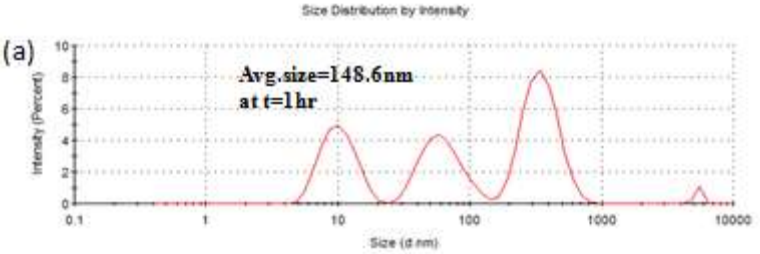
6.3 Lipase Degradation

The enzyme degradation was carried out as S-peg$_{750}$ (1mg) was solubilised by adding (100µl and 200 µl) Lipase and (50µl) NaN_3 in two different microcentrifuge tube and placed the sample on thermomixer at 37℃ and 350 rpm. The size of sample was measured by DLS after particular time interval and morphology and size of sample was also observed by TEM in dry state. These results were basically showed that how much time were taken by polymer for degradation on action of enzyme at different concentration and at different time interval. The results were showed that with increase in concentration of Lipase and time interval polymer size was also increase and as the hydrophobic and hydrophilic interaction were broken down and then there was no micelle formation.

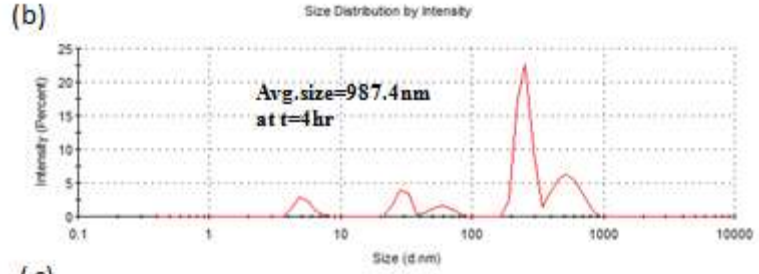
S. no	Time (in hrs)	Without Lipase			With Lipase 100µl			With Lipase 200µl		
		Average particle size (d.nm) ± S.D.	PDI ± S.D	count rate	Average particle size (d.nm) ± S.D.	PDI ± S.D	count rate	Average particle size (d.nm)± S.D.	PDI ± S.D	count rate
1.	1 hr	86.93± 0.24	0.186±0 .018	211 .5	148.6±8 1.57	0.453±0 .126	159 .7	91.11± 19.86	0.374± 0.090	198 .1
2.	4hr	128.7± 7.42	0.306±0 .065	209 .8	987.4±3 31.8	0.762±0 .192	372 .2	1308±2 72	0.879±0 .209	186 .1
3.	6hr	153.5± 8.25	0.245±0 .030	155 .2	1261±2 20.5	0.776±0 .21	174 .6	3168±2 25	1±0.0	285 .2
4.	23 hr	732.1±6 0.61	0.398±0 .066	79. 4	1036±9 3.70	0.482±0 .138	508 .3	1192±5 8.40	0.309±0 .125	111 .5
5.	26 hr	1276±1 80.1	0.635±0 .127	252 .7	1570±1 98.3	0.704±0 .177	265	1719±1 07.2	0.466±0 .123	294 .4
6.	29 hr	2109±4 78.5	1±0.0	189	1412±2 33.2	0.490±0 .274	144	2249±1 23.4	0.768±0 .221	217

Table 4: Size of S-PEG750 with and without action of Lipase at different concentration.

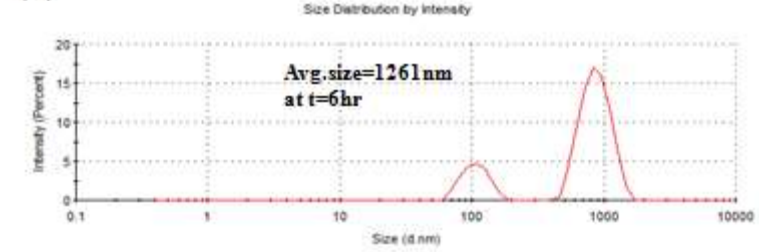
(a)


Size Distribution by Intensity
Avg.size=148.6nm
at t=1hr
Intensity (Percent)
Size (d.nm)

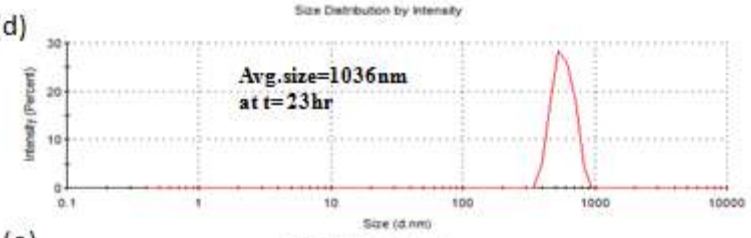
(b)


Size Distribution by Intensity
Avg.size=987.4nm
at t=4hr
Intensity (Percent)
Size (d.nm)

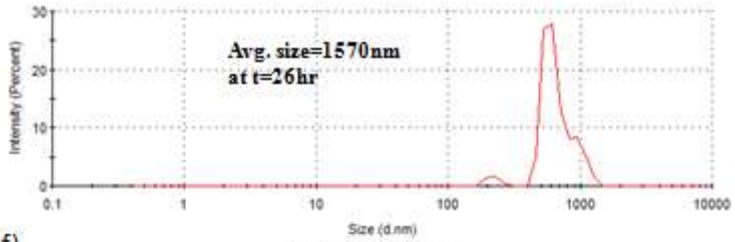
(c)


Size Distribution by Intensity
Avg.size=1261nm
at t=6hr
Intensity (Percent)
Size (d.nm)

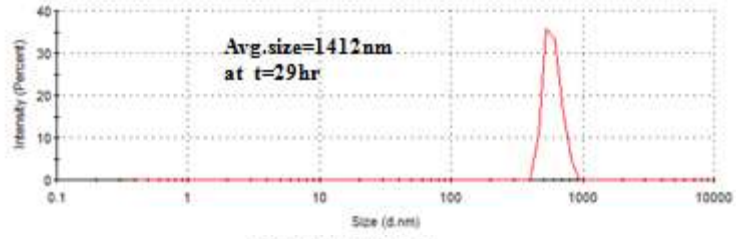
(d)


Size Distribution by Intensity
Avg.size=1036nm
at t=23hr
Intensity (Percent)
Size (d.nm)

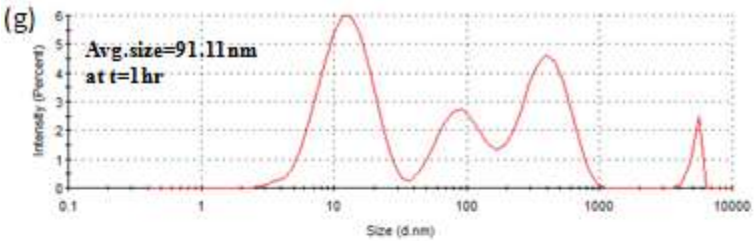
(e)


Size Distribution by Intensity
Avg. size=1570nm
at t=26hr
Intensity (Percent)
Size (d.nm)

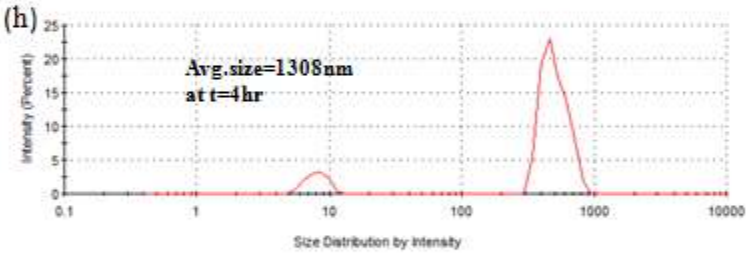
(f)


Size Distribution by Intensity
Avg.size=1412nm
at t=29hr
Intensity (Percent)
Size (d.nm)

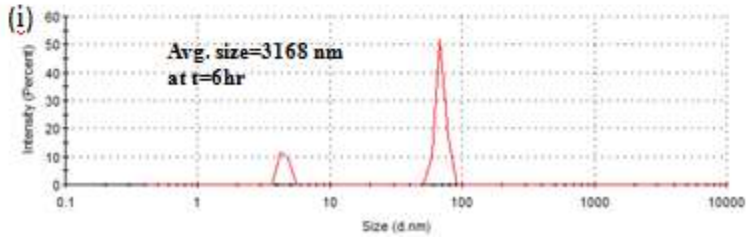
(g)


Size Distribution by Intensity
Avg.size=91.11nm
at t=1hr
Intensity (Percent)
Size (d.nm)

(h)


Size Distribution by Intensity
Avg.size=1308nm
at t=4hr
Intensity (Percent)
Size (d.nm)

(i)


Avg. size=3168 nm
at t=6hr
Intensity (Percent)
Size (d.nm)

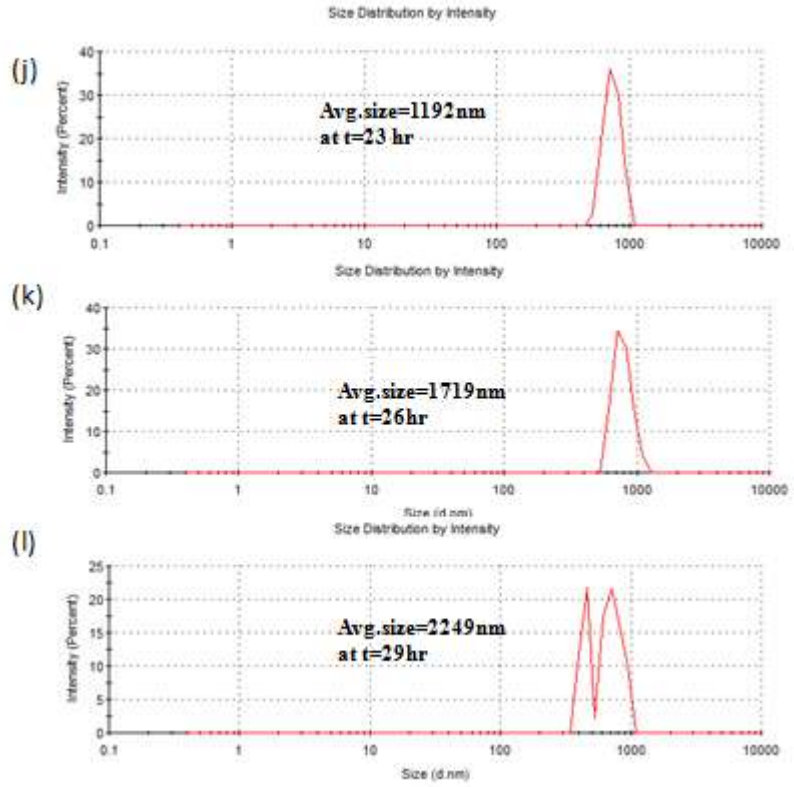


Figure 19:Size measurements of S-PEG750 by Lipase degradation at 100µl and 200µl (1mg ml^{-1}).(a) t=1hr (b) t=4hr (c)t= 6hr (d) t= 23hr (e) t= 26hr (f) t=29hr at 100 µl and (g) t=1hr (h) t=4hr (i) t=6hr (j) t=23hr (k) t=26hr (l) t=29hr at 200µl respectively.

6.4 Drug encapsulation

The evaluation of encapsulation of hydrophobic molecules in the self assembled polymer ornidazole and sulfasalazine were subjected to be encapsulated in polymer. DLS studies of the S-PEG after encapsulation were also carried out (Table 2). The size of loaded compound increases in comparison to unloaded compound and also depending upon the ratio of entrapment. The data showed that after loading , the size of the drug loaded compound increased from 212.9 nm to 300.4 nm when drug was loaded in ratio of 5:0.5 and increased from 212.9 nm to 230.3 nm when drug was loaded in the ratio of 5:1in case of ornidazole and in case of sulfasalazine, the size of drug loaded compound increased from 212.9nm to 265.4nm when drug was loaded in the ratio of 5:0.5 and increased from 212.9nm to 283.6 nm when drug was loaded in the ratio of 5:1. The encapsulation occurred due to the hydrophobic-hydrophobic interactions, which confirmed the presence of hydrophobic matrix in self assembled polymeric nanostructures. The loading of ornidazole drug was performed at different w/w ratios of polymer: drug and it was found to be 3.1% (w/w 5:1) and 2.78% (w/w 5:0.5). The % drug entrapment efficiency of nanostructures was found to be 18.06 (w/w 5:1) and 29.88% (w/w 5:0.5). The loading of sulfasalazine drug was performed at different w/w ratios of polymer:drug and it was found to be 5.38%(w/w 5:1) and 6.4%(w/w 5:0.5). The % drug entrapment efficiency of nanostructures was found to be 32.40% (w/w 5:1) and 60.29% (w/w 5:1). The morphology and size of S-PEG loaded with ornidazole and sulfasalazine were also observed by TEM which showed the spherical shaped self assembled structures of peptides that were loaded with ornidazole ~ nm (w/w 5:1) and ~ nm (w/w 5:0.5). A usual the average size of nanostructures is generally smaller in case of tem as compare to DLS.

S.no	Sample	Entrapment efficiency	Loading efficiency
1.	S-PEG loaded with ornidazole (5:1)	18.06%	3.1%
2.	S-PEG loaded with ornidazole (5:0.5)	29.88%	2.78%
3.	S-PEG loaded with sulfasalazine (5:1)	32.40%	5.38%
4.	S-PEG loaded with sulfasalazine (5:0.5)	60.29%	6.4%

Table 5: The entrappment and loading efficiency of S-PEG750 loaded with sulfasalazine and ornidazole at 5:1 and 5:0.5 ratio.

6.5 In vitro drug release

For evaluation of percentage release of encapsulated drug (w/w 5:1) inside the S-PEG the drug loaded S-PEG is equivalent to 2 mg ornidazole and sulfasalazine were put in a cellulose dialysis tube. The dialysis tube was put in 10 mL PBS solution (at varies pH 4.5 and 7.2 respectively) and mixed at 150 rpm under stirring. Aliquots of solution were taken out at regular time intervals and same volume of solution was replaced with fresh medium. The samples were analysed by UV-VIS spectrophotometer at 317 nm (ornidazole) and at 359nm (sulfasalazine) against the blank reagent. All the experiments were conducted in triplicates and the average sum up were considered for evaluation. The percentage release profile of both drug were showed that the release of drug were maximum in case of neutral pH.

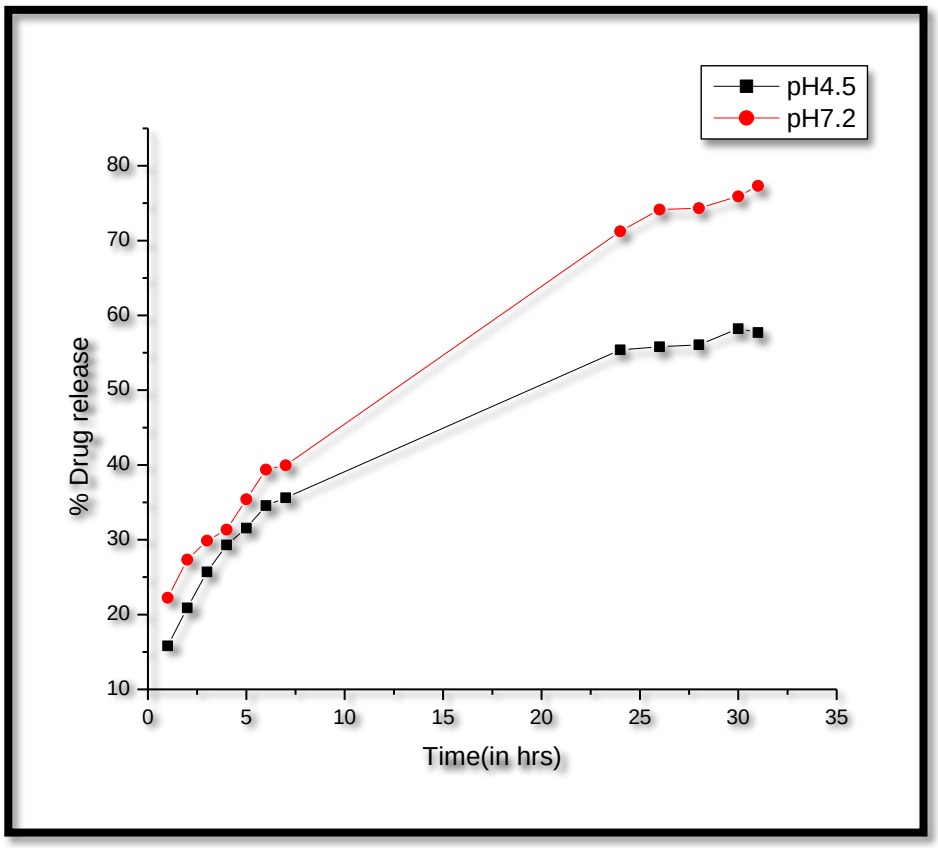


Figure 20: The % drug release profile of ornidazole w.r.t time from S-PEG750 at different pH (4.5 and 7.2).

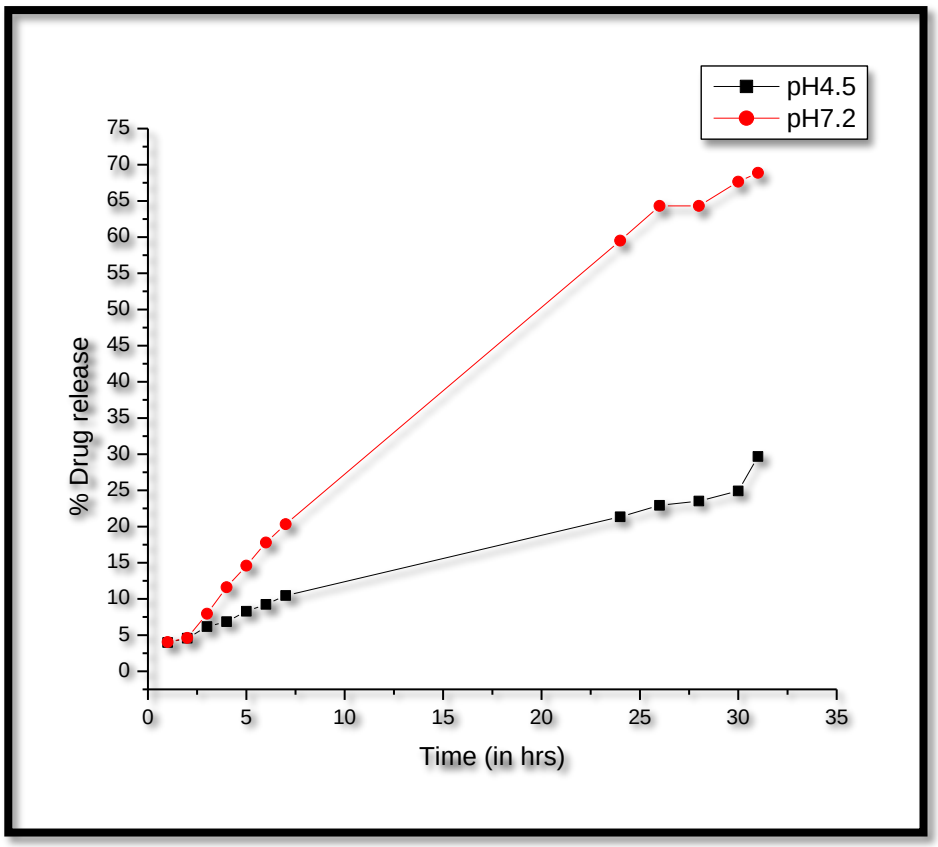


Figure 21: The % drug release profile of sulfasalazine w.r.t time from S-PEG750 at different pH (4.5 and 7.2).

6.6 *In vitro* cytotoxicity assay

The *in vitro* cytotoxic effect of sulfasalazine PEG polymer, sulfasalazine PEG encapsulated with drug, ornidazole drug were studied using a tetrazolium dye (MTT assay) in HEK 293 cells. The cytotoxicity of the polymer, drug and polymer loaded with drug were observed at different concentrations, upto 100µg/µL and the polymer, drug and polymer encapsulated with drug showed no significant cytotoxicity.

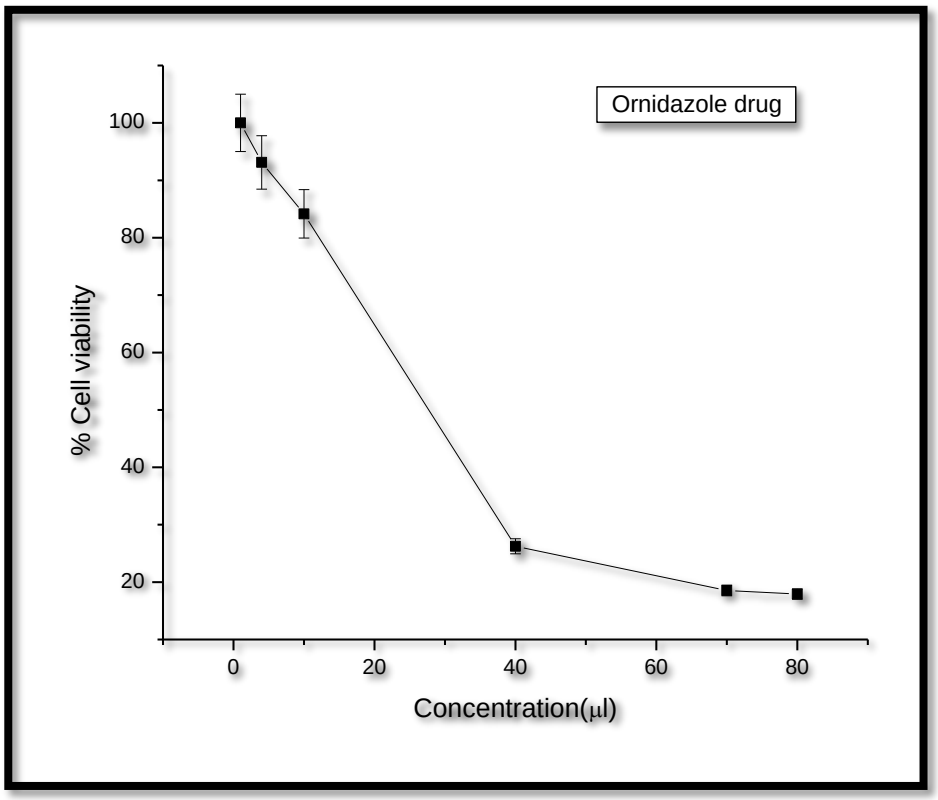


Figure 22: The % cell viability vs different concentration of ornidazole.

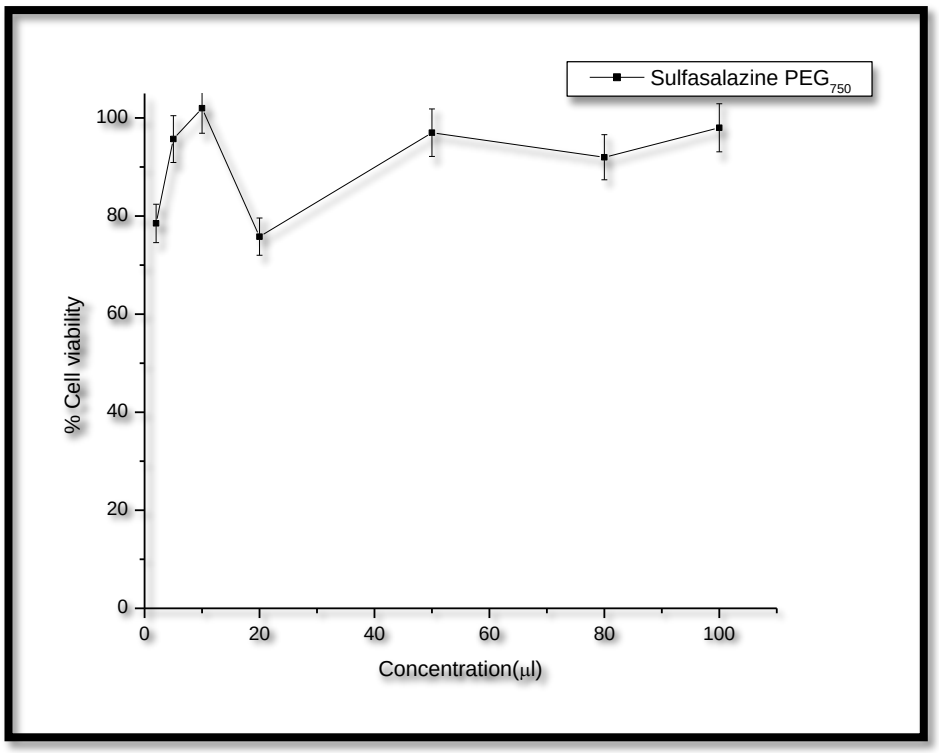


Figure 23: The % cell viability vs different concentration of S-PEG$_{750}$.

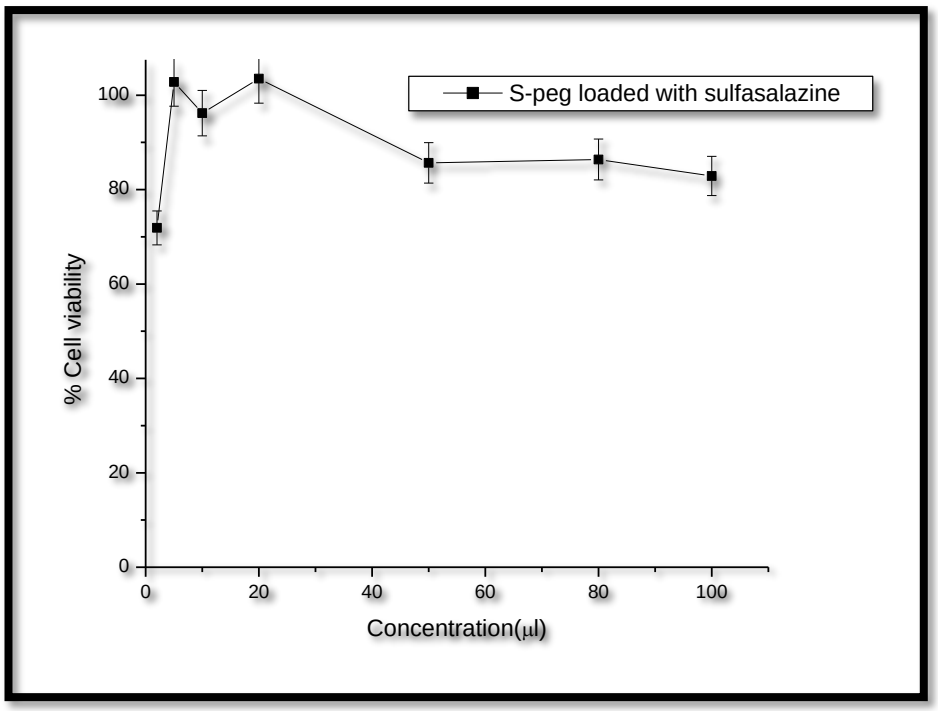


Figure 24 : The % cell viability vs different concentration of SPEG loaded with sulfasalazine drug.

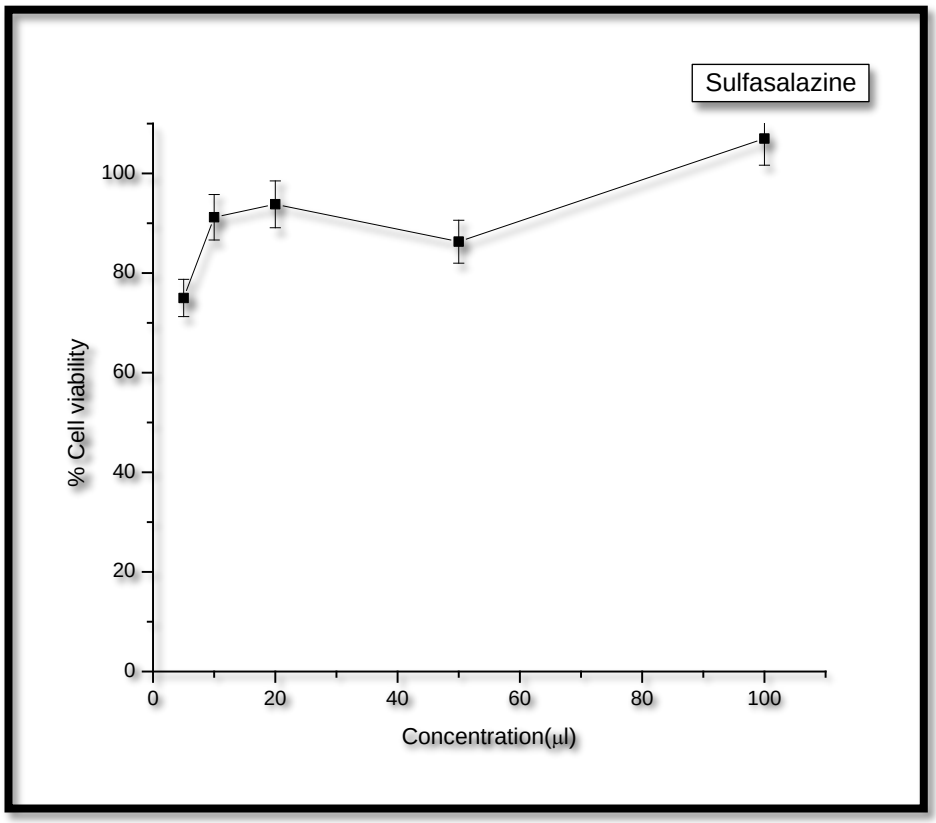


Figure 25 : The % cell viability vs different concentration of Sulfasalazine.

7. CONCLUSION

Nanoparticles from sulfasalazine and PEG_{750} were designed to load the hydrophobic drug Ornidazole and sulfasalazine. The nanoparticle was formed due to the formation of an ester linkage by the reaction of the alcoholic group of PEG_{750} and carboxylic acid group of sulfasalazine. Ornidazole and sulfasalazine were entrapped inside the hydrophobic core of the micelle formed. The basic characterization studies confirm the aforementioned. *In vitro* release studies reveals that the release of ornidazole is faster in neutral environment proving it to effectively target to colonic cancer and gastrointestinal diseases and the release of a sulfasalazine is faster in neutral environment proving it to effectively target to arthritis. Cytotoxicity assay for the ornidazole drug and sulfasalazine were also done revealing that polymer to be minimally toxic at a particular concentration. From these results, the novel nanoparticle synthesized has shown to be an ideal candidate for targeted delivery of drugs. *In vivo* studies still need to be done to check for the overall effect of these nanoparticles and drug inside the body of an organism.

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