*Original Article*

# Development, characterization, and stability assessment of Pegylated Bovine Serum Albumin Nanoparticles for prolonged pharmacological release

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## ABSTRACT:

**Background and Purpose:** The purpose of this study is to develop Pegylated albumin nanoparticles encapsulating the drug 5-Fluorouracil (5-FU) and evaluate their release kinetics and stability. A systematic investigation into the synthesis, characterization, optimization, and stability of modified albumin nanoparticles is necessary to assess their potential as efficient and reliable drug delivery systems.

**Methods:** The synthesis of Methoxy polyethylene glycol carboxylic acid (mPEG-COOH) and its coupling with albumin resulted in the formation of Pegylated albumin conjugates. Various physicochemical characterization techniques were used to confirm the successful formation of the conjugates. Optimization of process variables

for the preparation of mPEG-COOH conjugated BSA nanoparticles was conducted. In vitro release studies of 5-Fluorouracil from the nanoparticles were analyzed using mathematical models. Stability studies were performed under different conditions.

**Results:** The synthesis and characterization confirmed the successful formation of pegylated albumin conjugates. Optimal conditions for the preparation of mPEG-COOH conjugated BSA nanoparticles were determined. The drug-loaded pegylated albumin nanoparticles exhibited sustained and prolonged drug release patterns through diffusion and matrix erosion mechanisms. Stability studies showed maintenance of size and zeta potential, although slight particle aggregation and turbidity increase indicated polymer degradation over time.

**Conclusion:** This systematic study demonstrates the potential of Pegylated albumin nanoparticles as efficient and reliable drug delivery systems. The sustained and prolonged drug release supports their suitability for controlled drug delivery applications. The use of protein-based nanoparticles, particularly BSA, offers advantages in terms of biodegradability, non-toxicity, and non-antigenicity. Further optimization is needed to improve long-term stability and address polymer degradation. Future research should investigate the in-vivo release of the nanoparticles in target tissues and address any limitations identified in this study.

**Keywords:** Drug delivery, Pegylated Albumin Nanoparticles, Sustained release, polymeric nanoparticles

## INTRODUCTION:

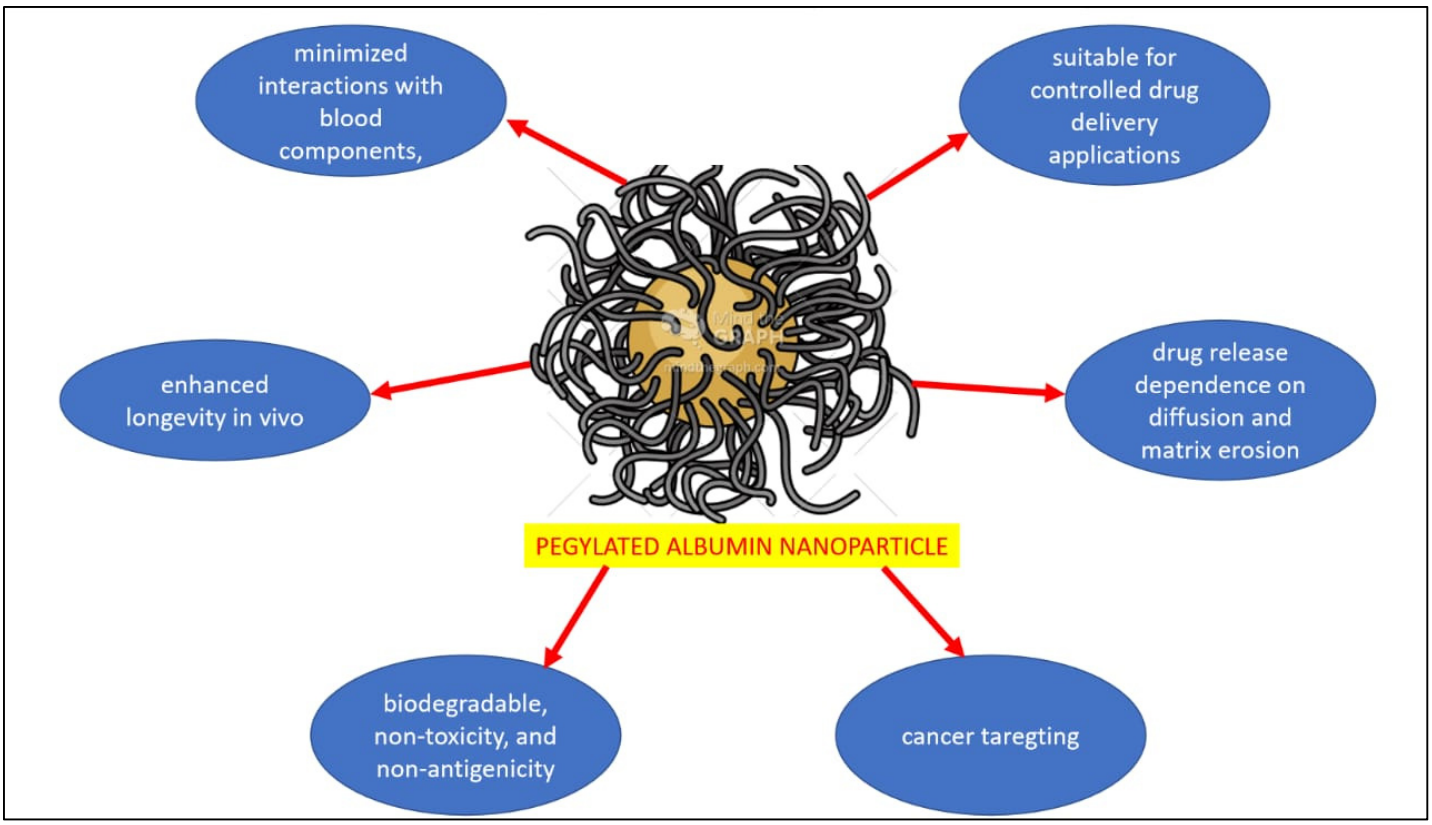
Nanoparticles, particulate dispersions or solid particles with sizes ranging from 10 to 1000 nanometer (nm), have gained significant attention as drug delivery systems in pharmaceutical and medical applications [1]. Polymeric nanoparticles, in particular, have shown promise due to their controlled and sustained release properties, subcellular size, and compatibility with tissue and cells [2].

Polymeric drug carriers employ various physicochemical mechanisms to release drugs at the target tissue site. These mechanisms include swelling of the polymer nanoparticles through hydration, followed by release through diffusion; enzymatic reactions leading to the rupture, cleavage, or degradation of the polymer at the delivery site, resulting in drug release; and dissociation of the drug from the polymer, followed by desorption or release from the swelled nanoparticles [3].

Nanoparticles have been utilized to enhance drug effects in target tissues, improve stability against enzymatic degradation, and facilitate solubilization for intravascular administration. To ensure safe administration via the intravenous route, nanoparticles are formulated as spherical amorphous particles in the form of injections [4].

Formulating nanoparticles involves considering several factors for the selection of matrix materials. These factors include the desired nanoparticle size, permeability, surface charge, biodegradability, biocompatibility, non-toxicity, preservation of drug solubility and stability, desired drug release profile, and avoidance of immunogenicity [5]. Several methods are employed for the formulation of nanoparticles, including dispersion of preformed polymers, polymerization methods, coacervation or ionic gelatin methods, and supercritical fluid technology [6].

Various methods can be employed for the formulation of drug-loaded nanoparticles, depending on the properties of the candidate drug and the desired polymer. Heat denaturation and cross-linking in a water-in-oil (w/o) emulsion can be utilized when the candidate drug is hydrophilic and requires a hydrophilic polymer such as albumin or gelatin. Desolvation and cross-linking in water are suitable for hydrophilic drugs with protein affinity, using hydrophilic polymers like albumin or gelatin. Cross-linking in water is another option for hydrophilic drugs with protein affinity, utilizing hydrophilic polymers like alginates and chitosan. Polymer precipitation in an organic solvent can be employed for hydrophilic drugs, and a suitable polymer is hydrophilic dextran. Emulsion polymerization is effective for hydrophilic drugs, while hydrophobic poly(alkylcyanoacrylates) are used as the polymer. Interfacial oil-in-water (o/w) polymerization is suitable for hydrophobic drugs, using hydrophobic poly(alkylcyanoacrylates) as the polymer. Solvent extraction evaporation is employed when the drug is soluble in polar solvents, both hydrophilic and hydrophobic, and polyesters such as poly(lactic acid) or poly(caprolactone) are utilized as polymers. Solvent displacement is an option for drugs soluble in polar solvents, both hydrophilic and hydrophobic, with polyesters such as poly(lactic acid) or poly(lactide-co-glycolide) as the polymers. Lastly, salting out is used when the drug is soluble in polar solvents, with polyesters such as poly(lactic acid) or poly(lactide-co-glycolide) as the polymers [7]. 5-FU (5-fluorouracil) is an anticancer drug categorized as an antimetabolite. It is a white crystalline powder that is practically odorless. It is soluble in water (12 g/L), ethanol (2.9 g/L), dimethyl sulfoxide (DMSO), and dimethyl formamide (DMF). 5-fluorouracil (5-FU) is stable but sensitive to light. For long-term storage, it can be stored for up to 2 years at -20˚C. The recommended dose of 5-FU is 12 mg/kg. It has a relatively short half-life of 15 minutes [24].




**Albumin as a drug carrier:**

Albumin, a macromolecular carrier, is a favorable choice for drug delivery due to its biodegradability, non-toxicity, ease of purification, and solubility in water. It is an ideal candidate for nanoparticle preparation because it can incorporate a significant amount of drug within its matrix [8]. Albumin-based nanoparticles offer various advantages, such as the ability to adsorb positively or negatively charged molecules without the need for additional compounds, ease of preparation using gentle methods like coacervation or emulsion formation, smaller size compared to microparticles, and controlled release properties superior to liposomes [9]. Bovine serum albumin (BSA), with its unique properties and wide acceptance in the pharmaceutical industry, is commonly used in drug delivery applications. BSA nanoparticles are biodegradable, reproducible, and allow for effective incorporation of various drugs due to their high protein binding capacity. The presence of functional groups on the nanoparticle surface enables covalent derivatization with drug targeting ligands. Clinical studies have demonstrated the tolerability of BSA-based particle formulations. Additionally, protein nanoparticles, particularly BSA, hold potential for gene therapy by minimizing unwanted interactions with serum following intravenous injection of transfection complexes [10]. Bovine serum albumin (BSA) is a powder with a white to

light tan color. It is a single polypeptide chain consisting of about 583 amino acid residues and no carbohydrates. BSA contains intrachain disulfide bridges and a sulfhydryl group. It has a molecular weight of 66,400 g/mol and a melting point of 212°C [21]. BSA is stable when stored at 2-8°C for at least 2.5 years. It is water-soluble and can be precipitated by high concentrations of neutral salts. BSA has excellent solution stability, especially when stored as frozen aliquots. It binds fatty acids, bilirubin, hormones, and drugs due to a hydrophobic cleft [22]. BSA is commonly used as a standard in protein calibration studies and for solubilizing lipids. It is also used as a blocking agent in Western blots and Enzyme-linked immunosorbent assay (ELISA) tests. Globulin-free albumins are suitable for applications where no other proteins are present [23].

**Pegylation:**

PEGylation, the addition of poly(ethylene glycol) (PEG) to nanoparticle surfaces, offers significant benefits in drug delivery and imaging applications. It reduces uptake by the reticuloendothelial system (RES), prolongs circulation time, mitigates aggregation, reduces association with non-targeted proteins and tissues, and enhances solubility. PEGylation also affects nanoparticle size and enhances tumor accumulation while reducing liver accumulation [12]. In pharmaceutical and biotechnical applications, PEGylation plays a crucial role in shielding antigenic epitopes, preventing uptake by RES, and protecting against recognition and degradation. It alters biodistribution and offers versatile PEGylation strategies through various functional groups [13].

PEG (polyethylene glycol) is a hydroxyl group-containing linear or branched polyether. It is synthesized through anionic ring opening polymerization of ethylene oxide. Monomethoxy polyethylene glycol (mPEG) is commonly used for polypeptide modification and is synthesized using methoxide ions. Commercial mPEG may contain diol PEG, which can be removed by converting PEGs to PEG-carboxylic acids. PEG has a narrow polydispersity, is soluble in both aqueous and organic solvents, and exhibits properties such as protein precipitation, exclusion from surfaces, reduced immunogenicity, and resistance to degradation. PEG is cleared rapidly in vivo without structural change, with clearance routes depending on its molecular weight. PEG has weak immunogenicity, but antibodies to PEG can be generated under specific immunization protocols [14].

PEG can be coupled to various molecules such as polypeptides, polysaccharides, polynucleotides, and small organic molecules by preparing a PEG derivative with a functional group. The choice of functional group depends on the reactive group on the molecule to be coupled. Reactive amino acids in proteins and glycoproteins with vicinal hydroxyl groups can be targeted for PEGylation [15].

**Nanoparticle PEGylation for therapy:**

Nanoparticles exhibit distinct behavior compared to other therapies and imaging agents due to their intermediate size between molecules and cells. They possess advantages in cancer treatment, such as preferential extravasation from leaky tumor vasculature and retention within the tumor area through the enhanced permeability and retention (EPR) effect. However, there are challenges that hinder their clinical application.

One challenge is the rapid uptake of nanoparticles by the reticuloendothelial system (RES), leading to their clearance to organs like the liver, spleen, or bone marrow, and nonspecific binding to non-targeted areas. These factors contribute to RES accumulation and potential toxicity concerns. Nanoparticle aggregation can also cause entrapment in organs, such as the liver and lungs, due to capillary occlusion [11].

Nanoparticles consist of a core and corona, and PEG chains modify the interface layer, increasing circulation time. The circulation half-time (t½) is important for reaching the target and maintaining drug delivery. The RES removes nanoparticles from circulation, but PEGylation reduces opsonization and nanoparticle-nanoparticle (NP-NP) interactions, enhancing stability. Factors such as nanoparticle type and PEG chain properties influence the behavior and increased half-life of polyethylen glycol-nanoparticle (PEG-NP) constructs

The stability and circulation half-life (t½) of nanoparticles are influenced by their type, size, charge, and composition. PEGylation or dextran coating can increase t½, particularly for Superparamagnetic iron oxide (SPIO) and Ultra-small superparamagnetic iron oxide (USPIO) nanoparticles. The nanoparticle scaffold helps stabilize therapeutic payloads, allowing controlled drug release and reducing toxicity. PEGylated polymers such as polylactic acid, poly(hexadecylcyanoacrylate), chitosan, and polycaprolactone are commonly used for drug-containing nanoparticles. The length of PEG chains, described by molecular weight (Mw), affects their conformation, with brush conformation providing longer circulation times by better shielding nanoparticles from the RES [16]. Opsonized nanoparticles without surface modifications are quickly taken up by the liver or spleen. The size of nanoparticles influences their biodistribution and blood clearance. Small molecules below 1500 daltons (Da) can be eliminated through the renal system. Increasing the molecular weight of PEG polymers extends the circulation half-life of particles in the bloodstream. Uncoated nanoparticles tend to accumulate in the liver and spleen, but PEGylation redirects the biodistribution towards the spleen. For instance, after 24 hours, 40% of PEGylated particles were found in the liver compared to 90% for uncoated particles. After 60 minutes, the concentration of PEGylated particles in the spleen was 12% compared to 2% for uncoated particles [17].

Covalent conjugation involves joining molecules using cross-linking reagents, including zero length, homobifunctional, and heterobifunctional cross-linkers. Non-covalent conjugation relies on interactions between colloidal particles and protein molecules, such as adsorption and binding to hydrophobic pockets. Non-specific covalent binding may occur between the carrier and free sulphydryl/amine/carboxyl groups on the ligand, if present [18] [19].

## MATERIALS AND METHODS:

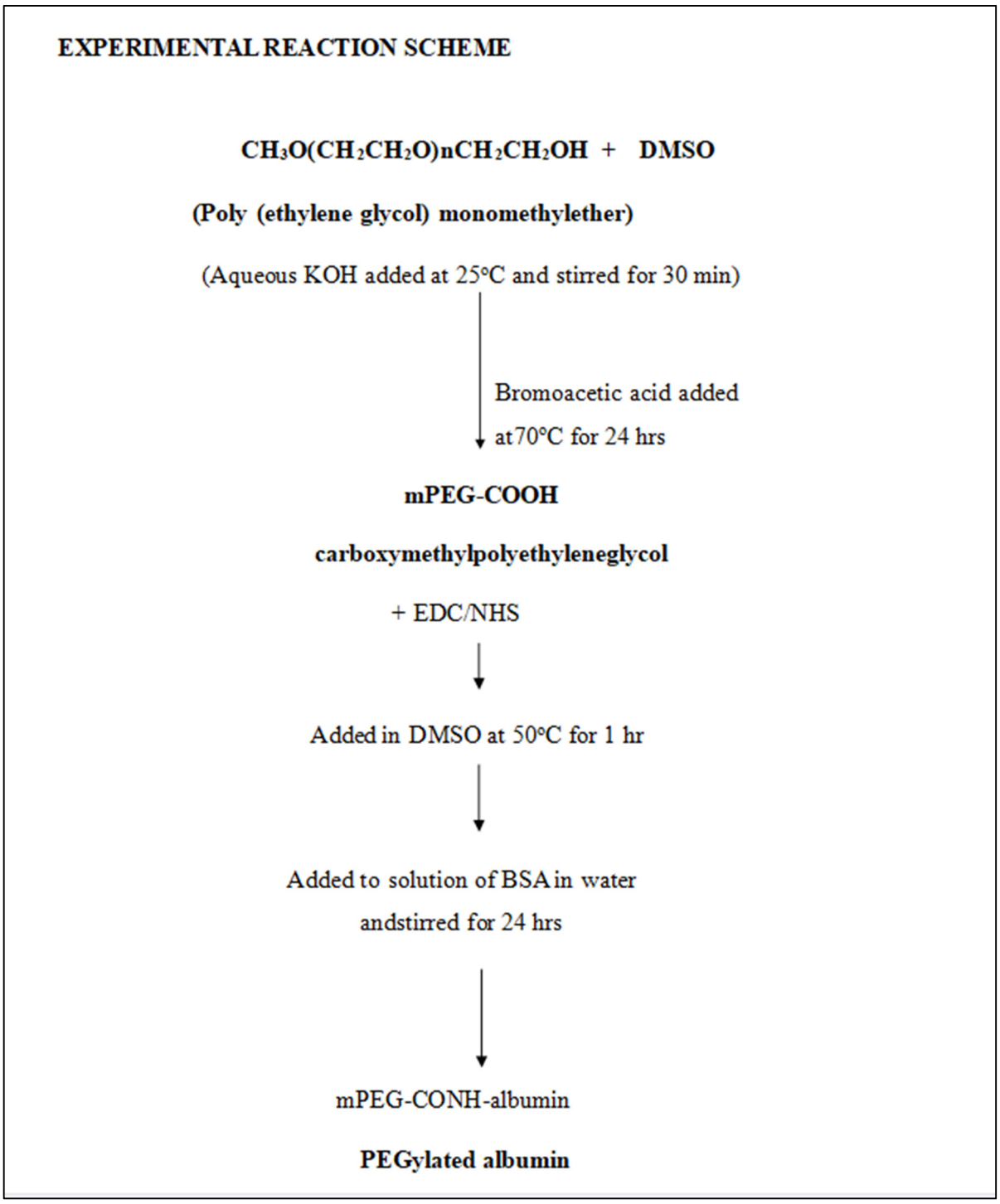
5-FU was purchased from sigma-aldrich. All other chemicals were purchased from local sources and were of analytical grade.

### Synthesis of mPEG-COOH

A solution of mPEG-1900 (5 g, 1 millimolar (mM)) in 30 ml of dimethylsulfoxide (DMSO) was mixed with an aqueous solution of potassium hydroxide (KOH) (40% w/v, 20 millileter (ml)). For 30 minutes, the solution was magnetically stirred at room temperature. The solution was then treated with bromoacetic acid (1.38 g, 10 mM) and kept at 70 $^{0}$C for an additional 24 hours. When the reaction was complete, one to two drops of a 38% hydrochloric acid (HCl) aqueous solution were added to neutralise the mixture, which was then tested with a potential of hydrogen (pH) paper. The residue was dissolved in dichloromethane and precipitated with excessive ethyl ether after successive removal of the resultant potassium chloride (KCl) and solvents. The precipitate mPEG-COOH was collected and dried under controlled conditions.

### Synthesis of mPEG-COOH coupled albumin

The carboxylic group of mPEG-COOH was combined with the free amine groups of albumin in the presence of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) to produce this. By heating at 50 $^{0}$C for 15 minutes, 20 mg of mPEG-COOH and 15 mg of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide (EDC) were dissolved in 1.25 ml of DMSO. After that, the mixture was completely cooled to room temperature. It was then mixed for 24 hours in a solution of 100 mg albumin dissolved in 2 ml of distilled water.




**Preparation of mPEG-COOH conjugated BSA Nanoparticles**

**Desolvation method**

mPEG-COOH conjugated BSA nanoparticles were prepared by a desolvation technique. Based on the optimization procedure, numbers of parameters were investigated by changing one parameter while keeping others constant. These parameters include concentration of mPEG-COOH

conjugated BSA solution, concentration of glutaraldehyde solution, speed of homogenizer, stirring time etc.

In order to prepare PEG conjugated BSA nanoparticles, 10 to 100 mg of modified BSA was dissolved in 2 ml of 0.2 N sodium chloride solution. The pH of the modified BSA solution was adjusted between 7 and 8. Prepared solutions were transformed into nanoparticles by the continuous addition of the desolvating agent ethanol under constant stirring at 900 revolutions per minute (rpm) at room temperature until the solution becomes turbid. After the desolvation process, 8% (v/v) glutaraldehyde in water was added to induce particle crosslinking. The crosslinking process was performed under stirring of the suspension over a time period of 3 hrs. The resultant nanoparticles were separated from the aqueous medium by centrifugation at 10,000 rpm for 1 hr at 4 0C. The supernatant was discarded and the pellets were washed with distilled water and dried.

**Characterization**

The synthesised conjugate was identified and characterised by the carboxylic group of mPEG-COOH was combined with the free amine groups of albumin in the presence of EDC/NHS to produce this. By heating at 50 $^0$C for 15 minutes, 20 mg of mPEG-COOH and 15 mg of EDC were dissolved in 1.25 ml of DMSO. After that, the mixture was completely cooled to room temperature. It was then mixed with a solution of 100 mg albumin dissolved in 2 ml of distilled water and stirred for 24 hours.

**Solubility**

The solubility of prepared conjugate was tested in different solvents like polar, semi polar and non polar. A definite quantity of compound (10 mg) was tried to dissolve in 10 ml of each investigated solvent at room temperature. Solubility of synthesized conjugate was checked in different solvents and mentioned in Table 4.

**Differential Scanning Calorimetry**

Thermogram of the synthesized conjugates, i.e methoxy PEG, mPEG-COOH, mPEG-COOH- BSA was obtained using Jade (Perkin Elmer) calibrated with indium. Sample (5mg) was placed onto a standard aluminium pan, crimped and heated from 40-400 $^o$C at a heating rate of 10 $^o$C/min with continuous purging of nitrogen (20 ml/min). An empty sealed pan was used as a reference.

### Infrared Spectroscopy

The infrared (IR) spectra of mPEG, mPEG-COOH, mPEG-COOH-BSA was obtained using Prestige- 21, Shimadzu FT-IR Spectrophotometer. The samples were prepared with potassium bromide (KBr) (Merck) which was previously dried in a hot air oven for 20 min before preparing samples.

### Preparation of modified albumin nanoparticles

Desolvation method was used for the preparation of PEG conjugated bovine serum albumin nanoparticles because this process does not require an increase in temperature and, therefore, may be useful when heat sensitive drugs are used. This process offers the advantage of producing nanoparticles directly in aqueous suspensions.

### Mean particle size and size distribution analysis

Mean particle size of drug loaded nanoparticles was determined by employing Malvern Zetasizer Dynamic Light Scattering (DTS version 4.10, Malvern, United kingdom). The samples were kept in poly styrene cuvettes and observations were made at 90$^{o}$C fixed angle. The sample of dispersion was diluted to 1:9 v/v with double distilled water to ensure that the light scattering intensity was within the instrument's sensitivity range.

**Polydispersity Index** (Size Distribution Uniformity)

The size distribution uniformity of nanoparticles was measured by Polydispersity index. The width of size distribution was indicated by Polydispersity Index (PI). The PI is calculated as the average molecular weight divided by the number average molecular weight. The PI of the modified nanoparticles was determined using a Zetasizer (DTS version 4.10, Malvern Instruments, U.K).

## OPTIMIZATION OF DRUG INCORPORATED MODIFIED BSA

### Encapsulation efficiency

The encapsulation efficiency of nanoparticles were determined by separation of nanoparticlesfrom the aqueous medium containing non-associated drug by centrifugation at 15000 rpm at 4 $^{o}$C for 30 min. The amount of free drug or unentrapped drug in the supernatant was measured in ultraviolet (U.V) spectrophotometer at 266 nm. The sediment obtained after centrifugation was resuspended in 1 ml of triton x-100 (0.2 % v/v). After appropriate dilution of the sample with PBS (pH 7.4), absorbance was recorded at 266 nm. The encapsulation efficiency can be calculated using the following formula:

Encapsulation Efficiency = (Amount of drug encapsulated / Total amount of drug used) × 100%

Loading Capacity

The loading capacity of nanoparticles was determined by separation of nanoparticles from the aqueous medium containing non-associated drug by centrifugation at 15000 rpm at 4ºC for 30 min. The sediment obtained after centrifugation was resuspended in 1 ml of triton x-100 (0.2 % v/v). After appropriate dilution of the sample with Phosphate-buffered saline (PBS) (pH 7.4), absorbance was recorded at 266 nm and loading capacity was calculated using the following formula:

Loading Capacity = (Amount of drug encapsulated in nanoparticles/ Weight of the nanoparticles) × 100%

Table 1. shows optimization of drug loaded formulation.

***Table 1.*** *Effect of drug to polymer ratio*

| Form code | Drug :Polymerr atio | Encapsulation efficiency | Loading Capacity in µg /mg | Process Yield in % | Mean Particle size | Mean PDI | Zeta Potential in mV |
|---|---|---|---|---|---|---|---|
| C1D1 | 1:1 | 67% | 455.78 | 73.5 | 165.3 | 0.213 | -23.1 |
| C1D2 | 1:3 | 74% | 496.6 | 74.5 | 245.4 | 0.123 | -26 |
| C1D3 | 1:5 | 85% | 515.15 | 82.5 | 170.7 | 0.251 | -21.8 |

**Surface morphology**

Electron microscope (EM) unit of High-Speed Atomic Force Microscopy Laboratory (HSADL) has a 120 kV class high-resolution transmission electron microscope (JEM-1400, Jeol, Japan). It is fitted with a digital Charge-Coupled Device (CCD) camera that enables focusing and image verification on the screen, providing filmless recording of images. The unit also has an ultramicrotome (Ultracut-UCT, Leica) and a knife maker (EMKMR2, Leica) for ultra-thin sectioning of tissue samples.

### *In-vitro* release of 5-Fluoro Uracil

The nanoparticles dispersion was placed in the donor compartment on dialysis membrane of diffusion cell. The dialysis membrane having a diameter of 17.5 millimeter (mm), average flat width 29.31 nm which was previously soaked for 24 hrs in the dissolution medium and stretched around one end of the tube was used. Then receptor chamber was filled with 50 ml phosphate buffer (pH 7.4). The entire system was incubated at 37 ºC under stirring at 100 rpm. At designated time interval 5 ml of the release medium was removed and replaced with same volume of fresh buffer solution to maintain the sink condition. The removed sample was suitably diluted and analyzed by U.V spectroscopy. The amount of drug in the release medium was determined and percentage cumulative release of the formulation was calculated.

### Calibration curve of drug in phosphate buffer (pH 7.4)

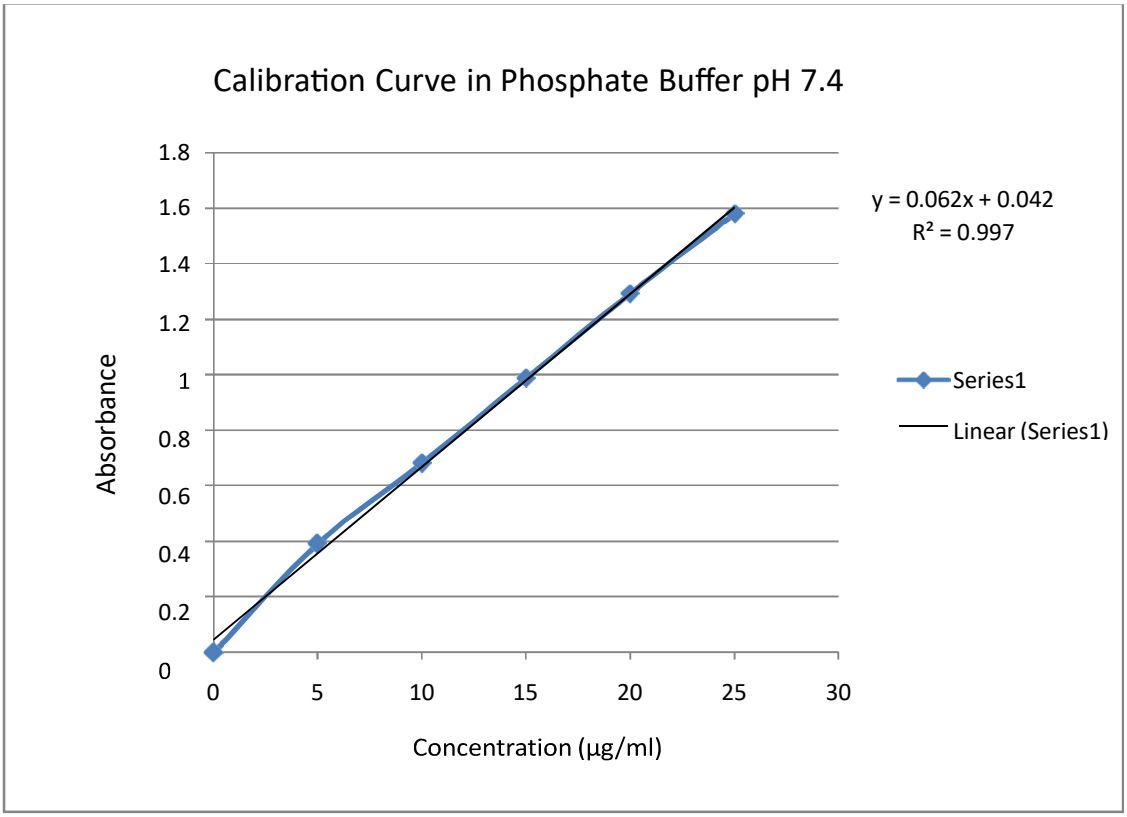
The drug was weighed accurately 10 mg and dissolved in 5 ml of phosphate buffer (pH 7.4)in a volumetric flask and volume was made upto 10 ml with phosphate buffer to obtain stock solution A. From stock solution A, 1 ml was withdrawn and diluted upto 10 ml with phosphate buffer in a separate volumetric flask, this gave stock solution B. From stock solution B, 1, 2, 3, 4 and 5 ml were withdrawn in 5 separate volumetric flask and volume of each was made upto 10 ml with phosphate buffer (pH 7.4) to obtain dilution of concentration 5, 10, 15, 20 and 25 µg/ml, respectively. The absorbances of these samples were obtained using Shimadzu 1800 UV/visible spectrophotometer at 266 nm.

Table 2. shows the calibration curve data of the drug at different concentrations in PBS pH 7.4 and Fig 1. shows the plotted graph

***Table 2.*** *Calibration Of 5-Fluorouracil in PBS pH 7.4 at Wavelength maximum (λmax) 266nm*

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0.000 |
| 2. | 5 | 0.391 |
| 3. | 10 | 0.681 |
| 4. | 15 | 0.987 |
| 5. | 20 | 1.292 |
| 6. | 25 | 1.580 |

***Fig 1.*** *Calibration curve graph in Phosphate Buffer pH 7.*



**Calibration curve of drug in distilled water**

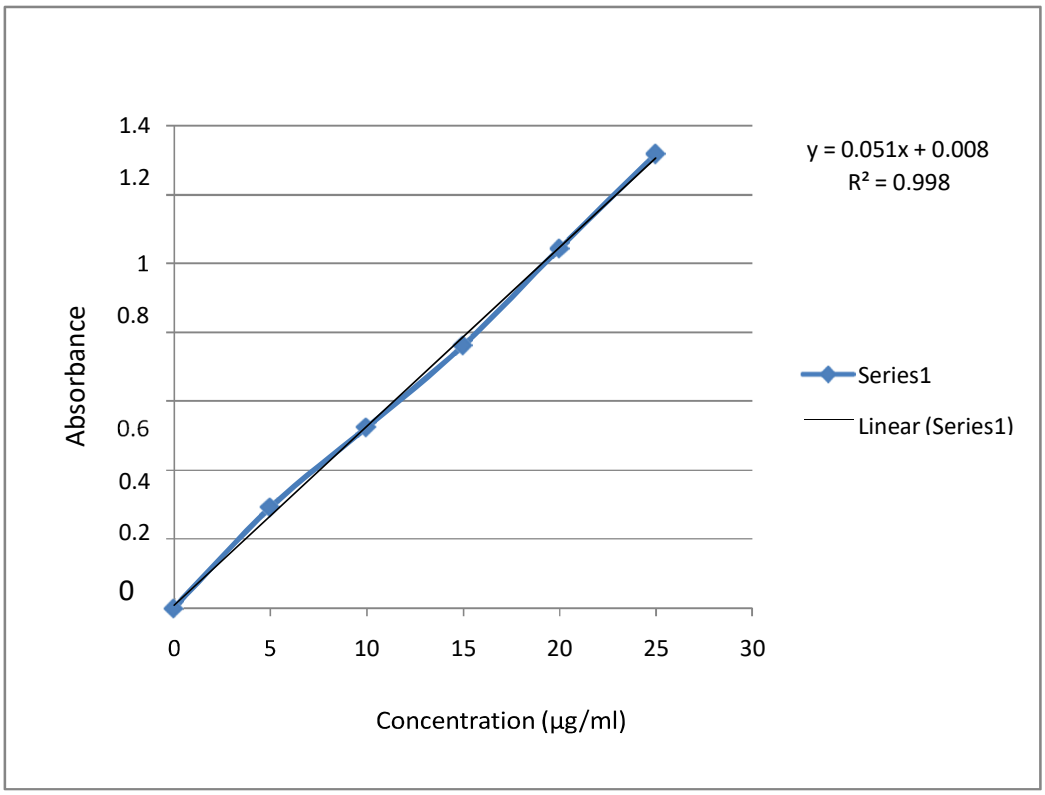
The drug was weighed accurately 10 mg and dissolved in 5 ml of distilled water in a volumetric flask and volume was made upto 10 ml with distilled water to obtain stock solution A. From stock solution A, 1 ml was withdrawn and diluted upto 10 ml with distilled water in a separate volumetric flask, this gave stock solution B. From stock solution B, 1, 2,3, 4 and 5 ml were withdrawn in 5 separate volumetric flask and volume of each was made upto 10 ml with distilled water to obtain dilution of concentration 5, 10, 15, 20 and 25 µg/ml, respectively. The absorbances of these samples were obtained using Shimadzu 1700 UV/visible spectrophotometer at 266 nm.

Table 3. shows the calibration curve data of the drug at different concentrations in distilled water and Fig 2. shows the plotted graph

***Table 3.*** *Calibration Curve Of 5-Fluorouracil in Distilled Water at λmax 266 nm*

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0.000 |
| 2. | 5 | 0.292 |
| 3. | 10 | 0.524 |
| 4. | 15 | 0.762 |
| 5. | 20 | 1.043 |
| 6. | 25 | 1.317 |

***Fig 2.*** *Calibration curve graph in Distilled water*


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
0
5
10
15
20
25
30
Concentration (µg/ml)
y = 0.051x + 0.008
R² = 0.998
Series1
Linear (Series1)

**Stability Studies**

Particle size, zeta potential and drug leakage were determined for the stability studies of PEGylated nanoparticles. Formulation were stored at cold conditions in a freezer (5°C ± 3°C) and room temperature (25°C ± 2°C/60 % relative humidity (RH) ± 5 % RH) for 3 month then after the change in particle size, zeta potential and leakage of drug were determined same as free drug in formulation by UV spectroscopy at 266 nm.

**Results:**

**Solubility Profile**

**Table 4. Solubility profile of synthesized conjugate**

| S.No. | SOLVENTS | SOLUBILITY |
|---|---|---|
| 1. | Methanol | Insoluble |
| 2. | Ethanol | Insoluble |
| 3. | Chloroform | Insoluble |
| 4. | Petroleum ether | Insoluble |
| 5. | Diethyl Ether | Insoluble |
| 6. | Dichloromethane | Insoluble |
| 7. | Ethyl Acetate | Insoluble |
| 8. | Distilled Water | Soluble |
| 9. | Phosphate buffer | Soluble |
| 10. | Dimethylsulfoxide | Insoluble |
| 11. | Acetone | Insoluble |
| 12. | THF | Insoluble |

**IR Interpretation of mPEG-COOH conjugate**

The IR spectra of mPEG, BSA and mPEG-COOH are shown in Fig 3. , Fig 4. and Fig 5. respectively. IR spectrum of mPEG did not show any peak in the region 1700-1750 Wavenumber (unit of measurement in infrared spectroscopy $cm^{-1)}$ indicating absence of carbonyl functional moiety, while appearance of a peak at 1732 $cm^{-1}$ in the spectra of mPEG-COOH indicates oxidation of terminal $–CH_2OH$ group to –COOH group.

The broad peak in the region of 3600-3300 $cm^{-1}$ for both mPEG (Table 5.) spectra and the mPEG-COOH (Table 7. ) spectra shows –OH stretching vibrations, while peak in the region of 1300-1100 $cm^{-1}$ shows characteristic ether group (C-O-C), also peak in the region at 2868 $cm^{-1}$ and 2885 $cm^{-1}$ in mPEG and mPEG-COOH spectra respectively shows C-H stretching.

The peak at 1654 $cm^{-1}$ in mPEG-COOH-BSA shows the formation of amide bond between carboxylic group and amine group. These data signifies the formation of conjugate.

***Fig 3.*** *IR spectra of mPEG*

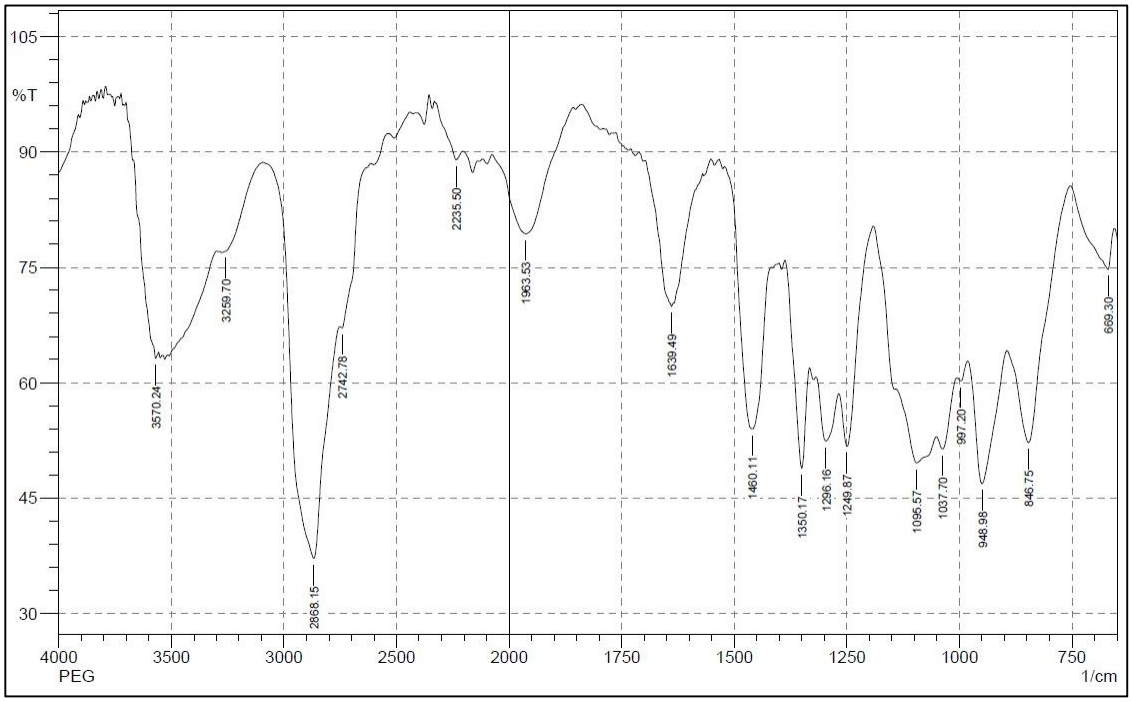



***Table 5.*** *Characteristic IR absorption bands of mPEG*

| Wave Number ($cm^{-1}$) | Characteristic Absorption |
|---|---|
| 3570.24 | -O-H stretching vibrations |
| 3259.70 | -O-H stretching vibrations |
| 2868.15 | -C-H stretching vibrations in methyl group |
| 1460.11 | C=C stretching vibrations |

***Fig 4.*** *IR spectra of BSA*

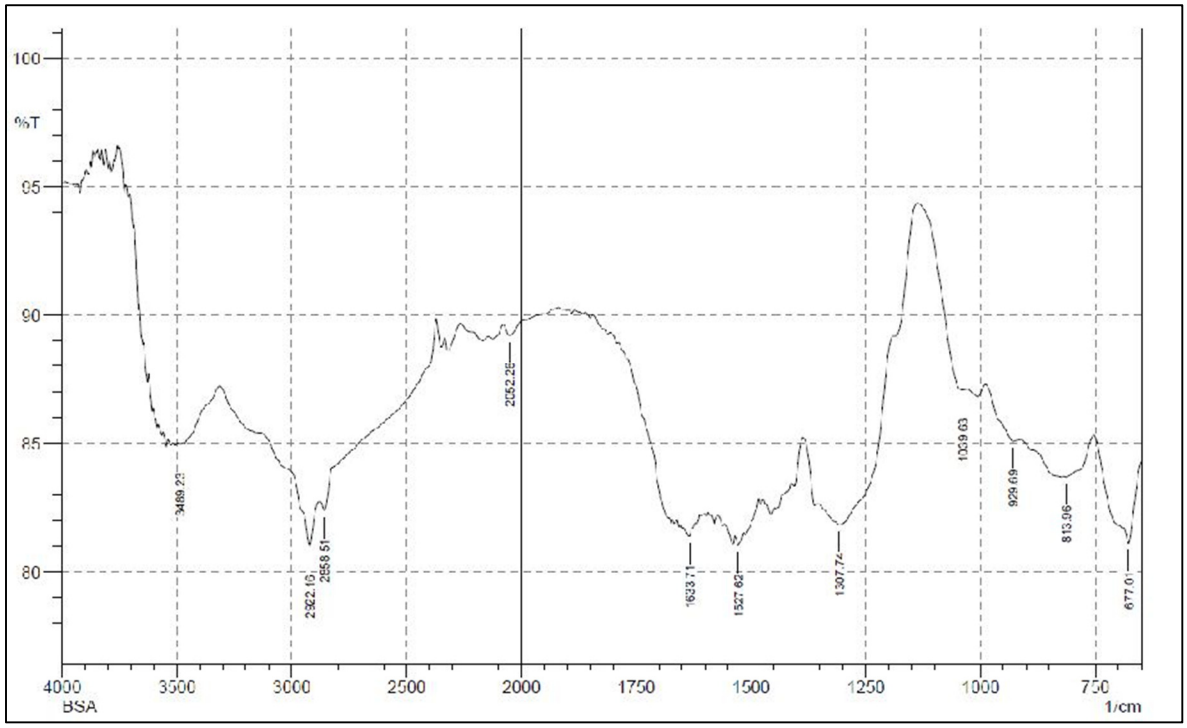



***Table 6.*** *Characteristic IR absorption bands of Bovine serum albumin*

| **Wave Number ($cm^{-1}$)** | **Characteristic Absorption** |
|---|---|
| 3489.23 | O-H stretching vibrations |
| 2922.16 | C-H stretching vibrations |
| 2052.26 | N-H stretching vibrations |
| 1633.71 | C=O stretching vibrations |

***Fig 5.*** *IR spectra of mPEG-COOH*

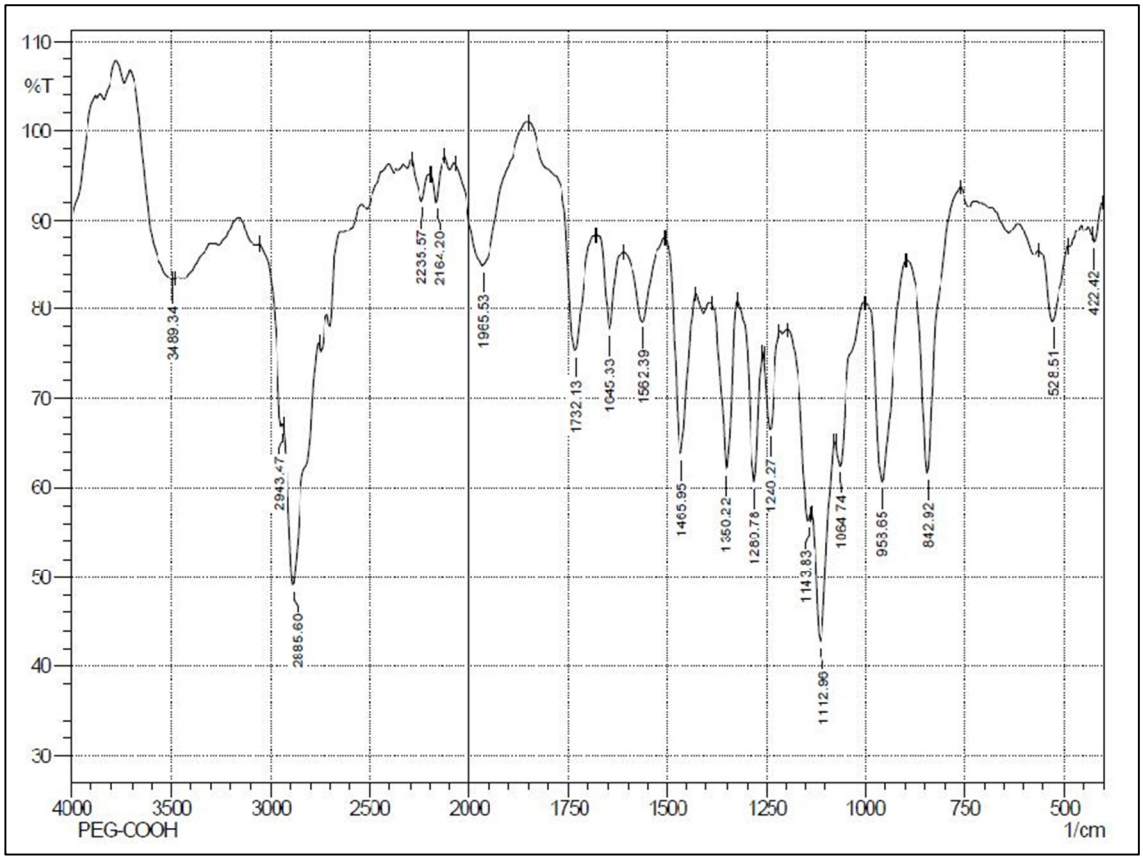



***Table 7.*** *Characteristic IR absorption bands of mPEG-COOH*

| **Wave Number ($cm^{-1}$)** | **Characteristic Absorption** |
|---|---|
| 1732.13 | -C=O (in carboxylic acids) |
| 3489.34 | O-H stretching vibrations |
| 2943.47 | C-H stretching vibrations |
| 2885.60 | C-H stretching vibrations |
| 1645.33 | C=O stretching vibrations |
| 1465.95 | C=C stretching vibrations |

**Fig 6. IR spectra of mPEG-COOH-BSA**

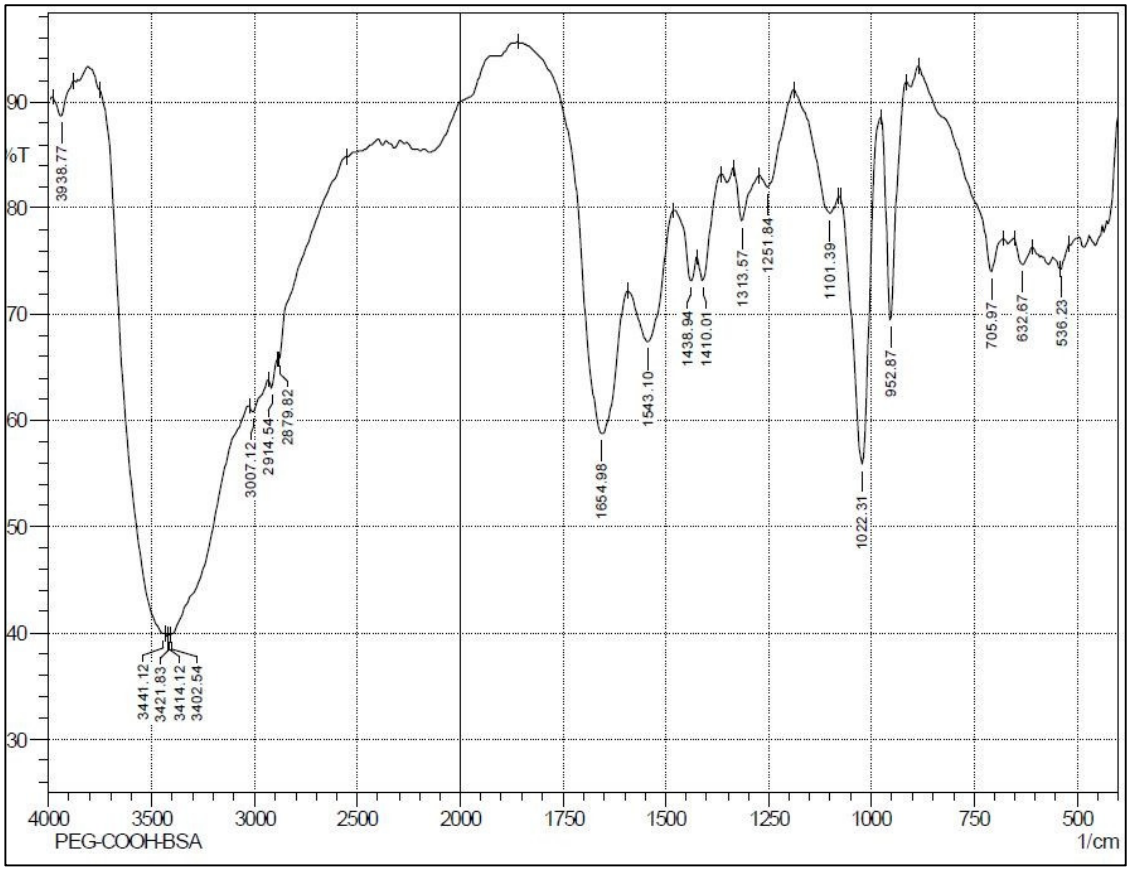



***Table 8.*** *Characteristic IR absorption bands of mPEG-COOH conjugated bovine serum albumin*

| **Wave Number ($cm^{-1}$)** | **Characteristic Absorption** |
|---|---|
| 3441.12 (bundle of peaks in this range) | Primary and secondary, amides |
| 2914.54 | C-H stretching vibrations |
| 1654.98 | C=O stretching vibrations |

## DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Thermogram of BSA (Fig 7.) and of the synthesized conjugate, mPEG-COOH (Fig 8.) mPEG-COOH-BSA (Fig 9.) with excipients was obtained using Jade (Perkin Elmer) calibrated with indium.

***Fig 7.*** *DSC Thermogram of BSA*

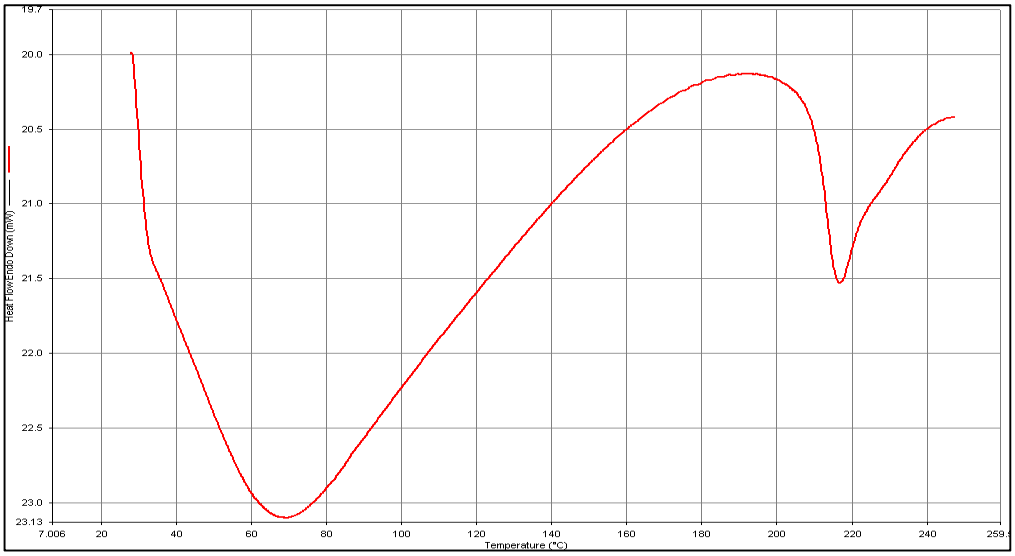



***Fig 8.*** *DSC Thermogram of mPEG-COOH*

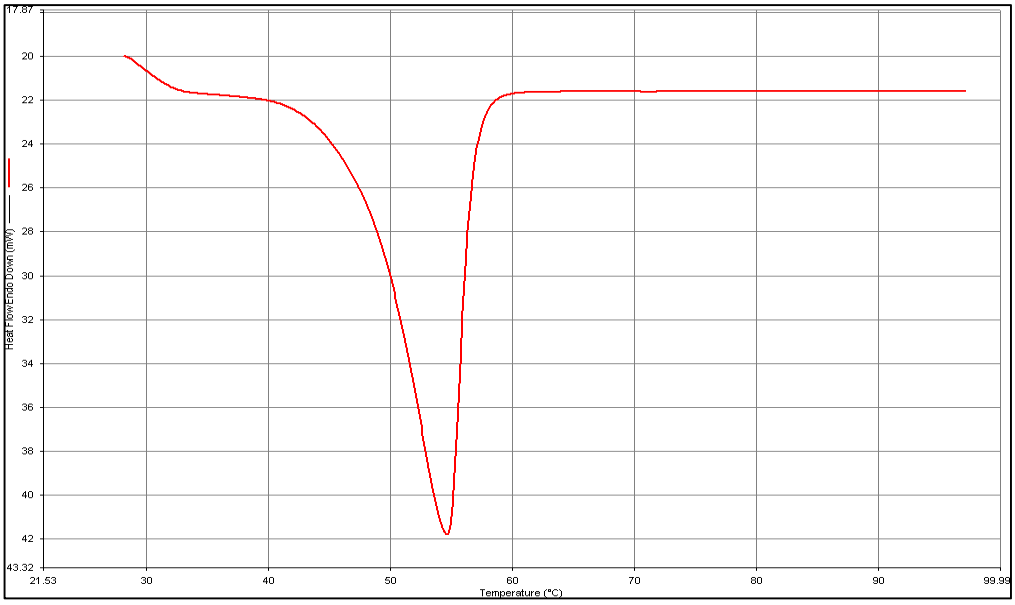
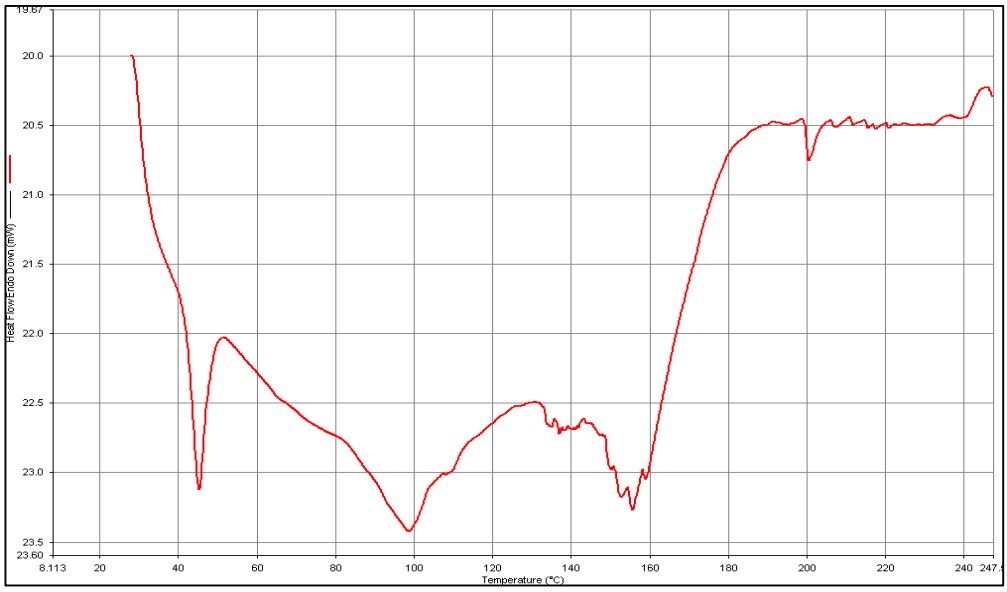


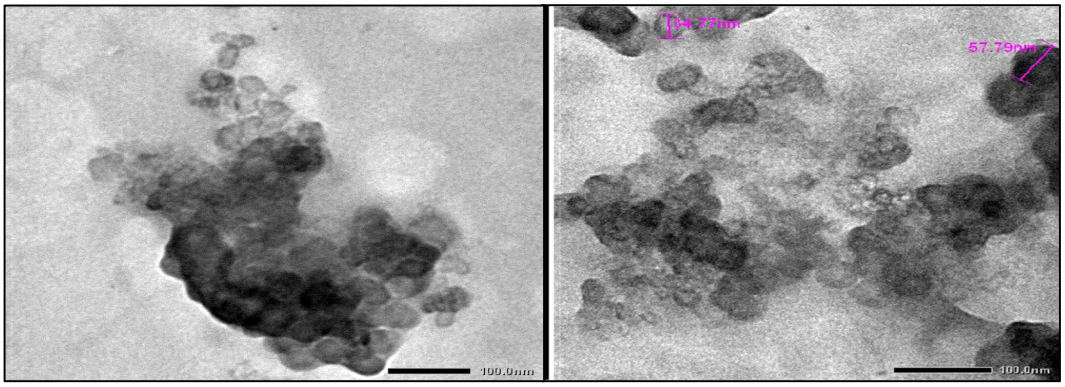

***Fig 9.*** *DSC thermogram of mPEG-COOH-BSA*




## Surface morphology

Transmission electron microscopy (TEM) revealed the spherical structure of modified nanoparticles. Fig 10. shows TEM images of modified nanoparticle formulation

***Fig 10.*** *TEM images of modified nanoparticle formulation*



**Size and size distribution**

Mean diameter of bare nanoparticles was 158 nm and PDI value of 0.088. In contrast, mean diameter of PEGylated nanoparticles under optimum condition was 190.7 nm and PDI valueof 0.134. As results reveal, PEG-BSA nanoparticles shows increase in size due to surface coating of nanoparticles by PEG molecules.

**Zeta Potential**

The zeta potential of bare nanoparticles was -29.5 mV. In contrast, Zeta potential of PEGylated nanoparticles under optimum condition was − 21.8 mV. The results revealed that PEG-BSA nanoparticles demonstrated reduction of the negative surface charge compared to the non-modified nanoparticles which shows reduction of the negative surface charge compared to the bare particles. Therefore, to some extent, PEG coating masked the negative charge of nanoparticles.

**Drug Loading and Encapsulation efficiency**

As the drug-to-polymer ratio increases from 1:1 to 1:5, as shown by Table 9 both the encapsulation efficiency and loading capacity also increase. This suggests that a higher amount of drug is being encapsulated and loaded into the polymer system as the ratio of drug to polymer increases. The highest encapsulation efficiency and loading capacity are observed at the 1:5 ratio, indicating the most efficient drug loading in this formulation.

***Table 9.*** *Encapsulation efficiency and loading capacity*

| S.No | Drug : Polymer Ratio | Encapsulation Efficiency | Loading Capacity |
|---|---|---|---|
| 1. | 1:1 | 67% | 455.78 |
| 2. | 1:3 | 74% | 496.6 |
| 3. | 1:5 | 85% | 515.15 |

### *In vitro* drug release studies

*In vitro* drug release from drug loaded, PEGylated nanoparticles is summarized in the cumulative percentage release shown in Table 10. The formulation produced an initial burst release upto 17 %. After this burst release, a constant release was observed. After 48 hrs 59.95 % of the drug was released, showing a typical sustained and prolonged drug release depending on drug diffusion and matrix erosion mechanisms.

***Table 10.*** *In Vitro release studies and percentage cumulative drug release*

| **Time In Hrs** | **Abs** | **Conc (µg/ml) [A]** | **Dilution [A]X 50 = [B]** | **Loss In Sample [A]X5** | **Cumulative Loss [D]** | **Cumulative Release [B] + [D] = [E]** | **Percentage Cumulative Release** |
|---|---|---|---|---|---|---|---|
| 1 | 1.12 | 17.38 | 869.35 | 0 | 0 | 869.35 | 10.22% |
| 2 | 1.73 | 27.22 | 1361 | 136.1 | 136.1 | 1497.1 | 17.6% |
| 3 | 1.78 | 28.03 | 1401.6 | 140.16 | 276.26 | 1677.86 | 19.74% |
| 4 | 1.825 | 28.76 | 1438 | 143.8 | 420.06 | 1858.06 | 21.85% |
| 5 | 1.87 | 29.48 | 1474 | 147.4 | 567.46 | 2041.46 | 24% |
| 6 | 1.925 | 30.37 | 1518.5 | 151.85 | 719.31 | 2237.81 | 26.32% |
| 7 | 1.98 | 31.26 | 1563 | 156.3 | 875.61 | 2438.61 | 28.69% |
| 8 | 2.04 | 32.22 | 1611 | 161.1 | 1036.71 | 2647.71 | 31.15% |
| 24 | 3.1 | 49.32 | 2466 | 246.6 | 1283.31 | 3749.31 | 44.11% |
| 48 | 4.34 | 69.32 | 3466 | 346.6 | 1629.91 | 5095.91 | 59.95% |

### Release kinetics

Table 11. presents the cumulative percentage of drug release and the log of the cumulative drug release profile over different time intervals. As time progresses, the cumulative percentage of drug release increases, indicating the amount of drug released from the formulation. Conversely, the percentage of drug remaining decreases. The log of the cumulative drug release and log of the drug unreleased are also provided, representing the logarithmic values of the cumulative drug release and the remaining drug, respectively.

These results provide insights into the release kinetics of the drug over time. The cumulative drug release increases gradually, suggesting a sustained release pattern. The logarithmic values can be useful for mathematical modeling and analyzing the drug release behavior. Fig 11. Fig 12. Fig 13. Fig 14. shows the Zero Order plot , First order, Higuchi diffusion plot, Korsmeyer peppas plot of nanoparticles with drug respectively

***Table 11.*** *Cumulative % drug releases and log % cumulative drug release profile*

| **Time in hrs** | **Square root of time** | **Log T** | **Cumulative % drugrelease** | **% Drug remain** | **Log %Cumulative Drug release** | **Log % Drug unreleased** |
|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 10.22% | 89.78% | 1.01 | 1.95 |
| 2 | 1.414 | 0.30 | 17.6% | 82.4% | 1.25 | 1.92 |
| 3 | 1.73 | 0.477 | 19.74% | 80.26% | 1.29 | 1.90 |
| 4 | 2 | 0.60 | 21.85% | 78.15% | 1.34 | 1.89 |
| 5 | 2.24 | 0.69 | 24% | 76% | 1.38 | 1.88 |
| 6 | 2.45 | 0.778 | 26.32% | 73.68% | 1.42 | 1.87 |
| 7 | 2.65 | 0.845 | 28.69% | 71.31 % | 1.46 | 1.85 |
| 8 | 2.828 | 0.903 | 31.15% | 68.85% | 1.49 | 1.84 |
| 24 | 4.898 | 1.380 | 44.11% | 55.89% | 1.644 | 1.74 |
| 48 | 6.928 | 1.681 | 59.95% | 40.05% | 1.78 | 1.60 |

***Fig 11.*** *Zero Order plot of nanoparticles with drug*


***Fig 12.*** *First Order plot of nanoparticles with drug*

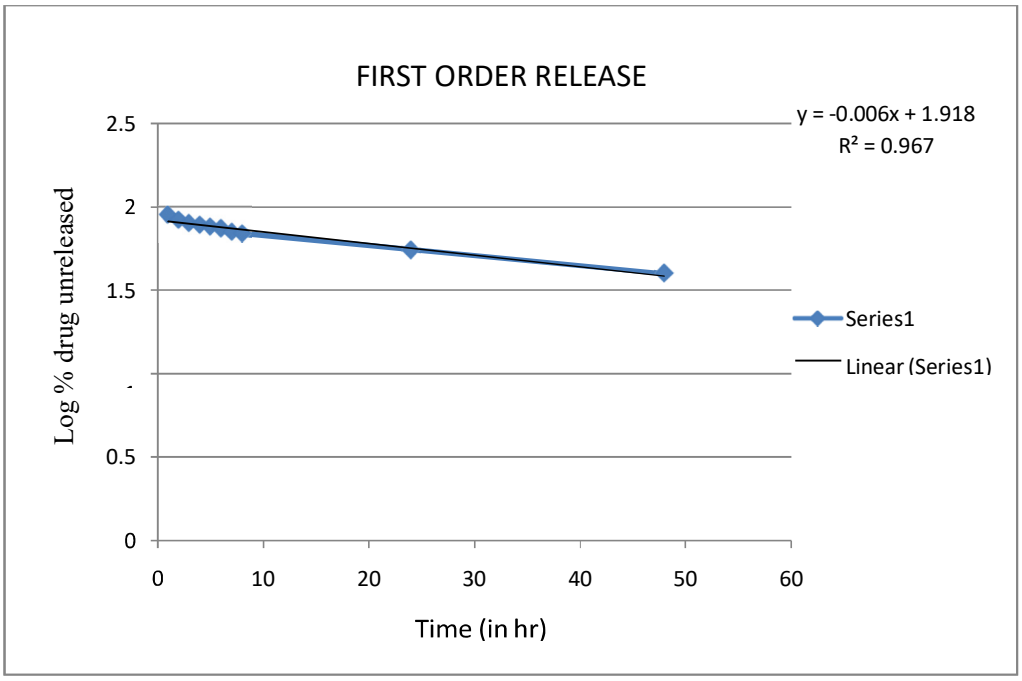


***Fig 13.*** *Higuchi diffusion plot of nanoparticles with drug*

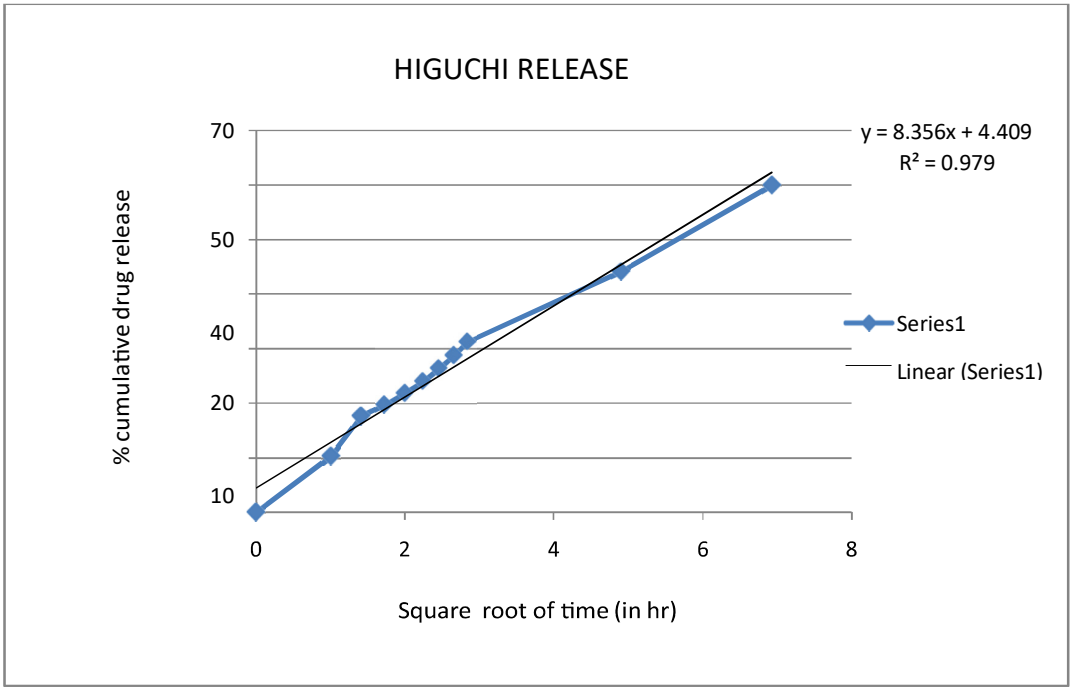
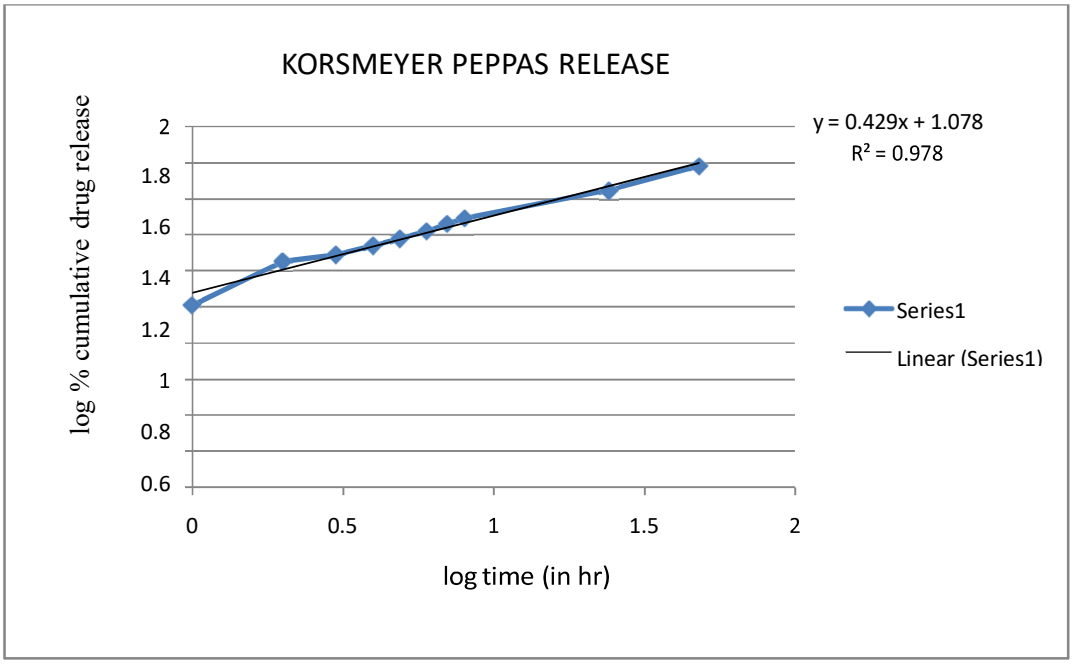



***Fig 14.*** *Korsmeyer peppas plot of nanoparticles with drug*



**Stability of Formulation**

At 5ºC ± 3ºC and room temperature 25ºC ± 2ºC/60 % RH ± 5 % RH there was a slight increase in size of the nanoparticles as those of freshly prepared samples, which can be contributed to the phenomenon of aggregation of particles. The zeta potential of the system remained nearly same indicating stability of the system. However, there was a slight increase in turbidity during the storage period. It is therefore proposed that in this type of nanoparticle, the major cause of instability is the degradation of the polymer used. Table 12. shows the stability study data

***Table 12.*** *Stability Study of the formulation*

| Time | Temperature and humidity | Size Distribution Average Diameter (nm) | Zeta potential in mV | Size Distribution PDI |
|---|---|---|---|---|
| 0 days | 5ºC ± 3°C | 195.4 | -22.4 | 0.233 |
| | 25ºC ± 2ºC/60 %RH ± 5 % RH | 214 | -23.5 | 0.243 |
| 3 month | 5ºC ± 3°C | 217 | -23.5 | 0.212 |
| | 25ºC ± 2ºC/60 %RH ± 5 % RH | 237 | -24 | 0.312 |

**RESULTS AND DISCUSSION**

The results of this study have a number of ramifications for ongoing studies on protein-based nanoparticle-based medication delivery systems. Bovine serum albumin (BSA) has the benefits of being biodegradable, non-toxic, and non-antigenic when used as a matrix for drug entrapment. Albumin nanoparticles are made more durable in vivo and have less interactions with blood components after being Pegylated with polyethylene glycol (PEG), which would make passive targeting possible. The Pegylated albumin nanoparticles' sustained and protracted drug release demonstrates their applicability for controlled drug delivery applications.

The effective synthesis and characterisation of pegylated albumin conjugates are highlighted in the paper, validating the conjugates' physicochemical characteristics. Protein-based nanoparticulate systems have piqued the interest of researchers due to their inherent

biodegradability, lack of toxicity, and nonantigenicity. Protein nanoparticles could be used as a matrix to physically entrap a wide range of drugs. They have the advantage, in comparison to other nanoparticulate systems, of carrying functional groups such as amino and carboxylic groups that can be used for surface modification. The enhanced permeability and retention effect is also known as passive targeting or accumulation via an impaired filtration mechanism. Furthermore, prolonged circulation can aid in the targeting of targeted drug carriers by allowing them more time to interact with the target. As was first proposed for liposomes, chemical modification of pharmaceutical nanocarriers with certain synthetic polymers, such as polyethylene glycol (PEG), is the most common way to impart in vivo longevity to drug carriers. This polymer is nontoxic, nonimmunogenic, nonantigenic, highly water soluble, and Food and Drug Administration (FDA) approved. Individual molecules and solid particulates have been shown to be protected from interaction with various solutes by hydrophilic polymers.

Because there has been no comprehensive study of the influence of process variables on the PEGylation of protein nanoparticles in the literature, the effect of process variables on PEGylation efficiency was investigated, and optimum conditions for preparing PEGylated nanoparticles were determined.

Then, 5-fluorouracil (5-FU) was used as a model drug to investigate the effect of PEGylation on drug release profile.

mPEG (methoxy polyethyleneglycol) was functionalized with a carboxylic group for conjugation with the amino group of bovine serum albumin in this approach.

The IR spectra of mPEG, mPEG-COOH, and mPEG-COOH-BSA were obtained using a Shimadzu Prestige-21 FT-IR Spectrophotometer. These materials' IR spectra revealed their characteristic absorption bands and the appearance of a characteristic peak at 1654.98 cm-1, indicating the formation of an amide bond in the final conjugate.

A desolvation process was then used to create nanoparticles from the prepared conjugate. The best conditions for producing PEGylated BSA nanoparticles were to prepare a 5mg/ml conjugate solution in 2% NaCl with 3.5 ml ethanol and 0.4 ml 8% glutaraldehyde homogenised at 1000 rpm for 1.5 hours. The pH of the solution was kept constant at 7.4.

The ideal size was determined to be 190.7 nm with a mean PDI of 0.134. The zeta potential was found to be -21.8 mV, indicating a slight decrease in negative charge; thus, PEG coating masked the negative charge of nanoparticles to some extent.

PBS pH 7.4 was used for in vitro release studies. The release data revealed that the system followed a sustained release pattern due to the presence of a PEG coating on the surface, which slowly released the drug over time.

The drug loading capacity was 515.15 and the encapsulation efficiency was 85%.

Stability studies of the optimized formulation were carried out for 3 months at (5°C + 3°C) and room temperature (25°C + 2°C/60% RH 5% RH) and analyzed for visual appearance, particle size, and zeta potential, with no significant changes in the results found.

The current study's findings show that PEG conjugated albumin nanoparticles are stable and can deliver active drugs to the target. Further research will be conducted to investigate its in-vivo release in the target.

**CONCLUSION**

Despite the valuable insights provided by this systematic study, it is important to acknowledge some limitations. The study focused on in vitro release and stability assessments, and further research is recommended to investigate the in-vivo release of the nanoparticles in target tissues. Additionally, the study used 5-FU as a model drug, and it would be beneficial to evaluate the performance of the nanoparticles with other drugs. Furthermore, while the study optimized process variables for preparing Pegylated nanoparticles, there may be other factors that could influence their efficiency and performance.

**List of Abbreviations:**

1. BSA - Bovine serum albumin
2. mPEG-COOH - Methoxy polyethylene glycol carboxylic acid
3. 5-FU - 5-Fluorouracil
4. nm - Nanometers
5. w/o - Water-in-oil
6. o/w - Oil-in-water
7. DMSO - Dimethyl sulfoxide
8. DMF - Dimethylformamide
9. ELISA - Enzyme-linked immunosorbent assay

10. PEG - Polyethylene glycol
11. RES - Reticuloendothelial system
12. mPEG - Monomethoxy polyethylene glycol
13. EPR - Enhanced permeability and retention
14. NP - Nanoparticle
15. t½ - Circulation half-time
16. SPIO - Superparamagnetic iron oxide
17. USPIO - Ultra-small superparamagnetic iron oxide
18. PEGylation - Polyethylene glycolylation
19. Mw - Molecular weight
20. Da - Daltons
21. KOH - Potassium hydroxide
22. mM - Millimolar
23. g - Gram
24. $^{0}$C - Degrees Celsius
25. HCl - Hydrochloric acid
26. KCl - Potassium chloride
27. EDC/NHS - N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide
28. mg - Milligram
29. ml - Milliliter
30. IR - Infrared
31. KBr - Potassium bromide
32. N - Normality
33. rpm - Revolutions per minute
34. DTS - Dynamic Light Scattering
35. v/v - Volume/volume
36. PI - Polydispersity IndexU.V
37. U.V - Ultraviolet
38. PBS - Phosphate-buffered saline
39. EM - Electron Microscope
40. HSADL - High-Speed Atomic Force Microscopy Laboratory
41. kV - Kilovolt
42. CCD - Charge-Coupled Device

43. JEM - Japan Electron Optics Laboratory
44. nm - Nanometer
45. pH - Potential of Hydrogen
46. ICH - International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
47. RH - Relative Humidity
48. Table - Tabulation
49. Fig - Figure
50. λmax - Wavelength maximum
51. mm - Millimeter
52. ml - Milliliter
53. FTIR - Fourier Transform Infrared Spectroscopy
54. cm-1 - Wavenumber (unit of measurement in infrared spectroscopy)
55. DSC - Differential scanning calorimetry
56. TEM - Transmission electron microscopy
57. FDA - Food and Drug Administration

**Figure Legend:**

Fig 1 - Calibration curve graph in Phosphate Buffer pH 7.4

Fig 2 - Calibration curve graph in Distilled water

Fig 3 - IR spectra of mPEG

Fig 4 - IR spectra of BSA

Fig 5 - IR spectra of mPEG-COOH

Fig 6 - IR spectra of mPEG-COOH-BSA

Fig 7 - DSC thermogram of BSA

Fig 8 - DSC thermogram of mPEG-COOH

Fig 9 - DSC thermogram of mPEG-COOH-BSA

Fig 10 - TEM images of modified nanoparticle formulation

Fig 11 - Zero Order plot of nanoparticles with drug

**List of tables:**

**References:**

1. Kreuter J. Encyclopaedia of Pharmaceutical Technology. Nanoparticles. Marcel Dekker Inc; 1994.

2. Herrero-Vanrell R, Rincón AC, Alonso M, et al. Self-assembled particles of an elastin-like polymer as vehicles for controlled drug release. J Control Release. 2005;102:113-122.

3. Kumar S, Dilbaghi N, Saharan R, et al. Nanotechnology as an emerging tool for enhancing solubility of poorly water-soluble drugs. Bionanoscience. 2012;2(4):227-250. doi: 10.1007/s12668-012-0060-7.

4. Ensign LM, Cone R, Hanes J. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. Adv Drug Deliv Rev. 2012;64(6):557-570. doi: 10.1016/j.addr.2011.12.009.

5. Obeid MA, Al Qaraghuli MM, Alsaadi M, et al. Delivering natural products and biotherapeutics to improve drug efficacy. Ther Deliv. 2017;8:947-956. doi: 10.4155/tde-2017-0060.

6. Krishnamoorthy K, Mahalingam M. Selection of a suitable method for the preparation of polymeric nanoparticles: multi-criteria decision making approach. Adv Pharm Bull. 2015;5(1):57-67. doi: 10.5681/apb.2015.008.

7. Dhanjal DS, Mehta M, Chopra C, et al. Proteins: emerging carrier for delivery of cancer therapeutics. Expert Opin Drug Deliv. 2013;10(10):1429-1448.

8. Yewale C, Baradia D, Vhora I, et al. Proteins: emerging carrier for delivery of cancer therapeutics. Expert Opin Drug Deliv. 2013;10(10):1429-1448.

9. An FF, Zhang XH. Strategies for Preparing Albumin-based Nanoparticles for Multifunctional Bioimaging and Drug Delivery. Theranostics. 2017;7(15):3667-3689. doi: 10.7150/thno.19365.

10. Elzoghby AO, Samy WM, Elgindy NA. Albumin-based nanoparticles as potential controlled release drug delivery systems. J Control Release. 2012;157(2):168-182. doi: 10.1016/j.jconrel.2011.07.031.

11. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2012;64(Suppl):116-127. doi: 10.1016/j.addr.2012.09.025.

12. Abuchowski A, McCoy JR, Palczuk NC, et al. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem. 1977;252:3582-3586.

13. Suk JS, Xu Q, Kim N, et al. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev. 2016;99(Pt A):28-51. doi: 10.1016/j.addr.2015.09.012.

14. Bentley MD, Harris JM, Kozlowski A. Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation. P.C.T. US99/23536.

15. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2012;64(Suppl):116-127. doi: 10.1016/j.addr.2012.09.025.

16. Jokerst JV, Lobovkina T, Zare RN, et al. Nanoparticle PEGylation for imaging and therapy. Nanomedicine (Lond). 2011;6(4):715-728. doi: 10.2217/nnm.11.19.

17. Suk JS, Xu Q, Kim N, et al. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev. 2016;99(Pt A):28-51. doi: 10.1016/j.addr.2015.09.012.

18. Ulbrich K, et al. Targeted drug delivery with polymers and magnetic nanoparticles: Covalent and noncovalent approaches, release control, and clinical studies. Chem Rev. 2016;116:5338-5431. doi: 10.1021/acs.chemrev.5b00414.

19. Li F, Mahato RI. Bioconjugate therapeutics: Current progress and future perspective. Mol Pharm. 2017;14(5):1321-1324. doi: 10.1021/acs.molpharmaceut.7b00263.

20. Anbarasu M, et al. Synthesis and characterization of polyethylene glycol (PEG) coated Fe3O4 nanoparticles by chemical co-precipitation method for biomedical applications. Spectrochim Acta A Mol Biomol Spectrosc. 2015;135:536-539. doi: 10.1016/j.saa.2014.07.059.

21. Topală T, et al. Bovine Serum Albumin Interactions with Metal Complexes. Clujul Med. 2014;87(4):215-219. doi: 10.15386/cjmed-357

22. Fullam S, et al. Competitive Binding of Bilirubin and Fatty Acid on Serum Albumin Affects Wear of UHMWPE. Lubricants. 2020;8(5):53. doi: 10.3390/lubricants8050053

23. Wingfield P. Protein precipitation using ammonium sulfate. Curr Protoc Protein Sci. 2001;Appendix 3:Appendix 3F. doi: 10.1002/0471140864.psa03fs13

24. Zorrilla-Veloz R, et al. Measurement and Correlation of the Solubility of 5-Fluorouracil in Pure and Binary Solvents. J Chem Eng Data. 2018;63. doi: 10.1021/acs.jced.8b00425

25. Lu JX, et al. University of Auckland; Creighton University School of Medicine; University of Florida COM - Jacksonville.