ORIGINAL ARTICLE

Nanoformulation of Antibacterial Antibiotics Cefpirome with Biocompatible Polymeric Nanoparticles and Evaluation for the Improved Antibacterial Activity and Nontarget Toxicity Studies

S. Karthick Raja Namasivayam, S. Samydurai, Trishna Chettia

Department of Biotechnology, Sathyabama University, Chennai, Tamil Nadu, India

Abstract

Background and Purpose: Nanotechnology principles are extensively utilized in the development of various drugs which exhibit the improved spectrum of activity with the less side effects. Among the various nanoparticles (NPs), protein-based nanomaterial gains recent attention because of the high rate of efficacy, delivery, and less side effects. In this study, the antibacterial antibiotics cefpirome has been formulated with biocompatible ovalbumin (OVA) NPs and the nanodrug conjugate was evaluated against improved antibacterial activity, *in vitro* control release and nontarget toxicity against Vero cells and blood cells. **Materials and Methods:** The methods for the preparation of phycocyanin functionalized OVA NPs were optimized with various parameters adapting coacervation technique. The nanodrug conjugate prepared using the optimized condition was characterized using electron microscopy studies, Fourier transform infrared spectroscopy and *in vitro* controlled drug release by continuous dialysis method, antibacterial activity against human pathogenic bacterial strains by well diffusion, liquid colorimetric assays, and biofilm inhibition assays. Nontarget toxicity study was conducted by determination of cytotoxicity against Vero cell line and human peripheral blood cells under laboratory condition. **Results:** Phycocyanin functionalized nanodrug conjugate was prepared under optimum condition characterized by electron microscopy studies reveals nanosphere is in the range of 100-200 nm. The drug loading efficiency and entrapment efficiency were found to be 85.0% and 90.5%. The *in vitro* drug release profile showed a steady rate of release and maximum release of 99.0% during 20 h. Antibacterial activity against human pathogenic bacteria showed improved spectrum of activity against all the tested pathogenic bacteria as dose-dependent manner. Microtiter plate and nitrocellulose membrane assay were adapted to study the biofilm inhibition of pathogenic bacteria which revealed all the tested bacterial biofilm was highly inhibited in both the tested assays as concentration-dependent manner. Effect of drug conjugate on dynamic growth curve of tested bacteria also showed the drastic reduction of growth inhibition. The antibacterial activity of nanodrug conjugate was found to be retained all the tested temperature against all the tested bacterial organism. Nontarget toxicity studies showed there was no distinct toxic effect on Vero and blood cells. **Conclusion:** Effective inhibition of pathogenic bacteria and best biocompatibility of nanoformulated cefpirome would suggest the possible utilization of the formulation as an antibacterial agent to fight against human pathogenic bacteria in modern medicine.

Key words: Antibacterial activity, cefpirome, nanoformulation, nontarget toxicity, ovalbumin, polymeric nanoparticles

INTRODUCTION

The development of antibiotics or other chemotherapeutics revolutionized human health, providing a simple cure for once dreaded diseases. However, widespread production, use, and misuse of antibiotics have contributed to the next generation concern for global public health: The emergence of multiple drug-resistant infectious organisms and cancer cells.[1,2] Nanotechnology offers tremendous

Address for correspondence:
Dr. S. Karthick Raja Namasivayam, Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Old Mamallapuram Road, Sholinganallur, Chennai - 600 119, Tamil Nadu, India.
Tel.: +91-44-2450 3145. Fax: +91-44-24501270.
E-mail: biologiask@gmail.com

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potential for medical diagnosis and therapy. Various types of nanoparticles (NPs) have been explored for biomedical applications having been widely employed in biological systems.[3] NPs have been suggested for imaging, screening, and biosensing not using only gold NP optical properties but also electrical and furthermore are known to carry higher payloads of drugs than other vehicles and are currently used in gene and drug delivery as well as in cancer diagnostics and therapeutic applications.[4]

Nanotechnology refers to the research and technological developments at atomic, molecular, and macromolecular scales, which lead to the controlled manipulation and study of structures and devices with length scales in the range of 1-100 nm biological NPs are mainly developed for drug delivery systems as an alternative to liposome technology, to overcome the problems related to the stability of these vesicles in biological fluids and during storage.[5] The NP technology used in the recent years has great significance in improving the efficacy of the drugs. The NPs fit into colloidal drug delivery systems, which offer advantages of drug targeting by modified body distribution well as the enhancement of the cellular uptake which benefits from reduction of undesired toxic side effects of the free drugs.[6-8] With their easy accessibility in the body, NPs can be transported via the circulation to different body sites, thus aiding in systemic treatments. NPs can be prepared from a variety of materials such as protein, polysaccharides, and synthetic polymers. Nanomaterials derived from polymers have gained increasing attention.[9]

Polymeric NPs have shown a certain degree of success for the delivery of proteins and vaccines to systematic circulation and to the immune system.[10] These NPs have many attractive properties for example size, surface potential, hydrophilic, and hydrophobic balance that make them suitable as potential carriers for bioactive component such as anticancer drug, vaccine, oligonucleotides, and peptides. Use of natural biodegradable polymers for production of NPs as drug delivery system has been developed recently. Among them proteins such as albumin, gelatin, gliadin, and legumin are play very important role in delivery system.[11,12]

Proteins are a class of natural molecules that have unique functionalities and potential applications in both biomedical and material sciences. They are ideal materials for NP preparation because of their amphiphilicity which allows them to interact well with both drug and solvent. Protein NPs have been recognized as carriers to deliver low-molecular-weight drugs, anticancer drug, DNA, vaccines, oligonucleotides, peptides, etc.[13] NPs derived from natural proteins are biodegradable, metabolizable and are easily amenable to surface modifications to allow attachment of drugs and targeting ligands. They have been successfully synthesized from various proteins, including water-soluble proteins (e.g., bovine serum albumin [BSA] and human serum albumin [HAS]) and insoluble proteins (e.g., zein and gliadin).[14]

Albumin is a protein that can be obtained from a variety of sources, including egg white (ovalbumin [OVA]), BSA, and HSA. Albumin is a major soluble protein of the circulating system and involved in the maintenance of osmotic pressure and binding and transport of nutrients to the cells. Many drugs and endogenous molecules are known to bind to albumin. Albumin serves as a depot and transporter protein. This protein is freely soluble in water and diluted salt solution. The high solubility of albumin (up to 40% w/v) at pH 7.4 makes it an attractive macromolecular carrier capable of accommodating a wide variety of drugs. It is stable in the pH range of 4-9 and can be heated at 60°C up to 10 h without any deleterious effects.[15] Albumin is widely used in the preparation of nanospheres and nanocapsules. These albumin nanocarriers are biodegradable, easy to prepare, and have well-defined sizes and reactive functional groups (thiol, amino, and carboxyl) on their surface that can be used for ligand binding and other surface modifications. Drug release from albumin NPs can be achieved naturally by protease digestion. Albumin is a versatile protein carrier for drug delivery. It is non-toxic, non-immunogenic, biocompatible, and biodegradable. Therefore, it is ideal material to fabricate NPs for drug delivery. Albumin NPs have high binding capacity for various drugs. Specialized nanotechnological techniques such as desolvation, emulsification, thermal gelation and recently nano spray drying, nanobiotechnology, and self-assembly have been investigated for fabrication of albumin NPs. The advantages of albumin NPs such as higher stability during storage and long shelf life, stability *in vivo*, ease of scale up during manufacture, biodegradable, non-antigenic, metabolizable and can also be easily amenable for surface modification and covalent attachment of drugs and ligands, ability to deliver proteins, peptides and gene, non-toxicity, increase the stability of drugs and useful controlled released properties, and synthetic protein nanostructure act as surrogate mimics such as viruses and plasmid for drug delivery systems which attacks the nanotechnologists to exploit the principles of albumin NPs in biomedicine and drug delivery.[16]

Among the albumin NPs, OVA NPs are non-toxic and non-antigenic in nature. Albumin NPs are easily metabolized in the body as well as the size of the particles, degree of stabilization. Thus, albumin being biodegradable, biocompatible and nontoxic was chosen as a matrix material for the preparation of NP and the simple method for the preparation like coacervation technique which play a key role in selection of OVA NPs in the field of medicine as the major material for drug delivery.[17] In this study, OVA NPs stabilized by phycocyanin loaded cephalosporin derivative cefpirome nanodrug conjugate was prepared under optimum condition and the synthesized nanodrug conjugate was evaluated for the improved antibacterial activity, *in vitro* drug release, nontarget toxicity studies. Among the pigments

produced by algae, phycocyanin is a pigment - protein complex is now being utilized in nanotechnology because of its distinct properties such as biodegradability, biocompatibility, photosensitivity, and poor immunogenicity. These unique properties will be created premier place for it in the field of nanotechnology as drug delivery agent.[18] Cefpirome is the cephalosporin derivative active against Gram-positive and Gram-negative bacteria. However, the continuous and high rate of usage causes various adverse side effects which progressively leads to severe health complications. Moreover, the poor solubility of the drug may causes less bioavailability and delivery to the target. Due to the unique biological effects based on the structure, size, biocompatibility, and stability of the OVA NPs, this study was aimed to formulate cephalosporin derivative cefpirome with the phycocyanin functionalized OVA NPs for the improved antibacterial activity, *in vitro* drug release profile, and biocompatibility.

MATERIALS AND METHODS

Chemicals and reagents

Egg albumin (very high purity) was obtained from Hi-Media, Mumbai, India. Reagents used for nanodrug conjugate including phycocyanin were supplied from SDFCL, Mumbai, India. All the reagents, chemicals were high quality analytical grade and used without purification. Media and reagents used for antibacterial and cytotoxicity studies were purchased from Sigma.

Preparation of free OVA NPs

Simple modified method of coacervation technique was adopted for the preparation of OVA NPs.[19] 125 mg of high purity analytical grade OVA was dissolved in 100 ml of 10 mM Tris/HCl followed by the dropwise addition of anhydrous ethanol, the mixture was kept under stirring at room temperature till the solution becomes turbid. 150 µl of 25% glutaraldehyde was added for cross-linking. The reaction was continued at room temperature (24°C). Ethanolamine was added to block the non-reacted aldehyde functional group. 0.1 ml of Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. The suspension was ultrasonicated for 30 min. Large aggregates were eliminated by centrifugation at 50,000 g for 30 min at 4°C. Collected pellet was lyophilized and used for further studies.

Nanoformulation of phycocyanin stabilized OVA NPs loaded cefpirome nanodrug conjugate

Nanoformulation of phycocyanin stabilized nanodrug conjugate preparation was carried out by cocervation method as described in our earlier studies.[20,21] In this method, optimization of three major parameters which determine the size and activity of nanodrug conjugate such as pH, ethanol to OVA ratio, and cross-linking time was done. Phycocyanin and cefpirome were used at 0.1% and 1.0 mg/ml (minimum inhibitory concentration [MIC]), respectively, in all the optimization studies.

Characterization

Characterization of lyophilized free OVA NPs and nanodrug conjugate was carried out by scanning electron microscopy (SEM). The sample was sputtered with palladium gold for 30 s under Polaron machine (BAL- TEC, model SCDOOS, Switzerland) followed by imaging with a Carl Zeiss Supra 55 (Germany). Field emission SEM with the upper detector at 15 kV. The magnification was set at 65,000. Fourier transform infrared radiation (FTIR) was studied using dried samples pelletized with potassium bromide (KBr). Transmission electron microscopy (TEM) images were obtained by HITACHI-H9500, 300 kV microscopes at an accelerating voltage of 120.0 kV. The samples for TEM studies were prepared by drying a drop of the aqueous suspension of films on carbon coated copper grid under ambient conditions. Before the TEM measurements, the samples were ground into small pieces at liquid nitrogen temperature to improve the depth of resolution.

Drug entrapment efficiency

Cefpirome concentration in the supernatant after the centrifugation of prepared nanodrug conjugate solution was determined using ultraviolet (UV) spectrophotometer at 391 nm. The drug encapsulation rate was determined by the following formula:

Entrapment efficiency (%) = Total cefpirome – Cefpirome in supernatant/Total cefpirome × 100

Drug loading efficiency

The nanosuspension with known amount of cefpirome was prepared with Millipore water, ultrasonicated for 30 min for disruption and filtered through the membrane. The drug contain in the suspension was determined by UV spectrophotometer to determine the drug in the conjugate and the total weight of nanodrug conjugate.

Loading efficiency (%) = Weight of drug in nanoconjugate/ total weight of nanodrug × 100

In vitro controlled drug release study

In vitro controlled drug release was done by dialysis bag method as described elsewhere. 10 mg of nanoformulation was dispersed in Millipore water and transferred into the dialysis bag (Hi-media, India) followed by dialysis at 37°C.

Dialysate thus obtained was filtered through sterile syringe filter and the absorbance was read at 300 nm.

Antibacterial activity

Bacterial strains and maintenance

Laboratory stock culture of *Pseudomonas aeruginosa, Klebsiella pneumonia, Serratia marcescens, Staphylococcus aureus, Salmonella typhi, Enterococcus faecalis, Escherichia coli,* and *Acinetobacter baumannii* was selected in this study. All the strains were maintained on nutrient agar slants.

Antibacterial assays

Well diffusion assay, colorimetric 3-(4, 5-dimethyl thiazol-2-yl)-2-5-dephenyl tetrazolium bromide (MTT) assay, dynamic growth curve assay, and biofilm inhibition assays were carried out to determine the improved antibacterial activity of nanodrug conjugate against selected bacterial strains.

Inoculum preparation

Mid-log phase culture of respective bacterial strain was used as the source of inocula for the antibacterial studies. A loopful of respective bacterial culture from the agar slant was transferred to the 10 ml of nutrient broth, incubated under shaking condition at 35°C for 12-16 h to reach the mid-log phase.

Well diffusion assay

Mid-log phase culture of the respective bacterial inoculum was spread uniformly with sterile cotton swab on Mueller-Hinton agar. The wells were made using cork borer, and aliquots of free cefpirome and nanodrug conjugate (aliquots of 25, 50, and 75 µg/ml were prepared from concentrated preparation) was loaded into the wells. The plates were incubated at 3°C for 24 h. After the incubation period, the zone of inhibition was measured. Improved antibacterial activity was determined by the following formula:

Improved activity (%) = Zone of inhibition in free cefpirome − Zone of inhibition in nanodrug conjugate ×

Effect of temperature on antibacterial activity of nanodrug conjugate

Known quantity of free cefpirome and nanodrug conjugate was dispersed into Eppendorf Tubes. The tubes were kept at 20°C, 30°C, 40°C, 50°C, and 60°C in a water bath for 1 h. To prevent evaporation, 100 µl of mineral oil (Tween 20) was over layered in all the Eppendorf Tubes. The respective bacterial culture was swabbed on Mueller-Hinton agar plates, and wells were made using sterile gel puncher (8 mm). 100 µl of free and nanodrug conjugate suspension treated at different temperature was added. The seeded plates were incubated at 37°C for 24 h. Zone of inhibition was recorded.

Microdilution colorimetric assay

Determination of antibacterial activity of phycocyanin stabilized nanodrug conjugate was studied by the microdilution calorimetric assay using the chromogenic reagent MTT which was used to study the MIC.[22] 0.9 ml of mid-log phase respective bacterial culture (10^6 CFU. ml) and different concentration of nanodrug conjugate (10-100 µg/ml) were added into each well of the 96-well microplate followed by incubation under a dark condition at 28°C for 24 h. After the incubation period, 10 µl of MTT (5 mg/mL in 0.2 mol/L, pH 7.2, and phosphate buffer saline [PBS]) was added into each well, and the plates were incubated another 4 h. Blue color appeared in the well after 4 h indicates the growth of the organisms. Only living microorganisms can convert MTT to formaldehyde and a blue color appeared in the well. The MIC value was defined as the lowest sample concentration that inhibited visible growth of the test bacterium, as indicated by MTT straining.

Biofilm inhibition assay

Microtiter plate spectrophotometric assay was carried out to determine the effect of nanodrug conjugate on the biofilm development of respective bacterial cultures. Microtiter plate was inoculated with 0.1 ml of respective bacterial culture and respective concentration of nanodrug conjugate followed by incubation at 37°C for 48 h. Whole content of the respective well was removed after the incubation period. Washed wells were stained with 0.1 ml of 0.1% aqueous solution of crystal violet and incubated at room temperature for 30 min. Stained contents were solubilized using 0.2 ml of ethanol (95%) absorbance of the solubilized content were studied at 540 nm to determine biofilm inhibition.

Biofilm inhibition assay on nitrocellulose membrane

Biofilm inhibition assay was also carried out by the method of nitrocellulose membrane filter assay as described in our earlier studies.[23] In this method, 0.1 ml of mid-log phase culture of respective bacterial strain was inoculated into the sterile nitrocellulose membrane filter (Rankem, New Delhi, India) with 47 mm diameter and 0.45 µm was followed by the addition of the respective concentration of free OVA NPs, OVA nanodrug conjugate, free cefpirome and the seeded filter was transferred to the 6 well tissue culture plate, kept at 37°C for 3 days. Four replications and control maintained. After the incubation period, the inoculated filter was taken, and the biofilm inhibition assay was carried out by the modified method of spectrophotometric inhibition assay. The filter was stained with 1.0% crystal violet and incubated for 1 h. After staining the filter was washed thoroughly with 1% ethanol and the washed solution was collected in sterile screw cap vial, and the reaction mixture was read at 570 nm.

Effect of nanodrug conjugate on dynamic growth curve

Nutrient broth was used for inocula preparation of bacterial strains. Cultures were inoculated from fresh slopes and incubated in 100 ml of sterile Luria broth (LB) with different concentration of OVA nanodrug conjugate and free cefpirome and kept in shaker at 37°C and every 5 h interval, the broth withdrawn from the flask and the optical density (OD) was measured at 640 nm.

Nontarget toxicity studies

Cytotoxicity against Vero cell line

Cytotoxicity of nanodrug conjugate was determined by inhibition of cell growth of Vero cell line using a tetrazolium dye (MTT) assay, and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the control cells.[24]

In vitro hemolysis assay

Nontoxic effect of nanodrug conjugate was studied against human peripheral blood cells under laboratory condition adopting spectrophotometric determination of plasma hemoglobin concentration which directly correlates blood lysis.[25]

RESULT AND DISCUSSION

The most important advantage of colloidal drug carrier systems is the possibility of drug targeting by a modified body distribution as well as the improvement of the cellular uptake of a number of substances. As a result, undesired toxic side effects of the free drug can be avoided, among of colloidal systems those based on proteins may be very capable. Proteins are a class of natural molecules that have unique functionalities and potential applications in both biological as well as material fields.[26] Protein-based nanomaterials are now extensively used as the drug delivery system because of high efficacy and biocompatibility. In this study, phycocyanin mediated influence on the improved antibacterial activity, controlled drug release pattern, and nontarget toxicity of OVA NPs loaded cefpirome nanodrug conjugate has been studied.

Free OVA NPs synthesis and characterization

OVA NPs were synthesized by simple cocervation method. Preparation of protein NPs is based on balancing the attractive and repulsive forces in the protein. It is generally accepted that increasing protein unfolding and decreasing intramolecular hydrophobic interactions are crucial to the formation of protein NPs. During such particle formation, the protein undergoes conformational changes depending on its composition, concentration, crosslinking and preparation conditions such as pH, ionic strength, and type of solvent. Usually, surfactants are required to stabilize the NPs of water-insoluble proteins coacervation/desolvation and emulsion-based methods are most commonly used for the preparation of protein NPs.[27] Coacervation or desolvation is based on the differential solubility of proteins in solvents as a function of solvent polarity, pH, ionic strength, and presence of electrolytes. The coacervation process reduces the solubility of the protein leading to phase separation. The addition of desolvating agent leads to conformation changes in protein structure resulting in coacervation or precipitation of the protein. By controlling processing variables, the size of NPs in the coacervate can be controlled. After NPs are formed, they are cross-linked by agents such as glutaraldehyde and glyoxal. Organic solvents such as acetone and ethanol have been used as antisolvents for the preparation of protein NPs.[8] In this study, controlled sized OVA NPs were synthesized with the size range of 70 nm uniform spherical particles confirmed by SEM and TEM studies [Figures 1 and 2].

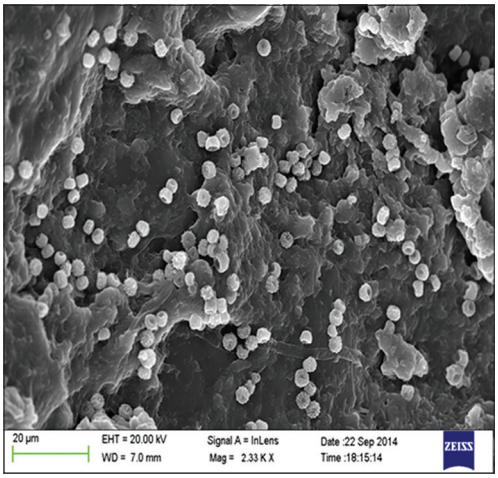

Figure 1: Scanning electron micrograph of free ovalbumin nanoparticles

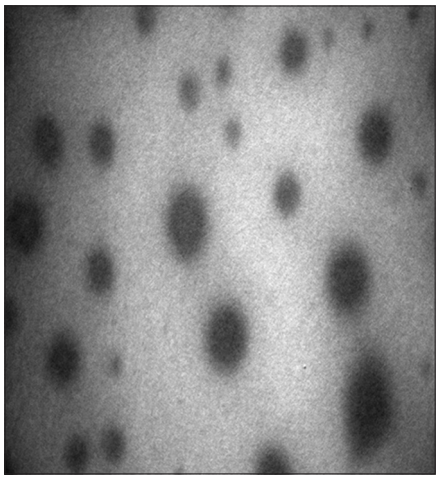

Figure 2: Transmission electron micrograph of free ovalbumin nanoparticles

Further characterization of synthesized particles was done by FTIR analysis. FTIR analysis helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the NPs. Figure 3 depicted the FTIR profile of free OVA NPs which reveals strong peaks at 3752.4 cm^{-1} (corresponds to OH), 3449.1 cm^{-1} (OH), medium peaks at 2429.9 cm^{-1} (CHO), 2344.8 cm^{-1} ($^{-}$CN), 2363.2 cm^{-1} (−CN), 2276.0 cm^{-1} (−CN), 2105.8 cm^{-1} (−CN), strong peak at 1644.5 cm^{-1} (C=O), variable peaks at 1400.2 cm^{-1} (C=C), 1245.9 cm^{-1} (C=C), strong peaks at 1115.5 cm^{-1} (N-O), 1076.7 cm^{-1} (N-O), and 674.9 cm^{-1} (C-Cl), respectively.

Nanoformulation

The process conditions for the synthesis of phycocyanin stabilized nanodrug conjugate were optimized based on the factors such as pH, cross-linking time, ethanol albumin ratio, and phycocyanin, and cefpirome were at the fixed rate [Table 1]. The initial formulation parameter selection showed that the pH and the dosage of ethanol significantly influenced the preparation of the NPs (P = 0.5%). At pH <8

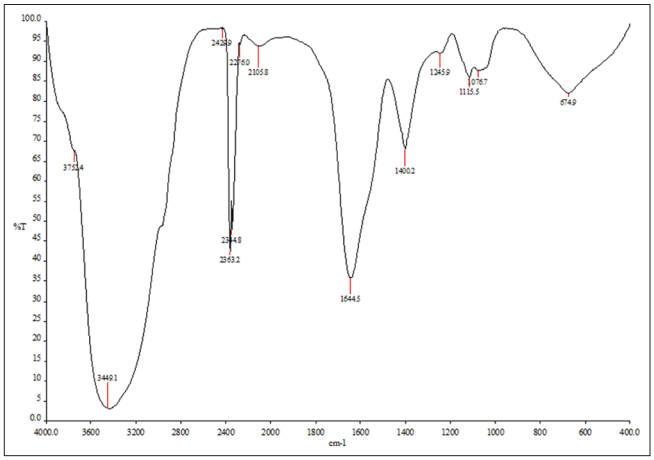

Figure 3: Fourier transform infrared spectra of free ovalbumin nanoparticles

Table 1: Optimal condition for nanodrug conjugate preparation

Parameters	Nanodrug conjugate
Ovalbumin	0.3 g
Cefpirome	1 mg/ml
Glutaraldehyde	125%
pH	9
Ethanol to ovalbumin ratio	4:1
Crosslinking time	12 h
Mean particle size	135 nm
Encapsulation rate	91.0
Drug loading	89.0

the formation of albumin NPs was less based on the yield. With increase in pH the mean diameter of the NPs decreased gradually and a significant increase in the yield percentage was also observed. Comparison of the pH values (6, 7, 8, and 9) showed that the pH of 8-9 was optimal and their yield was equal to 90%. The yield percentage was low at pH below 7 which were in a range of 50-65% which were comparatively lower than the yield percentages of pH above 7 which were >80%.

The ethanol concentration in the coacervation process is critical as it acts as the desolvating agent. The intermittent addition of desolvating agent improves the reproducibility of the BSA NPs preparation. It is noted from the optimization process is that the volume of ethanol added is key to the yield of controlled size NPs. When the ratio of ethanol to 2% BSA was >2.5:1, the yield of NPs was ≥80%. However, with an increase in ethanol to BSA the mean diameter of the NPs increased. The crosslinking of the particles by the glutaraldehyde is a critical factor in the synthesis of NPs. The time for crosslinking influences the yield and particle size of the BSA NPs. Crosslinking plays a major role in the stability and drug release of albumin NPs.[19] Herein, the crosslinking time was varied between 6 and 16 h for to synthesize a stable, high yielding process. The yield percentage of about 81% was obtained at a crosslinking time of 6 h while the yield increased during the increase in the crosslinking time. The optimal cross-linking time was found to be in the range of 8-12 h based on the comparison between the cross-linking times of 6-20 h [Table 1]. Hence, the optimal condition for the preparation of the blank BSA NPs was optimized to be as pH 8, ethanol:albumin - 4:1, cross-linking time of 8 h. Optimization of preparation process of vinblastine sulfate-loaded,[28] folate-decorated paclitaxel-loaded, and 5-fluorouracil loaded BSA NPs[29] has been reported. Size, surface charge and hydrophobicity,/hydrophilicity are parameters that affect the body distribution and interactions with the biological environment. The NP characterization by conventional light microscopy is not suitable because of its resolution limited to about 1 µm. Instead, techniques such as SEM and TEM are nowadays, studied with 5000-30,000-fold magnifications and provide visual and descriptive information about the NP population. In a SEM setup, the nanoparticulate sample, coated to be conductive (e.g., platinum), is scanned in a high vacuum chamber with a focused electron beam. SEM measurement on nanoformulation revealed that the particles were spherical shape with the size range of 80-90 nm [Figure 4]. Further confirmation of size was authenticated by TEM analysis. Figure 5 shows the TEM micrograph which reveals electron dense core shell particles with the size of 90 nm. FTIR spectra showed that the specific interaction of phycocyanin with the nanodrug conjugate [Figure 6]. When the FTIR spectrum of free OVA NPs and nanodrug conjugate was compared, it was found that absorbed peaks were modified after the functionalization with phycocyanin.

Drug loading and entrapment efficiency

The loading efficiency and the entrapment efficiency were studied by the spectrophotometric analysis of the nanodrug conjugate suspension. The unbound OVA NP concentration was determined by correlating the absorbance of the supernatant after the centrifugation with the standard absorbance concentration ratio. The drug loading and entrapment efficiency were in the range of 87.0%-89.0%, respectively.

In vitro drug release

In vitro drug release of the drug was studied using 1% PBS. *In vitro* drug release testing, a measure of release of the active pharmaceutical ingredient from the drug product matrix in controlled laboratory environment, is a key evaluation in drug development and quality control.[30] It involves subjecting the dosage form to a set of conditions that will induce drug release and quantitating the amount of drug released under those conditions. It is an important tool in evaluating drug product performance for most dosage forms and is known as dissolution testing, *in vitro* release testing, and elution testing. *In vitro* drug control release profile was studied against both free cefpirome and nanodrug conjugate adapting continuous dialysis bag method. In the case of free cefpirome, there was a continuous increase of release versus time period and maximum release percentage of 99.0% was observed during 14 h. In contrast with nanodrug conjugate, there was a steady state of release versus time period and maximum release percentage of 97.0% was recorded during 20 h [Figure 7]. The present findings were supported by our recent reports[20,21] which reveals sustained and controlled release profile of cephalosporin derivative loaded with phycocyanin based nanoformulation. The erosion and degradation of OVA NPs and the poor

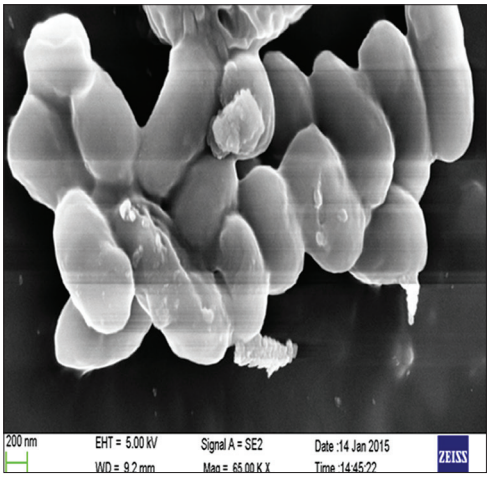

Figure 4: Scanning electron micrograph of phycocyanin stabilized nanodrug conjugate

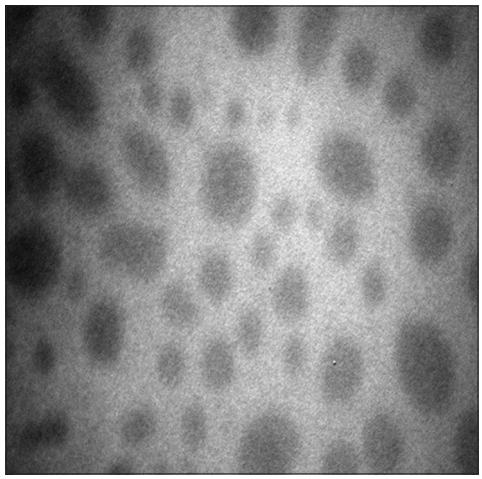

Figure 5: Transmission electron micrograph of phycocyanin stabilized nanodrug conjugate

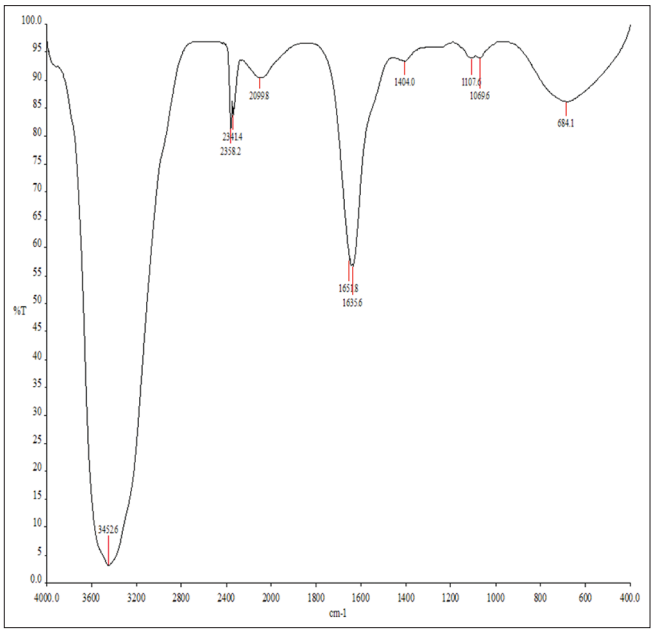

Figure 6: Fourier transform infrared spectra of phycocyanin stabilized nanodrug conjugate

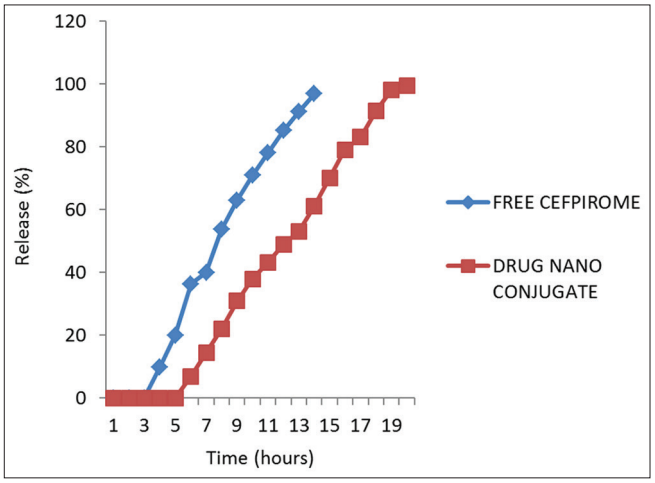

Figure 7: *In vitro* drug release profile of phycocyanin stabilized nanodrug conjugate

solubility of antibiotics in water were involved in the drug-release process. Different studies have shown that the rate and amount of drug release is inversely proportional to the hydroxypropyl methylcellulose percentage in formulations.[31] By increasing the polymer percentage, a viscose gel layer is formed, resisting to erosion and the diffusion of the drug is controlled primarily by the gel viscosity.[32-34]

Antibacterial activity

Well diffusion assay

Antibacterial activity was studied against tested human pathogenic bacteria adapting well diffusion assay and microdilution broth method. In well diffusion assay, all the tested bacteria were susceptible to nanodrug conjugate [Figures 8-12]. In general, all the tested bacteria showed improved spectrum of activity as dose-dependent manner. In the case of *P. aeruginosa,* 14.26%, 22.72%, 29.03%, and 36.47% of improved activity was recorded at respective concentration as 50, 100, 500, and 1000 µg/ml. 21.92%, 24%, 28.06%, and 31.81% of improved activity was noticed for *A. baumannii* at concentration of 50, 100, 500, and 1000 µg/ml. For *S. marcescens,* 7.4%, 11.11%, 21.87%, and 23.68% of improved activity was observed at different concentration of 50, 100, 500, and 1000 µg/ml. In the case of *S. aureus*, 4.15%, 8.57%, 18.8%, and 31.94% of improved activity was recorded at respective concentration of 50, 100, 500, and 1000 µg/ml. 8.51%, 17.5%, 21.87%, and 23.68%

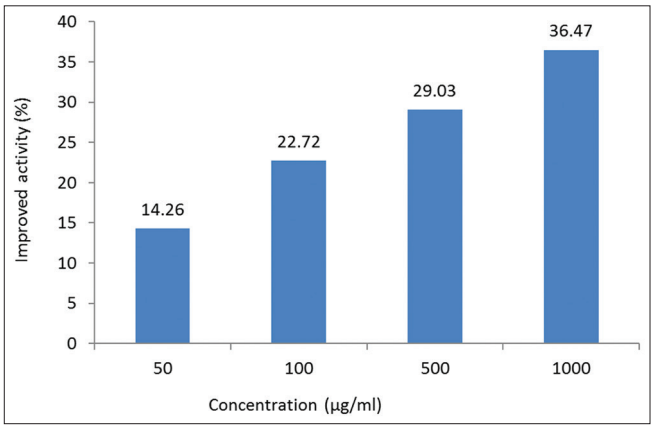

Figure 8: Improved antibacterial activity (%) of nanodrug conjugate against *Pseudomonas aeruginosa*

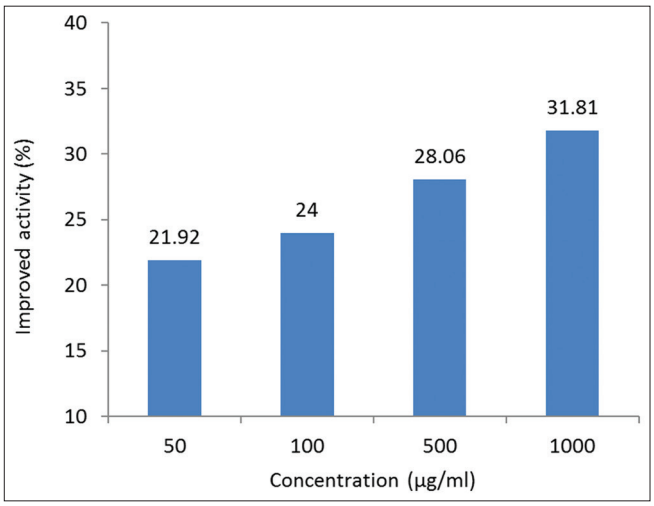

Figure 9: Improved antibacterial activity (%) of nanodrug conjugate against *Acinetobacter baumannii*

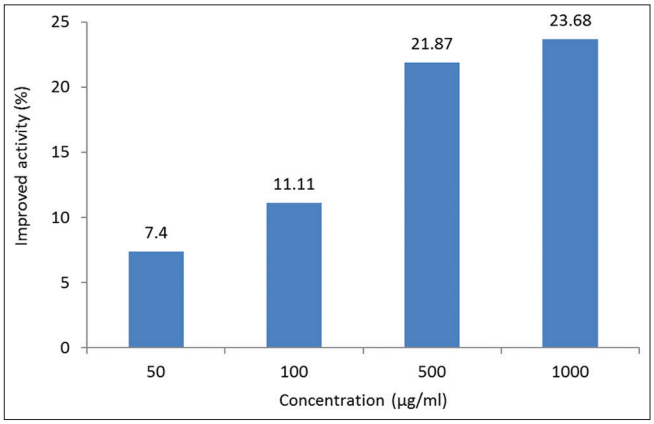

Figure 10: Improved antibacterial activity (%) of nanodrug conjugate against *Serratia marcescens*

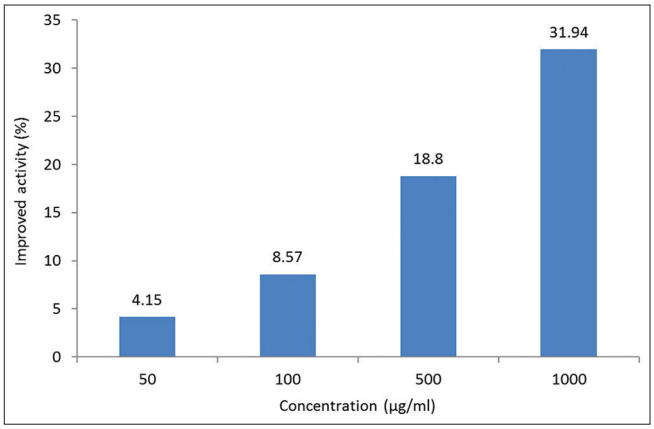

Figure 11: Improved antibacterial activity (%) of nanodrug conjugate against *Staphylococcus aureus*

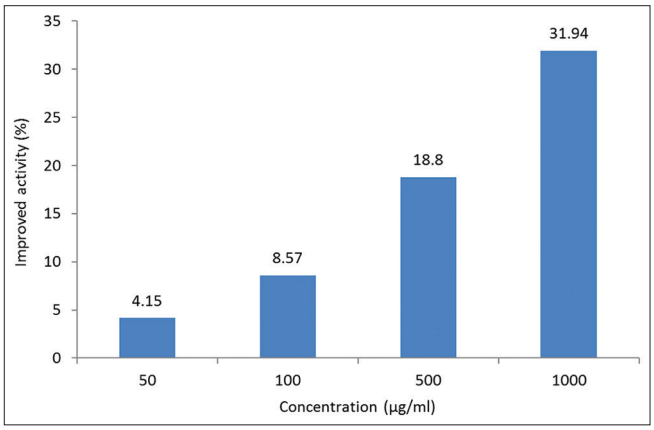

Figure 12: Improved antibacterial activity (%) of nanodrug conjugate against *Klebsiella pneumonia*

of improved activity at concentration of 50, 100, 500, and 1000 µg/ml was observed in *K. pneumonia*.

Biofilm inhibition studies

Biofilm is the assemblage of the microbial cells that is irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material.[3] Biofilm is composed primarily of microbial cells and extracellular polymeric substances. Adhesion to the surface provides considerable advantages such as protection against microbial agents, acquisition of new genetic traits, and the nutrient availability, and metabolic cooperability. Biofilm formation is quite important in bacterial lifestyle. It helps in protection from environment, nutrient availability, acquisition of new genetic trait, penetration of the antimicrobial agent, and in hydrodynamics. Inhibition of biofilm plays a major role in the development of antimicrobial agents. In this study, biofilm inhibition assay was carried out by microtiter plate and nitrocellulose membrane. In the case of microtiter plate assay, both free cefpirome and nanodrug conjugate inhibited the biofilm against both tested organism as dose-dependent manner but increased inhibition of biofilm was recorded in nanodrug conjugate treatment against all the tested organism [Figures 13-17]. Nanodrug conjugate treatment exhibited significant effect on the biofilm inhibition ($P = 5\%$). Nitrocellulose membrane filter assay also revealed same pattern of biofilm inhibition as in microtiter plate assay. Percent biofilm inhibition was shown in the Figures 18 and 19 which clearly indicated that nanodrug conjugate brought about maximum inhibition of biofilm in all the tested concentration against the tested bacterial strains.

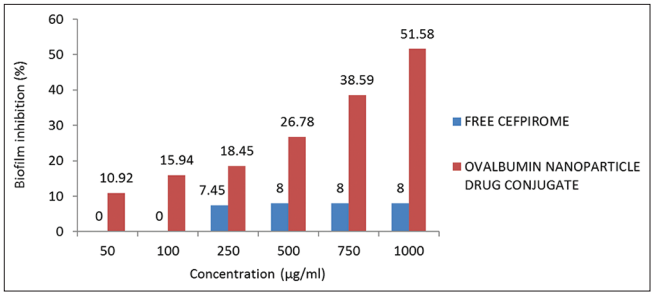

Figure 13: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Pseudomonas aeruginosa*

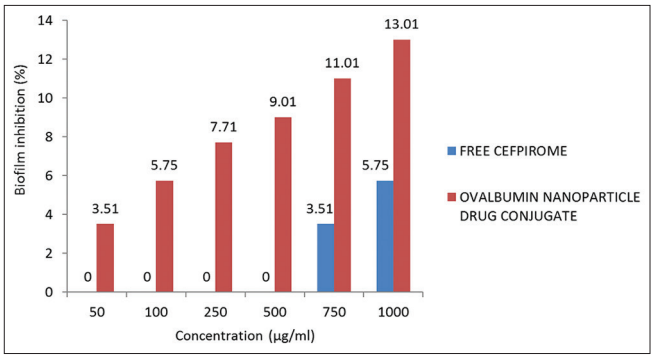

Figure 14: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Klebsiella pneumonia*

Effect of nanodrug conjugate on dynamic growth curve of pathogenic bacteria

Effect of nanodrug conjugate on dynamic growth curve of pathogenic bacteria was studied in LB inoculated with respective bacterial organism containing nanodrug conjugate by measuring OD at different time intervals. In all the tested organisms, the OD was found to be decreased drastically in nanodrug conjugate treatment [Figures 20-23]. Although

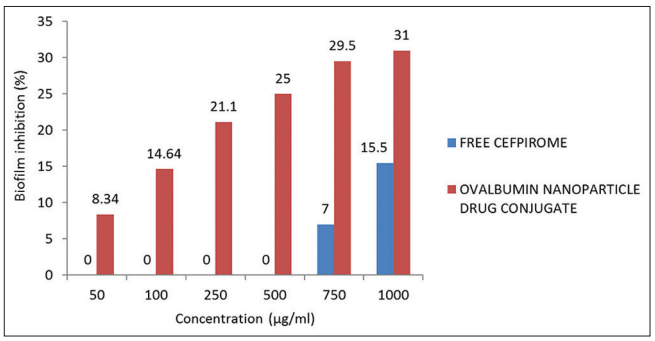

Figure 15: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Serratia marcescens*

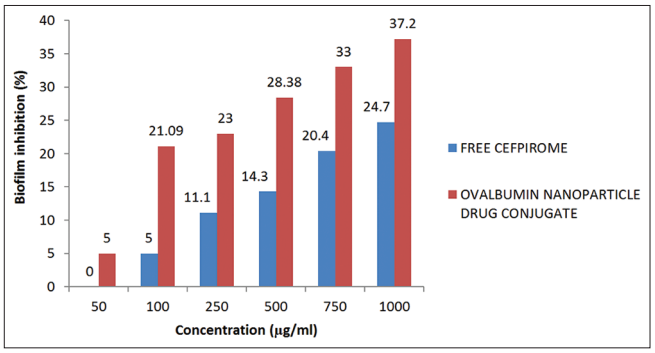

Figure 16: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Escherichia coli*

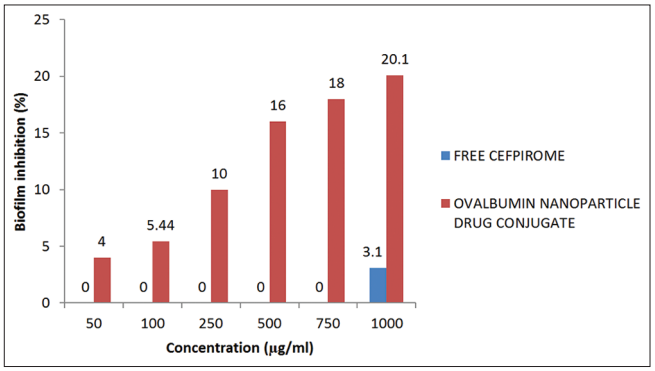

Figure 17: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Staphylococcus aureus*

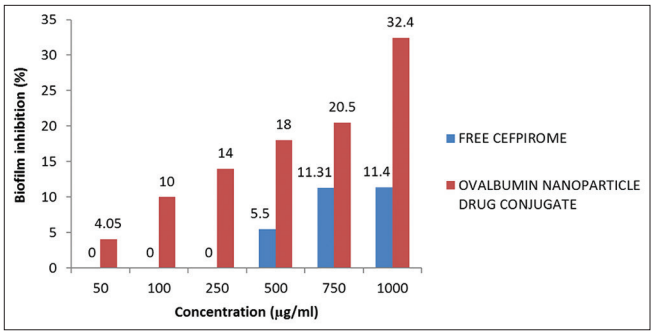

Figure 18: Effect of ovalbumin nanodrug conjugate on microtiter plate assay for biofilm inhibition of *Acinetobacter baumannii*

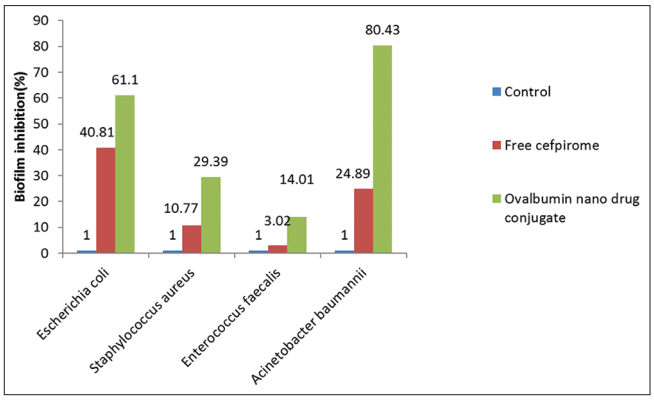

Figure 19: Effect of ovalbumin nanodrug conjugate on biofilm inhibition of pathogenic bacteria (nitrocellulose membrane assay)

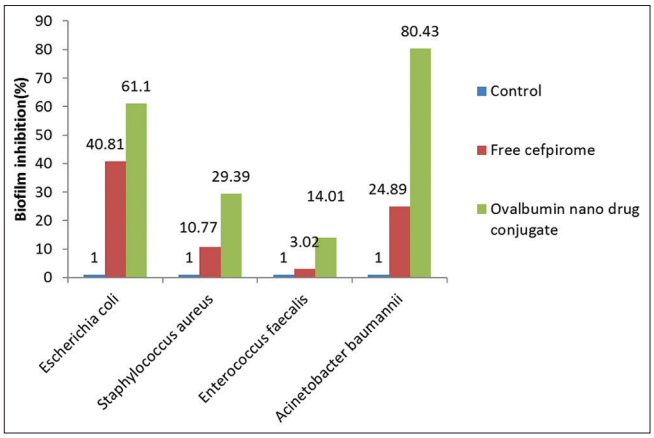

Figure 20: Effect of ovalbumin nanodrug conjugate on biofilm inhibition of pathogenic bacteria (nitrocellulose membrane assay)

the increasing concentration of free antibiotic progressively inhibited the growth of all the tested bacteria, the highest inhibition of growth has been inferred from the nanodrug conjugate treatment which reveals prolonged lag phase of all the tested bacteria than the control which confirms the stability and controlled or sustained release of the antibiotic form the nanoformulation which arrest of inhibits the growth of the organism.

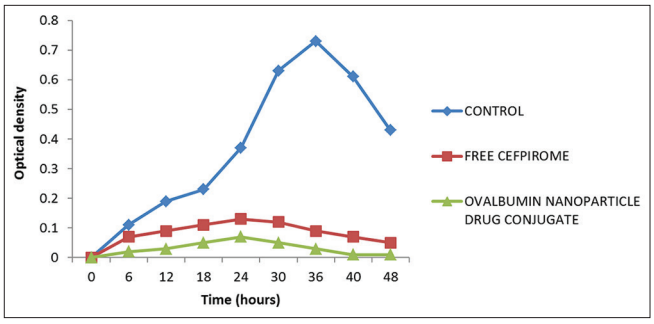

Figure 21: Effect of free cefpirome, ovalbumin nanodrug conjugate on optical density of *Pseudomonas aeruginosa* grown in Luria broth at different time intervals

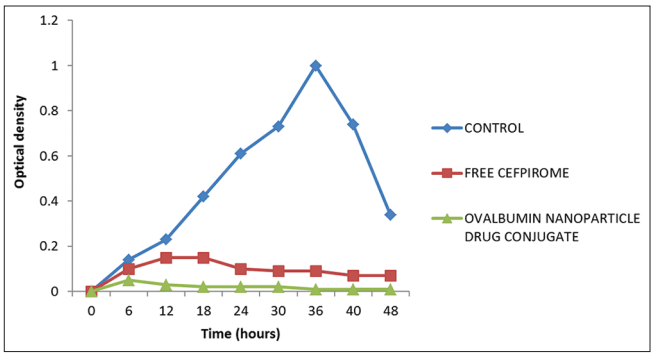

Figure 22: Effect of free cefpirome, ovalbumin nanodrug conjugate on optical density of *Acinetobacter baumannii* grown in Luria broth at different time intervals

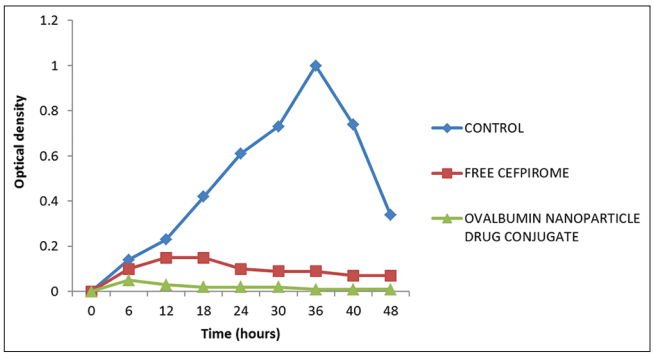

Figure 23: Effect of free cefpirome, ovalbumin nanodrug conjugate on optical density of *Escherichia coli* grown in Luria broth at different time intervals

Effect of temperature on growth inhibition (GI) (%)

Effect of temperature on GI of free cefpirome and nanodrug conjugate was studied by well diffusion assay. In general, GI was found to be maximum in all the tested temperature of nanodrug conjugate treatment against tested bacterial organism [Figures 24-28]. In *S. aureus,* maximum GI was recorded at 30°C followed by 40°C. Less inhibition was recorded in 20°C and 60°C. Free cefpirome revealed 87.86%, 59.91% of GI at 30°C and 40°C and 0.93%, 20.4% at 20°C and 60°C. In the case of OVA nanodrug conjugate, maximum GI was found at 30°C followed by 40°C which revealed 64.29% and 30.21% and less inhibition was found

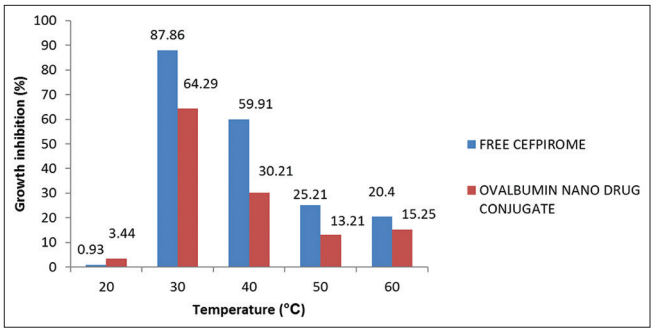

Figure 24: Effect of temperature on growth inhibition (%) of free cefpirome, ovalbumin nanodrug conjugate of *Staphylococcus aureus*

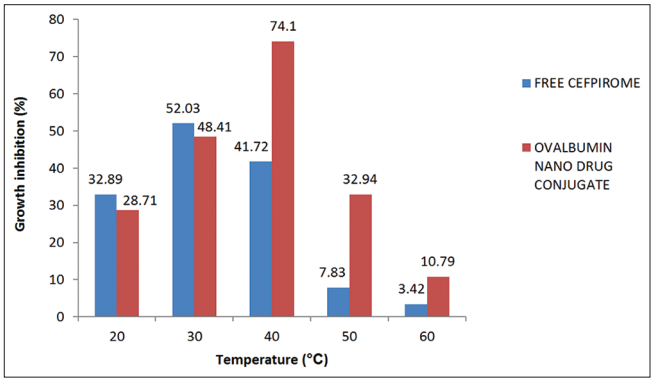

Figure 25: Effect of temperature on growth inhibition (%) of free cefpirome, ovalbumin nanodrug conjugate of *Pseudomonas aeruginosa*

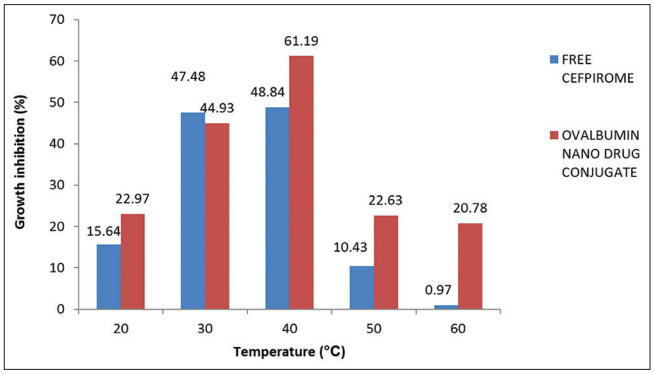

Figure 26: Effect of temperature on growth inhibition (%) of free cefpirome, ovalbumin nanodrug conjugate of *Acinetobacter baumannii*

at 50°C and 20°C which revealed 13.21% and 3.44%. Likewise in the case of *P. aeruginosa*, maximum GI of free cefpirome was found to be at 30°C followed by 40°C which revealed 52.03% and 41.72%. Less inhibition was recorded at 50°C followed by 60°C which revealed 7.83% and 3.42%. But in the case of OVA nanodrug conjugate, maximum growth was observed at 40°C followed by 30°C which revealed 74.1% and 48.41% and less inhibition was recorded as 28.71 % at 20°C and 10.79% at 60°C. For *A.*

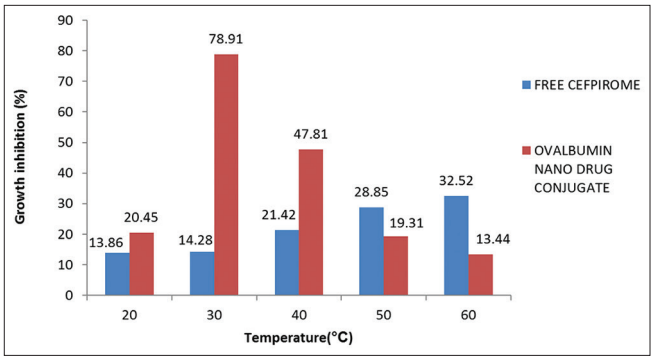

Figure 27: Effect of temperature on growth inhibition (%) of free cefpirome, ovalbumin nano drug conjugate of *Serratia marcescens*

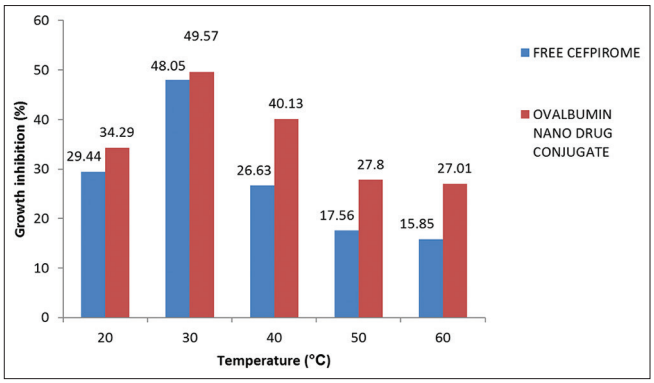

Figure 28: Effect of temperature on growth inhibition (%) of free cefpirome, ovalbumin nanodrug conjugate *Salmonella typhi*

baumannii, maximum GI of free cefpirome was found as 48.84% at 40°C followed by 47.48% at 30°C and less inhibition was recorded at 50°C followed by 60°C which revealed 10.43% and 0.97%. Similar results were obtained for nanodrug conjugate where maximum growth was found at 40°C followed by 30°C which revealed 61.19% and 44.93% and less inhibition was obtained at 50°C followed by 60°C which revealed 22.63% and 20.78%. In *S. marcescens,* maximum growth of free cefpirome was noticed at 60°C followed by 50°C which revealed 32.52% and 28.85% and less inhibition was recorded at 30°C followed by 20°C which revealed 14.28% and 13.26%. But in the case of nanodrug conjugate, maximum growth was observed at 30°C followed by 40°C which revealed 78.91% and 47.81% whereas less inhibition was found at 50°C and 60°C which revealed 19.31% and 13.44%. Likewise for *S. typhi,* maximum growth of free cefpirome was obtained as 48.05% and 29.44% at 30°C followed by 20°C and less inhibition was found at 50°C followed by 60°C which revealed 17.56% and 15.85%. In the case of OVA nanodrug conjugate, maximum growth was found at 30°C followed by 40°C which revealed 49.57% and 40.13% and less inhibition was observed at 50°C followed by 60°C which revealed 27.8% and 27.01%.

Nontarget toxicity studies

Cytotoxicity against vero cell line

Nontarget toxicity of the nanodrug conjugate was studied by the determination of cytotoxicity and haemocompatibility. Cytotoxicity study of nanodrug conjugate against Vero cell line adapting MTT assay reveals high concentration of drug conjugate reduced cell viability [Figure 29]. At 1000 and 750 µg concentration, 59.0% and 71.0% was recorded, respectively, but at least concentration, cell viability was not affected. 85.6%, 93.5%, and 97.3% of cell viability were observed in respective concentration. From the data, GI_{50} value was determined which reveals 1124.3 µg.

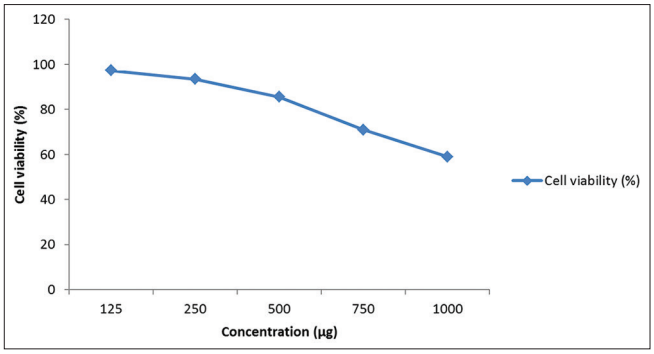

Figure 29: Cell viability of Vero cell line treated with nanodrug cefpirome conjugate

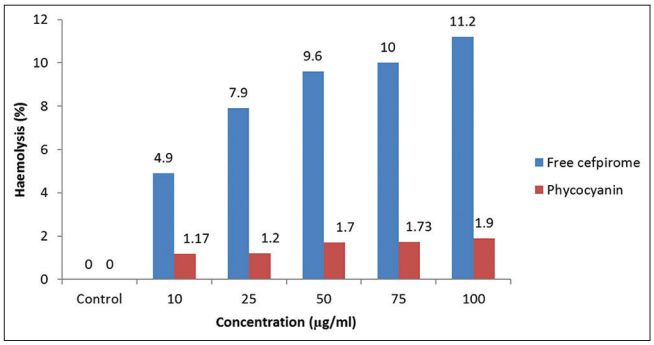

Figure 30: Effect of free cefpirome, phycocyanin on hemolysis of human peripheral blood

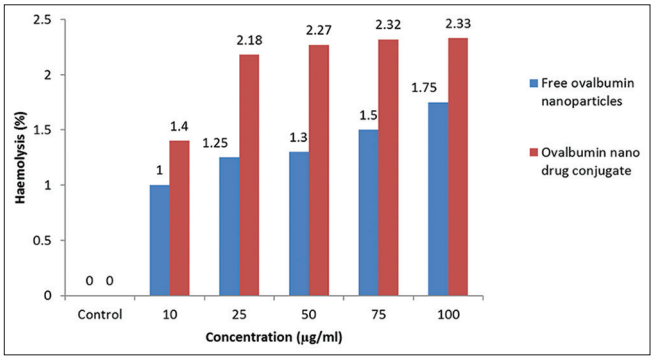

Figure 31: Effect of free ovalbumin nanoparticle, ovalbumin nanodrug conjugate on hemolysis of human peripheral blood

Haemocompatibility studies

Further confirmation of nontarget toxicity of nanodrug conjugate was studied using human peripheral blood under laboratory condition. The results shows that distinct effect of nanodrug conjugate was not observed on the blood by exhibiting no lysis of blood cells in all the tested concentration [Figures 30 and 31].

CONCLUSION

In therapeutics, a number of unexpected inventions have been done recently on polymer based NPs by the use of bio-nanotechnology, which has led to great attention in the field of smart drug delivery applications. The use of biodegradable polymeric NPs for controlled drug delivery has shown significant therapeutic potential. This study, nanoformulation of cephalosporin derivative cefpirome with phycocyanin stabilized OVA NPs prepared by cocervation showed distinct nanostructure, high spectrum of antibacterial activity against human pathogenic bacteria, high drug entrapment, loading efficiency, and controlled drug release profile. Biocompatibility of nanoformulation was also confirmed by less cytotoxic effect on Vero cells and blood cells. This study would suggests, the possible utilization of nanoformulated cefpirome as an effective and safe antibacterial agent in modern medicine to fight against life-threatening disease causing organisms.

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