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Research Article

PREPARATION, OPTIMIZATION AND CHARACTERIZATION OF BIOCOMPATIBLE NANOALBUMIN-OFLOXACIN(BSANP-OF) CONJUGATE AND EVALUATION OF CONTROL RELEASE , ANTI BACTERIAL ACTIVITY AGAINST CLINICAL ISOLATE OF PSEUDOMONAS AERUGINOSA

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ABSTRACT

Objective. The objective of the present study is to synthesize and optimize bovine serum albumin nanoparticles coated with ofloxacin drug nano conjugate and evaluation of control release and anti bacterial activity against clinical isolate of *Pseudomonas aeruginosa*,**Methods.**Simple coacervation technique was implemented for preparation of BSA nanoparticles and optimization was carried out with various parameters such as pH, Ethanol to BSA ratio and crosslinking time. The Nano-Drug conjugate was prepared using the optimized conditions along with ofloxacin. The Nanospheres thus formed were characterized by scanning electron microscopy and the control release, drug loading efficiency and entrapment efficiency was studied. Anti bacterial activity was studied with clinical isolate *Pseudomonas aeruginosa* adopting well diffusion assay.**Results**. The optimal pH was found to be 8.5 the ethanol to albumin ratio was fo be 5;1 and cross linking time of 8hrs which gives the higher yield of BSA nanoparticles.The Nanospheres thus formed were characterized using SEM which showed a particle size of nanosphere in the range of 160-230nm. The drug loading efficiency and entrapment efficiency was found to be 65 and 85% respectively. The *in vitro* drug release profiles show 81% release There was a controlled and a steady release of drugs from the nanoconjugate which showed distinct activity on *Pseudomonas aeruginosa* isolated from clinical samples. **Conclusion.** Nano albumin drug conjugate as an effective anti microbial agent against pathogenic bacteria would suggest the possible utilization of biocompatible non metallic nanoparticles –drug conjugate

Keywords: Control release, nano albumin,ofloxacin, *Pseudomonas aeruginosa*, nanosphere, anti bacterial activity

INTRODUCTION

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 [1]. Biological nanoparticles are mainly developed for drug delivery systems as an alternative to liposome technology, in order to overcome the problems related to the stability of these vesicles in biological fluids and during storage [2].The nanoparticle technology used in the recent years has great significance in improving the efficacy of the drugs. The nanoparticles fit into colloidal drug delivery systems, which offer advantages of drug targeting by modified body distribution[3] well as the enhancement of the cellular uptake [4] which benefits from reduction of undesired toxic side effects of the free drugs[5]. With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites, thus aiding in systemic treatments [6,7].Nanoparticles can be prepared from a variety of materials such as protein, polysaccharides and synthetic polymers. 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 [8].As a result undesired toxic side effects of the free drug can be avoided, for example with methotrexate [9].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 [10].Controlled drug release and subsequent biodegradation are important for developing successful formulations. Potential release mechanisms involve: (i) desorption of surface-bound /adsorbed drugs; (ii) diffusion through the carrier matrix; (iii) diffusion (in the case of nanocapsules) through the carrier wall; (iv) carrier matrix erosion; and (v) a combined erosion /diffusion process. The mode of delivery can be the difference between a drug's success and failure, as the choice of a drug is often influenced by the way the medicine is administered. Sustained (or continuous) release of a drug involves polymers that release the drug at a controlled rate due to diffusion out of the polymer or by degradation of the polymer over time. Pulsatile release is often the preferred method of drug delivery, as it closely mimics the way by which the body naturally produces hormones such as insulin. It is achieved by using drug-carrying polymers that respond to specific stimuli (e.g., exposure to light, changes in pH or temperature [11].

Nanomaterials derived from proteins, especially protein nanoparticles are biodegradable, non-antigenic, metabolizable and can also be easily amenable for surface modification and covalent attachment of drugs and ligands. Because of the defined primary structure of proteins the protein-based nanoparticles may suggest various possibilities for surface alteration and covalent drug attachment [12]. Protein nanoparticles can be utilized for the pulmonary delivery of protein therapeutics or can be incorporated into biodegradable polymer microspheres/nanospheres for controlled release depot or oral delivery [13,14].Nowadays active research is focused on the preparation of nanoparticles using proteins like albumin, gelatin, gliadin and legumin. Albumin, a protein found in blood plasma, has always been a remarkable molecule owing to its manifold functions and applications. Albumin is a biodegradable, biocompatible and less-immunogenic protein [15].The paramount function of albumin is in the circulatory system—to aid in transportation, metabolism, and distribution of exogenous and endogenous ligands[16].It also has an ability to act as an important extracellular antioxidant and to impart protection from free radicals and other harmful chemical agents. These unique attributes of albumin created a premier place for it in the drug therapy from time immemorial. Literature supports the use of modified serum albumin as a selective agent for tumor detection and/or therapy or as a delivery tool of toxic compounds for elimination of *Mycobacterium tuberculosis* via receptor-mediated drug delivery [17].Thus, nanotechnology era also employed the well-established properties of albumin, both human serum albumin

(HSA) and bovine serum albumin (BSA), for various purposes as the nanoparticle drug (antibodies, interferon gamma, antiviral compounds) targeting carriers, therapeutic enhancer of anti-cancer drugs, modified vehicles for drug delivery across the brain to the central nervous system and also across blood brain barriers[18].In the present study, bovine serum albumin (BSA) nnaoparticles were synthesized, under the optimum condition and the optimized nanoparticles were coated with ofloxacin. control release of bovine serum albumin (BSA) nanosphere with ofloxacin (BSA Np-OF) and the anti bacterial activity of nano drug conjugate against human pathogenic bacteria *Pseudomonas aeruginosa* was discussed.

MATERIALS AND METHODS

Preparation of BSA nanoparticles

Simple coacervation technique was implemented for preparation of BSA nanoparticles [18]. Anhydrous ethyl alcohol was added to 150 ml BSA (5 mg/l in 10 mM Tris/HCl contained 0.02% sodium azide) till the solution became turbid then 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. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation.The suspension is then ultrasonicated for 30 minutes. Large aggregates were eliminated by centrifuge (50,000 g, 30 min, 4°C). The pellet is then lyophilized to form fine powder.

Characterization of nanoparticles

The morphologies of the BSA nanoparticles were observed by scanning electron microscopy (SEM), The sample was sputtered with palladium gold for 30s under Polaron machine (BAL-TEC, Model SCDOOS, Switzerland). Afterwards, SEM was performed with a Carl Zeiss supra 55 (Germany) Field emission scanning electron microscope with the upper detector at 15 kV. The magnification was set at 65,000.

Optimization of the nanoparticles preparation

Determination of optimal pH

The optimization of BSA nanoparticles synthesis was performed over a pH range between 5 and 10 based on the method of Li *et al* [21]. For the analysis, the pH value of the suspension was automatically adjusted by the titration unit by addition of 0.1N hydrochloric acid or 0.1N sodium hydroxide solution, respectively. At 5 predefined pH values between 6 and 10, the yield percentage of the nanoparticles was measured and the particle size was determined by SEM.

Determination of optimal Ethanol to Albumin ratio

The optimization of the BSA nanoparticles preparation based on the ethanol concentration used for coacervation process. The ethanol concentration used for the desolvation influences the yield and the particle size of the nanoparticles. Five different ethanol to albumin ratios were preselected using earlier works on BSA nanoparticles synthesis. The different ratios include 2:1,2.5:1, 3:1, 4:1 and 5:1. optimal ethanol concentration was determined based on the particle size studied using SEM and yield percentage.

Determination of Crosslinking Time

The crosslinking of the ethanol coacervates takes place after the addition of glutaraldehye. The crosslinking time influences the particle size and yield percentage of the nanoparticles. The cross linking time at a range from 6h to 16h was predetermined and the optimal crosslinking time for the maximum synthesis was determined.

Preparation of BSA-Ofloxacin nanoconjugate

Ofloxacin-loaded BSA nanoparticles were prepared by a desolvation method . Briefly, 0.2 g BSA in 1.0-mL aqueous drug solution(1mg/ml), titrated to desired pH and incubated at room temperature, was converted to nanoparticles by addition of desolvating agent, ethanol, at the rate of 1.0 mL/min and under stirring (550 rpm) at room temperature. Then another 1ml of drug solution was added followed by 30 minutes of stirring. Subsequently, 8% glutaraldehyde aqueous solution was added to induce particle cross-linking. The cross-linking process was performed under stirring of the suspension over night. Experimental values of drug concentration, pH, drug–BSA incubation time, and volumes of ethanol and glutaraldehyde were variable in optimization trial.

Drug entrapment efficiency

Ofloxacin concentration in the supernatant after the centrifugation of the prepared nanosphere solution was detected using the UV-Vis Spectrophotometer at 291nm. The drug encapsulation rate of Ofloxacin-NSP is calculated using the formula.

$$Percent\ entrapment = \frac{((Total\ Ofloxacin) - (Ofloxacin\ in\ supernatant)) * 100}{Total\ Ofloxacin}$$

Drug Loading Efficiency

The nanosuspension with known amount of drug(10mg/20ml) was prepared with purified water. The suspension was then ultrasonicated for 30 mins for disruption and then filtered through a memberane. The drug content in the suspension was then detected by HPLC in order to calculate the drug in the conjugate and total weight of the Nab-Drug conjugate.

$$Loading\ Efficiency\ \% = \frac{Weight\ of\ drug\ in\ nanoconjugate * 100}{Total\ weight\ of\ nanoconjugate}$$

In vitro drug release study

The drug release studies were carried out in 1xPBS. Dialysis bag was used to investigate invitro release of ofl-Nab.Ofl-Nab was distributed in water and transferred into dialysis bag and dialyzed against physiological saline which was thermostated at 37°C and mechanically stirred at 75rpm. At designated intervals, a portion of the dialysis medium was taken for quantitation of Ofloxacin and the same volume of fresh medium added.The collected dialysis medium was syringe filtered and spectometrically read at 291nm.

Anti bacterial activity

The antimicrobial activity of blank albumin nanoparticles and the Ofloxacin coated nanoparticles were analysed on the clinically isolated pathogenic strains of *Pseudomonas aerogenosa*. The bacterial organisms were uniformly spread onto the sterile nutrient agar medium with sterile cotton swabs and blank Nab and Nab-Ofl of 100µl withdrawn at different time interval was loaded onto the wells that were made on culture seeded agar plates and incubated for 12-24hrs at 37C.The zone of inhibition after incubation period was observed and recorded.

RESULTS AND DISCUSSION

Preparation of BSA nanoparticles

Nanoalbumin was prepared based on the simple coacervation process as described earlier. the synthesized nanoparticles was in the size range of 110 nm (Fig 1) but the yield percentage of this initial formulation was found to be less which lead to the optimization of the process of nanoparticles synthesis.

Optimization of BSA nanoparticles synthesis

The process conditions for the synthesis of the nanoparticles was optimized based on the factors such as pH, cross linking time, ethanol albumin ratio. The initial formulation parameter selection with blank, nanoparticles indicated that the pH and the dosage of ethanol significantly influenced the preparation of the nanoparticles. At pH less than 8 the formation of albumin nanoparticles was less based on the yield. With increase in pH the mean diameter of the nanoparticles 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 to 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% to 65% which were comparatively lower than the yield percentages of pH above 7 which were greater than 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 nanoparticles preparation. It is noted from the optimization process is that the volume of ethanol added is key to the yield of controlled size nanoparticles. When the ratio of ethanol to 2% BSA was greater than 2.5:1,the yield of nanoparticles was greater or equal to 80 %. However with an increase in ethanol to BSA the mean diameter of the nanoparticles increased.

The crosslinking of the particles by the glutaraldehyde is a critical factor in the synthesis of nanoparticles. The time for crosslinking influences the yield and particle size of the BSA nanoparticles. Crosslinking plays a major role in the stability and drug release of albumin nanoparticles. Herein the crosslinking time was varied between 6h and 16 hrs for to synthesize a stable, high yielding process. The yield percentage of about 81% was obtained at a crosslinking time of 6h while the yield increased during the increase in the crosslinking time. The optimal cross linking time was found to be in the range of 8to 12 hrs based on the comparison between the cross linking times of 6hrs to 20 hrs(Table 1).

Hence the optimal condition for the preparation of the blank BSA nanoparticles was optimized to be as pH 8, Ethanol: Albumin 4:1, Cross linking time of 8hrs. (Table 2)

Table 1 shows

Parameter Selection for Nanoalbumin					
pH	6.0	7.0	8.0	9.0	10.0
Yield Percentage (%)	52.3±2.6	64.8±5.2	88.7±3.4	86.2±3.4	80.3±4
Ethanol/BSA(V/V)	2:1	2.5:1	3:1	4:1	5:1
Yield percentage	57.3±3.6	79.3±3.1	82.4±3.6	89.3±4.2	88.3±2.6
Crosslinking time	6h	8h	12h	16h	
Yield percentage	83.7	89.2	86.5	81.2	

Table 2 shows

	Nab	**Nab-Drug Conjugate**
BSA	2%	2%
Drug		1mg/ml
Glutaraldehyde	100%	100%
pH	9	9
Ethanol:BSA	4:1	4:1
Crosslinking time	12hr	12hr
Mean Diameter	100+nm	130+nm
Encapsulation rate		92%
Drug Loading		58%

Preparation of the Nab-Ofl nanospheres

The preparation of the albumin Ofloxacin was prepared based on the conditions optimized for the preparation of blank Nab (nano albumin).The drug concentration of 1mg/ml was added during nanoparticles preparation for to be coated with BSA nanoparticles. The prepared Nab-ofl was found to be highly stabled in water and cell medium. This method leads to uniform distribution of least aggregated particle. The further characterization was done using SEM (Fig 1).

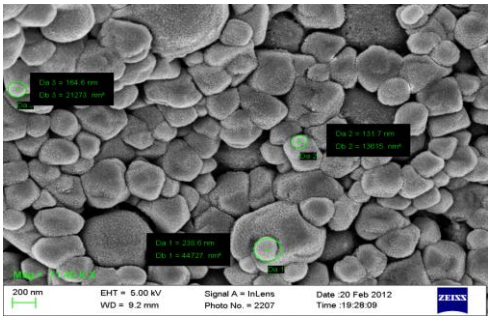


Figure 1:Scanning electron micrograph of BSA nanoparticles

Scanning electron microscopy was used to analyze the size and conformational features of BSA nanoparticles and Nab-ofl conjugate. the micrograph showed smooth surfaces , good dispersion and relatively uniform size distribution in all the optimally synthesized nanoparticles and the mean diameter of Ofloxacin loaded nanoparticles were in the range of 160-240nm (Fig.2)

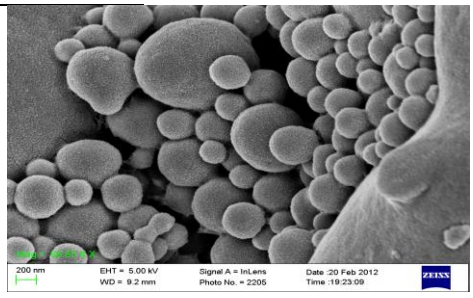


Figure 2: Scanning electron micrograph of nnaoalbumin-ofloxacin conjugate

Drug loading and entrapment efficiency

The loading efficiency and the entrapment efficiency of the drugs on to the BSA is found by the spectrophotometric analysis of the drug-BSA conjugate suspension. The unbound BSA concentration was found by correlating the absorbance of the supernatant after the centrifugation with the standard absorbance concentration ratio. The drug loading and entrapment efficiency of Ofloxacin was about

64% drug encapsulation rates were 93% and 89% for Ofloxacin and flutamide respectively. The drug loading is found to be much higher than earlier studies with BSA nanoparticles.

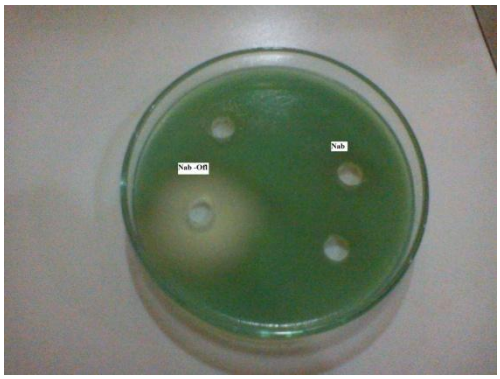
In vitro drug release and anti bacterial activity

In vitro drug release of the drug was studied using 1% PBS. The sample was taken at regular intervals and analysed spectrometrically. The release percentage was calculated using the intial drug concentration and the release at specified time. The drug release was calculated for 24 hours. There was a steady release of drug in the early hours and a total release of about 81.8% was observed (Table 3).

Table 3 shows

Time (hr)	Cumulative% of Ofloxacin
0	0
1	5.3
2	12.2
3	26.5
4	35.4
5	44.8
6	50.6
7	55.2
8	59.3
9	63.2
10	66.6
11	71.6
12	77.6
13	79.3
14	80.2
15	80.7
16	81.8
17	81.8
18	81.8

In the following 6 hours, cumulative release reached 66%, in a sustained manner, which provides distinct activity against tested bacteria..Cumulative release reached almost 81% during 16 hours, continued till 18 hours and showed an almost released ability of the nanoparticle formulation. The generally sustained and controlled release profile of ofloxacin facilitates the application of nanoparticles for the delivery of anti bacterial drugs. The crosslinking process with glutaraldehyde plays a major role in the stability and drug release from the desolvated BSANP. Anti bacterial activity against *Pseudomonas aeruginosa* revealed there was a sharp increase in zone of inhibition at increased time period and enhanced zone of inhibition was recorded during 18 hours with 21mm..(Figure 1).



This confirms the encapsulation of Ofloxacin by BSA nanospheres and also reveals the release of Ofloxacin from the nanosphere. Further studies are necessary for the Nab-OF system, such as prolonging the drug release time and enhancing the drug-loading rate, before the system meets clinical requirements

CONCLUSION

Nanotechnology has the power to radically change the way cancer is diagnosed, imaged and treated. Currently, there is a lot of research going on to design novel nanodevices capable of detecting cancer at its earliest stages, pinpointing it's location within the body and delivering anticancer drugs specifically to malignant cells. Our aim was to prepare BSA nanoparticles for the effective delivery of ofloxacin. BSA nanoparticles preparation process was optimized by varying various pH from 6 to 10 wherein pH 8 was found to be most favourable. The desolvating agent such as Ethanol concentration was varied for maximum yield which proved a ratio of 4:1 ratio to be optimal. The crosslinking time for the process was found to be 8h. The process conditions were used for the encapsulation and coating of drugs. ofloxacin was used as the drug for the study.Further study will helpful to develop the Nab-OF as the anti microbial drug.

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