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Optimal Synthesis OF Biocompatible Bovine Serum Nanoparticles- Incorporated Quercetin (BSA NPS-QT) Nano Drug Conjugate for the Controlled Release and Improved Anti Oxidative Activity.

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ABSTARCT

Application of nanotechnology in drug delivery systems has opened up new areas of research in sustained release of various drugs. Due to their size, nanoparticles have the advantage of reaching inaccessible sites in the body by escaping phagocytosis and entering tiny capillaries. Sustained release of the drug from the nanoparticles maintains the therapeutic concentration for long durations. Bovine serum albumin is an attractive macromolecular carrier and widely used to prepare nanospheres and nanocapsules, due to its availability in pure form and its biodegradability, nontoxicity and non immmunogenicity. In the present study,BSA nanoparticles was prepared by coacervation process and were optimized by various parameters such as temperature, pH, BSA concentration and glutaraldehyde concentration. The process conditions for the synthesis of the nanoparticles was optimized based on the factors such as pH, cross linking time, ethanol albumin ratio.Synthesized nanoparticles were characterized by scanning electron microscopy. The sizes of BSA nanoparticles were absolutely less than 100 nm. The antioxidant molecule quercetin was loaded in BSA nanoparticles by coaservation process and was characterized by SEM. Quercetin loaded BSA nanoparticles are smooth and spherical with an average size of 72.59 ±30 nm. Quercetin and quercetin loaded nanoparticles shows maximum free radicals scavenging properties that were confirmed by DPPH assay. The *in vitro* release assay of quercetin loaded BSA nanoparticles confirms its sustainable release and maximum release of quercetin was 91.0 % at 19 hours. These properties of quercetin loaded nanoparticles molecule pave way for encapsulating various therapeutically less useful highly active antioxidant molecules towards the development of better therapeutic compounds.

Keywords: Nanoparticles, BSA, quercetin, coacervation, antioxidant drug delivery,controlled release

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INTRODUCTION

Nanoparticulate delivery systems are extensively investigated as a drug delivery strategy in the pharmaceutical research. In general, nanocarriers may protect a drug from degradation, enhance drug absorption by facilitating diffusion through epithelium, modify harmacokinetic and drug tissue distribution profile and/or improve intracellular penetration and distribution. Furthermore, by modulating the surface properties, composition and milieu, the desired release pattern of the drug and its biodistribution can be achieved [1-3].Additionally, one of the major advantages associated with the anoparticulate systems is their ability to withstand physiological stress or improved biological stability and possibility of oral delivery which makes them more attractive as a drug delivery strategy than liposomes [4-6].The body distribution of colloidal drug carrier systems is mainly influenced by two physicochemical properties, particle size and surface characteristics [7].Concerning particle size, the particles should be small enough not to be removed by simple filtration mechanisms in a capillary bed after intravenous injection. With respect to the surface characteristics of a colloidal system, these characteristics may directly affect particle size. Above all, however, they represent the major determinant for protein adsorption in biological fluids and may modify particle interaction with specific plasma membrane receptors, thus leading to elimination of the particles from the systemic circulation. The mechanism of protein adsorption on particle surfaces in conjunction with the recognition of such coated particles by monocytes and macrophages is named opsonisation process. This opsonization process seems to be influenced by the surface curvature of the carrier system, smaller carriers leading to a reduced adsorption of proteins and opsonins and in turn to a reduced uptake of such systems by phagocytic cells [8].It was observed that the extent of opsonisation decreased with a decrease in particle size from 800 to 200 nm, and no enhancement of phagocytic uptake due to opsonization was recorded at particle sizes below 200 nm. For very small colloidal systems, with a size below 100 nm, it was described that after intravenous injection these carriers were able to cross the fenestration in the hepatic sinusoidal endothelium, leading to a hepaticaccumu lation instead of long intravasal circulation. Even systems such as liposomes, with a size range of about 400 nm and a highly deformable structure were able to cross the endothelial fenestration, whereas rigid systems of the same diameter did not [9,10].

Proteins represent good raw materials since they have the advantages of synthetic polymers together with the advantages of absorbability and low toxicity of the degradation end products [11,12].Among the available potential colloidal drug carrier systems, protein-based nanoparticles are particularly interesting as they hold certain advantages such as greater stability during storage and in vivo, being non-toxic and non-antigenic and their ease to scale up during manufacture over other drug delivery systems [13-17].Albumin is an attractive macromolecular carrier that has been shown to be biodegradable, nontoxic, metabolized in vivo to produce innocuous degradation products, non-immunogenic, easy to purify and soluble in water allowing ease of delivery by injection and thus an ideal candidate for nanoparticle preparation [18,19,20].Albumin-based nanoparticle carrier systems represent an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule [21].Due to the

defined albumin primary structure and high content of charged amino acids (e.g. lysine), albumin-based nanoparticles could allow the electrostatic adsorption of positively (e.g. ganciclovir) or negatively charged (e.g. oligonucleotide) molecules without the requirement of other compounds [22,23].In the present study, bovine serum albumin nanoparticles incorporated anti oxidant molecule quarcetin was prepared under optimum condition and the prepared nanoconjugate was evaluated for controlled release.

MATERIALS AND METHODS

Preparation of BSA nanoparticles

Simple coacervation technique was adopted for preparation of BSA nanoparticles. 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 mins. Large aggregates were eliminated by centrifugation at 50,000 g. for 30 minutes at 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. 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 Nps-Quercetin nanoconjugate:

Quercetin loaded BSA nanoparticles were prepared by a desolvation method. Briefly, 0.2 g BSA in 1.0-mL aqueous quercetin 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 followed by addition of 1ml of drug solution. 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

Quercetin 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 Flu-Nab is calculated using the formula.

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

Drug Loading Efficiency

The nanosuspension with known amount of quercetin (10mg/20ml) was prepared with purified water. The suspension was then ultrasonicated for 30 minutes 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 quercetin from nanoconjugate.Nano conjugate 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 quercetin and the same volume of fresh medium added. The collected dialysis medium was syringe filtered and spectrometrically read at 291nm.

Determination of antioxidant activity (Scavenging Activity of DPPH Radical)

The free radical scavenging capacity of free BSA Nps and BSA Np-QT was determined using DPPH assay. DPPH radical cation decolourization tests are spectrophotometric methods widely used to assess the antioxidant activity of various substances [24]..Different concentration of free BSA Np,free quercetin (F-QT) and BSA Np-QT (10, 50, 100, 500 and 1000 µg/ml) were used. Similar concentrations of ascorbic acid were used as positive control. The assay mixture contained in a total volume of 1 ml, 500 µl of respective concentration of nano formulation, 125 µl prepared DPPH (1 mM in methanol) and 375 µl solvent (methanol or 5% ethanol). After 30 min incubation at 25°C, the decrease in absorbance was measured at λ = 517 nm.

The radical scavenging activity was calculated from the equation:

$$\% \text{ of radical scavenging activity} = Abs_{control} - Abs_{sample}/Abs_{control} \times 100$$

RESULT AND DISCUSSION

Preparation and optimization 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. 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 1,2). 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..Optimization of preparation process of vinblastine sulfate (VBLS)-loaded [25],folate-decorated paclitaxel-loaded and 5-Fluorouracil loaded bovine serum albumin nanoparticles [26] and BSA Np-antibacterial antibiotic [27] has been reported.

Table 1. Parameter optimization for BSA nanoparticles synthesis

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. Characteristics of optimized formulation

	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%

Characterization

Scanning electron microscopy was used to analyze the size and conformational features of BSA nanoparticles and quercetin conjugate. the micrograph showed smooth surfaces , good dispersion and relatively uniform size distribution in all the optimally synthesized nanoparticles

mean diameters of the blank albumin nanoparticles were in the range of 100-110nm(Fig 1) and the mean diameter of quercetin loaded nanoparticles were in the range of 160-240nm (Fig 2).

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 was in the range of 85.0 and 83.7 % respectively. The drug loading is found to be much higher than earlier studies with BSA nanoparticles.

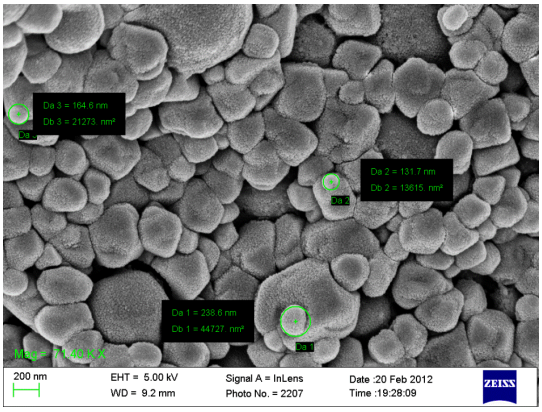


Figure 1: Scanning electron micrograph of BSA nanoparticles

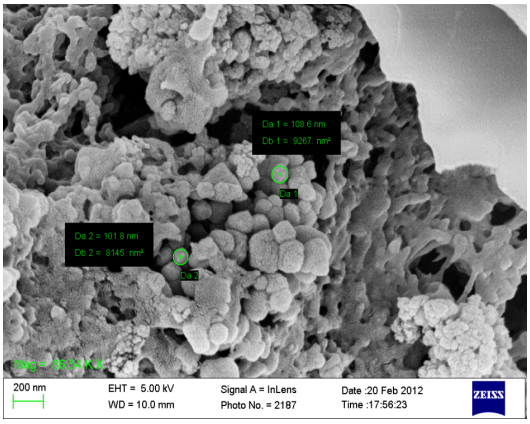
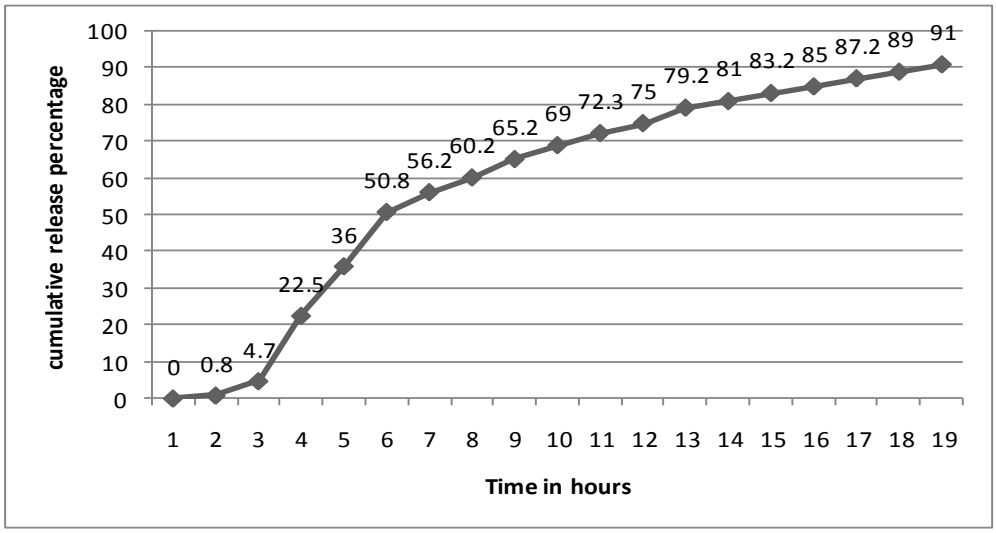


Figure 2: Scanning electron micrograph of BSA Np-QT

***In vitro* drug release**

Invitro 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 19 hours. There was a burst release of drug in the early hours and a total release of about 87.0 % was observed. An initial burst of 22.5% in the first 4 hours can be observed. In the following 6 hours, cumulative release reached 50.8%, in a sustained manner, which provides the distinct anti oxidative property. Cumulative release reached almost 91.0% at 19 hours and showed an almost released ability of the nanoparticle formulation (figure 3). The generally sustained and controlled release profile of the drug facilitates the application of nanoparticles for the delivery of drugs [28].The cross linking process with glutaraldehyde plays a major role in the stability and drug release from the desolvated BSANP. Different studies have shown that the rate and amount of drug release is inversely proportional to the HPMC percentage in formulations [29,30,31]. 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 [31]. BSA encapsulation increases the bioavailability and a good increase in the sustained release.



Anti oxidative activity

2,2-Diphenyl-1-picrylhydrazyl hydrate is a free radical, stable at room temperature, which produces a deep violet solution in organic solvents. It is reduced in the presence of quercetin molecules, giving rise to uncolored solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidant properties of quercetin. Quercetin shows an antioxidative effect that is mainly due to its phenolic hydroxyl groups. These phenolic hydroxyl groups are able to donatehydrogen to reduce the free radicals to prevent oxidation of lipids, proteins, and DNA [32]. The free radical scavenging properties of the free quercetin and quercetin loaded nanoparticles are shown in Figure 4. Free radical scavenging activity was found to be increased in BSA Np-QT (87.0%). Previous studies revealed that quercetin has a moderate DPPH activity.

Present study evaluates the antioxidant activity of quercetin bound with BSA nanoparticles. The results show that the BSA nanoparticles incorporated quercetin recorded a scavenging activity of 87 % which is higher than the previous studies. The comparative study on the free radical scavenging activity of free BSA Nps, free quercetin and BSA nanoparticles incorporated quercetin (BSA Np-QT) shows that quercetin in the nanoconjugate plays a vital role in scavenging while the BSA enhances its activity as a carrier.Further study will helpful to formulate BSA Np incorporated quercetin (BSA Np-QT as the effective biocompatible anti oxidant agent.

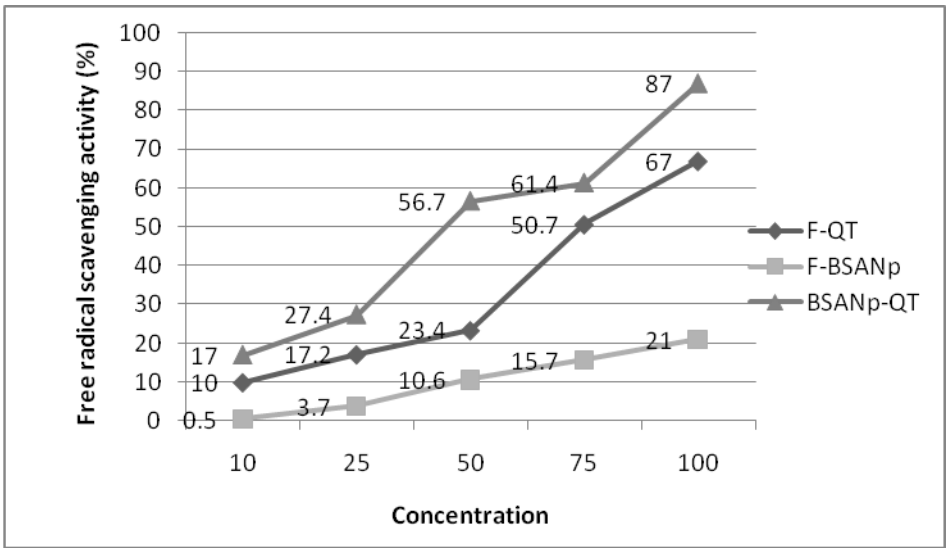


Figure 4: Free radical scavenging activity (%) of BSANp-QT, F-BSA and F-QT

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