

## A simple and reproducible method for production of protein nanoparticles at biological pH using egg white

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### ABSTRACT

Protein nanoparticles have been found to be of great interest as a carrier in a drug delivery system due to its biodegradability and non toxic nature. The purpose of the present investigation is to establish a simple and fast method for the preparation of stable egg white protein (EWP) nanoparticles. Desolvation process was adopted and the resulting nanoparticles were stabilized by a crosslinker, glutaraldehyde. To get the suitable and stable nanoparticles several process parameters such as pH, agitation speed, concentration of egg white, rate of addition of desolvating agent, glutaraldehyde concentration and addition of salt and buffer were examined. The minimum size of 112nm has been obtained at pH 9.0 when ethanol addition rate was 1ml/min at an agitation speed of 550 rpm. The size of nanoparticles is affected largely by pH of egg white while it is not significantly affected by the agitation speed and concentration of egg white and crosslinker. The SEM monochrome image of eggwhite nanoparticles displays the spherical shape with around 100nm size.

**Keywords:** Nanoparticles, Egg White, Desolvation technique, Drug delivery.

### 1. INTRODUCTION

In recent decades cancer is considered to be one of the leading causes of mortality across the globe. It is regarded as a group of disease characterized by an abnormal growth of cells. Chemotherapy, surgery and radiation therapy are the most common treatment for cancer. The first successful method used for the treatment of cancer was surgery. It is the only therapy to cure many common solid tumors [1]. Radiation therapy is a kind of treatment involving the use of high energy gamma radiation to shrink tumors, kill cancer cells and stop them from growing and dividing [2]. Chemotherapy is a method of modality used in the treatment of cancer which involves the administration of drug to attack cancer cells and it usually requires multiple cycles [3]. Chemotherapy may have some adverse side effect on normal tissues [4]. The major disadvantages with these treatments are hepatic and renal toxicity as well as lower availability of the drug at the target site. These problems can be overcome by using a biocompatible and target-specific drug delivery vehicle.

In order to improve the specificity and efficiency of drug action, different types of nanocarriers have been developed for targeted drug delivery. These involve polymeric and functionalized polymeric nanoparticles, magnetic nanoparticles, solid-lipid nanoparticles, metal and inorganic nanoparticles, ceramic nanoparticles, quantum dot, polymeric micelles, phospholipids micelles, dendrimers and colloidal nanoliposomes etc. [5,6]. The bottom up processes viz. desolvation, thermal gelation, emulsification, high pressure homogenization, salting out and nano spray drying is commonly adopted for the synthesis of nano carriers [5,5]. Polymer based nanoparticles can be fabricated from natural biopolymers, synthetic biodegradable polymers, lipids and polysaccharides. Nanocarriers based on protein including albumin, casein, collagen, whey protein and gelatin have been extensively studied for delivering bioactive molecules and drugs [7,8]. Due to low toxicity of degradation end product and high absorbability,

proteins are considered as good raw materials for the production of nanocarriers [9,10]. Albumin and modified albumin has been extensively used in patients having chronic liver disease [11-13]. Colloidal nanocarriers play a vital role in the area of drug delivery by improving the bioavailability and therapeutic index of the administered drug. The particle size and surface charge is crucial for retention of colloidal drug delivery system and distribution of drug in the human body [14]. Longmire [15] and Harashima [16] reported that the nanocarriers above 250nm diameter are identified by monocytes or macrophages and removed through opsonization process while smaller nanocarriers (<100nm) after intravenous injection cross the lymphatic capillaries and undergo clearance. Hence the colloidal drug carrier system should be in the range of 100-200nm with narrow size distribution.

Among the colloidal drug carrier systems available for the treatment of cancer, protein-based nanocarriers are very promising as they have certain benefits such as long shelf life and more stability during storage, non-antigenic and non toxic property, easy to scale up and biodegradable [17-19]. Albumin is a versatile drug carrier and a classic candidate for preparation of nanoparticles with the properties of being nontoxic, biodegradable, soluble in water, easy to purify and can be easily delivered through injection [20,21]. Nanoparticulate delivery systems using albumin as carrier represent a fascinating strategy. Due to the presence of carboxyl and amine groups in the albumin molecules, a significant amount of drug and other molecules can be conjugated to albumin nanoparticles through covalent bonding [22]. Depending on the pH of colloidal carrier, albumin nanoparticles will have positive and negative surface charge. Several reports are available on electrostatic adsorption of charged molecules on albumin nanoparticles [23,24]. Albumin being a polymer can also encapsulate the drug and delivers it at the site of action. Martinez [25] reported the tamoxifen- loaded folate

conjugate BSA nanoparticles for the controlled release of tamoxifen for the treatment of breast cancer. Curcumin loaded albumin nanoparticles synthesized by Delfiya [26] shows increased solubility in water as compared to free curcumin, more bioavailability and enhance tissue targetability of curcumin. Simple desolvation method has been successfully employed by Hu [27] for the synthesis of sodium ferulate entrapped bovine serum albumin nanoparticles as liver targeted drug delivery system. Gallic acid, an antioxidant and antiparkinson agent remains active during the preparation of gallic acid loaded polyethylenimine–HSA nanoparticles. These nanoparticles showed no significant toxicity and are good candidate for efficient and safe delivery of gallic acid to brain [28]. Wang [29] synthesized folate-conjugated human serum albumin magnetic nanoparticles loaded with cisplatin (anticancer drug). The half life release time and intake by tumor cells of cisplatin was significantly improved as compared to cisplatin solution. Numerous other reports are available for the efficient delivery of anticancerous drug using albumin as nanocarrier [30-38]. More over cancer cells are greedy for protein based constituents so albumin based nanocarriers will accumulate

on solid tumor and deliver the associated antitumor drug to the targeted site.

In recent decades several investigations are focused on production of albumin nanoparticles for targeted delivery [39-44]. Numerous reports are available on synthesis and process optimization of bovine and human serum albumin nanoparticles using the desolvation process [45-48]. The procedure adopted for the production of nanoparticles involves the use of tubing pumps and other designed apparatus for controlled addition of desolvating agent [49]. Furthermore the particle size around 100-150nm is obtained in acidic and alkaline condition. So it is worthwhile to develop a simple procedure for the synthesis of protein nanoparticle at biological pH. The present work is aimed at the development of simple and highly reproducible method for the cost effective production of EWP nanoparticles using egg white. Several preparation conditions viz. egg white concentration, pH, rate of ethanol addition, glutaraldehyde concentration, agitation speed and effect of salt and buffer solution were examined to optimize the preparation process for the production of EWP nanoparticles at biological pH.

## 2. MATERIALS AND METHODS

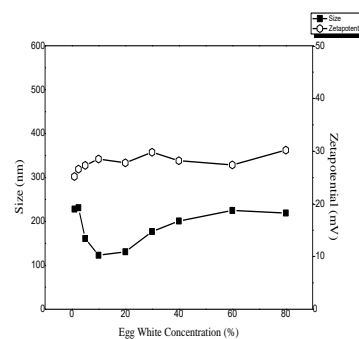
Chicken's egg was purchased from the local market; glutaraldehyde (25% aqueous solution) was commercially supplied by Merck. Ethanol and all other chemicals were procured from Sigma Aldrich. All the chemicals used were of analytical grade and used without further purification. Nanoparticles of egg white protein (EWP) were prepared by a simple desolvation process at room temperature. Hydration of 10% aqueous solution of egg white was performed at 550 rpm using a magnetic stirrer for an hour. The homogenized solution was then filtered off through Whatman filter paper. To 4 ml aqueous solution of egg white under stirring at 550 rpm, desolvating agent ethanol was added drop wise through burette with a constant flow rate (1ml/min) until the solution becomes just turbid. The turbidity indicates the formation of nanoparticles. The nanoparticles thus formed were stabilized by crosslinker (8% glutaraldehyde). The

stirring was continued for 6 hours at room temperature and left overnight to ensure the crosslinking of all amino acid residues. The yellow colored colloidal solution was purified with 3 cycles of centrifugation at 4000 rpm (Remi R-4C, Remi ElektrotechniK. Vasai, India) and supernatant was used for the study. The size of prepared nanoparticles and zetapotential were measured at 25<sup>0</sup>C using a Malvern zetasizer (Nano ZS series, Malvern Instruments Ltd., Malvern, UK) with a scattering angle of 173<sup>0</sup>. The data represented is the average of three run. The size of the prepared egg-white nanoparticles was examined by scanning electron microscope (Stereoscan model S360 brand SEM-Leica Cambridge, Cambridge, UK) under an accelerating voltage of 10 kV with a magnification of 15,000. The SEM monochrome image of eggwhite nanoparticles clearly indicates the spherical shape with around 100nm size.

## 3. RESULTS

**3.1. Effect of eggwhite concentration.** To access the requisite amount of egg white required for the production of nanoparticles of favorite sizes different concentrations of egg white were evaluated. The influence of egg white concentration on the particle size and zeta potential of the prepared samples is represented in Figure 1. Only a slight influence on particle size was observed in the egg white concentration range of 1% to 80% with a minimum particle diameter of around 100nm at 10% to 20% egg white concentration. This result is in good agreement with the earlier report of Langer et al. for HSA nanoparticles [19].

There was also no appreciable change on the surface charge of the resulting nanoparticles on increasing the egg white concentration. The decreasing trend in particle size of BSA [20], egg albumin [47] and gelatin [50] was observed with an increase in concentration up to a particular range.



**Figure 1.** Effect of egg white concentration on particle size and zetapotential.

**Preparation condition:** pH: 7.0, Ethanol Rate: 1ml/min., Stirring Rate: 550 rpm, Glutaraldehyde: 120  $\mu$ l, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

Few reports on BSA and HSA nanoparticles reveals the decreasing and then increasing trend in particle diameter with the increase in concentration [19,49]. High protein concentration may result in coagulation due to hydrophobic and electrostatic interactions subsequently resulting in larger particle size. Based on earlier reports and present investigation 10% aqueous solution of egg white was preferred for the production of EWP nanoparticles.

**3.2. Rate of addition and amount of desolvating agent .** For the production of nanoparticles of favorable size ethanol or acetone was used as a desolvating agent. To control the rate of addition of ethanol, pump control system set has been used by several researchers. To obtain smaller particle size Davaran et al. [49] used a self designed ethanol addition system containing a needle at the end resulting in uniform and small drops of desolvating agent. In the present study ethanol was added simply through burette with a constant flow rate. For the preparation of EWP nanoparticles ethanol was added drop wise to 10% aqueous solution of egg white until the solution become just turbid. The volume of desolvating agent required for denaturation of egg white is different from that required for BSA and HSA and it largely depends on the pH of egg white solution. Depending on pH, 2-16 ml of ethanol was required to denature 10% aqueous solution of egg white (Table 1).

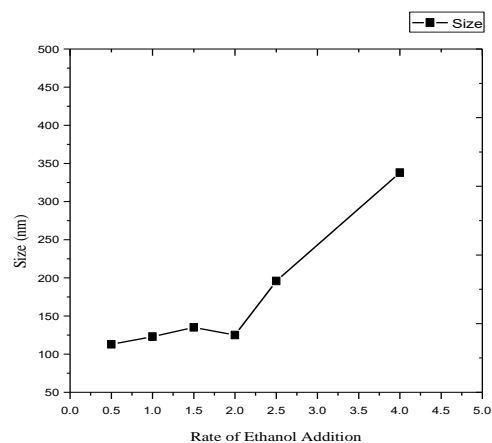
**Table 1.** Volume of ethanol required for denaturation of egg white.

S.No	pH of 10% aqueous egg white	Volume of ethanol required for turbidity (ml)
1	2	No turbidity up to 20
2	3	15-16
3	4	1.5-2
4	5	1.5-2
5	6	2-2.5
6	7	4-5
7	8	6-7
8	9	6-7
9	10	6-7
10	11	6-7
11	12	8-9

The influence of ethanol addition rate on particle size was checked by varying the ethanol addition rate from 0.5 – 4.0 ml/min (Fig. 2). At a rate of 0.5 to 2.0 ml/min nanoparticles with a diameter of about 120nm were obtained which increases with an increase in rate of ethanol addition. In the current investigation rate of ethanol addition was fixed at 1ml/min for the production of nanoparticles that also supports the report of Langer et al. [19] and Ko et al [48].

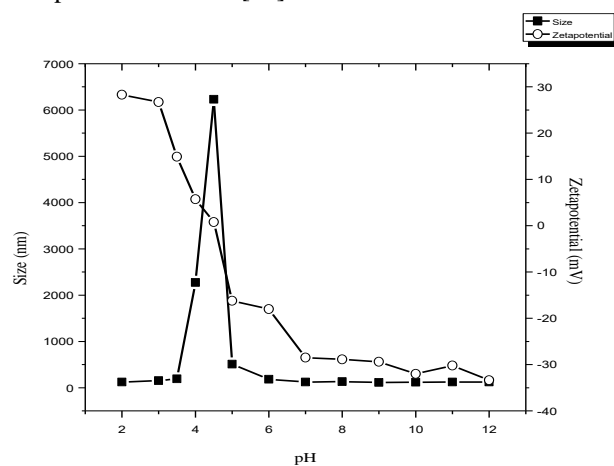
**3.3. Effect of pH.** The size of protein particles is highly influenced by the pH. Larger protein particles are expected near the isoelectric point (pI) which may be due to the enhanced hydrophobic interaction among protein particles leading to coagulation. The pI of egg white is 4.6 – 4.8.

The size and stability of egg white nanoparticles were evaluated over a pH range of 2-12 (Fig. 3). At pH 2 and 3 the particles of about 100nm were obtained with zeta potential of +28.3 mV and +26.7mV respectively. With increase in pH, particle size increases being maximum at pH 4.5, near the pI of egg white. In neutral and alkaline medium protein particles possessed negative surface charge with zeta potential of -28.5 mV to -33.4mV, resulted in formation of smaller particle size.



**Figure 2.** Effect of rate of ethanol addition on particle size  
**Preparation condition:** 10% Egg white: 4.0 ml, pH: 7.0, Stirring Rate: 550 rpm, Gluteraldehyde: 120 µl, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

Under acidic condition (pH 2-3) due to enhanced positive surface charge electrostatic interaction increases while hydrophobic interaction decreases which allowed smaller particle formation since the possibility of coagulation of protein molecules is reduced. Strong hydrophobic interaction with almost neutral surface charge resulted in larger particle size near the isoelectric point of egg white [51]. Hydrogen bonding decreases the hydrophobicity of protein molecules in basic medium. In higher pH medium nanoparticles exhibit negative surface charge. Strong electrostatic repulsion and reduced hydrophobicity among the protein molecules allowed them to stay apart which results in smaller particle diameter [52].

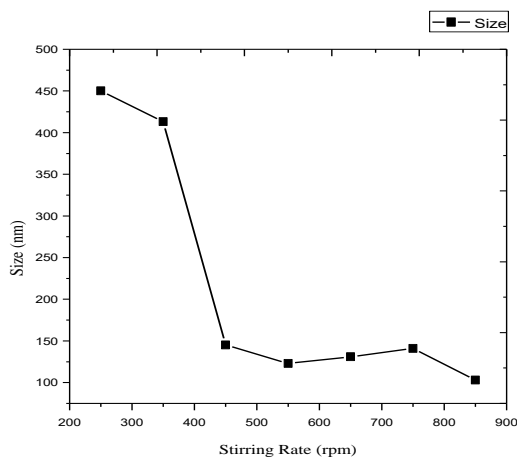


**Figure 3.** Effect of pH on particle size and zetapotential  
**Preparation condition:** 10% Egg white: 4.0 ml, Ethanol Rate: 1ml/min., Stirring Rate: 550 rpm, Gluteraldehyde: 120 µl, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

**3.4. Agitation speed.** As per previous reports, 500 rpm agitation speed is necessary for preparation of albumin nanoparticles [20,22]. To obtain EWP particles in nano range, different stirring rates ranging from 250 to 850 rpm with drop wise addition of ethanol to 10% aqueous solution of albumin was investigated (Fig. 4). Agitation speed from 450 to 850 rpm resulted in a smaller size while stirring rate below 450 rpm produced particles of relatively larger size, being maximum of 450 nm at 250 rpm. The aggregation of particles at low stirring may be the reason of large particle diameter. The same trend was also

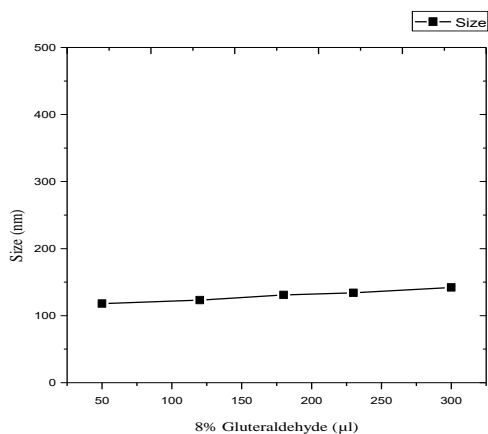
observed for BSA, HSA and gelatin nanoparticles while egg albumin shows inverse trend [20,22,50].

**3.5. Glutaraldehyde concentration.** Egg white is a colloidal suspension of proteins and water. To check the particle size of egg white protein, we prepared its 10% aqueous solution through stirring at 550 rpm for 1 hour. The particle diameter was about 500nm which further increases with time. Since a large amount of glutaraldehyde is toxic so we also checked whether we can get stable protein nanoparticles without using crosslinker. For that we added 8% glutaraldehyde at different time intervals to the denatured (using ethanol) egg white (Fig. 5). The figure clearly indicates that nanoparticles of favorable size are obtained only up to 20 minutes after that the particles size increases.



**Figure 4.** Effect of stirring rate on particle size.

**Preparation condition:** 10% Egg white: 4.0 ml, pH: 7.0, Ethanol Rate: 1ml/min., Glutaraldehyde: 120 µl, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

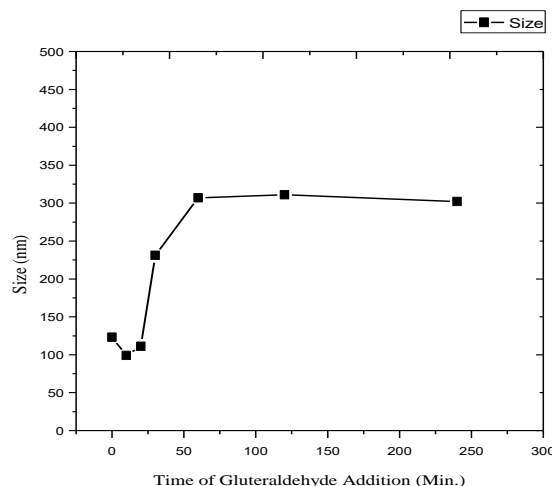


**Figure 5.** Effect of glutaraldehyde on particle size.

**Preparation condition:** 10% Egg white: 4.0 ml, pH: 7.0, Ethanol Rate: 1ml/min., Stirring rate: 550 rpm Glutaraldehyde: 120 µl, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

Nanoparticles obtained after desolvation process using ethanol were unstable and had a possibility of redissolving. To stabilize the formed nanoparticles glutaraldehyde was added as a cross-linker. Varying quantity of 8% glutaraldehyde were used to study the effect of cross-linker on particle size (Fig. 6). The figure clearly indicates that there is no effect of the cross-linking conditions on the resulting particle size while an excess of glutaraldehyde leads to larger particle size or even precipitation. Minimum amount of glutaraldehyde is required for the preparation of protein nanoparticles. The similar results were also reported by

Langer et al. [19] and Jahanshahi et al. [20] for HSA and BSA respectively.

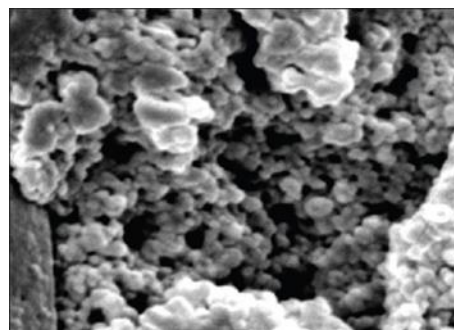


**Figure 6.** Effect of time of glutaraldehyde addition on particle size.

**Preparation condition:** 10% Egg white: 4.0 ml, pH: 7.0, Ethanol Rate: 1ml/min., Stirring rate: 550 rpm, Glutaraldehyde: 120 µl, Temperature: 25<sup>0</sup>C, Stirring Time: 6 Hours.

**Table 2.** Effect of salt and buffer on particle size.

S.No.	Salt / Buffer	Size(nm)	Zetapotential (mV)
1	Without Salt / Buffer	123	-28.5
2	KNO <sub>3</sub> (10mM)	207	-21.4
3	NaCl ( 10mM)	174	-23.7
4	Ca(NO <sub>3</sub> ) <sub>2</sub> (10mM)	225	-18.1
5	KCl (10mM)	176	-24.1
6	Phosphate Buffer (pH -7)	241	-23.4



**Figure 6.** SEM image of egg-white nanoparticles.

**3.6. Effect of salt and buffer.** The effect of added salt and buffer solution to the egg white solution in the process preparation of protein nanoparticles was investigated by dissolving 2ml of egg white to 18ml of KNO<sub>3</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, NaCl, KCl and phosphate buffer of pH 7 (Table. 2). The aqueous solution of egg white was stirred at 550 rpm for an hour. The nanoparticles obtained by ethanol addition were stabilized by glutaraldehyde. Only a slight change in particle diameter and zeta potential was observed on the addition of monovalent ions whereas in case of bivalent Ca<sup>2+</sup> ions zetapotential of nanoparticles increased from -28.5 to -18.1. The shielding of negative surface charge of protein particles by positively charged ions is responsible for the decreased surface charge. Due to decreased surface charge the enhanced hydrophobic interaction among the protein molecules leads to formation of larger particles. More reduction in surface charge was observed for Bivalent ions as compared to monovalent ions

which also supports the earlier report of Ko, et al. for BSA nanoparticles [45]. The addition of ethanol during desolvation process under phosphate buffered condition leads to formation of the precipitate. Nanoparticles with relatively larger particle size and reduced surface charge were observed in phosphate buffer condition this may be due to the decreased surface charge by

#### 4. CONCLUSIONS

The current study demonstrated that the simple and highly reproducible desolvation method can be adopted for the preparation of EWP nanoparticles at biological pH. The effect of various process parameters viz. egg white concentration, stirring rate, pH, glutaraldehyde amount and the addition of salt and buffer

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buffer salts. Successful preparation of albumin nanoparticles in presence of NaCl and in buffered condition has been reported by several authors [19,20,23]. Our investigation on egg white reveals that the size of nanoparticles increases in salt and buffered conditions.

on EWP nanoparticles indicated that pH is the key parameter which strongly influences the particle diameter. Except pH other parameters shows a negligible effect on EWP nanoparticles in a particular range. Drug loading and release pattern to the prepared EWP nanoparticles will be the subject for future study.

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