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PREPARATION OF SIZE CONTROLLED ALBUMIN NANOPARTICLES AND ITS *IN-VITRO* SAFETY EVALUATION

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ABSTRACT

Human serum albumin has gained popularity among researchers for the fabrication of nanoparticles (NPs) due to its biocompatible, biodegradable and non-toxic nature. Particle size is one of the critical parameter for determining the *in-vitro* cellular uptake and *in-vivo* behavior of NPs. The objective of the present study was to prepare the size controlled albumin NPs and study the impact of product and process related parameter on particle size and size distribution.The NPs were prepared by the desolvation technique. Influence of various factors including ethanol rate addition, stirring speed, amount of ethanol, albumin concentration and glutaraldehyde concentration on particle size and size distribution were extensively investigated. NPs were freeze-dried using mannitol (2%) as the cryoprotectant to inhibit the particle growth during lyophilization. The particle size and polydispersity index of the optimized albumin NPs were found to be 71.09 ± 3.12 nm and -24.5mV respectively. The images from scanning electron microscope demonstrated the spherical shape of NPs. The results from the stability studies did not show any significant change in the particle size up to 4 days at 4º and 25ºC. Moreover, the developed NPs exhibited cell viability more than 85% on HT29 and HCT cells indicating the non-toxic nature of the developed NPs.

KEYWORDS: desolvation, glutaraldehyde, cryoprotectant, lyophilization.

INTRODUCTION

Recent years have witnessed the intensification of the research in the field of nanoparticulate drug delivery systems for the treatment of various diseases mainly in cancer. Nanoparticles (NPs) are the colloidal particles consisted of polymer and vary in size from 10nm - 1000nm.[1] However, NPs > 200nm are not ideal for the nanomedicines and size < 200nm(i.e. diameter of microcapillaries) is generally preferred.[2] Moreover, NPs due to their small particle size can escape from reticuloendothelial system (RES) and have improved ability to reach the target site.[3] NPs by the mean of passive targeting exploits the enhanced permeability and retention (EPR) effect of diseased tissue due to change in physiological conditions and deliver the drug to the required site.[4] Among NPs made from different polymers, proteins are being extensively studied for the fabrication of drug delivery systems due to biocompatibility, biodegradability and low toxicity.[5]

In recent year NPs based on human serum albumin (HSA) has gained the interest of many researchers for the specific delivery of drug. Albumin is a multifunctional protein with plasma abundance of 35-50g/L of human serum and half life of 19 days.[6] HSA consists of 585 amino acid residues with relative molecular weight of 66.5kDa. HSA is stable in the pH range of 4-9 and can be heated at 60^0C up to 10 h without any deleterious effects.[7] On degradation, HSA is break down into the amino acids that can be used as nutrition by the peripheral tissues. This property allows the preferential uptake of HSA based NPs by tumor or inflamed tissues.[8] HSA can also accumulates in the tumor tissue by binding to gp60 receptors that further bind with caveolin-1 and form transcytocic vesicles.[9] In addition, it has multiple binding sites that can be used as the carrier of various drugs.[10] The familiarity of HSA based NPs are corroborated by the availability of commercial available products such as Abraxane®[11] and Albunex®[12] Abraxane® (paclitaxel HSA NPs) is approved by US FDA for the treatment of metastatic breast cancer[13] and first line treatment for pancreatic cancer in combination with gemcitabine.[14] Albunex® (HSA based air filled microsphere) is an ultrasound contrast agent for use in echocardiography and other ultrasound diagnostic procedures.[15] The clinical studies from these products have suggested that HSA based NPs have no adverse effects *in vivo*. Therefore, these properties including more bioavailability, non-immunogenicity, biodegradability and lack of toxicity makes HSA an ideal candidate for the fabrication of the NPs for drug delivery.[7]

Albumin NPs of various drugs such as paclitaxel,[16] obidoxime[17] and methotrexate[18] has been prepared by different methods. Methods for the preparation of albumin NPs includes nanoparticle albumin bound technology, thermal gelation, and self assembly.[19,20] However, desolvation is the most commonly used method as it leads to the production of NPs with size < 200 nm.[21,22] In the present study, we have prepared the HSA NPs by the modified desolvation technique followed by crosslinking with glutaraldehyde. Moreover, various factors were identified and investigated for influence of effective parameters on the particle size of HSA NPs. The effect of rate of addition of desolvating agent, stirring speed, amount of HSA, ethanol concentration, and amount of glutaraldehyde were strongly scrutinized. The NPs obtained were characterized for the particle size, polydispersity index, zeta potential and surface morphology. NPs were further freeze dried to enhance the stability of the product during storage. Furthermore, safety of the developed HSA NPs as a carrier was established by performing MTT(3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay on two colon cancer cell lines (HT29 and HCT-15) and % cell viability was calculated.

MATERIAL AND METHOD

Material

Human Serum albumin (Alburel 20%) was procured from Reliance Life Sciences, Mumbai, India. Absolute ethanol and glutaraldehyde (25%) were obtained from S.D Fine Chemicals Ltd., Mumbai, India. MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and DMSO(dimethyl sulfoxide) was purchased from Sigma-Aldrich, India. Human colon adenocarcinoma cell lines HT-29 and HCT-15 were obtained from National Centre for Cell Sciences (NCCS, Pune, India). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), Dulbecco's phosphate buffer saline (PBS) and 1 % of antibiotic antimycotic solution were procured from Himedia, India. Heat inactivated fetal bovine serum and 0.25% of trypsin EDTA solution were from Gibco, India.

Method

Preparation of human serum albumin nanoparticles (NPs)

NPs were prepared by the desolvation method with some modifications.[22] For the preparation of these NPs, 5 ml of albumin was diluted in 100 mL of water and ethanol was added at a predetermined rate with constant stirring. NPs formed were crosslinked by adding 100µL of glutaraldehyde (8%v/v) with continuous stirring for 4 h. The resulted NPs were washed twice with PBS and purified by centrifugation at 15,000rpm for 30 minutes.

Characterization of NPs

NPs were characterized for particle size, polydispersity index, zeta potential and surface morphology. To measure the particle size of NPs, samples were diluted with double distilled water and analyzed by dynamic light scattering (DLS) at the temperature of 25ºC and scattering angle of 90º using particle size analyzer (Malvern Instrument Ltd, UK). For the determination of zeta potential, NPs were dispersed in deionized water and measured by laser Doppler anemometry using Zetasizer (Malvern Instrument Ltd, UK). The particle size and zeta potential were measured in triplicates and results are presented as mean ± SEM. The surface morphology of NPs were characterized by surface electron microscope (JSM-7600F, JEOL, USA). Samples were dried overnight on copper chips and placed under scanning electron microscope and images were captured.

Freeze drying

For freeze drying, freshly prepared NPs were filled in the vials and were freeze dried in Epsilon 2- 4 LSC freeze dryer (Martin Christ, Germany) with a cycle of 24 hour. The primary drying was carried out at the shelf temperature of -55^0C for first 3 hours and at -25^0C for next 16 hours. Secondary drying was completed at 25^0C for another 5h. Cryoprotectants like mannitol and sucrose were added to facilitate freeze-drying.

Stability of NPs

To study the stability of prepared NPs, 5 ml of NPs were immediately stored in a glass vials at 4º and 25º C. Samples were withdrawn from vials over the period of fourdays and were analyzed for the particle size and polydispersity index.

Cell viability assay

Human colon adenocarcinoma cell lines HT-29 and HCT-15 were cultured in $75cm^2$ culture flask using Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI-1640) respectively added with 10% of heat inactivated fetal bovine serum (FBS) and 1 % of antibiotic antimycotic solution. Cells lines were maintained in the CO_2 incubator with 5% CO_2, temperature of 37˚C and relative humidity of 65%. After attaining confluency, cells were sub-cultured using trypsin-EDTA solution. MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay was used to assess the cell viability of developed NPs. For the assay, HT 29 and HCT-15 cells ($1X10^4$cells per well) were seeded in 96 well plate and were incubated overnight in CO_2 incubator at 37^0C. Next day, cells were treated with NPs at the concentrations of 50, 100, 150, 200, 250, 300 and 400µg/mL of HSA for 24 and 48 hr. After the given time intervals, NPs were removed and cells were washed with the PBS. Further, 100µL of MTT dye (500µg/mL) was added to each well and incubated for 4h. After incubation, MTT was removed and 100 µL of DMSO was added to dissolve the formazan crystal formed by MTT and were plates were kept on plate shaker for 5 minutes. The absorbance of the plates was read at 595 nm in microplate reader (Synergy-HT, Bio-Tek, USA) using Gen5 software. Cells were also incubated in their respective medias without any treatment and were considered as control for calculating the cell viability. The percentage cell

viability in treated wells was calculated using formula $Abs_{sample}/ Abs_{control}*100$. Where Abs_{sample} is the absorbance of treated wells and $Abs_{control}$ is the absorbance of control wells.

RESULT AND DISCUSSION

Preparation of NPs

NPs were prepared by the desolvation technique[23] with some modifications. Desolvation is the most common method used for the preparation of albumin NPs and produce the NPs with particle size less than 200nm.[21] Desolvation process includes dropwise addition of ethanol into the albumin solution at constant stirring speed until the turbidity appears. Ethanol addition to the albumin solution causes its precipitation due to reduced water solubility and results into the formation of NPs.[19] But the NPs formed by this process are not stabilized and can redissolve again on redispersion with water. So, formed NPs were crossslinked by the addition of glutaraldehyde and purified.

Influence of Parameters on Particle Size

In the present study we have identified the various parameters including rate of addition of ethanol, stirring speed, ethanol concentration, albumin concentration, glutaraldehyde concentration effecting the particle size and polydispersity index of NPs. These parameters were changed one at a time with other factors remaining constant and effect on particle size was investigated. Our aim was to optimize the process in such a way to obtain the NPs with minimum particle size and to study the influence of process parameters on particle size. As suggested in the earlier studies, submicron particles but not larger are preferentially up taken by many of the cell types.[24] Desai et al reported that the NPs with size of 100nm had 2.5 fold greater uptake rate than 1µm microparticles by Caco-2 cells.[25] Also, different NPs with smaller particle size are reported to have relatively higher cellular uptake in different cell lines via endocytocis.[26] Size of the NPs also drives several biological phenomenon including circulation time, extravasation through leaky vasculature and macrophage uptake. NPs with size less than 100nm are prove to be in circulation for the longest time.[27] Therefore, all material and process related parameters were optimized to obtain the NPs with the less particle size and more uniform distribution.

Influence of rate of addition of ethanol on particle size and size distribution

To understand the effect of rate of addition, ethanol was added to albumin solution at different rates of additions (0.5, 1, 1.5 and 2mL/min) and particle size was analyzed. As seen from Fig.1, the initial decrease in the particle size was observed with increase in the rate of addition from 0.5mL to 1mL/min. However, further increase in the rate of addition of ethanol resulted into the larger particle size. This might be due to the fast desolvation process that leads to the larger aggregates and precipitation of albumin. So, rate of addition of ethanol should be controlled and therefore it was kept at 1mL per minute for the further process. The results are in the agreement with the earlier report of increase in the particle size with rate of addition.[28] Moreover, more uniform particle size distribution was observed within the range of 0.5-1mL/min. Whereas, faster addition rate of 1.5 and 2mL/min leads to non-uniform particles due to insufficient time for the complete desolvation.

Influence of stirring speed on the particle size and size distribution

Stirring speed is one of the important factors in determining the particle size. Fig. 2 represents the initial decrease in the particle size with increase in the agitation speed from 100-500rpm. This is due to the high energy put into the system that is expected to cause the size reduction.[29,30] However further increase in the stirring speed higher than 500rpm did not cause further decrease in the particle size. Similar results were also obtained by Rahimnejad et al.[31] A more uniform size distribution was also observed with increase in the stirring speed.

Influence of ethanol HSA ratio on particle size and size distribution

Ethanol is considered to be an important parameter affecting the particle size of albumin NPs. Fig. 3 shows the effect of ethanol concentration (40-70%) with fixed amount of albumin on the particle size. The size of NPs and polydispersity index were found to be decrease with increase in the ethanol albumin ratio. The addition of ethanol reduces the availability of water to albumin leading to shrinkage of hydrated albumin chain. At a certain point, the hydration becomes too low and HSA chain gets precipitated as NPs and size decreases.[32] The results compliments the earlier study where increase in ethanol volume resulted in increase in size of bovine serum albumin NPs.[33]

Influence of HSA concentration on particle size and size distribution

The impact of different concentrations of albumin ranging from 10mg/mL to 40mg/mL on particle size and polydispersity index was also studied. Fig. 4 depicted that increase in the concentration of albumin lead to the reduction in the size of the NPs and polydispersity index as well. The results are substantiated with the earlier report of size reduction with increase in the concentration of bovine serum albumin.[34,35]

Influence of glutaraldehyde concentration on particle size and size distribution

The effect of glutaraldehyde concentration on particle size of NPs was studied by adding 50, 75 100 and 150µL of 8%v/v glutaraldehyde to NPs. As depicted from Fig. 5, no significant changes were observed in the particle size and size distribution on increasing the concentration of glutaraldehyde. The results are in agreement with the earlier reports saying little or no effect of glutaraldehyde concentration on particle size.[22,36]

Freeze Drying

Freeze drying is a process of removal of water from the product in which direct evaporation of water occurs from frozen solid state under vaccum.[37] It can convert the NPs into powder form that is more stable than the liquid product. However, freeze-drying can cause agglomeration of NPs due to water removal. So, in order to prevent the aggregation of NPs, cryoprotectants are generally used that inhibits the growth of particle size during the freeze drying process.[38] Mannitol and sucrose at the concentration of 2% and 4% were explored as the cryoprotectant during lyophilization.[39] The freeze-dried products were reconstituted with water and were analyzed for particle size and size distribution. Results of physical appearance, particle size and polydispersity index are summarized in Table1. The results obtained suggested that mannitol at the concentration of 2% was able to inhibit the growth of NPs more efficiently than sucrose. The photograph of the lyophilized NPs is presented in Fig. 6.

Characterization of NPs

The minimum particle size observed of the optimized NPs was found to be 71.09 ± 3.96nm. Polydispersity index of 0.134 indicated the uniform distribution of particles in the NPs.The zeta potential of the NPs was found to be -24.5mV. The high value of zeta potential recommends the strong repulsive forces between the particles and thus more stability. The representative plots of particle size and zeta potential are presented in Fig. 7a and b respectively.The images from scanning electron microscopy showed the spherical shape NPs (Fig. 8).

Stability of NPs

Stability is a crucial parameter to be evaluated as particles often becomes less stable due to precipitation of the particles caused by the gravitational force. However, precipitation is less likely to occur in the NPs since brownian movement provides constant motion and agitation of NPs[40]. In addition the repulsive forces among particles also avoid the agglomeration.As given Table 2, the particle size and size distribution analysis at various time points of storage suggested the good stability of the prepared NPs at both temperatures i.e. at 4º and 25ºC. Results showed that there was no significant increase in the particle size was observed after storage for 4 days.

Cell Viability

The prepared NPs were evaluated on HT29 and HCT-15 for their cell viability. The results from the cell viability of HT 29 and HCT-15 after treatment with NPs are presented in Fig.9a and b respectively. The calculated % cell viability of both HT 29 and HCT-15 cells after 24 and 48 of treatment was greater than 85%.From the results obtained it can be concluded that the NPs are non-toxic to the cells and can be safely used as the carrier for the various drugs. Results are also in agreement with the other reports showing non-toxicity of blank albuminNPs on different cells.[41]

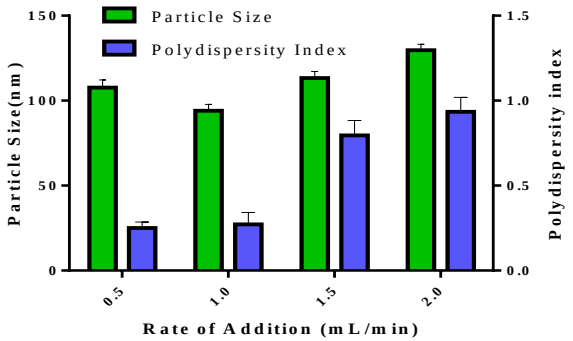


Fig 1: Influence of ethanol rate addition on particle size and polydispersity index.

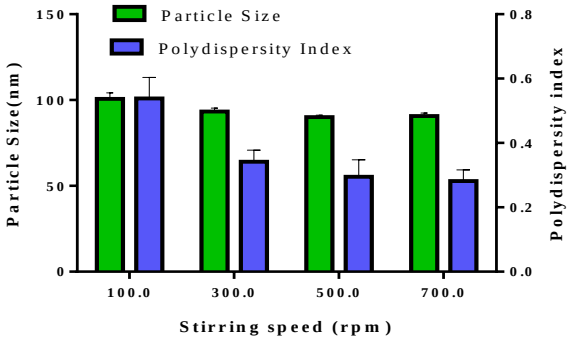


Fig 2: Influence of stirring speed on particle size and polydispersity index.

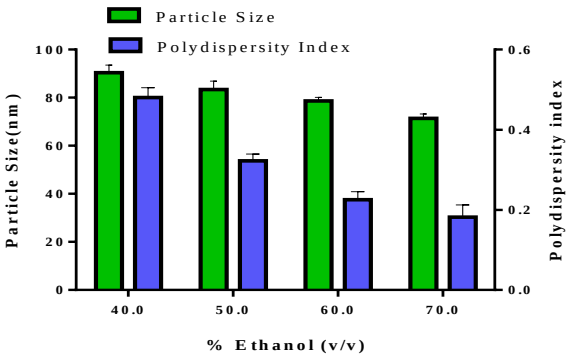


Fig 3: Influence of ethanol on particle size and polydispersity index.

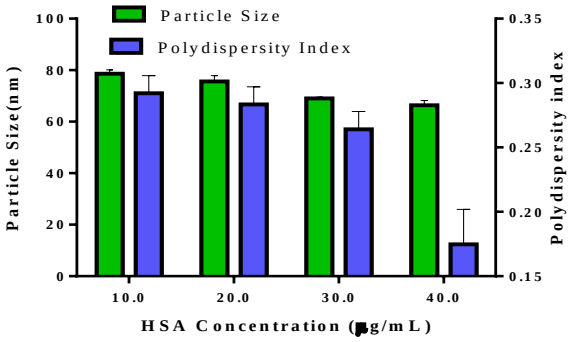


Fig 4: Influence of albumin concentration on particle size and polydispersity index.

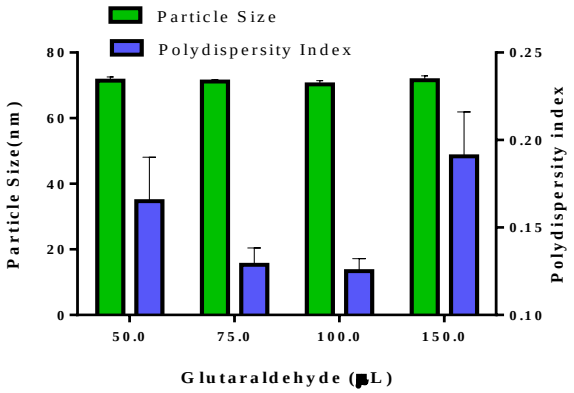
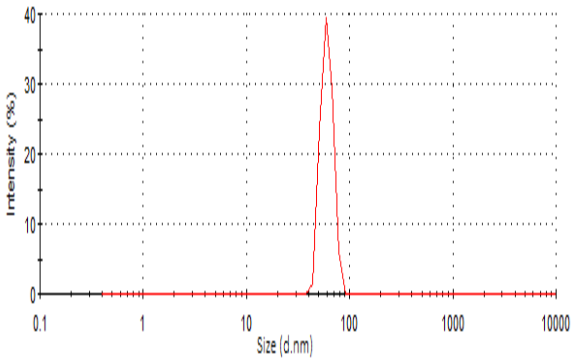


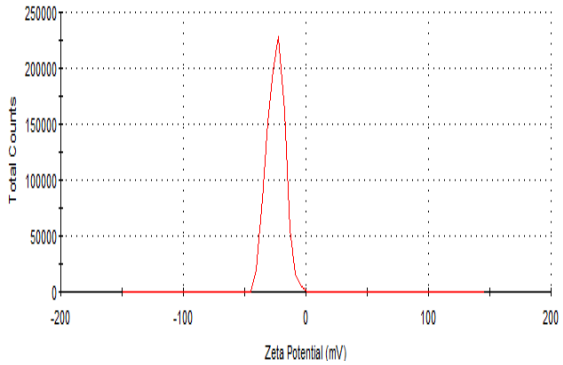
Fig 5: Influence of glutaraldehyde concentration on particle size and polydispersity index.

Lyophilized NPs

Fig 6: Photograph of lyophilized albumin NPs.



a.)



b.)

Fig. 7: a) Particle size and b) zeta potential of NPs.

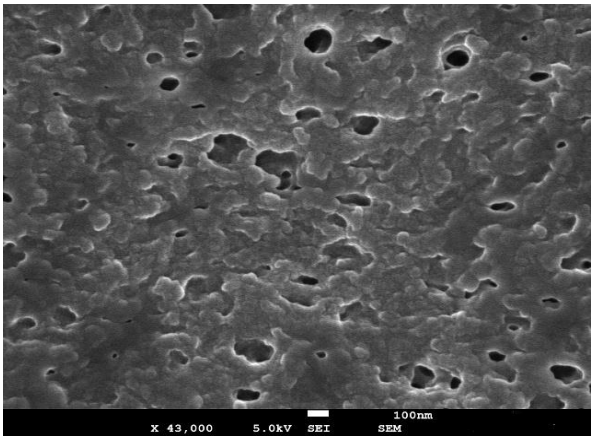
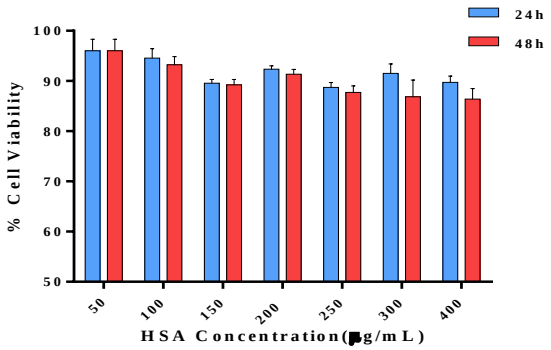
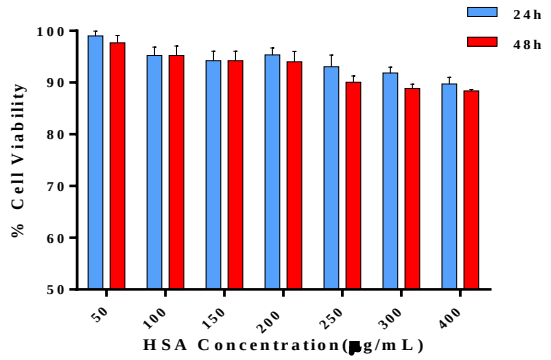


Fig. 8: Scanningelectron microscopic image of lyophilized NPs.



a)



b)

Fig. 9: Cell viability (%) of NPs after 24 and 48h of incubation with a) HT29 cells, b) HCT-15 cells.

Table 1: Cryoprotectant optimization for lyophilization of NPs.

Optimization of cryoprotectants for freeze drying of HSA-NPs			
	Cryoprotectant	**Particle Size (nm)**	**Polydispersity index**
Mannitol	-	75.12	0.260
	2%	70.2	0.210
	4%	69.7	0.149
Sucrose	-	75.32	0.140
	2%	74.8	0.419
	4%	73.4	0.216

Table 2: Stability study of NPs at 4º and 25º C.

Storage Time (days)	Particle Size (nm)		Polydispersity Index	
	4º	25º	4º	25º
1	67.43	65.30	0.276	0.216
2	69.01	70.12	0.248	0.318
4	71.90	70.19	0.120	0.301

CONCLUSION

The NPs were successfully prepared by the modified desolvation technique with particle size less than 100nm and uniform size distribution. The higher negative zeta potential value of NPs indicated more repulsion between the NPs and thus more stability of NPs. The detailed effect of various parameters was studied on particle size and size distribution of NPs. The minimum particle size and polydispersity index was achieved at the rate of addition of 1mL/min, stirring speed of 500 rpm, ethanol content 70%, albumin concentration 30µg/mL, crosslinking by 100µL of glutaraldehyde (8%v/v). The NPs were freeze-dried using 2% of mannitol with no increase in the particles size after reconstitution. The NPs were found to be stable for 4 days at both 4º and 25ºC. The cell viability of more than 85% of HT29 and HCT-15 cells after treatment with the developed NPs confirmed the non-toxicity of the NPs on the colon cancer cell lines. Thus developed NPs can be used further as nanocarrier platform for the safe and targeted delivery of drug. The modified desolvation method used and information derived from the experiments can be used further for the development of stable NPs of various drugs. Future plan includes the formulation of albumin NPs of anticancer drug for targeting colon cancer.

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Declaration of Interest
Authors declare no conflict(s) interest

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