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Albumin nanoparticles Preparation, Characterization and *In-Vitro* Safety Evaluation

Dina G.S. Youssef[a,*], Fatma A. Sallam[b], Taher A. Salaheldin[c], Samah F. Darwish[a], Abeer E. El-Metwally[d], Khaled Y. Farroh[e], Adel S. Amin[a]

a. Biotechnology Research Unit, Animal Reproduction Research Institute, Egypt.
b. Faculty of Women for Arts, Science and Education, Ain Shams University, Egypt.
c. Nanotechnology and Advanced Material Central Lab, Agriculture Research Center, Egypt; Mostafa Elsayed Nanotechnology Research Centre, British University in Egypt, Egypt.
d. Immunology Unit, Animal Reproduction Research Institute, Egypt.
e. Nanotechnology Department, Agricultural Research Center, Giza, Egypt.

Abstract:

The goal of this study was to prepare and characterize albumin nanoparticles to be later used as a drug delivery system. The utilization of nanoparticles as a delivery system for antimicrobial drugs has arisen recently which solve many problems and enhance the traditional treatment with this antimicrobial drugs. In this study, nanoparticles of bovine serum albumen were successfully obtained using a coacervation process (separation of proteins in two liquid phases in colloidal systems). The prepared nanoparticles were nearly spherical in shape and have smooth surface as determined by Transmission Electron Microscopy (TEM). The sizes of the obtained nanoparticles were 70 ± 10 nm with negative surface zeta potential. Additionally, the *in vitro* safety of albumin nanoparticles has been demonstrated. Both cytotoxicity and genotoxicity studies indicated that, there is no observed toxic effect of nano-albumin on lymphocyte cell line. Also, the results showed that the albumin nanoparticles enhances and promotes the response of immune system.

Keywords: albumin nanoparticles, cytotoxic, genotoxic, proliferation activity

1. Introduction:

Nanotechnology is the science dealing with the atomic, molecular and macromolecular levels of particles at the nano scale to provide a fundamental understanding of characters of these particles. Moreover, it is used to create and innovate many devices and systems that have innovative phenomena and functions because of their new very minute size [1, 2, 3].

Nanostructure of nanoparticles, biomaterials in particular, have new specific physicochemical properties such as large surface area verses mass ratio, ultra-small and controllable size, strong reactivity, and functionalize forms and these make them

***Corresponding author:** Dina G.S. Youssef**,** Biotechnology Research Unit, Animal Reproduction Research Institute, Egypt.
E-mail: dndn_77777@yahoo.com
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could be used in the applications of antimicrobial preparations, thus resolving problems encountered in conventional antimicrobial therapy [4,5]. Also the nanoparticles increase the therapeutic efficacy of these antimicrobials, incompetent delivery could lead to insufficient therapeutic index with localized and systemic side effects. So, the necessity for good antimicrobial drug delivery system to deliver the drug to the specific organ that harbors infection is critically important [6, 7]. Recently, utilization of nanoparticles as a delivery system for antimicrobial drugs has arisen as a new alternative that hopefully could not only minimizes the toxicity of these antimicrobial drugs but also enhances therapeutic efficacy [6].

Albumin is the most plentiful protein in human plasma; it constitutes more than 50% of human plasma proteins [8]. Albumen molecule consists from three parts working together to give albumin its discrete binding properties. Albumin contribute for 75%-80% of the normal colloid oncotic pressure of plasma. It contains long chain of solubilizing fatty acids, carriage of nutrients to cells, and also keeping the plasma pH in balance [9]. It can be used in many different formulations as nanocarrier, nanospheres and nanocapsules which all are biodegradable [10]. Many drugs and endogenous molecules can easily bind to albumin [11, 12], which acts as a depot and carrier system [10]. Its characteristic solubility which reaches up to 40% w/v at pH 7.4, nominate it as good macromolecular carrier having the ability to react with wide variety of drugs. Moreover, its stability at both wide-ranging of pH (4 to 9) and at heat (60° C for 10 hours) without any harmful effects [11, 13]. Bovine Serum Albumin (BSA) is one of the albumin proteins that is able to make complexes with different drugs and its non-toxic, biodegradable and non-immunogenic characters make it safely used as drug carrier [14, 15].

Our goal in this study was the preparation and characterization of albumin nanoparticles to be later used as a drug delivery system. Moreover, the cytotoxic and genotoxic effects of the prepared nano-albumin will be evaluated in vitro.

2. Materials and Methods:

2.1. Preparation of nano-albumin:

Bovine Serum Albumin (BSA) nanoparticles were prepared from Bovine serum albumin, Fraction V, purity min. 98% (BioShop Canada Inc) at room temperature using an ethanol desolvation technique [16]. In brief: 1g of BSA was dissolved in 5mL of 10 mM NaCl (BioShop Canada Inc) with stable stirring at 800

rpm for 10 min at room temperature. After total dissolution, the pH of the solution was adjusted to be 9.3 with 1N NaOH and stirred for 5 min. Then the additions of ethanol (BioShop Canada Inc) to aqueous phase drop-wisely to dissolve this of BSA solution with continuous stirring rate (800 rpm). The addition of the ethanol (1-2 mL/min) was until the BSA solution converted to turbid solution. Then addition of 8% glutaraldehyde (BioShop Canada Inc) as cross-linking agent was to form stable BSA particles. The achieved nano-albumin particles were washed three times by deionized water (dH_20) by centrifugation then the nanoparticles were re-suspended in an equal volume of phosphate buffered saline (BioShop Canada Inc). The given particles were lyophilized until used.

2.2. Characterization of resulted albumin nanoparticles:

Both preparation and characterization of albumen nanoparticles were performed at Nanotechnology and Advanced Materials Central Lab (NAMCL), Agricultural Research Center, Giza, Egypt.

2.2.1. Assessment of morphology of prepared nano-albumin:

To assess the morphology of the obtained albumen nanoparticles, the diluted nanoparticles solution was firstly ultra-sonicated to decrease the aggregation of particles for 5 min. Then, three drops of the solution were put on carbon coated-copper grid and left to dry at room temperature. The morphological evaluation of BSA nanoparticles was detected by capturing the deposited nanoparticles using High Resolution-Transmission Electron Microscope (HR-TEM) with voltage of 200 kV (Tecnai G2, FEI, Netherlands).

2.2.2. Assessment of the chemical properties of achieved nanoparticles:

X ray Diffraction (XRD) technique was used to assess the chemical structure of the prepared BSA nanoparticles. The XRD pattern was recorded in the scanning mode (X'Pert PRO, PANalytical, Netherlands) operated by Cu K radiation tube (= 1.54 A˚) at voltage of 40 kV and current of 30 mA. The resulted diffraction pattern was interpreted by the ICCD standard library installed in PDF4 software.

2.2.3. Estimation of the zeta potential and the particle size distribution:

Dynamic Light scattering (DLS) technique was utilized to detect the zeta potential and the average particle size distribution that were measured by Zeta Sizer (Malvern, ZS Nano, UK). The diameter and the zeta potential of the nanoparticles

were measured after dispersion in ultrapure water (1/10) at 25°C by dynamic light scattering angle of 90°C. Both particles sizing and zeta potential measurements were measured three times for each batch of particles and the results were the average of three measurements.

2.3. Assessment of the cytotoxicity effect of nano-albumin particles using *MTT* assay:

The cytotoxicity was assessed by a modified *MTT* assay [17]. Briefly, the lymphocytes were prepared [18]; lymphocyte cells were seeded into 96 well plates at a density of 5000 cells per well followed by overnight incubation. Then, the medium was aspirated and fresh RPMI 1640 medium containing different concentrations of nano-albumin ranging from (0.0001 to 10000µg/mL) per well was added. The plate was incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, 25µl of a 5mg/mL MTT (3-[4,5- Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added to each well, and the plates were incubated for an additional 4 h. After that, to dissolve the formazan crystals, 100 µL of Dimethyl sulfoxide (DMSO) (Sigma- Aldrich, St Louis, USA) was added into each well, stirred, and the absorbance of the cell suspension at 540 nm was determined using a FLUO star Omega Microplate Reader (BMG Labtech, Offenburg, Germany). Evaluation of cytotoxicity was done by comparing the absorbance of treated cells versus the untreated controls.

2.4. Assessment of Genotoxicity of prepared nano-albumin using comet assay:

The DNA integrity was evaluated using the alkaline comet assay [19]. Briefly, 20 µL whole blood was added to 1 mL RPMI 1640 in a microcentrifuge tube, then, 100 µL Ficoll histopaque (Sigma- Aldrich, St Louis, USA) was added below the blood/media mixture. After centrifugation for 30 min at 1300 x g, collect the middle layer (lymphocyte), wash with PBS for two times, the pellet was resuspended in ~1 ml of RPMI and counted over a Haemocytometer. 200 µl containing 5x 10^4 cells in RPMI-1640 culture medium were seeded into 96 well plates and 100 µl of different concentrations of nano-albumin ranging from 0.0001 to 10000µg/mL was added and incubated for 24 h. After incubation, the alkaline comet assay was proceeded to approximately 1x 10^4 cells/ 10 µl from the cell suspension. Visual scoring was performed according to tail size. Cells were classified into five classes ranging from undamaged (grade 0), to maximally damaged (grade 4) [20].

2.5. Assessment of the effect of the prepared nano-albumin on the immune response using Lymphocyte proliferation activity assay:

The proliferation activity of lymphocytes was determined by measuring mitochondrial activity using the *MTT* reduction method [18]. Briefly, lymphocyte proliferative response was estimated by stimulation of lymphocyte with phytohaemagglutinin (PHA, SIGMA) at concentration of (15ug/ml) and cultured in 96 well tissue culture plate with 10% fetal calf serum. Different concentrations of nano-albumin ranging from (0.0001 to 10000µg/mL) per well were added and incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. MTT (5mg/ml) was added as 1/10 of the media /each well followed by incubation at 37◦C for 4 hours in a humidified atmosphere with 5% CO2. Then, lyses of cells using lysis buffer and the absorbency was read at 590 nm in an enzyme immunoassay multi well photometer.

2.6. Statistical analysis:

Statistical analysis of results was determined using one way ANOVA employing a completely randomized design [21].

3. Results and Discussion:

3.1. Preparation of BSA nanoparticles:

Nano-albumin was successfully prepared based on the simple coacervation process (the desolvation technique) as described earlier. The technique was reported to be simple for preparation of nano-albumin. Moreover, the resultant nano-particles were stable in size, surface zeta potential and morphology. This technique involves the separation of the liquid phase of aqueous homogenous albumin solution. This separation leads to the formation of nanoparticles in the colloidal (or coacervate) phase [22]. In addition, the size of the nanoparticles formed by this technique can be changed based upon the various technique parameters including concentration, pH of BSA solution, volume and rate of ethanol addition [23, 24].

3.2. Characterization of nano-albumin:

3.2.1. High resolution transmission electron microscope (TEM):

The image of transmission electron microscopy (TEM) has revealed that the synthesized nano-particles are nearly spherical in shape and have smooth surface as shown in Fig. 1a. A similar conclusion was previously published by Rahimnejad. [25]

but this technique, the formed nanoparticles size can be altered as a result of the various parameters of the technique, such as pH and concentration of BSA solution, rate and volume of ethanol addition and he found that, as the concentration of BSA increased, the particle size was decreased and the minimum particle size was at BSA concentration (30 mg/ml). He also found that the particle size may not be influenced with the change of pH. Also, the rate of addition of ethanol on particle size was examined and it was found that there was no influence of ethanol concentration reflected on particle size and finally the size of the particles was expected to reduce with increasing the trend of agitation speed so the minimum size of particles was (138 nm) at agitation speed of 500 rpm. In the presented study, nanoparticles with average size 78.4 nm with the use of 20 mg/1mL BSA at pH 9.3 and ~1-2 mL of 100% ethanol with 500 rpm agitation were obtained.

3.2.2. X-ray diffraction:

X-Ray powder diffraction patterns of BSA nanoparticles are shown in Figure 1b. No peak was detected in the diffractograms. BSA nanoparticles are comprised of a dense network structure of interpenetrating polymer chains cross-linked to each other by glutaraldehyde counter ions [26]. The XRD showed greater disarray in chain alignment in the nanoparticles after crosslinks.

3.2.3. Determining Particle Size and Surface Zeta Potential:

Fig.1: 1c, 1d represents the particle size distribution curve as obtained by DLS measurements. The BSA nanoparticles surface charge, zeta potential, was (- 30 mV) and the size (69.9 nm) as measured by DLS technique [17, 26]. The bovine serum albumin (BSA) is an acidic protein where it carries a negative zeta potential in pH 9.3 and thus allows the positive molecules to bind to BSA nanoparticles [27, 28, 29].

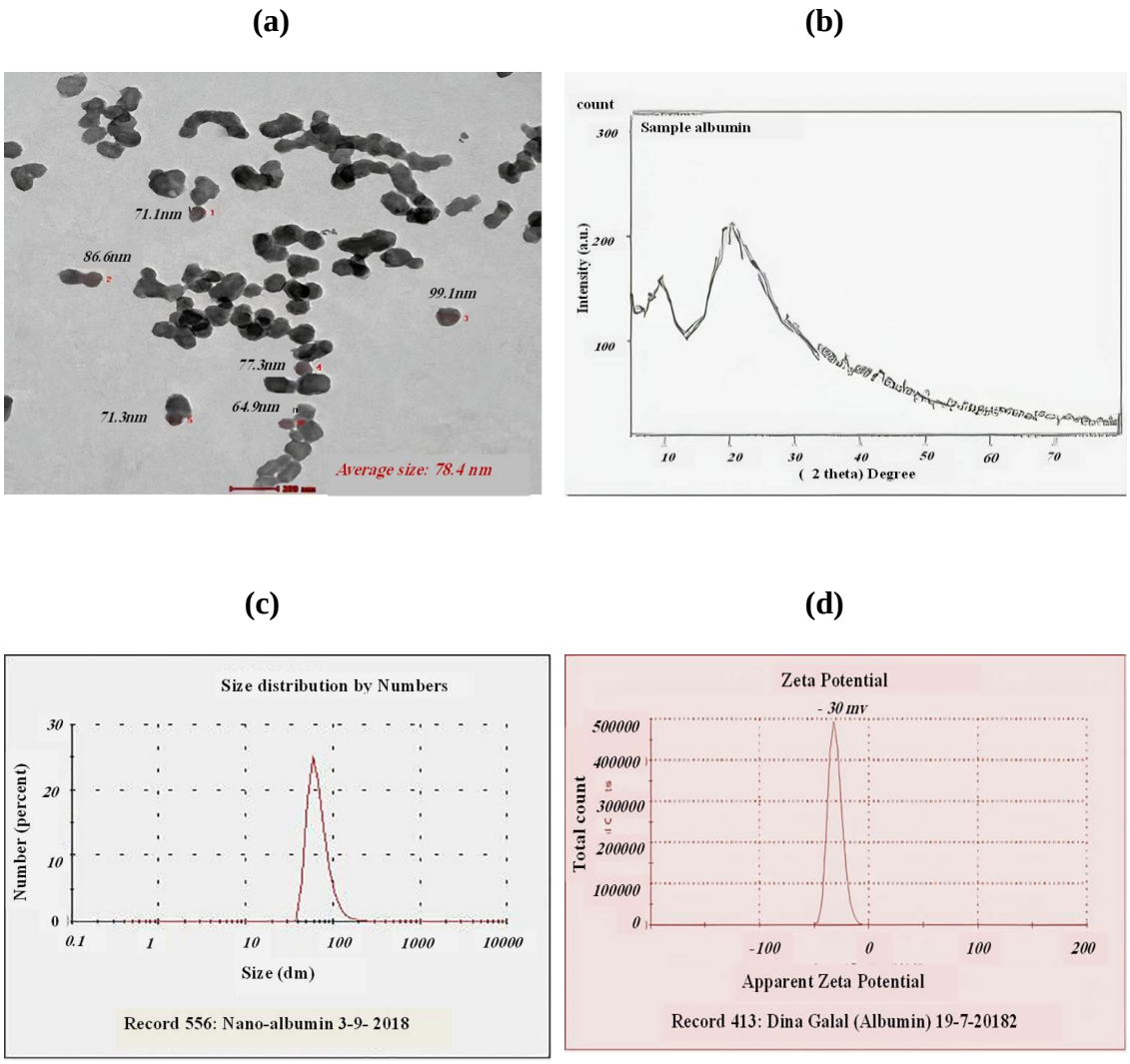

Fig. 1: Characterization of prepared nano-albumin (a) TEM image of prepared Nano-albumin (b) X-ray diffraction patterns of prepared nano-albumin, the intensity of XRD background is high at the corresponding degree of the amorphous structure of prepared nano-albumin.(c) Size determination of prepared nano-albumin (d) Zeta potential of prepared nano-albumin.

3.3. In vitro cytotoxicity (MTT assay):

The cytotoxicity of prepared albumin nanoparticles was evaluated using MTT assay using different concentration ranged from (0.0001 µg/ ml to 10000 µg /ml). As shown in table (1), it was concluded that nano-albumin has no cytotoxicity effect on lymphocytes. Similar findings were also reported by many authors [25, 30, 31]. This could be attributed to the fact that albumin is nontoxic, biodegradable and biocompatible which is also able to form noncovalent complexes with different molecules, natural and synthetic [32].

Table (1): Mean ± SE of MTT test readings of the cytotoxic effect of different concentrations of prepared nano-albumin on lymphocytes cell.

Conc. of nano-albumin µg /ml	**Mean ± SE**
Control	1.599 ± 0.026
10000	1.17 ± 0.04
1000	1.12 ± 0.041
100	1.25 ± 0.23
10	1.26 ± 0.21
1	1.07 ± 0.094
0.1	1.23 ± 0.09
0.01	1.15 ± 0.22
0.001	1.18 ± 0.031
0.0001	1.24± 0.08

3.4. In vitro genotoxicity (DNA Integrity test or comet test):

To exclude any genotoxic effect for the prepared nano-albumin, comet assay was used to detect any DNA degradation. The comet assay was used to detect the DNA fragmentation at the level of single cells (genotoxic effect). In our study, the comet assay didn't observe any significant genotoxic effects of nano-albumin with the different concentrations on the cultured lymphocyte Fig (2). The same results were previously published by Namasivayan and Robin [33] who evaluated the genotoxic effects of nano-albumin by incubating the prepared nanoparticles with the genomic DNA for 24h and then they subjected the DNA for electrophoresis. They found that the genomic DNA was not fragmented.

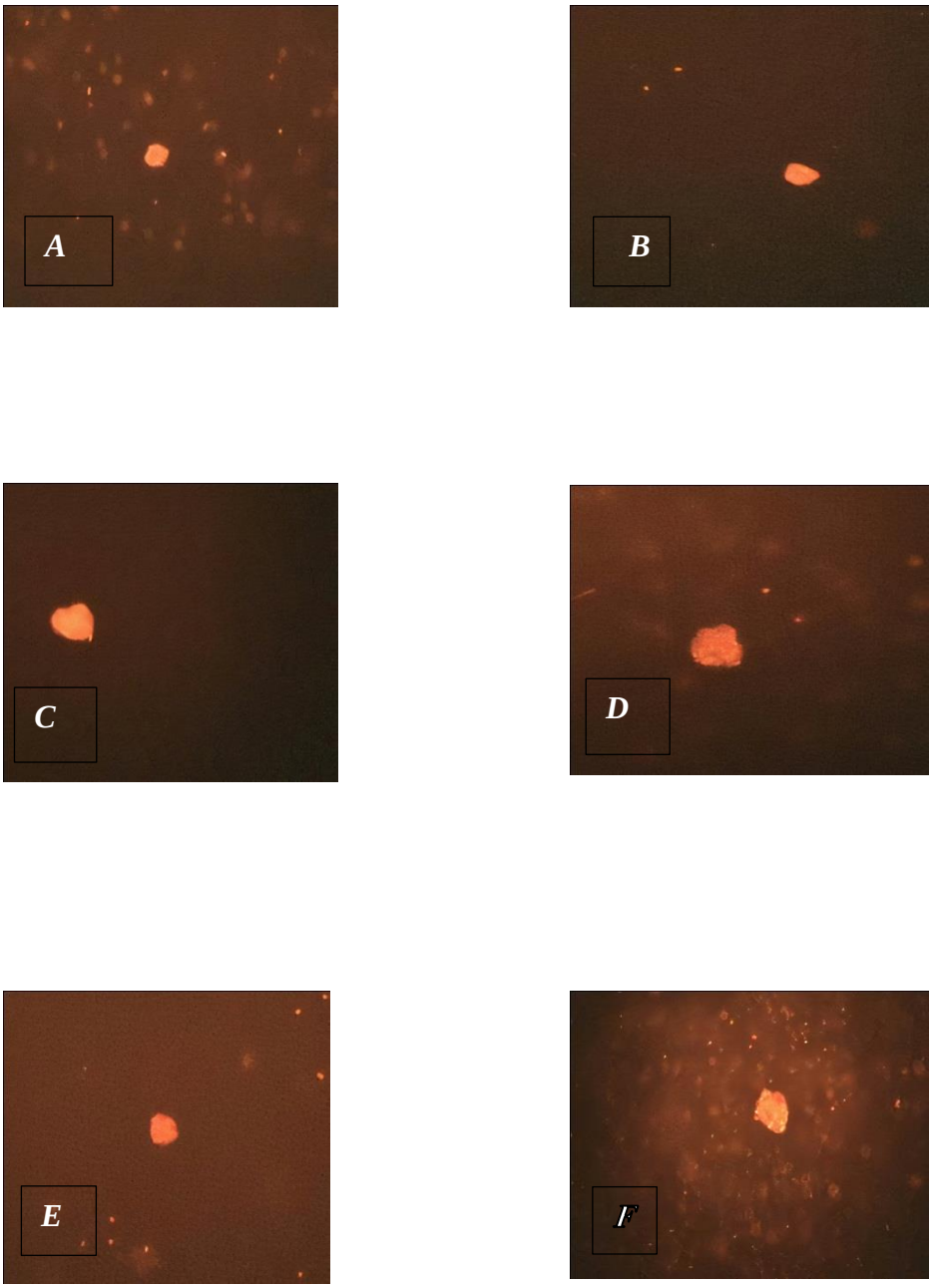

Fig. (2): Image of comet assay show no DNA damage in different groups since no DNA fragments outside the nucleus as in (A) control group and in all treated groups (B), (C), (D), (E) and (F) with different concentrations of prepared nano-albumin.

3.5. Lymphocyte proliferation activity using MTT reduction assay:

Lymphocyte proliferation activity assay showed significant stimulation of lymphocyte proliferation in case of both 100 µg/ ml and 0.0001 µg/ ml concentrations. Other evaluated concentrations of nano-albumin have no effect on lymphocyte as showed in table (2).

Because of their biodegradable properties and their ability to stimulate immune system, polymeric nanoparticles have been widely explored as new vaccine platforms which could provide continued antigen release after vaccine organization [32; 34]. Another publication reported that when the albumin has been incubated with osteoblastic cell at conc. (1mg/ml) for 24h, the cell proliferation was enhanced and this may be because different protein kinases that are partly mediated the signals of intracellular pathway [35]. So that, the albumin nanoparticles is suggestive for use as a carrier system to enhance antigen processing and/or as an immune stimulant adjuvant to enhance and promotes the response of immune system.

Table (2): Mean ± SE of Lymphocyte proliferation test readings of the effect immune reduclatory of different concentrations of prepared albumin nanoparticles on lymphocytes cell.

Conc. of nano-albumin µg/ ml	**Mean ± SE**
Control	1.814 ± 0.097
10000	1.07 ± 0.173
1000	0.998 ± 0.101
100	1.853 ± 0.284*
10	1.30 ± 0.386
1	1.73 ± 0.321
0.1	0.933 ± 0.006
0.01	1.067 ± 0.034
0.001	1.37 ± 0.194
0.0001	1.814 ± 0.127*

4. Conclusion:

This study utilized a method of obtaining Bovine Serum Albumin (BSA) nanoparticles. The *in vitro* safety of albumin nanoparticles has been demonstrated by the cytotoxicity and genotoxicity studies which indicated that there is no recorded toxic effect of albumin nanoparticles on living cell.

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الملخص العربى

الالبيومين النانومترى كحامل للمضاد الحيوى لعلاج العدوى البكتيرية

الملخص العربى:

كان الهدف من هذه الدراسة هو تحضير وتوصيف جزيئات الالبيومين النانونية لاستخدامها لاحقا كناقل للادوية وذلك لحل مشاكل كثيرة تواجهه الاستخدام التقليدى لبعض المضادات الحيوية والتعزيز من فرصة العلاج بهذه المضادات الحيوية المضادة للميكروبات. فى هذه الدراسة، تم الحصول على الجزيئات النانونية للالبيومين المستخلص من مصل صغار الابقار باستخدام طريقة الفصل التماسكى (حيث يتم فصل البروتين بين طبقتين سائليتين فى شكل غراوى). وقد كانت الجسيمات النانوية المحضرة كروية الشكل تقريبًا ذات سطح أملس كما تحدد باستخدام المجهر الاليكترونى الناقل، وحجمها يتراوح ما بين 70±10 نانومتر وسطحها يحمل شحنة سالبة. بالإضافة إلى ذلك، تم إثبات سلامة جسيمات الألبومين النانوية في المختبر، حيث أشارت كل من الدراسات السمية الخلوية والسمية الجينية إلى أنه لا يوجد تأثير سام ملاحظ لجزيئات الالبيومين النانونية على خلايا كرات الدم البيضاء الليمفاوية. كما أظهرت النتائج ان جزيئات الالبيومين النانونية تعزز من استجابة الجهاز المناعى.