Int Nano Lett (2016) 6:35–40
DOI 10.1007/s40089-015-0169-0

SHORT COMMUNICATION

Albumin microparticles as the carriers for allopurinol and applicable for the treatment of ischemic stroke

Hovsep Alexandr Aganyants[1] · Gayane Nikohosyan[1,2] · Kristine Edgar Danielyan[1]

Received: 7 April 2015 / Accepted: 18 September 2015 / Published online: 9 November 2015
© The Author(s) 2015. This article is published with open access at Springerlink.com

Abstract Albumin nanoparticles are already used for the treatment of the cancer. In our current work, it is presented the technique for the preparation of small-size 1- to 5-micron particles coated with the allopurinol. We propose that this combination of the compounds might be useful for the ischemic stroke treatment as the agent preventing formation of the brain edema, reactive oxygen species, and initiation of cells regeneration. Glutaraldehyde was used for the polymerization of albumin. Determination of the particle size was performed by the light as well as phase contrast microscopies and analyzed by Pixcavator 6.0 and Image Tool programs. Modification and establishment of iodine-based method served as the base for quantification of bound with the particles and free allopurinol. As a consequence of the experiments, the best formulation of glutaraldehyde ratio and albumin quantity as well as conditions for the formation of the smallest sized spheroid-shaped particles were found for the further in vivo application.

Keywords Albumin · Particles · Allopurinol · Cancer · Stroke

Abbreviations

ROS	Reactive oxygen species
GA	Glutaraldehyde

✉ Kristine Edgar Danielyan
kristine_danielyan@biochem.sci.am

[1] H. Buniatian Institute of Biochemistry, National Academy of Science, RA, 5/1 Paruyr Sevak street, 0014 Yerevan, Armenia

[2] Yerevan State University, Yerevan, Armenia

Introduction

In blood plasma, albumin is one of the multifunctional proteins which is found abundantly, 35–50 mg/ml [1]. Human serum albumin has a molecular weight equal to 66.348 kDa [2, 3]. Human albumin consists of three domains: I, II, and III. Each domain has A and B subdomains. Crystallographic analysis of HSA has established that this protein contains 67 % α-helical and no β-sheet [3]. The remaining structure, 33 %, consisting of the polypeptidic loop part, which makes the link between A and B subdomains, is as long as 20 amino acids. The helixes are locked together by 17 disulfide bonds, which, as believed, guarantee a thermal stability of the protein [4]. Fatty acid binding to HSA induces large-scale changes in the protein conformation, rotating domains I and III relative to domain II [4–6].

Based on Sudlow's early work, it was shown that drugs bind to two primary sites in subdomains, namely, IIA and IIIA [2, 7]. These sites are similar and contain six helixes [4]. However, recent work showed that the steroid antibiotic, fusidic acid binds specifically to subdomain IB [8].

The human albumin gene is located on the long arm of chromosome 4 at position q13.3 [3].

Albumin might be synthesized in the liver and its total amount is 13.9 mg/day [1]. The main function of the albumin is to support osmotic pressure [1]. This protein binds to various endogenous compounds including long-chain fatty acids, steroids, and L-tryptophan [9]. Albumin can also serve as a transporting agent for the delivery of copper, zinc, and calcium ions [1]. This plasma protein is able to carry medicines such as warfarin, ibuprofen, chlorpromazine, and naproxen. Binding of the compounds to albumin changes their targeting effects as well as the

circulation time [10]. Consequently, albumin might serve as a carrier not only for the medicines but also for the toxins, for instance bilirubin [1].

Compared to synthetic polymers, proteins possess several advantages: They might be degraded into the peptides by naturally occurring enzymes; in comparison with the chemically synthesized nanomolecules, they may accumulate in the body and result in toxic degradation products [11]. Electrostatic interactions, hydrophobic attractions, and covalent bindings are the mechanisms allowing attaching the drug with the carrier. Also, these nanoparticles are able to be modified and present the attached molecules to the surface of the targeted cells [11]. It was demonstrated that uptake of albumin–paclitaxel nanoparticles is presumably mediated by the gp60 transcytosis pathway and subsequent binding to secreted protein, acidic and rich in cysteine (SPARC) in the tumor extracellular matrix [12].

In our current work, we are presenting the method for the formation of the albumin particles, which are coated with the allopurinol solution.

Allopurinol is one of the classical inhibitors of Xanthine Oxidase (XO), and one of the last enzymes of purines catabolism responsible for the formation of uric acid. It has a wide utility for the treatment of podagra. The by-products of XO activity are the reactive oxygen species (ROS). Recently, allopurinol was used in the clinical trial with involvement of the stroke patients. The published results of these trials are contradictive [13, 14].

We think that albumin nanoparticles carrying allopurinol will not possess negative side effects, including triggering liver necrosis. Also, the frequency of the treatment after utility of the allopurinol-coated particles in comparison with the free allopurinol will be less, which will improve the efficiency of this medicine. Allopurinol is metabolized by the liver with the $t_{1/2}$ equal to 1–2 h. Binding with the albumin particles will prolong circulation time of this medicine and, consequently, will reduce the metabolic rate and prolong time of its gradual influence without necessity of second dosage uptake.

Materials and methods

Preparation of the nanoparticles

Different concentrations of serum albumin (Sigma) were dissolved in 1 ml of water. Numerous concentrations of glutaraldehyde and 14 ml of ethyl alcohol were added (GA; 25 %, Medisar, Armenia). We have used 20, 200, and 500 mg of the albumin for the formation of the particles as well as 50, 500, and 2000 µl of glutaraldehyde to be able to delineate the best composition for the formation of the small, spheroid-type particles. This mixture was incubated for 24 h. Also, these reactions were carried out in water vs ethanol solution. The mixture was centrifugated at 8000 RPM for 15 min. The precipitate was dissolved in 3 ml of water, and the particles were washed from the remaining parts of the glutaraldehyde. The remaining part of the precipitate was dissolved in 400 µl of 1 × PBS [15–19]. For better visualization, when the formation of particles was completed, they were mixed with 2 ml of 2 % solution of Evans Blue (Sigma).

Microscopy

The work was performed with the utility of light microscopy as well as phase contrast lenses system with 100× of objective magnification (Binocular Biological Microscope, Model BM-180/I/SP, Germany). The number of the particles as well as the size of the formed albumin polymers was assessed with the Pixcavator 6.0 as well as Image Tool programs.

Determination of the bound allopurinol with the particles

The method is based on the ability of the iodine ethylene solution (3 %) to oxidize allopurinol and to form the precipitate. The spectrophotometric method was developed, and absorption was measured at 480 nm. Particle suspension (50 µl) was dissolved in 910 µl of PBS (1×), and 40 µl of iodine solution was added (20 µl of 3 % iodine was dissolved in 10 ml PBS buffer). To measure the absorption, PerkinElmer Junior spectrophotometer was used [20].

Iodometric oxidation reaction for calculation of allopurinol

Purine derivatives such as the structures containing azo are able to form hardly dissolved complex compounds, which might be visualized by spectrophotometric techniques. The reaction was performed in acidic environment [21]. Twenty microliters of 3 % ethanol iodine solution was dissolved in 10 ml of buffer. As detecting agent, it was used 40 µl of above mentioned iodine solution. The quantity of the allopurinol/1 µl was calculated after normalization with the particles containing precipitate or supernatants control solutions based on the following equation, where X the concentration and Y is the absorption.

$$X = (Y/1.657)/50$$

Results

1. Determination of the albumin particles size utilizing Image Tool program.

The experiments were performed with three groups of particles. The total quantity of the protein, which was polymerizing, was equal to 200 mg. The analysis of the particles' size revealed that the group with the addition of 50 µl GA had a surface area equal to 62.946 ± 2.697 µ, as well as 162.775 ± 7.055 µ. Addition of 200 µl of the GA provokes particles formation with surface area equal to 78.004 ± 7.642 and 221.992 ± 39.859. Addition of 500 µl of GA results formation of the particles with the surface area equal to 62.449 ± 2.431 and 212.852 ± 15.482 µ (Fig. 1).

2. Determination of the formed particles number in the microscope field.
   By adding of 2000 µl of GA, the highest number of particles was formed and was equal to 23.3 ± 7.93. In 50- and 200-µl GA groups, the number of the particles in the statistically significant way was different from the previously mentioned group and was equal to 5.7 ± 1.136 and 6.6 ± 1.949 (Fig. 1).
3. Formation of the particles in the presence of different concentration of the protein and determination of the number of formed particles (Fig. 2).
   The number of the formed particles was evaluated after mild changes of the conditions of the experiment. After 24-h incubation, the mixture was centrifugated for 15 min, RPM = 8000. The precipitate was resuspended in water, and the samples were evaluated with the utility of light as well as phase contrast microscopy. The maximal number of the particles was observed in the samples with the addition of 20 mg of the protein 699.00 ± 35.87; in the samples with the addition of 200 mg of the albumin, the number was equal to 468.00 ± 51.87, whereas addition of 500 mg resulted in the consequential formation of 288.76 ± 17.83 particles. It is quite interesting to mention that the shape of the particles was spheroid and did not look like a flake as it is shown in the Fig. 3. To have better visualization, the particles were stained with Evans Blue.
4. Formation of the particles in the presence of different concentration of the protein and determination of size of formed particles.
   The size of the particles was gradually increasing with the increase in the concentration of the protein. In 20-mg samples the number was equal to 0.0155 ± 0.001, in 200 mg—0.036 ± 0.003, and the largest particles were formed after addition of 500 mg of protein (0.056 ± 0.003). The results of comparison of the groups were statistically significant.
   We also compared the shape of the particles before centrifugation in water vs ethyl alcohol. In ethyl alcohol solution, the particles were more condensed

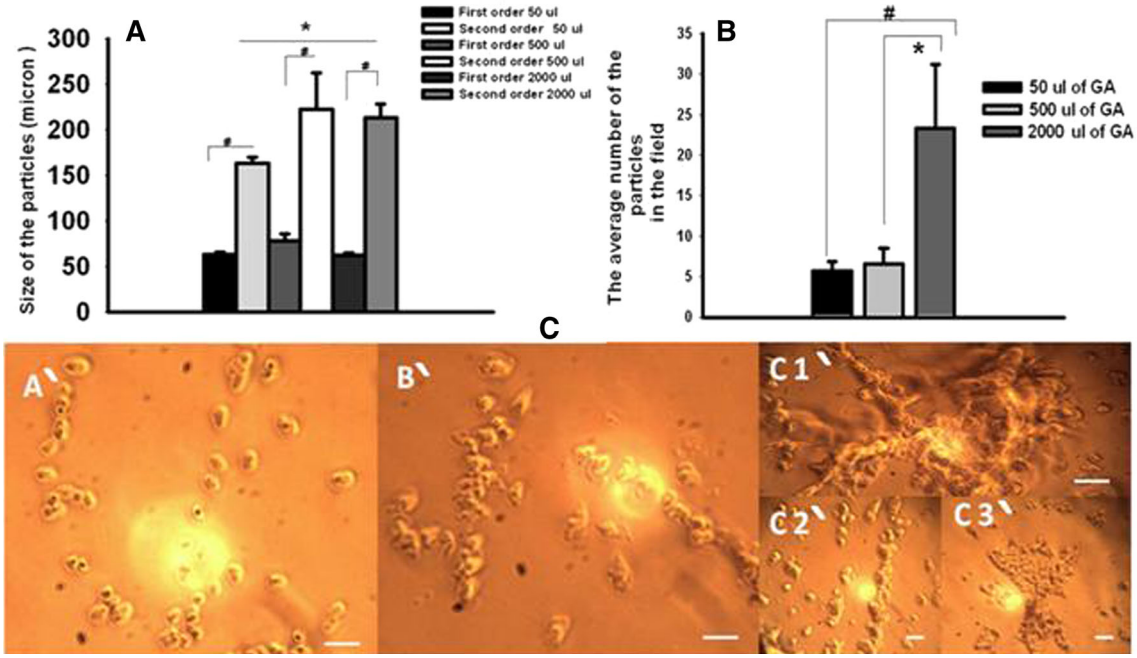

Fig. 1 Determination of the particle size after addition of different quantities of GA. After addition of 200 mg into the mixture, we have incubated the particles for 24 h. **a** The size of the particles, surface area, is presented as microns. The significance in the groups for 50, 500, 2000 µl of added GA was equal to $p = 1.62283E-27$, $p = 0.001327$ and $p = 1.72321E-18$, respectively. The difference between the size of the large particle in the groups was $p = 0.000304246$ and $p = 0.003935424$, for 50 µl GA vs 500 µl and 50 vs 2000 µl, respectively. We have used Image Tool program for the determination of particles sizes. **b** Representation of the pictures for 500 µl and 50 vs 2000 µl GA containing groups (respectively they are the pictures **a**′, **b**′ and **c1**′ + **c2**′ + **c3**′

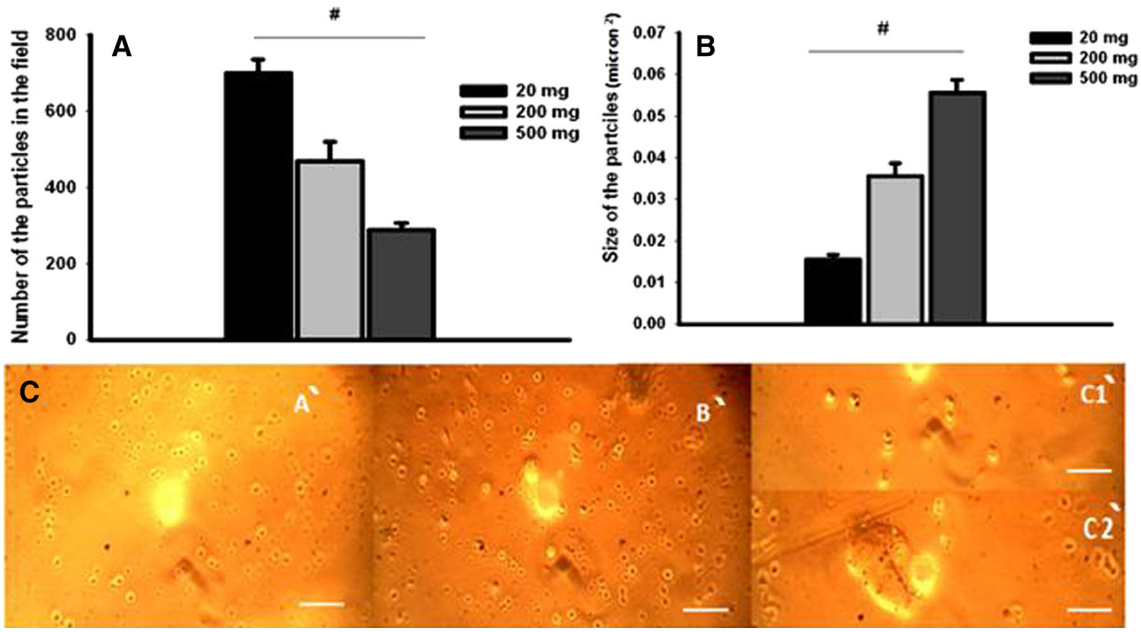

Fig. 2 Determination of the particle size after addition of different quantities of albumin. A. The number of the particles was calculated based on the micropictures and after utility of Pixcavator 6.0 program. The significances between groups for 20, 200, 500 mg of added albumin, reflected by *p*, were lower than 0.05 (one-way ANOVA). **b** The size of the particles, surface area, is presented as the μ^2. The significances between groups for 20, 200, 500 mg of added albumin, reflected by p, were lower than 0.05 (one-way ANOVA). **c** Representation of the pictures for 20, 200, 500 mg of albumin-containing groups (respectively they are the pictures **a**′, **b**′ and **c1**′ + **c2**′)

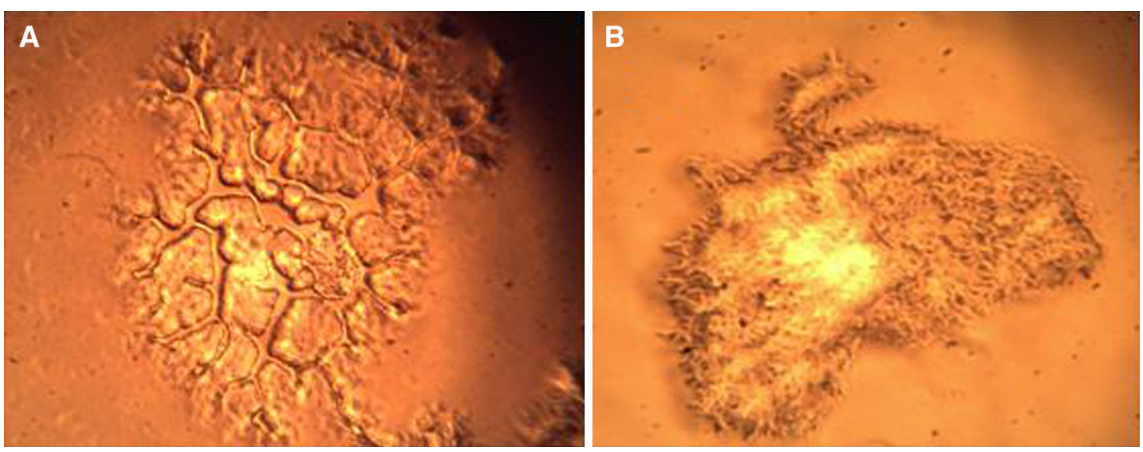

Fig. 3 Comparison of the particle shapes incubated in water vs ethanol. In **a** is represented the picture of the particle in water solution before centrifugation and, consequently, before formation of the spherical shape of it, whereas on the **b** it is represented the picture of the condensed in ethanol solution opened-shape particle

than in water solution. Thus, for the better coupling with the allopurinol, it was decided to use water instead of alcohol (Fig. 3).

5. It was also determined the quantity of allopurinol in 20-mg albumin-containing samples.
   We worked with DMSO vs water solutions of allopurinol with the concentration of 0.07 mg/ml. We have added 200, 400, 800 and 2000 µl of allopurinol solution for the incubation with the particles. Also, we evaluated the effectiveness of the binding normalized with the equal amount of allopurinol stock solutions. For 200 µl of DMSO, the percentile of bound allopurinol was equal to 110.04 ± 10.0, for 400—62.53 ± 1.25, for 800—31.26 ± 5.63 and for 2 ml—0.5 ± 0.75; for water solution of allopurinol: for 200 µl—70.03 ± 40.02, 400—57.52 ± 11.26, 800—13.76 ± 0.63, 2 ml—6.0 ± 0.0 (Fig. 4). *p* value in DMSO group was <0.05 (Kruskal–Wallis one-way ANOVA on ranks).
6. Stability experiments (Fig. 5).
   Particles were more stable until 15th h of incubation at 4 °C in DMSO solution rather than in water. The volume

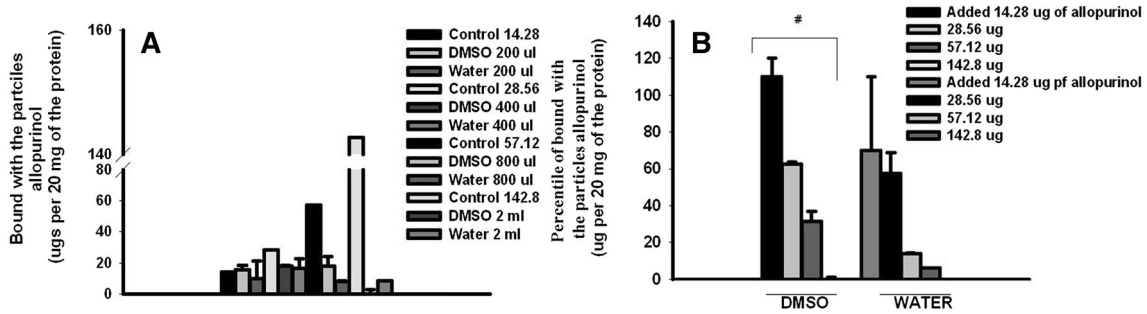

Fig. 4 Determination of bound with particle allopurinol quantity. PerkinElmer Junior spectrophotometer was used to estimate the quantity of the allopurinol in the samples. **a** Bound with the 20 mg of the particles allopurinol. Allopurinol was added as a water solution or as a dissolved in DMSO compound in the volumes equal to 200, 400, 800, 2000 µl (0.07 mg/ml). Controls point to the concentration of the allopurinol as stock solutions of comparable with the samples volumes. **b** Effectiveness (%) of allopurinol binding with the particles. The concentration of the equal volume stocks solution of the allopurinol was accounted as a 100 %

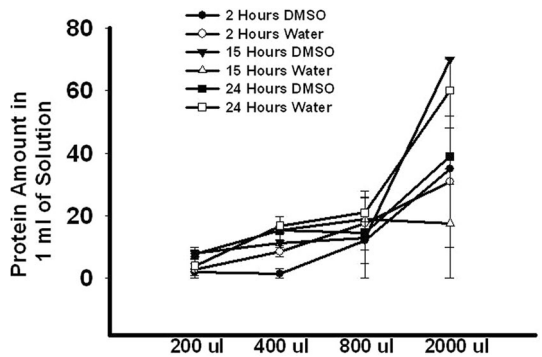

Fig. 5 Estimation of the particles stability over 24 h. Particles were suspended in water as well as in DMSO of different volumes (200, 400 µl and 800 and 2 ml). Three time points were chosen 2 h, 15 and 24 h of incubation. The suspensions were centrifugated, and the in supernatant the quantity of the proteins was assessed

of solution was also the defining factor for the stability experiments (larger volume was correlating with visualized less stability of the particles). The difference between 2-h incubation in 200 µl of DMSO and 24 h was statistically significant (2 ± 0; 39 ± 9; $p < 0.044153$); between 2 and 15 h for 400-µl volume in DMSO was also different in statistically significant way ($1.5 \pm 1{,}5$ vs 11.2 ± 1.2; $p < 0.037052$); between 2 and 24 h for 800 µl (12 ± 7.2 vs $14. \pm 0.6$, $p < 0.00226$, DMSO groups). Similar trend of dissolution of the particles was noticed for water suspensions (Fig. 5).

Discussion

There are numerous publications describing the formation of the albumin particles, which are very useful for the treatment of cancer because of specific accumulation of the albumin and because of its receptors existence in cancer tissue. The experimental mixtures might contain albumin and hypromellose acetate succinate [22], maleic anhydride molecules, resulting in disk-shaped particles, and BSA with N',N'-dimethylacrylamide in water-in-oil and using ammonium persulfate as an initiating agent [23], resulting in formation of spheroid like 1–5 µ particle [24]. We have used GA as described in the study of Zhao D. [25]. In the first set of the experiments, we were trying to evaluate the quantity of GA which will be efficiently conjugating albumin and will be forming the most useful shaped particles. After the first set of the experiments, we concluded that 50 µl of GA is the best quantity, which forms the smallest particles. During the second set of the experiments, it was found that the minimal quantity of the protein might force the formation of the small-size particle, and the number or quantity of the particles in these conditions was very high. Spheroid-type particles were chosen due to their large total surface which will be able to carry the highest concentration of the albumin. The allopurinol was not specifically conjugated with the albumin particles to guarantee the free possible penetration of the medicines in the capillaries after surface–surface tight interaction of them with the endothelial cells to provoke highly efficient penetration of allopurinol as the medicine the treatment of ischemic stroke.

Also, we noticed that the binding of allopurinol with the particles is much more effective, when DMSO and non-water solutions are used. It might be suggested that the higher effectiveness is based on abilities of allopurinol solubility in water vs DMSO. Also, from Fig. 4 it is clear that the binding process is much more effective in the smaller volumes. This process is reflecting stability of the particles in the solution. This means that in 24 h in aqueous solution or in the blood, these particles might be dissolved, which is the positive ability for these formulations and evidences about spontaneous biodegradable ability with the increase in albumin in plasma (Fig. 5). Increased plasma albumin by itself might serve as a wonderful compound in stroke treatment as the oncotic pressure-determining agent,

preventing diffusion of the water from blood via disrupted hematoencephalic barrier into the brain parenchyma.

In our studies, we have shown that inhibition of XO by its classical inhibitor might initiate regenerative processes in neuronal cell culture by the inhibition of purine catabolism by feedback mechanism as well as by the inhibition of XO-generated ROS [26–28]. On the other hand, we also propose the utility of such albumin particles for the treatment of stroke. Albumin is the compound, which might be useful in the near future for the treatment of stroke [29] as an antihemorrhagic and anti-ischemic agent. It is in clinical trials [30].

A method for the generation 1- to 5-micron spheroid particles has been adopted to assess the allopurinol quantity in the coated particles, which might be useful in the future for the treatment of ischemic stroke.

Acknowledgments The study was supported by the basic financing of National Academy of Science of Armenia.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

1. Peters, T.: All about albumin: biochemistry, genetics and medical applications. Academic Press Limited, San Diego (1996)
2. Sudlow, G., Birkett, D.J., et al.: Further characterization of specific drug binding sites on human serum albumin. Mol. Pharmacol. **12**, 1052–1061 (1976)
3. de Wolf, F.A., Brett, G.M.: Ligand-binding proteins: their potential for application in systems for controlled delivery and uptake of ligands. Pharmacol. Rev. **52**(2), 207–236 (2000)
4. Curry, S.: Lessons from the crystallographic analysis of small molecule binding to human serum albumin. Drug Metab. Pharmacokinet. **24**, 342–357 (2009)
5. Curry, S., Brick, P., et al.: Fatty acid binding to human serum albumin: new insights from crystallographic studies. Biochim. Biophys. Acta **1441**, 131–140 (1999)
6. Bhattacharya, A.A., Grüne, T., et al.: Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. J. Mol. Biol. **303**, 721–732 (2000)
7. Sudlow, G., Birkett, D.J., et al.: The characterization of two specific drug binding sites on human serum albumin. Mol. Pharmacol. **11**, 824–832 (1975)
8. Zunszain, P.A., Ghuman, J., et al.: Crystallographic analysis of human serum albumin complexed with 4Z, 15E-bilirubin-IXα. J. Mol. Biol. **381**(2), 394–406 (2008)
9. Kragh-Hansen, U.: Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev. **33**, 17–53 (1981)
10. Evans, T.W.: Review article: albumin as a drug-biological effects of albumin unrelated to oncotic pressure. Aliment. Pharmacol. Ther. **16**(Suppl. 5), 6–11 (2002)
11. Elzoghby, A.O., Samy, W.M., et al.: Protein-based nanocarriers as promising drug and gene delivery systems. J. Control. Release **161**, 38–49 (2012)
12. Nitta, S.K., Numata, K.: Biopolymer-based nanoparticles for drug/gene delivery and tissue engineering. Int. J. Mol. Sci. **14**(1), 1629–1654 (2013)
13. Dawson, J., Quinn, T.J., et al.: The effect of allopurinol on the cerebral vasculature of patients with subcortical stroke; a randomized trial. Br. J. Clin. Pharmacol. **68**(5), 662–668 (2009)
14. Taheraghdam, A.A., Sharifipour, E., et al.: Allopurinol as a preventive contrivance after acute ischemic stroke in patients with a high level of serum uric acid: a randomized, controlled trial. Med. Princ. Pract. **23**(2), 134–139 (2014)
15. Kim, T.H., Jiang, H.H., et al.: Preparation and characterization of Apo2L/TNF-related apoptosis-inducing ligand-loaded human serum albumin nanoparticles with improved stability and tumor distribution. J. Pharm. Sci. **100**(2), 482–491 (2011)
16. Sebak, S., Mirzaei, M., et al.: Human serum albumin nanoparticles as an efficient noscapine drug delivery system for potential use in breast cancer: preparation and in vitro analysis. Int. J. Nanomed. **5**, 525–532 (2010)
17. Zhang, L.K., Hou, S.X., et al.: Preparation, characterization, and in vivo evaluation of mitoxantrone-loaded, folate-conjugated albumin nanoparticles. Arch. Pharm. Res. **33**(8), 1193–1198 (2010)
18. Li, F.Q., Su, H., et al.: Preparation and characterization of sodium ferulate entrapped bovine serum albumin nanoparticles for liver targeting. Int. J. Pharm. **349**(1–2), 274–282 (2008)
19. Zhao, L., Su, R., et al.: Preparation of biocompatible heat-labile enterotoxin subunit B-bovine serum albumin nanoparticles for improving tumor-targeted drug delivery via heat-labile enterotoxin subunit B mediation. Int. J. Nanomed. **9**, 2149–2156 (2014)
20. Suprun, P.P.: Improvement of existing and elaboration of new technic of iodometric quantitative determination of xanthine derivatives by formation of periodide. Farm Zh. **26**(5), 65–69 (1971)
21. Arzamaszev, A.P.: Pharmchemistry course, 2nd edn, p. 319. Medicine, Moscow (1995)
22. Lee, K.N., Ye, Y., et al.: Formulation, pharmacokinetics and biodistribution of ofloxacin-loaded albumin microparticles and nanoparticles. J. Microencapsul. **28**(5), 363–369 (2011)
23. Sitta, D.L., Guilherme, M.R., et al.: Covalent albumin microparticles as an adjuvant for production of mucosal vaccines against hepatitis B. Biomacromolecules **14**(9), 3231–3237 (2013)
24. Sitta, D.L., Guilherme, M.R., et al.: Drug release mechanisms of chemically cross-linked albumin microparticles: effect of the matrix erosion. Colloids Surf. B Biointerfaces **122**, 404–413 (2014)
25. Zhao, D., Zhao, X., et al.: Preparation, characterization, and in vitro targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. Int. J. Nanomed. **5**, 669–677 (2010)
26. Danielyan, K.E.: Dependence of cells survival from xanthine oxidase and dihydopyrimidine dehydrogenase correlative activities in human brain derived cell culture. Cent. Nerv. Syst. Agents Med. Chem. **13**(2), 108–113 (2013)
27. Danielyan, K.E., Chailyan, S.G.: Xanthine dehydrogenase inhibition stimulates growth and development of human brain derived cells. Am. J. Med. Biol. Res. **1**(4), 95–98 (2013)
28. Gyongyan, S.A., Manucharyan, T.G., et al.: Xanthine oxidoreductase is a key enzyme of purine catabolism regulation. Electron. J. Nat. Sci. **5**(2), 17 (2013)
29. Belayev, L., Saul, I., et al.: Albumin treatment reduces neurological deficit and protects blood–brain barrier integrity after acute intracortical hematoma in the rat. Stroke **36**(2), 326–331 (2005)
30. Hill, M.D., Martin, R.H., et al.: The albumin in acute stroke part 1 trial: an exploratory efficacy analysis. ALIAS investigators; neurological emergencies treatment trials network. Stroke **42**(6), 1621–1625 (2011)