Biomedical Applications of Single Protein Nanoparticles

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Abstract: The drug carrier function of single protein nanoparticles, i.e., each individual protein molecule covered by a very thin, porous and few nanometer thick polymer layer, has been investigated. This layer around protein molecule is very thin, about 3-5 nm thick and highly porous, thus it does not reduce seriously the enzymatic function of protein molecule. The spatial structure of encapsulated protein molecule, which is essential in its function, can be stabilized by this polymer layer. Bovine serum albumin was used as protein drug molecule and it was encapsulated with acrylamide-bisacrylamide random copolymer. The polymerization, starting from the modified sites of the surface of bovine serum albumin molecules was initiated by TEMED (tetramethylethylenediamine). These single albumin nanoparticles were painted with fluorescein isothiocyanate. This material was then injected into the inferior vena cava of rats. The treated rats were decapitated after 1 to 10 minutes and its brain was investigated by fluorescent microscopy. It was proved that bovine serum albumin molecules as drugs encapsulated in polymer nano-layer with a reduced size (about 10 nm) can pass through the blood brain barrier. The results suggest that this method is capable of transformation of biomacromolecules to access the brain tissue via the blood.

Key words: Single protein nanoparticles, bovine serum albumin, drug carrier, blood-brain barrier.

Nomenclature

BSA	Bovine serum albumin
FITC	Fluorescein isothiocyanate
FITC-BSA	BSA conjugated with FITC
FITC-SPN-BSA	SPN from FITC-BSA
SPN	Single protein nanoparticles
TEMED	N,N,N',N'-tetramethylethylenediamine

1. Introduction¹

Proteins as drugs are widely applied therapeutic agents [1]. Proteins are very sensitive to a little change of their microenvironment (pH, ionic strength, temperature, etc.) [1]. Otherwise proteins have a limited biological lifetime in the systemic circulation because protease enzymes can break them down easily before protein drugs can perform its therapeutic function [2]. Proteins usually have enzymatic activity. The most effectively studied areas of enzymatic reactions are pharmaceuticals [3]. Increase of the

catalytic lifetime and stability of enzymes are crucial to widen usage of enzymes. Improvement in enzyme stability can reduce the amount of enzyme required, can extend the lifetime of enzymes and can increase the reuse of enzymes.

Protein modification is a covalent reaction between the functional groups on the surface of enzyme molecule and chemicals, e.g., polymers in order to change the chemical feature of enzyme surface, which can lead to greater stability of the pretreated enzyme [4-9]. Protein stabilization techniques, such as protein engineering [10-13], reaction medium engineering [7, 14, 15] and protein immobilization are often applied methods in order to stabilize enzyme catalytic activity, i.e., to increase its functional time [6], though immobilized enzymes often have lesser activity than the native ones [16, 17]. Protein or enzyme immobilization means an attachment of protein to the surface or onto the inner cavities of greater structures with adsorption or covalent linkage [4, 5]. Covalent linkage between the protein molecule and carrier

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material can reduce the unfolding mechanism of ternary structure of protein molecule.

Nowadays, researchers realize that the size reduction of carrier materials can increase the catalytic efficiency of enzymes. Enzyme carriers with a small size have greater active surface and to this greater surface can attach more enzymes, and it can lead to much higher amount of enzyme per unit mass. On the other hand, nanoparticles as drugs with reduced size tend to accumulate in tumors, with less side effect on other parts of the organism [18].

There are two main modes to stabilize enzymes: (1) “grafting onto” techniques which mean a two-step preparation: the nanoparticles are prepared at first and after it the enzyme conjugation comes as a second, separated process [19, 20]. (2) in the case of “grafting from” techniques, the stabilizing layer is synthesized directly from the surface of the enzyme molecules [21–27]. These methods are illustrated on Fig. 1.

Single protein nanoparticles which is applied here as nano-carrier, means that every individual protein molecule is covered with a very thin, few nanometers thick polymer layer. Stability features of single enzyme nanoparticles have been studied in the literature using organic/inorganic hybrid layer [28–32] and organic acrylic copolymer [33]. The nano-layer around the

enzymes has different functions, e.g., different types of stability increase (Fig. 2), drug carrier function, intracellular delivery agent, immune isolation function. The stabilization function can be divided as: (1) mechanical stability, when under mechanical stress (circular stirring at 150 rpm) the single enzyme nanoparticles seem to be more stable than control natural enzymes (enzymes without nano-layer) [29, 30, 32]. (2) Heat stability, when single enzyme nanoparticles have greater stability than natural enzymes at extremely high temperature (80 °C) [32]; (3) pH-stability, when the activity of single enzyme nanoparticles does not reduce under extremely acidic (pH = 1.5) or basic (pH = 12.0) pH-values, while the natural enzymes almost lose its activity [29, 32]. It can be assumed that the polymer nano-layer around the SPNs not only has stabilization function, but also has drug carrier function in living organisms. Recently, single protein nanoparticles were demonstrated as intracellular delivery platforms. The nano-layer protects the therapeutic protein against the degradation by proteases in the systemic circulation [34]. Furthermore, responsive polymer layers can release its protein cargoes in the cell under specific local intracellular conditions or events, e.g., (1) at low pH values in endosomes (pH~5.5) [34] or (2) enzyme digestion [35].

Fig. 1 Enzyme stabilization techniques using nano-sized carriers.

Fig. 2 Stability types of single protein nanoparticles.

The biocompatibility of haemoglobin nanocapsules covered by acrylamide-bisacrylamide random copolymer was investigated and good values were detected [36]. These results suggest immune isolation function of the polymer nanolayer but further investigations are needed.

Polymers are often used as protein drug carriers [37]. According to the literature, methacryloxypropyltrimethoxysilane [38] and acryl polymers [39] can go through biological membranes, have no toxicity and are biocompatible [40, 41]. These drug carriers have usually a greater size (in micrometer scale). Conventional polymeric drug carriers can not stabilize proteins, because protein drugs can be attached to the inner cavities of polymeric carriers by weak interactions only [37].

The investigation of the drug carrier function of SPNs (single protein nanoparticles) was started with studying the transport through the biological membranes. At first, the pass of SPNs through the blood-brain barrier was investigated. The blood-brain barrier is a metabolic barrier isolating the central nervous system from the systemic circulation [42-44]. The endothelial cells of blood-brain barrier are connected with very extensive tight junctions and hinder in the diffusion of hydrophilic molecules from the blood to the brain [42, 43]. Blood brain barrier normally does not allow the transport of proteins or peptide drugs [44]. For this reason, the facilitated

transport of peptide and protein drugs through the blood brain barrier means great challenges in pharmaceutical sciences [45, 46].

2. Materials and Methods

2.1 Materials

Chemicals: Acryloyl chloride (Sigma), disodium hydrogen phosphate, Potassium dihydrogen phosphate (Spektrum-3d, Scharlau), acrylamide, bisacrylamide (Sigma), 3,5-dinitrosalicylic acid (Sigma), sodium metabisulphite (Spektrum-3D), phenol (Sigma), ammonium peroxodisulphate (Sigma), fluorescein isothiocyanate (Fluka), bovine serum albumin (Sigma), TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma-Aldrich).

Instruments: Biochrom 4060 spectrophotometer (Pharmacia) was used for the detection of the protein concentration. Pictures were taken by a DP50 digital camera mounted on an Olympus BX-51 microscope (both from Olympus Optical Co. Ltd, Tokyo, Japan).

2.2 Methods

Preparation of SPNs (Fig. 3): A typical reaction contains the following steps: 250 mg of bovine serum albumin was solved in 25 ml of phosphate buffer (120 mM, pH = 7.15, with a three times ion exchanged water, specific conductivity: $\rho = 8.15 \mu\text{S}$) and 3 mg fluorescein isothiocyanate was added to the solution with a continuous stirring for 2 hours at room temperature. For the separation of the non-reacted FITC, dialysis was used (with a 12 mm diameter, 10 kDa cutoff dialysis tube, for 2×6 hours at 2°C with a 120 mM, pH = 7.15 phosphate buffer to 10,000 times solution).

Modification of BSA on the surface: the solution was cooled to 0°C and 100 μL acryloyl chloride was added and stirred over a half an hour. 152 μL of acrylamide/bisacrylamide (10:1 molar ratio of acrylamide: bisacrylamide) and 48.5 mg ammonium

peroxodisulphate and 2 μ L TEMED initiator was added into the solution under N_2 -atmosphere to initiate the polymerization reaction over 6 hours.

For the separation of the reagents and byproducts, dialysis was used again (with a 12 mm diameter, 10 kDa cutoff dialysis tube, for 2×6 hours at 2 $^{\circ}$ C with a 120 mM, pH = 7.15 phosphate buffer to 10,000 times solution).

Animal experiments: testing the permeability through the blood-brain barrier, the aforementioned material (5 mg/mL albumin nanoparticles or 5 mg/mL control) was injected (1 mL/100 g body weight, within 1 min) into the inferior vena cava of adult Wistar albino rats (200-250 g) of either sex. The vena was exposed by median laparotomy. Animals received a deep ketamine-xylazine narcosis (20 and 80 mg/kg body weight, respectively, intramuscularly) before operation. The animals were decapitated after 1 to 10 min, and the brains were fixed by immersion in 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (pH = 7.4) for 48 hours. Then coronal sections (50 μ m) were cut from the forebrains with a vibration microtome (vibratome). For negative controls, the same FITC-conjugated albumin was used but without coating by polymer layer. For positive controls, circumventricular organs (median eminence, subfornical organs, and area postrema) with 'leaky' blood-brain barrier [47-49] were studied. In some cases, the test-material was added together with 1% rhodamine solution.

3. Results and Discussion

For detection of the transport of SPNs through the blood brain barrier, fluorescent microscopy was used. FITC (Fluorescein isothiocyanate) was conjugated to bovine serum albumin (FITC-BSA) for the fluorescent images. This FITC-BSA conjugates was formed to single protein nanoparticles (FITC-SPN-BSA) as described above, and it was applied as test-material. For negative controls, the FITC-BSA was injected but without polymer layer. For positive controls,

circumventricular organs (median eminence, subfornical organs, and area postrema) with 'leaky' blood-brain barrier were studied [47-49]. The FITC-BSA delineates the cerebral vessels and shows contrast around them. The lumen of the vessels is bright (see the bright formations are pointed by white arrows on Fig. 4a) but their local environment (the brain tissue in the other side of the brain blood barrier) is dark. It means that the FITC-BSA conjugates could not be transported through the blood brain barrier (Fig. 4a).

Contrary to it, the FITC-SPN-BSA conjugates formed a fluorescent 'cloud' on the picture of the histological slide in the aforementioned circumventricular organs (Fig. 4b, area postrema). This phenomenon is a sign of exudation of FITC-SPN conjugates through from the brain vessels to the brain tissue. This experiment showed that the control material (FITC-SPN) can also diffuse to the brain in

Fig. 3 Synthesis of single protein nanoparticles. Before the synthesis of nano-layer BSA was labeled with FITC and then its surface was chemically modified (red points).

Fig. 4 (a) FITC-BSA without nano-layer was found inside the brain vessels only (arrows) (b) FITC-BSA without nano-layer covered in fluorescent "clouds" the areas of circumventricular organs (here the area postrema).

that case when the blood brain barrier is not complete.

There were different time periods between the injection of drug conjugates to the rats and the decapitation of rats (from 1 to 10 minutes). Different stages of the drug transport from the vein of the rats to the brain tissue could be detected when time periods between the injection and the decapitation were differed (Figs. 5 and 6).

When the time period between the drug injection and the decapitation of the rats was 1 min, the picture was still similar to that of FITC-BSA (Fig. 4a). The brain vessels contained FITC-SPN-BSA conjugates but the transport process from the lumen of the vessels to the brain tissue had not been finished yet. For this reason the lumen of the vessels is bright and contrast could be

Fig. 5 FITC-SPN-BSA forms clouds over the brain vessels (stars) when applied in 2-3 minutes before decapitation.

Fig. 6 FITC-SPN-BSA molecules can get through the BBB and colors green the brain tissue while rhodamine (red) remains on the vessels.

detected between the lumens of vessels and the brain tissue which remained dark.

When the decapitation of the rats followed the injection of (FITC-SPN-BSA) solutions within 2-3 minutes, around the vessels fluorescent “clouds” indicated that the exudation is just going on (Fig. 5; brain vessels are signed by white stars on the picture). No sharp contours of the vessels could be detected. Similar phenomena were found in similar post-injection time intervals by Nitta et al. [50].

In some cases (e.g., hypoxic stage of brain or inflammation) the tight junctions of the brain blood barrier are disrupted and the paracellular permeability is increased [51]. It means that free (not encapsulated) albumin molecules can also diffuse from the blood to the cerebral tissue. Rhodamine was used to check whether an occasional leakiness underlies the penetration of the test material through the blood-brain barrier. In the blood the rhodamine molecules are bound to albumin, and its free fraction is transported out of the cells by the P-glycoprotein [52]. When rhodamine was injected together with the FITC-SPN-BSA, the former one remained inside the vessels delineating them (see red lines or stripes on Fig. 6), whereas the FITC-SPN-BSA stained the cerebral tissue around (see homogenous green areas on Fig. 6).

4. Conclusion

Single protein nanoparticles were synthesized from bovine serum albumin. Acrylamide-bisacrylamide random copolymer layer was synthesized around the surface of each individual protein molecules. This polymerization was initiated from the previously modified (acrylated) sites of the surface of albumin molecules. The polymer layer is thin (about 3-5 nm thick) but highly porous and allow to maintain the enzymatic function of encapsulated protein molecules. The main function of this layer is to stabilize the structure of encapsulated protein molecule.

For the animal experiments the single albumin nanoparticles were painted by FITC. This

FITC-SPN-BSA conjugate was injected into the inferior vena cava of adult rats. Rats were decapitated after 1 to 10 minutes and histological sections of its brain were studied by fluorescent microscopy. The FITC-SPN-BSA conjugate was detected around the vessels of the rat brain within 2-3 minutes from the drug injection.

Rhodamine and FITC-SPN-BSA conjugate were also injected together and rats were decapitated after 10 minutes. In this case rhodamine remained inside the vessels delineating them, whereas the (FITC-SPN-BSA) conjugate stained the cerebral tissue around.

Results suggest that bovine serum albumin molecules as drugs encapsulated in polymer nano-layer with a small size (about 10 nm) can pass through the blood brain barrier.

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