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Human serum albumin nanoparticles for ocular delivery of bevacizumab

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ABSTRACT

Bevacizumab-loaded nanoparticles (B-NP) were prepared by a desolvation process followed by freeze-drying, without any chemical, physical or enzymatic cross-linkage. Compared with typical HSA nanoparticles cross-linked with glutaraldehyde (B-NP-GLU), B-NP displayed a significantly higher mean size (310 nm vs. 180 nm) and a lower negative zeta potential (−15 mV vs. −36 mV). On the contrary, B-NP displayed a high payload of approximately 13% when measured by a specific ELISA, whereas B-NP-GLU presented a very low bevacizumab loading (0.1 μg/mg). These results could be related to the inactivation of bevacizumab after reacting with glutaraldehyde. From B-NP, bevacizumab was released following an initial burst effect, proceeded by a continuous release of bevacizumab at a rate of 6 μg/h. Cytotoxicity studies in ARPE cells were carried out at a single dose up to 72 h and with repeated doses over a 5-day period. Neither bevacizumab nor B-NP altered cell viability even when repeated doses were used. Finally, B-NP were labeled with ^{99m}Tc and administered as eye drops in rats. ^{99m}Tc-B-NP remained in the eye for at least 4 h while ^{99m}Tc-HSA was rapidly drained from the administration point. In summary, HSA nanoparticles may be an appropriate candidate for ocular delivery of bevacizumab.

1. Introduction

Human serum albumin (HSA) is the most abundant protein in human blood, and is a natural and adequate material for the fabrication of nanoparticles for drug-delivery purposes. In recent decades albumin nanoparticles have gained considerable attention owing to their high capability to load a number of drugs in a non-specific way (Ghuman et al., 2005), as well as their tolerability when administered in vivo (Green et al., 2006). For the preparation of these nanocarriers a great variety of physico-chemical processes have been proposed, including thermal gelation (Qi et al., 2010; Yu et al., 2006a,b), emulsification (Crisante et al., 2009; Patil, 2003; Yang et al., 2007) and desolvation (coacervation) (Merodio et al., 2001; Weber et al., 2000; Wilson et al., 2012). In any case, desolvation-based procedures appear to be most suitable due to their simplicity and repeatability. However, the just obtained albumin nanoparticles are unstable and a supplementary step of stabilization or cross-linkage has to be performed in order to prolong their half-life in an aqueous environment and/or prevent the formation of macro-aggregates of the protein.

In general, cross-linkage with glutaraldehyde (GLU) is one of the most frequently implemented strategies to stabilize albumin nanoparticles. While it is highly effective for this purpose, the use of GLU (and other derivatives) is questionable due mainly to its toxicity and reactivity against some functional groups (e.g. primary amine residues) (McGregor et al., 2006; Van Miller et al., 2002). Thus, for the delivery of biomacromolecules (e.g. antibodies, proteins, peptides), this dialdehyde may also react with the biologically-active compound, resulting in an important loss of their activity and efficacy (Zhang et al., 2008). In addition, the potential toxicity of GLU is a concern for in vivo delivery (McGregor et al., 2006; Van Miller et al., 2002; Zhang et al., 2008). In order to overcome these drawbacks, different strategies have been proposed to harden the just formed albumin nanoparticles without the need of using toxic reagents. Amongst others, this stabilization of nanoparticles from proteins can be obtained through thermal treatment (Yang et al., 2007), high hydrodynamic pressure (Desai, 2006), enzymatic cross-linkage with genipin (Elzoghby et al., 2013; Kang et al., 2003) or transglutaminase (Huppertz and de Kruif, 2008).

Bevacizumab is a G immunoglobulin (MW of 149 kDa) composed of two 214-residue light chains and two 453-residue heavy chains that contain an N-linked oligosaccharide. This monoclonal antibody,

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commercially available as Avastin®, targets and blocks the binding of vascular endothelial growth factor (VEGF-A) to its receptor (Weiner et al., 2010). It was approved by the FDA in 2004 for first-line treatment of metastatic colorectal cancer, and later was approved for other cancers, such as non-small-cell lung cancer and metastatic breast cancer in combination with cytotoxic chemotherapy (Gray et al., 2009; McCormack and Keam, 2008; Perren et al., 2011).

On the other hand, bevacizumab is employed off-label in the treatment of proliferative (neovascular) eye diseases, including corneal (Chang et al., 2012) and retinal neovascularization (Campochiaro, 2013), diabetic retinopathy (Arevalo et al., 2017), and age-related macular degeneration (Chappelow and Kaiser, 2008). Regarding corneal neovascularization, bevacizumab is administered as eye drops, alone (Bock et al., 2008) or in combination with other drugs such as suramin (Lopez et al., 2017). However, topical administration of bevacizumab appears to be associated with an increased risk of corneal epithelial defects that are dependent on the dose and duration of treatment (Kim et al., 2008). One possible strategy to minimize these drawbacks would be the design of nanoparticles with mucoadhesive properties, able to prolong their residence in close contact with the corneal epithelium, in order to decrease the frequency of administration and the amount of medication given. As a result, this strategy is expected to improve patient adherence as well as the safety profile and efficacy of the treatment.

The aim of this work was to prepare and characterize human serum albumin nanoparticles as carriers for ocular delivery of bevacizumab. In addition, this work also includes the evaluation of the cytotoxicity on retinal pigment epithelium cells (ARPE-19) as well as the biodistribution of the resulting nanoparticles after ocular application as eye drops in laboratory animals.

2. Materials and methods

2.1. Materials

Human serum albumin or HSA (fraction V, purity 96–99%) and glutaraldehyde (GLU) 25% aqueous solution were obtained from Sigma (Madrid, Spain). Bevacizumab (Avastin®) was purchased from Roche (Madrid, Spain). Avastin® is provided as a concentrate for solution for infusion in a single-use vial, which contains a nominal amount of either 100 mg of bevacizumab in 4 mL or 400 mg of bevacizumab in 16 mL (concentration of 25 mg/mL). The Micro BCA protein assay kit was purchased from Pierce (Thermo Fisher Scientific Inc., Illinois, USA). The Shikari Q-beva Enzyme immunoassay used for the detection of bevacizumab was purchased from Matriks Biotech (Gölbaşı, Turkey). Absolute ethanol and dimethyl sulfoxide (DMSO) were purchased from Panreac Pharma (Barcelona, Spain). All other reagents and chemicals used were of analytical grade. The cacodylate buffer, osmium tetroxide and uranyl acetate were purchased from Sigma–Aldrich (St Louis, USA). Formvar® films were purchased from Agar Scientific (Stansted, UK). Dubelcco's modified Eagle's medium (DMEM; Sigma–Aldrich, St Louis, MO, USA), phosphate buffered saline (PBS), fetal bovine serum (FBS), fungizone and L-glutamine penicillin-streptomycin (Invitrogen) were purchased from Thermo Fisher Scientific Inc. (Illinois, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) was provided by Sigma-Life Science (Mannheim, Germany). Acuolens® eye drops were purchase from Alconcusí (Barcelona, Spain). Tin-chloride dihydrate and chlorhydric acid were supplied by Panreac (Barcelona, Spain).

2.2. Preparation of human serum albumin nanoparticles with GLU (NP-GLU)

Human serum albumin (HSA) nanoparticles were prepared by a desolvation method as previously described (Merodio et al., 2001) with some modifications. Briefly, 100 mg of HSA was dissolved in 7.5 mL purified water and the solution was titrated to pH 4.9 with HCl 1 M. Then, nanoparticles were formed by the continuous addition of 16 mL ethanol under magnetic stirring. After the desolvation process, nanoparticles were cross-linked with GLU (12.5 µg glutaraldehyde in 300 µL ethanol) by incubation under stirring for 5 min. The organic solvents were eliminated under reduced pressure (Büchi Rotavapor R-144; Büchi, Postfach, Switzerland) and the nanoparticle suspension was purified twice by centrifugation at 41,000*g* for 20 min at 4 °C (Sigma 3 K30 Osterodeam Harz, Germany). Finally, the nanoparticles were freeze-dried (Genesis 12EL, Virtis, NewYork, USA) using a 5% sucrose solution as a cryoprotector.

2.3. Preparation of bevacizumab-loaded nanoparticles

For the preparation of bevacizumab-loaded nanoparticles, the monoclonal antibody was added to an aqueous solution containing 100 mg HSA, adjusted to a pH 4.9 with HCl 1 M, and incubated for 10 min. Then, nanoparticles were obtained by the continuous addition of 16 mL ethanol under magnetic stirring. The organic solvents were eliminated under reduced pressure (Büchi Rotavapor R-144; Büchi, Postfach, Switzerland) and the resulting suspensions were purified and freeze-dried as described above. This formulation was identified as B-NP.

As control, bevacizumab-loaded nanoparticles cross-linked with glutaraldehyde (B-NP-GLU) were also prepared. For this purpose, the just formed bevacizumab-loaded nanoparticles were incubated with 12.5 µg glutaraldehyde for 5 min at room temperature. Then, these nanoparticle suspensions were purified and freeze-dried as described above.

2.4. Physico-chemical characterization of nanoparticles

2.4.1. Size, zeta potential and morphology

The particle size and zeta potential of loaded or empty HSA nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zeta Plus analyzer system (Brookhaven Inst. Corp., NY, USA). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1/10) and measured at 25 °C by dynamic light scattering angle of 90 °C. The zeta potential was determined as follows: 200 µL of the samples were diluted in 2 mL of a 0.1 mM KCl solution adjusted to pH 7.4.

The morphological characteristics of the nanoparticles were studied by transmission electron microscopy (TEM) using copper grids covered with Formvar film for negative staining. Then, 5 µL of each sample were absorbed to the grid for 30 s, followed by washing with Milli-Q water (3 times). Samples were then exposed to 2% uranyl acetate solution in water for 5 min. After removing excess stain with filter paper, the samples were air-dried and examined at 80 kV on a Zeiss Libra 120 transmission electron microscope (Stuttgart, Germany).

2.4.2. Yield

The amount of protein transformed into nanoparticles was calculated by micro-BCA analysis and microfluidic electrophoresis. Briefly, 10 mg of the nanoparticles were dispersed in 10 mL of ultra-pure water and centrifuged at 21,000*g* for 15 min at 4 °C (Biofuge Heraeus, Hanau, Germany). Then, the pellet was digested with 1 mL of 0.02 N NaOH and the mixture centrifuged. Samples of these supernatants were analyzed with the Protein Assay Reagent Kit (Pierce, Rockford, USA), following the manufacturer's instructions.

In parallel, for bevacizumab-loaded nanoparticles, the amounts of albumin and monoclonal antibody were calculated by microfluidic electrophoresis in an Experion™ Automated Electrophoresis System (Bio Rad, Hercules, USA). Again, samples from supernatants in 0.02 N NaOH were treated in non-reducing conditions following the manufacture's specification (Experion System Pro260 Analysis Kit; Bio-Rad Lab.,