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The protective effect of albumin on bevacizumab activity and stability in PLGA nanoparticles intended for retinal and choroidal neovascularization treatments

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ABSTRACT

The rapidly growing applications of antibody-based therapeutics requires novel approaches to develop efficient drug delivery systems in which biodegradable polymeric nanoparticles are amongst the best candidates. In the present study bevacizumab loaded PLGA nanoparticles were formulated by water-in-oil-in-water emulsion method. Protein inactivation and aggregation are the major drawbacks of this technique. Therefore protective ability of various stabilizers was studied during entrapment process. Probable changes in $VEGF_{165}$ binding capability of bevacizumab was assayed by ELISA which portrays the antibody's bio-efficiency. Probable breakage of bevacizumab and its secondary and tertiary structural integrity upon entrapment were analyzed by SDS–PAGE and circular dichroism spectroscopy, respectively. *In vitro* and *ex vivo* released bevacizumab from the prepared nanoparticles was also investigated. Results revealed that the protein interfacial adsorption is the foremost destabilizing factor in the double emulsion method and incorporation of appropriate concentrations of albumin could protect bevacizumab against entrapment stress. *Ex vivo* release results, in rabbit vitreous, indicated the ability of prepared nanoparticles in prolonged release of the active antibody. Consequently this approach was an attempt to achieve sustained release PLGA nanoparticle formulation with the aim of protecting integrity and performance of entrapped bevacizumab.

1. Introduction

Considering the remarkable progress in biotechnology, biological agents such as proteins, peptides, aptamers and oligonucleotides have attracted much interest (Forbes and Peppas, 2012; Leader et al., 2008). However, their pace to reach the clinical phase has faced difficulties. This may be due to certain hurdles including proteins fragility and their short half-life in the body. In order to overcome such obstacles novel controlled delivery systems have been proposed (Bilati et al., 2005). Among numerous carriers applied for this purpose, biodegradable polymers have obtained increasing interest due to their biocompatibility, non-toxicity and diversity in physico-chemical properties (Bysell et al., 2011; Mora-Huertasa et al., 2010).

Long clinical experiences have made poly(lactic-co-glycolic) acid (PLGA) one of the most popular biodegradable polymers (Danhier et al., 2012). Particulate polymeric carriers including micro and nanoparticles have shown immense potentials in the field of drug delivery (Dai et al., 2005; Parveen et al., 2012). It should be kept in mind, however, that all the formulations designed for protein delivery regardless of their shape or matter, should be able to release intact and biologically active entities. Moreover physico-chemical reactions between carriers and loaded proteins, not to mention the safety of each component of formulation, should be considered crucially (Bilati et al., 2005). Despite considerable progress in this field, the main issue still remains: preserving protein stability during system preparation and release inside the body. Denaturation and aggregation during these processes would not only therapeutically inactivate the proteins, but relevant outcomes

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could also cause immunogenicity or toxicity (van de Weert et al., 2000a; Yadav et al., 2011). To avoid these problems application of additives and protein stabilizers have been suggested. Unfortunately due to individual differences in the types of proteins, there is no general stabilizer capable of protecting all types of proteins. Therefore their feasibility should be investigated case by case (Bilati et al., 2005; van de Weert et al., 2000a).

Among proteins, the use of antibody-derived therapeutics has been growing significantly in recent years. Therapeutic antibodies are mostly used in cancer therapy; however, other diseases such as immunopathies and ophthalmopathies also act as targets for antibody therapy. The approval of more than 30 monoclonal antibodies (mAb) and mAb mediated therapeutics by the US. Food and Drug Administration (FDA) in recent 20 years and presence of a large number of these entities in the early or late phases of clinical trials portrays the remarkable progress in this area, reviewed in (Reichert, 2011). Owning to such effort, innovative approaches to develop efficient antibody delivery systems are urgently required.

Bevacizumab (Avastin®) is a humanized recombinant whole antibody against vascular endothelial growth factor (VEGF) which is clinically used to inhibit angiogenesis in the cancer tissues as well as abnormal neovascularizations. The drug has FDA approval for the treatment of metastatic colorectal cancer and it has demonstrated positive effects in other cancers in clinical trials as well (Dorrell et al., 2007). Introduction of VEGF inhibitors has revolutionized the field of ophthalmology. In recent years bevacizumab has been successfully used by ophthalmologist as an off-label drug for treatment of retinal and choroidal neovascularizations (Spaide et al., 2006). Unfortunately due to its short half-life in the vitreous, repetitive intravitreal injections are necessary to maintain the drug efficiency (Krohne et al., 2008). To address this problem, here we have prepared bevacizumab loaded PLGA nanoparticles to achieve a controlled protein delivery system. Studies demonstrated that double emulsion technique is one of the most successful methods for incorporation of hydrophilic drugs such as proteins in hydrophobic polymers like PLGA. Unfortunately it is one of the most deleterious methods as well (van de Weert et al., 2000a). There are some controversial studies which report on the less damaging conditions of other encapsulation techniques such as solid-in-oil-in-water (s/o/w) method in comparison with water-in-oil-in-water (w/o/w) method (Castellanos et al., 2002; Giteau et al., 2008a). Actually in those approaches, microparticles have been obtained rather than nanoparticles (Carrasquillo et al., 2001; Wang et al., 2004). Contrary to microparticles, it has been reported that nanoparticles with sizes of 200 nm or less could penetrate in the retina following their injection into the vitreous cavity, indicating the potential of nanoparticles for drug targeting to the retina (Yasukawa et al., 2004).

Accordingly in present research we have employed w/o/w double emulsion technique to attain PLGA nanoparticles containing bevacizumab. Although some studies have reported similar efforts (Pan et al., 2011; Li et al., 2012), to the best of our knowledge, the undesirable impacts of emulsification process on the performance and integrity of this antibody have not been considered. In this regard, current study is an attempt to investigate the protective capabilities of several additives on bevacizumab activity during incorporation into PLGA nanoparticles and optimize the obtained formulation.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic) acid (RG502H, glycolide:lactide ratio of 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Bevacizumab (Avastin®) was from Genentech/Roche (San Francisco, CA) and recombinant human VEGF 165 was from R&D Systems (Minneapolis, MN). Rabbit anti-human IgG, Fc fragment specific, biotin conjugated was bought from Thermoscientific (Waltham, MA) and horseradish peroxidase streptavidin conjugated (HRP-streptavidin) was from Invitrogen (Grand Island, NY). Rabbit anti-human Immunoglobulin was gifted by Avicenna Research Institute (ACECR) (Tehran, Iran). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from PishtazTebZaman (Tehran, Iran). Bovine and human serum albumin (BSA and HSA) and poly vinyl alcohol (PVA 22,000) were purchased from Sigma Aldrich (St. Louis, MO). All other solvents and materials were analytical grade.

2.2. Bevacizumab concentration and activity assay

Two kinds of enzyme-linked immunosorbent assays (ELISA) were developed to evaluate the concentration and activity of bevacizumab. Although usually there is a correlation between the concentration and activity of antibodies, in specific conditions due to probable conformational alterations, the antibody might lose its activity. Thus two kinds of ELISA tests were developed to distinguish between the concentration and the activity of bevacizumab. Concentration assay ELISA (CA-ELISA) was designed by anti-human immunoglobulin coated strips whereas $VEGF_{165}$ coated strips made activity assay ELISA (AA-ELISA).

To determine the concentration of bevacizumab rabbit anti-human IgG was immobilized on Maxisorp® plates (Nunc, Roskilde, Denmark) at a concentration of 1.0 μg/ml in PBS buffer (0.15 M, pH 7.4), overnight at 4 °C (100 μl/well). Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS/T) and then blocked with 2% BSA solution in PBS/T for 4 h at 4 °C (300 μl/well). After washing 3 times with PBS/T wells were dried at room temperature and stored at 4 °C for later use.

For activity evaluation of bevacizumab Maxisorp® plates were coated with the 165 amino acid variant of human recombinant VEGF at a concentration of 0.125 μg/ml in carbonate buffer (0.05 M, pH 9.6), overnight at 4 °C (100 μl/well). In continuation after washing 3 times strips were blocked, washed, and dried as same as the CA-ELISA and finally stored at 4 °C for later use.

In both ELISA testes samples with proper dilution in PBS/T containing 0.1% BSA were added into the wells (100 μl/well) followed by 1.5 h incubation at 37 °C. A standard curve was plotted with bevacizumab ranging from 2.5 to 80 ng/ml. The bound bevacizumab was detected by rabbit anti-human IgG (Fc fragment specific)-biotin conjugated at dilution of 1/80,000 in 1% BSA–PBS/T for 1.5 h at 37 °C, followed by 20 min incubation with HRP-conjugated streptavidin at dilution of 1/10,000 in PBS at room temperature. Color development was performed with TMB and the reaction was stopped by adding sulfuric acid (20%). Optical density (OD) was recorded at 450 nm using a plate reader (ELX800, BioTec, Winooski, VT)).

In addition to proving the specificity of the developed AA-ELISA for bevacizumab, its performance for an irrelevant antibody -trastuzumab (Herceptin®, Hoffmann-La Roche Inc, Nutley, NJ) – was studied.

2.3. Preliminary studies

To investigate the effects of double-emulsion nanoparticle preparation technique on the performance and structure of bevacizumab, some preliminary studies seemed to be necessary. In this regard, a nanoparticle formulation screening was performed to obtain a pilot pattern for nanoparticle preparation. Following preliminary studies were conducted under the resulted conditions of the pilot formulation.