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Arming embolic beads with anti-VEGF antibodies and controlling their release using LbL technology

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ABSTRACT

Transarterial chemoembolization (TACE) is used to treat various types of hypervascular tumors such as hepatocellular carcinoma and renal cancer. However, embolization and blocking of blood vessels nourishing a tumor mass evokes an angiogenic response due to the secretion of vascular endothelial growth factor (VEGF), which results in the formation of new blood vessels and eventually limitation in therapeutic efficacy. The presented work investigates the feasibility of loading the clinically used embolic beads (DC Bead®) with Bevacizumab (BEV), an anti-VEGF antibody, and control its release kinetics via Layer-by-Layer (LbL) coating. This strategy has the aim to achieve high, localized and sustained concentrations of BEV at the tumor site and reduce drug exposure in the systemic circulation. High loading of BEV on lyophilized beads of about 76 mg BEV/bead vial was achieved. LbL coating was carried out by depositing alternating layers of the biocompatible polymers alginate and poly-L-lysine. Coating was proven successful by monitoring the reversal of zeta potential after addition of each layer. Morphological changes of the bead surface before and after coating were illustrated using SEM imaging. Moreover, release profiles from different formulations were studied and results showed that optimizing the number of deposited layers effectively slows the release of BEV for three days. Activity of released BEV was studied in different 2D and 3D cell based assays. Released BEV fractions showed comparable activity to fresh BEV solution used as control after 3 days. In conclusion, our results suggest the opportunity for loading anti-VEGF antibodies on commercially available embolic beads to increase the efficacy of TACE of hypervascular tumors.

1. Introduction

Therapeutic embolization is the selective transcatheter blockage of blood vessels or diseased vascular structures by administration of embolic beads to occlude the arterial blood supply to hypervascular tumors as hepatocellular carcinoma (HCC) and renal cancer. This results in an infarct and subsequently necrosis of the tumor, ideally maintaining normal blood supply to surrounding healthy tissues [1–4]. The clinical effects are even enhanced by the injection of a chemotherapeutic agent followed by embolic beads into the feeding artery. This technique is called transarterial chemoembolization (TACE), a clinically established technique often recommended as the preferred treatment option for unresectable, intermediate stage HCC as a palliative therapy [3,5]. TACE is particularly useful in the treatment of liver cancer because of the dual nature of hepatic blood supply from both the hepatic artery (25%), and the portal vein (70%). Most liver tumors derive their blood supply predominantly from the hepatic artery, such that blood flow through these vessels can be occluded without subsequent complete ischemia of the whole organ [3]. TACE has been found to be more effective than non-active treatment. A meta-analysis, which included five randomized controlled trials showed that TACE significantly reduced the overall 2-year mortality rate compared with non-active treatment [6]. This was likely due to the dual effect of embolization and anticancer drug activity, besides resulting in a higher local concentration of drug in the target tissue, leading to less systemic side effects [7]. Today, transarterial chemoembolization (TACE) has become the most common approach for the management of HCC without curative options [8].

Conventional TACE (cTACE) technique based on concurrent injection of beads and chemotherapeutic solution was improved recently with the introduction of drug-eluting beads (DEBs). This is because DEBs allow for the controlled release of the drug in a loco-regional fashion at the site of the targeted tumor tissue, thereby minimizing leakage of the drug to the systemic circulation [9]. Recent clinical results comparing the use of doxorubicin-loaded beads versus conventional doxorubicin formulations for transarterial chemoembolization suggest a significant benefit for drug-eluting beads in terms of reduced side effects and increased efficacy for patients with advanced disease [10]. Among DEBs, DC Bead® microspheres (Biocompatibles, Farnham, United Kingdom, also called LC Beads in the United States), have been extensively characterized for doxorubicin [11] and irinotecan [12] delivery. They have received marketing

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authorization in Europe for the treatment of malignant hypervascular tumors and loading with doxorubicin. DC Bead® microspheres consist of a poly(vinyl alcohol) hydrogel modified with sulfonate groups imparting a significant number of negative charges on the microsphere surface and allowing for the loading of positively charged molecules [13].

Nevertheless, one major limitation of TACE is that the residual viable tumor responds by activating the expression of numerous hypoxia-responsive genes, inducing a sustained expression of vascular endothelial growth factor (VEGF) [14]. This leads to the formation of new blood vessels (angiogenesis) and increased microvessel density (MVD), resulting eventually in a limitation in therapeutic efficacy of treatment [15]. Different strategies are available to counteract this phenomenon, among which is the use of anti-angiogenic drugs (e.g., tyrosine kinase inhibitors (TKIs) such as sunitinib) and anti-VEGF monoclonal antibodies (e.g., bevacizumab, ranibizumab), which have proven efficacy and are already available in the market [16]. Our group has previously proposed the use of sunitinib-eluting beads for TACE [17]. In this present study, we explored the approach of arming embolic beads with the anti-VEGF monoclonal antibody, bevacizumab.

Bevacizumab (BEV; 149 kDa, pI = 8.3), marketed as Avastin® (Roche), is an anti-VEGF, recombinant humanized monoclonal IgG1 antibody, approved by the FDA for intravenous use for treatment of metastatic colorectal cancer [18,19]. Given the role of VEGF in HCC, and the changes in VEGF expression associated with TACE, combination of TACE with intravenous bevacizumab was an interesting treatment option to test, and recent clinical studies have shown this combination to be safe and effective for HCC patients [5,14]. However, VEGF plays important physiological roles in other parts of the body and systematic inhibition of VEGF would disturb these functions. Common adverse events associated with systemic administration of BEV are hypertension, hemorrhage, and arterial/venous thromboembolism [20,21]. These findings highlight the need for a delivery system that can achieve high, localized and sustained concentration of BEV exclusively at the tumor site and reduce exposure in the systemic circulation. Therefore, formulation of embolic DEBs releasing BEV to the tumor mass after blocking its blood supply may be a promising strategy [22].

Very few publications addressed the topic of loading BEV onto embolic beads. Only very recently, Bedouet et al. [23] published on loading and release of BEV from degradable embolization microspheres. In these studies, BEV was released over the course of 6 h. To the best of our knowledge, no work has been published on loading BEV onto marketed beads such as DC Beads®. As shown by kinetic studies [14], the increase in VEGF serum level after TACE is continuous for at least one month. Therefore, our strategy was to load the beads with BEV followed by applying a coating film, using Layer-by-Layer (LbL) technology that would lead to a sustained release profile of the protein content. We also aimed at minimizing the burst release phase usually encountered with protein formulations, while maintaining maximum possible activity.

LbL deposition is an established method for the fabrication of multicomposite ultrathin films on solid surfaces. Typically, this technique is based on the use of polyelectrolytes of opposite charges assembled layer-wise on the surface of interest, thereby building up a layered system of tunable characteristics in terms of composition, nanometer range thickness, surface charge, permeability, and elasticity. More particularly relevant to protein formulation, LbL deposition has the advantage of utilizing mild conditions (e.g., aqueous solutions), which are more favorable to preserve fragile protein folding and activity in contrast to organic solvents typically employed in the fabrication of many other protein formulations [24]. The choice of polyelectrolytes is a critical process and should be ultimately based on the target product profile. As the case at hand represents an injectable product, biocompatibility and/or biodegradability were of great concern. Accordingly, alginate sodium (Alg) as a biocompatible negative polyelectrolyte and poly-L-lysine (PLL) as a positive biodegradable polyelectrolyte were chosen as building blocks.

In this report, different strategies were adopted to load BEV on beads and loading kinetics were studied. Deposition of polyelectrolyte layers was followed and proven by zeta potential measurements and the coating was visualized using scanning electron microscopy (SEM) imaging. The ability to extend BEV release up to three days was demonstrated. Finally, the activity of BEV released from beads was tested using conventional MTT assay and a fibrin bead assay, a 3D anti-angiogenic test developed in-house. Such studies are crucial to develop a better understanding of protein interactions with embolic beads as well as to judge the feasibility of using LbL technology to deliver proteins in a controlled manner.

2. Materials and methods

2.1. Materials

DC Bead® microspheres, hereafter referred to as "beads", diameter range of 100–300 μm were purchased from Biocompatibles Ltd. (Farnham, United Kingdom). Bevacizumab (BEV, Avastin®, Roche) was bought from a local pharmacy. Alginic acid sodium salt from brown algae (Alg, low viscosity powder, 120,000–190,000 g/mol, mannuronic acid to guluronic acid (M/G ratio) 1.56) and poly-L-lysine (PLL, 43,300 Da) were purchased from Sigma Aldrich (Hannover, Germany). Micro BCA Protein Assay kit was obtained from Thermo Scientific (Weil am Rhein, Germany) and used according to the manufacturer's instructions. Other chemicals were of analytical grade and were used as received without further purification.

2.1.1. Cell based studies

Human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts (NHDFs) were purchased from PromoCell (Boston, MA, USA). HUVECs were cultured in EGM-2 (endothelial growth medium-2). The supplements added to EBM-2 (endothelial basal medium-2) to yield EGM-2 were fetal bovine serum (FBS, 2% [*v*/*v*]), human fibroblast growth factor-B (hFGF-B) at 10 ng/ml, human epidermal growth factor (hEGF) at 5 ng/ml, human vascular endothelial cell growth factor (hVEGF) at 0.5 ng/ml, long R insulin like growth factor-1 (R3-IGF-1) at 20 ng/ml, ascorbic acid at 1 μg/ml, hydrocortisone at 0.2 μg/ml and heparin at 22.5 μg/ml. NHDFs were cultured in FGM. The supplements added to FBM (fibroblast basal medium) to yield FGM (fibroblast growth medium) were basic fibroblast growth factor (recombinant human) at 1 ng/ml and insulin (recombinant human) at 5 μg/ml. Cells at passages 2 to 6 were used for MTT and fibrin bead assays. All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air (HERAcell, Heraeus, Zurich, Switzerland).

2.2. Methods

2.2.1. Loading kinetics of BEV on beads

One bead vial contains about 2 ml of beads in a total volume of 6 ml saline. To weigh the beads, the concept of wet weight was adopted, where a vial of bead suspension in saline was mixed well, then aliquots of 150 μl suspension were transferred into pre-weighed small glass vials and beads were left to settle completely. Afterwards, supernatant was carefully removed as much as possible using a syringe, and the weight of swollen beads (wet weight) was determined [25]. Two distinct strategies were tested to load BEV on beads. The first approach was simply incubating about 100 mg of wet beads in 1 ml of a 2 mg/ml BEV solution, prepared by diluting 80 μl of commercial Avastin® solution (25 mg/ml) to 1 ml of pH 5.5 MilliQ water. In the second approach, beads were first lyophilized in the presence of 2% sucrose as a cryoprotective. Then, lyophilized beads were rehydrated with the same BEV solution described before. In both cases, the bead suspension was maintained under gentle shaking for 120 min at room temperature using a rotor shaker at 20 rpm. Loading kinetics were studied by withdrawing 150 μl of the bead-free supernatant at preselected time points (after 5, 30, 60 and 120 min), which was replaced with equal amounts of MilliQ water at the same pH. BEV samples were analyzed for protein content using