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Double-targeted polymersomes and liposomes for multiple barrier crossing

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ABSTRACT

In order to treat metastasis in the brain, drug delivery systems must overcome multiple physical barriers between the point of administration and the target, such as the Blood-brain barrier, that hinder their free access across them. Multiple targeting approaches arise as a promising alternative to this barrier and target certain tissues inside the brain at a time. Herein, two surface modification methods are presented to obtain dual-targeted vesicle-like carriers functionalized with an MCF-7-specific phage protein and a BBB-specific peptide, providing the system the ability to cross a BBB model, target breast cancer cells and deliver its payload. The aim of this study was to compare new designed polymersomes with liposomes, a well-established delivery vehicle, in terms of drug loading, targeting, release and tumor cell killing. The bilayer structure of both systems allowed the conjugation with different ligands both by insertion and covalent binding. Different behaviour was observed in release, uptake and tumor cell killing corresponding to differences in membrane permeability of both vehicles and type of targeting and ligands' combination. Preliminary results showed that both formulations were able to cross the BBB monolayer without harming it, showing cytotoxic activity in the abluminal compartment.

1. Introduction

Targeting has been one of the most important topics in drug delivery research throughout the years, as it is a crucial factor to guarantee their delivery to the disease site at a sufficient concentration. However, before reaching their targets, nanocarriers must also face other obstacles, such as physical barriers between the bloodstream and cells (Debbage, 2009). Overcoming these obstacles becomes particularly challenging when it comes to the blood-brain barrier (BBB), which is the most restrictive biological barrier in the body as it protects the CNS structures from intrusion of pathogens and large molecules, hence the BBB is the main bottleneck in brain drug development (Pardridge, 2005). For this reason, the brain is regarded as a sanctuary site for many diseases, such as metastatic tumor cells, where they exist partially protected from drugs (Palmieri et al., 2007).

Brain metastases occur late in the progression of multiple types of solid tumors in up to 30% of patients (Pestalozzi, 2009) and is associated with poor patient survival (Palmieri et al., 2007). Because of this high incidence of brain metastases in systemic malignancies and their inaccessibility, metastasis to the CNS remains a major cause of mortality in patients with advanced cancer (Kenchappa et al., 2013; Rahmathulla et al., 2012). The incidence of brain colonization strongly depends on the tumor type and molecular subtype, arising up to a 15–25% when coming from breast primary tumors. Current therapeutic approaches for brain metastasis include surgery, radiotherapy, chemotherapy or a combination of them, which have to be tailored to each individual patient and achieve from 4 to 24 months of patient survival. Recently, molecular targeted therapies have gained increasing interest because of the elevated expression of several receptors in metastatic progression that could serve as targets for treatment (Caffo et al., 2013). Several monoclonal antibodies and small molecules that inhibit key receptors such as HER-2 or VEGF, have demonstrated improvement in clinical trials delaying breast cancer metastasis progression (trastuzumab and lapatinib) or

Abbreviations: 3B, polymersomes from amphiphilic triblock copolymers; DMPG, phage fusion coat protein pVIII bearing DMPGTVLP; CHOL, methacryloylated cholesterol derivative; REG, regulon peptide; PMOXA-PDMS-PMOXA, poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline).

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increasing overall survival (bevacizumab) (Caffo et al., 2013; Kodack et al., 2012). However, most of these drugs are not conceived to treat breast cancer metastasis concretely in the brain, like trastuzumab, which is not able to cross the BBB (Caffo et al., 2013).

Aiming to design more effective therapies, targeted drug delivery is a promising tool to explore. It plays an important role providing materials that can potentially be designed to carry out multiple specific functions at once. The first generation of these systems mainly aimed to address single challenges, such as the need to target a drug to a specific tissue. Now, research has led to the development of systems that can perform two or more functions (either simultaneously or sequentially) to overcome multiple physiological barriers to optimize delivery to the required target sites (Torchilin, 2009). Similarly, the ability to cross the blood-brain barrier while potentially targeting a specific group of cells requires several things to happen together, this means being able to find the CNS, cross the BBB without harming its integrity, target cancer cells inside the brain and release the therapeutic agent (Silva, 2008). Indeed, dual-targeting strategies using several ligands have already been used to treat malignancies in the brain. An example of this is a drug delivery system bearing two ligands, which present affinity for two different receptors that demonstrate an increase in drug concentration in the brain (Ying et al., 2011). On one hand, 4-aminophenyl-α-D-manno-pyranoside (MAN), that shows high affinity for GLUT1, a receptor present in the BBB involved in the transport of glucose into the brain, would allow the system to firstly cross the barrier, and on the other hand, the use of transferrin as the second ligand, would help the binding to glioma cells inside the brain thanks to the presence of transferrin receptor, whose expression is much higher in tumour cells than in healthy tissue (Ying et al., 2010).

Likewise, in this work, a dual-targeted approach is presented to treat metastatic breast cancer into the brain, combining the use of an MCF-7-specific phage fusion pVIII coat protein and a BBB-specific peptide providing the system the ability to cross the blood-brain barrier and address breast cancer cells (MCF-7) in a metastasis in the brain. On one hand, one of the most recurrent mechanisms to go through the BBB is receptor-mediated transcytosis, taking advantage of different transporters and receptors present at the BBB. This is the case of low-density lipoprotein receptor-related protein (LRP-1), a multifunctional endocytic receptor that mediates the internalization and degradation of multiple ligands involved in diverse metabolic pathways (Gabathuler, 2010). Herein, a 59-residue novel peptide ligand of LRP-1, developed by Regulon Inc., was used as the targeting moiety to penetrate a BBB model, as the intravenous administration of a liposomal formulation of this peptide into animals resulted in higher concentrations in the brain rather than in the liver (Borros et al., 2014). On the other hand, the use of a phage fusion coat protein pVIII bearing DMPGTVLP peptide as targeted delivery ligand in doxorubicin-loaded PEGylated liposomes (Wang et al., 2010a) and in Paclitaxel-loaded polymeric micelles (Wang et al., 2010c) have shown an increase in binding and enhanced cytotoxicity in MCF-7 (Fagbohun et al., 2012), thanks to its specificity for this cell line and endosomal escape ability (Wang et al., 2010b). The acid groups present in its N-terminus, aspartic and glutamic acid, act absorbing protons like a “proton sponge” in an acidic environment which results in a swelling and rupture of the endosomal membrane.

In order to perform this dual-targeting approach, the selected nanocarrier should have a specific structure, so that its surface modification to attach both targeting ligands can be achieved in a fast and easy way. Vesicle-like carriers arise as promising candidates thanks to their lipid bilayer, as amphiphilic proteins such as phage proteins, can be spontaneously inserted into bacterial membranes and lipid bilayers of liposomes (Kuhn, 1995). On the other hand, these systems can be easily functionalized with specific ligands to obtain bioconjugates (Torchilin, 2009). A simple and commonly used approach to form bioconjugates is the post-polymerization conjugation of functionalized lipids or polymers and biomolecules (Boyer et al., 2009b), thus, the selection of lipids or polymers with functional groups suitable for the conjugation of biomolecules under mild conditions is required (Boyer et al., 2009a). These groups are amenable to a wide range of simple transformation procedures to achieve reactive groups towards peptides and proteins. Among the most commonly used there is aminolysis followed by thiol coupling (Boyer et al., 2009a) or carboxylic acid activation using *N*-hydroxysuccinimide (NHS) to perform carboxyl-to-amine crosslinking (Bulmus, 2011). In addition, these systems also have the ability to encapsulate both hydrophobic and hydrophilic species. Among vesicle-like platforms, liposomes have been one of the most widely studied systems in the last 40 years, thanks to their biocompatible character and the permeability of their bilayer membranes (Bergstrand, 2003; Le Meins et al., 2011). However, they may suffer from a lack of mechanical and chemical stability. Their polymeric analogue, named polymersomes and made from the self-assembly of amphiphilic block copolymers, present a rather high mechanical stability, elastic behaviour, higher membrane viscosity and larger resistance to bending (Le Meins et al., 2011).

The aim of this work was to comparatively study both systems in terms of drug loading efficiency, easy surface modification, targeting efficacy, stability and tumor cell killing capacity, in order to determine which system would fulfil the best the requirements of this application. Thus, the system should firstly be able to cross the blood-brain barrier loosing the minimum amount of their payload and preserving its integrity, target breast cancer cells and, last but not least, accomplish their therapeutic function (Fig. 1).

2. Methods

2.1. Materials and reagents

N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt was purchased from Corden Pharma, Switzerland. Phage fusion coat protein (ADMPGTVLPDPAKAAFDSLQASATEYIGYAWAMVVVIVGATI-GIKLFKKFTSKAS) (Brigati et al., 2008; Petrenko et al., 1996) was provided by the Department of Pathobiology, at the College of Veterinary Medicine of Auburn University, AL (US).

MCF-7, U87MG and C166 cells were purchased from ATCC, VA (US). Cell culture media and reagents such as MEM, DMEM, FBS and PBS were all purchased from CellGro, NY (US). Fibronectin, Lucifer Yellow, dexamethasone, Ringer tablets and sodium cholate were purchased from Sigma-Aldrich, Germany. Transferrin alexa fluor 488 was purchased from Invitrogen, RO-20-1724 from Calbiochem, 8-(4-CPT) from Santa Cruz Biotechnology and Cell Titer Blue assay reagent from Promega, WI (US).

Bovine brain microvascular endothelial cells (BBMVECs), Bovine brain endothelial growth media and Attachment Factor Solution (AFS) were all purchased from Cell Applications, CA (US). Regulon peptide (—COOH) was provided by Regulon AE (Greece).

2.2. Liposomes and polymersomes preparation

Synthesis of the amphiphilic triblock copolymer is described in Supplementary data. Both formulations were obtained by the lipid film hydration technique. A dry film of lipid or 3B was prepared by rotary evaporation of a chloroform solution of MPEG-2000-DSPE or 3B followed by freeze-drying for 4 h. The film was hydrated in 300 mM ammonium citrate buffer (pH 4) at a concentration of