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Engineering hepatitis B virus core particles for targeting HER2 receptors *in vitro* and *in vivo*

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ABSTRACT

Hepatitis B Virus core (HBc) particles have been studied for their potential as drug delivery vehicles for cancer therapy. HBc particles are hollow nano-particles of 30–34 nm diameter and 7 nm thick envelopes, consisting of 180–240 units of 21 kDa core monomers. They have the capacity to assemble/dis-assemble in a controlled manner allowing encapsulation of various drugs and other biomolecules. Moreover, other functional motifs, i.e. receptors, receptor binding sequences, peptides and proteins can be expressed. This study focuses on the development of genetically modified HBc particles to specifically recognise and target human epidermal growth factor receptor-2 (HER2)-expressing cancer cells, *in vitro* and *in vivo*, for future cancer therapy. The non-specific binding capacity of wild type HBc particles was reduced by genetic deletion of the sequence encoding arginine-rich domains. A specific HER2-targeting was achieved by expressing the Z_{HER2} affibodies on the HBc particles surface. *In vitro* studies showed specific uptake of Z_{HER2}-ΔHBc particles in HER2 expressing cancer cells. *In vivo* studies confirmed positive uptake of Z_{HER2}-ΔHBc particles in HER2-expressing tumours, compared to non-targeted ΔHBc particles in intraperitoneal tumour-bearing mice models. The present results highlight the potential of these nanocarriers in targeting HER2-positive metastatic abdominal cancer following intra-peritoneal administration.

1. Introduction

The effectiveness of detecting and treating cancer has remained a challenge for many researchers [1]. Additionally, delivering anti-tumour drugs to achieve a therapeutic effect without causing severe systemic side effects has proven to be challenging [2]. To address these issues, many efforts have been put into developing specific targeted carriers that can deliver the desired cargo selectively to tumour sites.

Virus-like particles (VLPs) provide an alternative platform for cell-targeted therapeutic delivery. VLPs are attractive as they are self-assembled, uniform, have well-defined geometry and are able to be tailored at an amino acid level by genetic modification [3]. Moreover, they form a closed structure that defines an interior environment capable of housing therapeutic or imaging agents and an exterior surface capable of multivalent presentation of targeting moieties [4,5]. Hepatitis B Virus core (HBc) particles, examples of VLPs, have attracted many researchers as promising nanocarriers

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for cancer therapeutic studies [6].

HBc particles are hollow nanoparticles, 30–34 nm in diameter with 7 nm thickness envelopes, consisting of 180–240 units of 21 kDa core monomers [7,8]. They are capable of non-specific binding to various cell types *via* the action of positively-charged arginine-rich domain. However, the arginine-rich domain of the core protein is not critical for the particle assembly [7,9,10]. The major immunodominant region (MIR) of HBc particles, located at the 78–83 amino acids (aa), is able to express immunological epitopes [11]. It has been shown that other functional motifs i.e., receptors [12], proteins [13] and element recognising low molecular mass substrates [14], can be expressed by genetic modification to this region.

Recently, affibody molecules, a new class of affinity ligands derived from the Z-domain in the binding region of *Staphylococcus aureus* protein A [15], have been the focus of researchers as a viable alternative to antibodies. Among the types of affibodies, monomeric $Z_{HER2:342}$ (hereafter Z_{HER2}) can specifically bind the HER2 over-expressed on the cell membrane surface of breast cancer and ovarian cancer cells [16]. Due to this attractive property, Z_{HER2} affibody makes a suitable targeting moiety to facilitate HER2 targeting by the nanocarriers.

In this study, we focused on the development of genetically modified HBc particles to specifically recognise and target HER2-expressing cancer cells *in vitro* and *in vivo*, qualitatively and quantitatively, for nucleic acid delivery applications. HER2 targeting was achieved by expression of Z_{HER2} affibodies in the HBc monomer. HBc particles were prepared using the *E. coli* expression system. HBc particles morphology was confirmed by atomic force microscopy (AFM) and cryo transmission electron microscopy (Cryo-TEM). Protein specificity was confirmed by Western blotting. A range of cells expressing different levels of HER2 were treated with fluorescently labelled HBc particles and the cell uptake was assessed using flow cytometry. HBc particles were then radiolabelled with technetium-99m (^{99m}Tc), using the previously reported hexahistidine sequence (His-tag) labelling protocol [17]. Single-photon emission computed tomography/computerised tomography (SPECT/CT) imaging and quantitative gamma counting were performed to characterise the organ biodistribution profile of the HER2 specific-targeting HBc particles in tumour-bearing mice.

2. Materials and methods

2.1. Materials

Please refer to Supporting information for the list of materials used.

2.2. Methods

2.2.1. Expression, purification and assembly of HBc particles

E. coli BL21 (DE3) was transformed with plasmids for expression of wild type HBc, ΔHBc or Z_{HER2}-ΔHBc cultured in 10 mL of Auto-Induction Media Terrific broth (AIM-TB) media in the presence of 100 μg/mL ampicillin and grown at 37 °C for 16 h using an incubator shaker. The culture was then diluted with 500 mL of fresh AIM-TB media in the presence of 100 μg/mL ampicillin and grown at 25 °C for 72 h. Cells were harvested at 5000 rpm, 4 °C for 15 min. Pelleted cells were re-suspended in the 30 ml of lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 1x cOmplete™ protease inhibitor pH 8.0). The cells were treated with RNase A at final concentration 5 μg/mL at 4 °C for overnight. The lysate was sonicated using a probe sonicator on ice by three cycles for 1 min each with 1 min intervals to avoid heating the material. The supernatant was removed by centrifugation at 12,000 rpm, 4 °C for 30 min. The core particles in the cell pellet were washed in 30 ml of lysis buffer and collected by centrifugation at 12,000 rpm, 4 °C for 30 min. The cell pellet containing HBc, ΔHBc or Z_{HER2}-ΔHBc particles was denatured in 40 ml of dissociation buffer (8 M urea, 200 mM NaCl, 50 mM sodium carbonate, 10 mM 2-mercaptoethanol, pH 9.5) by overnight incubation at 4 °C. Then, the pellet was discarded by centrifugation at 12,000 rpm, 4 °C for 30 min.

Soluble fraction containing-contaminating proteins were separated from HBc particle proteins using Ni^{2+}-chelate affinity chromatography. A column with 6 mL of cOmplete™ His-Tag Purification Resin (Roche, Germany) was equilibrated with 3-times bed-volume (18 mL) of dissociation buffer. The column was loaded with the protein probe and washed with 18 mL of dissociation buffer. Bound HBc particle proteins were eluted with 14 mL of elution buffer (2 M urea, 200 mM NaCl, 50 mM sodium carbonate, 10 mM 2-mercaptoethanol, 1 M imidazole, pH 9.5). The eluted material was collected in 1 mL fractions. The aliquots of each fraction were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB) to analyse their purity.

Fractions containing the HBc protein were re-assembled to particles by the removal of the urea. Specifically, protein fractions were dialysed against 2 L of dialysis buffer 1 (0.5 M urea, 100 mM Tris, 150 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM $CaCl_2$, pH 8.0) using SnakeSkin™ Dialysis Tubing, 10K MWCO (Thermo Scientific, USA) at 4 °C for 4 h, allowing HBc protein to start assembling. Then, the solution was dialysed against dialysis buffer 2 (100 mM Tris, 150 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 8.0) to completely removed urea and DTT, to fully assemble the HBc particles. HBc particles were filtered using 0.44 μm filter to remove any aggregates. All protein concentrations were measured using NanoDrop™ ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, USA).

2.2.2. Atomic force microscopy (AFM) analysis of purified HBc particles

One hundred microliter of 10 μg/mL of purified wild type HBc, ΔHBc or Z_{HER2}-ΔHBc particles was deposited on mica surfaces for 5 min and then flushed with air. Tapping mode AFM analysis (TM-AFM) on the mica substrates were carried out in air at 25 °C using a Bruker Dimension ICON with Scan Assist. The surfaces were imaged with a general purpose-tapping tip made by MikroMasch in Estonia (NSC15/no Al, tip radius < 10 nm; tip height = 20–25 μm; cone angle < 40°; cantilever thickness = 3.5–4.5 μm; cantilever width = 32 -28 μm; cantilever length = 120–130 μm; frequency f_0 = 265–400 kHz; force constant k = 20–75 N m^{-1}, VEECO, USA). The statistical analysis of the AFM images was carried out using WSxM v5.0 Developed 6.2 software (Spain).

2.2.3. Cryo-transmission electron microscopy (Cryo-TEM) of HBc particles

The shape, morphology and size distribution of HBc particles was evaluated using low electron dose cryo-transmission electron microscopy (cryo-TEM). Cryo-TEM enables the investigation of colloidal dispersion [18] and biological origin samples like viruses [19] and proteins [20], close to their native state. A drop of solution (3 μl) was applied on hydrophilic TEM Quantifoil grids. The grids were then blotted for 2 s and plunged into the liquid ethane pool using a FEI Vitrobot mark IV, in order to produce a thin vitreous ice layer with embedded assemblies in the holes of the grid. Digital cryo-TEM images were acquired using a FEI Tecnai Spirit operated at 120 kV using a Gatan 626 cryo-transfer tomography holder.

2.2.4. SDS-PAGE and western blot analysis of WT-HBc particles

The expression of each WT-HBc, ΔHBc or Z_{HER2}-ΔHBc monomer