Surface engineering of exosomes led to an enhanced uptake by triple negative breast cancer cells

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Background

Triple negative breast cancer (TNBC) represents 10-20% of all breast cancer cases and is characterized by an aggressive progression with metastatic nature and high rates of relapse [1]. Due to a lack of known specific molecular biomarkers for this breast cancer subtype, there are no targeted therapies available, which results in the worst prognosis of all breast cancer subtypes. Hence, the identification of novel tumor-homing ligands, such as peptides, for this particular type of breast cancer is highly important for the development of novel targeted therapies.

Over the past few years, exosomes have increasingly earned attention as candidate drug delivery platforms due to their distinctive features. The exosome natural role as a transporter of functional molecules is one of the main characteristics that drive their use as drug delivery vehicles [2,3]. Furthermore, their therapeutic potential can be greatly improved by lipid, protein or even nucleic acid content engineering. Based on this rationale, we hypothesized that the decoration of exosomes with TNBC-specific peptides could lead to an increased uptake.

Methods

In this work, we propose the identification of peptides for the specific recognition of MDA-MB-231, a cell line representative of TNBC, using phage display. Binding assays were performed to select the most interesting peptides and bioinformatics approaches were applied to putatively identify the biomarkers to which these peptides bind. Moreover, an insightful characterization of exosomes derived from the normal human foreskin fibroblast cultures (BJ cells) isolated using the gold standard of exosome isolation, the ultracentrifugation using well known protocols described in the literature was performed [4]. For exosome engineering, one of the phage display selected peptides with higher affinity for MDA-MB-231 cells (231Pep1) was conjugated to PEG-phospholipid micelles and subsequently introduced into exosomes using a post-insertion method previously applied to functionalize liposomes. Uptake of BJ-derived exosomes by several breast cancer cells lines was also assessed.

Results

Five peptides from the phage pools (using two different initial libraries) were selected to evaluate their binding affinities through different experimental assays [5]. Both phage forming units (PFUs) and ELISA (**Figure 1**) assays suggest that all peptides exhibit specificity to the MDA-MB-231 cells with a lower binding capacity to MDA-MB-435 cells, as expected since this latter cell line was used in the counter-selection.

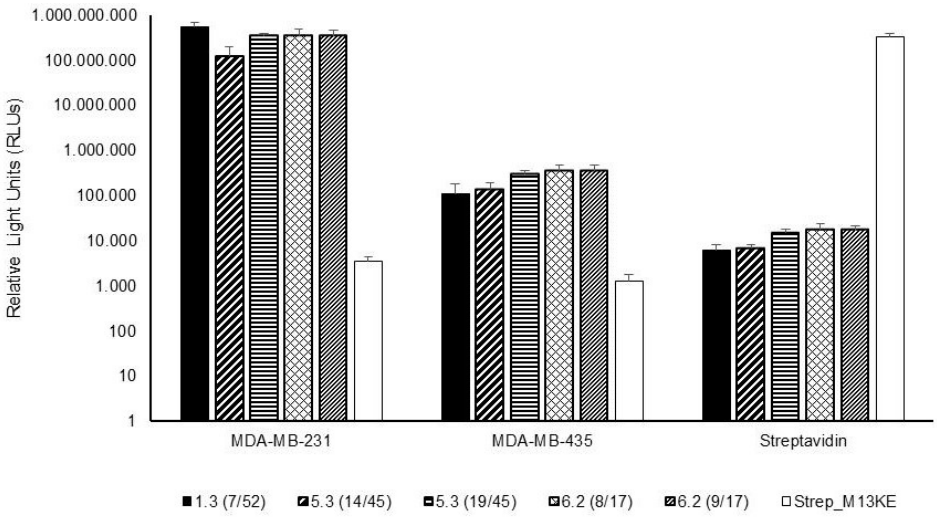

Figure 1: Relative light units (RLUs) obtained for the selected peptides assessed by ELISA against MDA-MB-231 and MDA-MB-435 cell lines, as well as against the control streptavidin, according to the New England BioLabs phage display manual.

Among the peptides, 1.3(7/52) (231Pep1) and 6.2(9/17) (231Pep2) present the most promising results for targeted therapies against TNBC due to the higher binding strengths and selectivity for the MDA-MB-231 cells. Bioinformatics analysis indicate that peptides 231Pep1 and 231Pep2 specifically target TIMP-1 and PAI1, respectively, with both biomarkers being related to breast cancer and to MDA-MB-231 cells.

Exosomes have been exploited as promising delivery vehicles due to their outstanding biocompatibility, as well as natural role in intracellular communication [3], being already reported as efficient delivery platforms for several different molecules. Based on this, we isolated exosomes from BJ cells and a distribution pattern characteristic of exosomes with a mode size of 110±7 nm was observed by nanoparticle tracking analysis (**Figure 2A**) and electron microscopy, showing a typical cup-shaped morphology (**Figure 2B**). Moreover, they exhibited the surface expression of tetraspanin markers that are characteristic of exosomes, including CD63, CD9 and CD81, as assessed by flow cytometry (**Figure 2C**), as well as by western blot (**Figure 2D**).

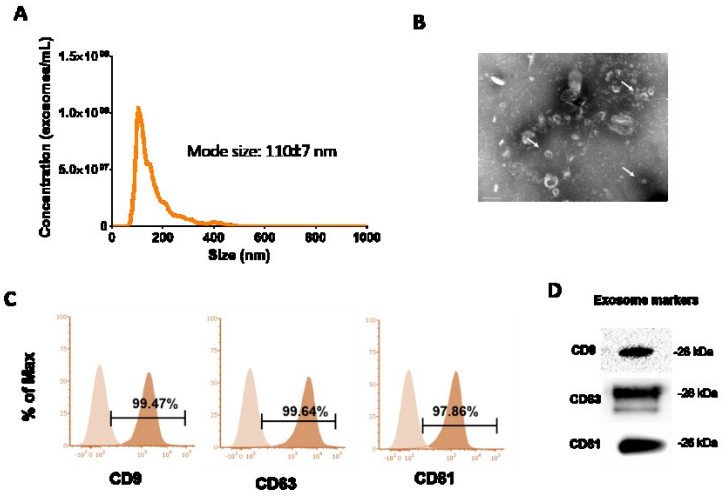

Figure 2: Generation of BJ-derived exosomes. A) Concentration (exosomes/mL) and size distribution (nm) of purified BJ exosomes**. B)** Negative-staining transmission electron microscopy of purified BJ exosomes. Scale bar, 200 nm. White arrows point exosomes. **C)** Representative cytometry histograms for CD63, CD9 and CD81 of purified BJ exosomes. **D)** Representative images of western blot analysis of the protein expression level of CD63, CD9 and CD81 of BJ-derived exosomes.

Various studies reported the use of exosomes as successful targeted drug delivery vehicles by engineering them with targeting ligands, including antibodies, aptamers and peptides [6,7]. Targeting peptides have been extensively used in cancer research given their small size and facilitated synthesis in large amounts [5,8,9]. Therefore, we hypothesized that exosome engineering with a peptide with a strong specificity for the TNBC MDA-MB-231 cell line would confer targeting ability to such cell line [5]. To unveil this hypothesis, herein we conjugated the 231Pep1 to PEG-phospholipid micelles and subsequently introduced into BJ-derived exosomes (**Figure 3**). Our preliminary results showed no size distribution differences for all the conditions assessed (**Figure 3A**). This is in line with previous reports [10]. However, an increased MDA-MB-231 cell uptake was observed for the Exo@231Pep1 comparing to the controls (**Figure 3A** and **3B**).

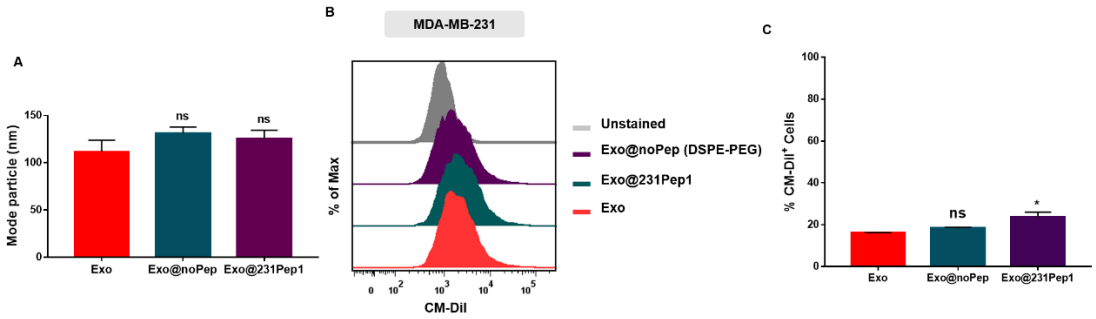

Figure 3: CM-DiI labelled exosomes modified with 231Pep1 uptake by MDA-MB-231 cells. **A)** Mode particle diameter (nm) of purified BJ exosomes (Exo), BJ exosomes coupled with PEG micelles without 231Pep1 (Exo@noPep) and BJ exosomes coupled with PEG micelles with 231Pep1 (Exo@231Pep1). **B)** Representative cytometry histograms of CM-DiI labelled Exo, Exo@noPep and

Exo@231Pep1 (amicon-based purification) uptake up to 24 h incubation by MDA-MB-231 cells. **C)** Quantification of CM-DiI labelled Exo, Exo@noPep and Exo@231Pep1 positive cells of B). Data from **B** and **C** are expressed as the mean ± SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: *$\rho \leq 0.05$.

To potentially increase this uptake capability, different ratios of exosome:231Pep1-PEG micelles are currently being tested, as well as different number of exosomes per cell.

Conclusion

The engineered delivery platform herein scrutinized may contribute for the development of more individualized therapies for TNBC.

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