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The fusogenic peptide HA2 from influenza virus hemagglutinin-2 impairs selectivity of CXCR4-targeted protein nanoparticles

L. Sánchez-García [a†], N. Serna [a†], M. Mattanovich [a,b], P. Cazzanelli [a,b], A. Sánchez-Chardi [c], O. Conchillo-Solé [a], F. Cortés [d], X. Daura [a, e], U. Unzueta [f,g ¥], R. Mangues [f,g], A. Villaverde [a,g ¥], E. Vázquez [a,g]

[a] *Institut de Biotecnologia i de Biomedicina and Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*

[b] *Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU), Muthgasse 18, 1190 Vienna, Austria*

[c] *Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*

[d] *Servei de Cultius Cel·lulars, Producció d'Anticossos i Citometria, (SCAC), Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*

[e] *Catalan Institution for Research and Advanced Studies (ICREA), 08010 Barcelona, Spain*

[f] *Institut d'Investigacions Biomèdiques Sant Pau and Josep Carreras Research Institute, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain.*

[g] *CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Spain.*

† Equally contributed.

¥ Corresponding authors.

Experimental procedures

Protein design, production and purification

The pET22b-derivatives encoding proteins T22-HA2-GFP-H6 and T22-GFP-HA2-H6 were designed in house and produced by GeneArt. These plasmids were transformed into the *Escherichia coli* Origami B strain (BL21, $OmpT^-$, Lon^-, $TrxB^-$, Gor^-; Novagen) by conventional heat-shock procedures as described [1]. Cells were cultured in Lysogenic Broth (LB) medium [2] and the gene expression was induced at an OD of ~0.5 by the addition of 0.1 mM and 0.01 mM isopropyl-β-thiogalactopyronaside (IPTG) respectively. Cells were harvested after subsequent overnight culture at 16°C (T22-HA2-GFP-H6) or 20°C (T22-GFP-HA2-H6). After centrifugation at 5,000 g (4 °C, 15 min), the cell pellet was resuspended in Tris buffer (Tris 20 mM, pH 8.0, NaCl 500 mM, imidazole 10 mM) with the addition of ethylenediamine tetra-acetic acid-free protease-inhibitor (Complete EDTA-Free, Roche, Basel, Switzerland). Cells were disrupted by 3 rounds at 1,200 psi in a French Press (Thermo FA-078A) and subsequently purified by IMAC affinity chromatography using HiTrap Chelating HP 1 mL and 5 mL columns (GE Healthcare, Piscataway, NJ, USA) in an ÄKTA purifier (GE Healthcare). The proteins were eluted by a linear gradient of elution buffer (Tris 20 mM, pH 8.0, 500 mM NaCl and 500 mM imidazole) and dialyzed against 166 mM $NaHCO_3$ buffer (T22-HA2-GFP-H6) or 166 mM $NaHCO_3$ + 333 mM NaCl buffer (T22-GFP-HA2-H6). Protein amounts were determined by Bradford's assay [3].

Protein and nanoparticle characterization

Protein integrity and purity was determined by SDS-PAGE protein electrophoresis and western-blot analysis using an anti-His (Santa Cruz) mouse monoclonal primary antibody (1:500), using a secondary anti-mouse (Santa Cruz) for visualizing (1:1000). The molecular masses of T22-HA2-GFP-H6 and T22-GFP-HA2-H6 proteins were determined by MALDI-TOF mass spectrometry, while the size of the resulting nanoparticles was determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Limited, Malvern, Worcestershire, UK), performed at 633 nm. Fluorescence of the nanoparticles was determined using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Agilent Technologies, Santa Clara, CA, USA). The nanoparticles were excited at a wavelength of 450 nm and detected at 510 nm.

Field emission scanning electron microscopy (FESEM)

To evaluate the native ultrastructure of the nanoparticles, drops of 3 µl of each sample were directly deposited on conductive silicon wafers (Ted Pella Inc., Reading, CA, USA) for 1 min, cleared for a few

seconds in deionized water, excess blotted with Whatman filter paper number 1 (GE Healthcare), air dried, and finally observed without coating in a FESEM Merlin (Zeiss, Oberkochen, Germany) operating at 1 kV. Images were acquired with a high resolution in-lens secondary electron detector.

Cell culture

$CXCR4^+$ HeLa cells were cultivated in MEM Alpha (Minimum Essential Medium α, Gibco, Rockville, MD, USA) supplemented with 10 % fetal calf serum (Gibco) at 37 °C and 5 % CO_2 in a humidified atmosphere. The media was exchanged for serum-free Optipro medium (Gibco) prior to the addition of nanoparticles. Internalization was analysed by detaching the cells with 1 mg/mL trypsin (for 15 min). This harsh proteolytic treatment has been specifically designed to remove externally attached protein [4]. Fluorescence emission was determined by a 488 nm laser in a FACS-Canto system (Becton Dickinson, Franklin Lakes, NJ, USA) with a detector D (530/30 nm band pass filter).

For the internalization assay, cells were incubated for 24 h in Optipro medium containing 0.1 µM and 2 µM of protein nanoparticles. The uptake kinetics were recorded by exposing the cells to nanoparticles for 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5.5 h and 24 h prior to fluorescence measurement. Fluorescence data recorded by cytometry was corrected by the specific fluorescence of the protein, previously determined by fluorescence spectrophotometry, to render comparative units in terms of protein amount.

In competition assays, cells were incubated with protein nanoparticles in Optipro medium in presence of the specific ligand of CXCR4, AMD3100 (octahydrochloride hydrate). This drug was used at 1 µM and added 1 h before protein exposure (at 0.1 µM). For the determination of endosomal escape, cells were incubated in absence and in presence of 100 µM chloroquine for 4 h before the addition of the protein (at 1 µ M).

Cell viability

Viability of HeLa cells incubated with nanoparticles was determined through a CellTiter-Glo Luminescent Cell Viability Assay (PROMEGA), as described elsewhere [2].

Fluorescence spectroscopy

Conformational variations in the tertiary structure of recombinant proteins were analysed by intrinsic Trp-fluorescence in 0.5 mg/ml protein samples. Temperature-dependent emission was monitored

every 20 ºC from 20 to 100 ºC at 330 nm, with an excitation wavelength of 280 nm. Scans were taken along the 290-400 nm range after 8 min of temperature equilibration. A total of ten scans were averaged in each plot.

Confocal laser scanning microscopy

For confocal analysis, HeLa cells were grown on Mat-Teck culture dishes (Mat Teck Corp., Ashland, Massachusetts, United States) and nanoparticles were added at 0.5 μM. After 24 h, nuclei were labelled with 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, Oregon, United States) and the plasma membrane was labelled with 2.5 μg/ml CellMask™ Deep Red (Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 5 min in the dark. Cells were washed in PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Live cells were recorded with a TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) using a Plan Apo 63x / 1.4 (oil HC x PL APO lambda blue) objective. Hoechst 33342 DNA labels was excited with a blue diode (405 nm) and detected in the 415-460 nm range. GFP-proteins were excited with an Ar laser (488 nm) and detected in the 525-545 nm range. CellMask was excited with a HeNe laser (633 nm) and detected in the 650-775 nm range. To determine the protein localization inside the cells, stacks of 20 to 30 sections every 0.5 μm along the cell thickness were collected. The projections of the series obtained were generated with Leica LAS AF software, and three-dimensional models were generated using Imaris v. 7.2.1 software (Bitplane; Zürich, Switzerland).

Statistical analysis

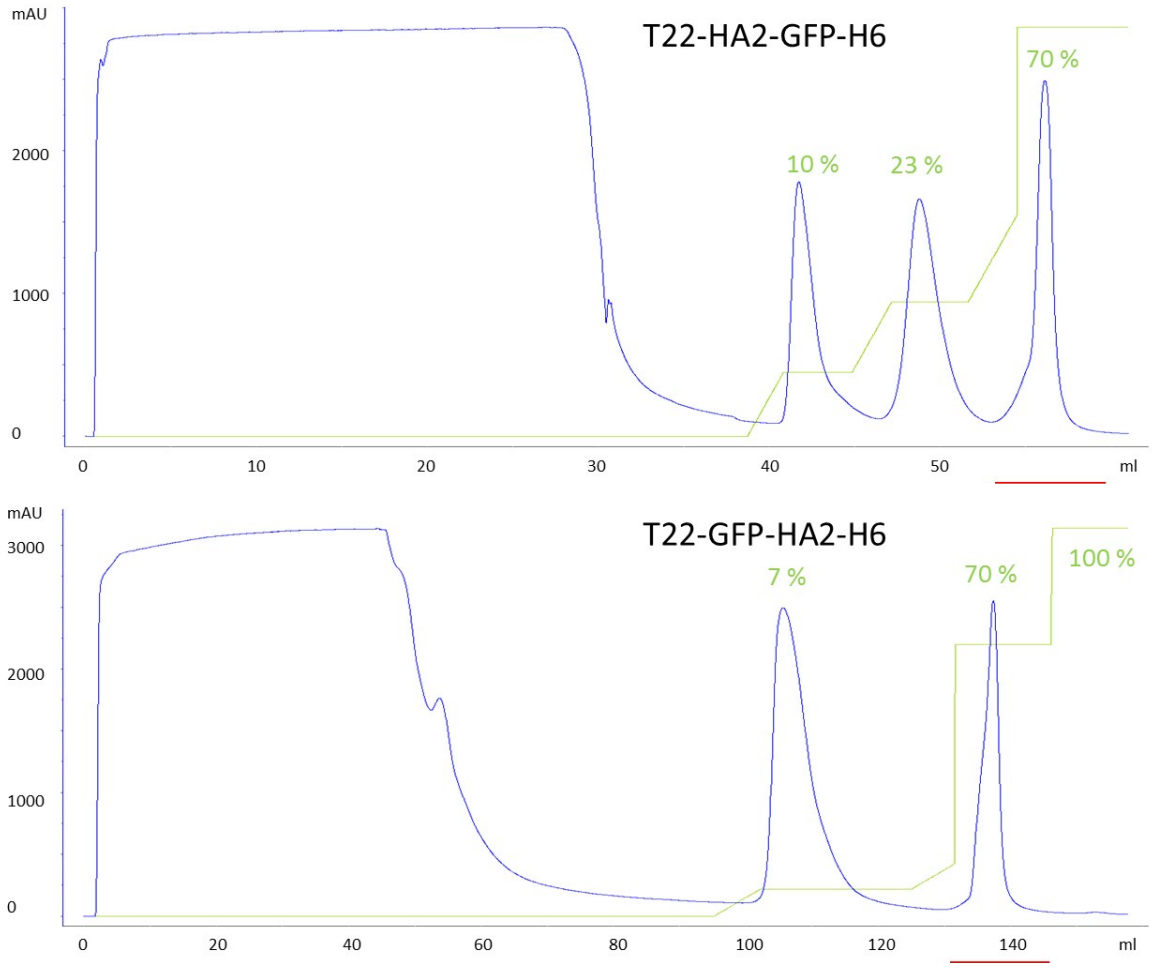
All the numerical data (mean values, standard deviations and errors) were calculated using Microsoft Office Excel 2003 (Microsoft) and visualized by Sigmaplot 10.0. One way ANOVA followed by Fisher's least significant difference (LSD) method was used for multiple comparisons. Pairwise comparisons were performed using Student- t test. Statistical differences were assumed at $p<0.05$.

Molecular modelling

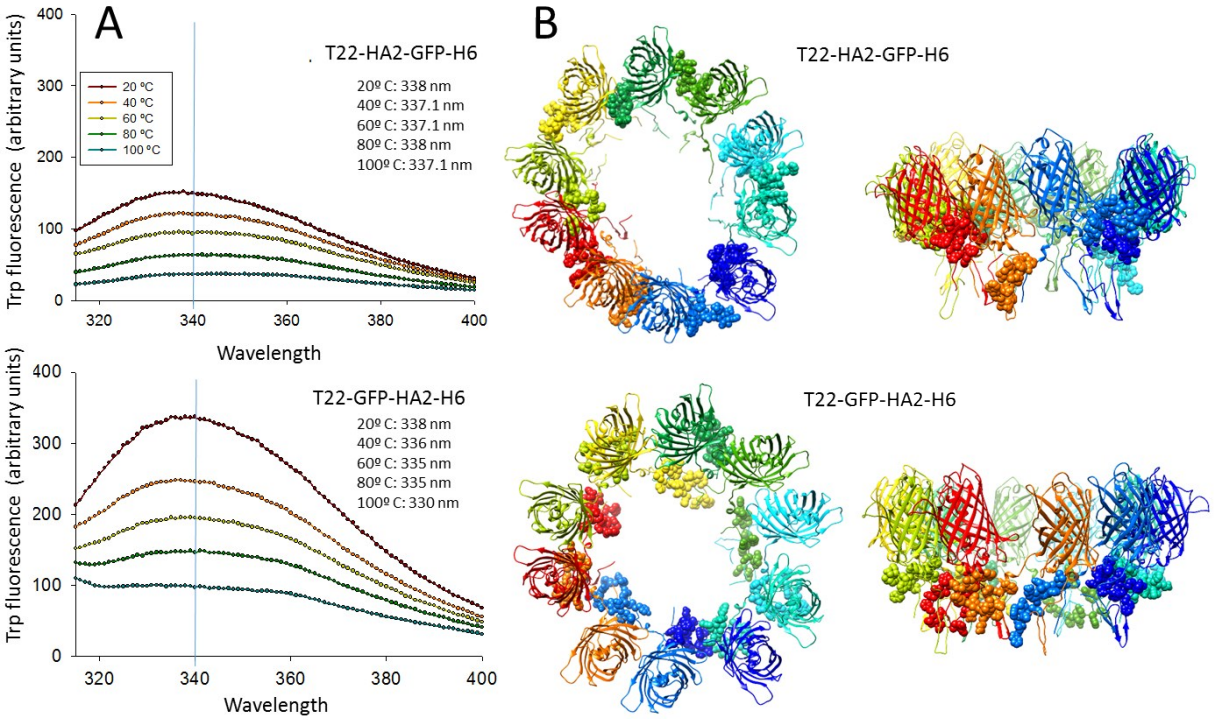
Models for T22-HA2-GFP-H6 and T22-GFP-HA2-H6 where build by homology using the Modeller software [5]. Two structures where used as templates, namely T22-GFP-H6 nanoparticles (those in the peak 2 from IMAC purification, 100 % identity, [6]) for the nanoparticle core, and the pdb code 4wa1 [7] including residues from 330 to 352 for the inserted HA2 segment (91.3 % identity). Five hundred

models were generated with very thorough VTFM optimization and MD refinement. Final models were selected based on their DOPE scores [8].

Supplementary Figures



Supplementary Figure 1. IMAC chromatograms of HA2-containing nanoparticles. Blue lines correspond to ultraviolet signal expressed as milliAbsorbance Units (mAU). Green lines and numbers indicate the percentage of the elution buffer. Red lines represent the protein fractions that were eluted and collected for further experiments.



Supplementary Figure 2. Conformational and structural analysis of HA2-containing protein nanoparticles. A. Analysis of the tertiary structure of T22-HA2-GFP-H6 and T22-GFP-HA2-H6 nanoparticles by Trp-fluorescence spectroscopy, recorded at different temperatures. The maximum of each spectra is indicated by a blue line. The blueshift from 340 nm to 330 nm shown by T22-GFP-HA2-H6 upon temperature increase (but not by T22-HA2-GFP-H6) indicates a comparatively higher letvel of compactness of the material. B. Models of T22-HA2-GFP-H6 and T22-GFP-HA2-H6. Nanoparticles in which each monomer is different colored. All atoms in the HA2 segment are represented as spheres while the rest of the protein is shown as ribbons.

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