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Formulating tumor-homing peptides as regular nanoparticles enhances receptor-mediated cell penetrability

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ABSTRACT

Homing peptides are exploited in nanomedicine to functionalize either free drugs or nanostructured materials used as drug carriers. However, the influence of multivalent versus monovalent peptide presentation on the interaction with the receptor and on the consequent intracellular delivery of the associated cargo remains poorly explored. By using a tumor-homing peptide (T22) with regulatable self-assembling properties we have investigated here if its display in a either a monomeric form or as multimeric, self-assembled protein nanoparticles might determine the efficacy of receptor-mediated penetrability into target cells. This has been monitored by using a fluorescent cargo protein (iRFP), which when fused to the homing peptide acts as convenient reporter. The results indicate that the nanoparticulate protein versions are significantly more efficient in mediating receptor-dependent uptake than their unassembled counterparts. These finding stresses an additional benefit of nanostructured materials based on repetitive building blocks, regarding the multivalent presentation of cell ligands that facilitate cell penetration in drug delivery applications.

1. Introduction

Cell targeted drug delivery is expected to result in high concentration levels of drugs in desired cells and tissues, aiming to minimize side effects of chemotherapies and to thereby improve the patient's quality of life. Tumor-homing peptides specifically internalize cancer cells via specific binding to over-expressed cell-surface receptors. Therefore, they have been explored for use as targeting tools in drug delivery, either fused to therapeutic proteins [5], coupled to drugs or therapeutic nucleic acids [2,17], or as functionalizing agents of different types of nanoparticles intended as drug carriers [1,8]. Under the combined development of materials sciences and nanomedicine it is however unclear whether tumor-homing peptides are more effective in promoting passenger drug penetration when formulated as plain molecular preparations or as multimeric presentations on nanoparticulate entities.

Recently, we have developed a new protein engineering principle to construct protein-only nanoparticles for targeted drug and DNA delivery in cancer [12,14,15]. This has been possible through the construction of self-assembling building blocks containing tumor homing peptides, which organized as highly stable nanoscale entities. The size of these constructs (>8 nm) allow them to escape renal filtration [4], thus exhibiting a convenient biodistribution upon administration and exclusively accumulating in primary tumor and metastatic foci [13]. Among the set of different building blocks generated under this principle, the protein T22-IRFP-H6 exhibits unique assembling properties. In a standard physiological buffer, it organizes as toroidal nanoparticles but in presence of high salt content, the building blocks remain fully disassembled. The possibility to regulate the assembling of T22-IRFP-H6 offers an exceptional opportunity to comparatively explore, in the same molecular species, whether the cell targeting

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and internalization properties of T22, a potent ligand of CXCR4 [13], are influenced by its presentation as either free protein molecules or as regular assembled, protein-only nanoparticles.

2. Materials and methods

Protein production and characterization: T22-IRFP-H6 was produced by standard procedures [4]. After purification, the protein was collected and dialyzed against two alternative buffers: $NaHCO_3$ 166 mM, pH=7.4 and $NaHCO_3$ 166 mM, NaCl 333 mM, pH=7.8, overnight at 4 °C. Fluorescence emission spectrum was measured at 710 nm, by using a JASCO FP-8000 spectrofluorometer (JASCO, US), with an excitation wavelength of 635 nm. Volume size distribution of nanoparticles and monomeric proteins were determined by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments Limited, Malvern, UK), at 633 nm. Size exclusion chromatography (SEC) was done in a calibrated Superdex200 10/300GL (Tricorn) column (GE Healthcare).

Cell culture, flow cytometry and competition assay: HeLa cells (ATCC-CCL-2) were grown on 24 wells plate for 24 h in OptiPRO™ SFM medium (Life technologies) supplemented with 3 mM L-Glutamine. Recombinant proteins were added to each well at a final concentration of 2 μM and further incubated for 24 h. After that, the medium was removed and cells washed with PBS and incubated with 1 mg/ml trypsin for 15 min to remove protein bound to cell surface. Cells were centrifuged at 400*g* for 5 min to remove trypsin, and collected and resuspended in PBS. Cells were analyzed on a FACSCanto system (Becton Dickinson), using a 15 W air-cooled argon-ion laser at 635 nm excitation. IRFP fluorescence was measured with detector A (780/60 nm band pass filter). For the competition assay, AMD3100 was added 1 h before proteins. Protein internalization was determined 24 h later by flow cytometry.

Confocal microscopy: HeLa cells were cultured on Mat-Teck culture dishes (Mat Teck Corp., Ashland, MA, USA) with serum-free medium for 24 h. Proteins were added with a final concentration of 2 μM. After 24 h, cells were washed with PBS and nuclei and membranes stained with Hoechst 33342 and concanavalin A respectively for 10 min. After washing with PBS complete medium was added and stained cells were examined using a TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) with a Plan Apo 63 × /1.4 (oil HC × PL APO l blue) objective.

Cryo transmission electron microscopy (CryoTEM): Microdrops of purified proteins (3 μL) were deposited on Quantifoil R1.2/1.3 grids, put in liquid ethane in a Leica EM CPC and immediately placed in a Gatan cryo-transfer specimen holder. Samples were observed in a Jeol JEM 2011 transmission electron microscope, operating at 200 kV and equipped with a CCD Gatan 895 USC 4000 camera.

Field emission scanning electron microscopy (FESEM): Microdrops (5 μl) of protein sample were added to a silicon wafer and air-dried at room temperature. Native structure of samples was observed in a FESEM Zeiss Merlin operating at 2 kV equipped with a high-resolution in-lens secondary electron detector.

3. Results and discussion

Features of protein materials: Upon production in bacteria, T22-IRFP-H6 occurred as an individual molecular species of 38.6 kDa that partially adopted a dimeric form (77.2 kDa) (Fig. 1A), the natural organization of iRFP [6]. Further dialysis in front a physiological buffer or a buffer containing 500 mM salt, did not alter the integrity of the protein (Fig. 1B). However, in low salt buffer, T22-IRFP-H6 organized as supramolecular entities formed by around 10 monomers (Fig. 1C), that were seen as regular nanoparticles of 15 nm by DLS (Fig. 1D). Particle formation was fully confirmed by FESEM (Fig. 1E) and by cryo-TEM (Fig. 1F). No

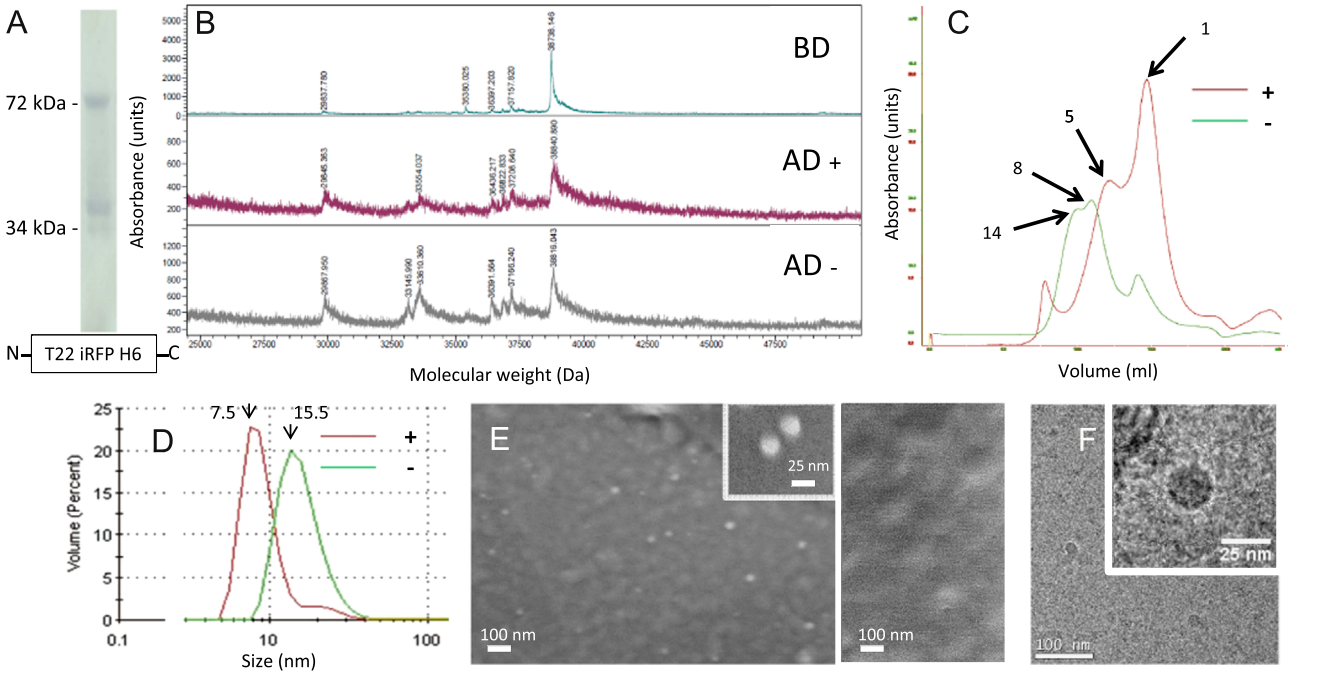

Fig. 1. Characterization of T22-iRFP-H6-based materials. (A) Western blot of purified T22-iRFP-H6 revealed with an anti-His antibody, indicating the molecular weight of markers. At the bottom, scheme of the modular composition of T22-iRFP-H6. (B) Mass spectrometry of purified T22-iRFP-H6 before dialysis (BD), and after dialysis (AD) against low salt (−) and high salt buffers (+). (C) SEC of T22-iRFP-H6 in low salt (green line) and in high salt (red) buffers. Figures indicate the approximate number of monomers forming the plotted structures. (D) DLS plots of the same samples. (E) FESEM images of T22-iRFP-H6 nanoparticles (left) at two magnification levels. No nanoparticles were observed in high salt buffer (right). (F) Cryo-TEM images of T22-IRFP-H6 nanoparticles at two magnification levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)