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Intracellular targeting of CD44^{+} cells with self-assembling, protein only nanoparticles

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Abstract

CD44 is a multifunctional cell surface protein involved in proliferation and differentiation, angiogenesis and signaling. The expression of CD44 is up-regulated in several types of human tumors and particularly in cancer stem cells, representing an appealing target for drug delivery in the treatment of cancer. We have explored here several protein ligands of CD44 for the construction of self-assembling modular proteins designed to bind and internalize target cells. Among five tested ligands, two of them (A5G27 and FNI/II/V) drive the formation of protein-only, ring-shaped nanoparticles of about 14 nm that efficiently bind and penetrate $CD44^+$ cells by an endosomal route. The potential of these newly designed nanoparticles is evaluated regarding the need of biocompatible nanostructured materials for drug delivery in CD44-linked conditions.

Keywords: CD44**;** Multifunctional protein; Nanoparticle; Self-assembling; Biomaterials; Drug delivery

1. Introduction

CD44 is a transmembrane adhesion glycoprotein which participates in cell-cell and cell-extracellular matrix interactions, being hyaluronic acid (HA), fibronectin and laminin its natural ligands. CD44 is expressed in many cell lines including leukocytes and fibroblasts and it participates in a wide range of physiological processes such as cell migration, lymphocyte homing, cell activation and hematopoiesis (Gee et al. 2004). Among several surface receptors overexpressed in cancer stem cells (CSCs), including CD133, CD44, CD49 and ITGA6, CD44 is the most frequent molecular marker, being present in a large variety of tumor types. It is prevalent in highly recurrent colon, liver, prostate or breast cancers (Zoller, 2011).The tumorigenic and metastatic potential of CSCs have been associated to CD44 expression. Many evidences strongly support that an alteration of CD44 expression levels promotes tumor cell survival and aggressiveness and it also induces tumorogenesis and metastasis. In this regard, cell lines which highly express CD44 are capable of forming more aggressive tumors in the invading tissue (Goodison, Urquidi et al., 1999). In breast cancer, CD44 is not only a useful stem cell marker but also a promising therapeutic target (Marangoni, Lecomte et al., 2009; Sauter, Kloft et al., 2007; Tijink, Buter et al., 2006), and targeting to CD44 reduces tumor growth and prevents post-chemotherapy relapse of human breast cancer xenografts (Marangoni, Lecomte et al., 2009). In humans, anti-CD44 antibodies used as targeting agents for either radiolabels or anticancer chemotherapeutics have shown promise in clinical trials, and disease stabilization was observed in patients with breast or head and neck tumors treated with anti-CD44-based conjugates (Sauter, Kloft et al., 2007; Tijink, Buter et al., 2006).

In the drug delivery scenario, a diversity of materials including natural polymers, carbon nanotubes and lipid-based and inorganic nanoparticles have been proposed for the specific targeting CD44-expressing cells, upon convenient functionalization. Most of them have been formulated by the conjugation of a given nano-vehicle with HA acid

(Peer, Karp et al., 2007), but in general, preliminary results have been not dramatically promising. Apart from toxicity issues (Goodison, Urquidi et al., 1999), not all CD44 + cells constitutively bind HA, as the binding capacity appears to be influenced by structural variation and/or CD44 glycosilation patterns (Gee, Kryworuchko et al., 2004; Zoller, 2011). In this regard, CD44-binding proteins are promising alternatives to HA as targeting agents, and the use of these proteins as functionalizing agents would offer the flexibility of protein engineering in nanoparticle design. However, while CD44 has been described to undergo receptor-mediated endocytosis when bound to HA and collagen (Koo, Huh et al., 2011; Rezler, Khan et al., 2007), evidences supporting internalisation of laminin and fibronectin are still missing.

In the present study and by applying a nano-architectonic principle based on the combined use of end terminal cationic peptides and polyhistidines (Ferrer-Miralles, Corchero et al., 2011; Unzueta, Cespedes et al., 2012; Unzueta, Ferrer-Miralles et al., 2012), we have explored five CD44-specific protein ligands as components of multifunctional chimerical proteins, with potential for oligomerization. Among them, two peptides from laminin and fibronectin, respectively, efficiently target CD44+ cells and in addition, they promote the self-assembling of the carrier protein as functional nanoparticles of 14 nm. Such particulate organization confers added value properties to protein constructs, favouring cellular penetrability and opening a plethora of possibilities for the rational design of protein-based, fully biocompatible nano-medicines, that in form of viral biomimetics, target CD44-overexpressing cells.

2. Materials and methods

2.1. Cell lines and media. MDA-MB-231 cell line was maintained in RPMI 1640 supplemented with 10 % foetal calf serum (FBS) and 6 mM GlutaMAX (Invitrogen), and MCF-7 in Dulbecco's Modified Eagle Medium (DMEM) F12 supplemented with 10 % foetal bovine serum (FBS) and 2 mM GlutaMAX (Invitrogen). HepG2 (ATCC HB-8065) cell line was maintained in MEM-α (Invitrogen) supplemented with 10 % FBS and 2 mMGlutaMAX. All cell types were incubated at 37°C and 5 % CO_2 except HEK-293-T, which was maintained at 10% CO_2 in DMEM supplemented with 10% FBS and 2 mMGlutaMAX.

2.2. Protein design, production, purification, and characterization. Five chimeric genes were designed in-house and provided by GenScript (Hong Kong, China). The resulting fusion proteins (Figure 1 A) were named according to their modular organization as N_{term}-Peptide-GFP- H6-$C_{term,}$ being all N terminal peptides known CD44 ligands (Table 1). Using *Nde*I/*Hind*III restriction sites, segments of these genes were inserted into pET22b expression plasmid (Novagen 69744-3). Fibroblast growth factor 2 (FGF2-H6), as CD44 overexpression activator, was produced as described for the proteins above. All the encoded proteins were produced in *Escherichia coli* BL21 (F^- *ompThsdS*$_B$(r_B^- m_B^-) *gal dcm*DE3, Novagen) overnight at 16°C for A5G27-GFP-H6,FNI/II/V-GFP-H6 and FGF2-H6, overnight at 20ºC for FNI-GFP-H6 and P7-GFP-H6 and during 3 h at 37ºC for FNV-GFP-H6. Gene expression was induced upon the addition of 1 mM IPTG. Bacterial cells were then centrifuged for 45 min (5000 g at 4°C) and resuspended in Tris buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole) in the presence of protease inhibitor (Complete EDTA-Free, Roche, Basel, Switzerland). The cells were disrupted at 1200 psi in a French press (Thermo Scioentific). Lysates were centrifuged at 15,000g for 45 min and soluble fractions were filtrated before His-tag affinity chromatography using HiTrap Chelating HP 1 mL

columns (GE Healthcare, Piscataway, NJ) with an AKTA purifier FPLC (GE Healthcare). Elution was achieved by a linear gradient of 20 mM Tris, pH 8.0, 500 mM NaCl, and 500 mM imidazole, and the eluted material was analyzed by Western Blotting with an anti-His monoclonal antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) to observe the presence of the protein of interest. An anion-exchange chromatography was additionally performed for A5G27-GFP-H6 and FNI/II/V-GFP-H6 proteins purification using DEAE HP and QFF HP 1 ml columns (GE Healthcare) respectively and a linear gradient of 10 mM Tris-HCl pH 8.0, 1 M NaCl for A5G27-GFP-H6 and 166 mM$NaHCO_3$, 1 M NaCl for FNI/II/V-GFP-H6. Proteins were finally dialyzed overnight at 4ºC against sodium bicarbonate buffer (166 mM $NaHCO_3$ at pH 7.4) except for FGF2-H6 that was dialysed with phosphate buffer (0.1 M $NaPO_3$, pH 6.3). Protein integrity and purity were checked by Coomassie Brilliant Blue staining, by mass spectrometry (MALDI-TOF) and N-terminal sequencing using the Edman degradation method. Protein concentration was determined by Bradford assay as described elsewhere (Bradford, 1976). A5G27-GFP-H6, FNV-GFP-H6 and FNI/II/V-GFP-H6 had been preliminarily screened for self-assembling in a previous study (Unzueta, Ferrer-Miralles et al., 2012). Protein production was supported by Protein Production Platform (CIBER-BBN – UAB, http://www.ciber-bbn.es/en/programas/89-plataforma-de-produccion-de-proteinas-ppp).

2.3. Atomic force microscopy, fluorescence determination, and dynamic light scattering. The fluorescence of chimerical proteins was determined in a Cary Eclipse fluorescence spectrophotometer (Varian Inc, Palo Alto, CA) at 510 nm using an excitation wavelength of 450 nm. Volume size distribution of nanoparticles was determined by dynamic light scattering at 633 nm (Zetasizer Nano ZS, Malvern Instruments Limited, Malvern, UK). Atomic force microscopy (AFM) analyses were performed with a commercial atomic force microscope (PicoSPM 5100 from Molecular Imaging Agilent Technologies, Inc., Santa Clara, CA, USA) operating in acoustic mode.

Proteins suspended in 166 mM $NaCO_3H$ at pH 7.4 were dropped onto a freshly cleaved mica surface. The substrate was rinsed gently with miliQ water to eliminate the salts of the buffer solution and let to air dry before imaged. For the acoustic mode measurements, a monolithic supersharp silicon SSS-NCH-50 (Nanosensors, Inc.) tip, with a radius of 2 nm, a nominal spring constant of 10–130 N/m and a resonance frequency of 204–497 kHz were used.

2.4. Flow cytometry. MDA-MB-231, MCF-7, HEK-293-T and HepG2 cells were cultured on a 24-well plate at $8 \cdot 10^4$, $1 \cdot 10^5$, $8 \cdot 10^4$ and $1.2 \cdot 10^5$ cells/well respectively with their correspondent medium for 24 h until reaching 70 % confluence. Medium was removed and cells were washed twice with PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and then OptiPro medium supplemented with L-glutamine and peptide at the desired concentration was added and incubated for 24 h at 37ºC and 5% CO_2 in a humidified atmosphere. Cells were detached using 1 mg/mL trypsin for 15 min followed by the addition of complete medium and centrifuged at 1200 rpm for 15 min. After supernatant was removed, the cell pellet was resuspended in 300 µl DPBS Ca^{2+}, Mg^{2+}-free (Invitrogen). Protein internalization was analyzed using a FACS-Canto system (Becton Dickinson, Franklin Lakes, NJ) using a 15 mW air-cooled argon ion laser at 488 nm excitation. Experiments were performed in duplicate.

2.5. Protein internalisation monitored by confocal laser scanning microscopy. MDA-MB-231 cells were plated on a MatTek culture dish (MatTek Corporation, Ashland, MA) at 200.000 cells/plate for 24 h. Medium was removed and cells were washed with DPBS, OptiPro medium supplemented with L-glutamine and peptide at 1.5 µM was added and incubated for 24 h at 37ºC and 5 % CO_2 in a humidified atmosphere before confocal analysis. Plasma membranes were labelled with 2.5 µg/mL CellMask™ Deep Red (Molecular Probes, Eugene, OR) and cell nuclei with 0.2 µg/mL Hoechst 33342 (Molecular Probes) for 10 min. in the dark before confocal analysis. Cells were washed in PBS and complete medium was added. Analysis was

performed using a TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) using a Plan Apo 63 ×/1.4 (oil HC × PL APO lambda blue) objective as described elsewhere (Vazquez, Roldan et al., 2010). Images were processed using Imaris version 6.1.0 software (Bitplane, Zürich, Switzerland).

2.6. Analysis of protein stability and cytoxicity. Stability of A5G27-GFP-H6 and FNI/II/V-GFP-H6 was analysed in duplicate in human serum (S2257-5ML, Sigma, St Louis, MO) at 37°C, with agitation and at a final concentration of 0.115 µg/µL and 0.055 µg/µL respectively. Fluorescence was determined as previously described. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using VICTOR3V 1420 (Waltham, USA).

2.7. Determination of CD44 expression. $1.5 \cdot 10^6$ of each MDA-MB-231, MCF-7, HEK 293 T and HepG2 cells were tripsinised and centrifuged (1,200 rpm, 5 min, 4ºC) and fixed in 100 µl of 2 % formaldehyde for 10 min at room temperature. The pellet was washed with PBS-BSA. Cells were then resuspended in 75 µl of blocking solution (Human Ig 200 µg/mL PBS) for 15 min at room temperature, and aliquoted into 3x25 µl fractions. 5 µl of APC Mouse IgG2b κ Isotype control (BD Pharmingen, 555745) and 5 µl of APC Mouse Anti-Human CD44 (BD Pharmingen, 559942) were added to two of these samples respectively. 45 min after incubation at 4ºC out from light, samples were centrifuged (8,000 rpm, 15 sec, 4ºC) and washed with 1 ml PBS-BSA (0.5 %). Pellet was resuspended in 500 µl PBS. Samples were analysed in duplicate with a FacsCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.9. Peptide internalisation under CD44 deregulation and competition assays. MDA-MB-231 cells were cultured on a 24 well-plate at $8 \cdot 10^4$ c/w for 24 h until 70 % confluence. Medium was removed and cells washed twice with PBS. 250 µl of OptiPro medium supplemented with L-glutamine with the CD44 expression regulator/competitor was added. Competitor anti-CD44 and downregulator anti-IL-10 polyclonal antibodies

(HCAM sc-7946 and IL-10 sc-7888 respectively, Santa Cruz Biotechnology Inc., Heidelberg, Germanyand) were used at 0.3 µM whereas positive regulatorFGF2 was added at different ratios (1:1, 1:10, 1:30). After 1 h incubation of regulator/competitor at 37ºC, proteins were added at 0.3 µM. Cells were detached and prepared for cytometry analysis as described above.

2.10. Statistical analysis. Mean data and other statistics were calculated with Sigmaplot 10.0.

202 **Table 1. CD44 peptidic ligands used to tag GFP-H6 monomers.**

Source protein and reference	CD44 ligand protein segment	Length (in amino acids)	Number of positively charged residues (arg + lys)	Amino acid sequence	Particle formation and size of monomer or oligomer (nm/PDI[a])	Construct name. The N-terminal module corresponds to the peptide ligand.
Fibronectin, HBFN-fragment I, (Jalkanen and Jalkanen, 1992; Yasuda, Poole et al., 2003)	1977-1991	15	5	KNNQKSEPLIGRKKT	No, 6.3/0.7	FNI-GFP-H6
Fibronectin, HBFN-fragment V, (Jalkanen and Jalkanen, 1992; Yasuda, Poole et al., 2003)	1923-1930	8	2	WQPPRARI	No, 5.5/0.8	FNV-GFP-H6
Fibronectin, HBFN-I/II/V containing fragment, (Jalkanen and Jalkanen, 1992; Yasuda, Poole et al., 2003)	1923-1991	69	12	WQPPRARITGYIIKY EKPGSPPREVVPRP RPGVTEATITGLEPG TEYTIYVIALKNNQKS EPLIGRKKT	Yes, 13.7/0.4	FNI/II/V-GFP-H6
Laminin α5 chain, peptide A5G27, (Hibino, Shibuya et al., 2004; Hibino, Shibuya et al., 2005)	2975-2987	13	2	RLVSYNGIIFFLK	Yes, 13.8/0.4 [b]	A5G27-GFP-H6

P7, phage display derived peptide, (Park, Lee et al., 2012)	-	12	2	FNLPLPSRPLLR	No, 6.5/0.4	P7-GFP-H6

[a] The indicated size refers to the peak determined by DLS. PDI is the polydispersity index in the DLS measurements.

[b] Slight discrepancies between the size of A5G27-GFP-H6 nanoparticles determined here and in a previous report (Unzueta, Cespedes et al., 2012) are due to the different composition of the buffers used in these studies.

3. Results

Five known peptidic ligands of CD44 (FNI, FNV, FNI/II/V, A5G27 and P7, Table 1) were fused to C-terminal H6-tagged GFP following a previously described approach (Figure 1 A, (Unzueta, Ferrer-Miralles et al., 2012)), to construct multidomain GFP protein versions with affinity for CD44+ cells. All these constructs were efficiently produced in bacteria, resulting in full length polypeptides with expected mass and predicted N-terminal amino acid sequence (see Supplementary Figure 1). Before testing for biological properties, the potential self-assembling of protein monomers into higher order entities was explored. According to a previously proposed model (Unzueta, Ferrer-Miralles et al., 2012), the highly cationic peptide FNI/II/V was expected to promote the formation of ordered oligomers within the nanoscale. In this regard, FNI/II/V-GFP-H6 in solution peaked at around 14 nm by DLS (Figure 1 B, Table 1), indicative of nanoparticle formation. The unassembled GFP-H6 protein showed a size of 6 nm (Vazquez, Roldan et al., 2010), and FNI, FNV and P7-empowered GFP proteins peaked at the same size, indicating that they remained unassembled like the parental GFP-H6 (Figure 1 B, Table 1). Unexpectedly, A5G27, even being poorly cationic (Table 1), also promoted the formation of supramolecular entities of 14 nm (Figure 1 B, Table 1).

When the nanoparticulate architecture of FNI/II/V-GFP-H6 and A5G27-GFP-H6 was assessed by AFM, these proteins clearly organized as regular nanoparticles with a size fully compatible with DLS determinations (Figure 1C-G). Some A5G27-GFP-H6 particles appeared as rings or pseudorings with a centred cavity (Figure 1E), and such a ring-based architecture was generic in FNI/II/V-GFP-H6 samples, in which some of the nanoparticles showed a pentameric organization (Figure 1F). In this regard, molecular modelling of R9-GFP-H6 nanoparticles indicated a pentameric organization of the constructs (Unzueta, Ferrer-Miralles et al., 2012; Vazquez, Cubarsi et al., 2010; Vazquez, Roldan et al., 2010) and size-exclusion chromatography of several GFP and

iRFP-based nanoparticles also showed pentamers as a basic module resulting from self-assembling in protein particles empowered by cationic stretches plus H6 (Cespedes, Unzueta et al., 2014).

To evaluate the biological properties of the fusion proteins, namely cell penetration and eventual intracellular trafficking, we first checked the specific fluorescence of all fusion proteins, as fluorescence emission is a convenient reporter to monitor cell binding and internalization. As observed (Figure 2 A), fluorescence emission of the enhanced GFP was not straightforward affected by protein fusion but it was reduced up to around 30-40 % of the parental protein in A5G27-GFP-H6 and FNI/II/V-GFP-H6. The coincidence between fluorescence dropping and nanoparticle formation might be indicative of a slight quenching effect associated to oligomer formation. Irrespectively of the precise cause, fluorescence levels were well acceptable and sufficient for further analyses. An important fraction of $CD44^+$ MDA-MB-231 cells were fluorescent when exposed to A5G27-GFP-H6 and FNI/II/V-GFP-H6 (around 80 % *vs* 40 % or less in the case of other fusions, Figure 2B), and a higher global fluorescence emission was observed in cell cultures exposed to these proteins when compared with alternative GFP versions (between 1000 and 2000 *vs* up to 600, Figure 2C). In both experimental approaches and as it was expected, GFP-H6 only rendered background values. In agreement with quantitative data, confocal analyses of protein-exposed MDA-MB-231 cell cultures confirmed the high penetrability of A5G27-GFP-H6 and FNI/II/V-GFP-H6 *vs* alternative GFP versions, mostly showing a background uptake (Figure 2D). Note that as indicated above, the lower specific fluorescence of these two constructs resulted in an underestimation of the internalized material when compared to alternative ligands. Considering the values presented in the Figure 2B, A5G27-GFP-H6 and FNI/II/V-GFP-H6 internalized 20 times more efficiently than the alternative fusion proteins. Again, the parental, H6-tagged GFP was seen as totally excluded from cultured cells. The

incorporation of the internalized recombinant proteins into membranous vesicles was clearly observed and it was indicative of endosomal uptake (Figure 2D).

Internalization of A5G27-GFP-H6 and FNI/II/V-GFP-H6 occurred very fast, and the fraction of target cells and the amount of intracellular protein reached a plateau at about 10 h (Figure 3A). Interestingly, the penetration of these nanoparticles did take place without compromising cell viability (Figure 3B), again in agreement with the occurrence of an endosomal route and supporting the full biocompatibility nature of protein-only nanoparticles. In this context, both A5G27-GFP-H6 and FNI/II/V-GFP-H6 were fully stable when incubated in human serum (Figure 3C), a fact that confirmed the structural robustness of the particles and prompted us to envisage a potential for proper biodistribution of these materials in targeted drug delivery or diagnostic applications.

To assess the receptor-driven specificity of the cell penetration, the uptake of protein nanoparticles was explored in several cell lines, expressing and not expressing CD44, namely MDA-MB-231, MCF-7, Hep G2 and HEK-293T. A differential cell penetrability of A5G27-GFP-H6 and FNI/II/V-GFP-H6 was observed (Figure 4A), coincident with the amount of cellular CD44 in the target cells (Figure 4B). This fact strongly supported the CD44-dependence of cell binding. In this context, a commercial polyclonal anti-CD44 serum inhibited the entrance of both type of nanoparticles (Figure 5). Finally, we wanted to determine if the externally mediated up- or down-regulation of CD44 expression could have enhancing or inhibiting effects on the penetration of nanoparticles, as expected. The fibroblast growth factor 2 (FGF-2), a positive regulator of CD44 (Grimme, Termeer et al., 1999; Jones, Tussey et al., 2000), stimulated the cell penetrability of A5G27-GFP-H6 (Figure 6A), while the blocking of interleukin-10 (IL-10; also a positive regulator of CD44, (Gee, Kryworuchko et al., 2004)) by a specific antibody reduced the uptake of the nanoparticle (Figure 6B). By the combination of

these data, the CD44-targeting of the protein constructs developed here was fully demonstrated.

4. Discussion

Developing tools for targeted drug delivery is a priority in targeted medicines of cancer and other prevalent diseases (Ruoslahti, Bhatia et al., 2010; Vicent and Duncan, 2006). While of the number of cell surface proteins identified as valuable markers are rapidly expanding (Gonzalez-Angulo, Hennessy et al., 2010; Klonisch, Wiechec et al., 2008; Mocellin, Lise et al., 2005; Nguyen and Massague, 2007; Ruoslahti, Bhatia et al., 2010; Sawyers, 2008; Tjalsma, 2010), efficient and secure vehicles are still missing. While consensus exists in that nanoscale containers are ideal for competent systemic transport, diffusion in the tissue and cell penetrability (Mastrobattista, van der Aa et al., 2006), a fully biocompatible material remains to be developed. In this regard, the number of nanomedicines so far approved by the medicament agencies is still limited (Duncan and Gaspar, 2011).

A promising route to the generation of efficient vehicles for drug delivery is conventional genetic engineering, since proteins are fully biocompatible and functional macromolecules. The ability to recruit diverse peptides from distinct origins and with different complementing activities in a single chain protein offers promise to generate constructs showing the biological properties exhibited by viruses during infection. These include stable systemic circulation, receptor targeting, internalization, intracellular trafficking and accumulation into the appropriate compartment (Aris and Villaverde, 2004; Ferrer-Miralles, Vazquez et al., 2008; Vazquez, Ferrer-Miralles et al., 2009; Vazquez, Ferrer-Miralles et al., 2008). Despite this potential, the exploration of how protein-protein contacts could be engineered to construct protein-only nanoparticles has been in general neglected. The adaptation of virus-like particles (VLP) (Ma, Nolte et al., 2012), bacterial micro compartments (BMC) (Corchero and Cedano, 2011), eukaryotic vaults (Rome and Kickhoefer, 2012) or other natural protein constructs (Rodriguez-Carmona and Villaverde, 2010) poses severe limitations, as conferring new tropisms might in general alters the stability of the particle. However,

315 recent lessons about how protein-protein interactions can be engineered for self-
316 assembling in fully *de novo* designed protein constructs {Neus Ferrer-Miralles, 2013
317 1106 /id;Unzueta, 2014 1308 /id;Villaverde, 2012 7494 /id} should permit to approach
318 an 'artificial virus' strategy for the design of novel nanomedicines (Mastrobattista, van
319 der Aa et al., 2006). Importantly, the recent advances in systems and synthetic
320 biotechnology and industrial microbiology (Lee, Mattanovich et al., 2012) allow the
321 large scale biosynthesis of natural proteins and protein constructs, being biological
322 biofabrication highly versatile (Vazquez and Villaverde, 2013) and progressively more
323 competitive with regard to conventional chemical synthesis (Chen, 2012).

324 We have here constructed a set of five modular polypeptides intended to target CD44+
325 cells (Table 1). Among them, two constructs (empowered by peptides A5G27 and
326 FNI/II/V respectively), self-organize as stable nanoparticles (Figure 1 and 3C) that
327 efficiently bind and internalize CD44+ target cells (Figure 4), accumulating in the
328 perinuclear and nuclear regions (Figure 2). Since the five peptides used here are very
329 well known ligands of CD44 (Table 1), the coincidence between nanoparticle formation
330 and internalization strongly suggests that the presentation of a given protein in form of
331 nanoparticle (versus the plain monomeric form) stimulates cell penetration. This is
332 probably because of the multiple ligand display and multivalent cross-linking at the cell
333 surface, favoring membrane wrapping (Jiang, Kim et al., 2008), and in the line of
334 nanoparticle size being a main determinant of interactions with cells (Jiang, Kim et al.,
335 2008). Also, this is in agreement with the high penetrability found in natural oligomers
336 when displaying cell-binding peptides (Aris and Villaverde, 2003; Villaverde, Feliu et al.,
337 1998).

338 While other engineering principles have been recently proposed to control protein self-
339 assembling (Bai, Luo et al., 2013) (King, Sheffler et al., 2012), the approach based on
340 the end terminal fusion of a cationic peptide and a polyhistidine is not restricted to a
341 unique core protein. This versatility would be convenient to avoid immunogenicity of the

342 constructs by selecting homologous proteins in next generation-constructions. By using
343 the end-terminal peptide-pair strategy, the formation of nanoparticles with pentameric,
344 toroid-like organization had been previously predicted (Unzueta, Ferrer-Miralles et al.,
345 2012; Vazquez, Roldan et al., 2010) and demonstrated by FESEM for a certain
346 category of proteins (those empowered by the cationic peptide T22) (Cespedes,
347 Unzueta et al., 2014). The highly resolutive AFM has confirmed this particular
348 architecture also for A5G27-GFP-H6 and FNI/II/V- GFP-H6 (Figure 1E,F), indicative
349 that the ring shaped distribution of the building blocks is not restricted to an unique type
350 of end-terminal tags. Being highly cationic (Table 1), the oligomerization of FNI/II/V-
351 GFP-H6 was fully anticipated at the upstream stage, while the formation of A5G27-
352 GFP-H6-based nanoparticles (being A5G27 poorly cationic) was unexpected. Acting
353 the amino terminal stretch both as architectonic tag and cell ligand, the promotion of
354 protein self-assembling by a non-cationic peptide expands, in any case, the spectrum
355 of potential ligands usable for the formation of cell-targeted nanoparticles, so far
356 restricted to cationic protein regions.

357 Laminin and fibronectin ligands bind CD44 through chondroitin and heparin-like GAG
358 side chains, especially by the heparin-sulphate found in CD44v3 and CD44v6 isoforms.
359 On the other hand, the alternative CD44 ligand HA binds to a binding site termed the
360 “link module” which is a domain expressed in all CD44 isoforms and it is located on
361 CD44 most exposed region (far from V3 and v6 variant regions) (Peach, Hollenbaugh
362 et al., 1993). Therefore, although HA could not be used as a competitor to probe the
363 CD44 targeting of the protein constructs developed here, the specificity in binding and
364 internalization was successfully demonstrated by the coincidence between CD44 levels
365 and penetrability (Figure 4), by inhibition mediated by a polyclonal anti-CD44 serum
366 (Figure 5), and though the external alternate regulation of CD44 levels and the
367 consequent variation in the efficiency of nanoparticle uptake (Figure 6).

In summary, we have developed smart and stable protein-only nanoparticles (A5G27-GFP-H6 and FNI/II/V-GFP-H6) as plastic agents that bind specifically CD44+ cells and that are efficiently internalized by receptor-mediated endocytosis in absence of cell toxicity. Interestingly, the vehicle itself is composed by fully functional (fluorescent, in our model system) proteins, what opens a plethora of possibilities regarding the targeted delivery of therapeutic polypeptides in form of nanoparticles. The nanostructure gained by these proteins would desirably allow them escaping from renal clearance, as the final size of the constructs is largely over the 6-7 nm cut-off. In addition, protein nanoparticles could be loaded with chemically coupled conventional drugs, as the principle of protein-drug coupling has been largely proved in already licensed drugs (Elzoghby, Samy et al., 2012). Altogether, and according to recent data proving the high stability *in vivo* (Cespedes, Unzueta et al., 2014) and efficient biodistribution of similar protein-only modular constructs (Unzueta, Cespedes et al., 2012), the tools generated here are promising platforms as vehicles for drugs and imaging agents, in the context of emerging nanomedines for breast cancer and other metastatic CD44-linked tumours based on biocompatible and versatile protein materials.

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553 Figure legends:

554

555 **Figure 1.** Construction and nanoscale characterization of CD44-targeted protein
556 nanoparticles. A) Schematic representation of the gene fusion scheme used in this
557 study. L represents a CD44 ligand that in some cases also has an architectonic role. B)
558 Size distribution of the protein constructs determined by DLS. Numerical values are
559 given in Table 1. C) AFM images of randomly selected A5G27-GFP-H6 nanoparticles.
560 D) Topography cross- section of one randomly selected isolated A5G27-GFP-H6
561 nanoparticle. E) Topography cross- section of two ring shaped A5G27-GFP-H6 nano
562 particles. F) AFM images of randomly selected FNI/II/V-GFP-H6 nanoparticles,
563 showing the topography cross- section of one isolated particle. G) Images of a
564 pentameric particle are shown. Measurements have been done with a tip radius of 2
565 nm and thus the width (but not the high) of the particles is inherently overestimated. An
566 AFM image is a convolution of the imaging tip shape/size with the actual shape of the
567 imaged object (Allen, Hud et al., 1992). Thus, one will observe broadening of the
568 sample features.

569

570 **Figure 2.** Internalization of CD44-targeted protein nanoparticles. A) Specific
571 fluorescence of the different protein constructs in comparison to that of the parental
572 GFP-H6 (in green). The specific green fluorescence of the parental protein is 1,021
573 fluorescence units (FU)/ug. B) Percentage of MDA-MB-231 cells that are fluorescent
574 after 24 h exposure to the multidomain proteins. C) Green fluorescence emitted by
575 MDA-MB-231 cells after 24 h exposure to multidomain proteins. D) Confocal sections
576 or projections of MDA-MB-231 cells upon 24 h of exposure to multidomain proteins.
577 Bars indicate 20 μm. E) Details of target cells during the uptake of fusion proteins,
578 indicating the exogenous material (in green) included in endosomes (red signal).

579 Merging into yellow is evident in some cases. A 3D projection is included in the case of
580 FNI/II/V-GFP-H6.

581

582 **Figure 3.** Kinetics of cellular internalization of CD44-targeted protein nanoparticles,
583 cytotoxicity and protein serum stability. A) Time course cell penetration of protein-only
584 nanoparticles at 1.5 µM. Percentatge of protein-internalised cells (left) and cell EGFP
585 fluorescence intensity (right). B) MTT viability analysis of MDA-MB-231cells upon
586 exposure to different doses of protein nanoparticles for 24,48 and 72h.. C) Stability of
587 protein nanoparticles in human. Fluorescence emission of serum samples with
588 nanoparticle incubation at different time point up to 24 hours. Soluble 24h corresponds
589 to fluorescence of soluble fraction from 24 hours sample after centrifugation to discard
590 nanoparticle aggregation/precipitation

591 **Figure 4:** Specific internalization of CD44-targeted protein nanoparticles in CD44-
592 expressing and not expressing cells. A) Percentage of cells uptaking A5-GFP-H6 (left)
593 and FNI/II/V-GFP-H6 (right) in CD44-overexpressing MDA-MB-231 and MCF-7 lines
594 and in Hep G2 and HEK 293 T lines. B) Histograms of CD44-expressing cell population
595 from $CD44^+$ cell lines MDA-MB-231 and MCF-7 and $CD44^-$ cell lines HepG2 and HEK-
596 293-T. APC-anti-CD44 marked cells (red) are compared with isotopic control cells
597 (black). FL4-H axis corresponds to APC intensity. The percentage of $CD44^+$ cells is
598 indicated.

599 **Figure 5:** Anti-CD44 mediated inhibition of nanoparticle internalization. Decrease on
600 the intensity of intracellular fluorescence mediated by 0.3 µM of either A5G27-GFP-H6
601 or FNI/II/V-GFP-H6 (control: back bar) when adding 0.3 µM polyclonal anti-CD44 (ratio
602 1:1). A control of GFP-H6 incubation with anti-CD44 is presented.

603

Figure 6: Modulation of nanoparticle internalization through CD44 regulation. A) Enhanced cell entry (left) and fluorescence intensity (right) of A5G27-GFP-H6 incubation at 0.3 µM after 1 h of cell exposure to increasing amounts of FGF2 (ratios 1:1, 1:10 and 1:30) due to CD44 receptor overexpression mediated by FGF2-H6. B) Reduced cell entry (left) and fluorescence intensity (right) of A5G27-GFP-H6 incubation at 0.3 µM after 1 h of cell exposure to anti-IL10 antibody due to an inhibition of IL-10-induced CD44 expression mediated by polyclonal anti-IL10.