Engineering a recombinant chlorotoxin as cell-targeted cytotoxic nanoparticles

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ABSTRACT

The controlled oligomerization of functional proteins at the nanoscale offers the possibility to design and produce, by recombinant DNA technologies and in cell factories, improved materials and drugs in form of nanoparticles. A recombinant version of the scorpion toxin chlorotoxin (CTX), which has attracted interest due to its ability to preferentially bind cancer cells, has been engineered to self-assemble as regular 12 nm-nanoparticles that penetrate cultured cells with the same receptor-specificity than the natural toxin. These materials, that appear as promising, biocompatible and biodegradable drug carriers for cell-targeted therapy of glioma also exhibit a mild but still significant cytotoxic activity associated to the recombinant toxin, that simultaneously acts as both driver and therapeutic agent. In addition, the manipulation of the CTX-flanking regions shows a potent impact on the performance of the nanoparticles, supporting a high functional versatility of CTX-based constructs, regulatable by conventional genetic engineering and adaptable to specific therapeutic situations.

Keywords: Recombinant proteins; nanoparticles; cancer therapies; targeted therapies

Cytotoxic proteins have a wide applicability in human therapies, especially in those conditions that require efficient and selective cell killing, such as cancer [1]. Chlorotoxin (CTX) is a small (4 kDa) basic peptide from the venom of the yellow scorpion *Leiurus quinquestriatus* [2], which blocks small-conductance chloride channels [3] thus paralyzing the scorpion prey. Being not extremely potent as a cytotoxin (for instance when compared to ribosome-inactivating proteins), it has gained interest as targeting agent, as the peptide shows a preferential binding to glioma cells mediated by the cell surface matrix metalloproteinase-2 (MMP-2) and the annexin-2. The expression of these proteins is increased in gliomas and other cancer cell types [4]. Upon exposure, CTX blocks the chloride channel activity but it also inhibits and downregulates MMP-2 [5], hampering the glioma tissue migration and invasion potency and inhibiting the metastasis [6]. Despite the efforts to develop chlorotoxin-derivatives and analogues that may enhance the cytotoxic effect of the natural peptide, the most promising strategy to improve patient mean survival time appears to be the use of chlorotoxin as a targeting agent for the delivery of anti-tumor agents. In this context, CTX has been explored in drug delivery as a component of drug formulations that have entered in clinical trials or are already FDA-approved [7]. Indeed, CTX has been largely explored as a partner in drug conjugates [8] or in form of fusion proteins [9] for the treatment and diagnosis of gliomas and other malignant tumors.

Recently, we have developed a protein engineering platform based on functional recruitment [10] to promote the self-assembly of reporter proteins such as the green fluorescent proteins (GFP) [11], and of therapeutic proteins such as pro-apoptotic factors [12] or microbial [13] and plant toxins [14] in form of therapeutic or theranostic nanoparticles [15]. These category of constructs, based on the fusion of N-terminal cationic stretches [16], form fully functional non-amyloid nanoparticles (ranging from ~10 to 60 nm) [17], that are highly stable upon *in vivo* administration and show a proper biodistrubution and accumulation in tumoral tissues [15, 18]. Lacking natural cell-targeting properties, these constructs have been genetically empowered to bind $CXCR4^+$

cells by the addition of the CXCR4-binding peptide T22 [19]. We were interested in knowing how a protein-only nanostructured version of CTX would keep the cell binding and internalization abilities of this peptide.

In this context, we designed the modular protein CTX-GFP-H6 (Figure 1; see all used methods in the Supplementary Information). Being cationic, CTX was expected to act as an architectonic tag in combination with the carboxy terminal histidine tail. In addition, we were interested in investigating whether CTX might retain its natural biological activities as a targeting agent in such a macromolecular organization. Since the cationic character of CTX is only moderate, we generated the alternative fusion CTX-KRKRK-GFP-H6, in which additional cationic residues were inserted between CTX and GFP (Figure 1A), to favour nanoparticle formation. Such strategy was previously observed as useful to promote oligomerization of blood-brain-barrier (BBB) crossing peptides as brain-targeted, protein-only nanoparticles [20]. These two CTX-containing proteins were produced and stored in carbonate buffer, which had been previously shown to be optimal for the stability of self-assembling protein nanoparticles in cell cultures [13]. We also tested two salt concentrations, as the ionic strength might have a significant role in nanoparticle formation [18]. As observed in the inset (Figure 1 A), both proteins were produced in bacteria as a single molecular species of the expected molecular mass, and spontaneously assembling as regular nanoparticles of ~12 nm (Figure 1 B, C). The addition of SDS, that promotes the disassembly of protein-only nanoparticles, revealed the actual size of the building blocks (around 3.8-5 nm, probably protein monomers and/or dimers), very similar to that of the parental GFP-H6 (5.4 nm, probably dimers, Figure 1 D). The salt content did not have any detectable impact on the particle size and stability but the buffer with salt seemed to promote or increase nanoparticle density or amount (Figure 1 B, C). A rich culture media such as Optipro did not show any significant impact on the size of the materials, while 10 % BSA decreased the particle size, probably by slightly destabilizing protein-protein contacts without inducing their full disassembling

(Figure 1 D). The resulting nanoparticles were fully fluorescent, with specific emission values of 2550.6 ± 2.8 units/µg and 2027.8 ± 8.1 units/µg for CTX-GFP-H6 and CTX-KRKRK-GFP-H6 respectively (not shown). Such intrinsic fluorescence emission allowed the monitoring of the materials in subsequent assays in cell cultures.

Figure 1. Modular organization of CTX-based building blocks and nanoparticle characterization. A. Schematic representation of the fusion proteins showing the amino acid sequences, where CTX (green) is placed at the amino termini and a hexahistidine tail (H6, blue) at the carboxy termini. Linker regions (purple) were placed in both cases between CTX and GFP (grey), to ensure fluorescence emission of the fusion protein. A cationic (red) region was intersected in CTX-KRKRK-GFP-H6 downstream the CTX. Siding amino acid sequences, we show the Comassie blue staining of proteins upon elution from affinity chromatography and PAGE. Relevant molecular weight markers are indicated. At the bottom, the molecular weights of the whole constructs as determined by MALDI. B. FESEM images of purified protein, showing their nanoarchitecture. Particles were diluted in two buffers, in which nanoparticles were tested for stability, namely carbonate buffer (C) and carbonate buffer plus 333 mM NaCl (C+S). Bar size is 20 nm in all panels. C. DLS plots showing the hydrodynamic size of nanoparticles. The peak value and the polydispersion index (Pdi) are indicated. Determinations were done on the material dissolved in carbonate buffer (C) and carbonate buffer plus 333 mM NaCl (C+S). D. The hydrodynamic size of the particles in these buffers was also determined in presence of BSA 10 % and in Optipro cell culture medium. SDS (at 1 %), that promotes the disassembling of protein-only nanoparticles was alternatively added to the buffer to identify the size of the building blocks. The size of the parental GFP-H6 is also indicated in nm. Untreated nanoparticles are shown by coloured plots. All the experiments were performed at pH 8. The peak value of the samples in SDS, BSA and Optipro are specified over the respective plots.

The spontaneous self-assembling of the engineered CTX, in both versions, prompted us to further investigate whether the toxin, in such oligomeric form, could mediate cell binding and internalization. Two cell lines previously identified as targets for CTX, namely HeLa (overexpressing annexin-2) and U87MG (overexpressing MMP2) [21-24], were selected to examine cell penetrability of the nanoconstructs, using the intrinsic green fluorescence as a monitoring tool. As observed (Figure 2A), CTX-KRKRK-GFP-H6 nanoparticles were much more efficient than CTX-GFP-H6 in cell internalization, in both cell lines. Moreover, regarding to the CTX-KRKRK-GFP-H6 protein version, a high salt content was significantly improving cell penetration of the material, in particular when

observing the uptake in U87MG cultures. In this cell line, penetrability of the protein nanoparticles was globally much higher than in HeLa cell line.

To ensure that CTX, in form of nanoparticles, had not lost its cell targeting activities we explored the selectivity of cell penetrability by inhibiting annexin-2 binding during cell interaction. As observed (Figure 2B), cell uptake in HeLa cells was significantly reduced by both a monoclonal antibody and a polyclonal serum against the cell surface protein acting as a CTX receptor. Since the antibodies acted over the penetration of both proteins in both buffers, we deduced that both the cationic stretch added to CTX-KRKRK-GFP-H6 and the high salt content enhanced the penetrability of the protein (Figure 2A) by a receptor-dependent mechanism (Figure 2B). Both cell penetrability and receptor specificity were observed at levels comparable to those shown by T22-GFP-H6 (Figure 2 C). This protein contains the peptide T22 that selectively and uniquely binds the cell surface cytokine receptor CXCR4, expressed in HeLa cells [19]. In that case, inhibition of cell uptake by the CXCR4 antagonist AMD3100 [25] was more effective than the mediated by the anti-annexin-2 antibodies over CTX-carrying constructs probably because the unique target of T22 compared to the dual binding sites of CTX. On the other hand, GFP-H6 was unable to penetrate cultured cells (Figure 2 C), supporting again the role of CTX in the penetrability of the nanoparticles.

Figure 2. Cell penetrability of CTX-based nanoparticles. A. Internalized nanoparticles in two alternative cell lines, namely HeLa and U87MG cells, 24 h after exposure to different protein amounts. Intracellular fluorescence was corrected by the specific emission to result in data representative of protein amounts. Cells were submitted to a harsh trypsin treatment before measurements to remove externally attached protein as described [26]. Nanoparticles were administered as dissolved in either carbonate (C) or carbonate with NaCl (C +S) buffer. Y axis scales might be not precisely comparable. B. Selective antibody-mediated inhibition of nanoparticle uptake in HeLa cells, by an anti-annexin-2 monoclonal antibody (mAb) and polyclonal antibody (pAb). The statistical analysis was performed using an ANOVA Tukey's multiple comparisons test (*$p < 0.05$; **$p < 0.01$). Normality was confirmed by Shapiro-Wilk W where $p>0.05$. Comparisons were done always with samples without antibody. C. Internalization of the related CXCR4-binding T22-GFP-H6 nanoparticles in ($CXCR4^+$) HeLa cells, and inhibition by the CXCR4 antagonist AMD3100 at an excess molar ratio 10:1. The parental GFP-H6 protein is

unable to enter cultured cells. All the experiments using HeLa cells were performed at pH 7.0-7.4, and those using U87 cells at pH 6.8-7.2.

At this stage, we determined the viability of cells exposed to CTX nanoparticles. Although classified as a toxin, chlorotoxin has displayed no obvious cytoxicity when administered to humans, which is important for drug development. Indeed, biological activities of chlorotoxin are mainly related with targeting ability, inhibition of migration and invasion of glioma cells and also, with antiangiogenic properties [27].

Unexpectedly (Figure 3), the nanostructured CTX-GFP-H6 had a significant cytotoxic impact on both lines, being the CTX-KRKRK-GFP-H6 version more cytotoxic than its counterpart CTX-GFP-H6, and U87MG cells more sensitive than HeLa. CTX-GFP-H6, when added to 0.1 μM, had a surprising but robust positive impact on cell viability, the number of viable cells reaching 120 % of the control samples. This fact was not observed in U87MG cells (Figure 3 B), in which cell death was only moderate, dose-independent, protein-independent, although significantly modulated by the salt content of the protein storage buffer. The toxicity of CTX was milder than that of the potent microbial toxin PE24 from *Pseudomonas aeruginosa* [13] (Figure 3 C), while the CXT-less GFP-H6 showed no effect on cultured cells (Figure 3 C).

Figure 3. Cell viability upon exposure to CTX-based nanoparticles. HeLa cells (A) and U87MG cells (B) were exposed to protein nanoparticles for 72 h. Nanoparticles were administered as dissolved in either carbonate (C) or carbonate with NaCl (C +S) buffer. The statistical analysis was performed using an ANOVA Tukey's multiple comparisons test (*$p < 0.05$; **$p < 0.01$). Normality was confirmed by Shapiro-Wilk W where $p>0.05$. Symbols at the top of the bars indicate the comparison with the control (100 %). Symbols at the left of the bars indicate comparisons between protein pairs, indicated by withe linkers. C) HeLa cell viability upon exposure to control, non-toxic GFP-H6 protein and cytotoxic T22-PE24-H6 nanoparticles. All the experiments using HeLa cells were performed at pH 7.0-7.4, and those using U87 cells at pH 6.8-7.2.

In summary, we have constructed two recombinant versions of CTX, that fused to a His-tagged GFP assemble as stable, fully fluorescent protein nanoparticles of regular size (12 nm, Figure 1). In this oligomeric form, the protein retains its ability to penetrate target cells, as determined here in two cell lines that display suitable receptors for CTX, namely HeLa and U87MG [21, 23, 24, 28]. The cell uptake is receptor-dependent, as it is inhibited when annexin-2 is sterically blocked by both a monoclonal antibody and an anti-annexin-2 sera (Figure 2 B). The CTX version that contains some additional cationic residues (KRKRK, Figure 1) inserted between the targeting peptide and GFP, shows an enhanced cell penetrability when compared with the plain CTX fusion (Figure 2 A). Since the uptake of the cationic construct is still receptor-mediated (Figure 2 B), it cannot be merely attributed to a higher electrostatic affinity of the nanoparticles to the cell membrane. In fact, it has been reported that single amino acid substitutions (Lys to Arg) that enhance the cationic nature of CTX result in a more stable version of the peptide and in an enhanced cell penetrability, probably associated to such higher structural stability [7]. Interestingly, the presence of salt dramatically enhances up to three fold the already improved cell penetrability of the cationic CTX version (Figure 2 A) that is accompanied by a slight tendency to an increased cytotoxicity *in vitro,* at least in HeLa cells (Figure 3).

Importantly, the nanostructured version of CTX retains the tumor cell-targeting properties of this protein, with high cell level of specificity, and excellent cell penetrability. In addition, a mild but significant cytotoxicity is associated to the constructs. The cell killing properties of the CTX nanoparticles appear to slightly be cell line dependent, and also influenced by the engineered cationic segments and by the salt content of the media (Figure 3). In this regard, both CTX nanoparticle versions developed here appear as promising biocompatible and biodegradable carrier systems to load anticancer drugs or therapeutic proteins for targeted therapy of glioma. However, in addition, the unexpected dual role of CTX as driver and cell killing agent is highly promising for a true functional

recruitment in the generation of nanostructured, multifunctional and smart therapeutics [10]. This is also in the line of designing chemically homogeneous vehicle-free drugs, at the nanoscale, that is now an emerging and appealing concept in the context of innovative tumor targeted drugs [29].

ACKNOWLEDGMENTS

This study has been funded by the Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER) (grant BIO2016-76063-R, AEI/FEDER, UE), AGAUR (2017SGR-229) and CIBER-BBN (project VENOM4CANCER) granted to AV, ISCIII (PI15/00272 co-founding FEDER) to EV. Protein production and DLS have been partially performed by the ICTS "NANBIOSIS", more specifically by the Protein Production Platform of CIBER-BBN/IBB (http://www.nanbiosis.es/unit/u1-protein-production-platform-ppp/) and the Biomaterial Processing and Nanostructuring Unit (http://www.nanbiosis.es/portfolio/u6-biomaterial-processing-and-nanostructuring-unit/). Cytometry and cell culture experiments were performed at the Cytometry and Cell Culture Unit of the UAB (SCAC). RD received an overseas predoctoral fellowship from Conacyt (Gobierno de México, 2016). LSG was supported by predoctoral fellowship from AGAUR (2018FI_B2_00051), NS was supported by a predoctoral fellowship from the Government of Navarra, and UU is supported by PERIS program from the health department of la Generalitat de Cataluña. AV received an ICREA ACADEMIA award.

AUTHOR CONTRIBUTIONS

Díaz R performed most of the protein production and characterization experiments assisted by Sánchez-Garcia L, Serna N, Cano-Garrido O and Sánchez, J. Serna N and Sánchez-García designed the fusion proteins and Sánchez-Chardi A performed the electron microscopy studies. Unzueta U, Vázquez E and Villaverde A conceived the study, supervised the experimental and prepared the figures. The manuscript was mainly written by Villaverde A.

CONFLICT OF INTEREST

The authors do not appreciate any conflict of interest.

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