Biomaterials 34 (2013) 4360–4367

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Enhancing cellular uptake of GFP *via* unfolded supercharged protein tags

Diego Pesce [a,1], Yuzhou Wu [b,1], Anke Kolbe [a], Tanja Weil [b,*], Andreas Herrmann [a,*]

[a] Department of Polymer Chemistry, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[b] Macromolecular Chemistry & Biomaterials, Institute of Organic Chemistry III, University of Ulm, Albert-Einstein-Allee 11, D-89069 Ulm, Germany

ARTICLE INFO

Article history:
Received 28 November 2012
Accepted 13 February 2013
Available online 9 March 2013

Keywords:
GFP
Supercharged protein
Protein therapeutic
Fusion protein
Elastin-like polypeptide

ABSTRACT

One of the barriers to the development of protein therapeutics is effective delivery to mammalian cells. The proteins must maintain a careful balance of polar moieties to enable administration and distribution and hydrophobic character to minimize cell toxicity. Numerous strategies have been applied to this end, from appending additional cationic peptides to supercharging the protein itself, sometimes with limited success. Here we present a strategy that combines these methods, by equipping a protein with supercharged elastin-like polypeptide (ELP) tags. We monitored cellular uptake and cell viability for GFP reporter proteins outfitted with a range of ELP tags and demonstrated enhanced uptake that correlates with the number of positive charges, while maintaining remarkably low cytotoxicity and resistance to degradation in the cell. GFP uptake proceeded mainly through caveolae-mediated endocytosis and we observed GFP emission inside the cells over extended time (up to 48 h). Low toxicity combined with high molecular weights of the tag opens the way to simultaneously optimize cell uptake and pharmacokinetic parameters. Thus, cationic supercharged ELP tags show great potential to improve the therapeutic profile of protein drugs leading to more efficient and safer biotherapeutics.

1. Introduction

The application of proteins for therapy represents an emerging area and until now, around 180 protein drugs have been clinically approved including insulin, erythropoietin, interferons, and a variety of antibodies [1]. In contrast to small molecule drugs, therapeutic proteins are generally considered as biocompatible with higher specificity for their respective target. Until now, most protein therapeutics mainly address extracellular targets since exogenous proteins are typically not able to diffuse into cells [2]. The pharmacokinetic properties and bioavailability of drugs critically depend on their molecular weight and their ability to overcome cellular membranes. As a consequence, many promising drug candidates fail to advance in the clinic since they do not fulfill the structural requirements needed for cellular uptake; either they are too hydrophobic for administration and distribution or they are too polar or of too high molecular weight for passive cellular entry. The latter holds particularly true for protein therapeutics.

Over the past decade, a variety of reagents have been developed to allow the delivery of proteins into mammalian cells including lipid-linked compounds [3], nanoparticles [4], cationic peptides [5] and fusions to receptor ligands [6] like the protein transduction domains (PTDs) including the HIV-1 transactivator of transcription (Tat) peptide, oligoarginine, the Drosophila Antennapedia-derived penetratin peptide [7] and the (antiapoptotic) pentapeptides derived from the Bax binding domain of Ku70 [8]. These cell penetrating peptides (CPPs) can trigger the transfer of a cargo across the cell membrane into the cytoplasm. They are typically characterized by an amino acid composition that either contains a high abundance of positively charged amino acids such as lysine or arginine (polycationic) or exhibits an alternating pattern of polar/charged and non-polar, hydrophobic residues (amphiphilic). A typical polycationic peptide is poly-L-lysine (PLL), which has been used to efficiently deliver a range of biomolecules into cells including albumin and horseradish peroxidase [9]. Poly-L-arginine (PLA), a motif inspired by the naturally occurring CPPs, has been widely used both in vitro for the transfer of peptides [10] and in vivo for the delivery of proteins [11]. Arg7 and Arg9 have been used in vivo for the transfer of cyclosporine [12] and human catalase [13], respectively. Several experimental and theoretical studies were carried out to elucidate the mechanism for translocation of CPPs and to investigate the role of different positively charged amino acid residues [14,15]. However, it was also demonstrated that polycations can induce toxicity such as mitochondria-mediated apoptosis [16].

Rather than appending a polycationic peptide, another strategy for introducing positive charges is increasing the net charge of a

* Corresponding authors.
E-mail addresses: Tanja.Weil@uni-ulm.de (T. Weil), a.herrmann@rug.nl (A. Herrmann).
[1] Both authors have contributed equally.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.02.038

polypeptide chain (henceforward called "supercharging"). This process enhances solubility and causes intermolecular repulsion even in the unfolded state, which in turn prevents aggregation [17]. Fusion to supercharged polypeptides enhances protein delivery in vitro and in vivo in a process that is clathrin- and energy-dependent, involving binding to anionic cell surface proteoglycans and endocytosis [2]. Using this approach, potent delivery vectors were realized by a "supercharged" variant of GFP [18], and by using a diverse class of naturally occurring supercharged human proteins [19]. Nevertheless, when rational supercharging was applied to other functional proteins like binding proteins (streptavidin) and enzymes (glutathione S-transferase) the modifications conferred increased thermal resistance but diminished function [20]. The "supercharging" of a protein by genetic mutation of surface-exposed amino acids requires, indeed, careful examination of the crystal structure to identify charged amino acid residues on the protein surface, mutation and shuffling of the gene sequence, and expression and screening for functional variants [2]. Moreover, it is not guaranteed that this process creates a functional variant with a certain desired surface charge.

We have previously reported the fabrication of unfolded supercharged polypeptides designed with elastin-like polypeptide (ELP) as the basic sequence [21]. These materials can be positively and negatively "supercharged" in a straightforward manner by introducing a lysine or glutamic acid residue at the second position of the repetitive pentapeptide sequence (GVGVP) [21]. This enables us to tune the overall charge of the protein by simply adjusting the length of the tag while obviating modification of the protein itself. At the same time, the lower charge density of the backbone in these ELP tags compared to other polycationic polymers such as polyarginine decreases cytotoxicity [21]. The suitability of neutral or weakly charged ELPs as materials for biomedical applications has been demonstrated in a number of publications. ELPs were employed as hydrogels for biosurface and tissue engineering [22] and for controlled release [23,24]. Furthermore, ELPs have been already used for delivery of drugs, peptides, proteins and DNA [24–26] and included in modified liposomes they enhance cellular uptake into tumor cells [27].

Thus positively supercharged tags based on ELP could be a suitable tool for facilitating cellular uptake of active proteins. In this contribution we tested whether positively "supercharged" ELP tags can serve as a platform for the delivery of proteins into mammalian cells, without altering their properties. Therefore, GFP was selected as a reporter protein and the cellular uptake of a functional variant of GFP fused to positively charged cationic ELP tags, with different numbers of charges (ranging from 9 to 72), was studied using an in vitro cell culture lung cell model (A549). Moreover, cell toxicity and cellular uptake mechanisms were investigated.

2. Materials and methods

2.1. Cloning and expression of GFP equipped with supercharged ELP tags

Monomers of the cationic and anionic ELP genes (K9 and E9) were ordered from Entelechon and were delivered in the pEN vector. Gene sequences and respective amino acid sequences of monomers are shown in Fig. S1. As the recognition sites of the restriction enzymes PflMI and BglI had to be preserved, one valine residue per ten pentapeptide repeats was incorporated instead of a lysine or glutamic acid residue. The ELP gene was excised from the pEN vector by digestion with EcoRI and HinDIII (Fermentas, St. Leon-Rot, Germany) and run on a 1% agarose gel (Sigma–Aldrich, St. Louis, MO) in TAE buffer (per 1 L, 108 g Tris base, 57.1 mL glacial acetic acid, 0.05 M EDTA, pH 8.0). The band containing the ELP gene was excised from the gel and purified using a spin column purification kit (QIAGEN). PUC19 (Fermentas, St. Leon-Rot, Germany) was digested with EcoRI and HinDIII and dephosphorylated. The vector was purified by agarose gel extraction following gel electrophoresis. The linearized pUC vector and the ELP-encoding gene were ligated and transformed into XL1-Blue cells (Stratagene, Cedar Creek, TX). For transformation, 20 μL of chemically competent *Escherichia coli* XL1-Blue cells were combined with 5 μL of the ligation mixture and further treated according to the manufacturer's protocol. Cells were spread on LB agar plates (for 1 L, 10 g Bacto™ tryptone, 5 g BBL™ yeast extract, 5 g NaCl, 15 g agar) supplemented with 100 μg/mL carbenicillin (Carl Roth, Karlsruhe, Germany), and incubated o/n at 37 °C. Colonies were picked and grown in 6 mL LB media (for 1 L, 10 g Bacto™ tryptone, 5 g BBL™ yeast extract, 5 g NaCl) (Becton, Dickinson and Co. Sparks, MD) supplemented with 100 μg/mL carbenicillin o/n, and plasmids were isolated using the Plasmid Miniprep kit (Fermentas, St. Leon-Rot, Germany). Positive clones were verified by plasmid digestion with EcoRI and HinDIII and the DNA sequence of putative inserts was further verified by DNA sequencing (SequenceXS, Leiden, The Netherlands). Gene oligomerization was performed as described by Chilkoti and co-workers (Fig. S2) [28].

The expression vector pET 25b(+) (Novagen Inc., San Diego, CA) was modified by cassette mutagenesis for incorporation of a unique SfiI recognition site as described before [21,28]. The modified pET 25b(+) vector (henceforward called pET-SfiI) was further digested with XbaI and NdeI (Fermentas, St. Leon-Rot, Germany), dephosphorylated and purified by a spin column purification kit. The GFP gene including the ribosomal binding site was excised from the pGFP vector (pGFP was a kind gift from Prof. D. Hilvert, Federal Institute of Technology, Zurich, Switzerland) by digestion with XbaI and SacI (Fermentas, St. Leon-Rot, Germany), and the excised gene (747 bp) was purified by agarose gel electrophoresis. A linker sequence that connects GFP and the SfiI restriction site was constructed in the following way: Oligonucleotides (Sigma–Aldrich, St. Louis, MO) linker_sens (cggtgtagtc ggtttagttc ccagaggaag tca) and linker_antisens (tatgacttcc tctgggaact aaaccgacta caccgagct), both 5′-phosphorylated, were mixed in equimolar ratios, incubated at 90 °C for 1 h and then cooled down stepwise to 20 °C for annealing (1 °C per 3 min). The resulting linker contained overhangs corresponding to a SacI and an NdeI restriction site, respectively. pET-SfiI, the insert containing GFP and the linker were ligated, yielding pET-GFP-SfiI. For insertion of ELP genes, pET-GFP-SfiI was digested with SfiI, dephosphorylated and purified using a microcentrifuge spin column kit. The respective ELP gene was excised from the pUC19 vector by digestion with PflMI and BglI, and the linearized vector and the insert containing the ELP gene were ligated, transformed into XL1-Blue cells, and screened as described above.

E. coli BLR (DE3) cells (Novagen) were transformed with the pET-SfiI expression vectors containing the respective ELP genes. For protein production, Terrific Broth medium (for 1 L, 12 g tryptone and 24 g yeast extract) enriched with phosphate buffer (for 1 L, 2.31 g potassium phosphate monobasic and 12.54 g potassium phosphate dibasic) (Merck KGaA, Darmstadt, Germany) and glycerol (4 mL per 1 L TB) (Merck) and supplemented with 100 μg/mL ampicillin (Roth), was inoculated with an o/n starter culture to an initial optical density at 600 nm (OD_{600}) of 0.1 and incubated at 37 °C with orbital agitation at 250 rpm until OD_{600} reached 0.7. Protein production was induced by a temperature shift to 30 °C. Cultures were then continued for additional 16 h post-induction. Cells were subsequently harvested by centrifugation (7000× *g*, 20 min, 4 °C), resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole (Carl Roth, Karlsruhe, Germany) for E variants or 10 mM Tris–HCl buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole for K variants) to an OD_{600} of 100 and disrupted with a constant cell disrupter (Constant Systems Ltd., Northands, UK). Cell debris was removed by centrifugation (40,000× *g*, 90 min, 4 °C). Proteins were purified from the supernatant under native conditions by Ni-sepharose chromatography (GE Healthcare). Product-containing fractions were pooled and dialyzed against ultrapure water (>18 MΩ). K variants were further purified by affinity chromatography using a Heparin HP column (GE Healthcare), and E variants by anion exchange chromatography using a Q HP column (GE Healthcare). Protein-containing fractions were dialyzed extensively against ultrapure water (>18 MΩ). Purified proteins were frozen in liquid nitrogen, lyophilized and stored at −20 °C until further use.

2.2. Protein characterization

The concentrations of the purified ELPs were determined by measuring absorbance at 280 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Protein purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). Gels were stained with Coomassie staining solution (40% methanol, 10% glacial acetic acid, 1 g/L Brilliant Blue R250). Photographs of the gels were taken with a LAS-3000 Image Reader (Fuji Photo Film (Europe) GmbH, Dusseldorf, Germany).

Mass spectrometric analysis was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) in the linear positive mode. The protein samples were mixed 1:1 *v*/*v* with a recrystallized α-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% ACN and 0.1% TFA, LaserBio Labs). Mass spectra were analyzed and calibrated internally with the Data Explorer software, version 4.9 (Applied Biosystems, Foster City, CA, USA). Trypsinogen (M_w = 23,980), enolase (M_w = 46,672) (LaserBio Labs, Sophia-Antipolis, France) and bovine serum albumin (M_w = 66,431) (Carl Roth, Karlsruhe, Germany) were used as calibration standards.

Fluorescence spectra of GFP, GFP-K72 and GFP-E72 were recorded on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). GFP concentration in phosphate buffer solution (PBS) was determined by measuring absorbance at 488 nm. Volumes were adjusted with PBS until absorbance at 488 nm was between 0.82 and 0.85. Samples were diluted ten times in PBS for fluorescence measurements. Fluorescence