Designing Cartilage Targeting and Drug Depot-Forming Cationic Fusion Protein of Insulin-Like Growth Factor 1

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INTRODUCTION: Drug delivery into cartilage is challenging due to rapid clearance by synovial fluid (SF) and hindrance from the dense cartilage matrix composed of anionic chondroitin sulfate-glycosaminoglycan (CS-GAG) and collagen II. We recently designed an arginine-rich short-length cationic peptide carrier (CPC) with a distributed net charge of +14 that exhibited a superior transport into cartilage **[1]**. CPC transport relies on electrostatic interaction with GAG that showed ~300x greater cartilage uptake compared to its neutral counterpart yet localized only in anionic tissue, offering the potential of optimum targeted drug delivery **[2]**. Although the effect of net charge and hydrophobicity on cartilage transport has been studied, the effect of their different arrangement has not been elucidated yet. Thus, we investigated the contribution of charged and hydrophobic residue arrangement on CPC cartilage transport to optimize it. The optimized CPC is fused into IGF-1, a pro-anabolic drug candidate for osteoarthritis treatment, forming a cationic fusion protein called CPC-IGF-1.

METHODS: Peptide syntheses: Six configurations of labeled CPC with a net charge of +14 were synthesized with varying numbers of charge clusters **(alternating (C-AL), end (C-EN), and block (C-BL)**; **Fig. 1A)** and hydrophobic clusters **(alternating (H-AL), end (H-EN), and block (H-BL);** all hydrophobic peptide has the same number of charged clusters as C-AL **Fig. 2A)** as the measure of distribution. Cartilage disks were harvested from femoropatellar grooves of bovine calf knees. To simulate late-stage OA, disks were digested in 0.1 mg/ml of trypsin-EDTA in PBS for 14h to induce ~90% GAG depletion. Equilibrium transport: Cartilage disks were equilibrated for 24h in 30μM CPCs in PBS or synovial fluid (SF). Uptake ratio was measured by normalizing intra-cartilage CPC concentration by bath concentration at equilibrium. Equilibrated disks were desorbed in 1X and 10X PBS for 7 days to estimate % intra-cartilage retention. Transient transport: Using a custom transport setup, CPCs were allowed to diffuse in 1D through cartilage's superficial (SZ) to deep zone (DZ) for 4h and then confocal imaged. CPC effective (D_{eff}) and steady state (D_{ss}) diffusivities were estimated using a transport chamber. Binding interactions: Microscale thermophoresis (MST) was used to measure the microscopic dissociation constant (K_d) and co-operativity (n) of CPCs to CS-GAG using the Hill equation. CPC-IGF-1 design: C-AL is fused to the C-terminus of R_3-IGF1 (Glu3→Arg3 substitution) using a rigid linker (LAEAAAKAAA); its structure is predicted using Alphafold and then docked into IGF-1R (PDB: 5U8Q). A similar procedure is done on R_3-IGF-1 as a comparison.

RESULTS: Higher number of charge clusters enabled faster intra-cartilage transport. Peptides with the same net charge but varying arrangements of charged residues exhibited similar equilibrium uptake ratios in healthy cartilage that were reduced by ~4x in late-stage OA explants—this is expected owing to the loss of anionic CS-GAG. C-BL exhibited the highest uptake in OA cartilage **(Fig. 1B)**. The majority of three peptides, despite their varying number of charge clusters, were retained within the healthy and OA cartilage over 7 days of desorption in 1X PBS; when charge-based binding was shielded using 10X PBS, 80% of uptaken C-AL and C-EN desorbed out while 60% of C-BL remained. Peptides with a greater number of charged clusters showed deeper 1-D cartilage penetration and higher D_{eff}, implying faster intra-cartilage transport **(Fig. 1C-D)**. These findings are supported by MST data that showed stronger interactions of the peptide with a lesser number of charged clusters to CS-GAG, which can slow down intra-cartilage transport **(Fig. 1E)**. Hydrophobic residues did not aid uptake into OA cartilage and slowed down intra-cartilage transport. All hydrophobic peptides except for H-BL showed similar uptake in healthy and OA cartilage compared to their hydrophilic counterpart, C-AL **(Fig. 2B)**. Hydrophobic peptides exhibited higher retention in 10X PBS desorption after 7 days in healthy cartilage and 2 days in OA cartilage. H-BL showed the highest retention in both conditions. However, in 1X PBS, these peptides were retained similarly over 7 days of desorption. The presence of hydrophobic residues slowed down intra-cartilage as seen in their 1D penetration and D_{eff} compared to C-AL **(Fig. 2C & 2E)**. This is also supported by their stronger binding interaction with CS-GAG **(Fig. 2D)**. In the presence of SF, H-AL and H-EN showed lower uptake in healthy and OA cartilage compared to C-AL **(Fig. 2F)** . MST measurement and uptake in SF were not conducted on H-BL due to aggregation issues. CPC-IGF1 computational assessment: Alphafold predicted CPC-IGF-1 conformation with a predicted local distance difference test (pLDDT) score of 81.8 which indicates high confidence in the structure. R_3-IGF1-domain of CPC-IGF-1 showed a close conformation alignment to R_3-IGF1 (RMSD: 2.49 Å).

DISCUSSION: We report that CPC with higher number of charge clusters exhibit faster intra-cartilage transport. This is owing to weak and reversible electrostatic binding interactions with the CS-GAGs that facilitate rapid and full-thickness intra-cartilage transport of cationic peptides. Hydrophobic interactions can also synergistically stabilize intra-cartilage charge-based binding; we thus, in three configurations, added phenylalanine into the most efficient C-AL design to harness any advantage from short-range hydrophobic interactions in stabilizing binding within OA cartilage. However, its binding within the SF was enhanced which slowed down intra-cartilage transport limiting any expected benefits. Thus, C-AL was chosen as CPC for R_3-IGF1 **(Fig. 3)**. C-AL was added to the C-terminus of R_3-IGF-1 using a rigid linker to ensure minimum interference to IGF-1R binding and proper R_3-IGF-1-domain folding. R_3-IGF-1 was chosen instead of native IGF-1 to minimize inhibition by IGF-BP which is elevated in OA **[3]**. Fusion protein's R_3-IGF-1-domain showed a close conformation alignment with R_3-IGF-1. These results indicate that CPC-IGF-1 could retain R_3-IGF-1 binding properties and bioactivity.

SIGNIFICANCE: We investigated the effect of the spatial configuration of charged and/or hydrophobic residue on CPC cartilage transport and used that knowledge to rationally design cationic IGF-1 fusion protein. Rapid and targeted transport of cationic IGF-I fusion protein in joints can enable its clinical translation for OA treatment. The same methods can be used for designing cartilage targeting cationic fusions of other disease-modifying OA drugs.

REF.: [1] Vedadghavami+, Acta Biomater, 2019 [2] Vedadghavami+, Acta Biomater, 2022 [3] Tardif+, Arthritis Rheumatol, 1996.

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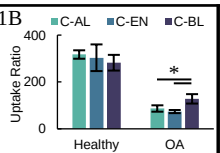
1A

CPC +14	CPC Sequence	Charge Cluster
C-AL	$(RN)_{14}$	14
C-EN	$R_7N_{14}R_7$	2
C-BL	$R_{14}N_{14}$	1

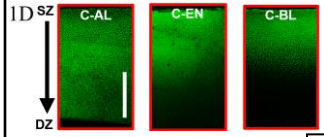
1B



1C

CPC +14	D_{EFF} ($\times 10^{-8}$ $\frac{cm^2}{s}$)	KD_{SS} ($\times 10^{-4}$ $\frac{cm^2}{s}$)
C-AL	15.3 ± 0.9	3.38 ± 0.3
C-EN	9.33 ± 0.4	2.29 ± 0.2
C-BL	4.43 ± 0.6	1.21 ± 0.2

1D



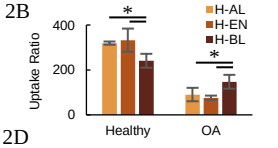
1E

CPC +14	K_d (μM)	n	ΔG ($\frac{kJ}{mol}$)
C-AL	1.49 ± 0.24	0.79	-26.26
C-EN	0.87 ± 0.19	1.20	-41.38
C-BL	6.01 ± 0.67	1.97	-58.68

2A

CPC +14	CPC Sequence	Hydrophobic Cluster
H-AL	$(RFRN)_2(RNRF)(RN)_2$ $(RFRN)(RNRF)_2$	6
H-EN	$(RF)_3(RN)_8(RF)_3$	2
H-BL	$(RN)_8(RF)_6$	1

2B



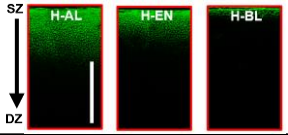
2C

CPC +14	D_{EFF} ($\times 10^{-8}$ $\frac{cm^2}{s}$)	KD_{SS} ($\times 10^{-4}$ $\frac{cm^2}{s}$)
H-AL	7.45 ± 1.0	4.18 ± 0.2
H-EN	5.23 ± 0.9	3.22 ± 0.1
H-BL	5.82 ± 0.4	2.33 ± 0.5

2D

CPC +14	K_d (μM)	n	ΔG ($\frac{kJ}{mol}$)
H-AL	482 ± 58	1.81	-34.21
H-EN	2748 ± 205	2.40	-35.26

2E



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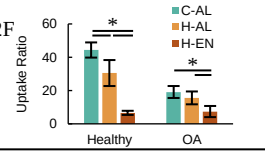

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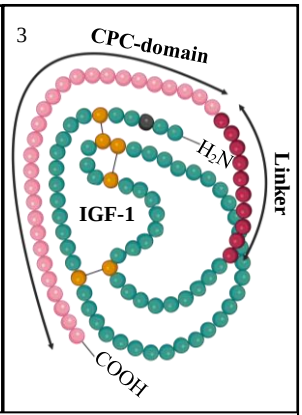

Fig. 1 Results on peptide with different charged residue arrangement. **A.** List of peptides with their # of charge cluster. **B.** 24h uptake into healthy and OA cartilage in PBS. **C.** Diffusivity data. **D.** 1D penetration after 4h. **E.** Binding properties into CS-GAG. **Fig. 2** Results on peptide with different hydrophobic residue arrangement. **A.** List of peptides with their # of hydrophobic cluster. **B.** 24h uptake into healthy and OA cartilage in PBS. **C.** Diffusivity data. **D.** Binding properties into CS-GAG. **E.** 1D penetration after 4h. **F.** 24h uptake into healthy and OA cartilage in SF. **Fig. 3** CPC-IGF1 fusion protein. Black bead represents Glu3→Arg3 substitution and yellow beads represent cysteine residues. Scale bar = 500 μm. *p<0.05.