Platform Reagent Development for the Intracellular Delivery of Therapeutic Peptides

B. C. Evans[*,**], C. Lander[**] and C. L. Duvall[***]

[*] Division of Vascular Surgery, Vanderbilt University Medical Center, Nashville, TN, USA, brian.c.evans@vanderbilt.edu
[**] Moerae Matrix, Inc., Morristown, NJ, USA, clander@moeraematrix.com
[***] Department of Biomedical Engineering, Vanderbilt University, Nashville, TN, USA, craig.duvall@vanderbilt.edu

ABSTRACT

Peptides hold many advantages over small molecule drugs in terms of specificity and ability to selectively modulate otherwise undruggable targets. The primary challenge of peptide therapeutics is permeation of the cell and endolysosomal compartment membranes, both of which must be traversed to access intracellular targets. Positively charged cell penetrating peptides (CPPs) are commonly used to improve peptide uptake by cells, but endosome entrapment remains an issue for most CPPs . We recently developed a peptide delivery technology comprising electrostatically complexed, endosomolytic nano-polyplexes (NPs) containing the pH-responsive polymer poly(propylacrylic acid) (PPAA). This formulation promotes greater internalization, endosome escape, and longer intracellular retention of a cell penetrant, intracellular-acting anti-inflammatory peptide. This work explores the generalizability of this nanopolyplex approach as a user-friendly reagent that further enhances CPP-mediated peptide uptake and enables escape from intracellular compartments.

Keywords: drug delivery, intracellular, cell penetrating peptide, endosomal escape, nanoparticle

1 INTRODUCTION

Intracellular-acting peptides have the potential to be applied as powerful therapeutic or research tools to modulate kinase activity, alter protein-protein interactions, and elucidate specific protein functions. Peptides can modulate targets not druggable by conventional small molecules and can be rationally designed to have more predictable and specific activity. The majority of peptides used in biomedical research are modified with cationic cell penetrating peptides (CPPs) to facilitate cell uptake, but CPP-based peptides lack potency due to entrapment and degradation in endolysosomal compartments inside of cells. Other currently available delivery reagents that purport the ability to deliver peptides are all based upon polycationic (i.e., positively charged) lipids or polymers that lack the ability to efficiently package and deliver cationic cargo. Collaborative efforts between the Vanderbilt Advanced Therapeutics Laboratory and Moerae Matrix, Inc., which specializes in the development of peptide therapeutics for inflammatory and fibrotic disorders, have yielded a proprietary technology termed Cytospear designed to facilitate CPP-based peptide intracellular bioavailability by both increasing peptide cell internalization and promoting intracellular bioavailability and long-term retention by enabling endosomal escape. Cytospear is an anionic, pH-responsive polymer formulation that, when mixed with cationic peptides, triggers the formation of electrostatically complexed nanoparticles (**fig. 1**).

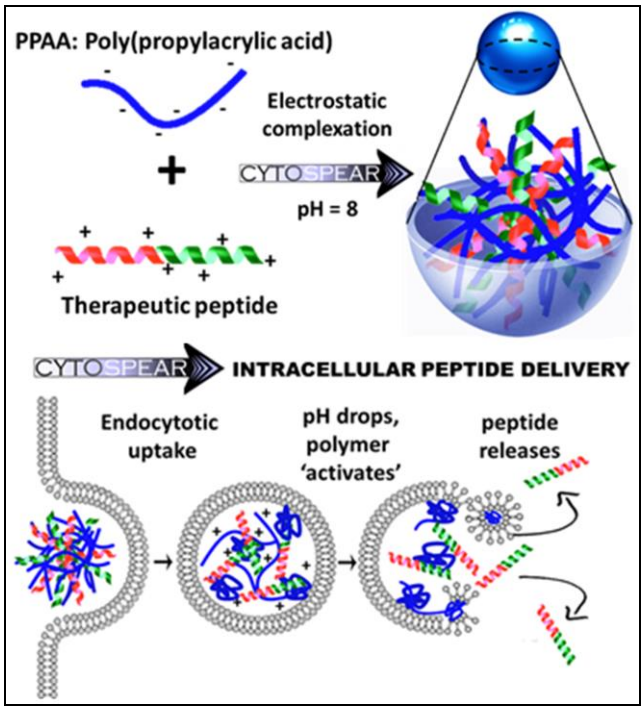

Figure 1: Cytospear-peptide nanopolyplex formulation and mechanism of peptide endosomal escape and cytosplasmic delivery

In high impact publications in *Science Translational Medicine* [1] and *ACS Nano* [2] the Cytospear technology was shown to significantly increase the potency, longevity of action, and therapeutic efficacy of two intracellularly active peptides that contain a cationic CPP sequence. The primary goal of this work is to establish greater breadth of this approach in terms of delivering a wide variety of CPP-

based peptides across a broad range of cell types. Achieving this goal will validate the general utility of this technology as a research reagent that enhances the uptake of cationic, CPP modified peptides and overcomes the delivery barrier of endosomal entrapment.

2 MATERIALS & METHODS

2.1 Monomer and Polymer Synthesis

All reagents were purchased from Sigma and were of analytical grade unless otherwise stated. 2- propylacrylic acid was synthesized according to [3] using diethyl propylmalonate as a precursor. The 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid chain transfer agent (CTA) was synthesized as described previously [4]. RAFT polymerization, purification, and characterization of the poly(propylacrylic acid) (PPAA) homopolymer was carried out as previously described [1].

2.2 Peptides and Delivery Reagents

Competing delivery reagents were obtained from the vendors specified in **table 1**. A library of seven therapeutic peptides fused to a variety of CPP sequences (**table 2**) was obtained from EZBiolab, where MK2i is a proprietary therapeutic MAPKAP Kinase 2 inhibtory peptide and VASP is a proprietary phospho-mimetic peptide of the vasodilator-stimulated phospho-protein.

Manufacturer	Delivery reagent
Pierce Biotechnology	Pro-ject
Polyplus (VWR)	PULSin
EMD Biosciences	ProeteoJuice
Genlantis	BioPorter
Targeting Systems	Profect-1
Targeting Systems	Profect-2
Clontech	Xfect

Table 1: Commercially available peptide / protein delivery reagents utilized in competitive uptake assays.

2.3 Cell Culture

Primary human coronary artery vascular smooth muscle cells (HCAVSMCs) and human cardiac microvascular endothelial cells (HMVECs) were obtained from Lonza. Mouse macrophages (RAW 264.7) and human mammary gland epithelial breast cancer cells (MCF7) were obtained from ATCC. All cells were maintained per suppliers' instructions and all cell media was supplemented with 1% penicillin-streptomycin and 50 ug/mL plasmocin (Invivogen).

2.4 Peptide Uptake

An amine-reactive Alexa-488 succinimidyl ester (Life Technologies) was dissolved in DMSO and mixed at a 1 to 3 molar ratio with each peptide in 100 mM sodium bicarbonate buffer and allowed to react for 4 hours. Labeled peptides were then purified using a PD-10 miditrap G-10 desalting column and lyophilized. Cells were grown to 80-90% confluence, harvested, and seeded at 60,000 cells/well in a 12 well plate and allowed to adhere overnight. For Cytospear formulation optimization, cells were treated with Cytospear reagent and labeled peptide at mass ratios of 3:1, 1:1, 1:3, 1:5, 1:10, and 1:20 peptide:polymer for 30 minutes then harvested for flow cytometric analysis. For comparative uptake and retention studies, cells were treated with fluorescent peptide with and without each delivery reagent (formulated according to the respective manufacturer's protocol), or PBS -/- as a negative control at a concentration of 5 µM peptide in Opti-MEM medium supplemented with 1% penicillin-streptomycin and 1% FBS for 30 minutes. Cells were then immediately harvested or washed and cultured for an additional 12, 24, 72, or 120 hours. For sequential delivery experiments, cells were treated for 30 minutes with PPAA alone, washed, and subsequently treated with fluorescently labeled peptide alone. Following treatment, cells were washed 2x in PBS -/, and harvested with 0.05% trypsin-EDTA, centrifuged, and suspended in 300 µL of 0.05% Trypan blue in PBS (-/-) for analysis on a FACSCalibur flow cytometer (Becton Dickinson) with BD CellQuestTM Pro software (V 5.2). Data was exported and analyzed with FlowJo software (V 10.1). All samples were run in triplicate.

Peptide	CPP Sequence (CPP-peptide)	pI	MW (Da)	CPP type
YARA-MK2i	YARAAARQARA-MK2i	pH 12.41	2283.7	cationic, non-amphipathic
TAT-MK2i	GRKKRRQRRRPPQ-MK2i	pH 12.89	2798.31	cationic, non-amphipathic
R6-MK2i	RRRRRR-MK2i	pH 13.18	2034.5	cationic, non-amphipathic
Penetratin-MK2i	RQIKIWFQNRRMKWKK-MK2i	pH 12.89	3326.1	primary amphipathic
Transportan-MK2i	GWTLNSAGYLLGKINLKALAALAKKIL-MK2i	pH 12.61	7228.86	secondary amphipathic
YARA-VASP	YARAAARQARA-VASP	pH 12.15	2124.4	cationic, non-amphipathic
VASP (no CPP)	VASP	pH 11.67	938.06	none

Table 2: Model CPP-modified peptides used in cellular uptake studies. Red fotn indicates cell penetrating peptides and their corresponding seuqences. The proprietary MK2i and VASP peptide sequences are not shown. pI = isoelectric point, MW = molecular weight.

2.5 Cytotoxicity Assay

200 µL of HMVEC cell suspension (at 10,000 cells/well) was seeded onto 96-well plates to yield an approximate 70% confluence per well. Cells were allowed to adhere to the plate overnight. Cells were then treated with 1.25, 2.5, 5, and 10 uM PPAA, or PBS -/- as a control treatment for 2 hours in Opti-MEM medium supplemented with 1% penicillin-streptomycin. Treatments were subsequently removed and the cells were cultured in fresh complete growth medium for 24 hours. Cells were then washed 2x with PBS +/+ and cell viability was then determined by a CytoTox-ONE Homogenous Membrane Integrity assay (Promega) according to the manufacturer's protocol.

3 RESULTS

We first investigated what factors influence optimal formulation of the Cytospear reagent with CPP containing peptides in terms of peptide uptake. With all cationic, non-amphipathic CPP containing peptides, optimal Cytospear-mediated peptide uptake was found to be dependent on the concentration of the polymeric delivery reagent alone (i.e., peak uptake at ~2.5 µM PPAA in vascular smooth muscle cells) and independent of peptide dose or the ratio of peptide to polymer (**fig. 2**).

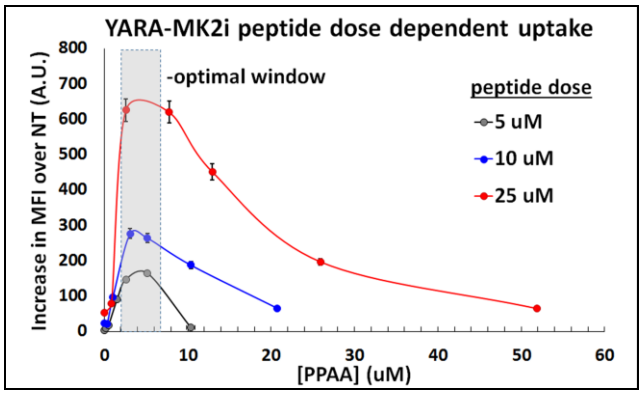

Figure 2: PPAA polymer dose dependent uptake of 5, 10, and 25 µM YARA-MK2i peptide in HCAVSMCs.

We then performed an analogous study looking at uptake of the MK2i peptide with two amphiphilic CPPs: the primary amphipathic, PTD-derived CPP penetratin and the secondary amphipathic, chemieric CPP transportan. Interestingly, Cytospear mediated delivery of these amphipathic CPP modified peptides showed the opposite trend to the cationic CPPs in that peptide uptake decreased as the amount of Cytospear reagent was increased (**fig. 3**). We hypothesized that when electrostatically complexed, the hydrophobic propyl moiety of the Cytospear polymer competitively interacts with and binds to the hydrophobic domain of these amphiphilic CPPs, thereby preventing them from interacting with the cell membrane.

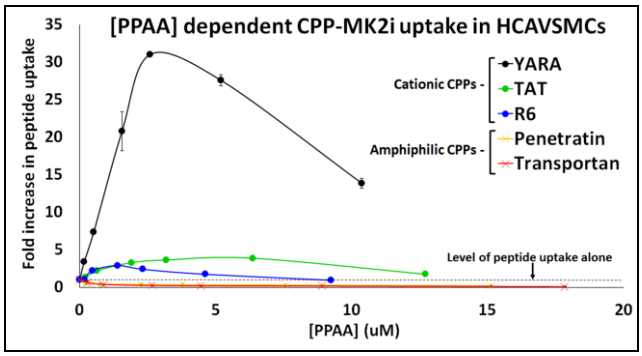

Figure 3: CPP-MK2i peptide uptake as a function of the dose of Cytospear polymer.

To test this hypotheses we performed another uptake assay where we compared pre-complexed treatments (i.e., co-delivery) to sequential delivery of the polymer then peptides. Our results demonstrated that although co-delivery decreased uptake, sequential delivery of the Cytospear polymer then amphiphilic CPP modified peptide was found to increase peptide uptake. We then investigated whether sequential delivery was also effective with cationic, non-amphipathic CPP modifed peptides, Sequential delivery was found to also enhance uptake, although less so than co-delivery (e.g., 12-fold increase at a 10 µM dose of polymer for sequential delivery vs. a 31-fold increase in uptake at a 2.5 µM dose of polymer for co-delivery of the YARA-MK2i peptide in HCAVSMCs). These results suggests that the delivery reagent coats cell membranes through hydrophobic interactions, resulting in a net increase in the net negative charge at the cell surface that increases cell surface affinity for cationic CPPs (**fig. 4**).

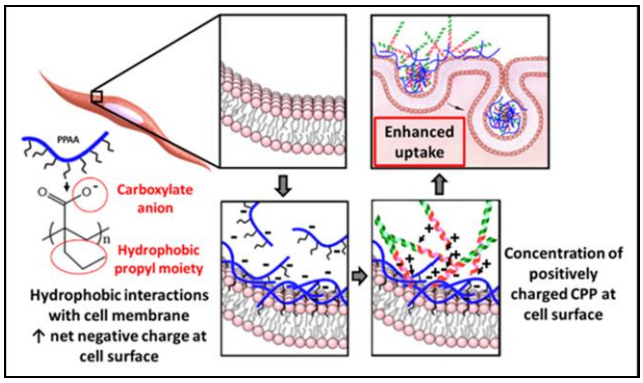

Figure 4: Schematic of proposed Cytospear mode of action for enhancing CPP-peptide uptake.

Considering the dependency of Cytospear-mediated cell internalization on the type of CPP, we then performed an uptake study utilizing a VASP peptide with and without the cationic CPP YARA to both verify the role of the CPP in Cytospear mediated peptide uptake and to investigate if the peptide sequence influenced uptake independent of the CPP sequence. No apparent differences were seen in VASP

peptide uptake without the inclusion of the CPP, whereas Cytospear enhanced uptake of the VASP peptide with the YARA-CPP in the same manner as the cationic CPP modified MK2i peptides tested (data not shown). These results indicate that the inclusion of the CPP is necessary for and that the therapeutic peptide sequence does not significantly impact Cytospear-mediated peptide uptake.

The ability of the Cytospear delivery reagent to enhance peptide uptake across a range of cell types was investigated, demonstrating enhanced uptake across all cell types tested (i.e., ≥ 30-fold increase in uptake in vascular smooth muscle and microvascular endothelial cells and a ~10-fold increase in uptake in macrophage and epithelial cancer cells; **fig. 5**). Furthermore, the optimal Cytospear dose range (i.e., 2-5 µM PPAA) was consistent among all cell types.

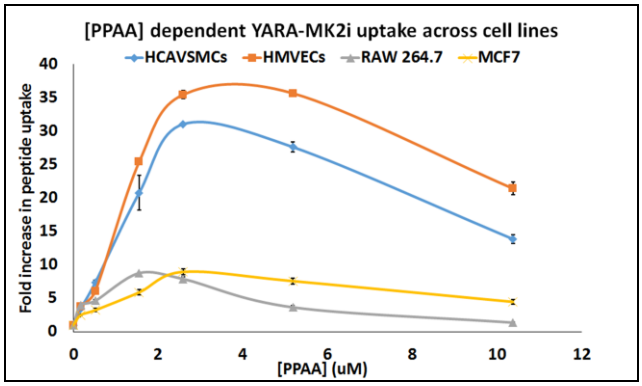

Figure 5: Cytospear polymer dose dependent fold increase in YARA-MK2i peptide uptake across a range of cell types compared to cells treated with peptide alone.

Dose dependent cytotoxicity of the Cytospear polymer, PPAA, was invgestigated in HMVECs revealing an LC50 = 5.07 µM PPAA (data not shown). This result suggests that the decreases in peptide uptake seen at polymer concentrations above the optimal 2-5 µM dose range is potentially due to toxicity and informs a maximal dose at which the Cytospear delivery reagent can be utilized.

To assess the commercial viability of the Cytospear delivery reagent, we compared Cytospear-mediated peptide delivery to seven commercially available protein/peptide delivery reagents (shown in **table 1**). For this study, all treatments were applied for 30 minutes and then removed. The cells were then immediately harvested or incubated in fresh medium for an additional 12, 24, 72, or 120 hours prior to harvesting for flow cytometric analysis. The Cytospear reagent (PPAA) demonstrated superior peptide delivery capability compared to all competing reagents, achieving a > 20 fold increase in peptide uptake following a 30 minute treatment (**fig. 6**). Furthermore, the Cytospear reagent demonstrated superior peptide retention over 5 days following treatment removal compared to all other delivery reagents. Interestingly, several reagents [i.e., Cytospear (PPAA), Xfect, Bioporter, Pro-ject, and PULSin] showed increases in internalized peptide following treatment removal. This finding suggests that peptide-delivery reagent complexes either 1) remain adhered to the tissue culture plate following treatment removal and continue to be internalized by cells or 2) that peptide-delivery reagent complexes adhere to the extracellular membrane and are internalized at later timepoints after treatment removal.

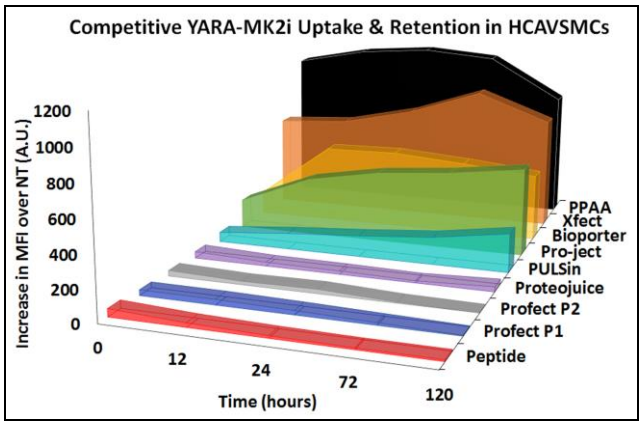

Figure 6: Comparative analysis of delivery reagent mediated YARA-MK2i peptide uptake and retention in HCAVSMCs.

4 CONCLUSION

These findings support that the Cytospear technology is an effective peptide delivery reagent that can be utilized with peptides modified with a wide array of cell penetrating peptides, although the treatment approach must be tailored to the type of CPP (i.e., sequential vs. co delivery for amphipathic vs. non-amphipathic CPPs, respectively). Furthermore, our results suggest that the Cytospear reagent is broadly applicable across a wide array of cell types. Finally, our results thus far have demonstrated the superiority of the Cytospear reagent to other commercially-available delivery technologies in delivering CPP modified peptides. Taken together, these results demonstrate the potential and commercial viability of Cytospear and support continued research and development of the technology as a peptide delivery reagent.

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