Published in final edited form as:

Cell Mol Bioeng. 2016 September ; 9(3): 368–381. doi:10.1007/s12195-016-0446-7.

Mechanism of Enhanced Cellular Uptake and Cytosolic Retention of MK2 Inhibitory Peptide Nano-polyplexes

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Abstract

Electrostatic complexation of a cationic MAPKAP kinase 2 inhibitory (MK2i) peptide with the anionic, pH-responsive polymer poly(propylacrylic acid) (PPAA) yields MK2i nano-polyplexes (MK2i-NPs) that significantly increase peptide uptake and intracellular retention. This study focused on elucidating the mechanism of MK2i-NP cellular uptake and intracellular trafficking in vascular smooth muscle cells. Small molecule inhibition of various endocytic pathways showed that MK2i-NP cellular uptake involves both macropinocytosis and clathrin mediated endocytosis, whereas the free peptide exclusively utilizes clathrin mediated endocytosis for cell entry. Scanning electron microscopy studies revealed that MK2i-NPs, but not free MK2i peptide, induce cellular membrane ruffling consistent with macropinocytosis. TEM confirmed that MK2i-NPs induce macropinosome formation and achieve MK2i endo-lysosomal escape and cytosolic delivery. Finally, a novel technique based on recruitment of Galectin-8-YFP was utilized to demonstrate that MK2i-NPs cause endosomal disruption within 30 minutes of uptake. These new insights on the relationship between NP physicochemical properties and cellular uptake and trafficking can potentially be applied to further optimize the MK2i-NP system and more broadly toward the rational engineering of nano-scale constructs for the intracellular delivery of biologic drugs.

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ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: xxxxx) contains supplementary material, which is available to authorized users.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for the completion of this work.

CONFLICTS OF INTEREST

KVK, BCE, CMB, and CLD report grant support from the National Institutes of Health and the American Heart Association; KVK additionally reports grant support from National Science Foundation Graduate Research Fellowship Program. During the conduct of the study, authors disclose non-financial support from Moerae Matrix, Inc., outside the submitted work. CMB is chief scientific officer and a shareholder of Moerae Matrix, Inc. BCE, CMB, and CLD are inventors listed on patent PCT/US2014/033873, licensed by Moerae Matrix, Inc. MK2i is known commercially known as MMI-0100 and is being developed by Moerae Matrix, Inc. for clinical use (ClinicalTrials.gov Identifier: NCT02515396).

Keywords

Drug delivery; nanoparticle; endosome escape; pH-responsive polymer; macropinocytosis; vascular therapeutic

INTRODUCTION

Intracellular-acting peptides have the potential to be applied as powerful research and therapeutic tools to modulate kinase activity, alter protein-protein interactions, and elucidate specific protein functions^{6,29,32,43}. Peptides can modulate targets not druggable by conventional small molecules and can be rationally designed to have more predictable and specific activity. However, clinical use of peptides is limited due to lack of intracellular bioavailability, arising from their hydrophilic nature and inability to escape endo-lysosomal pathways. To overcome this barrier, the majority of peptides used in research are modified with cationic cell penetrating peptides (CPPs) to facilitate plasma membrane interactions and transduction^{14,16,24}. Initial reports suggested that some CPPs may directly translocate the cell membrane, but later studies³⁷ showed this effect to be an artefact of microscopy. Upon endocytosis, CPPs typically traffic through early and late endosomes before ultimately becoming entrapped in lysosomes^{22,28}, sequestering most of the drug from its intracellular binding target. Many research groups have used polymeric nano-carriers to enable endo-lysosomal escape of biologic drugs like nucleic acids and peptides^{12,17}. Although debated, the “proton sponge” mechanism of endosomal escape has been extensively described for nucleic acid delivery systems like polyethyleneimine^{2,7}; however, the effects of endosomal escape on the subsequent trafficking of the drug cargo remain unclear⁴². Anionic, pH-responsive carriers like the one described herein have been shown to reduce trafficking into acidifying compartments following CD22-dependent receptor mediated uptake³. While the pharmacodynamics of anionic, polymer-based nano-polyplexes (NPs) used for the delivery of both an intracellular acting MAPKAP kinase 2 inhibitory (MK2i) peptide and a peptide mimetic of phosphorylated heat shock protein 20 (p-HSP20 peptide) have been thoroughly investigated^{18,19}, the mechanisms and kinetics of NP uptake and trafficking have not been rigorously studied. Because of the promise of MK2i-NPs as a prophylactic therapy to inhibit intimal hyperplasia and vasospasm of vascular grafts¹⁸, this specific formulation is the focus of the current studies designed to better elucidate endosomolytic NP intracellular pharmacokinetics.

Bioactivity of MK2i is dependent on access to the cellular cytoplasm because the molecular target of MK2i, activated MAPKAP kinase 2 (MK2), translocates from the nucleus to the cytosol upon phosphorylation by its primary upstream signaling kinase p38. In the cytosol, phospho-MK2 phosphorylates downstream mediators in the p38/MAPK signaling axis such as CREB, SRF, hnRNP A0, and HSP25/27 which are responsible for pro-inflammatory cytokine production, cellular proliferation, migration, and actin stress fiber formation^{30,34}. MK2i binds to and blocks the active site used by MK2 to phosphorylate these downstream mediators^{25,33} of pathologic vascular smooth muscle cell behavior.

Here, we sought to provide a comprehensive cellular pharmacokinetics analysis of the MK2i-NP formulation, which our group previously developed for the cytosolic delivery of MK2i to vascular smooth muscle cells to prevent intimal hyperplasia and vasospasm^{18,19}. A previous cell trafficking study by Flynn et al. found that the p-HSP20 peptide (therein known as AZX100)²², which shares the same CPP sequence with MK2i^{18,19}, was internalized rapidly *via* a lipid raft dependent, caveolae mediated uptake process that was not significantly influenced by actin or dynamin inhibition. This work also demonstrated that the majority of internalized peptide was sequestered within the endo-lysosomal system, preventing the peptide from efficiently binding to its intracellular target. Because the MK2i-NP formulation has significantly higher cell uptake and longevity of action relative to the free MK2i peptide¹⁸ (which utilizes the same CPP and suffers from the same endolysosomal entrapment fate as p-HSP20 peptide^{18,19,22}), we hypothesized that formulation of MK2i into NPs alters the peptide uptake mechanism and subsequent efficiency of intracellular trafficking to the cytosol.

MK2i-NPs^{18,19}, formed by the simple mixing of the cationic MK2i peptide with the anionic, endosomolytic polymer poly(propylacrylic acid) (PPAA) at pH 8, are electrostatically complexed nano-sized structures with a negative zeta potential ($\zeta = \sim -12$ mV). A long-standing view in the drug delivery field is that excess cationic charge enhances intracellular delivery of CPPs²⁷, polymeric micelles^{13,15}, and lipoplexes²¹ *via* electrostatic interaction between cationic carriers and anionic external cell membranes. However, formulation of the cationic MK2i peptide into net negatively charged NPs significantly enhances the cellular uptake of CPP-based peptides (e.g., both MK2i and p-HSP20 peptides)¹⁹. MK2i-NPs demonstrate a longer duration of intracellular retention relative to free MK2i and demonstrated equivalent bioactivity at an order of magnitude lower dose than the free peptide or control, non-endosomolytic nano-polyplexes formed with poly(acrylic acid) (PAA)^{18,19}. Both PPAA and PAA contain pH-responsive carboxylate moieties that drive electrostatic complexation with cationic MK2i peptide, however, due to the α -alkyl substitution of a pendant propyl chain (Fig. 1a), the carboxylate of PPAA has an effective acid dissociation constant (pK_a) of ~ 6.8 (whereas the pK_a of the pendant carboxylate of PAA is ~ 4.3). This difference results in PPAA, but not PAA, demonstrating pH-dependent membrane disruptive activity at pH values encountered during endo-lysosomal trafficking (i.e., pH 4.5 – pH 7.4)³⁹. Although formulation with both polymers at an optimized charge ratio formed NPs with statistically equivalent size and surface charge, control NPs formulated with non-endosomolytic PAA did not enhance cellular uptake or bioactivity. These findings motivated exploration into how the physicochemical properties of PPAA-based MK2i-NPs affect uptake, cellular processing, and intracellular trafficking of the therapeutic MK2i payload. Understanding the cellular mechanisms underlying the enhanced cellular uptake and altered cellular trafficking of MK2i-NPs may provide insights generalizable to intracellular delivery of peptides and other biomacromolecular drugs.

MATERIALS AND METHODS

Materials

Poly(propylacrylic acid) and MK2i were synthesized as previously reported^{18,19}. MK2i-NPs were formulated from these two components and characterized as previously described¹⁸. Endocytic inhibitors were obtained from Sigma-Aldrich. MK2i peptide was labeled with Alexa 488-NHS or Alexa 568-NHS (ThermoFisher Scientific) according to manufacturer instructions and purified with a desalting column (PD10 Minitrap, GE). Conjugation efficiency and conjugate purity were verified by UV-VIS spectroscopy. Fluorescently labeled MK2i peptide was used to formulate fluorescently labeled MK2i-NPs. To formulate gold labeled MK2i-NPs (Au-MK2i-NPs), a solution of 10 nm gold nanoparticles stabilized in PBS (Sigma-Aldrich 752584) was first added to PPAA, to which MK2i was subsequently added. Au loading into MK2i-NPs was confirmed by the complete disappearance of the 10 nm gold peak in DLS measurements (Fig. S2).

Cell Culture

Cell culture reagents were purchased from Gibco/Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise specified. Human coronary artery vascular smooth muscle cells were obtained from Lonza and grown in ATCC Vascular Cell Basal Medium supplemented with ATCC Vascular Smooth Muscle Cell Growth Kit (ATCC, Manassas, VA, USA), 1% Penicillin-Streptomycin, and Plasmocin prophylactic (Invivogen, San Diego, CA USA). All cultures were maintained in 75 cm² polystyrene tissue culture flasks in a sterile, 37 °C incubator with a humidified atmosphere supplemented to 5% CO₂. Cells were passaged 1:3 when they reached 75–90% confluence, with media otherwise replaced every other day. Primary cells were used at passages less than 9. The embryonic rat aortic smooth muscle cell line A7r5 was used for TEM and confocal studies. A7r5, HEK 239T, and their stable derivatives were grown in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1× ciprofloxacin (GenHunter, Nashville, TN, USA), and supplemented as noted with selection antibiotics to select cells expressing transgenes.

Flow Cytometry Quantification of Peptide Uptake Inhibition

Flow cytometry was performed as previously reported¹⁹, except that before treatment with MK2i-NPs or MK2i peptide, HCAVSMCs were pre-treated with endocytic inhibitors for 30 minutes, before being co-treated with inhibitor and MK2i-NP or peptide treatment. Doses for these inhibitors were verified to be above the published IC₅₀ literature values, consistent with other published inhibition studies, and to be in a range that did not cause significant toxicity in our hands in HCAVSMC pilot studies. Briefly, cells were seeded in triplicate in a 24 well plate at 30,000 cells *per* well and allowed to adhere overnight. Cell culture media was replaced with OptiMEM supplemented with 1% FBS, 1% penn-strep, and appropriate doses of each small molecule inhibitor: dynamin, 50 or 100 nM; Nystatin, 50 µg/mL; methyl-β-cyclodextrin, 5 mM; cytochalasin D, 5 µM; 5-(N-ethyl-N-isopropyl)amiloride (EIPA), 50 µM; wortmannin, 10, 50, and 100 nM; amiloride, 50 µM; polyinosinic acid, 50 µg/mL; and dextran sulfate, 100 µg/mL. Fluorescently labeled MK2i-NPs (10 µM) or free MK2i peptide (10 µM) were added to co-treat the cells for 30 minutes. Cells were then washed twice with PBS –/–, trypsinized, and resuspended in 0.1% Trypan blue in PBS (to

quench extracellular fluorescence) for analysis on a FACSCalibur flow cytometer equipped with BD CellQuest Pro software (v 5.2). Data was exported and analyzed with FlowJo software (V 7.6.4). Mean fluorescence intensity (MFI) was calculated by gating the cell population *via* forward and side scatter and subtracting the baseline MFI of untreated cells. Relative uptake was calculated by the following equation where $MFI_{Inhibition}$ and $MFI_{No\ Inhibition}$ are the mean fluorescence intensities calculated for cells pre-treated with inhibitors and solely treated with MK2i-NPs or MK2i peptide, respectively:

$$\text{Relative Uptake} = \left(\frac{MFI_{Inhibition}}{MFI_{No\ Inhibitor}} \right) \quad \text{Eq. 1}$$

Confocal Microscopy

Cells were plated at low confluence (4,000–10,000 cells/well) on an 8-well Nunc Lab-Tek chambered coverslip for confocal microscopy studies. For time course experiments, cells were maintained in a heated chamber with a humidified atmosphere and supplemented with 25 mM HEPES to provide physiologic pH buffering. Confocal image analysis was performed using the Nikon C1si+ system on a Nikon Eclipse Ti-0E inverted microscopy base, Plan Apo VC 20× differential interference contrast N2 objective, 0.75NA, Galvano scanner, and 408/488/543 dichroic mirror. The PMT HV gain, laser power, and display settings were set for maximal SNR based on control biological samples such that negative control samples lacking label had no background fluorescence and positive control samples had no saturated pixels. To ensure no crosstalk between fluorophores, image were acquired sequentially line by line (i.e., line 1 was imaged first with 405 ex / 450 em, then with 488 ex / 515 em before proceeding to line 2). Lack of crosstalk was verified with fluorescence-minus-one controls for all fluorescence channels. Nikon Perfect Focus System was used to ensure image focus. Image acquisition and analysis were performing using Nikon NIS-Elements AR version 4.30.01.

Scanning Electron Microscopy Imaging of Cellular Membrane Morphology

A7r5 cells were plated on acid washed glass coverslips at 50% confluence and allowed to adhere overnight. Cells were then treated for 20 minutes with MK2i-NP, MK2i peptide, or PBS, then thoroughly washed 1× with media and 5× with PBS. Samples were then either immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 37 ° C for 1 hour and then transferred to 4 ° C overnight. The samples were washed in 0.1 M cacodylate buffer, incubated for 1 hour in 1% osmium tetroxide at room temperature (RT), and then washed with 0.1 M cacodylate buffer. Subsequently, the samples were dehydrated through a graded ethanol series followed by 3 exchanges of 100% ethanol. Samples were then critical point dried on a Tousimis samdri-PVT-3D critical point dryer and mounted on carbon adhesive attached to aluminum stubs. Samples were then sputter coated with gold/palladium for 90 seconds. Images were taken on FEI Quanta Q250 SEM.

Transmission Electron Microscopy Imaging of Cellular Uptake and Trafficking

A7r5 cells were plated in 100 mm dishes at 75% confluence and allowed to adhere overnight. Cells were then treated for 30 minutes with Au-MK2i-NP or dose matched free

Au, then thoroughly washed 1× with media and 5× with PBS. Samples were then either immediately fixed or incubated in fresh media for an additional 24 hours prior to fixation and processing. Samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 37 ° C for 1 hour then transferred to 4 ° C, overnight. The samples were washed in 0.1 M cacodylate buffer, then incubated 1 hour in 1% osmium tetroxide at RT then washed with 0.1 M cacodylate buffer. Subsequently, the samples were dehydrated through a graded ethanol series, followed by 3 exchanges of 100% ethanol and 2 exchanges of pure propylene oxide (PO). Dehydrated samples were infiltrated with 25% Epon 812 resin and 75% PO for 30 minutes at RT, followed by infiltration with Epon 812 resin and PO [1:1] for 1 hour at RT and subsequent infiltration with fresh Epon 812 resin and PO [1:1] overnight at RT. The samples were subsequently infiltrated with resin for 48 hours and then allowed to polymerize at 60°C for 48 hours. Samples were cut to 500 – 1000 nm thick sections using a Leica Ultracut microtome. Thick sections were contrast stained with 1% toluidine blue and imaged with a Nikon AZ100 microscope to locate cells. 70–80 nm ultra-thin sections were cut and collected on 300-mesh copper grids and then post-stained with 2% uranyl acetate followed by Reynolds' lead citrate. Thin samples were imaged on a Philips/FEI Tecnai T12 electron microscope.

Generation of Stably Transfected Cell Lines

Retroviral transfer plasmids encoding galectin/yellow fluorescent protein fusion proteins were kindly gifted by Felix Randow⁴¹. The retroviral packaging and pseudo-typing vectors pUMVC and pCMV-VSV-G (Addgene.com plasmid #8449 and #8454, respectively) were kind gifts of Bob Weinberg⁴⁰. Galectin-8-YFP, pUMVC, and pCMV-VSV-G (9:8:1 mass ratio) were transfected into HEK 239T cells at 50% confluence in T-75 plates using FuGENE 6 according to the manufacturer's instructions to produce pseudotyped MMLV retroviral particles. Cell culture media was changed after 24 hours; viral supernatant collected between hours 24–48 and 48–72 hours post-transfection were syringe filtered and frozen at –20 °C until use. A7r5 cells were transduced overnight with 1:10 (v/v) viral supernatant:media and allowed to incubate for 24 hours, at which point cells were transferred into selection media containing Blasticidin (10 µg/ml). After 2 weeks, cells were plated by limiting dilution for single clonal selection and expansion to ensure homogenous expression levels for downstream assays. Successful selection was validated by fluorescence microscopy using non-transfected cells as a control.

Time Lapse Galectin-8 Microscopy

Single cell clonal populations were plated at 5,000 cells/well in chambered coverslips as described for confocal microscopy. Cells were monitored for 3–5 minutes to establish baseline Gal8 fluorescence. Cells were then treated for 30 minutes with MK2i or MK2i-NPs, after which the media was replaced. Live imaging was continued post-wash for at least 3 hours. Images were processed using the “spot detection” algorithm within Nikon NIS-Elements AR version 4.30.01 (Build 1021), exported to Prism GraphPad, and normalized to pre-treatment baseline fluorescence.

Statistics

Statistical analysis was performed *via* one-way ANOVA followed by Tukey's post-hoc test to compare experimental groups. Analyses were done with Microsoft Excel or GraphPad Prism 6 software. Statistical significance was accepted within a 95% confidence limit with a significance level of $\alpha = 0.05$. Results are presented as arithmetic mean $\pm$ SEM graphically. For time-lapse Gal8 recruitment data, statistical significance was defined by non-overlapping 95% confidence intervals.

RESULTS

Characterization of Cellular Uptake Mechanisms

To confirm that the A7r5 rat aortic smooth muscle cell line recapitulates the uptake behavior of human coronary artery smooth muscle cells as previously reported^{18,19}, A7r5 cells were treated with 10 μ M fluorescently labeled MK2i-NPs or MK2i peptide. MK2i-NP and MK2i treated cells both show higher mean fluorescence intensity relative to no treatment cells, and MK2i-NP treated A7r5 cells show a 49-fold increase in mean fluorescence intensity compared to cells treated with free MK2i peptide (Fig. 1b). These data recapitulate our previous results and validate that formulation of therapeutic CPPs into NPs increases peptide uptake by over an order of magnitude in vascular smooth muscle cells. To confirm these differences qualitatively, we visualized cell peptide uptake by fluorescence confocal microscopy (Fig. 1c and 1d).

To further understand the effects of NP formulation on MK2i uptake, a library of small molecule inhibitors of different endocytic pathways was used (see inhibitor descriptions in Table 1). Specifically, we sought to investigate: macropinocytosis, which has been implicated in CPP and nanoparticle internalization²⁷ and can be upregulated by cell surface receptor crosslinking^{20,36}; clathrin and caveolin mediated endocytosis, which has been implicated in MK2i internalization^{10,22}; lipid raft mediated endocytosis, which has been implicated in MK2i internalization in some cell types¹⁰; and scavenger receptor mediated uptake, which is known to be highly utilized in smooth muscle cells for uptake of negatively charged, hydrophobic particles like low-density lipoprotein that have similar physicochemical properties to PPAA-based MK2i-NPs^{9,11,31}.

Both MK2i-NP and MK2i peptide uptake were significantly inhibited by dynasore, indicating that clathrin and/or caveolae mediated endocytosis plays a key role in their uptake, consistent with the results previously reported by Brugnano and colleagues for MK2i peptide uptake (which they denote "YARA")¹⁰. Uptake of MK2i-NPs, but not MK2i, was significantly inhibited by all inhibitors implicated in macropinocytosis (i.e., wortmannin, cytochalasin D, and EIPA) (Fig. 2a). We initially hypothesized that scavenger receptors may play a role in MK2i-NP and/or MK2i uptake due to their negative and positive charge, respectively^{5,9,11,31}. However, treatment with the scavenger receptor inhibitors polyinosinic acid or dextran sulfate (DxSO₄) had no significant effects on MK2i-NP or MK2i uptake. Furthermore, inhibition of lipid-raft systems *via* nystatin or methyl- β -cyclodextrin pretreatment also showed no significant effects on MK2i-NP uptake (Fig. S1). Collectively, these data suggest that MK2i-NPs are internalized through macropinocytic

mechanisms in addition to the clathrin and/or caveolae mediated endocytic mechanisms responsible for uptake of the free MK2i peptide. The modest (and not statistically significant) increases in internalization of MK2i peptide with wortmannin, cytochalasin D, EIPA, and nystatin treatment are likely due to compensatory uptake mechanisms as previously shown by Brugnano et al.¹⁰

To confirm these results, live cells were treated with macropinocytosis inhibitors and visualized through confocal microscopy imaging (Fig. 2b). Cells treated with any of the macropinocytosis inhibitors showed a marked decrease in the amount of large / macropinosome-like vesicles positive for MK2i-NP fluorescence (white arrows in Fig. 2b). Because macropinocytosis is a process that involves re-organization of the actin cytoskeleton to form membrane protrusions (i.e., pseudopodia and membrane ruffling / blebbing) that engulf extracellular fluid, we sought to visualize this mechanism by SEM as previously reported³⁵. Cells were treated with 10 μ M MK2i-NPs, MK2i, or PBS. As expected, MK2i-NPs, but not MK2i or PBS, induced a high degree of visible membrane ruffling (Fig. 2c).

Transmission Electron Microscopy

TEM imaging was utilized for high-resolution visualization of cellular ultrastructure during uptake and intracellular trafficking of gold-labeled MK2i-NPs (Au-MK2i-NPs). Au-MK2i-NP size equivalence with MK2i-NPs and gold loading (~98%) were confirmed by dynamic light scattering (DLS, Fig. S2) which showed disappearance of ~10 nm free Au peak. Gold-labeled MK2i peptide was excluded from TEM studies because of concerns that 10 nm gold would significantly affect trafficking of the individual peptide molecules, which are very small relative to the gold (i.e., 2 nm compared to 10 nm diameters for the peptide and gold, respectively). Furthermore, irreversible aggregation and precipitation were apparent upon mixing of the gold label with free MK2i peptide. Therefore, free gold was used as a control. Cells were treated for 30 minutes and thoroughly washed 1 $\times$ with media and 5 $\times$ with PBS. Samples were then either immediately fixed or incubated in fresh media for an additional 24 hours prior to fixation and processing.

TEM imaging showed that Au-MK2i-NPs were visibly clustered at the plasma membrane during uptake in cells fixed immediately after 30 minutes of treatment (Fig. 3.a.i. & 3.a.ii.). TEM images also evinced structures consistent with macropinocytosis (Fig. 3.a.iii & 3.a.iv.), which were not found in untreated or gold only samples (Fig. 3.d.i. & 3.d.iii.). Au-MK2i-NPs were also found both contained within vesicular structures (Fig. 3.b., black arrows) and in the cytosol at 30 minutes (Fig. 3.b.ii. & 3.b.iv., white arrows). At 24 hr. post-treatment, Au-MK2i-NPs were found within the cell both inside vesicular structures consistent with the endo-lysosomal system (Fig. 3.c.i.–iii., black arrows) and outside membrane bound vesicles (Fig. 3.c.ii–iv., white arrows), suggesting endosomal escape and cytosolic MK2i delivery. Although some Au-MK2i-NPs were found to reside within vesicles with clearly visible and intact membranes (Fig. 3.c.i., black arrows), they were also commonly associated with vesicles with what appeared to be disrupted membranes [i.e., only partial membranes (Fig. 3.c.ii., black arrows) or fragmented membranes (Fig. 3.c.iii., all particles)]. These damaged and swollen vesicles found in Au-MK2i-NP treated cells are in clear contrast to untreated or gold only treated control cells (Figs. 3.d.i–iv.). These are the first high-resolution electron

micrographs, to our knowledge, that enable visualization of the endosomal disruption activity of the highly-utilized endosomolytic polymer PPAA.

Real-time Monitoring of Endosomal Escape

To determine the temporal kinetics of endosomal escape revealed by TEM, a novel live-cell fluorescent imaging methodology based on intracellular localization of galectin-8 (Gal8) was performed to assess endosomal membrane damage during MK2i-NP treatment. Gal8 is a β -galactoside-binding lectin of the galectin family, and it is normally localized diffusely throughout the cytosol and secreted, binding to glycans in the extracellular space to influence cell behavior¹. They also serve as part of the innate immune system to sense exposed intracellular glycans, which has been shown to occur during intracellular invasion by pathogens⁴¹. Use of the Gal8-YFP reporter to directly assay endosomal escape is possible because glycans are naturally located on the external plasma membrane and on the luminal surfaces inside vesicles (endosomes, macropinosomes, etc.). When endosomes are disrupted by pathogens (e.g., *Salmonella*⁴¹) or transfection reagents (e.g., Lipofectamine⁴⁴), Gal8 binds to and is concentrated onto glycans on the exposed luminal surface of the endosomal membrane where it induces macroautophagy (sequestration in a *de novo* generated double-membraned autophagosome⁸ that fuses with lysosomes to facilitate degradation of the autophagosomal contents). In 2015, Wittrup et al.⁴⁴ showed that Lipofectamine lipoplexes induce endosomal damage at the Rab 5 to Rab 7 conversion step, at which point, nucleic acids are released into the cytosol. These studies also showed that the transient membrane permeability of damaged endosomes results in detectable recruitment of Gal8-YFP, due to exposure of glycans within the endosomal lumen to cytosolic Gal8-YFP. Based on this method, A7r5 cells were generated that stably express Gal8-YFP, enabling real time monitoring and quantification of endosomal disruption in vascular smooth muscle cells (Fig. 4a). Treatment with MK2i-NPs, but not free MK2i peptide, rapidly induced recruitment of Gal8 to intracellular vesicles (bright punctate staining in Fig. 4b), whereas treatment with free MK2i peptide triggered no appreciable changes over time. In MK2i-NP-treated cells, we also located double membrane structures consistent with autophagosomes merging with electron dense vesicles consistent with lysosomes 30 minutes after treatment (Fig. 5).

DISCUSSION

This work focused on investigating the cellular uptake and intracellular trafficking mechanisms of a novel nano-polyplex formulation for the intracellular delivery of a therapeutic, anti-inflammatory MK2 inhibitory peptide. This MK2i-NP formulation was initially developed to circumvent peptide endo-lysosomal degradation and consequently increase the intracellular bioavailability of MK2i in vascular smooth muscle cells, thereby increasing potency and longevity of action of MK2i-NPs as a prophylactic therapy for blocking vascular bypass graft failure¹⁸. Formulation into net negatively-charged NPs was unexpectedly found to significantly increase MK2i peptide uptake in addition to enabling endosomal escape and increasing intracellular half-life. The clinical translatability of the MK2i-NP formulation was then validated in a pre-clinical animal model of bypass grafting, where treatment with MK2i-NPs was found to significantly enhance the ability of the MK2i peptide to prevent intimal hyperplasia in vein transplants *in vivo*¹⁸. Thus, the studies herein

were designed to gain mechanistic insight into how NP formulation influences the cellular uptake and intracellular trafficking of the MK2i peptide to realize a more effective peptide-based therapy. By elucidating the mechanism of MK2i-NP uptake and trafficking, we can gain insight into how to synthesize new drug delivery vehicles that utilize these efficient delivery pathways and also potentially identify strategies to further optimize the specific MK2i-NP prophylactic therapy.

Flow cytometry based analysis of MK2i-NP uptake verified that NP formulation significantly increased MK2i uptake in A7r5 rat aortic smooth muscle cells 49-fold (Fig. 1b), recapitulating the uptake effects found in primary human coronary artery vascular smooth muscle cells. Confocal microscopy imaging revealed that MK2i-NPs rapidly associated with the cellular membrane (Fig. S3), likely due to interactions of the hydrophobic/lipophilic propyl moiety of the PPAA polymer with the hydrophobic tails of phospholipids in the cellular membrane. To understand whether formulation into PPAA-based MK2i-NPs influences the mechanism of cellular internalization of the MK2i peptide, uptake studies were performed in conjunction with a library of small molecule inhibitors of the key endocytic pathways. Although the MK2i peptide is positively charged, MK2i-NPs have a negative ζ -potential (i.e., surface charge) due to the relative excess of anionic PPAA polymer (charge ratio of MK2i-NP formulation is one cationic primary amine on the MK2i peptide per three anionic carboxylate groups on PPAA). Considering that scavenger receptors are implicated in the uptake of negatively charged, oxidized low-density lipoprotein particles in vascular smooth muscle cells^{5,31}, we initially hypothesized that scavenger receptors may be responsible for the observed increase in peptide uptake. However, two separate scavenger receptor inhibitors, polyinosinic acid and dextran sulfate, were found to have no influence on MK2i-NP uptake (Fig. S1). In agreement with previous studies^{10,22} on MK2i and the related p-HSP20 peptide in other cell types, uptake of the MK2i peptide in smooth muscle cells appeared to be dependent on clathrin and/or caveolae mediated endocytosis and independent of macropinocytosis. In contrast, MK2i-NP uptake was dependent on both macropinocytosis and clathrin and/or caveolae mediated endocytosis. It is worth noting that the effect of fluorophore label could alter uptake and trafficking of MK2i in these studies. However, the Alexa family of fluorophores is among the least membrane interactive of all small molecule, water-soluble fluorophores²⁶ and is much smaller and has less charge than the MK2i peptide. Additionally, the effect of the fluorophore on cellular uptake and trafficking was minimized by using the same batch of labeled MK2i for both MK2i and MK2i-NP in every experiment and by ensuring that the molar ratio of dye to peptide was always less than 1.

Electron microscopy studies confirmed macropinocytosis as an underlying mechanism of enhanced uptake of MK2i-NPs. SEM analysis of MK2i-NP treated cells confirmed the induction of macropinocytosis as evinced by appearance of membrane ruffling, blebbing, and protrusions that were not present in untreated cells or cells treated with the free MK2i peptide (Fig. 2c). TEM analysis further supported macropinocytosis as a differential mechanism of MK2i-NP uptake. Gold-labeled Au-MK2i-NPs were found to strongly associated with areas of the cell membrane that displayed membrane ruffling, membrane blebbing, and pseudopodia. Furthermore, Au-MK2i-NPs were found within large diameter

(i.e., ~500 nm) vesicular compartments consistent with morphology of macropinosomes (Fig. 3.b.i–iv., 3.c.i–iii.).

Macropinosomes have been reported to be inherently more leaky than other types of endosomes²⁸, so we aimed to determine the trafficking and ultimate fate of MK2i-NPs following cellular internalization. Previous studies demonstrated that MK2i-NPs display switch-like pH-dependent membrane disruption ideally tuned for escape from acidified endo-lysosomal compartments. TEM analysis of Au-MK2i-NP uptake further support that NP formulation enables endosomal disruption and cytosolic delivery. TEM imaging revealed Au-MK2i-NPs associated with disrupted intracellular membranes and also showed Au-MK2i-NPs in the cytosol of treated cells, no longer bound by a clear endo-lysosomal membrane (Fig. 3.b.ii, iv; Fig. 3.c.ii–iv). The apparent endosomal escape and cytosolic delivery of Au-MK2i-NPs can be potentially attributed to both the pH-dependent membrane disruptive properties of PPAA and the leakiness of macropinosomes.

To further investigate membrane disruption as the mechanism of cytosolic MK2i delivery, a novel assay using A7r5 smooth muscle cells transduced with a stably integrated, fluorescent Gal8-YFP reporter was utilized. In contrast to untreated control cells and cells treated with free MK2i peptide, MK2i-NP cell treatment triggered significant recruitment of Gal8 to intracellular vesicles (Fig. 4), indicating active endosomal membrane disruption. Interestingly, TEM images also evinced double membrane autophagosomal structures (Fig. 5) in Au-MK2i-NP treated cells that were not found in control cells and that were consistent with the trafficking of damaged endosomes to lysosomes. This result is in agreement with the observations of Wittrup et al.⁴⁴ who found that Lipofectamine lipoplexes traffic to autophagosomes following Gal8 recruitment. In turn, Gal8 recruits NDP52 and LC3 proteins that are responsible for the formation of a secondary containment membrane around the damaged endosome^{41,44}, which is then rapidly trafficked to terminal lysosomes^{4,8,23,38}. Our data further supports the importance of identifying the role autophagosomal encapsulation of damaged endosomes plays in the delivery of biomacromolecular therapeutics and may help to explain why we see a 49-fold enhancement in cell uptake but only an approximately 10-fold enhancement in bioactivity^{18,19}.

In sum, our data suggest that formulation of a therapeutic MK2i peptide into PPAA-based pH-responsive NPs increases uptake through enhanced cellular membrane association and macropinocytosis, as summarized in Figure 6. This enhanced membrane association is hypothesized to be a result of the hydrophobic nature of the PPAA polymer causing interactions with lipids in the cell membrane. The pH-responsive behavior of the PPAA polymer also provided environmentally-triggered endo-lysosomal disruption following cellular internalization as supported by the observation of Au-MK2i-NPs in the cell cytoplasm and the robust recruitment of Gal8. However, endosomolytic carriers entering via macropinocytosis still suffer from partial lysosomal entrapment due to autophagosomal encapsulation of damaged endosomes followed by lysosomal trafficking. This result suggests that autophagosomal trafficking and sequestration may be another critical barrier in the delivery of cytosolic therapeutics. Inhibiting autophagosomal trafficking may represent a high potential target for further enhancements to intracellular bioavailability of cytosolic-acting biologic therapeutics.

Finally, further research into the structure-function relationships of peptide nano-polyplexes is warranted. Complexation with PPAA has been shown to enhance multiple peptides¹⁹, though it is unknown whether PPAA alters intracellular trafficking for all biomacromolecular cargo in similar ways. Efficient delivery of protein and peptide drugs to cytosolic targets remains a critical barrier in the clinical translation of this powerful class of therapeutics. Even the strongest endosomolytic agents are only partially efficient at cytosolic delivery and endosomal escape and may be ultimately limited in their delivery efficiency by autophagosome sequestration mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Felix Randow and Dr. Bob Weinburg for kind gifts of plasmids *via* AddGene.com. We thank Dr. Janice Williams for imaging support and electron microscopy expertise. Confocal imaging, transmission electron microscopy, and scanning electron microscopy were performed in part through the use of the Vanderbilt University Medical Center Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, DK59637 and EY08126). Dynamic light scattering was conducted at the Vanderbilt Institute of Nanoscale Sciences and Engineering. This work was supported by the American Heart Association (11SDG4890030), National Institutes of Health / National Heart, Lung, and Blood Institute (R21 HL110056 and R01 HL122347), and a National Science Foundation Graduate Research Fellowship to K.V.K. (0909667 and 1445197).

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Biography

Dr. Duvall is an Associate Professor of Biomedical Engineering (BME) at Vanderbilt University. He completed his undergraduate studies at the University of Kentucky in 2001 and immediately started his doctoral studies in BME at Georgia Tech and Emory University. Robert Guldberg, a mechanical/biomedical engineer from Georgia Tech, and W. Robert Taylor, a cardiologist from Emory, jointly directed Dr. Duvall's Ph.D. work. In 2007, Dr. Duvall joined the Bioengineering laboratories of Patrick Stayton and Allan Hoffman at the University of Washington developing polymeric drug delivery technologies as a postdoctoral researcher. Based on the foundations built from these combined experiences, the Duvall Advanced Therapeutics Laboratory (ATL) was launched at Vanderbilt in 2010. The ATL is funded by grants from NIH, DOD, NSF, AHA, and ADA and focuses on the development of novel drug delivery technologies for applications in regenerative medicine and breast cancer therapy.

Figure 1. NP formulation of MK2i peptide significantly enhances cell internalization in A7r5 VSMCs

(a) Chemical structure of poly(propylacrylic acid) and sequence of MK2i peptide. (b) Flow cytometric mean fluorescence intensity of cells treated with Alexa-488-labeled 10 μM MK2i peptide either as free peptide or formulated into MK2i-NPs. (c, d). Fluorescence microscopy images of A7r5 cells after 30 minutes of treatment with 10 μM Alexa-488-MK2i either as free peptide or formulated into MK2i-NPs.; scale bar is 10 μm; blue is nuclei, green is MK2i peptide.

Figure 2. MK2i-NPs enter cells through macropinocytosis while free MK2i exclusively utilizes clathrin mediated endocytosis

(a) Uptake inhibition of MK2i during inhibition of macropinocytosis, dynamin, and lipid rafts as measured by flow cytometry. Data is presented as mean fluorescence intensity relative to no treatment control. (b) Confocal micrographs of cells treated with MK2i-NP 10 μM and macropinocytosis inhibitors. (c) Scanning electron micrographs showing induction of cell surface ruffling indicating macropinocytosis of MK2i-NPs.

Figure 3a. NP binds to cell membrane, inducing macropinosome formation at t = 30 min. Cells treated 30 minutes with 10 μ M Au-MK2i-NP and immediately fixed.

500 nm

Figure 3b. NP found both inside vesicles and in cytosol near vesicles at t = 30 min. Cells treated 30 minutes with 10 μ M Au-MK2i-NP and immediately fixed.

500 nm

Figure 3c. NP found inside vesicles and in cytosol at t = 24 hr.

Cells treated 30 minutes, incubated 24 hr with medium, and immediately fixed.

500 nm

Figure 3d. Untreated control cells or gold only control as marked.

Cells were treated with PBS or dose-matched gold as controls.

500 nm

Key:

▲ Vesicular
 ▲ Cytosolic

Figure 3. TEM analysis supports MK2i-NP uptake by macropinocytosis and escape from endo-lysosomal vesicles

(a) Au-MK2i-NP binds to cell membrane, inducing macropinosome formation. (b) Au-MK2i-NPs are apparent both inside vesicles and in cytosol near vesicles at t=30 minutes. (c) Au-MK2i-NPs are also visualized inside of vesicles and in the cytosol at t=24 hours. (d) Untreated cells and Au-alone treated cells showing lack of membrane binding and low intracellular accumulation.

Figure 4. MK2i-NP treatment rapidly triggers endosomal disruption as measured by Gal8-YFP recruitment

(a) Endosomal disruption kinetics are plotted as fold change in Gal8 punctation. From top to bottom, blue circles represent 10 μ M MK2i-NP, green circles represent no treatment, and red circles indicate 10 μ M MK2i. Treatments were removed at $t = 30$ minutes and replaced with fresh medium. Asterisk (*) indicates $p < 0.05$ for MK2i-NP vs MK2i and NT. (b)

Representative images are shown for no treatment, MK2i-NP 10 μ M, and MK2i peptide 10 μ M at $t = 0, 15, 30$, and 60 minutes.

Figure 5. TEM image showing a structure consistent with autophagosome morphology merging with a lysosome

Time = 30 minutes. L marks electron dense structure consistent with lysosome, A marks a double membrane structure consistent with a damaged vesicle sealed inside an autophagosome.

Cellular Mechanisms of Endocytosis

Figure 6. Proposed mechanism of endocytosis

Macropinocytosis is a process of nonspecific internalization of large amounts of extracellular fluid characterized by actin-rich protrusions from the cell surface which close in a phosphoinositide 3-kinase (PI3K) dependent step and rely on Rac/Cdc42 signaling. Clathrin mediated endocytosis and caveolin mediated endocytosis rely on a dynamin dependent closure step for internalization. MK2i-NPs are proposed to enhance uptake by binding to external plasma membrane and internalizing *via* macropinocytosis.

Table 1

Selected inhibitors of endocytosis

Inhibitor	Pathway Inhibited	Mechanism
Wortmannin	Macropinocytosis, phagocytosis	an inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase [PI3K], which is required for closure of macropinosomes
Cytochalasin D	Macropinocytosis, phagocytosis	an inhibitor of actin polymerization, which is required for membrane ruffling and macropinosome formation
5-(N-Ethyl-N-isopropyl)amiloride (EIPA)	Macropinocytosis	inhibitor of Rac1/Cdc42 signaling that is required for macropinocytosis
Dynasore	Clathrin mediated endocytosis, caveolin mediated endocytosis	an inhibitor of dynamin, a GTPase responsible for clathrin mediated endocytosis, caveolin mediated endocytosis, and the related lipid raft mediated endocytosis
Nystatin	Lipid raft mediated endocytosis	a molecule that binds to and sequesters membrane cholesterol thereby inhibiting lipid raft mediated endocytosis
Methyl- β -cyclodextrin	Lipid-raft mediated endocytosis	Chelates plasma membrane cholesterol, thereby inhibiting lipid-raft mediated endocytosis
Polyinosinic acid	Scavenger receptor mediated endocytosis	Depletes cell surface of scavenger receptors by saturating binding and causing receptor internalization; competitive agonist
Dextran Sulfate (DxSO ₄)	Scavenger receptor mediated endocytosis	Depletes cell surface of scavenger receptors by saturating binding and causing receptor internalization; competitive agonist