

PROTAMINE MOLECULES AND USES THEREOF

FIELD OF INVENTION

[0001] The present invention relates to protamine molecules. More specifically, the present invention relates to protamine molecules and their use in delivery of other molecules.

BACKGROUND OF THE INVENTION

[0002] Protein drugs have gained substantial traction in the global pharmaceutical market, capturing a 20% share due to their increased selectivity and prolonged half-life compared to small molecule drugs [1]. Although oral administration can be desirable, the harsh gastrointestinal environment exhibits formidable biological barriers, including extreme acidity (pH 1.0-2.0) and active digestive enzymes, which minimizes oral absorption of protein drugs [2]. Limited by their inherently poor membrane permeability and stability in the gastrointestinal tract, protein and peptide drugs are often delivered via parenteral routes, such as intravenous (i.v.), subcutaneous (s.c.), and intramuscular (i.m.) injection, which is associated with discomfort and injection site reactions [3]. This poses challenges to patient compliance, particularly in chronic conditions requiring long-term medications [4].

[0003] Relative to oral delivery, the nasal environment is less harsh and allows for bypassing first-pass metabolism [5]. Nevertheless, several barriers have been reported for nasal delivery of proteins and peptides, including the mucosal layer, a physical barrier limiting diffusion of macromolecules, and the epithelial barrier that contains cellular junctions impeding paracellular penetration [2]. Disruption of adherens junctions has been shown to result in increased penetration of pathogens that lead to nasal inflammation [7].

[0004] Sublingual administration has emerged as a promising option, characterized by increased patient preference [8] and reduced formulation requirements, such as pH and osmolarity [9]. Sublingual delivery also boasts convenience and rapid absorption, which is particularly beneficial for emergent conditions [9]. However, thick, mucinous fluid secreted by sublingual glands and the tight epithelial layer under the mucus minimize drug absorption, especially for large molecules [10].

[0005] There have been various technologies developed to enhance transmucosal and transcellular delivery of biomolecules [11]. Absorption enhancers, such as surfactants that rearrange membrane lipids and proteins or the conformation of cellular junctions, have been shown to promote transmucosal delivery of macromolecules [12] and particles [13, 14]. However, absorption enhancers may risk irreversible junctional damage in the epithelial layer [15].

[0006] Mucoadhesive materials, such as chitosan (CS), Zein, and CS-N-arginine/alginate, can be included in the formulation to improve drug retention at the mucosal membrane for enhanced absorption [16-18]. Due to the rapid mucociliary clearance (7-15 min) in the nasal cavity, mucoadhesive materials and penetration enhancers are often used in drug formulations to enhance nasal absorption [19]. However, these materials exhibit limited activity in penetrating the epithelium [20, 21].

[0007] Cell-penetrating peptides (CPPs) have been studied for improving transmembrane delivery of macromolecules [22] including via the nasal route [78]. CPPs rich in arginine have been used for facilitating transmucosal delivery of macromolecules through formation of transient pores in the cell membrane and promotion of cellular internalization [23].

[0008] CPPs comprise a maximum of 30 amino acids, most of which are positively charged, such as arginine and lysine. Commonly used CPPs include poly(arginine)₈ (R8), TAT, and iRGD. The cationic CPPs have been implicated in interactions with anionic cell membranes, facilitating several mechanisms for intracellular delivery of macromolecules, including direct translocation across the membrane, formation of transient pores in the membrane, rearrangement of actin to reduce the membrane mechanical tension and increase the membrane permeability [24], and promotion of cellular tubulation and endocytosis [25, 26]. At low concentrations ($\leq 5 \mu\text{M}$), CPPs were internalized by cells mainly through endocytosis and accumulated in the lysosomes, whereas rapid cytoplasmic release occurs at higher concentrations ($\geq 10 \mu\text{M}$) [27]. Arginine-rich CPPs induced membrane multilamellarity and subsequently entered into cells via the formation of a fusion pore [28]. When CPP was chemically attached to a protein, the conjugate at high concentrations (50-150 μM) was shown to interact with the anionic cellular cytoskeleton components, leading to irreversible actin and tubulin aggregation [29] and subsequently an irreversible change in the cell cytoskeleton and cell ruffling [26, 30]. An intact actin cytoskeleton is required for cellular internalization, and CPPs can modify the actin cytoskeleton to influence cellular processes including CPP-mediated

intracellular delivery [31]. Additionally, actin rearrangement is a common mechanism of action for absorption enhancers for oral delivery [13]. A recent study showed that cell membrane tension was coordinated and maintained by the actin network. A decrease of F-actin alignment induced membrane invagination and reduced membrane tension, which facilitated cellular endocytosis and penetration of nanoparticles [24]. Furthermore, the reduced cortical tension favored the formation of tubulation, allowing entry of macromolecules [31]. Aggregation of F-actin has been discovered in cells treated with arginine-rich CPPs [29] and is associated with increased trafficking of endosomes and lysosomes [32]. Formation of microtubules in cells has been associated between CPP treatment and CPP-mediated endocytosis and intracellular drug delivery [31, 33].

[0009] However, CPP-mediated protein delivery may be constrained by the payload size and susceptible to endosomal entrapment [34, 35]. Fusing a protein payload with a CPP increases mucoadhesion and enhances endosomal escape [36, 37] but such methods require delicate chemistry or protein engineering to ensure consistent fabrication of the conjugate and release of the active drug [38, 39, 77].

[0010] Protamine is an FDA-approved injectable pharmaceutical ingredient and an antidote for heparin overdose. It is isolated from the sperm of fish, usually salmon, with an average molecular weight of 4,000 to 5,000 Da. Although protamine and low molecular weight protamine (LMWP) prepared by enzymatic digestion have been used to promote intracellular delivery of biomolecules, including nucleic acids and proteins [40], the strategies have been focused on forming complexes with nucleic acids via charge-charge interaction or chemical conjugation to a protein to facilitate its delivery [41-44]. Protamine is arginine rich and contains a nuclear translocation sequence [45].

[0011] Chronic rhinosinusitis with nasal polyps (CRSwNP) is classified as a type 2 inflammatory disease affecting up to 4% of people in the United States [46, 47]. If not treated properly, the disease can lead to significant inflammation in the lower airway. The current standard of care includes sinus surgery, antibiotics and oral corticosteroids [48]; however, the recurrence rate is high, and the patients experience many side effects [49]. IL-4 and IL-13 are the major cytokines mediating the progression of nasal polyposis; consequently, monoclonal antibodies (mAbs) blocking the IL-4, IL-13 or both pathways have become emerging therapeutics for CRSwNP, including Omalizumab, Reslizumab, Mepolizumab, and Dupilumab [50]. Dupilumab, which is administered via weekly subcutaneous injections over 16 weeks

[50] or twice a week for 52 weeks [51], blocks the IL-4 receptor α subunit (IL-4R α). This leads to inhibition of both IL-4 and IL-13 inflammatory pathways [52]; however, these frequent long-term injections cause injection site reactions in 40% of the patients [53]. Additionally, a high dose of mAb is required to achieve effective local concentrations in the nasal tissue. This not only raises the risk of systemic side effects, but also increases the cost of therapy [54].

SUMMARY

[0012] The present invention relates to protamine peptides and molecules and their use.

[0013] In one aspect, there is provided a protamine molecule including a first protamine peptide conjugated to one or more of a lipid, a hydrophobic moiety, a polymer including an amino group or an additional protamine peptide.

[0014] In some embodiments, the lipid may be a fatty acid, a lipid-amine, or a lipid-carboxyl and may further include a linker, such as polyethylene glycol (PEG) or N-Hydroxysuccinimide (NHS).

[0015] In some embodiments, the lipid may be palmitic acid, stearic acid, 1,2-phosphatidylethanolamine (PE), dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), DPPE-NHS, DSPE-NHS, or DOPE-NHS.

[0016] In some embodiments, the hydrophobic moiety may be allyl glycidyl ether (AGE).

[0017] In some embodiments, the polymer including an amino group may be poly(amidoamine) (PAMAM), polyethyleneimine (PEI), or polylysine (PLL).

[0018] In some embodiments, the polymer including an amino group may include comprising a crosslinker, such as is N-Succinimidyl S-Acetylthioacetate (SATA) or N- γ -Maleimidobutyryloxysuccinimide (GMBS), or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC).

[0019] In some embodiments, the protamine molecule may be protamine-stearic acid, protamine-PAMAM, protamine-DMPE, protamine-GMBS-PAMAM, protamine-

DMPE, protamine-AGE, protamine-SATA-PAMAM, protamine-C18, protamine-C16, protamine-PEI, or protamine-PLL.

[0020] In some embodiments, the first protamine peptide may be conjugated to one or two additional protamine peptides resulting in a protamine dimer or a protamine trimer.

[0021] In some embodiments, the first protamine peptide and the additional protamine peptide may be the same or different.

[0022] In some embodiments, the first protamine peptide and/or the additional protamine peptide may be a protamine salt, such as a protamine sulfate salt from salmon.

[0023] In some embodiments, the first protamine peptide or the additional protamine peptide may include the amino acid sequence of any one of SEQ ID NOs: 1-4 or a conservative substitution thereof.

[0024] In some embodiments, the protamine molecule may include an alpha-helix and/or a beta sheet.

[0025] In some embodiments, the protamine molecule may self-assemble into a nanostructure.

[0026] In some embodiments, the protamine molecule may increase the density at the cell membrane, increase the concentration of protamine at the cell membrane, and/or increase the interaction of protamine with the cell membrane.

[0027] A protamine molecule, as described herein, may be provided in a composition, for example, a pharmaceutical composition.

[0028] In an alternative aspect, there is provided a method for delivery of a payload molecule to a cell, by contacting the cell with the payload molecule in combination with the protamine molecule as described herein. In some embodiments, the delivery may be non-parenteral delivery.

[0029] In an alternative aspect, there is provided a method for non-parenteral delivery of a large payload molecule to a cell, by contacting the cell with the payload molecule in combination with a protamine peptide or a protamine molecule.

[0030] In some embodiments, the non-parenteral delivery may be intranasal, sublingual, oral, buccal, rectal, vaginal, intravitreal, topical, or to the skin, eye, brain, or lungs. In some embodiments, the non-parenteral delivery may be transmucosal, such as transepithelial or is to the lamina propria, or transcellular. In some embodiments, the transmucosal delivery is not by needle-based injection.

[0031] In some embodiments, the payload molecule may be delivered to the nucleus of the cell and/or substantially bypass the lysosomes of the cell.

[0032] In some embodiments, the payload molecule may be a protein, a peptide, a peptide analogue or a small molecule. In some embodiments, the payload molecule may be provided in a physical mixture with the protamine molecule. In some embodiments, the payload molecule may not be complexed with the protamine molecule. In some embodiments, the payload molecule may be an antibody, a growth hormone, insulin, or semaglutide.

[0033] In an alternative aspect, there is provided a method of permeabilizing the membrane of a cell by contacting the membrane of a cell with a protamine peptide and/or the protamine molecule as described herein. In some embodiments, the permeabilizing may be transient and/or reversible.

[0034] In an alternative aspect, there is provided a method of transfecting a cell by contacting the membrane of a cell with a protamine peptide and/or the protamine molecule as described herein.

[0035] In an alternative aspect, there is provided a method of treating or preventing a condition benefited by non-parenteral delivery of a payload molecule, by administering the payload molecule with a protamine peptide and/or the protamine as described herein to a subject in need thereof.

[0036] In some embodiments, the payload molecule may be administered at the same time or at a different time from the protamine peptide and/or the protamine molecule. In some embodiments, the protamine peptide and/or the protamine molecule may be administered prior to administration of the payload molecule.

[0037] In an alternative aspect, there is provided the use of a protamine peptide and/or the protamine molecule as described herein for treating or preventing a condition benefited by non-parenteral delivery of a payload molecule.

[0038] In some embodiments, the condition may be diabetes, rhinosinusitis, an eye condition or a skin condition.

[0039] This summary does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

[0041] **Figures 1A-B** are graphs showing cytotoxicity of protamine in Caco2 (**A**) and RPMI2650 (**B**) cells.

[0042] **Figure 2** is a graph showing FITC-BSA uptake by RPMI2650 cells in the presence or absence of different sources of protamine. FITC positive cells were quantified by FACS. (Protamine 1: protamine sulfate salt from salmon; protamine 2: protamine sulfate salt from herring; protamine 3: protamine from salmon; protamine 4: protamine chloride from salmon). Data = mean $\pm$ SD. ($n \geq 3$). **** $p < 0.0001$.

[0043] **Figures 3A-D** are graphs showing intracellular delivery of FITC-BSA in RPMI2650 (**A**, **C**) and Caco2 (**B**, **D**) cells. (**A**, **B**) Quantitative analysis of intracellular FITC-BSA and (**C**, **D**) FITC-BSA in different sites within the cytosol by ImageJ. Data = mean $\pm$ SD. ($n \geq 5$). Kruskal-Wallis test followed by Dunn's post hoc test was used to determine the statistical significance (* $p < 0.05$; ** $p < 0.01$).

[0044] **Figures 4A-B** are graphs showing Alexa488-Anti-SIRT1 monoclonal antibody (mAb) delivery to RPMI2650 cells in the presence or absence of protamine or R8. (**A**) Quantitative analysis of intracellular fluorescence of mAb ($n \geq 4$). Kruskal-Wallis test followed by Dunn's post hoc test was used to determine the statistical significance (* $p < 0.05$; ** $p < 0.01$). (**B**) Percentages of mAb fluorescence in the lysosomes, nucleus, and cytosol analyzed by ImageJ ($n \geq 5$). Data = mean $\pm$ SD.

[0045] **Figures 5A-C** are graphs showing actin cytoskeleton in RPMI2650 cells after different treatments. (**A**) Overall F-actin expression ($n \geq 9$). (**B**) Quantification of cellular spread area by ImageJ ($n \geq 6$). (**C**) Quantification of %cell with F-actin aggregates ($n \geq 8$). Significance determined by Kruskal-Wallis test followed by Dunn's post hoc test. Data = mean $\pm$ SD. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and n.s., no significant difference.

[0046] **Figures 6A-B** are graphs showing characterization of microtubule formation in RPMI2650 after R8 and protamine treatment. **(A)** Microtubule number per cell ($n \geq 5$); **(B)** Quantitative analysis of total length of microtubules ($n \geq 5$). Significance determined by one-way ANOVA followed by Tukey's post hoc test. Data = mean $\pm$ SD. Statistical significance: * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$, and n.s., not significant difference.

[0047] **Figures 7A-B** are graphs showing transcellular delivery of FITC-BSA *in vitro* and *in vivo*. **(A)** Cells were dissociated from the spheroids and analyzed by FACS ($n \geq 4$). **(B)** Cy7-BSA concentrations in plasma after intranasal administration of Cy7-BSA in the presence of protamine or R8. Saline and Cy7-BSA only were included as controls ($n \geq 6$). Significance determined by one-way ANOVA followed by Tukey's post hoc test. Data = mean $\pm$ SD. Statistical significance: ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

[0048] **Figures 8A-E** are graphs showing blood glucose (BG) levels in STZ-induced diabetic mice after intranasal (i.n.) treatment of different protamine/insulin formulations. **(A)** Insulin was dissolved in water at a pH of 3.2 and mixed with protamine at different ratios before being intranasally instilled (insulin dose 1 mg/kg). **(B)** Insulin was dissolved in water at various pHs and mixed with protamine at a weight ratio of 1:3 (insulin: protamine) before being intranasally instilled (insulin dose 1 mg/kg). **(C)** The optimal protamine/insulin formulation was prepared as described above and i.n. delivered to mice at 0.5, 1 and 5 mg/kg. **(D)** Comparison of the BG lowering effect of various formulations (insulin dose 1 mg/kg). **(E)** Serum insulin levels in STZ-induced diabetic mice after i.n. treatment of various formulations at 0, 0.5, 1, 2, and 4 h (insulin dose 1 mg/kg). Data = mean $\pm$ SD ($n \geq 4$). Arrows indicate formulation administration.

[0049] **Figure 9** is a graph showing sublingual delivery of Semaglutide.

[0050] **Figure 10** is a graph showing sublingual delivery of Semaglutide.

[0051] **Figures 11A-C** are graphs showing sublingual delivery of GLP-1/protamine provided better control of BG compared to GLP-1 alone delivered by i.v. or sublingual.

[0052] **Figure 12** is a graph showing Cy7-BSA concentration in plasma at different time points.

- [0053] **Figure 13** is a graph showing 647-Dupilumab concentration in plasma at different time points.
- [0054] **Figure 14** is a graph showing symptoms in a mouse model of chronic rhinosinusitis.
- [0055] **Figure 15** is a graph showing polypoid lesions and epithelial disruption in a mouse model of chronic rhinosinusitis.
- [0056] **Figures 16A-D** are graphs showing protamine/CD124 treated mice displayed decreased levels of INF gamma, IL-1 β , IL-17a, and TNF- α in the nose compared to the PBS control.
- [0057] **Figure 17** is a graph showing critical micelle concentration (CMC) of DMPE-Protamine.
- [0058] **Figure 18** is a graph showing cellular uptake of CRISPR/Cas9-gRNA.
- [0059] **Figure 19** is a schematic showing a two step reaction scheme for the preparation of protamine dimer.
- [0060] **Figure 20** is a schematic showing a two step reaction scheme for the preparation of Protamine- PAMAM.
- [0061] **Figure 21** is a schematic showing a reaction scheme for the preparation of Protamine trimer.
- [0062] **Figure 22** is a graph showing Protamine-PAMAM and (Protamine)₂ increased FITC-BSA delivery into the cells compared to protamine.
- [0063] **Figure 23** is a graph showing Protamine-PAMAM and Protamine-AGE increased GFP pDNA transfection efficiency in HEK 293T cells compared to protamine.
- [0064] **Figures 24A-D** are graphs showing DMPE-Protamine, (Protamine)₂ and (Protamine)₃ formulations for insulin can better decrease blood glucose levels compared to protamine/insulin formulation.
- [0065] **Figures 25A-F** are graphs showing PAMAM-Protamine-SATA and Protamine-AGE formulations for intranasal delivery of insulin performed more

consistently in lowering the BG in STZ-diabetic mice compared to the protamine formulation.

[0066] **Figure 26** is a graph showing Protamine-AGE and PAMAM-AGE_{0.35}-Protamine enhances systemic absorption of Alexa647-Dupilumab through intranasal delivery.

[0067] **Figures 27A-B** are graphs showing delivery into cells by sequences identified from protamine sulfate.

[0068] **Figure 28** is a graph showing sequences identified from protamine sulfate in lysosomes.

[0069] **Figures 29A-B** are graphs showing Cas9 RNP/sgRNA delivery to HEK 293 GFP cells in the presence or absence of Protamine or Protamine-AGE. A,B), quantitative analysis of intracellular fluorescence of EGFP and Cas9 RNP, respectively, analyzed by Image J. Data = mean $\pm$ SD. ($n \geq 3$). * $p < 0.05$ ** $p < 0.01$, **** $p < 0.0001$.

[0070] **Figure 30** is a graph showing protamine-based formulations enhanced nasal retention of mAb.

[0071] **Figures 31A-G** show the characterization of protamine and the derivatives. Synthetic scheme of C18-P (A) and P2 (B). Mass spectra of protamine (C), C18-P (D) and P2 (E). Insulin (5.7 kDa) was used as the internal standard. FTIR (F) and CD (G) spectra of protamine, C18-P and P2.

[0072] **Figure 32** is a graph showing cytotoxicity of protamine, C18-P and P2 in human primary gingiva keratinocytes.

[0073] **Figures 33A-B** are graphs showing effects of protamine, C18-P and P2 on cell membrane and intracellular delivery of IgG. (A) Quantitative analysis of intracellular AF647-IgG and (B) AF647-IgG colocalized with lysosomes by ImageJ. Data = mean $\pm$ SD. ($n \geq 5$). (** $p < 0.01$; **** $p < 0.0001$; n.s. no significant difference).

[0074] **Figure 34** is a graph showing AF647-IgG uptake by primary human gingiva keratinocytes in the presence or absence of protamine, C18-P and P2. AF647 positive cells were quantified by FACS. Data = mean $\pm$ SD. ($n \geq 3$). **** $p < 0.0001$.

[0075] **Figures 35A-B** are graphs showing intracellular delivery of FITC-BSA in human primary gingival keratinocytes. (A) Quantitative analysis of intracellular FITC-BSA by image J and (B) FITC-BSA positive cells quantified by FCM.

Data = mean $\pm$ SD. ($n \geq 5$). (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

[0076] **Figures 36A-B** are graphs showing spheroid penetration of AF647-IgG delivered by protamine, C18-P and P2. (A) Heat map of fluorescence intensity of AF647-IgG distributed from the spheroid periphery. (B) Cells were dissociated from the spheroids and analyzed by FCM for AF647-IgG+ cells. Data = mean $\pm$ SD.

($n \geq 5$). (** $p < 0.01$; **** $p < 0.0001$).

[0077] **Figures 37A-B** are graphs showing spheroid penetration of FITC-BSA delivered by protamine, C18-P and P2. (A) Heat map of fluorescence intensity of FITC-BSA distributed from the spheroid periphery. (B) Cells were dissociated from the spheroids and analyzed by FACS for FITC-BSA + cells. Data = mean $\pm$ SD.

($n \geq 5$). (** $p < 0.01$; **** $p < 0.0001$).

[0078] **Figures 38A-B** are graphs showing gene editing efficiency mediated by Cas9-RNP delivered by protamine, C18-P and P2 in eGFP HEK 293 cells. Cells were dissociated from the spheroids, and Cas9-RFP-RNP positive cells (A) and RNP+ and eGFP- cells (B) were quantified by FCM. Data = mean $\pm$ SD. ($n \geq 5$). (** $p < 0.01$; **** $p < 0.0001$).

[0079] **Figures 39A-B** are graphs showing gene editing efficiency mediated by protamine C18-P and P2 in eGFP HEK 293 cell lines. (A) eGFP intensity from Figure 4A was quantified by image J. (B) eGFP knockdown efficiency was analyzed by FCM.

[0080] **Figures 40A-B** are graphs showing penetration of AF647-IgG in a human sublingual tissue substitute delivered by protamine, C18-P, or P2. Confocal images were analyzed in ImageJ for the overall fluorescence intensity of AF647-IgG (A) and the fluorescence intensity of AF647-IgG in relation to distance from the apical surface (B). Data = mean $\pm$ SD. ($n \geq 5$). (** $p < 0.01$; *** $p < 0.001$).

[0081] **Figures 41A-C** are graphs showing sublingual penetration of AF647-IgG in mice delivered by protamine, C18-P and P2. Fluorescence intensity of AF647-IgG in relation to distance from the tissue surface in sublingual tissues collected at 0.5 (A), 2 (B) and 4 h (C).

[0082] **Figures 42A-B** are graphs showing blood glucose (BG) controlling effect in mice. (A) Comparison of blood glucose levels in STZ-induced diabetic mice after sublingual (sub.) or subcutaneous (s.c.) treatment with various insulin formulations. Insulin was dissolved in water at a pH of 3.2 and mixed with protamine, C18-P, or P2 before being sublingually administered (insulin dose = 5 mg/kg for sub. and 1 mg/kg for s.c.). Black arrow indicates formulation administration. (B) Comparison of the BG controlling effect of various semaglutide formulations (drug dose = 60 µg/kg for s.c. and 500 µg/kg for sub.) Black arrow indicates formulation administration; green arrows indicate glucose injection at 1.5 g/kg. Data = mean ± SD ($n \geq 4$). Statistical analyses: protamine vs. C18-P (blue asterisk); protamine vs. P2 (green asterisk). * $p < 0.05$, ** $p < 0.01$.

[0083] **Figures 43A-C** are graphs showing the pharmacokinetics of various sublingually (sub.) delivered protein formulations compared with subcutaneous (s.c.) protein administration. Plasma was collected at 0, 0.5, 2 and 4 h. Proteins were dissolved in PBS and mixed with protamine, C18-P, or P2 before being sublingually administered (Dose of protamine, C18-P and P2 is 6 mg/kg). (A) Recombinant human growth hormone (rhGH). (rhGH dose = 500 µg/kg). (B) Cy7-BSA. (Cy7-BSA dose = 20 mg/kg.) (C) AF647-IgG. (AF647-IgG dose = 1.3 mg/kg.) Data are presented as mean ± SD ($n \geq 3$). Statistical analyses in area under the curves (AUCs): Payload only vs. s.c. (black asterisk); Payload only vs. C18-P (blue asterisk); Payload only vs. P2 (green asterisk). * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$. No significant difference was observed between CPP formulations and s.c. control.

[0084] **Figure 44** is a graph showing in vivo distribution of ^{111}In -IgG delivered intranasally by different formulations. Quantitative analysis of the central nasal cavity as the Volume of Interest (VOI) for both experimental groups. The results are given as the standard uptake values (SUVs) +/- their standard deviation.

[0085] **Figures 45A-F** are graphs showing in vivo efficacy of different αCD124 formulations in mice with moderate CRSwNP. (A) Number of nose touches in 10 min for mice ($N \geq 5$). (B) Surface area ratios (airway/bone) obtained from micro-CT images. (C) Numbers of nasal polypoid lesions per mouse was counted from the H&E images ($N \geq 5$). (D) Numbers of epithelial disruptions per field (magnification 40X) were counted ($N \geq 10$). IgE concentrations in plasma (E) and nasal tissue homogenate (F) from moderate CRSwNP mice after different treatments ($N \geq 3-7$).

[0086] **Figures 46A-K** are graphs showing levels of type 2 inflammatory biomarkers in moderate CRSwNP mice treated with various formulations.

[0087] **Figure 47** is a schematic showing a reaction scheme for the preparation of Dendri-P.

[0088] **Figure 48** is a graph showing FTIR spectra of protamine, Nano-P and Dendri-P.

[0089] **Figures 49A-B** are graphs showing penetration of AF- α CD124 in the nasal tissue in mice delivered by protamine, Nano-P, and Dendri-P. Confocal images were analyzed by ImageJ for the overall fluorescence intensity of AF- α CD124 in the nasal tissue (A) and the fluorescence intensity of AF- α CD124 in relation to distance from the apical surface (B). Data = mean $\pm$ SD. ($n \geq 5$). (** $p < 0.01$; **** $p < 0.0001$).

[0090] **Figure 50** is a graph showing quantification of epithelium thickness per field from different treatment groups. $N \geq 15$ fields from 4-7 mice per group.

[0091] **Figures 51A-B** are graphs showing in vivo efficacy of different α CD124 formulations in severe CRSwNP mice. The number of nasal polyp-like lesions (A) per field ($N \geq 4$) and epithelial disruptions per field (magnification 40X) (b) ($N \geq 10$) were counted.

[0092] **Figure 52** is a graph showing sinonasal tissue infiltration of eosinophils in severe CRSwNP mice after treatments with various formulations. Counts of eosinophils per field in tissue sections collected from CRSwNP mice after various treatments. $N \geq 15$ fields from 4-7 mice per group.

[0093] **Figures 53A-B** are graphs showing Masson's Trichrome staining and goblet cells in the nasal epithelium of mice in the nasal tissues of CRSwNP mice after various treatments. (A) Quantification of collagen area per field from different treatment groups. $N \geq 15$ fields from 4-7 mice per group. (B) Quantification of goblet cells (blue) per image field from different treatment groups. $N \geq 15$ fields from 4-7 mice per group.

[0094] **Figures 54A-N** are graphs showing levels of type 2 inflammatory biomarkers in CRSwNP mice treated with various formulations.

[0095] **Figures 55A-U** are graphs showing long-term in vivo safety evaluation for protamine, Nano-P and Dendri-P in mice. (A) Organ/body weight ratios of different

groups of experimental animals. (B-U) Effect of protamine, Nano-P and Dendri-P on hematological and blood biochemistry parameters.

DETAILED DESCRIPTION

[0096] In general, the present disclosure provides, in part, protamine molecules and uses thereof.

[0097] Accordingly, the present disclosure provides, in part, protamine molecules, including multivalent protamine peptides and protamine peptides conjugated to a lipid, a hydrophobic moiety, and/or a polymer including an amino group.

[0098] Protamine is a clinically used peptide rich in arginine and is an FDA-approved injectable pharmaceutical ingredient and an antidote for heparin overdose.

[0099] A "protamine peptide," as used herein refers to a protamine peptide isolated from natural sources or artificially synthesized, as well as fragments thereof and/or mixtures thereof. In some embodiments, a protamine peptide may be in the form of a suitable salt, such as a sulfate from, for example, salmon. In some embodiments, a protamine peptide may be commercially available.

[00100] In some embodiments, a protamine peptide isolated from natural sources ("native protamine peptide") may be isolated from the sperm and/or fertilized eggs of fish, such as salmon (salmine), rainbow trout (iridine), herring (clupeine), sturgeon (sturine), or spanish mackerel or tuna (thynnine).

[00101] A protamine peptide may be generally basic ($pK_a > 10$). In some embodiments, a protamine peptide may contain up to about 70% arginine or other basic amino acids, such as lysine. In some embodiments, a protamine peptide may be about 30 to about 110 amino acids, or any integer in between, in length. In some embodiments, a protamine peptide may be about 20 to about 150 amino acids, or any integer in between, in length. In some embodiments, a protamine peptide may be about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 135, 140, 145, 150 or more amino acids, and may contain at least 20 %, 30 %, 40 %, 50%, 60%, 70% arginine and/or basic amino acid. In some embodiments, a protamine peptide may have 30-32 amino acids, of which 21-22 amino acids may be arginine. In some embodiments, a protamine peptide may have 32 amino acids, of which 21 amino acids may be arginine. In some embodiments, a protamine peptide may have an average

molecular weight of about 4,000 to about 5,000 Da. It is to be understood that the composition of native protamine may vary depending on the source. Protamine nucleic and amino acid sequences have been identified from a number of species. In some embodiments, a protamine peptide may be a mouse or a human protamine.

[00102] In some embodiments, a protamine peptide may have an amino acid sequence as set forth in, without limitation, any one of GenBank accession nos. AAG27965.1, AAG27962, AAG27961.1, AAG27960.1, AAG27964.1, AAG27963.1, AAG27959.1, AAG27952.1, AAG27958.1, AAG27956.1, AAG27954.1, AAG27953.1, AAG27957.1, AAG27951.1, AAG27955.1, AAG27950.1, AAC15630.1, AAC15629.1, AAC15628, AAC15627.1, AAB35760.1, AAB19741.1, AAB34978.1, AAB34977.1, NP_002752.1.

[00103] In some embodiments, a protamine peptide may be encoded by a nucleic acid sequence as set forth in, without limitation, GenBank accession nos. X07511 or X01204.

[00104] In some embodiments, a protamine peptide may have an amino acid sequence as set forth in any one of SEQ ID NOs: 1-4 or a conservative substitution thereof, as well as fragments thereof and/or mixtures thereof.

[00105] As used herein, the term “conservative amino acid substitution” or “conservative substitution” refers to the substitution of one amino acid for another at a given location in the peptide, where the substitution can be made without substantial loss of the relevant function.

[00106] Conservative substitutions may include the following:

Original Residue	Substitutions 1	Substitutions 2
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; gln	arg
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp

Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

[00107] In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like, and such substitutions may be assayed for their effect on the function of the peptide by routine testing.

[00108] Peptides or peptide analogues can be synthesised by standard chemical techniques, for example, by automated synthesis using solution or solid phase synthesis methodology. Automated peptide synthesisers are commercially available and use techniques well known in the art. Peptides and peptide analogues can also be prepared using recombinant DNA technology using standard methods.

[00109] A "protamine molecule," as used herein refers to a protamine peptide that is conjugated to another molecule, such as a lipid, a hydrophobic moiety, a polymer or an additional protamine peptide. In some embodiments, a protamine molecule as described herein may contain a secondary structure, such as an alpha helix and/or a beta sheet. In some embodiments, a protamine molecule as described herein is not substantially in a random coil configuration e.g., less than 1%, 5%, 10%, 15%, 20%,

25%, 30%, 35%, or 40%, may be in a random coil configuration. In this regard, and without being bound to any particular hypothesis, α -helices have been associated with the formation of pores in the cell membrane [55] and β -sheets have been associated with peptide self-assembling [56, 57], while random coils have been associated with disorganized orientation [58].

[00110] A protamine molecule as described herein may be capable of self-assembly into a nanostructure such as a sphere or a rod. Without being bound to any particular theory, such a nanostructure may increase the local density and concentration of the protamine molecule and/or increase interaction of the protamine molecule with the cell membrane.

[00111] A protamine molecule as described herein may exhibit increased interaction with the membrane of a cell and/or increased cell penetration and/or increased cell permeability.

[00112] A "lipid," as used herein, refers to compounds that are soluble in nonpolar organic solvents. It is to be understood that a person of ordinary skill in the art would readily be able to determine a suitable lipid or conjugate thereof for use in a protamine molecule as described herein. In some embodiments, a lipid as described herein contains an amine or carboxylate group. A suitable lipid includes without limitation a fatty acid, a lipid-amine, a lipid-carboxyl group, *etc.* In some embodiments, a lipid as described herein may be modified with a linker, such as polyethylene glycol (PEG) [59] or N-Hydroxysuccinimide (NHS), resulting in a lipid-PEG-amine, a lipid-PEG containing carboxyl group, a lipid containing NHS or a lipid-PEG containing NHS *etc.* A lipid-conjugated protamine molecule may exhibit increased interaction with the membrane of a cell and/or increased cell penetration and/or increased cell permeability. Exemplary lipids include without limitation palmitic acid, stearic acid, 1,2- phosphatidylethanolamine (PE), dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), DPPE-NHS, DSPE-NHS, DOPE-NHS, *etc.*

[00113] A "hydrophobic moiety," as used herein, refers to a compound that is insoluble in water or other polar solvents. It is to be understood that a person of ordinary skill in the art would readily be able to determine a suitable hydrophobic moiety for use in a protamine molecule as described herein, for example, a hydrophobic compound with a terminal double bond. A hydrophobic moiety when

conjugated to a protamine peptide, which is substantially hydrophilic, may create an amphipathic protamine molecule capable of self-assembly into micelles. Without being bound to any particular hypothesis, such an amphipathic protamine molecule may exhibit increased interaction with the membrane of a cell and/or increased cell penetration and/or increased cell permeability. An exemplary hydrophobic moiety includes, without limitation, allyl glycidyl ether (AGE) [60].

[00114] Multivalent protamine molecules may be prepared by conjugating protamine peptides. Without being bound to any particular hypothesis, multivalent protamine molecules may mimic protamine aggregation. In some embodiments, a first protamine peptide may be conjugated to a crosslinker and/or a polymer which can then be conjugated to additional protamine peptides. The protamine peptides may have the same amino acid sequence or may have different amino acid sequences. The protamine peptides may be from the same source and/or the same form (such as protamine sulfate salt from salmon) or may be from different sources.

[00115] A “crosslinker,” as used herein, refers to a compound that is capable of coupling a protein, such as a protamine peptide, to for example, prepare multivalent protamine molecules. It is to be understood that a person of ordinary skill in the art would readily be able to determine a suitable crosslinker for use in a protamine molecule as described herein. Exemplary crosslinkers include without limitation N-Succinimidyl S-Acetylthioacetate (SATA), N-γ-Maleimidobutyryloxysuccinimide (GMBS), or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC).

[00116] A “polymer,” as used herein, refers to a compound that is composed of smaller, repeating chemical units. A “dendrimer,” as used herein, refers to structurally defined, hyperbranched polymers containing functional moieties on their surface permitting multivalency. It is to be understood that a person of ordinary skill in the art would readily be able to determine a suitable polymer or dendrimer for use in a protamine molecule as described herein, for example, a polymer or dendrimer containing an amino group. Accordingly, multivalent protamine molecules may include 2, 3, 4, 5, 6, 7, 8, 9, 10 or more protamine peptides, depending on the number of available amino groups in the polymer. Exemplary polymers include without limitation poly(amidoamine) (PAMAM) G1-G5, polyethyleneimine (PEI), or polylysine (PLL).

[00117] Without being bound to any particular hypothesis, a multivalent protamine molecule may exhibit increased interaction with the membrane of a cell and/or

increased cell penetration and/or increased cell permeability due the increased number of charged (arginine, lysine, *etc.*) amino acids.

[00118] Exemplary protamine molecules include without limitation protamine-GMBS-PAMAM, protamine-DMPE, protamine-AGE, protamine-SATA-PAMAM, protamine-C18, protamine-C16, protamine-PEI, protamine-PLL *etc.*

[00119] By “conjugating,” as used herein, is meant joining a peptide to another molecule, such as a lipid, a hydrophobic moiety, a polymer, a crosslinker or a peptide by covalent chemical bonds to form a conjugated molecule. In some embodiments, conjugation as used herein excludes electrostatic interactions.

[00120] A protamine molecule may be prepared as described herein or known in the art and characterized by a variety of techniques, including but not limited to nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD), transmission electron microscopy (TEM), atomic force microscopy (AFM), mass spectrometry (MS) and dynamic light scattering (DLS).

[00121] In some embodiments, a protamine molecule may be provided in a composition, such as composition suitable for cell transfection, cell penetration, cell permeabilization, or delivery to a cell nucleus. In some embodiments, the composition may be a pharmaceutical composition.

[00122] Accordingly, protamine molecule, as described herein, may be provided alone or in combination with other compounds (such as a “payload,” for example, small molecules, proteins, peptides, or peptide analogues), in the presence of any suitable carrier, for example, a carrier suitable for cell permeabilization, penetration or transfection or delivery to a cell nucleus.

[00123] In alternative embodiments, protamine molecules, as described herein, may be provided alone or in combination with other compounds (such as a “payload,” for example, small molecules, proteins, peptides, or peptide analogues), in the presence of a pharmaceutically acceptable carrier, in a form suitable for administration to a subject, such as a mammal, for example, humans, cattle, sheep, *etc.* Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions for administration. In some embodiments, a pharmaceutical composition as described herein may specifically exclude an absorption enhancer.

[00124] In alternative embodiments, protamine molecules, as described herein, may be used without causing substantial toxicity. Toxicity can be determined using standard techniques, for example, by testing in cell cultures or experimental animals and determining the therapeutic index, *i.e.*, the ratio between the LD50 (the dose lethal to 50% of the population) and the LD100 (the dose lethal to 100% of the population), or ED₅₀, or median effective dose, *i.e.*, the dose that produces a specified effect (“response”) in 50% of the subjects under study.

[00125] In some embodiments, a protamine molecule, as described herein, may be used in a method for delivery of a payload molecule to a cell, by contacting the cell with the payload molecule in combination with the protamine molecule.

[00126] In alternative embodiments, a protamine peptide or a protamine molecule, as described herein, may be used in a method for non-parenteral delivery of a large payload molecule to a cell, by contacting the cell with the payload molecule in combination with the protamine peptide or the protamine molecule.

[00127] Any appropriate route of delivery or administration may be employed, for example, a non-parenteral route. Non-parenteral routes include intranasal, sublingual, oral, buccal, rectal, vaginal, intravitreal, or topical routes. Non-parenteral routes may target the skin, eye, brain, lungs, *etc.* The non-parenteral delivery may be transmucosal or transcellular (for example, transepithelial or to the lamina propria). Transmucosal routes may include intranasal, sublingual or intravaginal delivery. In some embodiments, a non-parenteral delivery route includes injection (needle)-free transmucosal delivery of a payload molecule, for example by nasal, sublingual, and topical routes.

[00128] Parenteral routes include intravenous, subcutaneous, intramuscular routes, for example, by injection with a needle. In some embodiments, parenteral administration methods are specifically excluded. In some embodiments, administration methods involving injection with a needle are specifically excluded.

[00129] By “payload” molecule, as used herein, is meant any molecule that can be delivered to a cell in combination with a protamine peptide or a protamine molecule, as described herein. Accordingly, a payload molecule may include without limitation, a small molecule, a protein, a peptide, or a peptide analogue. In some embodiments, a payload molecule is not a nucleic acid molecule. In some embodiments, the payload molecule may be about 0.8 or more in size to about 1000 KDa, or any range

or value in between, such as about 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 KDa, or about 50 to about 150 KDa, or about 150 to about 800 KDa, *etc.* In some embodiments, the payload molecule may be about 0.8 KDa to about 800 KDa in size, or any range or value in between, such as about 0.8, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, or 800 KDa, or about 50 to about 150 KDa, or about 150 to about 800 KDa, *etc.* In some embodiments, the payload molecule may be about 20 KDa to about 150 KDa in size, or any range or value in between, as about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 KDa or about 20 to about 100 KDa, or about 50 to about 150 KDa, *etc.* In some embodiments, a “large” payload molecule may be at least about 3 KDa in size. In some embodiments, a “large” payload molecule may be about 3 KDa to about 1000 KDa in size, or any range or value in between, such as about 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 KDa or about 50 to about 150 KDa, or about 150 to about 800 KDa, *etc.* In some embodiments, a “large” payload molecule may be at least about 200 KDa in size. In some embodiments, the large payload molecule may be about 200 KDa to about 1000 KDa in size, or any range or value in between, such as about 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 KDa or about 250 to about 450 KDa, or about 550 to about 800 KDa, *etc.* In some embodiments, the payload molecule may be about 1000 KDa or more in size. In some embodiments, a payload molecule may block the IL-4 and/or IL-13 pathway, such as a monoclonal antibody that blocks the IL-4 and/or IL-13 pathways. Exemplary payload molecules include without limitation growth hormone (*e.g.*, human recombinant growth hormone (rhGH)), antibodies (*e.g.*, monoclonal antibodies such as Omalizumab, Reslizumab, Mepolizumab, and Dupilumab), Immunoglobulin G (IgG), insulin, or semaglutide.

[00130] In some embodiments, the large payload molecule may be delivered sublingually. In some embodiments, the payload molecule may be insulin, where the protamine to insulin ratio may be at least 3:1, and the delivery may be intranasal and/or the pH may be acidic. In some embodiments, the payload molecule may be insulin, where the protamine to insulin ratio may be between about 3:1 and about 10:1, and the delivery may be intranasal and/or the pH may be acidic.

[00131] The payload molecule and the protamine peptide or protamine molecule may be combined by physical mixing. In some embodiments, the payload molecule may be released upon delivery, for example, after transcellular or transmucosal delivery or after delivery to the cell nucleus. In some embodiments, the payload molecule and the protamine peptide or protamine molecule are not conjugated to each other. In some embodiments, the payload molecule and the protamine peptide or protamine molecule do not form a complex, for example, by covalent bonds and/or electrostatic interaction. It is to be understood that, while there may be some interaction between the payload molecule and the protamine peptide or protamine molecule, the interaction should not substantially hinder cell permeabilization, penetration or transfection or delivery to a cell nucleus, or hinder release of the payload. By “substantially hinder,” as used herein, is meant that the interaction between the payload molecule and the protamine peptide or protamine molecule is dynamic *i.e.*, not permanent.

[00132] The payload molecule and the protamine peptide or protamine molecule may be administered at the same time (for example, in the same composition or formulation) or may be administered separately (for example, in different compositions or formulations). For example, in the case of topical administration, the protamine peptide or protamine molecule may be administered to the skin of a subject, and the payload molecule may be administered subsequently. Similarly, a cell can be contacted first with the protamine peptide or protamine molecule, and subsequently with the payload molecule. It is to be understood that, where the protamine peptide or protamine molecule is provided separately from the payload molecule, the timing of provision of the payload molecule will depend on the specific application and mode of delivery.

[00133] In some embodiments, a protamine peptide or a protamine molecule, as described herein, may be used in a method for permeabilizing the membrane of a cell. The permeabilization may be transient and/or reversible.

[00134] In some embodiments, a protamine peptide or a protamine molecule, as described herein, may be used in a method for transfecting a cell.

[00135] In some embodiments, a protamine peptide or a protamine molecule, as described herein, optionally in combination with a payload molecule, may be delivered to the nucleus of a cell. In some embodiments, a protamine peptide or a protamine molecule, as described herein, in combination with a payload molecule,

may be delivered to the nucleus of a cell. In some embodiments, a protamine peptide or a protamine molecule, as described herein, in combination with a payload molecule, may substantially bypass the lysosomes of the cell, *e.g.*, less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, or 40%, of the protamine peptide or protamine molecule, in combination with the payload molecule, may be found in the lysosomes of the cell. In some embodiments, a protamine peptide or a protamine molecule, as described herein, optionally in combination with a payload molecule, may substantially bypass the lysosomes of the cell, *e.g.*, less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, or 40%, of the protamine peptide or protamine molecule, optionally in combination with the payload molecule, may be found in the lysosomes of the cell.

[00136] A “cell” may be any cell isolated from an animal, such as an invertebrate (*e.g.*, an insect or a worm) or a vertebrate, such as a mammal. For example, a cell can include, without limitation, cells or tissue (*e.g.*, from a biopsy or autopsy) from bone, brain, breast, colon, muscle, nerve, ovary, prostate, retina, skin, skeletal muscle, intestine, testes, heart, liver, lung, kidney, stomach, pancreas, uterus, adrenal gland, tonsil, spleen, soft tissue, blood, semen, *etc.* A cell may include cultured cells, such as a cell or cell line created under experimental conditions.

[00137] In alternative embodiments, a protamine peptide or a protamine molecule, as described herein, may be used in a method for treating a condition benefited by non-parenteral delivery of a payload molecule, by administering the payload molecule with a protamine peptide and/or the protamine molecule to a subject in need thereof.

[00138] In some embodiments, the payload molecule may be administered at the same time or at a different time from the protamine peptide and/or the protamine molecule.

[00139] In some embodiments, the protamine peptide and/or the protamine molecule may be administered prior to administration of the payload molecule.

[00140] In some embodiments, the protamine peptide and/or the protamine molecule and/or the payload molecule may be administered in an effective amount. An “effective amount” includes a therapeutically effective amount or a prophylactically effective amount. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic

result. A therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects are outweighed by the therapeutically beneficial effects. A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, a prophylactic dose is used in subjects prior to or at an earlier stage of disease, so that a prophylactically effective amount may be less than a therapeutically effective amount. A range for therapeutically or prophylactically effective amounts of a compound may be any value from 0.1 nM-0.1M, 0.1 nM-0.05M, 0.05 nM-15 μ M or 0.01 nM-10 μ M.

[00141] It is to be noted that dosage values may vary with the severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that may be selected by medical practitioners. The amount of active compound(s) in a composition may vary according to factors such as the disease state, age, sex, and weight of the individual. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It may be advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage.

[00142] It is to be understood that the condition may be any condition benefited by non-parenteral delivery of a payload molecule. In some embodiments, a condition benefited by non-parenteral delivery of a payload molecule may be, without limitation, a condition in which standard modes of treatment or prophylaxis are by needle-based injection. In some embodiments, a condition benefited by non-parenteral delivery of a payload molecule may be, without limitation, a skin condition *e.g.*, psoriasis; an eye condition; an inflammatory condition, such as a type 2 inflammatory disease, *e.g.*, rhinosinusitis *e.g.*, chronic rhinosinusitis; diabetes, *etc.*

[00143] As used herein, a subject may be a human, non-human primate, rat, mouse, cow, horse, pig, sheep, goat, dog, cat, *etc.* The subject may be a clinical patient, a clinical trial volunteer, an experimental animal, *etc.* The subject may be

suspected of having or at risk for having a condition as described herein, be diagnosed with a condition as described herein, or be a control subject that is confirmed to not have a condition as described herein.

[00144] In some embodiments, the protamine peptide and/or the protamine molecule, or compositions thereof, may be provided in a first container of a kit, together with instructions for use. The first container may further include a payload molecule as described herein, together with instructions for use. Alternatively, the kit may include a second container including a payload molecule as described herein, together with instructions for use.

[00145] The present disclosure will be further illustrated in the following examples.

[00146] **Examples**

[00147] ***Materials and methods***

[00148] **Compounds and antibodies:** In the studies relating to protamine, Alexa Fluor 488 Phalloidin, Alexa Fluor 647 protein labeling kit, LysoTracker Red DND-99, Tubulin Tracker, FITC-conjugate of bovine serum albumin (FITC-BSA), and all the ELISA kits were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). Rabbit polyclonal antibody (pAb) against E-Cadherin was purchased from Abclonal. Goat Anti-Rabbit IgG (Alexa-Fluor-594 conjugated) was purchased from Elabscience. Human recombinant insulin, streptozotocin (STZ), Hoechst 33342, protamine and FITC-insulin were purchased from Sigma-Aldrich. Dialysis membrane (molecular weight cutoff MWCO = 10 or 3.5 kDa) was purchased from Spectrum Laboratories (Waltham, MA). Hydrophobic fluorescent dye DiI (DiI_{C18}(3); 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine) and DiD (DiI_{C18}(5); 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate salt) were purchased from Cedarlane Labs. Cyanine7-NHS was purchased from Abcam. All other general laboratory chemicals were purchased from Fisher Scientific and VWR Scientific (Mississauga, ON, Canada).

[00149] **Cell culture:** In the studies relating to protamine, RPMI2650 (human nasal epithelium) and Caco2 (human intestinal epithelium) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 units/mL penicillin, 100 µg/mL streptomycin, Gibco). All cells were cultured at 37 °C under 5% CO₂ and passaged when becoming confluent for up to 15 passages.

[00150] **XTT assay for cell viability:** In the studies relating to protamine, cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 h (37 °C, 5% CO₂, humidified). The medium was then changed to a fresh complete medium, and the cells were treated with protamine or R8 at different concentrations. After 24 h of treatment, cell viability was analyzed by the XTT assay as described previously [61].

[00151] **Cellular uptake of fluorescently tagged proteins and cellular changes in the presence of protamine or R8:** In the studies relating to protamine, cells were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluence. For the Alexa488-anti-SIRT1 monoclonal antibody (mAb) uptake study, the cell suspension was first incubated with a membrane staining dye DiD at 1 µg/mL for 30 min at 37°C and then centrifuged and washed with PBS three times before being seeded. The medium was replaced with serum-free medium containing 20 µg/mL FITC-BSA or 1 µg/mL Alexa488-anti-SIRT1 in the presence or absence of either 37.5 µg/mL protamine or R8 for 2–4 h at 12 or 37°C. Different sources of protamine were used and their efficacy was compared, including protamine sulfate salt from Salmon Grade X, protamine sulfate salt from herring Grade III, protamine sulfate salt from Salmon Grade IV and protamine chloride from Salmon. Cells were washed with PBS 3 times and then stained with Hoechst 33342 and LysoTracker for the nuclei and lysosomes, respectively, according to the manufacturer's protocols. The stained cells (without fixation) were immediately imaged by confocal laser scanning microscopy (CLSM) and analyzed with ZEN software (Carl Zeiss, Oberkochen, Germany) and ImageJ.

[00152] For cellular change studies, after incubation with protamine or R8 at 37.5 µg/mL, cells were washed with PBS three times, then stained with Hoechst 33342 and Tubulin Tracker according to the manufacturer's protocols. Live cells were then imaged by CLSM. Alternatively, cells were fixed with 10% formalin at room temperature for 10 min, treated with 0.1% Triton X-100 in PBS at room temperature for 2 min, and washed with PBS. Cellular F-actin was stained with Alexa Fluor 488 Phalloidin according to the manufacturer's protocol, followed by PBS washes, mounting onto a glass slide with a drop of Fluoroshield containing DAPI (Sigma-Aldrich), and imaging by CLSM. F-actin expression and cellular spread area were quantified by ImageJ through analyzing cellular fluorescence and morphology, respectively. F-actin aggregation in dots was quantified and cells containing ≥ 20 F-actin dots were counted positive of actin aggregation. Microtubule quantification was

performed using Fiji: the images were first processed using a Gaussian smoothing method ($\sigma = 0.100$) with an intensity threshold of 140. The "Skeletonize" feature was then used to trace the microtubules to determine the quantities and total lengths of microtubules per cell.

[00153] **FITC-BSA uptake by 3D cell spheroids:** In the studies relating to protamine, cell suspension (1×10^5 /mL) was first incubated with 1 $\mu\text{g/mL}$ Dil for 30 min at 37°C for cell membrane staining. Cells were centrifuged at 400 g for 3 min and then washed with PBS three times. Cells were seeded in a U-bottom 96-well plate (FaCellitate) at 1×10^4 /well. Two days later, spheroids were collected and treated with FITC-BSA at 40 $\mu\text{g/mL}$ in the presence or absence of either protamine or R8 at 0.1-0.2 mg/mL. After 3 h, spheroids were washed with PBS 3 times and then imaged under CLSM. Alternatively, spheroids were trypsinized for 5 min, resuspended in complete medium, centrifuged at 400 g for 3 min, washed with PBS 3 times, resuspended in PBS, and analyzed by Fluorescence-activated Cell Sorting (FACS).

[00154] **Cellular uptake of FITC-BSA in the presence of different sources of protamine:** In the studies relating to protamine, cells were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluency. The medium was replaced with serum-free medium containing 10 $\mu\text{g/mL}$ FITC-BSA in the presence or absence of 37.5 $\mu\text{g/mL}$ of different sources of protamine, including protamine sulfate salt from Salmon Grade X, protamine sulfate salt from herring Grade III, protamine sulfate salt from Salmon Grade IV and protamine chloride from Salmon 4 h at 37°C . After that, the cells were trypsinized for 3 min, resuspended in complete medium, centrifuged at 400 g for 3 min, washed with PBS 3 times, resuspended in PBS, and analyzed by Fluorescence-activated Cell Sorting (FACS).

[00155] **Protamine uptake study.** In the studies relating to protamine, protamine was first labeled with Alexa Fluor 647 (AF647) protein labeling kit according to the manufacturer's protocol and then added to the culture medium at 37.5 $\mu\text{g/mL}$. Live cells were imaged under confocal laser scanning microscopy (CLSM) for 60 min.

[00156] **Propidium iodide (PI) uptake study.** In the studies relating to protamine, cells were treated with 40 μM PI, a membrane impermeable dye, for 60 min, then protamine was added at a final concentration of 37.5 $\mu\text{g/mL}$ and cells were imaged by CLSM under the live-cell image mode. Alternatively, cells were treated with AF647-protamine at 37.5 $\mu\text{g/mL}$ for 10 mins, washed with PBS three times, and

incubated in complete medium for 2 min. Then, PI was added at a final concentration of 40 μ M, and cells were imaged by CLSM under the live-cell image mode.

[00157] **Cellular uptake of FITC-BSA at 12 or 37°C:** In the studies relating to protamine, cells were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluency. Cells were stained with Hoechst 33342, LysoTracker, and Tubulin Tracker for the nuclei, lysosomes, and tubulin respectively, according to the manufacturer's protocols. The medium was replaced with serum-free medium containing 20 μ g/mL FITC-BSA in the presence of 37.5 μ g/mL protamine for 4 h at 12 or 37°C. The stained cells (without fixation) were immediately imaged by CLSM and analyzed with ZEN software (Carl Zeiss, Oberkochen, Germany) and image J.

[00158] **Animal:** In the studies relating to protamine, four-week old male CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). *In vivo* experiments were performed following the established experimental protocols approved by the Animal Care Committee of the University of British Columbia (Vancouver, BC, Canada). Animal approval number: A22-0141.

[00159] **Intranasal delivery of Cy7-BSA:** In the studies relating to protamine, Cy7-BSA was synthesized by reacting Cyanine7-NHS with BSA at a molar ratio 10:1. The reaction was carried out in Milli Q water at room temperature for 1 h. The reaction solution was then dialyzed for 3 days and lyophilized. Cy7-BSA was mixed with protamine or R8 in water and intranasally (i.n.) delivered to mice (dose = 20 mg/kg Cy7-BSA; 3 mg/kg protamine or R8). Conscious mice were restrained by the non-dominant hand, and a small droplet (~ 5 μ l) of the Cy7-BSA formulations was delivered close to one of the nostrils at a 45 degree angle through a 10 μ l micro-pipette tip. After the droplet was inhaled, another droplet was administered to the different nostril. The procedures were repeated up to 5 times to deliver a total volume of up to 24 μ l of the formulations. Saline and Cy7-BSA only were included as controls. Blood was collected at 0.5 and 2 h. Plasma was isolated by centrifugation (10,000 g, 5 min) and analyzed for Cy7 fluorescence using a plate reader.

[00160] **Streptozotocin (STZ) induced diabetic mice model:** In the studies relating to protamine, mice were fasted for 6 h and then i.p. injected with 200 mg/kg STZ. Three days later, blood glucose (BG) was measured using a glucose meter (ONE Touch Ultra 2 Blood Monitoring System). Mice with BG $\geq$ 300 mg/dL were regarded as diabetic.

[00161] **Preparation of insulin-protamine formulations:** In the studies relating to protamine, protamine was dissolved in water at 10 mg/mL without pH adjustment, and insulin was dissolved in water at 5 mg/mL with pH adjustment to either 3.2, 6.2, 7.4, 8.2, or 11.2 using 1 N HCl or 1 N NaOH. Protamine was then mixed with insulin at a range of weight ratios: 0.2, 1.5, 2.5, 3.0, and 3.75 (w/w). The mixture was used immediately.

[00162] **BG lowering efficacy study in STZ-induced diabetic mice:** In the studies relating to protamine, BG of STZ-diabetic mice was measured before one dose treatment of either s.c. insulin or intranasal administration (i.n.) of various protamine-insulin formulations at 0.5, 1, or 5 mg insulin/kg. The volume of protamine-insulin formulations for intranasal delivery was from 10 to 20 μ L per mouse. BG was determined after 0.5, 1, 2, 4, and 6 h.

[00163] **FITC-Insulin penetration in the nostrils of diabetic mice:** In the studies relating to protamine, the protamine-FITC-insulin formulation was prepared as described above at a weight ratio of 3 and a pH at 3.2, and was i.n. delivered to mice at 1 mg FITC-insulin/kg. FITC-Insulin dissolved in saline was included as a control. Two h later, mice were euthanized, and their nasal turbinates were collected and fixed in 10% formalin for 48 h. The nasal turbinates were washed with PBS 10 times and decalcified in an EDTA solution (143 g/L distilled water) for 1 week. The EDTA solution was changed freshly every day. The nasal turbinates were then incubated in PBS for 1 h 4 times, followed by incubation in 30% sucrose for 24 h, and then embedded with OCT. The samples were sectioned using a Leica Cryostat. Sections were incubated in PBS for 10 min to remove OCT followed by incubation in 10 % formalin in PBS for 10 min. Next, sections were washed 3 times with PBS, permeabilized with 0.1% Triton X-100 for 5 min, and washed 3 times with PBS. Sections were blocked with 1% BSA in PBST (PBS +0.1% Tween 20) for 45 min and then washed with PBS 5 times. The phalloidin staining solution was applied to the sections and incubated for 45 min, followed by 5 washes with PBS and incubation with E-Cadherin Rabbit pAb (abclonal, A3044) (1:200) for 1 h at room temperature. The sections were washed with PBS 5 times (10 min each), and were stained with Goat anti-Rabbit IgG (Alexa 594 conjugated) at 1 μ g/mL at room temperature for 1 h. The sections were washed with PBS 5 times (10 min each) and stained with DAPI for 30 min before CLSM.

[00164] **Pharmacokinetic study:** In the studies relating to protamine, different insulin formulations were delivered to mice via either i.n. or s.c.. Blood was collected

into ethylenediaminetetraacetic acid (EDTA)-coated tubes (Microvette, Sarstedt AG & Co., Nümbrecht, Germany) at different time points from the saphenous vein or through cardiac puncture. Plasma was isolated by centrifugation (10,000 g, 5 min) and analyzed for insulin by the human insulin ELISA kit (Thermo) according to the manufacturer's protocol.

[00165] **Histological analysis:** In the studies relating to protamine, mice received the protamine-insulin mixture daily for 7 consecutive days and then were euthanized one day after the final dose. The nasal turbinates, trachea, and lungs were collected, sectioned, stained with hematoxylin and eosin, imaged, and independently analyzed by Dr. Ian Walch, a board-certified pathologist at UBC.

[00166] **Statistical analysis:** In the studies relating to protamine, normality of data distribution was first assessed with Shapiro-Wilk test ($p < 0.05$). For data that passed the test, the parametric one-way ANOVA with Tukey's post-hoc test with an adjusted P value was used for multiple comparisons. For data that failed the Shapiro-Wilk test, comparisons between groups were made by non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons post-hoc test. A p-value < 0.05 was considered statistically significant. Data were plotted with GraphPad Prism version 8.0 (GraphPad Software). All data are presented as mean $\pm$ standard deviation (SD). All statistical analysis was performed with R software (version 4.0.2)

[00167] **Compounds and antibodies:** In the studies relating to protamine derivatives, Alexa Fluor 647 protein labeling kit, FITC-conjugate of bovine serum albumin (FITC-BSA), EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), NHS (N-hydroxysuccinimide), 2-mercaptoethanol, LysotrackerTM green DND-26, and all the ELISA kits were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). Recombinant human growth hormone (rhGH), streptozotocin (STZ), Hoechst 33342, D-glucose, stearic acid, and protamine were purchased from Sigma-Aldrich. Ultra-LEAFTM purified IgG was purchased from Biolegend. Dialysis membrane (molecular weight cutoff (MWCO) = 10 or 15 kDa) was purchased from Spectrum Laboratories (Waltham, MA). Hydrophobic fluorescent dye DiI (DiI_{C18}(3); 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine) was purchased from Cedarlane Labs. NHS-PEG-NHS was purchased from Biopharma PEG. Cyanine7-NHS was purchased from Abcam. PrOZEMPIC[®] (1.34 mg semaglutide/ml) was purchased from Novo Nordisk[®]. Type I bovine collagen was purchased from Advanced BioMatrix. Transwell insert for 24-well plates with a growth area of 0.33 cm² was purchased from Corning. Cas9-RFP-RNP and sgRNA were ordered from

Integrated DNA Technologies. The sequence of sgRNA was 5'-GUCGCCCUCGAACUUCACCU-3' (SEQ ID NO: 5). All other general laboratory chemicals were purchased from Fisher Scientific and VWR Scientific (Mississauga, ON, Canada).

[00168] **Synthesis of protamine derivatives**

[00169] **Stearic acid-conjugated protamine (C18-P):** In the studies relating to protamine derivatives, protamine (10 mg/ml) and EDC were dissolved at a molar ratio of 1:5 in PBS and incubated for 15 min. NHS was then added at 7.5 molar equivalence to protamine, and the reaction was continued for 30 min. After that, 2-mercaptoethanol was added at a final concentration of 20 mM to quench the EDC. Stearic acid was dissolved in chloroform (75 mg/ml) and added at 10 molar equivalence to protamine and allowed to react overnight. The product was dialyzed against Milli-Q water at room temperature and then filtered with 0.22 µm filter for lyophilization.

[00170] **Protamine dimer (P2):** In the studies relating to protamine derivatives, protamine (10 mg/ml) and NHS-PEG-NHS were dissolved at a molar ratio of 1:10 in PBS (pH 7.2). The solution was incubated at room temperature for 24 h, followed by dialysis against Milli-Q water for 48 h and then lyophilization.

[00171] **Cell culture:** In the studies relating to protamine derivatives, human primary gingival keratinocytes (PCS-200-014™, ATCC) were cultured in dermal cell basal medium (PCS-200-030™, ATCC) supplemented with a keratinocyte kit (PCS-200-040™, ATCC). Primary human gingival fibroblasts (PCS-201-018, ATCC) were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). eGFP stable expression Flp-In™ T-REx™ 293 cell line (HEK 293 eGFP) was obtained from Dr. Colin Ross's lab at UBC. Cells were cultured with DMEM supplemented with 10% FBS and 0.2 mg/mL hygromycin B. All cells were cultured at 37 °C under 5% CO₂ and passaged when confluent for up to 7 passages.

[00172] **Cellular uptake of fluorescently tagged proteins in the presence of protamine, C18-P or P2:** In the studies relating to protamine derivatives, cells were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of culture medium for 18–20 h to achieve 70–80% confluence. IgG was labeled with Alexa647 according to the manufacturer's protocol. The medium was replaced with serum-free medium

containing 20 µg/mL FITC-BSA or 20 µg/mL AF647-IgG in the presence or absence of either protamine (10 µg/mL), C18-P (10 µg/mL) or P2 (5 µg/mL), followed by incubation overnight at 37°C. Cells were washed with PBS 3 times and then stained with Hoechst 33342 and LysoTracker according to the manufacturer's protocols for the nuclei and lysosomes respectively. The stained cells (without fixation) were immediately imaged by confocal laser scanning microscopy (CLSM) and analyzed with ZEN software (Carl Zeiss, Oberkochen, Germany) and ImageJ. Alternatively, cells were trypsinized for 5 min, resuspended in complete medium, centrifuged at 125 g for 10 min, washed with PBS 3 times, resuspended in PBS, and analyzed by flow cytometry (FCM). Alternatively, cells were trypsinized for 5 min, resuspended in complete medium, centrifuged at 125 g for 10 min, washed with PBS 3 times, resuspended in PBS, and analyzed by fluorescence-activated cell sorting (FACS).

[00173] **XTT assay for cell viability:** In the studies relating to protamine derivatives, human primary gingival keratinocytes were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 h (37 °C, 5% CO₂, humidified). The medium was then changed to a fresh complete medium, and the cells were treated with protamine/C₁₈-P or P₂ at different concentrations. After 24 h of treatment, cell viability was analyzed by the XTT assay as described previously.

[00174] **Scanning Electron Microscopy (SEM):** In the studies relating to protamine derivatives, primary human gingival keratinocytes (1×10^5 cells on a 20-mm glass coverslip) were incubated with protamine (10 µg/mL), C18-P (10 µg/mL) or P2 (5 µg/mL) at 37°C for 4 h. Cells were washed three times with PBS, fixed with glutaraldehyde and dehydrated, followed by coating with iridium (10 nm thickness) in a Leica EM high vacuum sputter coater and dried in a Tousimis Samdri ®-795 critical point dryer (30.98 °C and 73.8 bar). Imaging was done with a Helios NanoLab 650 Focused Ion Beam SEM at 15 kv.

[00175] **FITC-BSA and AF647-IgG penetration and cellular uptake in 3D cell spheroids:** In the studies relating to protamine derivatives, the primary human gingival keratinocytes cell suspension (1×10^5 /mL) was first incubated with 1 µg/mL Dil for 30 min at 37°C for cell membrane staining. Cells were centrifuged at 125 g for 10 min and then washed with PBS three times. Cells were then seeded in a U-bottom 96-well plate (FaCellitate) at 1×10^4 cells per well. Two days later, spheroids were collected, treated with FITC-BSA or AF647-IgG at 20 µg/mL in the presence or absence of either protamine, C18-P or P2 at 0.1 mg/mL, and incubated overnight. Spheroids were then washed with PBS three times, and the distribution of FITC-

BSA/AF647-IgG within the spheroids was analyzed using CLSM under Z-stack imaging with 20 μm intervals. The distance from the boundary of the spheroid to the center of penetrating fluorescence was analyzed using ImageJ. Alternatively, spheroids were trypsinized for 5 min, resuspended in complete medium, centrifuged at 125 g for 10 min, washed with PBS three times, resuspended in PBS, and analyzed by FCM. Alternatively, spheroids were trypsinized for 5 min, resuspended in complete medium, centrifuged at 125 g for 10 min, washed with PBS three times, resuspended in PBS, and analyzed by FACS.

[00176] **Human sublingual tissue substitute:** In the studies relating to protamine derivatives, the human sublingual tissue substitute was prepared according to previously established procedures with slight modifications [62]. In brief, primary human gingival fibroblasts (2×10^4 cells/cm²), FBS (9.5 $\mu\text{L}/\text{model}$), and type I bovine collagen were brought to neutral pH and poured into a transwell insert in a 24-well plate (growth area = 0.33 cm²). After incubation for 1 h at room temperature and another hour at 37°C, 200 μL of keratinocyte growth kit supplemented with dermal cell basal medium was added and the system was transferred to a 37°C incubator with 5% (v/v) CO₂ and 95% (v/v) humidity for another 2 h. Subsequently, 2×10^5 cells/cm² primary human gingival keratinocytes were seeded on top of the collagen matrix. After 24 h, the model was lifted to the air-liquid interface and the medium was changed to the gingival keratinocyte differentiation medium. The tissue was cultured for 14 days (37°C, 5% CO₂, and 95% humidity) with a medium change every other day.

[00177] **AF647-IgG penetration in human sublingual tissue substitute:** In the studies relating to protamine derivatives, protamine, C18-P, or P2 was dissolved in 5% glucose at 10 mg/mL. AF647-IgG was used at a concentration of 2 mg/mL in PBS. Eight μL of protamine or derivative was mixed with 4 μL of AF647-IgG, and the volume was then brought to 20 μL with PBS. The formulation was applied to the apical side of the tissue. After 4 h of treatment, the tissue was harvested and then embedded with OCT. After keeping them frozen in a -80 °C freezer overnight, the sample was sectioned to a thickness of 10 μm using a Leica Cryostat. The section was incubated in PBS for 10 min to remove OCT and stained with DAPI for 30 min before CLSM.

[00178] **Delivery of Cas9-RNP/sgRNA:** In the studies relating to protamine derivatives, HEK 293 eGFP cells (2×10^5 cells) were seeded in a confocal dish for 18–20 h to achieve 70–80% confluence. Cells were then treated with either

protamine (10 µg/mL), C18-P (10 µg/mL) or P2 (5 µg/mL) for 40 min. Next, 3.5 µL Cas9-RNP-RFP (52 µmol/L) was diluted with 20 µL nuclease-free water, and then mixed with 2 µL sgRNA (100 µmol/L) and incubated for 5 min at room temperature. The Cas9-RNP/sgRNA mixture was then added to the cells and incubated overnight. Two days later, the cells were stained with Hoechst 33342 and imaged by CLSM. Alternatively, cells were trypsinized for 3 min, resuspended in complete medium, centrifuged at 1000 g for 3 min, washed with PBS three times, resuspended in PBS, and analyzed by FCM. Alternatively, cells were trypsinized for 3 min, resuspended in complete medium, centrifuged at 1000 g for 3 min, washed with PBS three times, resuspended in PBS, and analyzed by FACS.

[00179] Data was analyzed using FCS Express 7 (De Novo Software, Pasadena, CA, USA) and FlowJo (BD Ashland, OR, USA). Cells were first gated to analyze only individual single cells, using negative controls (HEK 293 eGFP). A second gate for the cells with RFP violet (red channel) was then applied to determine Cas9-RFP-RNP positive cells. Lastly, a gate for eGFP (green channel) was applied to calculate the percentages of green negative cells.

[00180] Additionally, cells were seeded in a U-bottom 96-well plate (FaCellitate) at 1×10^4 cells per well. Two days later, spheroids were pretreated with protamine, C18-P or P2 at 0.1 mg/mL for 40 min, and then the Cas9-RNP/sgRNA mixture was added and incubated for 48 h. Spheroids were then washed with PBS three times, and the distribution of Cas9-RNP-RFP within the spheroid was analyzed using CLSM under Z-stack imaging with 10 µm intervals. Alternatively, spheroids were trypsinized for 5 min, resuspended in complete medium, centrifuged at 1000 g for 3 min, washed with PBS three times, resuspended in PBS, and analyzed by FCM. Alternatively, spheroids were trypsinized for 5 min, resuspended in complete medium, centrifuged at 1000 g for 3 min, washed with PBS three times, resuspended in PBS, and analyzed by FACS. Data was analyzed as mentioned above.

[00181] **Animal:** In the studies relating to protamine derivatives, four-week-old male/female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). In vivo experiments were performed following the protocol (A22-0141) approved by the Animal Care Committee of the University of British Columbia (Vancouver, BC, Canada).

[00182] **Streptozotocin (STZ) induced diabetic mice model:** In the studies relating to protamine derivatives, mice were fasted for 6 h and then intraperitoneally

(i.p.) injected with 200 mg/kg STZ. Three days later, blood glucose (BG) was measured using a glucose meter (ONE Touch Ultra 2 Blood Monitoring System). Mice with BG $\geq$ 300 mg/dL were regarded as diabetic.

[00183] Preparation of sublingual insulin formulations and the animal study:

In the studies relating to protamine derivatives, protamine, C18-P or P2 was dissolved in water at 10 mg/mL, and insulin was dissolved in water at 20 mg/mL with the pH adjusted to 3.2 with 1 N HCl. The two solutions were mixed and used for animal studies immediately. The dose for protamine or the derivatives was 3 mg/kg, while the dose for insulin was 5 mg/kg. The insulin dose for the subcutaneous (s.c.) control was 1 mg/kg. Different insulin formulations were s.c. or sublingually (sub.) delivered to STZ-mice. BG was measured before and 0.5-6 h after the treatment.

[00184] Preparation of sublingual semaglutide formulations and the animal study:

In the studies relating to protamine derivatives, protamine, C18-P, or P2 was dissolved in water at 10 mg/mL, which was then mixed with Ozempic. The mixture was used immediately. The dose of protamine or the derivatives was 6 mg/kg, while the dose of semaglutide was 500 μ g/kg for sub. administration. The semaglutide dose for the s.c. control was 60 μ g/kg. After mice received different semaglutide formulations, a glucose solution was given to mice at 1.5 mg/kg through i.p. injection. Blood samples were collected at different time points for BG measurement.

[00185] Pharmacokinetic study: In the studies relating to protamine derivatives, C18-P, or P2 was mixed with either rhGH, Cy7-BSA, or AF647-IgG to prepare formulations for sublingual delivery. The dose of novel peptides was 6 mg/kg, while the doses of rhGH, Cy7-BSA, and AF647-IgG were 500 μ g/kg, 20 mg/kg, and 1.3 mg/kg respectively. Plasma was collected from mice and analyzed for payload concentrations by ELISA (rhGH) or a fluorescence plate reader (Cy7-BSA and AF647-IgG). Tissue penetration of AF647-IgG was analyzed by CLSM imaging of sublingual tissue sections collected from the base of the tongue of the mice 4 h post-treatment.

[00186] Safety analysis: In the studies relating to protamine derivatives, mice received protamine or the derivatives (6 mg/kg) via the sublingual route at days 0, 7, 14 and 21 and were euthanized on day 22. Whole blood was collected into an ethylenediaminetetraacetic acid (EDTA)-coated tube (Microvette, Sarstedt AG & Co., Nümbrecht, Germany) through cardiac puncture. Plasma was isolated by centrifugation of blood (1,000 g, 5 min). Whole blood was used for hematology analysis. Liver and kidney function was analyzed by performing blood chemistry

analysis (IDEXX) at UBC. Body weight of mice was monitored throughout the study. Major organs were collected, weighed and stained with hematoxylin and eosin for imaging and toxicity analysis.

[00187] **Statistical analysis:** In the studies relating to protamine derivatives, two-group analysis was performed using a two-tailed unpaired Student's *t* test. One-way ANOVA with Tukey's test was used for multiple comparisons. A *p*-value <0.05 was considered statistically significant. Data were plotted with GraphPad Prism version 9.0 (GraphPad Software). All data are presented as mean $\pm$ standard deviation (SD). All statistical analysis was performed with GraphPad Prism version 9.0.

[00188] **Transmission electron microscopy (TEM):** In the studies relating to protamine derivatives, C18-P and P2 were prepared at a concentration of 0.1 mg/mL in deionized water. Prior to analysis, samples were stained with 2% uranyl acetate (aq.) before deposition onto 400-mesh Formvar-coated TEM grids (Ted Pella). C18-P and P2 were then imaged with 120 kV Tecnai Spirit electron microscope.

[00189] **Atomic force microscopy (AFM):** In the studies relating to protamine derivatives, protamine, C18-P and P2 were prepared at a concentration of 0.1 mg/mL in MilliQ and one drop of each sample solution was placed on a silicon wafer and dried overnight. The images were captured with SUPERSHARPSILICON™-Silicon SPM-Sensor (SSS-NCL-10) under Asylum research molecular force probe 3D controller. The images were then analyzed by Gwyddion software.

[00190] **Compounds and antibodies:** In the studies relating to protamine nanostructures, Alexa Fluor 647 protein labeling kit, Traut's Reagent (2-Iminoethanol-HCl), Inject™ Alum Adjuvant, Pierce™ Rapid Gold BCA Protein Assay Kit, ProcartaPlex™ Mouse and Rat Mix & Match Panels, and all ELISA kits were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). Streptozotocin (STZ), Hoechst 33342, allyl glycidyl ether (AGE), PAMAM G4, dimethyl sulfoxide (DMSO), albumin from chicken egg white (OVA), Staphylococcus aureus enterotoxin B (SEB) and protamine were purchased from Sigma-Aldrich. Ultra-LEAF™ purified α CD124 was purchased from Biolegend (San Diego, USA). RIPA buffer (10X) was purchased from New England Biolabs (Ipswich, MA, USA). Dialysis membrane (molecular weight cutoff MWCO = 10 and 15 kDa) was purchased from Spectrum Laboratories (Waltham, MA). Hydrophobic fluorescent dye DiI (DiI_{C₁₈(3)}; 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) was purchased from Cedarlane Labs (Burlington, ON, Canada). GMBS (N- γ -maleimidobutyryl-oxysuccinimide ester)

was purchased from Biopharma PEG (Watertown, MA, USA). Indium-111 labeled IgG (^{111}In -IgG) was provided by Dr. Urs Hafeli from UBC. All other general laboratory chemicals were purchased from Fisher Scientific and VWR Scientific (Mississauga, ON, Canada).

[00191] **Synthesis of protamine nanostructures**

[00192] **AGE conjugated protamine (Nano-P):** In the studies relating to protamine nanostructures, allyl glycidyl ether (AGE, 1.5 g) was added dropwise into 20 mL of protamine solution (10 mg/mL in PBS) and stirred at 300 rpm for 3.5 h at room temperature. The product was purified using dialysis (molecular weight cutoff, 10 kDa) against Milli-Q water for 48h. Nano-P was obtained after freeze-drying.

[00193] **PAMAM-Protamine (Dendri-P):** In the studies relating to protamine nanostructures, ten mL of protamine (10 mg/mL in PBS) and 1 mL Traut's Reagent (2-Iminothiolane•HCl) (50 mg/mL in PBS) were mixed, followed by addition of 1.2 mL EDTA solution (50 mM). The mixture was incubated at room temperature for 1 h, followed by dialysis against 5 mM EDTA for 1 h to obtain solution A. One hundred μL of GMBS (280 mg/mL in DMSO) and 3 mL PAMAM G4 (4 mg/mL in PBS) were mixed. The mixture was incubated at room temperature for 1 h, followed by dialysis against Milli Q water for 1 h to obtain solution B. Solution A and solution B were reacted at room temperature for 1 h, followed by dialysis against Milli Q water for 3 days and lyophilization.

[00194] **Transmission electron microscopy (TEM):** In the studies relating to protamine nanostructures, nano-P and Dendri-P were dissolved at a concentration of 0.1 mg/mL in deionized water. Prior to analysis, samples were stained with 2% uranyl acetate (aq.) before deposition onto 400-mesh Formvar-coated TEM grids (Ted Pella). Nano-P and Dendri-P were then imaged with an 80 kV Tecnai Spirit electron microscope.

[00195] **Animals:** In the studies relating to protamine nanostructures, four-week-old female CD-1 and BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). *In vivo* experiments were performed following the established experimental protocols (A22-0141 and A20-0132) approved by the Animal Care Committee of the University of British Columbia (Vancouver, BC, Canada).

[00196] **^{111}In -IgG in vivo distribution:** In the studies relating to protamine nanostructures, ^{111}In -IgG was mixed with protamine and then intranasally

delivered to naïve four-week-old CD-1 mice (dose = 13 mg/kg for ^{111}In -IgG; 3 mg/kg for protamine). Conscious mice were restrained by the non-dominant hand, and a small droplet ($\sim 5 \mu\text{L}$) of the ^{111}In -IgG formulations was delivered close to one of the nostrils at a 45-degree angle through a $10 \mu\text{L}$ micro-pipette tip. After the droplet was inhaled, another droplet was administered to the other nostril. The procedures were repeated up to 5 times to deliver a total volume of up to $24 \mu\text{L}$ of the formulations¹². Mice were imaged by SPECT/CT under anesthesia at 0, 0.5, 2, 4 and 6 h after intranasal administration. For the SPECT/CT imaging sessions, mice were anesthetized using isoflurane (1–3% for maintenance, up to 5% for induction) delivered in oxygen from a precision vaporizer. We used a VECTOr/CT multimodal preclinical scanner (MILabs in the Netherlands), equipped with an extra ultra-high sensitivity (XUHS) pinhole collimator (2 mm)[63, 64]. Throughout the imaging process, constant monitoring of the mice's respiratory rate and body temperature was maintained. All the animals successfully recovered after each imaging scan. After the final SPECT/CT scans at 24 hours post-administration, mice were kept under isoflurane and promptly euthanized by CO_2 asphyxiation followed by cardiac puncture. SPECT data were reconstructed with the pixel-based ordered subset expectation maximisation (POSEM) algorithm, with a voxel size of 0.4 mm^3 , 16 subsets, and 6 iterations. Reconstructions were carried out using the 171 keV photopeak of ^{111}In , with a spectral width of 20%. Each SPECT scan was decay corrected to the time of injection and recorded counts were adjusted using attenuation factors determined from CT scans at each time point. A point source measurement of a known ^{111}In sample was scanned to establish a calibration factor, linking counts/voxel to activity concentration. In each SPECT image, we manually outlined a volume of interest around the nasal cavity area to account for activity concentration in that specific region. Subsequently, we calculated mean Standardized Uptake Values (SUVs) using the formula $\text{SUV mean (g/mL)} = [\text{radioactivity concentration (MBq/mL)}] / [\text{injected dose (MBq)} / \text{animal weight (g)}]$ for comparing different groups. We used AMIDE software(v. 1.0.6)to visualize and analyse all the images [65].

[00197] **In vivo distribution of AF- αCD124 in mice:** In the studies relating to protamine nanostructures, AF- αCD124 was mixed with protamine, Nano-P, or Dendri-P and then intranasally delivered as described above to naïve four-week-old CD-1 mice (dose = 1.3 mg/kg for AF- αCD124 ; 3 mg/kg for protamine, Nano-P, or Dendri-P). Two hours later, mice were euthanized and the noses were collected. Noses were processed as previously reported¹³. Briefly, the nasal turbinates were

washed with PBS and decalcified in an EDTA solution (143 g/L in distilled water) for at least 1 week. Samples were moved into a fresh EDTA solution daily. The nasal turbinates were then incubated in PBS for 25 min 4 times, followed by incubation in 30% sucrose for 24 h. These samples were then embedded in OCT, and sectioned using a Leica Cryostat at a thickness of 10 μ m. Slides were then stained with DAPI for 30 min before CLSM.

[00198] **CRSwNP model:** In the studies relating to protamine nanostructures, naïve four-week-old BALB/c mice were systemically sensitized with an intraperitoneal injection of 25 μ g OVA mixed with 2 mg of aluminum hydroxide gel in 100 μ l saline on days 0 and 5. After general sensitization, 20 μ l of 6% OVA in saline was intranasally administered to mice daily from day 12 to day 19. From day 19 to day 47 or 107, 6% OVA was intranasally administered three times per week to establish moderate or severe CRSwNP models, respectively. After induction of allergic rhinosinusitis, 6% OVA with *Staphylococcus aureus* enterotoxin B (SEB, 20 ng) was administered into the nasal cavity three times per week for 8 weeks. Different formulations containing α CD124 were given thrice per week for 8 weeks starting from the week of the SEB treatment. For moderate CRSwNP, the dose of α CD124 was 1 mg/kg for both the intranasal and subcutaneous routes. For severe CRSwNP, the dose was 2 mg/kg for the intranasal route and 25 mg/kg for the subcutaneous route. Protamine, Nano-P, and Dendri-P were given at 3 mg/kg for both models. One day after the final treatment, mice were euthanized and whole blood was collected for IgE analysis. The noses were collected and imaged using a micro-CT specimen scanner (Scanco Medical μ CT100) at 90 kVp, 0.2 mA and then reconstructed with 17 μ m voxel size. One third of the nose was excised and homogenized in 500 μ l RIPA lysis buffer per 100 mg of tissue and placed in a homogenization tube equipped with 0.5 mm zirconium oxide beads. The tube was placed in a Bullet Blender® (Next Advance), with controls set to Speed 8 and Time 5. The samples were then centrifuged at 16,000 rpm for 10 min at 4 °C. The supernatant was collected and Pierce™ Rapid Gold BCA Protein Assay Kit was used to determine protein concentrations. The samples were assayed for murine Immunoglobulin E (IgE), eotaxin, thymus and activation-regulated chemokine (TARC), interleukin-13 (IL-13), tumor necrosis factor α (TNF- α), IL-2, IL-1 β , thymic stromal lymphopoietin (TSLP), IL-17A, IL-25, IL-33, interferon gamma (INF- γ) and IL-12p70 using ELISA or ProcartaPlex™ Mouse and Rat Mix & Match Panels. The rest of the nose was decalcified as mentioned, sectioned at a thickness of 6 μ m, and stained with

hematoxylin and eosin (H&E), Giemsa, Masson's Trichrome (MT), or Alcina Blue-Periodic Schiff (AB-PAS).

[00199] **In vivo safety evaluation of Protamine, Nano-P, and Dendri-P:** In the studies relating to protamine nanostructures, healthy mice received protamine, Nano-P, or Dendri-P at 3 mg/kg twice per day for one month and were euthanized one day after the final dose. Whole blood was collected into ethylenediaminetetraacetic acid (EDTA)-coated tubes (Microvette, Sarstedt AG & Co., Nümbrecht, Germany) through cardiac puncture. Plasma was isolated by centrifugation (10,000 x g, 5 min). Whole blood was used for hematology analysis. Liver and kidney functions were analyzed through blood chemistry analysis (IDEXX) at UBC. The body weight of each mouse was monitored throughout the study. Major organs including the nose and trachea were collected, weighed, and stained with hematoxylin and eosin for imaging and toxicity analysis. Animals were observed for nasal symptoms (nasal secretion) [66], tear secretion, eye irritation, excessive blinking, salivation, cyanosis, lethargy, piloerection, paralysis, skin irritation, erythema, nose twitching, as well as directed and non-directed movements within the cage prior to, during, and immediately following each exposure throughout the experiment [66]. A 5-min video for each mouse was recorded after the final dose of intranasal administration.

[00200] **Statistical analysis:** In the studies relating to protamine nanostructures, two-group analysis was performed using a two-tailed unpaired Student's t test or Welch's t test. One-way ANOVA with Tukey's test was used for multiple comparisons. A p-value <0.05 was considered statistically significant. Data were plotted with GraphPad Prism version 9.0 (GraphPad Software). All data are presented as mean $\pm$ standard deviation (SD). All statistical analysis was performed with GraphPad Prism version 9.0.

[00201] RESULTS

[00202] Characteristics of protamine-mediated cellular penetration

[00203] We first showed that below 37.5 $\mu\text{g}/\text{mL}$, protamine did not cause any toxicity to cells (**Figure 1A-B**). We then compared cellular uptake of FITC-BSA in the presence of protamine of different sources at 37.5 $\mu\text{g}/\text{mL}$ after 4 h incubation using FACS. As shown in **Figure 2**, FACS revealed that protamine 1 (protamine sulfate salt from salmon) displayed superior activity in promoting cellular delivery of FITC-BSA compared to other sources of protamine in this study, and approximately 85% of cells

were positive of FITC with the protamine 1 formulation. Protamine 2 (protamine sulfate salt from herring) and 4 (protamine chloride salt from salmon) delivered FITC-BSA to 70-75% of the cells, while protamine 3 (protamine from salmon) was ineffective. Based on the above data, protamine sulfate from salmon was used for the rest of this research.

[00204] We then utilized confocal live-cell imaging to examine cells during the treatment with protamine to characterize protamine-mediated cellular delivery. Live-cell images of RPMI2650 and Caco2 cells under CLSM after incubation with AF647-protamine for up to 60 min were examined. Protamine was first labeled with Alexa Flour 647 (AF647) protein labeling kit according to the manufacturer's protocol and then added to the culture medium at 37.5 µg/ml. We found that AF647 labeled protamine adhered to the cell surface within 4-8 min post-incubation, and the adsorption to the cell surface increased over time. Protamine translocated to the nucleus in 20-30 min, and there was little accumulation of protamine in the cytosol at any given time point. This suggests that protamine rapidly transferred from the cell membrane to the nucleus.

[00205] Uptake of a membrane impermeable dye, propidium iodide (PI) by RPMI2650 cells in the absence and presence of AF647-protamine under live CLSM was examined. While no PI uptake by RPMI2650 cells was observed even after 60 min of incubation in the absence of protamine, significant PI uptake in the nuclei was detected two min after protamine addition. The data further indicated that once protamine was adsorbed to the cell membrane, the cell membrane permeability increased instantly, allowing cell permeation of PI.

[00206] RPMI2650 cell membrane permeability to PI after removal of protamine was examined. Cells were treated with AF647-protamine for 10 min, followed by wash and fresh medium replacement. Cells were incubated with PI for up to 30 min and imaged under CLSM. The results indicated that the effect of protamine in increasing cell membrane permeability was reversible. Once the protamine-containing medium was replaced with fresh medium, the cells became impermeable to PI again. Specifically, limited cellular uptake of PI was detected after protamine removal, suggesting the cell penetrating effect of protamine was transient and reversible.

[00207] Protamine increased intracellular delivery of proteins compared to R8.

[00208] We then compared FITC-BSA delivery efficiency by protamine and R8 in cell culture. Confocal microscopy images of cells after treated with FITC-BSA in the presence or absence of protamine or R8 were examined. The confocal images showed increased intracellular delivery of FITC-BSA when physically mixed with protamine, while the R8 effect was mild. The intracellular FITC-BSA fluorescence was quantified by ImageJ, demonstrating 20-fold (RPMI2650) and 5-fold (Caco2) increased intracellular delivery with protamine compared to R8 (**Figure 3A-B**). Protamine facilitated nuclear delivery of FITC-BSA for both RPMI2650 and Caco2 cell lines, and about 60-75% of FITC-BSA was delivered to the nuclei (**Figure 3C-D**). Furthermore, in R8/FITC-BSA treated cells, FITC-BSA was almost exclusively (~95%) detected within the lysosomes in both cell lines, while in the protamine treated cells, lysosomes were barely detected. The data suggest that protamine and R8 exhibited distinct cell penetrating mechanisms, and that protamine delivery could reduce lysosomal trapping and degradation of biomolecules.

[00209] In a follow-up study, confocal microscopic images of cells after treatment with the mAb in the presence or absence of protamine or R8 were examined. An antibody targeting SIRT1 located in the nucleus (Alexa488-anti-SIRT1) showed that protamine improved intracellular delivery of this monoclonal antibody (mAb) in RPMI2650 cells by 11-fold compared to R8 (**Figure 4A**), while the free mAb displayed little cellular delivery. The almost exclusive localization of this antibody in the nucleus (~80%) delivered by protamine suggests that the antibody was delivered in the active form with antigen targeting ability. On the other hand, almost all the Alexa488-anti-SIRT1 delivered by R8 accumulated in the lysosomes. As shown in **Figure 4B**, over 95% of the mAb overlaid with the lysosomes in the R8 treated cells, and >80% of the mAb in the cells accumulated in the nuclei in the protamine group with <10% in the lysosomes. Protamine delivery provided significant advantages over R8 for this mAb, increasing cellular uptake and improving delivery to the target organelle.

[00210] Protamine displayed increased activity in modulating cellular F-actin rearrangement and tubulation compared to R8

[00211] We characterized and compared cellular actin and tubulation after R8 and protamine treatment. Confocal microscopic images of cellular actin cytoskeleton after

treatment with protamine or R8 were examined with respect to extension of F-actin and increased cellular spread or F-actin aggregation in dots. When a cell expressed ≥ 20 F-actin dots, the cell was considered positive of actin aggregates. As shown in **Figure 5A-C**, there was no significant difference in overall F-actin expression. However, cells treated with R8 and protamine exhibited increased spread areas: R8 and protamine increased the cellular spread areas by 1.5-fold and 2.3-fold compared to control, respectively. The protamine treated cells exhibited the largest cytoplasm to nucleus ratio, and cells were flattened. The data suggest that the R8- and protamine-treated cells rearranged their intracellular actin to make the cells thinner, which could reduce the membrane tension [13, 25] and increase the membrane permeability. In the R8 treated group, 40% of the cells displayed F-actin aggregates whereas only 10-15% of cells in the control and protamine-treated groups showed such aggregates. The R8-treated cells displayed increased actin aggregates and lysosomal accumulation of cargos. However, increased actin aggregation and lysosomal accumulation were not observed with protamine-treated cells, suggesting a different mechanism of action.

[00212] Confocal microscopic images of cellular microtubules after treatment with protamine or R8 were examined. R8 and protamine appeared to affect the polymerization of tubulin into microtubules as the TubulinTracker staining revealed distinct morphologies. To quantify the tubulation, the TubulinTracker-stained microtubule skeleton was quantitatively analyzed in Fiji to obtain the mean microtubule number per cell and the total length of the microtubules. Protamine increased the microtubule number per cell and the total length of microtubules per cell by ~ 2 -fold and 3.5-fold compared to control, respectively, while R8 displayed no such effect (**Figures 6A-B**). These data indicate that protamine displayed enhanced effects in promoting cellular tubulation that have been linked to the mechanism for transcellular delivery and CPP activity. The data are consistent with the results that protamine exhibited increased intracellular delivery efficiency of BSA and anti-SIRT1.

[00213] Live-cell CLSM imaging of FITC-BSA uptake by RPMI2650 and Caco2 cells in the presence of protamine at 12 or 37 °C for 4 h was performed. The results showed that protamine mediated cellular uptake of protein was dependent on the temperature (increased with higher temperatures), suggesting an energy-dependent mechanism. When cellular tubulation was reduced by lowering the incubation temperature, the effect of protamine on intracellular delivery of FITC-BSA was

decreased, supporting that tubulation was one of the key mechanisms of protamine's CPP activity.

[00214] Protamine increased protein penetration in cell spheroids compared to R8

[00215] We then examined whether protamine could enhance FITC-BSA penetration through multiple cell layers compared to R8 using cell spheroid models. RPMI2650 spheroids were incubated with FITC-BSA in the presence or absence of protamine or R8 for 3 h, and were washed and imaged by CLSM at different depths from the top. Confocal microscopy analysis of penetration of FITC-BSA in the presence of protamine or R8 in RPMI2650 spheroids was performed. Spheroids were imaged at different depths from the surface. Protamine treated spheroids (Dil labeled) showed significant FITC-BSA penetration with FITC signal strongly exhibiting in every layer scanned, while FITC-BSA only and R8/FITC-BSA displayed little spheroid penetration. It is noted that due to the limited light penetration, fluorescence in the spheroid core was not detectable. Although R8 increased FITC-BSA delivery in 2D cell culture compared to the protein only group, it did not show any effect in this 3D spheroid model. The results were further confirmed by FACS (**Figures 7A**), showing that 94% of the cells in the spheroid were positive with FITC in the protamine group and that only <10% of the cells in the FITC-BSA group and R8/FITC-BSA group were associated with FITC fluorescence. Similar results were obtained with the Caco2 spheroid model in which confocal images of FITC-BSA penetration in Caco2 spheroids in the presence or absence of protamine or R8 were examined. Spheroids were imaged at different depths from the surface. The results indicated that protamine increased FITC-BSA penetration into the spheroid compared to R8.

[00216] Protamine increased systemic absorption of protein via the intranasal route compared to R8

[00217] We further examined the effect of protamine in an *in vivo* model compared to R8. Cy7-BSA was mixed with protamine or R8 and intranasally delivered to mice, and 0.5-2 h later, Cy7-BSA concentrations in the plasma were measured by fluorescence. As shown in **Figure 7B**, at 2 h, the protamine group displayed 3-fold increased Cy7-BSA absorption compared to the R8 formulation and the protein only group. The results were consistent with those in the 2D culture and spheroid models.

[00218] Protamine enabled systemic absorption of insulin by intranasal delivery, normalizing blood sugar levels in diabetic mice

[00219] Next, we explored a medical utility of this non-invasive protein delivery technology and selected insulin for further studies. We first focused on optimizing the protamine-insulin formulation by changing the ratio between protamine and insulin. Insulin was first dissolved in water at a pH of 3.2 and mixed with different amounts of protamine, followed by i.n. delivery to STZ-diabetic mice. As indicated in **Figure 8A**, no BG lowering effect was detected when the protamine-to-insulin ratio was ≤ 2.5 . When the ratio increased to ≥ 3 , the formulations significantly decreased BG in 1 h from 400 mg/dL to 200 mg/dL after i.n. delivery. The BG was further decreased to 100-150 mg/dL at 2-4 h post i.n., but rebounded to 300 mg/dL in 6 h. Hence, we used the protamine-to-insulin weight ratio at 3 for further studies.

[00220] We then focused on optimizing the pH of the formulation (**Figure 8B**). Although there was no significant difference among the different pHs due to the large variation of the data, the pH 3.2 formulation appeared to be the most effective and was selected to prepare the optimal formulation. The normal human nasal mucosa is in an acidic environment (pH ~ 5) [67], and nasal formulations with acidic pHs (2.5-3.5) have been demonstrated to be safe in clinical trials, including Taffix [68, 69] and a preservative-free acidified saline nasal spray [70].

[00221] As shown in **Figure 8C**, the BG lowering efficacy of the optimal protamine/insulin formulation was dose dependent: the efficacy increased with the dose. **Figure 8D** shows that the BG lowering effect of the optimal protamine/insulin formulation was comparable to s.c. delivery of insulin, except that the effect of s.c. insulin appeared to be stronger and longer lasting, although statistically insignificant, maintaining the BG at ~ 50 mg/dL at 2-6 h post injection. It is noted that the efficacy comparison in this study was based on the same low dose, and the effects of our nasal formulation and the standard s.c. insulin were statistically comparable. In previous studies, much higher doses were required for non-parenteral formulations for insulin to achieve significant activity in animals [13, 71]. This supports the high efficiency of protamine for intranasal delivery of insulin. The pharmacokinetic (PK) results are consistent with the efficacy data (**Figure 8E**): s.c. insulin was rapidly absorbed into the plasma, reaching a peak concentration at 300 μ U/mL in 0.5 h and rapidly declining thereafter to undetectable in 4 h. The i.n. protamine/insulin formulation produced peak concentrations around ~ 50 μ U/mL at 0.5-2 h, and the concentration was under the detection limit at 4 h. The area under the curve in the

s.c. insulin group was 3.5-fold higher than that in the i.n. protamine/insulin treated mice. The relative nasal bioavailability of protamine-insulin was calculated by comparing the areas under the curves (AUCs) in **Figure 8E** and was 28.6%.

[00222] The optimal protamine formulation for insulin reported in this study is significantly different from the Neutral Protamine Hagedorn (NPH) insulin used clinically. First, NPH insulin still requires s.c. delivery, and protamine in the NPH formulation is to control the release of insulin to produce prolonged BG lowering effect. Second, the protamine-to-insulin ratio in NPH is 0.2, while our formulation had a ratio of 3 to achieve systemic absorption through the nasal delivery. Third, the pH of NPH is neutral and the formulation appearance is milky. On other hand, our formulation had a pH of 3.2 and appeared clear. Fourth, in addition to insulin and protamine, the NPH formulation contains phenol for pH adjustment and zinc ion for controlled release of insulin.

[00223] **Protamine overcame mucosal and epithelial barriers for intranasal delivery of insulin and remodeled the adherens junctions in the nasal epithelium**

[00224] FITC-Insulin penetration in the nose in the presence and absence of protamine was examined at earlier and later stages of FITC-Insulin distribution. The results indicate protamine induced E-cadherin rearrangement in the epithelium, and E-cadherin expression was lost in some cell junctions. The BG and PK data suggest that protamine facilitated insulin transport from the nasal cavity through the mucosal and epithelial barriers to the micro-vessels in the lamina propria layer for systemic absorption. To provide visual evidence, the nasal turbinates from mice were collected at 2 h after i.n. delivery of protamine/FITC-Insulin. FITC-Insulin alone was included as a control in this study. Within the same nasal turbinate sample, different insulin absorption stages were observed, and images of early and later stages are reported separately. Without protamine, no FITC-Insulin penetration to the mucosal (the layer above the epithelium) or the epithelial (labeled with E-cadherin, a marker for adherens junctions) layer could be detected. However, when mixed with protamine, FITC-Insulin penetrated through the mucosal and epithelial layers, reaching the lamina propria (the layer below the epithelium). At this early stage, some FITC-Insulin was also detected in the mucosal/epithelial layers in the protamine group. It is also noted that during this early stage, protamine inhibited E-cadherin expression in the epithelial layer as E-cadherin was not detectable between some epithelial cells. To quantify this phenomenon, we counted the number of nuclei and E-cadherin loops in

both groups. The nasal epithelium exposed to protamine had a significantly higher ratio of nuclei to E-cadherin enclosures (1.4 ± 0.28) than the saline treated group (0.95 ± 0.11). The data suggest that protamine could reduce epithelial adherence to facilitate paraepithelial delivery of insulin. However, such an effect was transient and reversible, as at a later stage of insulin absorption when all FITC-Insulin had left the epithelial layer and reached the lamina propria, E-cadherin expression in the epithelium was back to normal in the protamine group. Nevertheless, at this later stage in the saline/FITC-Insulin group, only a low level of FITC-Insulin was detected in the mucosal layer without further penetration to the epithelial and lamina propria layers.

[00225] **Short-term and long-term safety of the protamine formulation**

[00226] Pathological analysis of the main airway tissues from mice that received protamine once, protamine once daily for 7 consecutive days, or PBS, was performed. We also examined short-term (one dose) and long term (once daily for 7 consecutive days) safety of the insulin/protamine formulation. One day after the final dose, the nasal cavity, trachea and lungs were harvested and sectioned. On H&E staining, no abnormalities were found in the trachea, lungs, or nose among the groups by a board-certified pathologist. We also employed the Periodic Acid-Schiff (PAS) method to stain goblet cells in the tissues, which over-proliferate to produce mucin in inflammatory conditions. The goblet cells were stained in purple by PAS and differentiated from other types of cells with their cylinder morphology. The images show that there was no increased proliferation of goblet cells nor increased expression of mucin in the nasal tissue after protamine treatments compared to control. No PAS positive goblet cells were detected in the trachea and lungs. Toluidine Blue was used to stain acidic mast cells and the dye developed a dark purple color in granules within acidic mast cells when reacting with heparin and histamine, which is a main mediator in tissue inflammation. No significant mast cell infiltration in the nose, trachea and lungs was observed after protamine treatments compared to the PBS control. Finally, TNF- α , the main inflammatory cytokine, was stained by immunohistochemistry in the tissues and no significant upregulation of TNF- α after protamine treatments was detected compared to the PBS control. All the tissues showed background staining of TNF- α when compared to the control sample stained without the primary antibody. Overall, the tissue histology results support that there was no inflammatory toxicity induced by the protamine treatments.

[00227] The protamine formulation was shown to offer a few advantages for non-parenteral transcellular and transmucosal delivery of proteins and peptides compared to previously reported technologies. First, the method was simple, requiring only physical mixing of protamine with the protein cargo. Second, protamine has been approved by the FDA for human use with a track record of safety. Third, protamine facilitated transcellular and transmucosal penetration of proteins and peptides up to 110KDa with reduced lysosomal accumulation. Fourth, protamine-mediated changes to cells were reversible, including transiently increased membrane permeability and disrupted adherens junctions in the nasal epithelium. Therefore, repeated treatments with the protamine formulation were shown to be safe. Fifth, protamine-mediated intranasal delivery of insulin was effective and the effect in diabetic animals was comparable to s.c. insulin at the same dose.

[00228] We showed that by simple physical mixing, protamine significantly increased intracellular delivery of proteins into cells in both 2D and 3D models compared to R8. Within the cells, protamine-mediated delivery displayed reduced accumulation in the lysosomes and increased targeting to the nuclei compared to R8. The mechanistic studies revealed that protamine exerted stronger effects than R8 on cellular actin rearrangement and tubulation, which have been associated with increased cellular permeability and uptake. The protamine formulation was optimized for intranasal delivery of insulin, promoting systemic absorption. The optimal protamine/insulin formulation displayed comparable BG lowering activity compared to s.c. insulin. Tissue histology and confocal microscopy results showed that protamine mediated rearrangement of the adherens junction in the nasal epithelium, facilitating insulin penetration to the lamina propria layer for systemic absorption. Repeated treatments of this formulation were safe in the mice model.

[00229] **Protamine-enhanced FITC-BSA penetration in human skin.**

[00230] DAPI and protamine sulfate salt from Salmon Grade X were purchased from Sigma-Aldrich. Poly(arginine)₈ (R8) and FITC-conjugate of bovine serum albumin (FITC-BSA) were purchased from Thermo Fisher Scientific. Protamine solution (10mg/ml) was mixed with FITC-BSA (2mg/ml) at a weight ratio of 1.5:1 at a final volume of 100ul and was dropped to the surface of the human skin sample and incubated for 24hr. The bottom of human skin was moistened with flowing PBS. After 24hr, the human skin was collected and cryo-sectioned. The section was then stained by DAPI and imaged by confocal laser scanning microscopy (CLSM). The results indicated that protamine enhanced FITC-BSA penetration in human skin.

[00231] **Pig skin penetration**

[00232] FITC-CM-Dextran (average mol wt 150,000), Sigma, was dissolved at 2 mg/ml in 5% glucose. Protamine was dissolved at 10 mg/ml in 5% glucose. Hyaluronic acid was dissolved in water at 0.1 mg/ml. FITC-BSA was dissolved in water at 5 mg/ml.

[00233] Pig skin was inserted into Franz cells device. The bottom of Franz cells was filled with saline and kept at 37 °C. Solution **A**, 50 µL FITC-CM-Dextran + 45 µL protamine+ 25 µL 5% glucose; **B**, 50 µL FITC-CM-Dextran +70 µL 5% glucose as control; **C**, 25 µL FITC-CM-Dextran + 75 µL protamine+ 25 µL 0.1 mg/ml Hyaluronic acid; **D**, 25 µL FITC-CM-Dextran + 75 µL 5% glucose+ 25 µL 0.1 mg/ml Hyaluronic acid as control; **E**, 20 µL FITC-BSA + 90 µL protamine; **F**, 20 µL FITC-BSA + 90 µL 5% glucose water as control, or PBS was applied on top of the pig skin and incubated for 24 h.

[00234] Pig skin incubated with PBS for 24 h was used as a control.

[00235] Pig skin incubated with **A** or **B** for 24 h showed that FITC-CD-Dextran was delivered to the epidermal layer by protamine, while FITC-CD-Dextran alone showed no penetration.

[00236] Pig skin incubated with **C** or **D** for 24 h showed that FITC-CD-Dextran/HA was delivered to the epidermal layer by protamine, while FITC-CD-Dextran/HA only did not penetrate.

[00237] Pig skin incubated with **E** or **F** for 24 h showed that FITC-BSA was delivered to the epidermal and dermal layers by protamine, while FITC-BSA alone did not penetrate.

[00238] **Protamine enhanced sublingual delivery of Semaglutide (GLP-1 receptor agonist)**

[00239] Mice received glucose at 1.5 g/kg by i.p injection (Glucose tolerance test, GTT) right after Semaglutide delivery. Blood glucose (BG) was monitored at 0, 5 min, 10 min, 20 min, 30 min, 50 min, 80 min and 120 min. Another run of GTT was conducted 1-3 days after semaglutide delivery.

[00240] Semaglutide delivered by s.c. or sublingually when mixed with protamine effectively controlled the BG for 2 days, while semaglutide alone delivered sublingually was ineffective (**Figure 9**).

[00241] The areas under the BG curve for groups of semaglutide (s.c.) and semaglutide + protamine (sublingual) were significantly lower compared to the control (Figure 10 and Table 1).

[00242] **Table 1**

	AUC
Glucose i.p. + Semaglutide 60 µg/kg s.c.	32648
Glucose i.p. + Prot 6 mg/kg + Semaglutide 670 µg/kg sublingual	34871
Glucose i.p. + Prot 6 mg/kg + Semaglutide 500 µg/kg sublingual	40221
Glucose i.p. + Semaglutide 670 µg/kg sublingual alone	43633
Glucose i.p.	54329

[00243] **Protamine enhanced GLP-1 sublingual delivery.**

[00244] Mice received glucose at 1.5 g/kg by i.v. (Glucose tolerance test, GTT) right after GLP-1 delivery. Blood glucose (BG) was monitored at 0, 5 min, 10 min, 20 min, 30 min, 50 min, 80 min and 120 min.

[00245] Sublingual delivery of GLP-1/protamine provided better control of BG compared to GLP-1 alone delivered by i.v. or sublingual (Figures 11A-C).

[00246] **Sublingual Cy7-BSA penetration.**

[00247] Cy7-BSA was dissolved in water at 60mg/ml. Protamine was dissolved in water at 10 mg/ml.

[00248] Cy7-BSA was mixed with protamine solution or water. The mixture was given to mice sublingually. Mice were anaesthetized during the formulation administration and kept under anaesthesia for another 5 min to allow absorption.

[00249] The doses for protamine and Cy7-BSA were 6 mg/kg and 20 mg/kg respectively. Mice were euthanized at 0, 0.5h, 2h, and 4 h respectively. Blood and tongues were collected. Plasma was isolated by centrifugation (10,000 g, 5 min) and analyzed for Cy7 fluorescence using a plate reader. Tongues were sectioned and analyzed by confocal.

[00250] Protamine showed enhanced sublingually delivery of Cy7-BSA, which is comparable to s.c. injection (**Figure 12**).

[00251] Protamine also enhanced Cy7-BSA penetration in tongue at 0.5 h.

[00252] Protamine showed enhanced sublingually delivery of 647-Dupilumab (**Figure 13** and **Table 2**).

[00253] **Table 2**

	AUC
647-Dupilumab s.c	1.685
647-Dupilumab only sublingual	0.2117
Protamine/647-Dupilumab sublingual	0.5176

[00254] Protamine enhanced 647-Dupilumab penetration and retention in tongue at 0.5, 2, 4 and 24 h.

[00255] Lymph nodes were collected at 0.5, 2, 4, and 24 h. 647-Dupilumab delivered by s.c. or protamine through sublingual showed accumulation in the lymph nodes.

[00256] **Mouse model of chronic rhinosinusitis with nasal polyps (CRSwNP)**

[00257] Naïve Four-week-old BALB/c mice were systemically sensitized with an intraperitoneal injection of 25 µg OVA (grade V; Sigma, St. Louis, MO) mixed with 2 mg of aluminum hydroxide gel on Days 0 and 5. After general sensitization, conscious intranasal administration of 3% OVA in sterile saline was given daily from Day 12 to Day 19. After induction of allergic rhinosinusitis, 3% OVA in 40 µL PBS with Staphylococcus aureus enterotoxin B (SEB; 20 ng per mouse) was instilled into the nasal cavity of conscious mice three times per week for 8 weeks. Different drug formulations were given thrice per week for 8 weeks starting from the week of SEB treatment. After that, mice were euthanized and the noses were collected for analysis.

[00258] Treatment groups included:

[00259] Group A: Intranasal instillation of sterile PBS

[00260] Group B: Intranasal instillation of CD124 (1.5 mg/kg)

[00261] Group C: Intranasal instillation of Protamine (3 mg/kg) + CD124 (1.5mg/kg)

[00262] Group D: Healthy mice

[00263] Symptoms were evaluated by counting the number of rubs that occurred in a 10 min timeframe 24 h after the last dose. Protamine/CD124 treated mice showed significantly decreased number of nose touching compared to the PBS control, while CD124 i.n. and CD124 s.c. displayed no activity (**Figure 14**).

[00264] Polypoid lesions and epithelial disruption were counted from the H&E staining slides. Protamine/CD124 treated mice showed significantly decreased numbers of polypoid lesions and epithelial disruption compared to the CD124 only group (**Figure 15**).

[00265] The nasal cavity of mice was imaged by MicroCT. Protamine/CD124 treated mice showed the least extent of mucosal thickening and adhesion in nasal tubercles compared to treatment with PBS, or CD124 only i.n.

[00266] ELISA result from nasal homogenization. Protamine/CD124 treated mice displayed decreased levels of INF gamma, IL-1 β , IL-17a, and TNF- α in the nose compared to the PBS control (**Figures 16A-D**).

[00267] **Lipid conjugate of protamine: DMPE-protamine**

[00268] **Materials**

[00269] DMPE-PEG2k-NHS was purchased from Biochempeg Scientific Inc. Hoechst 33342 and protamine sulfate salt from Salmon Grade X were purchased from Sigma-Aldrich. Dialysis membrane (molecular weight cutoff MWCO = 10 or 3.5 kDa) was purchased from Spectrum Laboratories (Waltham, MA). Hydrophobic fluorescent dye Dil (DiI C18(3); 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) was purchased from Cedarlane Labs. All other general laboratory chemicals were purchased from Fisher Scientific and VWR Scientific (Mississauga, ON, Canada).

[00270] **DMPE-Protamine**

[00271] DMPE-protamine was synthesized by reacting DMPE-PEG-NHS with protamine at a molar ratio 10:1. The reaction was carried out in Milli Q water at room temperature for 1 h. Then the reaction solution was dialyzed for 3 days and lyophilized. DMPE-Protamine was characterized by NMR.

[00272] The critical micelle concentration (CMC) of DMPE-Protamine was found to be 0.376 mg/ml, size 107.4 ± 3.8 nm; polydispersity index: 0.188 ± 0.02 . DMPE-protamine molecules self-assembled into nanoparticles in an aqueous phase (**Figure 17**).

[00273] **Cellular uptake**

[00274] For a GFP-CRISPR/Cas9-gRNA uptake study, cell suspension was first incubated with a membrane staining dye Dil at 1 μ g/mL for 30 min at 37°C and then centrifuged and washed with PBS three times before being seeded. Cells (RPMI2650) were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluency. The medium was replaced with serum-free medium containing 0.1 μ M Oligo (sgRNA) and 0.1 μ M Cas9 protein in the presence or absence of either 37.5 μ g/mL protamine or DMPE-protamine for 4 h at 37°C. Cells were washed with PBS 3 times and then stained with Hoechst 33342 and LysoTracker for the nuclei and lysosomes, respectively, according to the manufacturer's protocols. The stained cells (without fixation) were immediately imaged by confocal laser scanning microscopy (CLSM) and analyzed with ZEN software (Carl Zeiss, Oberkochen, Germany) and image J.

[00275] The results indicated that DMPE-Protamine showed better nuclear delivery of CRISPR/Cas9-gRNA compared to protamine (**Figure 18**).

[00276] **Multivalent (or cross-linked) protamine: (Protamine)₂, (Protamine)₃ and PAMAM dendrimer-protamine conjugate**

[00277] **Materials**

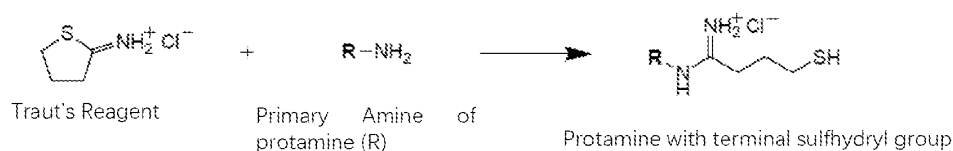
[00278] SULFO-SMCC and Succinimidyl 4-Maleimidobutyrate (98%) (GMBS) were purchased from Biochempeg Scientific Inc. PAMAM G4 was purchased from Sigma. Traut's Reagent (2- Iminothiolane•HCl), EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), NHS (N-hydroxysuccinimide) and 2-mercaptoethanol were purchased from Thermo. LysoTracker Red DND-99, FITC-conjugate of bovine serum albumin (FITC- BSA) were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). Hoechst 33342, Citric Acid, Anhydrous, and protamine sulfate salt from Salmon Grade X were purchased from Sigma-Aldrich. Dialysis membrane (molecular weight cutoff MWCO = 10 or 3.5 kDa) was purchased from Spectrum Laboratories (Waltham, MA). All other general laboratory chemicals

were purchased from Fisher Scientific and VWR Scientific (Mississauga, ON, Canada).

[00279] **(Protamine)₂**

[00280] Protamine was cross-linked through two-step reaction by using Traut's Reagent and SULFO-SMCC (**Figure 19**).

[00281] Solution A: Protamine and Traut's Reagent (2-Iminoethiolane•HCl) were dissolved in 5 mM EDTA at a molar ratio 1:20. The solution was incubated at room temperature for 1 h, followed by dialysis against 5 mM EDTA for 1 h.

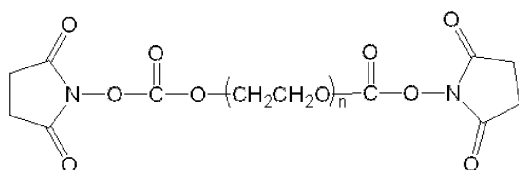


[00282] Solution B: Protamine and SULFO-SMCC were dissolved in Milli Q water at a molar ratio 1:10. The solution was incubated at room temperature for 1 h, followed by dialysis against Milli Q water for 1 h.

[00283] Solution A was added to solution B and reacted at room temperature for 1 h. Then the reaction solution was dialyzed against Milli Q water for 3 days and lyophilized.

[00284] **(Protamine)₂**

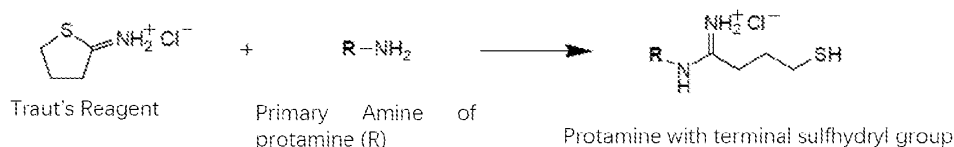
[00285] Protamine was cross-linked through a one-step reaction by using NHS-PEG-NHS:



[00286] Protamine and NHS-PEG-NHS were dissolved at a molar ratio of 1:10 in PBS (protamine 10 mg/ml). The solution was incubated at room temperature for 24 h, followed by dialysis against water for 48 h and lyophilized.

[00287] **PAMAM dendrimer-Protamine**[00288] Protamine was linked to PAMAM dendrimer (**Figure 20**).

[00289] Solution A: Protamine and Traut's Reagent (2-Iminoethanol-HCl) were dissolved in 5 mM EDTA at a molar ratio 1:20. The solution was incubated at room temperature for 1 h, followed by dialysis against 5 mM EDTA for 1 h.



[00290] Solution B: GMBS was dissolved in Dimethylacetamide (DMAC) and was added to PAMAM G4 (10% in methanol) at a molar ratio of 640:1. The solution was incubated at room temperature for 1 h, followed by dialysis against Milli Q water for 1 h.

[00291] Solution A was added to solution B and reacted at room temperature for 1 h. Then the reaction solution was dialyzed against Milli Q water for 3 days and lyophilized

[00292] **(Protamine)₃**[00293] Protamine was linked to citric acid through the EDC/NHS reactions (**Figure 21**).

[00294] Solution A: 10 μ mol Citric acid, 300 μ mol EDC, and 750 μ mol NHS were dissolved in 2 ml water and the pH was adjusted to 4.5~6 by using 1N NaOH. The mixture was allowed to react for 15 min. Next, 2-mercaptoethanol was added to a final concentration of 20 mM to quench the EDC and then the pH was adjusted to 7.

[00295] Solution B: 200 mg protamine was dissolved in PBS at 10 mg/ml and the pH was adjusted to 7~7.5.

[00296] Solution A was added to solution B and reacted at room temperature for 2 h. Then the reaction solution was dialyzed against Milli Q water for 2 days.

[00297] Cellular uptake

[00298] Cells (RPMI2650) were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluency. The medium was replaced with fresh 10% FBS-containing medium including 20 $\mu\text{g/mL}$ FITC-BSA in the presence or absence of either 37.5 $\mu\text{g/mL}$ protamine, (Protamine)₂ or PAMAM dendrimer-protamine for 2.5 h at 37°C. Cells were washed with PBS 3 times and then trypsinized for 3 min, resuspended in complete medium, centrifuged at 400 g for 3 min, washed with PBS 3 times, resuspended in PBS, and analyzed by Fluorescence-activated Cell Sorting (FACS).

[00299] Second generation of protamine (Protamine-PAMAM and (Protamine)₂) increased FITC-BSA delivery into the cells compared to protamine. (**Figure 22**)

[00300] Cellular uptake

[00301] Cells (HEK 293T) were seeded in a 24-well plate at 5×10^4 cells per well in 0.5 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 50–60% confluency. The medium was replaced with fresh FBS-free medium including 2 $\mu\text{g/mL}$ GFP pDNA in the presence or absence of either 60 or 120 $\mu\text{g/mL}$ protamine, Protamine-AGE, PAMAM or PAMAM dendrimer-protamine for 48 h at 37°C. Cells were washed with PBS 3 times and then trypsinized for 3 min, resuspended in complete medium, centrifuged at 400 g for 3 min, washed with PBS 3 times, resuspended in PBS, and analyzed by Fluorescence-activated Cell Sorting (FACS). (slide 39)

[00302] Second generation of protamine (Protamine-PAMAM and Protamine-AGE) increased GFP pDNA transfection efficiency in HEK 293T cells compared to protamine (**Figure 23**).

[00303] Streptozotocin (STZ) induced diabetic mice model

[00304] Mice were fasted for 6 h and then i.p. injected with 200 mg/kg STZ. Three days later, blood glucose (BG) was measured using a glucose meter (ONE Touch Ultra 2 Blood Monitoring System). Mice with BG ≥ 300 mg/dL were regarded as diabetic.

[00305] Preparation of insulin/DMPE-protamine and insulin/(protamine)₂ formulations

[00306] DMPE-Protamine, (Protamine)₂ and (Protamine)₃ were dissolved in water at 3~4 mg/mL without pH adjustment, and insulin was dissolved in water at 5 mg/mL with pH adjustment to 3.2 using 1 N HCl. DMPE-Protamine, (Protamine)₂ or (Protamine)₃ was then mixed with insulin at a weight ratio of 1.5 (w/w). The mixture was used immediately.

[00307] The results indicated that DMPE-Protamine, (Protamine)₂ and (Protamine)₃ formulations for insulin can better decrease blood glucose levels compared to protamine/insulin formulation.

[00308] **Protamine and protamine-PAMAM increased insulin delivery to the brain via intra-nasal delivery.**

[00309] Alexa Fluor 647 (AF647) protein labeling kit was purchased from Thermo Fisher Scientific. Human recombinant insulin, protamine sulfate salt from Salmon Grade X, DAPI, and FITC-insulin were purchased from Sigma-Aldrich. Dialysis membrane (molecular weight cutoff MWCO – 10 or 3.5 kDa) was purchased from Spectrum Laboratories. All other general laboratory chemicals were purchased from Fisher Scientific and VWR Scientific.

[00310] Four-week old female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). In vivo experiments were performed following the established experimental protocols approved by the Animal Care Committee of the University of British Columbia.

[00311] Insulin was labeled with AF647 protein labeling kit according to the manufacturer's protocol to produce 647-insulin. FITC-insulin or 647-insulin was mixed with protamine or protamine-PAMAM in water and intranasally (i.n.) delivered to mice (dose = 1mg/kg FITC-insulin or 647-insulin; 3mg/kg protamine). Saline, FITC-insulin, and 647-insulin were included as controls. Brains were collected at 2hr and frozen overnight and then cryo-sectioned. The cryo-section was mounted onto a glass slide with a drop of Fluorshield containing DAPI (Sigma-Aldrich) and imaged by CLSM. The results indicated that protamine-PAMAM displayed enhanced efficiency for brain delivery of insulin.

[00312] Preparation of insulin/DMPE-protamine and insulin/(protamine)₂ formulations

[00313] DMPE-Protamine, (Protamine)₂ and (Protamine)₃ were dissolved in water at 3~4 mg/mL without pH adjustment, and insulin was dissolved in water at 5 mg/mL with pH adjustment to 3.2 using 1 N HCl. DMPE-Protamine, (Protamine)₂ or (Protamine)₃ was then mixed with insulin at a weight ratio of 1.5 (w/w). The mixture was administrated intranasally immediately. (slide 41)

[00314] The results indicated that intranasal DMPE-Protamine, (Protamine)₂ and (Protamine)₃ formulations for insulin performed more consistently in decreasing the BG in STZ-mice compared to the protamine group. (Figure 24A-D).

[00315] PAMAM-Protamine-SATA

[00316] Solution A:

[00317] 1. Dissolve 10-15 mg SATA (N-Succinimidyl S-Acetylthioacetate) in 50 μ L of DMSO.

[00318] 2. Dissolve protamine in MilliQ at 10 mg/mL. Combine 10 mL of protamine solution with 50 μ L of the SATA solution. Incubate at room temperature for 30 minutes.

[00319] 3. Dialyzed against MilliQ for 30 min.

[00320] 4. Combine 1 mL of SATA-modified (acetylated) protamine with 100 μ L of the Deacetylation Solution.

[00321] Deacetylation Solution: 0.5M Hydroxylamine, 25mM EDTA in PBS, pH 7.2-7.5. Dissolve 1.74g hydroxylamine•HCl and EDTA (0.475g of tetrasodium salt or 0.365g of disodium salt) in 40mL of MilliQ. Add ultrapure water to a final volume of 50mL and adjust pH to 7.2-7.5 with NaOH.

[00322] 5. Dialyzed against MilliQ for 30 min.

[00323] Solution B: GMBS was dissolved in DMSO and was added to PAMAM G4 (10% in methanol) at a molar ratio of 10:1. The solution was incubated at room temperature for 1 h, followed by dialysis against Milli Q water for 30 min.

[00324] Solution A was added to solution B and reacted at room temperature for 1 h. Then the reaction solution was dialyzed against Milli Q water for 3 days and lyophilized.

[00325] Protamine-AGE : Allyl glycidyl ether (AGE, 1.5 g) was added dropwise into 15 mL protamine water solution (10 mg/ml) for a 3.5 h reaction at 28 °C. The product Protamine-AGE was purified using dialysis (molecular weight cutoff, 10 KDa) against water for 48h. Protamine-AGE was finally obtained using freeze-drying under vacuum. Protamine-AGE was characterized by NMR.

[00326] Protamine-2AGE: Allyl glycidyl ether (AGE, 1.84 g) was added dropwise into 18 mL protamine water solution (10 mg/ml, pH 8.56) for a 2.5 h reaction at 28 °C. The product Protamine-2AGE was purified using dialysis (molecular weight cutoff, 10 KDa) against water for 48h. Protamine-2AGE was finally obtained using freeze-drying under vacuum.

[00327] **Streptozotocin (STZ) induced diabetic mice model**

[00328] Mice were fasted for 6 h and then i.p. injected with 200 mg/kg STZ. Three days later, blood glucose (BG) was measured using a glucose meter (ONE Touch Ultra 2 Blood Monitoring System). Mice with BG $\geq$ 300 mg/dL were regarded as diabetic.

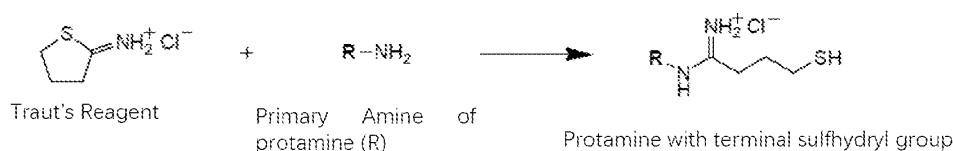
[00329] **Preparation of insulin/PAMAM-Protamine-SATA and insulin/Protamine-AGE formulations**

[00330] PAMAM-Protamine-SATA and Protamine-AGE were dissolved in water at 10 mg/mL without pH adjustment, and insulin was dissolved in water at 5 mg/mL or 20 mg/mL with pH adjustment to 3.2 using 1 N HCl. The dose of PAMAM-Protamine-SATA and Protamine-AGE was 1.5 mg/kg and insulin was 1 mg/kg for intranasal delivery. For sublingual delivery, the dose for PAMAM-Protamine-SATA and Protamine-AGE was 3 mg/kg and insulin was 5 mg/kg. (slide 47)

[00331] PAMAM-Protamine-SATA and Protamine-AGE formulations for intranasal delivery of insulin performed more consistently in lowering the BG in STZ-diabetic mice compared to the protamine formulation (**Figures 25A-F**).

[00332] **PAMAM-AGE-Protamine**

[00333] Solution A: Protamine and Traut's Reagent (2-Iminothiolane•HCl) were dissolved in 5 mM EDTA at a molar ratio 1:20. The solution was incubated at room temperature for 1 h, followed by dialysis against 5 mM EDTA for 1 h.



[00334] Solution B: Allyl glycidyl ether (AGE, 1.5g or 0.35g) was dissolved in 7.5 mL of methanol/MilliQ water (3/4, v/v). Next, PAMAM G4 10% in methanol (700 μ L) was added dropwise into the AGE solution for a 3 h reaction at 25 °C. The product PAMAM-AGE was purified using dialysis (molecular weight cutoff, 10 KDa) against water for 1h.

[00335] Solution A was added to solution B in the presence of the catalyst azobisisobutyronitrile (AIBN, 18 mg, 0.1 mmol). After reaction at 65 °C for 24 h, the mixture was transferred to a dialysis tube (molecular weight cutoff, 10 KDa) and then dialyzed against MilliQ for 48 h. PAMAM-AGE_{1.5}-Protamine and PAMAM-AGE_{0.35}-Protamine was finally obtained using freeze-drying under vacuum.

[00336] Alexa647-Dupilumab intranasal delivery (IN): Dupilumab was first labeled with Alexa Fluor 647 (AF647) protein labeling kit according to the manufacturer's protocol. Alexa647-Dupilumab was mixed with different protamine (prot) analogues in water and intranasally (i.n.) delivered to mice (dose = 3 mg/kg protamine, protamine-AGE, PAMAM-AGE-Protamine unless specified otherwise; 20 μ L of 3 mg/mL Alexa647-Dupilumab). Saline and Alexa647-Dupilumab only were included as controls. Plasma was isolated by centrifugation of blood (10,000 g, 5 min) and analyzed for Alexa647 fluorescence using a plate reader 4 h after intranasal delivery.

[00337] Protamine-AGE and PAMAM-AGE_{0.35}-Protamine enhanced systematic absorption of Alexa647-Dupilumab through intranasal delivery. (**Figure 26**)

[00338] **Human Nasal Epithelial Cells (HNEpC) models**

[00339] HNEpC models were established by using method developed by PromoCell (<https://promocell.com/wp-content/uploads/2022/07/AppNote-HNEpC-and-HTEpC-ALI-web.pdf>).

[00340] After 10 days of culture, 8 μ L of Protamine or Protamine-AGE (dissolved in 5% glucose at 10 mg/mL) plus 2 μ L Alexa647-Dupilumab (2 mg/mL) and 10 μ L 5% glucose was added dropwise onto the HNEpC model. After 2 h, the HNEpC model was frozen and then sectioned for confocal microscopy analysis.

[00341] Protamine enhanced 647-Dupilumab penetration in HNEpC models compared to 647-Dupilumab only group. Protamine-AGE further enhanced 647-Dupilumab penetration compared to protamine.

[00342] Protamine enhanced 647-Dupilumab penetration in HNEpC models compared to 647-Dupilumab only group. Protamine-AGE and PAMAM-Protamine further enhanced 647-Dupilumab penetration compared to protamine.

[00343] Cellular uptake: Cells (RPMI2650) were seeded in a 12-well plate at 1×10^5 cells per well in 1 mL of medium containing 10% FBS and incubated for 18–20 h to achieve 70–80% confluency. The medium was replaced with fresh 10% FBS-containing medium including 20 μ g/mL FITC-BSA in the presence or absence of either 37.5 μ g/mL protamine or different CPP sequences isolated from protamine for 4 h at 37°C. Cells were washed with PBS 3 times and then stained with lysotracker for 30 min followed by 3 times PBS wash. After that, 2 mL of FBS-free medium was added and cells were observed under confocal. Alternatively, after 4 h incubation, Cells were washed with PBS 3 times and then trypsinized for 3 min, resuspended in complete medium, centrifuged at 400 g for 3 min, washed with PBS 3 times, resuspended in PBS, and analyzed by Fluorescence-activated Cell Sorting (FACS).

[00344] Different CPP sequences identified from protamine sulfate were examined.

[00345] **S1**: PRRRRRSSSRPIRRRRRPRASRRRRRGGRRRR (SEQ ID NO: 1)

[00346] **S2**: PRRRRSSRRPVRRRRRPRVSRRRRRRGGRRRR (SEQ ID NO: 2)

[00347] **S3**: PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR (SEQ ID NO: 3)

[00348] **S4**: PRRRRASRRIRRRRRPRVSRRRRRRGGRRRR (SEQ ID NO: 4)

[00349] **S2** and **S4** were superior to **S1** and **S3** in delivering FITC-BSA into the nucleus of cells.

[00350] **S2** and **S4** were superior to **S1** and **S3** in delivering FITC-BSA into the cells. (Figures 27A-B)

[00351] S2, S3 and S4 treated cells displayed fewer lysosomes compared to S1. (Figure 28)

[00352] Protamine-AGE was characterized by NMR.

[00353] Protamine-AGE size was detected under PBS at pH 7.4. Particle size and zeta potential were determined with a particle analyzer (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK). The size results are expressed as Z-Ave diameter.

Table 3: Particle size and zeta potential of Protamine-AGE and Protamine at pH 7.4, n=3

	Z-average (d. nm)	PDI	Zeta potential (mV)
Protamine-AGE	170.1 ± 4.0	0.223 ± 0.003	3.0 ± 1.8
Protamine	-- ^a	-- ^a	2.05 ± 0.54

^a Unreliable data report.

[00354] Cas9 RNP/sgRNA delivery to HEK 293 T EGFP cells: Cells (HEK 293T EGFP) were seeded in a 3.5mm confocal dish at 1 × 10⁵ cells per well in 2 mL of medium containing 10% FBS and 0.2 mg/mL Hygromycin B and incubated for 18–20 h to achieve 70–80% confluency. The medium was replaced with fresh FBS-Free medium in the presence or absence of either 37.5 µg/mL protamine or Protamine-AGE for 40 min at 37°C. 3.5 µL Cas9-RNP-RFP (52 µM) was added to 20 µL nuclease free water followed by 2 µL of sgRNA (100 µM) and then incubated for 10 min. The cell culture medium was replaced with FBS-free medium containing Cas9-RNP-RFP/sgRNA mixture with either 37.5 µg/mL protamine or Protamine-AGE. Cells were keep at 37°C for 48 h. After that, cells were treated with 2 µg/mL of tetracycline hydrochloride for 24 h to active EGFP expression. Cells were washed with PBS 3 times and then 2 mL of FBS-free medium was added and cells were observed under confocal. (slide 62)

[00355] Protamine-AGE increased Cas9-RNP/sgRNA delivery for EGFP knockdown. (Figures 29A-B)

[00356] **647-CD124 monoclonal antibody (mAb) penetration in mice nose**

[00357] 647-CD124 mAb was dissolved in PBS at 2mg/ml, and 20 µL of the mAb solution was mixed with different protamine solutions (Protamine, Protamine-2AGE,

Protamine-AGE and PAMAM-Protamine) to achieve a final protamine concentration of 3.3 mg/ml. The mixture was intranasally at a dose of 1.3 mg/kg for mAb and 3 mg/kg for protamine. The total delivery volume was up to 32 μ L. Twenty μ L of 647-CD124 mAb solution was subcutaneously administrated as control (dose = 1.3 mg/kg).

[00358] Mice were observed under IVIS at different time points (0, 5 min, 30 min, 1 h, 2 h, 3 h and 4 h). Mice were euthanized at 4 h and noses were collected and sectioned for confocal.

[00359] **647-CD124 retention in mice nose**

[00360] The protamine-based formulations enhanced nasal retention of mAb. (Figure 30)

[00361] **Table 4**

Treatment	AUC
647-CD124 s.c.	349760000
647-CD124 i.n.	543047500
Protamine/647-CD124	737950000
Protamine-AGE/647-CD124	713800000
Protamine-2AGE/647-CD124	714000000
PAMAM-Protamine/647-CD124	655250000

[00362] **647-CD124 penetration in mice nose**

[00363] Second generation of protamine enhanced 647-CD124 mAb penetration in the nose of mice compared to protamine. The results were consistent with IVIS.

[00364] **Eye drops**

[00365] FITC-BSA was dissolved in water at 5 mg/ml. Protamine, Protamine-AGE or PAMAM-Protamine was dissolved in 5% glucose at 10 mg/ml.

[00366] 5 μ L of the FITC-BSA solution was mixed with 22.5 μ L of the Protamine, Protamine-AGE or PAMAM-Protamine solution (or 5% glucose as a control) and was

applied to the eye. 5 μ L of the mixture was applied to the eye each time. After 5 min, another drop was applied. The whole procedure was finished in 30 min.

[00367] 2-24 h later, the eye was collected and fixed in 10% formalin for 24 h and then change to 10% sucrose in water for 24 h. Lastly, eyes were kept in 30% sucrose for 24 h and then sectioned and imaged under confocal.

[00368] Eyes were collected 2 h after treatment. Protamine enhanced FITC-BSA retention on the mouse eyes.

[00369] Eyes collected 4 and 24 h after treatment. Protamine-based formulation enhanced FITC-BSA penetration in lens.

[00370] **Synthesis and characterization of novel peptides**

[00371] C18-P was synthesized by linking a steric acid to the terminal amino group of protamine via the EDC/NHS method (**Figure 31A**).

[00372] C18-P, protamine and stearic acid was characterized by ^1H NMR and ^{13}C NMR. The ^1H NMR analysis indicated a distinctive peak at 2.5 ppm, corresponding to C18 from stearic acid. The ^{13}C NMR spectrum showed the presence of all stearic acid peaks, alongside those from protamine. The shift of the protamine peak in the MS from 4.1 kDa (**Figure 31C**) to 4.4 kDa (**Figure 31D**) indicated conjugation of stearic acid to protamine.

[00373] P2 was prepared by cross-linking two terminal amino groups of protamine using NHS-PEG-NHS (**Figure 31B**).

[00374] P2, protamine and NHS-PEG-NHS was characterized by ^1H NMR. A prominent 3.5 ppm peak in the ^1H NMR spectrum confirmed the linkage of PEG to protamine, while the mass result exhibited a peak at 8.2 kDa (**Figure 31E**). These collective data affirmed the successful synthesis of C18-P and P2. FTIR spectroscopy further confirmed the reactions through the appearance of a peak at 1300 cm^{-1} , indicative of amide bonds (**Figure 31F**).

[00375] CD analysis, employed to study the secondary structure of novel peptides, revealed noteworthy insights. Protamine exhibited a pattern consistent with the existing literature [72], marked with a dominant negative signal peak at 210 nm (**Figure 31G**). This suggested the presence of a random coil structure, which can be attributed to the high arginine content fostering positive charge-driven electrostatic

repulsions. Modification with stearic acid or a PEG linker led to distinct changes in the CD spectra, which were analyzed by BeStSel to quantify secondary structures, including α -helices, β -sheets, β -(anti) parallel, turns, and random coils (**Table 5**). The data confirmed that protamine was mainly in the random coil conformation, while α -helix and β -sheet structures dominated in C18-P and P2. Without being bound to any particular hypothesis, these structural changes may stem from the hydrophobic impact of the stearic acid tail or the influence of the PEG linker.

Table 5. BeStSel analysis of the percentage of α -helix, β -sheet, and random coil from protamine, C18 and P2 from CD spectra.				
	α -helix	β -sheet		Random coil
		Anti-parallel β -sheet	β -turn	
Protamine	15.8%	21.7%	6.7%	55.9%
C18-P	38.6%	30.7%	21.2%	9.5%
P2	37.1%	27.3%	15.9%	19.6%

[00376] When dissolved in water, protamine did not form any aggregated structures as shown by AFM and DLS, while C18-P and P2 self-assembled into spherical and rod-shaped (400 nm x 5 nm) nano-structures respectively, as shown by TEM and AFM. DLS also confirmed the presence of nano-aggregates in samples prepared with C18-P and P2 (**Table 6**).

[00377] **Table 6.** Particle size and polydispersity index (PDI) of C18-P and P2. Data = mean $\pm$ SEM (n = 3).

	Z-average (d. nm)	PDI
C18-P	121.6 $\pm$ 55.9	0.284 $\pm$ 0.007
P2	453.4 $\pm$ 3.6	0.17 $\pm$ 0.004

[00378] These data are consistent with the increased presence of β -sheets in C18-P and P2. C18-P is an amphiphilic compound, containing a hydrophobic acyl chain and a hydrophilic peptide, which is anticipated to self-assemble into micelles. While the β -sheet content in P2 was similar to that of C18-P, P2 displayed a distinct nanorod structure. Without being bound to any particular hypothesis, this difference might be attributed to steric hindrance from the PEG linker, increasing the separation between the two protamine molecules and reducing the density of the β -sheet structure.

[00379] C18-P and P2 increased intracellular delivery of proteins via increased pore generation in the cell membrane

[00380] We first showed that below 10 $\mu\text{g/ml}$, C18-P and protamine did not cause any toxicity to primary human gingival keratinocytes, while P2 needed to be decreased to 5 $\mu\text{g/ml}$ (**Figure 32**). To investigate how protamine and the derivatives interacted with cells, human primary gingival keratinocytes were imaged by SEM after treatment at non-toxic concentrations. SEM images of human primary gingival keratinocytes after treatment with protamine, C18-P, or P2 were examined and, in the untreated group, the cell membrane was relatively smooth without any large aggregates on the surface. In the protamine treated group, some small aggregates were found on the surface with pores (black areas) underneath. Increased amounts and size of aggregates and membrane pores were seen on cells treated with C18-P and P2. ImageJ was employed to analyze the SEM images to better outline the membrane pores. Analysis of pores on the cell membrane using ImageJ was performed and pore area relative to total cell membrane area quantified. The percentage of the pore area to total cell surface quantified in the protamine-treated group was only 0.1%, compared to 1.4 % and 2.0 % in the C18-P and P2 groups respectively. It is noted that these numbers could be underestimated, as some pores might be covered by the aggregates and not shown. Nevertheless, the data support our hypothesis that these structural modifications to protamine increased its cell permeation activity. Interestingly, although protamine and the derivatives caused defects in the cell membrane, no toxicity to cells was induced, likely because the effect was transient.

[00381] The SEM data suggested that the novel peptides would increase intracellular drug delivery compared to protamine. Next, AF647-IgG (150 kDa) was used as a model protein payload and was incubated with human primary gingival keratinocytes in the presence of protamine, C18-P, or P2 at 5-10 $\mu\text{g/ml}$ for 12 h, followed by washing and confocal imaging. Confocal microscopy images of cells after

treatment with AF647-IgG in the presence of protamine, C18-P, or P2 were examined), C18-P and P2 treated cells displayed increased cytosolic delivery of IgG compared to protamine. ImageJ analysis revealed 8- and 21-fold increased cellular uptake of IgG in the C18-P and P2 treated cells, respectively compared to protamine (**Figure 33A**). Notably, IgG delivered by protamine and the derivatives displayed minimal overlay with lysosomes (**Figure 33B**), supporting that membrane pore formation was the main mechanism of delivery. FCM data were consistent with the confocal results (**Figure 34**). Comparable results were also obtained with another payload, FITC-BSA (66 kDa). Confocal microscopy images of cells after treated with FITC-BSA in the presence or absence of protamine, C18-P, or P2 were examined. Quantitative analysis was performed (**Figure 35**).

[00382] Collectively, these data suggest that novel peptides promoted intracellular delivery of proteins up to 150 kDa in size and bypassed lysosomal degradation.

[00383] **C18-P and P2 enhanced protein penetration into cell spheroids**

[00384] We next investigated whether protamine and the derivatives could enhance penetration of proteins through multiple cell layers. Spheroids prepared with Dil labeled human primary gingival keratinocytes were incubated with AF647-IgG mixed with protamine, C18-P, or P2 overnight, and were then washed and imaged by CLSM at a depth of 40 μm from the top of the spheroids. Confocal microscopy analysis of AF647-IgG penetration in the presence of protamine, C18-P, or P2 in human primary gingival keratinocyte spheroids was performed. Spheroids were imaged at a depth of 40 μm from the surface), delivery of free AF647-IgG into the spheroid was limited, while protamine significantly increased the penetration. The spheroids treated with C18-P and P2 showed further enhanced uptake and penetration of AF647-IgG compared to protamine. The images were analyzed by ImageJ to compare AF647-IgG penetration within the spheroids delivered by different CPPs. The majority of the IgG was limited to a distance of 30 μm from the periphery in the protamine group, while in the C18-P and P2 treated groups, the IgG was detected at up to 68 μm from the periphery (**Figure 36A**). To further confirm delivery of the IgG in the spheroids, single cells were collected from the spheroids and analyzed by FCM. As shown in **Figure 36B**, the AF647-positive cells represented 10.6, 46.3, 52.1, and 71.3% of total cells in the PBS, protamine, C18-P, and P2 treated groups respectively. C18-P and P2 displayed enhanced activity in promoting IgG penetration in the spheroids compared to protamine, and P2 was superior to C18-P. Comparable results were obtained with another protein payload, FITC-BSA. Confocal microscopy analysis of FITC-BSA penetration in the presence of

protamine, C18-P, or P2 in human primary gingival keratinocyte spheroids was performed. Spheroids were imaged at a depth of 60 μm from the surface (**Figures 37A-B**).

[00385] C18-P and P2 increased Cas9-RNP delivery compared to protamine in 2D and 3D cell models

[00386] We next evaluated whether the protein payload delivered by protamine and the derivatives remained active to exert its biological activity in cell models. HEK293-eGFP cells were incubated with protamine, C18-P or P2 for 40 min, followed by the addition of Cas9-RNA/sgRNA. Cells were cultured for another 48 h before being washed and imaged by CLSM. Confocal microscopy images of cells treated with Cas9-RNP-RFP/sgRNA in the presence of protamine, C18-P, or P2 were examined and the results indicated that delivery of free Cas9-RFP-RNP was limited and eGFP remained strongly expressed. Protamine significantly increased the RNP delivery, and the eGFP expression was reduced compared to the cells treated with free RNP. RNP delivery was further increased by C18-P and P2, accompanied with further reduced eGFP expression compared to the protamine group (**Figures 38A-B**).

[00387] The images were analyzed using ImageJ (**Figures 39A-B**), which showed that the eGFP fluorescence intensities were 3118, 2058.4, 1180, 938 a.u. for free RNP, protamine, C18-P and P2 respectively. The FCM analysis showed that cells that were RNP+ eGFP- represented 10.1, 12.8, 15.3, and 30.2% of cells treated with free RNP, protamine, C18-P and P2 respectively. In the 2D cell model, the RNP delivered by the CPPs were active and effectively silenced eGFP, and the delivery efficiency followed the order of P2>C18-P>protamine. We next examined and compared these delivery systems in a cell spheroid model. Penetration of Cas9-RFP-RNP/sgRNA in the presence of protamine, C18-P, or P2 in eGFP HEK 293 spheroids was examined. Cells were dissociated from the spheroids, and Cas9-RFP-RNP positive cells (C) and RNP+ and eGFP- cells (D) were quantified by FCM. Data = mean $\pm$ SD. ($n \geq 5$). (** $p < 0.001$; **** $p < 0.0001$).), most RNP was detected at the periphery of the spheroids when treated with free RNP or protamine/RNP. The protamine group displayed moderately improved penetration of RNP compared to the free RNP group. On the other hand, cells treated with C18-P and P2 showed significantly enhanced RNP penetration to the core of spheroids compared to protamine. To further confirm the delivery of RNP in the spheroids as well as eGFP knockdown efficiency, single cells were collected and analyzed by FCM. As shown in **Figure 38**, the protamine treated group showed a 1.6-fold increase of RNP uptake, while C18-P and P2

increased the delivery to 4- and 3.5-fold respectively compared to the free RNP group. Cells that were RNP+ and eGFP- in the spheroids treated with free RNP, protamine, C18-P, and P2 were 6.9, 13.44, 20.14, and 20.34% of the total cells analyzed (**Figure 38**), indicating the superiority of C18-P and P2 in RNP delivery. No difference was found between C18-P and P2 in the spheroid model.

[00388] C18-P and P2 enhanced protein penetration in a human sublingual tissue substitute

[00389] Keratinocytes in cell spheroids do not sufficiently differentiate to develop the structural barriers present in sublingual tissues that impede drug penetration; therefore, we next compared protein payload penetration mediated by protamine and the derivatives in a human sublingual tissue substitute. Section of the human sublingual tissue substitute stained with H&E was examined and confirmed the successful establishment of a human sublingual tissue substitute with distinct anatomical layers, which included a basal layer of collagen-based stroma embedded with fibroblasts and an apical layer composed of stratified keratinocytes. Protamine, C18-P, or P2 mixed with AF-647-IgG was applied to the apical site. The tissue was then harvested after 4 h and imaged by CLSM. Confocal microscopy analysis of AF647-IgG tissue penetration in the presence of protamine, C18-P, or P2 was performed), delivery of free AF647-IgG into the sublingual substitute was limited, while protamine increased adhesion of the IgG to the surface of the tissue. C18-P and P2 facilitated IgG penetration into the tissue compared to protamine. The images were analyzed using ImageJ (**Figure 40A**), indicating that C18-P and P2 showed 3.7-fold and 4.1-fold respective increases in AF647-IgG delivery into the tissue compared to protamine. Furthermore, AF647-IgG delivered by protamine displayed the farthest travel distance of 60 μm from the tissue surface, while those in the C18-P and P2 treated groups reached up to 80 μm and 190 μm , respectively with significantly increased fluorescence intensity within the tissue (**Figure 40B**). The data confirmed that both novel peptides were superior in enhancing IgG penetration in the human sublingual tissue substitute compared to protamine, while the difference between C18-P and P2 was insignificant.

[00390] C18-P and P2 enhanced sublingual penetration of AF647-IgG in mice compared to protamine

[00391] We then compared sublingual penetration of AF647-IgG mediated by protamine, C18-P, and P2 in mice. AF647-IgG was mixed with protamine, C18-P, or

P2, and sublingually delivered to isoflurane-anesthetized mice, which were returned to their cages for recovery directly after the procedure. Mice were then euthanized, and the base of the tongue containing sublingual tissue was collected, sectioned and imaged by CLSM. Sublingual AF647-IgG alone was included as a control. Confocal microscopy analysis of AF647-IgG penetration in the presence of protamine, C18-P, or P2 at 0.5, 2 and 4 h post-administration was performed), without any delivery system, no AF647-IgG penetration was detected in the sublingual tissue. However, with protamine, C18-P or P2, mucoadhesion of AF647-IgG was significantly enhanced for at least 2 h. Especially in the groups treated with C18-P and P2, increased sublingual penetration of the IgG to the epithelium (C18-P and P2) and lamina propria (P2) was detected compared to protamine. This is further evidenced by the quantitative data in **Figures 41A-C**, showing that IgG delivered by protamine was found exclusively within 10 μm from the surface (*i.e.*, in the mucus), while C18-P and P2 significantly enhanced the tissue penetration, reaching up to 40 μm and 190 μm from the surface **respectively**. The tissue penetration of IgG mediated by C18-P and P2 occurred as early as 0.5 h post administration, persisted for at least 2 h, and largely disappeared at 4 h, likely due to swallowing or sublingual absorption and degradation. Notably, P2 displayed increased IgG penetration compared to C18-P, which might be due to the increased mucoadhesion mediated by hydrogen bonds between the PEG linker and the carbohydrates of the mucin.

[00392] C18-P and P2 enabled efficient sublingual delivery of insulin and semaglutide for enhanced blood glucose control in mice

[00393] To demonstrate potential medical utilities of the delivery technologies, we mixed protamine, C18-P, or P2 with either insulin or semaglutide, sublingually administered the formulations to mice, and compared their efficacy in blood glucose control. The sublingual dose for protamine and the derivatives was fixed at 3 mg/kg, while the doses for insulin and semaglutide were 5 mg/kg and 0.5 mg/kg respectively. As shown in **Figure 42A**, s.c. insulin at 1 mg/kg was effective in decreasing the BG of STZ-induced diabetic mice; the effect took place as early as 0.5-1 h and persisted for at least 6 h, keeping the BG at ~ 100 mg/dL. Sublingual insulin at 5 mg/kg showed little efficacy, and including protamine in the formulation barely improved the effect, exhibiting no significant difference. On the other hand, both C18-P and P2 sublingual formulations effectively suppressed the BG with comparable onset, potency and duration to s.c. insulin. In the study of semaglutide, 1.5 mg/kg glucose was intraperitoneally injected into normal mice immediately after administration of

semaglutide at 60 µg/kg (s.c.) or 500 µg/kg (sublingual). As shown in **Figure 42B**, the BG increased rapidly after glucose injection from ~150 mg/dL to 250 mg/dL in 0.25 h, followed by gradual decline to the basal level of BG. When treated with s.c. semaglutide, the BG only increased to 200 mg/dL and rapidly dropped to the baseline in 50 min, and the BG control effect was maintained on the second day. The effect was gone on the third day. Sublingual semaglutide showed efficacy in suppressing the BG surge during the initial 50 min after glucose intake, but the effect became insignificant after 1 h. On the contrary, the protamine sublingual formulation did not display any effect in controlling BG in the initial phase, but the effect kicked in by 1 h with the BG returning to the baseline at 1.25 h. However, the effect of the protamine sublingual formulation on the second day was only moderate. These results suggest that free semaglutide could be rapidly absorbed sublingually, but the absorption was too low to sustain the efficacy beyond 1 h. On the other hand, protamine might slow down semaglutide absorption through the binding with the drug, leading to delayed onset, and the extent of absorption was suboptimal to maintain the same level of efficacy on the second day. The superior C18-P and P2 formulations showed comparable onset, potency, and duration to s.c. semaglutide, indicating that C18-P and P2 increased sublingual delivery of semaglutide compared to protamine. Additionally, there was no BG fluctuation on the second day in the groups treated with C18-P and P2 formulations. It is to be noted that in order to achieve the same levels of efficacy, the doses for the sublingual formulations of C18-P and P2 were 5- to 8-fold higher than those of the s.c. formulations. Without being bound to any particular hypothesis, this could be attributed to saliva dilution and rapid removal of the sublingual formulations by swallowing. Nevertheless, compared with Rybelsus®, an oral formulation of semaglutide that requires up to a 100-fold increase in overall dose due to a low oral bioavailability (0.8%) and takes 4 weeks of daily doses to start working [73], the C18-P and P2 sublingual formulations offer significant advantages. The nausea side effect from semaglutide also poses challenges to patients taking oral medications consistently [74].

[00394] C18-P and P2 enabled efficient sublingual absorption of proteins up to 150 kDa

[00395] After demonstrating that C18-P and P2 were superior to protamine in delivering peptides in mice, we further investigated whether C18-P and P2 would work for sublingual delivery of larger proteins in mice. Protein payloads, including recombinant human growth hormone (rhGH, 22 kDa), Cy7-BSA (66 kDa), and AF647-

IgG (150 kDa), were mixed with either C18-P or P2 and sublingually delivered to mice, followed by plasma collection and measurement of payload concentrations using ELISA or fluorescence spectrometry. As seen in **Figures 43A-C**, sublingual absorption of all three protein payloads in the absence of a delivery system was limited, except for Cy7-BSA at 4 h displaying minimal yet significant plasma concentrations. Albumin has been shown to cross epithelial barriers via gp60-mediated transcytosis [75]. C18-P and P2 displayed comparable activity in promoting sublingual absorption of rhGH, Cy7-BSA, and AF647-IgG with similar PK profiles. Both CPP sublingual formulations yielded similar systemic absorption as the s.c. formulations for both rhGH and Cy7-BSA, indicating efficient delivery. However, sublingual delivery of 150 kDa IgG by these CPP formulations was inferior to the s.c. formulation. Despite the fact that C18-P and P2 increased IgG penetration in the sublingual tissue of mice (**Figure 41A-C**), a significant portion of the IgG penetrating the tissue might not be systemically absorbed. C18-P and P2 were effective in overcoming *in vivo* delivery barriers for smaller compounds like insulin, semaglutide, rhGH and BSA, but need further optimization for larger molecules, such as IgG. Nevertheless, both CPPs enabled efficient sublingual absorption of macromolecules that could previously only be delivered by needle-based injections. In particular, the relative bioavailabilities (BA) of IgG for the C18-P and P2 groups were 51.2% and 63.2% respectively, which were significantly higher than the 5% reported with other transmucosal delivery systems [76].

[00396] Safety study of protamine, C18-P and P2

[00397] Mice received sublingual CPPs at 6 mg/kg once weekly for one month and were then euthanized one day after the last dose. The blood and organs (including the base of the tongue (sublingual), heart, liver, spleen, lungs, kidneys, and trachea) were collected and analyzed. There were no significant differences observed in the organ/body weight ratios of treated groups compared to the saline treated control. The values of RBC, hematocrit, hemoglobin, MCV, MCH, RDW, WBC and lymphocytes were within the normal ranges in the treated animals, and there were no significant changes compared to the saline treated group. Liver and kidney function, indicated by serum levels of Urea (BUN), ALT, AST and creatine kinase, were within the normal ranges. H&E staining of the major tissues of concern from mice that received protamine, C18-P and P2 was performed. Tissue histology of the sublingual tissue and major organs showed no abnormalities compared to those collected from the saline treated mice. Collectively, these data indicate that the three CPP formulations given sublingually were safe in mice.

[00398] To summarize, two novel peptides were synthesized: C18-P was a conjugate of protamine and steric acid, and P2 was a protamine dimer with a PEG linker. The content of α -helix and β -sheet conformations was increased by 1.5- to 2-fold in C18-P and P2 compared to protamine, leading to increased peptide aggregation on the cell surface to permeate the cell membrane and up to 21-fold increased cytosolic delivery compared to protamine. RNP delivery in 2D cell culture and spheroid models confirmed that protamine, C18-P, and P2 effectively overcame multiple layers of membrane barriers to deliver the payload for gene knockdown, and C18-P and P2 were superior to protamine. C18-P and P2 were more efficient than protamine in promoting IgG penetration in the human sublingual tissue substitute and murine sublingual tissue. In mice, C18-P and P2 enabled efficient sublingual absorption of insulin and semaglutide, leading to enhanced BG control compared to protamine. Pharmacokinetic results supported that C18-P and P2 also enabled efficient sublingual absorption of larger proteins up to 150 kDa in mice with a relative BA of >50%. Finally, repeated treatments of these CPPs were safe in mice.

[00399] **Protamine enhanced mAb retention in the nasal cavity**

[00400] To demonstrate the capability of protamine in promoting the retention and penetrating of larger proteins, we acquired SPECT/CT scans at 0.5 h, 2 h, 4 h and 6 h after mice intranasally administrated of ^{111}In -IgG alone or in a mixture of protamine and ^{111}In -IgG. ^{111}In -IgG was mixed with saline or protamine and intranasally delivered to mice. Head of mice was scanned by SPECT/CT at 0.5 h, 2 h, 4 h and 6 h after intranasal administration, and the whole body was scanned at 6 h. N =3. As shown in **Figure 44**, radioactivity was detected in the nasal cavity for both treated groups at 0.5 h and 2 h. However, starting from 4 h onwards, the IgG-alone group exhibited less radioactivity in the central nasal cavity compared to the protamine group. Axial scan images showed that the signal detected in the IgG-alone group diminished and shifted from the region near the hard palate to cranium over time. This observation suggests that the IgG remained on the surface of the nasal epithelium and was swallowed by mice start from 2 h. This was confirmed by the presence of IgG in the gastrointestinal tract of the IgG-alone mice at 6 h. In contrast, in the protamine-treated mice group, most of the IgG radioactivity was concentrated on the nasal septum during the entire time of the experiment without significant fading. The radioactivity was exclusively detected in the nasal cavity throughout the 0.5-6 h. Quantification of the radioactivity in the central nasal cavity was performed using SPECT/CT images, calculating the SUV_{mean} values in the specific region. The signal of IgG given alone diminished rapidly

(**Figure 44**) within 2 h, decreasing from a standard uptake value (SUV) of 200 to 100 g/mL, until almost no signal remained at 4 h. The protamine-containing radiolabeled IgG formulation in mice maintained its radioactivity with an SUV of ~400 g/mL over the entire 6 h (**Figure 44**). These results confirm that protamine enhanced the retention of IgG in the nasal cavity. Inspired by this mAb retention ability, our subsequent experiments aimed to investigate whether protamine could be used for intranasal delivery of α CD124 to enhance its efficacy in CRSwNP mice models.

[00401] In vivo efficacy of intranasal protamine/ α CD124 in moderate CRSwNP mice

[00402] The progression of CRSwNP led to itchiness in the nasal cavity, which in turn resulted in frequent nose touching [79]. As shown in **Figure 45A**, the number of nose touches in 10 minutes in the CRSwNP mice after PBS treatments averaged ~30 times, while healthy mice averaged ~15 times. In the protamine/ α CD124-treated CRSwNP mice, the number of nose touches was significantly decreased to 25 times in 10 min compared to the PBS, α CD124 i.n. (~35 times), and α CD124 s.c. (~40 times) groups [79]. There was no significant difference among the PBS, α CD124 i.n. and α CD124 s.c. groups.

[00403] We further imaged the nasal cavity by micro-CT to examine smoothness of the mucosal surface and thickness of the mucus. Micro-CT scanning of noses from CRSwNP mice one day after the final dose ($N \geq 5$) was performed and, in the PBS, α CD124 i.n. and s.c.-treated groups, diffuse mucosal thickening of the nasal septum, ethmoid labyrinths and maxillary sinuses were observed, while the protamine/ α CD124 group exhibited a smooth, thin mucosal lining similar to that of the healthy control group. The nasal cavity was also reconstructed into a 3D model to measure the surface areas of the airway (A_{Airway}) and bone (A_{Bone}) using 3D Slicer. A decreased $A_{\text{Airway}}/A_{\text{Bone}}$ ratio indicates increased nasal cavity blockage. As shown in **Figure 45B**, the ratio in the protamine/ α CD124 i.n. group (0.49) was significantly increased compared to that in the PBS group (0.41), while the other α CD124-treated mice (ratio ~0.45) showed no improvements.

[00404] Representative H&E-stained images of sinonasal cavity treated with various formulations were examined with respect to polypoid lesions, epithelium disruptions, bone erosion, inflammatory infiltration to the ethmoturbinates and increased mucosal thickness), aggressive inflammation was induced in the nasal cavity in the mice with CRSwNP, evidenced by bone erosion (indicated by red arrows), inflammatory

infiltration to the ethmoturbinates, and increased mucosal thickness. These inflammatory characteristics were significantly reduced in all α CD124-treated groups, especially the protamine/ α CD124 group. Numbers of nasal polypoid lesions (indicated by *) and epithelial disruption lesions (indicated by #) in these images were counted and compared. As shown in **Figure 45C, D**, protamine/ α CD124-treated mice achieved a ~50% reduction in nasal polyps and a 50% decrease in epithelium disruptions compared to the PBS-treated group, and the effects were superior to other α CD124 treatments. Without protamine, α CD124 delivered intranasally was ineffective, while s.c. α CD124 treatment achieved a 25% reduction in epithelium disruptions at the same dose.

[00405] Increased IgE production is typically found in both systemic circulation and within the nasal polyps in CRSwNP patients due to active IL-4 and IL-13 pathways [80]. The increased IgE production results in migration of eosinophils to the inflammatory sites, exacerbating inflammation. Blocking IL-4R α by α CD124 has been shown to reduce IgE production [81]. As shown in **Figure 45E**, protamine/ α CD124 treatments significantly decreased the systemic IgE level compared to the α CD124 only groups (i.n. and s.c.) and significantly decreased the nasal IgE compared to PBS-treated mice (**Figure 45F**). A decrease of IFN- γ in the nasal tissue was also measured in the protamine/ α CD124 group (**Figure 46A**). However, no significant difference was found for other type 2 inflammatory biomarkers (**Figures 46B-K**). This might be because either the 8-week treatment period was not long enough to yield enhanced efficacy [82] or the delivery of α CD124 was suboptimal.

[00406] **Nano-P and Dendri-P enhanced protein penetration in nasal turbinates in mice**

[00407] A hydrophobic AGE moiety was conjugated to hydrophilic protamine, creating an amphipathic conjugate that could self-assemble into micelles (Nano-P). To synthesize this structure, AGE was linked to the N-terminal amino group of protamine via the amine-epoxide coupling.

[00408] Second, multiple protamine molecules were linked to a PAMAM dendrimer (Dendri-P). To synthesize Dendri-P, the primary N-terminal amino group of protamine was transformed to a thiol group by the Trout's reagent, and the surface amino groups at PAMAM were reacted with GMBS to present maleimide groups. The thiol-protamine was then conjugated to the maleimide-PAMAM through the Michael Addition reaction (reaction scheme shown in **Figure 47**).

[00409] Several techniques were employed to confirm the successful synthesis of these structures. The ¹H NMR analysis of Nano-P displayed distinctive peaks at 2.85, 3.90, 4.1, 5.2 and 5.8 ppm, corresponding to AGE. For Dendri-P, a prominent peak at 3.4 ppm indicated the presence of 2-Iminothiolane•HCl bound to protamine. Peaks of b, c, and d illustrated terminal sulfhydryl groups, and the presence of GMBS linker was supported by peaks of e, f, and g. Additionally, peaks observed at 3.2 ppm (methylene groups in the branch chains of PAMAM) and 2.51 ppm (methylene groups in the core and terminal chains of PAMAM) confirmed the linkage of PAMAM to protamine.

[00410] FTIR spectroscopy (**Figure 48**) further confirmed the Nano-P identity through the appearance of peaks at 1050 cm⁻¹ (C-O stretching), 1510 cm⁻¹ (C=C), and 3200 cm⁻¹ (-OH). The peak at 1300 cm⁻¹ was indicative of amide bonds in Dendri-P. Finally, TEM showed that Nano-P self-assembled into a spherical shape with a mean diameter of ~60 nm, and Dendri-P displayed a leaf-like structure with a mean size around 500 nm. Penetration of AF-αCD124 in the nasal tissue in mice delivered by protamine, Nano-P, and Dendri-P with respect to morphology of Nano-P and Dendri-P under TEM). The results were consistent with DLS (**Table 7**).

Table 7. Size of Nano-P and Dendri-P detected by DLS.

	Z-average (d. nm)	PDI
Nano-P	94.6 ± 37.8	0.138 ± 0.005
Dendri-P	459.2 ± 2.0	0.200 ± 0.027

[00411] Two hours after administration of various AF-αCD124 formulations, the nasal turbinates were collected from the mice and then sectioned and imaged by CLSM. Confocal microscopy analysis of tissue penetration of AF-αCD124 in the presence of protamine, Nano-P, or Dendri-P was performed. Confocal images were analyzed by ImageJ for the overall fluorescence intensity of AF-αCD124 in the nasal tissue, in the s.c. injection group, AF-αCD124 was barely detected in the nasal tissue, indicating limited distribution of AF-αCD124 from systemic circulation to the nasal turbinates. Little AF-αCD124 was detected in the tissue after intranasal delivery without any delivery system, likely because of the efficient ciliary clearance. However, in the protamine-treated group, significant retention of AF-αCD124 in the mucus layer and

penetration into the epithelial layer were observed. In the Nano-P-treated group, AF- α CD124 was found in the epithelium (minor) and lamina propria (major) without significant retention in the mucus, suggesting that Nano-P effectively permeated nasal delivery barriers, including the mucus and epithelium. On the other hand, in the Dendri-P-treated mice, AF- α CD124 was found in the epithelium (major) and the lamina propria (minor). The images were analyzed by ImageJ, and the results shown in **Figure 49A** demonstrate that Nano-P and Dendri-P increased AF- α CD124 delivery into the nasal turbinates by 3.5 and 7.8-fold compared to protamine, respectively. In the Nano-P group, both a farther distance of up to 90 μ m and higher intensity of AF- α CD124 were measured relative to the protamine-treated group (**Figure 49B**). On the contrary, no difference in the farthest travel distance (60 μ m) of AF- α CD124 from the tissue surface was observed between the protamine and Dendri-P groups. However, Dendri-P showed much stronger AF- α CD124 intensity in the epithelial layer (2 to 35 μ m) compared to protamine. Collectively, these data suggest that protamine, Nano-P, and Dendri-P effectively overcame delivery barriers in the mucus and epithelium for AF- α CD124. Nano-P was superior in delivering AF- α CD124 to the lamina propria (stronger AF- α CD124 intensity was observed from 25 to 90 μ m), and Dendri-P displayed enhanced epithelium retention. This may be attributed to the shape and size differences between Nano-P and Dendri-P. Nano-P was spherical with a small diameter of ~60 nm, which might allow Nano-P to penetrate deeper. The 8-fold increased size of Dendri-P (~500 nm) might restrict its tissue penetration, resulting in AF- α CD124 accumulation in the epithelium. As nasal polyps usually occur in the epithelium, Dendri-P might offer advantages over Nano-P for improving nasal histology.

[00412] In vivo efficacy of α CD124 formulations in severe CRSwNP mice

[00413] We focused the rest of our efficacy studies on a severe CRSwNP model, as human patients are typically diagnosed at an advanced stage that turns most patients to mAb therapy due to uncontrolled disease progression. In this severe model, an increased amount of mucus was produced in the nasal cavity that could further impede penetration of therapeutic agents, providing an opportunity to better differentiate different delivery systems. To establish a severe CRSwNP model, mice were sensitized with OVA for 137 days. At day 137 after the first instillation of OVA and SEB, α CD124 formulations were prepared by mixing α CD124 with protamine, Nano-P, or Dendri-P and were then intranasally administered thrice a week until the end point of the study (day 197). Subcutaneous administration of α CD124 thrice per week was

included as a control. A much higher mAb dose was used for s.c. delivery (25 mg/kg) compared to intranasal formulations (2 mg/kg)⁸. One day after the final dose, mice that received different treatments were compared for their disease burdens.

[00414] Formulations with Nano-P and Dendri-P relieved symptoms in severe CRSwNP mice and reduced sinus opacification compared to the protamine formulation.

[00415] As shown in **Table 8**, the number of nose touches in PBS-treated mice reached an average of 111 times in 10 min, whereas the healthy mice averaged only 10 times in 10 min. The nose touching frequencies significantly decreased to 50 times in 10 min in the s.c. high-dose αCD124 group compared to the PBS-treated mice. Intranasal αCD124 without any delivery system showed no significant activity, while intranasal αCD124 mixed with protamine, Nano-P, and Dendri-P decreased the nose touches per 10 min to 37, 32, and 26, respectively. Dendri-P was superior to protamine in suppressing nose-touching, while Nano-P and protamine showed no significant difference in this study.

[00416] **Table 8.** Nose touching frequencies for mice under different treatments.

Group		Number of nose touches per 10 min
Healthy		10 ± 4
CRSwNP	PBS i.n.	111 ± 41
	αCD124 s.c.	50 ± 21
	αCD124 i.n.	89 ± 37
	Protamine/αCD124 i.n.	37 ± 8
	Nano-P/αCD124 i.n.	32 ± 8
	Dendri-P/αCD124 i.n.	26 ± 4

[00417] Representative micro-CT scan images of sinonasal cavity treated with various formulations and showing uneven and diffusely swollen mucosa were examined, coronal CT scans revealed diffuse mucosal thickening of various parts of the nasal cavity in the CRSwNP mice, and the majority of left nasal cavity was blocked due to extensive swelling of the mucosa and filling with mucus. In s.c. (high-dose) and i.n. αCD124-treated groups, the diffuse mucosal thickening was found to be varied in the nasal septum, ethmoid labyrinths, and true maxillary sinuses. These mice also

exhibited an uneven thickness throughout the mucosal surface, whereas the healthy mice exhibited a smooth, thin mucosal lining. In the protamine/ α CD124-treated mice, reduced mucosal thickening was found compared to the s.c. and i.n. α CD124-treated mice. In Nano-P and Dendri-P-treated mice, further decreased mucosal thickening was observed compared to the protamine treated group.

[00418] CT images of nasal cavity cross-sections were also collected in sequence and reconstructed to three-dimensional (3D) models. 3D reconstruction of sinonasal cavity from mice treated with various formulations, showing blockade, were examined. In healthy mice, the airway was well defined and connected, while in the PBS-treated CRSwNP mice, area significant portion of the nasal airway was blocked. Moderate improvements in sinonasal airway morphology were seen with treatments of s.c. (high-dose) α CD124 and protamine/ α CD124, while i.n. α CD124 displayed little effect. Nano-P, and Dendri-P treatments preserved the majority of the nasal airway, appearing to be superior to other treatments.

[00419] H&E was used to further visualize epithelium thickening. Representative H&E images of sinonasal tissues after treatments with various formulations, showing increased epithelium thickness, were examined **Figure 50**. The nasal mucosal thickness in the healthy mice was around 20 μ m, while that of the CRSwNP mice was at least 4-fold increased. High-dose s.c. α CD124 significantly decreased the nasal epithelium thickness compared to mice receiving PBS or i.n. α CD124 (no delivery system). The effect of protamine/ α CD124 given at 2 mg/kg was comparable to that of the high-dose s.c. α CD124 (25 mg/kg). Nano-P and Dendri-P formulations further reduced thickening of the nasal epithelium compared to the protamine formulation. These results support the superior nasal penetration efficiency of the three protamine formulations compared to s.c. delivery and display that Nano-P and Dendri-P are more efficacious compared to protamine.

[00420] Nano-P and Dendri-P enhanced anti-CRSwNP efficacy of α CD124 compared to protamine

[00421] Polypoid lesions and epithelium disruptions were examined in the H&E images of nasal tissues collected from severe CRSwNP mice after different treatments. Representative images of H&E-stained sections of sinonasal tissues after various treatments, showing classical polyp-like lesions and epithelium disruptions, were examined). As shown in **Figure 51A**, the number of polypoid lesions in PBS-treated CRSwNP mice was 1.3-fold higher than that in the α CD124 s.c.-treated mice (30%

polypoid lesion reduction) and in the protamine formulation-treated mice (32% polypoid lesion reduction). α CD124 i.n. without any delivery system did not have any anti-polypoid effect. The data once again confirmed that protamine-mediated intranasal delivery of α CD124 achieved a similar effect to the s.c. preparation, but at a 12.5-fold lower dose. By using the same low-dose of α CD124, Nano-P and Dendri-P formulations achieved 47% and 58% polypoid lesion reductions compared to the PBS-treated mice, respectively, which were superior to the protamine formulation. Similar results were obtained with the epithelium disruptions in the nasal tissue. As shown in Figure 5C, s.c. α CD124-treated mice had an average of 21 epithelium disruptions per field, which was significantly decreased compared to the average for the PBS-treated mice (38 per field) and comparable to the average for the protamine formulation-treated mice (24 per field). Nano-P and Dendri-P formulations further decreased the disruptions to 20 and 12 per field, respectively, and the effects were better than the protamine formulation. Notably, Dendri-P achieved a significantly lower number of epithelium disruptions compared to Nano-P, which may be due to the increased delivery to the epithelial layer as shown in **Figure 51B**.

[00422] CRSwNP is diagnosed by the enrichment of eosinophils in the mucosal tissue. These cells release type 2 inflammatory cytokines including IL-4 and IL-13 in the nasal epithelium to activate and transform the surrounding cells, leading to increased inflammation and polyposis. Giemsa staining was employed to stain eosinophils in the nasal tissues. Representative Giemsa-stained images of sinonasal tissue sections from CRSwNP mice after treatments with various formulations, showing eosinophils, were examined, and the eosinophil numbers per field were identified and quantified using Trainable Weka Segmentation in ImageJ [83]. Increased tissue infiltration of eosinophils was detected in the PBS (37 per field) and α CD124 i.n. (34 per field) groups compared to the others. The high-dose s.c. α CD124-treated group displayed reduced eosinophil infiltration (24 per field), which was similar to the protamine group (27 per field). Nano-P and Dendri-P formulations were more efficacious than protamine, further decreasing the numbers of eosinophils to 17 and 16 per field, respectively (**Figure 52**).

[00423] Persistent tissue inflammation often results in increased deposition of extracellular matrix (ECM), which leads to increased stiffness of the tissue, impaired movement of the cilia and decreased clearance of mucus. These pathological changes worsen inflammation and increase the chance of infections. Representative Masson's Trichrome-stained images of sinonasal tissue sections from CRSwNP mice after

various treatments, showing cartilage and collagen-based ECM, were examined), Masson's Trichrome (MT) was used to stain collagen deposition and was isolated with Trainable Weka Segmentation in ImageJ [83] (**Figure 53A**). In healthy mice, little collagen deposition was detected in the nasal tissue, while intensive ECM was stained in the CRSwNP tissue. The high-dose s.c. α CD124-treated mice showed a significant decrease in the nasal tissue ECM compared to the PBS-treated CRSwNP mice. The nasal ECM in the protamine group was 23% of that in the PBS-treated mice, and 43% of that in the α CD124 i.n. group, suggesting that i.n. protamine/ α CD124 suppressed fibrosis of the nasal tissue in the CRSwNP mice. The high-dose s.c. α CD124 displayed comparable anti-fibrosis efficacy to the protamine formulation. In the Nano-P and Dendri-P groups, the areas of ECM were significantly decreased to 11.6% and 14.5% of the PBS group, and the effects were superior to the protamine formulation.

[00424] Goblet cell hyperplasia and mucus hypersecretion induced by type 2 eosinophilic inflammation are often characterized in CRSwNP. Next, the goblet cells were stained purple by the Alcina Blue-Periodic Schiff (AB-PAS) staining. Representative PAS-stained images of sinonasal tissues of mice treated with various formulations were examined), and the numbers per field were quantified by ImageJ. Increased numbers of goblet cells were detected in the PBS (32 per field) and α CD124 i.n. (30 per field) groups compared to others. The high-dose s.c. α CD124-treated group displayed reduced goblet cells staining (24 per field), which was similar to the protamine group (20 per field). Nano-P and Dendri-P formulations were more efficacious than the high-dose s.c. α CD124, further decreasing the numbers of goblet cells to 15 and 12 per field, respectively (**Figure 53B**). Dendri-P formulation was superior to protamine, and this might be due to the enhanced delivery and retention of α CD124 in nasal epithelium.

[00425] **Protamine, Nano-P, and Dendri-P-mediated intranasal delivery of α CD124 suppressed type 2 inflammatory biomarkers in CRSwNP mice**

[00426] Human patients with CRSwNP receiving Dupilumab show reduced levels of total IgE, eotaxin, IL-13 [84] and thymus and activation-regulated chemokine (TARC). Among these, IgE is the key mediator for eosinophil migration to the polyps, facilitating the disease progression. As shown in **Figure 54A-B**, CRSwNP mice had high IgE levels in the plasma (~100 ng/mL) and the nasal tissue (~400 ng/mL), while those in the healthy mice were undetectable. Protamine, Nano-P, and Dendri-P formulations significantly decreased the IgE levels in the plasma and the nasal tissues by 50-75%. Particularly in the nasal tissues, but not in the plasma, Nano-P and Dendri-P

formulations displayed enhanced activity in decreasing IgE release compared to protamine. This might be due to the increased local delivery of α CD124 by Nano-P and Dendri-P. Without a delivery system, α CD124 given by i.n. or s.c. did not have any significant effect on IgE production in the plasma or the nasal tissues.

[00427] These three CPP formulations also showed significant efficacy in suppressing type 2 inflammatory cytokines in the nasal tissues. For example, treatments with the protamine, Nano-P, and Dendri-P formulations significantly decreased eotaxin, IL-13 and TARC in the nasal tissues by 25-100% (**Figure 54C-E**). TARC and eotaxin are major chemokines attracting inflammatory cells, and the decreases of eotaxin and TARC in the nasal tissues were consistent with the reduced infiltration of eosinophils. IL-13 is an inflammatory cytokine that promotes tissue remodeling, leading to hypersecretion of mucus by goblet cells, subepithelial fibrosis, and increased airway hyperreactivity. IL-13 also promotes migration of eosinophils by inducing production of eosinophil-promoting factors, such eotaxins, from Type 2 helper T (Th2) cells and epithelial cells. The reduced numbers of eosinophils, suppressed collagen deposition and decreased numbers of goblet cells in the nasal tissues are consistent with the decreased levels of IL-13.

[00428] TNF- α causes loss of ciliated cells, epithelial metaplasia, and further accumulation of inflammatory cells in the subepithelial layer [85]. IL-2 is associated with reduced olfactory function in CRS patients and is significantly elevated in CRSwNP patients [86-88]. As shown in **Figure 54F-G**, levels of TNF- α and IL-2 in the nasal tissues were decreased by 50-75% after treatment with the protamine, Nano-P, and Dendri-P formulations.

[00429] IL-25 and IL-33 are innate lymphoid type 2 cells (ILC2)-activating cytokines that contribute to the production of IL-4 and IL-13, and INF- γ is involved in the inflammation perpetuation and aggravation [89]. These cytokines and IL-12p70 are increased in human CRSwNP patients. As shown in **Figure 54H-K**, levels of IL-25, IL-33, INF- γ , and IL-12p70 in the nasal tissues were decreased in the Nano-P and Dendri-P groups, but not in other groups.

[00430] Elevated levels of IL-1 β and IL-17A are correlated with increased infiltration of inflammatory cells such as eosinophils in human CRSwNP patients [90, 91]. Additionally, IL-1 β increases secretion of Chemokine (C-C motif) ligand 5 (CCL5) from the nasal polyp fibroblasts and contributes to glucocorticoid resistance, and thymic stromal lymphopoietin (TSLP) promotes production of IL-4 and IL-13 and expands the

type 2 inflammation [92]. IL-1 β , IL-17A and TSLP were significantly decreased in the protamine, Nano-P and Dendri-P groups compared to the PBS control (**Figure 54L-N**) [91].

[00431] Without a delivery system, α CD124 i.n. did not suppress any cytokines compared to the PBS control. High-dose s.c. α CD124 and i.n. protamine/ α CD124 decreased 5/12 and 8/12 cytokines tested, respectively, while Nano-P and Dendri-P suppressed all 12 cytokines in the nasal tissues. IL4R α is the shared receptor for both IL-4 and IL-13. Blocking IL4R α on eosinophils, mast cells, basophils, goblet cells, lymphocytes and ILC2 will deactivate these inflammatory immune cells, leading to decreased type 2 cytokine production and eosinophil recruitment. Both Nano-P and Dendri-P facilitated penetration of α CD124 in the nasal mucosae, which would block IL4R α on the inflammatory cells that had migrated to the nasal tissue. Therefore, Nano-P and Dendri-P were more consistent in suppressing the Th2 cytokines compared to protamine, while there was no difference between Nano-P and Dendri-P.

[00432] Nasal goblet cells are located in the epithelium, and goblet cell hyperplasia is featured by the loss of mucociliary clearance, accumulation of mucin gel and eventually polyp formation in the nasal cavity [93]. Dendri-P accumulated an increased amount of α CD124 in the epithelium compared to other protamine-based delivery system, and this might lead to enhanced blocking on the IL4R α on the goblet cells, resulting in enhanced stabilization of the goblet cells and improved nasal histology among different treatment groups.

[00433] The results indicate that the anti-polypoid effect was highly dependent on the delivery of α CD124 to the nasal epithelium and lamina propria, blocking both IL-4 and IL-13 pathways and type 2 inflammation, leading to reduced production of IgE, migration of eosinophils, hyperplasia of the goblet cells, and secretion of mucus.

[00434] **Safety of protamine, Nano-P, and Dendri-P after long-term use**

[00435] Mice received intranasal protamine, Nano-P and Dendri-P at 3 mg/kg twice daily for one month, and no nasal symptoms were observed in all the mice during the treatments. One day after the final dose, mice were euthanized. The blood and tissues, including the nose, heart, liver, spleen, lung, kidney, and trachea were collected and analyzed. There were no significant differences in organ weights among the treated groups and control (**Figure 55A**). Tissue histology of the nasal tissues and major

organs showed no abnormality. Representative images of H&E staining of the main airway tissue sections from mice that received protamine, Nano-P, or Dendri-P treatment were examined). The values of RBC, hematocrit, hemoglobin, MCV, MCH, RDW, WBC and lymphocytes were within the normal ranges (**Figures 55B-U**). Liver and kidney functions indicated by serum levels of urea (BUN), ALT, AST and creatine kinase were also within the normal ranges (**Figures 55B-U**). Collectively, these data indicated that the three formulations given intranasally were safe in mice.

[00436] The results demonstrate a successful nasal delivery system for α CD124 to treat CRSwNP. The formulations were prepared by physically mixing α CD124 with the protamine-based delivery systems, providing convenience and flexibility for optimization of the drug-to-carrier ratio. α CD124 delivered by these protamine-based systems were given at a 12.5-fold lower dose, but were superior to high-dose s.c. α CD124, the standard therapy for CRSwNP. Furthermore, we achieved anti-polypsis within 8-week treatments. Among these protamine-based formulations, nanostructural Nano-P and Dendri-P enhanced the penetration of α CD124 in the nasal mucosa and thus improved the effects of anti-polypsis and type 2 inflammation. In particular, Nano-P facilitated increased penetration of α CD124 to the nasal lamina propria, and appeared to be the most consistent in suppressing the type 2 inflammation. Dendri-P promoted accumulation of α CD124 in the nasal epithelium, the site of histological lesions, and thus showed enhanced inhibition of epithelium disruptions among different treatments.

[00437] To summarize, high-dose s.c. α CD124, the standard therapy for advanced CRSwNP, yielded significant but moderate efficacy in symptom relief, anti-polypsis, and anti-Th2 inflammation in severe CRSwNP mice. Nano-P and Dendri-P are nano-derivatives of protamine synthesized to promote nasal penetration of α CD124 for improved therapy of CRSwNP. Protamine increased α CD124 penetration to the nasal epithelium, while Nano-P and Dendri-P further enhanced the penetration to the epithelium and lamina propria, respectively, compared to protamine. The three protamine-based delivery systems were physically mixed with α CD124 and given intranasally at a 12.5-fold lower dose than the high-dose s.c. α CD124. The protamine formulation was generally comparable to the high-dose s.c. α CD124, but slightly more consistent in suppressing type 2 inflammation. Both nano-formulations outperformed protamine and high-dose s.c. α CD124 in suppressing the CRSwNP symptom, polypsis, and type 2 inflammation. Nano-P displayed increased penetration to the nasal lamina propria and the most consistent efficacy in reducing type 2 inflammation,

while Dendri-P tended to accumulated in the nasal epithelium and exerted superior efficacy in improving the nasal histology.

[00438] **CPP Intravitreal data + MM**

[00439] Protamine was dissolved in sterilized water at 10 mg/ml

[00440] AF-647 IgG was dissolved in PBS at 2 mg/ml

[00441] Mice were anesthetized by isoflurane. Neuros syringes (50 μ L, Neuros Syringe, Model 1705 RN, 33 gauge, Point Style 4 (65460-16 | Laboratory | Hamilton Company)) was used.

[00442] The 33 gauge needle was positioned at 90 degree angle of sclera and inject around 1 mm depth.

[00443] For IgG delivery (10 μ L of IgG + 5 μ L of water or 5 μ L of protamine) were given slowly over 30 s to allow for diffusion of the liquid.

[00444] 2h later, mice were euthanized and eye balls was collected. Eyes were then fixed with 10% formalin overnight.

[00445] Perform cryopreservation with sucrose for fixed eyes prior to OCT embedding.

[00446] Place eye in 10% sucrose in PBS until eye sinks, usually overnight.

[00447] Then transfer the eye to 30% sucrose in PBS until sinks, overnight.

[00448] **Embedding eye in OCT**

[00449] Samples were sectioned to a thickness of 10 μ m using Leica Cryostat.

[00450] Protamine increases IgG delivery to ganglion cell layer (GCL), inner nuclear layer (INL), retinal pigment epithelium (RPE) and choroid after intravitreal injection in mice.

[00451] **Penetration for 4 h for human skin substitute**

[00452] Protamine was dissolved in water at 10 mg/ml.

[00453] Alexa647-IgG (AF-IgG) was dissolved in PBS at 2 mg/ml.

[00454] For Protamine/ AF-IgG treated group, 40 ul AF-IgG was mixed with 80 ul Protamine.

[00455] For AF-IgG alone treated group, 40 ul AF-IgG was mixed with 80 ul water.

[00456] Formulations were applied topically and incubated on 37 °C for 4 h.

[00457] After 4 h, human skin substitute were mounted by OCT and section at 10 um followed by DAPI staining.

[00458] After healthy human skin substitute IgG penetration for 4 h, protamine enhanced AF-IgG retention and penetration.

[00459] **Penetration for 4 h for Psoriasis human skin substitute**

[00460] Protamine was dissolved in water at 10 mg/ml.

[00461] Alexa647-IgG (AF-IgG) was dissolved in PBS at 2 mg/ml.

[00462] For Protamine/ AF-IgG treated group, 60 ul AF-IgG was mixed with 120 ul Protamine.

[00463] For AF-IgG alone treated group, 60 ul AF-IgG was mixed with 120 ul water.

[00464] Formulations were applied topically and incubated on 37 °C for 4 h.

[00465] After 4 h, psoriasis human skin substitutes were mounted by OCT and section at 10 um followed by DAPI staining.

[00466] After psoriasis human skin substitute IgG penetration for 4 h, protamine enhanced AF-IgG retention and penetration.

[00467] **References**

1. Kintzing, J.R., Filsinger Interrante, M.V. & Cochran, J.R. Emerging Strategies for Developing Next-Generation Protein Therapeutics for Cancer Treatment. *Trends Pharmacol Sci* **37**, 993-1008 (2016).
2. Renukuntla, J., Vadlapudi, A.D., Patel, A., Boddu, S.H. & Mitra, A.K. Approaches for enhancing oral bioavailability of peptides and proteins. *Int J Pharm* **447**, 75-93 (2013).
3. Jeong, W.Y., Kwon, M., Choi, H.E. & Kim, K.S. Recent advances in transdermal drug delivery systems: a review. *Biomater Res* **25**, 24 (2021).

4. Varmus, H. et al. Public health. Grand Challenges in Global Health. *Science* **302**, 398-399 (2003).
5. J.K.W. Lam, C.C.K. Cheung, M.Y.T. Chow, E. Harrop, S. Lapwood, S.I.G. Barclay, I.C.K. Wong, Transmucosal drug administration as an alternative route in palliative and end-of-life care during the COVID-19 pandemic, *Adv Drug Deliv Rev* **160** (2020) 234-243.
6. [Z. Luo, N. Paunovic, J.C. Leroux, Physical methods for enhancing drug absorption from the gastrointestinal tract, *Adv Drug Deliv Rev* **175** (2021) 113814.
7. Y. Kim, S. Hwang, R. Khalmuratova, S. Kang, M. Lee, Y. Song, J.W. Park, J. Yu, H.W. Shin, Y. Lee, alpha-Helical cell-penetrating peptide-mediated nasal delivery of resveratrol for inhibition of epithelial-to-mesenchymal transition, *J Control Release* **317** (2020) 181-194.
8. Schworer, S.A. & Kim, E.H. Sublingual immunotherapy for food allergy and its future directions. *Immunotherapy* **12**, 921-931 (2020).
9. Hua, S. Advances in Nanoparticulate Drug Delivery Approaches for Sublingual and Buccal Administration. *Front Pharmacol* **10**, 1328 (2019).
10. Patel, V.F., Liu, F. & Brown, M.B. Advances in oral transmucosal drug delivery. *J Control Release* **153**, 106-116 (2011).
11. O. Kammona, C. Kiparissides, Recent advances in nanocarrier-based mucosal delivery of biomolecules, *J Control Release* **161** (2012) 781-794.
12. [A.S. Macedo, P.M. Castro, L. Roque, N.G. Thome, C.P. Reis, M.E. Pintado, P. Fonte, Novel and revisited approaches in nanoparticle systems for buccal drug delivery, *J Control Release* **320** (2020) 125-141.
13. N.G. Lamson, A. Berger, K.C. Fein, K.A. Whitehead, Anionic nanoparticles enable the oral delivery of proteins by enhancing intestinal permeability, *Nat Biomed Eng* **4** (2020) 84-96.
14. X. Han, Y. Lu, J. Xie, E. Zhang, H. Zhu, H. Du, K. Wang, B. Song, C. Yang, Y. Shi, Z. Cao, Zwitterionic micelles efficiently deliver oral insulin without opening tight junctions, *Nat Nanotechnol* **15** (2020) 605-614.
15. Deli, M.A. Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. *Biochim Biophys Acta* **1788**, 892-910 (2009).
16. D. Esposito, C. Conte, I. d'Angelo, A. Miro, F. Ungaro, F. Quaglia, Mucoadhesive zein/beta-cyclodextrin nanoparticles for the buccal delivery of curcumin, *Int J Pharm* **586** (2020) 119587.
17. Z. Sang, J. Qian, J. Han, X. Deng, J. Shen, G. Li, Y. Xie, Comparison of three water-soluble polyphosphate tripolyphosphate, phytic acid, and sodium hexametaphosphate as crosslinking agents in chitosan nanoparticle formulation, *Carbohydr Polym* **230** (2020) 115577.

18. M.C. Gómez-Guillén, M.P. Montero, Enhancement of oral bioavailability of natural compounds and probiotics by mucoadhesive tailored biopolymer-based nanoparticles: A review, *Food Hydrocolloids* 118 (2021).
19. Ouellette, M. et al. Insights into gold nanoparticles as a mucoadhesive system. *Sci Rep* 8, 14357 (2018).
20. I. Pepic, J. Lovric, J. Filipovic-Grcic, How do polymeric micelles cross epithelial barriers?, *Eur J Pharm Sci* 50 (2013) 42-55.
21. Dave, R.S. et al. Ocular drug delivery to the anterior segment using nanocarriers: A mucoadhesive/mucopenetrative perspective. *J Control Release* 336, 71-88 (2021).
22. D. Zhang, J. Wang, D. Xu, Cell-penetrating peptides as noninvasive transmembrane vectors for the development of novel multifunctional drug-delivery systems, *J Control Release* 229 (2016) 130-139.
23. Chen, N. et al. Approaches and materials for endocytosis-independent intracellular delivery of proteins. *Biomaterials* 286, 121567 (2022).
24. D. Zhang, G. Wang, X. Yu, T. Wei, L. Farbiak, L.T. Johnson, A.M. Taylor, J. Xu, Y. Hong, H. Zhu, D.J. Siegwart, Enhancing CRISPR/Cas gene editing through modulating cellular mechanical properties for cancer therapy, *Nat Nanotechnol* (2022).
25. D.J. Drucker, Advances in oral peptide therapeutics, *Nat Rev Drug Discov* 19 (2020) 277-289.
26. W.B. Kauffman, T. Fuselier, J. He, W.C. Wimley, Mechanism Matters: A Taxonomy of Cell Penetrating Peptides, *Trends Biochem Sci* 40 (2015) 749-764.
27. I. Ruseska, A. Zimmer, Internalization mechanisms of cell-penetrating peptides, *Beilstein J Nanotechnol* 11 (2020) 101-123.
28. C. Allolio, A. Magarkar, P. Jurkiewicz, K. Baxova, M. Javanainen, P.E. Mason, R. Sachl, M. Cebecauer, M. Hof, D. Horinek, V. Heinz, R. Rachel, C.M. Ziegler, A. Schrofel, P. Jungwirth, Arginine-rich cell-penetrating peptides induce membrane multilamellarity and subsequently enter via formation of a fusion pore, *Proc Natl Acad Sci U S A* 115 (2018) 11923-11928.
29. N. Schmidt, A. Mishra, G.H. Lai, G.C. Wong, Arginine-rich cell-penetrating peptides, *FEBS Lett* 584 (2010) 1806-1813.
30. I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, M. Takehashi, S. Tanaka, K. Ueda, J.C. Simpson, A.T. Jones, Y. Sugiura, S. Futaki, Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement, *Mol Ther* 10 (2004) 1011-1022.
31. L. He, E.J. Sayers, P. Watson, A.T. Jones, Contrasting roles for actin in the cellular uptake of cell penetrating peptide conjugates, *Sci Rep* 8 (2018) 7318.
32. T. Takeuchi, S. Futaki, Current Understanding of Direct Translocation of Arginine-Rich Cell-Penetrating Peptides and Its Internalization Mechanisms, *Chem Pharm Bull (Tokyo)* 64 (2016) 1431-1437.

33. I. Nakase, G. Tanaka, S. Futaki, Cell-penetrating peptides (CPPs) as a vector for the delivery of siRNAs into cells, *Mol Biosyst* **9** (2013) 855-861.
34. Khafagy el, S. & Morishita, M. Oral biodrug delivery using cell-penetrating peptide. *Adv Drug Deliv Rev* **64**, 531-539 (2012).
35. Räägel, H., Säälilik, P., Hansen, M., Langel, U. & Pooga, M. CPP-protein constructs induce a population of non-acidic vesicles during trafficking through endo-lysosomal pathway. *J Control Release* **139**, 108-117 (2009).
36. Chen, K. & Pei, D. Engineering Cell-Permeable Proteins through Insertion of Cell-Penetrating Motifs into Surface Loops. *ACS Chem Biol* **15**, 2568-2576 (2020).
37. Dinca, A., Chien, W.M. & Chin, M.T. Intracellular Delivery of Proteins with Cell-Penetrating Peptides for Therapeutic Uses in Human Disease. *Int J Mol Sci* **17**, 263 (2016).
38. Jo, J., Hong, S., Choi, W.Y. & Lee, D.R. Cell-penetrating peptide (CPP)-conjugated proteins is an efficient tool for manipulation of human mesenchymal stromal cells. *Sci Rep* **4**, 4378 (2014).
39. Yu, M., Wu, J., Shi, J. & Farokhzad, O.C. Nanotechnology for protein delivery: Overview and perspectives. *J Control Release* **240**, 24-37 (2016).
40. Kim, H.S., Kim, J.S., Lee, Y.K., Koo, K.H. & Park, Y.S. An efficient liposomal gene delivery vehicle using Sendai F/HN proteins and protamine. *Cancer Gene Ther* **15**, 214-224 (2008).
41. E. Song, P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, J. Lieberman, Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, *Nat Biotechnol* **23** (2005) 709-717.
42. N. Baumer, W.E. Berdel, S. Baumer, Immunoprotein-Mediated siRNA Delivery, *Mol Pharm* **14** (2017) 1339-1351.
43. N. Baumer, J. Tiemann, A. Scheller, T. Meyer, L. Wittmann, M.E.G. Suburu, L. Greune, M. Peipp, N. Kellmann, A. Gumnior, C. Brand, W. Hartmann, C. Rossig, C. Muller-Tidow, D. Neri, C.A. Strassert, C. Ruter, P. Dersch, G. Lenz, H.P. Koeffler, W.E. Berdel, S. Baumer, Targeted siRNA nanocarrier: a platform technology for cancer treatment, *Oncogene* **41** (2022) 2210-2224.
44. H. He, J. Ye, E. Liu, Q. Liang, Q. Liu, V.C. Yang, Low molecular weight protamine (LMWP): a nontoxic protamine substitute and an effective cell-penetrating peptide, *J Control Release* **193** (2014) 63-73.
45. F.L. Sorgi, S. Bhattacharya, L. Huang, Protamine sulfate enhances lipid-mediated gene transfer, *Gene Ther* **4** (1997) 961-968.
46. Bandi, S., Stephen, E., Bansal, K. & Mahdavinia, M. Understanding the CRSwNP Patient as Whole. *Am J Rhinol Allergy* **37**, 140-146 (2023).
47. Grose, E., Li, A.Y. & Lee, J.M. Clinical outcomes of dupilumab therapy in chronic rhinosinusitis with nasal polyps in a Canadian tertiary care rhinology practice. *Allergy Asthma Clin Immunol* **19**, 26 (2023).

48. Stevens, W.W., Schleimer, R.P. & Kern, R.C. Chronic Rhinosinusitis with Nasal Polyps. *J Allergy Clin Immunol Pract* **4**, 565-572 (2016).
49. Hopkins, C. Chronic Rhinosinusitis with Nasal Polyps. *N Engl J Med* **381**, 55-63 (2019)
50. Bachert, C. et al. Effect of Subcutaneous Dupilumab on Nasal Polyp Burden in Patients With Chronic Sinusitis and Nasal Polyposis: A Randomized Clinical Trial. *Jama* **315**, 469-479 (2016).
51. Bachert, C. et al. Rapid and Continuing Improvements in Nasal Symptoms with Dupilumab in Patients with Severe CRSwNP. *J Asthma Allergy* **15**, 557-563 (2022).
52. Kariyawasam, H.H. Chronic rhinosinusitis with nasal polyps: mechanistic insights from targeting IL-4 and IL-13 via IL-4R α inhibition with dupilumab. *Expert Rev Clin Immunol* **16**, 1115-1125 (2020).
53. Kim, H.C., Lim, J.Y., Kim, S., Kim, J.H. & Jang, Y.J. Development of a mouse model of eosinophilic chronic rhinosinusitis with nasal polyp by nasal instillation of an Aspergillus protease and ovalbumin. *Eur Arch Otorhinolaryngol* **274**, 3899-3906 (2017).
54. Keller, L.A., Merkel, O. & Popp, A. Intranasal drug delivery: opportunities and toxicologic challenges during drug development. *Drug Deliv Transl Res* **12**, 735-757 (2022).
55. Deshayes, S. et al. Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. *Biochemistry* **43**, 1449-1457 (2004).
56. Chen, J. & Zou, X. Self-assemble peptide biomaterials and their biomedical applications. *Bioact Mater* **4**, 120-131 (2019).
57. Wychowaniec, J.K. et al. Role of Sheet-Edge Interactions in β -sheet Self-Assembling Peptide Hydrogels. *Biomacromolecules* **21**, 2285-2297 (2020).
58. Smith, L.J., Fiebig, K.M., Schwalbe, H. & Dobson, C.M. The concept of a random coil. Residual structure in peptides and denatured proteins. *Fold Des* **1**, R95-106 (1996).
59. Elsayed, A., Al-Remawi, M., Jaber, N. & Abu-Salah, K.M. Advances in buccal and oral delivery of insulin. *Int J Pharm* **633**, 122623 (2023).
60. Ji, Z., Xie, Z., Zhang, Z., Gong, T. & Sun, X. Engineering intravaginal vaccines to overcome mucosal and epithelial barriers. *Biomaterials* **128**, 8-18 (2017).
61. Yang, Y., Roy, A., Zhao, Y., Undzys, E. & Li, S.D. Comparison of Tumor Penetration of Podophyllotoxin-Carboxymethylcellulose Conjugates with Various Chemical Compositions in Tumor Spheroid Culture and In Vivo Solid Tumor. *Bioconjug Chem* **28**, 1505-1518 (2017).
62. Delcourt-Huard, A., Corlu, A., Joffre, A., Magloire, H. & Bonnaure-Mallet, M. Reconstituted human gingival epithelium: nonsubmerged in vitro model. *In Vitro Cell Dev Biol Anim* **33**, 30-36 (1997)

63. Ivashchenko, O., van der Have, F., Goorden, M.C., Ramakers, R.M. & Beekman, F.J. Ultra-high-sensitivity submillimeter mouse SPECT. *J Nucl Med* **56**, 470-475 (2015).
64. Goorden, M.C. et al. VECTor: a preclinical imaging system for simultaneous submillimeter SPECT and PET. *J Nucl Med* **54**, 306-312 (2013).
65. Loening, A.M. & Gambhir, S.S. AMIDE: a free software tool for multimodality medical image analysis. *Mol Imaging* **2**, 131-137 (2003).
66. Bhavna et al. Donepezil nanosuspension intended for nose to brain targeting: In vitro and in vivo safety evaluation. *Int J Biol Macromol* **67**, 418-425 (2014).
67. R.J. England, J.J. Homer, L.C. Knight, S.R. Ell, Nasal pH measurement: a reliable and repeatable parameter, *Clin Otolaryngol Allied Sci* **24** (1999) 67-68.
68. K. Shmuel, M. Dalia, L. Tair, N. Yaakov, Low pH Hypromellose (Taffix) nasal powder spray could reduce SARS-CoV-2 infection rate post mass-gathering event at a highly endemic community: an observational prospective open label user survey, *Expert Rev Anti Infect Ther* **19** (2021) 1325-1330.
69. J.E. Gern, A.G. Mosser, C.A. Swenson, P.J. Rennie, R.J. England, J. Shaffer, H. Mizoguchi, Inhibition of rhinovirus replication in vitro and in vivo by acid-buffered saline, *J Infect Dis* **195** (2007) 1137-1143.
70. W.R. Ryan, P.H. Hwang, Safety of a preservative-free acidified saline nasal spray: a randomized, double-blind, placebo-controlled, crossover clinical trial, *Arch Otolaryngol Head Neck Surg* **136** (2010) 1099-1103.
71. J. Yu, J. Wang, Y. Zhang, G. Chen, W. Mao, Y. Ye, A.R. Kahkoska, J.B. Buse, R. Langer, Z. Gu, Glucose-responsive insulin patch for the regulation of blood glucose in mice and minipigs, *Nat Biomed Eng* **4** (2020) 499-506.
72. Liu, Y.Y. et al. Purification and Characterization of Protamine, the Allergen from the Milt of Large Yellow Croaker (*Pseudosciaena crocea*), and Its Components. *J Agric Food Chem* **64**, 1999-2011 (2016).
73. Overgaard, R.V., Navarria, A., Ingwersen, S.H., Bækdal, T.A. & Kildemoes, R.J. Clinical Pharmacokinetics of Oral Semaglutide: Analyses of Data from Clinical Pharmacology Trials. *Clin Pharmacokinet* **60**, 1335-1348 (2021).
74. Kane, M.P., Triplitt, C.L. & Solis-Herrera, C.D. Management of type 2 diabetes with oral semaglutide: Practical guidance for pharmacists. *Am J Health Syst Pharm* **78**, 556-567 (2021).
75. Monks, J. & Neville, M.C. Albumin transcytosis across the epithelium of the lactating mouse mammary gland. *J Physiol* **560**, 267-280 (2004).
76. Zhu, W. et al. Oral Delivery of Therapeutic Antibodies with a Transmucosal Polymeric Carrier. *ACS Nano* **17**, 4373-4386 (2023).
77. Räägel, H., Säälk, P., Hansen, M., Langel, U. & Pooga, M. CPP-protein constructs induce a population of non-acidic vesicles during trafficking through endo-lysosomal pathway. *J Control Release* **139**, 108-117 (2009).

78. Lin, T. et al. Nose-to-brain delivery of macromolecules mediated by cell-penetrating peptides. *Acta Pharm Sin B* **6**, 352-358 (2016).
79. Nam, S.Y. et al. The therapeutic efficacy of α -pinene in an experimental mouse model of allergic rhinitis. *Int Immunopharmacol* **23**, 273-282 (2014).
80. Shen, Y., Zhang, N., Yang, Y., Hong, S. & Bachert, C. Local Immunoglobulin E in nasal polyps: Role and modulation. *Front Immunol* **13**, 961503 (2022).
81. Le Floch, A. et al. Dual blockade of IL-4 and IL-13 with dupilumab, an IL-4R α antibody, is required to broadly inhibit type 2 inflammation. *Allergy* **75**, 1188-1204 (2020).
82. Hamilton, J.D. et al. Dupilumab suppresses type 2 inflammatory biomarkers across multiple atopic, allergic diseases. *Clin Exp Allergy* **51**, 915-931 (2021).
83. Arganda-Carreras, I. et al. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. *Bioinformatics* **33**, 2424-2426 (2017).
84. Jonstam, K. et al. Dupilumab reduces local type 2 pro-inflammatory biomarkers in chronic rhinosinusitis with nasal polyposis. *Allergy* **74**, 743-752 (2019).
85. Karosi, T., Csomor, P. & Sziklai, I. Tumor necrosis factor- α receptor expression correlates with mucosal changes and biofilm presence in chronic rhinosinusitis with nasal polyposis. *Laryngoscope* **122**, 504-510 (2012).
86. Morse, J.C. et al. Patterns of olfactory dysfunction in chronic rhinosinusitis identified by hierarchical cluster analysis and machine learning algorithms. *Int Forum Allergy Rhinol* **9**, 255-264 (2019).
87. Vaitkus, J. et al. Chronic Rhinosinusitis with Nasal Polyps: Age and Disease Severity Differences in the Levels of Inflammatory Markers. *Medicina (Kaunas)* **57** (2021).
88. Wu, J., Chandra, R.K., Li, P., Hull, B.P. & Turner, J.H. Olfactory and middle meatal cytokine levels correlate with olfactory function in chronic rhinosinusitis. *Laryngoscope* **128**, E304-e310 (2018).
89. Lu, X. et al. The cytokine-driven regulation of secretoglobins in normal human upper airway and their expression, particularly that of uteroglobin-related protein 1, in chronic rhinosinusitis. *Respir Res* **12**, 28 (2011).
90. Ryu, G. et al. Role of IL-17A in Chronic Rhinosinusitis With Nasal Polyp. *Allergy Asthma Immunol Res* **12**, 507-522 (2020).
91. Scheckenbach, K. & Wagenmann, M. Cytokine Patterns and Endotypes in Acute and Chronic Rhinosinusitis. *Curr Allergy Asthma Rep* **16**, 3 (2016).
92. Fokkens, W., Van Der Lans, R. & Reitsma, S. Dupilumab for the treatment of chronic rhinosinusitis with nasal polyposis. *Expert Opin Biol Ther* **21**, 575-585 (2021).
93. Cho, H.J. et al. IL-4 drastically decreases deuterosomal and multiciliated cells via alteration in progenitor cell differentiation. *Allergy* **78**, 1866-1877 (2023).

[00468] All citations are hereby incorporated by reference.

[00469] The present invention has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims. Therefore, although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. Elements listed with specific embodiments, are understood to be subject to combination in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

[00470] In the specification, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to," and the word "comprises" has a corresponding meaning. It is to be however understood that, where the words "comprising" or "comprises," or a variation having the same root, are used herein, variation or modification to "consisting" or "consists," which excludes any element, step, or ingredient not specified, or to "consisting essentially of" or "consists essentially of," which limits to the specified materials or recited steps together with those that do not materially affect the basic and novel characteristics of the claimed invention, is also contemplated. By "about" is meant a variance (plus or minus) from a value or range of 5% or less, for example, 0.5%, 1%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, *etc.* Citation of references herein shall not be construed as an admission that such references are prior art to the present invention. All publications are incorporated herein by reference as if each individual publication was specifically and individually indicated to be incorporated by reference herein and as though fully set forth herein. The invention includes all embodiments and variations substantially as hereinbefore described and with reference to the examples and drawings.

WHAT IS CLAIMED IS:

1. A protamine molecule comprising a first protamine peptide conjugated to one or more of a lipid, a hydrophobic moiety, a polymer comprising an amino group or an additional protamine peptide.
2. The protamine molecule of claim 1 wherein the lipid is a fatty acid, a lipid-amine, or a lipid-carboxyl.
3. The protamine molecule of claim 2 wherein the lipid further comprises a linker.
4. The protamine molecule of claim 3 wherein the linker is polyethylene glycol (PEG) or N-Hydroxysuccinimide (NHS).
5. The protamine molecule of claim 1 wherein the lipid is palmitic acid, stearic acid, 1,2- phosphatidylethanolamine (PE), dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), DPPE-NHS, DSPE-NHS, or DOPE-NHS.
6. The protamine molecule of claim 1 wherein the hydrophobic moiety is allyl glycidyl ether (AGE).
7. The protamine molecule of claim 1 wherein the polymer comprising an amino group is poly(amidoamine) (PAMAM), polyethyleneimine (PEI), or polylysine (PLL).
8. The protamine molecule of claim 4 further comprising a crosslinker.
9. The protamine molecule of claim 4 wherein the crosslinker is N-Succinimidyl S-Acetylthioacetate (SATA) or N-γ-Maleimidobutyryloxysuccinimide (GMBS), or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC).
10. The protamine molecule of claim 1 wherein the protamine molecule is protamine-stearic acid, protamine-PAMAM, protamine-DMPE, protamine-GMBS-PAMAM, protamine-DMPE, protamine-AGE, protamine-SATA-PAMAM, protamine-C18, protamine-C16, protamine-PEI, or protamine-PLL.
11. The protamine molecule of claim 1 wherein the first protamine peptide is conjugated to one or two additional protamine peptides resulting in a protamine dimer or a protamine trimer.
12. The protamine molecule of claim 1 wherein the first protamine peptide and the additional protamine peptide are the same or different.
13. The protamine molecule of any one of claims 1 to 12 wherein the first protamine peptide and/or the additional protamine peptide is a protamine salt.

14. The protamine molecule of claim 13 wherein the protamine salt is a protamine sulfate salt from salmon.
15. The protamine molecule of any one of claims 1 to 14 wherein the first protamine peptide or the additional protamine peptide comprises the amino acid sequence of any one of SEQ ID NOs: 1-4 or a conservative substitution thereof.
16. The protamine molecule of any one of claims 1 to 15 wherein the protamine molecule comprises an alpha-helix and/or a beta sheet.
17. The protamine molecule of any one of claims 1 to 16 wherein the protamine molecule self-assembles into a nanostructure.
18. The protamine molecule of any one of claims 1 to 17 wherein the protamine molecule increases the density at the cell membrane, increases the concentration of protamine at the cell membrane, and/or increases the interaction of protamine with the cell membrane.
19. A composition comprising the protamine molecule of any one of claims 1 to 18.
20. A pharmaceutical composition comprising the protamine molecule of any one of claims 1 to 18.
21. A method for delivery of a payload molecule to a cell, comprising contacting the cell with the payload molecule in combination with the protamine molecule of any one of claims 1-18.
22. The method of claim 21 wherein the delivery is non-parenteral delivery.
23. A method for non-parenteral delivery of a large payload molecule to a cell, comprising contacting the cell with the payload molecule in combination with a protamine peptide or a protamine molecule.
24. The method of claim 22 or 23 wherein the non-parenteral delivery is intranasal, sublingual, oral, buccal, rectal, vaginal, intravitreal, topical, or to the skin, eye, brain, or lungs.
25. The method of any one of claims 22 to 24 wherein the non-parenteral delivery is transmucosal or transcellular.
26. The method of claim 25 wherein the transmucosal delivery does not comprise an injection.
27. The method of claim 25 wherein the transcellular delivery is transepithelial or is to the lamina propria.
28. The method of any one of claims 21 to 27 wherein the payload molecule is delivered to the nucleus of the cell and/or substantially bypasses the lysosomes of the cell.

29. The method of any one of claims 21 to 28 wherein the payload molecule is a protein, a peptide, a peptide analogue, a nucleic acid molecule, or a small molecule.
30. The method of any one of claims 21 to 29 wherein the payload molecule is provided in a physical mixture with the protamine molecule.
31. The method of any one of claims 21 to 30 wherein the payload molecule is not complexed with the protamine molecule.
32. The method of any one of claims 21 to 31 wherein the payload molecule is an antibody, a growth hormone, insulin, or semaglutide.
33. A method of permeabilizing the membrane of a cell comprising contacting the membrane of a cell with a protamine peptide and/or the protamine molecule of any one of claims 1-18.
34. The method of claim 33 wherein the permeabilizing is transient and/or reversible.
35. A method of transfecting a cell comprising contacting the membrane of a cell with a protamine peptide and/or the protamine molecule of any one of claims 1-18.
36. A method of treating or preventing a condition benefited by non-parenteral delivery of a payload molecule, the method comprising administering the payload molecule with a protamine peptide and/or the protamine molecule of any one of claims 1-18 to a subject in need thereof.
37. The method of claim 36 wherein the payload molecule is administered at the same time or at a different time from the protamine peptide and/or the protamine molecule.
38. The method of claim 36 wherein the protamine peptide and/or the protamine molecule is administered prior to administration of the payload molecule.
39. The method of any one of claims 36 to 38 wherein the condition is diabetes, rhinosinusitis, an eye condition or a skin condition.
40. Use of a protamine peptide and/or the protamine molecule of any one of claims 1-18 for treating or preventing a condition benefited by non-parenteral delivery of a payload molecule.