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Mucoadhesive chitosan- and cellulose derivative-based nanofiber-on-foam-on-film system for non-invasive peptide delivery

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ABSTRACT

Oromucosal administration is an attractive non-invasive route. However, drug absorption is challenged by salivary flow and the mucosa being a significant permeability barrier. The aim of this study was to design and investigate a multi-layered nanofiber-on-foam-on-film (NFF) drug delivery system with unique properties and based on polysaccharides combined as i) mucoadhesive chitosan-based nanofibers, ii) a peptide loaded hydroxypropyl methylcellulose foam, and iii) a saliva-repelling backing film based on ethylcellulose. NFF displays optimal mechanical properties shown by dynamic mechanical analysis, and biocompatibility demonstrated after exposure to a TR146 cell monolayer. Chitosan-based nanofibers provided the NFF with improved mucoadhesion compared to that of the foam alone. After 1 h, >80 % of the peptide desmopressin was released from the NFF. *Ex vivo* permeation studies across porcine buccal mucosa indicated that NFF improved the permeation of desmopressin compared to a commercial freeze-dried tablet. The findings demonstrate the potential of the NFF as a biocompatible drug delivery system.

1. Introduction

Therapeutic peptides are used in the treatment of chronic and often life-threatening or debilitating diseases such as diabetes and osteoporosis (Maher et al., 2016; Walsh, 2018). The most common route of administration for therapeutic peptides is by injection as the more convenient oral route of administration associates with inherent limitations for successful therapeutic peptide delivery such as degradation by the low gastric pH and/or gastric and intestinal enzymes, and poor absorption across the digestive tract mucosa (Maher et al., 2016). Thus, daily injections are often required, which can be inconvenient and associated with discomfort by the patient (Mitragotri et al., 2014). The complex structure of therapeutic peptides is related to their high specificity and potency, but also represents a challenge for formulation and delivery, as they have poor physicochemical stability, high molecular weight, and often a high degree of hydrophilicity. These properties result in poor permeation across biological barriers such as mucosae (Frokjaer & Otzen, 2005). The oral cavity mucosa is easily accessible, and dosing of drugs *via* the oral cavity leads to high patient compliance in general (Rathbone et al., 1994). Especially the buccal and sublingual regions of the oral cavity are promising routes for non-invasive peptide delivery as these mucosae are non-keratinized and the underlying tissue is highly vascularized. Further, the sublingual tissue in particular consists of a limited number of epithelial cell layers (Rathbone et al., 1994).

Although the number of drugs of biological origin approved by the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) is increasing each year, most of the newly approved therapeutic peptides and proteins formulations are administered by injection (Maher et al., 2016). Indeed, because of the many challenges still associated with non-invasive peptide delivery, only a single therapeutic peptide, desmopressin, to the best knowledge of the authors is currently approved by the EMA and FDA for oromucosal administration (Gleeson et al., 2021). Desmopressin is a synthetic analogue of the natural antidiuretic hormone vasopressin and is 10 times more potent (with regards

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to antidiuretic action) than the natural hormone (Sharman & Low, 2008). Despite its small size of 1069 Da, the bioavailability of desmopressin is nevertheless only 0.25 % after sublingual administration of a lyophilized tablet containing desmopressin (van Kerrebroeck & Nørgaard, 2009). Desmopressin-containing tablets intended for swallowing result in a very low desmopressin bioavailability of 0.08–0.16 % (Hashim & Abrams, 2008). Desmopressin (as desmopressin acetate) is also available in nasal formulations (sprays and drops). Despite their reported high bioavailability of around 5–10 %, administration *via* the nasal route may be less advantageous and come with side effects. Recently, desmopressin (as desmopressin acetate) was also formulated as minitablets attached to a mucoadhesive bilayered film to form a composite system in comparison to traditional minitablets applied for buccal drug delivery (Kottke et al., 2021).

The oral route of administration is the most preferred by patients. Nevertheless, in a recent study, it was reported that ~10 % were non-adherent to their treatment because of swallowing difficulties and that this is especially prevalent in the young and elderly population (Schiele et al., 2013). Accordingly, as alternatives, orodispersible films have gained popularity because of their ease of use and due to the important fact that they can be administrated without water and do not require swallowing of the intact dosage form (Hoffmann et al., 2011). Because of their fast disintegration when in contact with saliva, the active pharmaceutical ingredient is often released fast from the dosage form and then easily swallowed. Significant dilution of the therapeutic peptide in the pool of saliva, subsequent swallowing, and degradation in the gastro-intestinal (GI) tract make these types of formulations less suitable for systemic delivery of therapeutic peptides. Pleasant taste and palatability are required for good patient acceptance as a significant part of the oral cavity is exposed to the constituents of the dosage form. Hence, there is a demand for new and innovative drug delivery systems (DDS) to facilitate transmucosal absorption of therapeutic peptides by non-invasive means.

DDS for oromucosal application benefit from the advantages of oral administration, *e.g.*, high acceptance of this particular route of administration and ease of use as they do not require swallowing. Strong mucoadhesion and unidirectional drug release can result in minimal drug exposure to, *e.g.*, the gastric tissue and fluids, which minimize the risk of side effects, improves the bioavailability of the peptide as it is not degraded in the harsh conditions of the stomach upon swallowing, and may provide a more rapid onset of the therapeutic effect as compared to the conventional oral dosage forms even if the drug is absorbed efficiently from the gastro-intestinal tract. Mucoadhesive formulations that adhere to the oral mucosa can also improve the drug absorption by maintaining a high concentration of the drug at the site of application. Different multi-layered systems have been developed for applications in the field of *e.g.*, tissue regeneration and drug delivery (Eleftheriadis et al., 2020; Mašek et al., 2017; Neves et al., 2020). Specifically for oromucosal drug delivery, Mašek et al. (Mašek et al., 2017) presented a multi-layered nanofibrous mucoadhesive film for the administration of nanoparticles for oromucosal vaccination. Very recently, Kottke et al. (Kottke et al., 2020) described a composite system for local pain relief consisting of lidocaine-loaded mini-tablets and a mucoadhesive buccal film to ensure high local penetration of the drug into the tissue. Fiber-based systems can be developed with tunable functionalities and their preparation is easily scalable. The adhesiveness of electrospun chitosan/polyethylene oxide (PEO) nanofibers to the oral mucosa was recently evaluated (Stie et al., 2020). Facilitated by swelling of the nanofibers and dehydration of the mucosal tissue upon contact, electrospun chitosan/PEO nanofibers adhered strongly to the oral mucosa (Stie et al., 2020). In general, nanofiber-based systems benefit from the combined properties of their individual components or layers, yet may display limitations in drug loading capacity. Freeze-dried porous foams/wafers are also promising carriers for oromucosal application of drugs, including peptides, because of their good mechanical properties, high drug loading capacity, tunable release, mild fabrication conditions and potential for industrial scale-up (Ayensu et al., 2012; Boateng et al., 2009; Iftimi et al., 2019). The drug can be loaded in various amounts, concurrent with the freeze-drying process or, for example, by imprinting the freeze-dried foam, utilizing inkjet printing (Iftimi et al., 2019).

The aim of this study was to develop a biocompatible multi-layered DDS from hereon denoted nanofiber-on-foam-on-film (NFF) for oromucosal delivery of therapeutic peptides consisting of i) mucoadhesive electrospun chitosan-based nanofibers with strong adherence to the oral mucosa, ii) a peptide-loaded foam, and iii) a saliva-repelling backing film to ensure unidirectional peptide release towards the oral mucosa. To demonstrate proof of concept, desmopressin was chosen as the therapeutic peptide to be loaded due to its clinical relevance, but also to enable benchmarking against a marketed product, MiniRin®, containing between 60 and 240 μg desmopressin per dose for sublingual administration. We hypothesize that by exploiting the physical properties of each of the individual layers in the NFF, the proposed multi-layered DDS can adhere to the mucosa and efficiently deliver the therapeutic peptide desmopressin across the oral mucosa. We expect the chitosan nanofibers to facilitate strong mucoadhesion, whereas the hydrophilic foam and hydrophobic backing layer will allow efficient peptide loading and unidirectional peptide release, respectively, contributing to efficient peptide permeation by keeping a high concentration of peptide on the mucosa (on the site of application). Having multiple layers and several methods of their preparation expands the potential usability of a dosage form such as NFF in terms of the drugs that can be delivered. NFF is a triple-layered system, where the drug-containing layer is the middle layer. This is beneficial because the system then (i) provides protection of the drug against some harsh environmental conditions (*e.g.*, direct sun light), (ii) avoids direct contact of the end-user with the drug during application and handling, and (iii) avoids direct contact of the drug with the container, thereby minimizing adsorption of peptide molecules to plastic packing material. To the best of our knowledge, the NFF system is the first multi-layered system based on freeze-dried foam made primarily of the cellulose ether, and mucoadhesive chitosan-based electrospun nanofibers, intended for oromucosal delivery of therapeutic peptides.

2. Materials and methods

2.1. Materials

Chitoceuticals chitosan 95/100 (degree of deacetylation 96 %, Mw 100–250 kDa, chitosan-96) was purchased from Heppe Medical Chitosan (Halle, Germany). Polyethylene oxide (Mw 900 kDa, PEO), bovine serum albumin (BSA), acetic acid anhydride, Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin, streptomycin, phenazine methosulfate (PMS), glycerol (≥99 %), tributyl citrate, poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly (ethylene glycol) (Lutrol® F68), formic acid, trifluoroacetic acid (TFA), acetonitrile and ethyl cellulose were obtained from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from PAA laboratories (Brøndby, Denmark). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was obtained from Promega (Madison, WI, USA). N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (hepes) was obtained from PanReac AppliChem (Damstadt, Germany). Polyethylene glycol 4000 (PEG 4000) and polyoxyethylene sorbitan monolaurate (Tween® 20) was from Emprove Merck (Darmstadt, Germany). Iron(*III*)oxide (Secovit® E172) was from BASF (Copenhagen, Denmark). Hydroxypropyl methylcellulose (HPMC) (Metolose® 60SH-4000) was kindly provided by Shin-Etsu (Chiyoda, Tokyo, Japan). The human buccal epithelial cell line TR146 was obtained from European Collection of Authenticated Cell Cultures (ECACC) (Public Health England, Porton Down, UK) and purchased from Sigma Aldrich (St. Louis, MO, USA). Desmopressin as TFA salt (purity >98 %) was obtained from SynPeptide (Shanghai, China). MiniRin® contains desmopressin acetate but for research purposes, the

TFA salt of desmopressin was purchased. We do not expect this to affect the results. Freshly prepared ultrapure water (18.2 MΩ × cm) purified by a PURELAB flex 4 (ELGA High Wycombe, UK) was used if not otherwise stated.

2.2. Freeze-drying of peptide-loaded porous foam

The polymer dispersion for the fabrication of the foam was prepared according to Iftimi et al., (Iftimi et al., 2019) with slight modification in the composition of the formulation and manufacturing procedure. In short, 2.5 g HPMC, -0.0825 g poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol), 0.25 g polyxyethylene sorbitan monolaurate, 0.25 g PEG 4000, and 0.25 g glycerol were dispersed in 50 mL ultrapure water preheated to 70 °C. The mixture was stirred for 5 min and 50 mL ultrapure water (room temperature (RT)) was added. This mixture was stirred on a magnetic stirrer until a clear viscous dispersion was obtained. The dispersion was stored at least overnight at 2–8 °C prior to use. A total of 7.28 mg desmopressin-TFA (equal to 6 mg desmopressin) was added to 6.2 g of the prepared dispersion. For samples used for the *ex vivo* permeation study, 29.12 mg desmopressin-TFA (equal to 24 mg desmopressin) was added. Subsequently, 5.1 g of the peptide-containing dispersion was cast in a glass petri dish (area 66.6 cm^2) and freeze-dried to yield the foam with a theoretical dose of either 58 μg or 232 μg desmopressin per patch with a diameter of 10 mm. The freeze-drying was carried out on an Epsilon 2-4 LSC shelf apparatus (Martin Christ, Osterode am Harz, Germany). The casted formulation was cooled to −30 °C over 3 h and kept at this temperature for the next 3 h. After that, the pressure was reduced to 0.12 mbar over 10 min and the temperature was hereafter increased to 0 °C for 1 h 20 min. At this setting, the primary drying was conducted for 16.5 h. The obtained solid foams were removed from the petri dish and stored in zipper bags over silica at 2–8 °C before use.

2.3. Electrospinning a mucoadhesive layer of nanofibers onto foam

The mucoadhesive electrospun chitosan/PEO nanofibers were prepared by electrospinning according to Stie et al., (Stie et al., 2020) directly onto the freeze-dried foam. Briefly, a square of approximately 2 cm × 2 cm was cut from the mat of freeze-dried foam and secured with adhesive tape on the aluminum foil on the stainless steel electrospinning collector on which the fibers were collected. Aqueous dispersions of 2 % (w/w) chitosan with 0.7 % (w/w) acetic acid and 4 % (w/w) PEO in ultrapure water were stirred for two days at RT. Information on the properties of the polymer dispersions, *e.g.*, viscosity, surface tension and conductivity was published previously (Stie et al., 2020). The polymer dispersions were mixed to obtain a 1:1 (w/w) ratio of the chitosan to PEO in the dry nanofibers (assuming total evaporation of water during electrospinning). After stirring for 30 min, chitosan/PEO dispersion was electrospun (20 kV, ES50P-10 W high voltage source, Gemma High Voltage Research, Ormond Beach, FL, USA) at low humidity (<20 %) for 2 h from a 20 G blunt needle (Photo-Advantage, Ancaster, ON, Canada) positioned 15 cm from the collectors plate.

2.4. Spraying a water-repelling backing film on foam and nanofiber-on-foam

A hydrophobic backing film was applied on either the rough or the smooth (oriented towards the petri dish during freeze-drying) surface of the foam. The backing film was prepared as follows; 750 mg ethyl cellulose, and 141 mg acetyl tributyl citrate and 47 mg glycerol as plasticizers were dispersed in 15 mL ethanol (absolute). After stirring for at least 3 h at RT, 10 mg iron(*III*)oxide pigment was added and the dispersion was hereafter stirred for at least another 30 min. Round patches (10 mm diameter) of the foam or fiber-on-foam were punched out using a biopsy puncher, and the backing film was applied by spraying of the dispersion using an air brush (Model BD-134, Custom Colors, Jyderup, Denmark). During spraying, the patches were kept in place on a custom-made metal plate with small holes. Using a pump (1HAE-25-M104X, Gast Manufacturing, Benton Harbor, MI, USA), suction was applied through the holes to keep the patches in place during spraying.

2.5. Evaluation of morphology by scanning electron microscopy (SEM)

The morphology of the foam, nanofibers, and multi-layered NFF was visualized by SEM. The foam and the backing film were visualized using a TM3030 SEM (Hitachi, Tokyo, Japan) at 5.0 kV. For high-resolution SEM imaging of the electrospun nanofiber surface and cross-section of the multi-layered NFF, samples were visualized with a Quanta FEG 3D microscope (Thermo Fischer Scientific, Hillsboro, OR, USA) at 2.0 kV. Prior to analysis, the samples were mounted on aluminum stubs on carbon tape and sputter-coated with gold (108 Auto sputter coater, Cressington Scientific Instruments, Watford, UK). ImageJ software version 1.53 k (National Institute of Health, Bethesda, MD, USA) was used for the analysis of nanofiber diameter.

2.6. Evaluation of the mechanical properties of foam and nanofibers

To prepare the mats for mechanical analysis, the chitosan-PEO dispersion was spun for 2 h, using the same process parameters as stated above. The electrospun mats and foams were stored in a desiccator over silica at 5–8 °C and were let to equilibrate at ambient conditions (21–24 °C) prior to analysis. The mechanical properties of the electrospun nanofibers as well as peptide-free, plasticizer-free (contained only HPMC), and peptide-loaded foams, respectively, were studied using a dynamic mechanical analyzer (DMA) (Q800, New Castle, DE, USA). The samples were prepared by cutting out rectangular shapes in a dimension of 6.4 mm × 30.0 mm from the electrospun mats or freeze-dried foams. Width and thickness of each of the cut-out samples were measured at three different points using a digital caliper, and the average values were reported. The samples were mounted using the film tension clamps. A preload force of 0.01 N and initial displacement of 0.01 % were set up before the actual analysis. The samples were subjected to a displacement ramp of 200 μm/min for a total length of 5000 μm. The obtained stress-strain curves were analyzed in Thermal Advantage Software v 5.5.2 (TA Instruments, New Castle, DE, USA) to determine Young's modulus as the slope of the curve in the initial linear region (0–1.0 % strain for the foam samples, and 0–0.4 % and 0.6–1.0 % strain for nanofibers due to the shape of the curve). Furthermore, the ultimate tensile strength (UTS) was determined as the maximum stress that the material could withstand before breaking, and the elongation at break was used to determine the strain at which the material could not stretch any further.

2.7. Evaluation of the mucoadhesion of foam and nanofiber-on-foam

The mucoadhesion of the foam and the NFF multi-layered system without the saliva-repelling backing film (nanofiber-on-foam) to *ex vivo* porcine buccal mucosa was evaluated according to Stie et al. (Stie et al., 2020) with few modifications. In short, cheeks from healthy experimental pigs (approximately 30–60 kg, Danish Landrace/Yorkshire/Duroc) were collected immediately after euthanization and kept in PBS on ice until use on the same day as the tissue was isolated. The cheeks were trimmed to remove the underlying tissue and cut to a thickness of 0.50–0.75 mm with an electric dermatome (Zimmer Biomet, Albertslund, Denmark). The buccal mucosa was immediately mounted on microscopy glass slides using Loctite® Power Flex gel (Henkel, Ballerup, Denmark) and kept submerged in PBS on ice until use; measurements were conducted on the same day as tissue isolation. The force of adhesion of round patches (10 mm in diameter) to *ex vivo* porcine buccal mucosa was determined at RT by a TA.XT plus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell. The

samples were in contact with the buccal tissue for 10 s by applying a force of 500 g, and withdrawn with a speed of 10 mm/s. The work of adhesion was determined as the area under the recorded force *versus* distance curve using the Exponent software (Stable Micro Systems, Godalming, UK).

2.8. Release of desmopressin from foam and multi-layered NFF

Round patches (10 mm in diameter) of foam and nanofiber-on-foam with and without water-repelling backing film were fixed in Ussing chamber sliders (diffusion area of 0.4 cm^2) and placed in EM-CSY-8 Ussing chambers (Physiologic Instruments, Santiago, CA, USA) as described in Stie et al., 2022. 2 mL warm (37 °C) 10 mM hepes in HBSS pH 6.8 with 0.05 % (w/v) BSA (hereafter named hHBSS) was added to each chamber. The samples were incubated for 3 h at 37 °C and aliquots of 100 μL were withdrawn from each of the diffusion cells at specific time points and replenished with 100 μL warm (37 °C) hHBSS. The exact peptide dose (the peptide content) was determined by disintegrating a 10 mm foam patch of known weight in 1 mL ultrapure water for at least 1 h at RT. All samples were centrifuged (10,000 rpm/9279 ×g, 10 min, 4 °C) and the concentration of desmopressin in the supernatant was determined by reversed phase high performance liquid chromatography using ultra-violet (UV) absorbance detection (RP-HPLC-UV).

2.9. Quantification of desmopressin by RP-HPLC-UV

The analysis was conducted on a Shimadzu Prominence system (Kyoto, Japan) with a Kinetex XB-C18 column (100 × 2.1 mm, 3.6 μm, Phenomenex, Torrance, CA, USA). Desmopressin was eluted using a mobile phase consisting of eluent A [95:5 % (v/v) acetonitrile:ultrapure water, 0.1 % (v/v) TFA] and eluent B [5:95 % (v/v) acetonitrile:water, 0.1 % (v/v) TFA]. Samples were run with a gradient of 0 → 40 % eluent B for 8 min at a flow rate of 0.8 mL/min at 40 °C. Injection volume was 10 μL. Desmopressin was detected at a retention time of 5.3 min at a wavelength of 218 nm. The limit of detection (LOD) and limit of quantification (LOQ) were 0.6 μg/mL and 1.7 μg/mL respectively.

2.10. In vitro compatibility testing of foam and NFF

TR146 cells were cultured in DMEM supplemented with FBS (10 % (v/v)), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL) in Corning Costar® polystyrene culture flasks (175 cm^2, Sigma Aldrich, St. Louis, MO, USA) at 37 °C with 5 % CO_2 in a humidified environment. A total of 85,000 TR146 cells/well were seeded in flat-bottom, transparent 12-well Nunclon™ delta cell culture-treated plates (3.5 cm^2, Thermo Scientific, Roskilde, Denmark) and cultured for three days at the aforementioned conditions attaining a confluence of 70–90 % before use. The cells were washed twice in 2 mL 37 °C hHBSS without BSA. The cells were exposed to desmopressin (60 μg/well), foam, foam with backing film, NFF, or a MiniRin® (60 μg desmopressin) freeze-dried tablet submerged in 2 mL hHBSS and incubated for 3 h at 37 °C with mild agitation (50 rpm on a Thermo MaxQ 2000 (Thermo Fischer Scientific, West Palm Beach, FL, USA)). After exposure, remnants of the formulations were removed, and the cells were washed twice with 2 mL warm (37 °C) hHBSS without BSA. The cells were then incubated at 37 °C for up to 2 h with 1 mL solution containing 240 μg/mL MTS and 2.4 mg/mL PMS in hHBSS without BSA. Subsequently, 100 μL samples in quadruplicate of the solution with metabolized MTS were transferred from each well to a transparent 96-well plate and the absorbance at 492 nm was measured in a plate reader (POLARstar OPTIMA, BMG LABTECH, Ortenberg, Germany). The absorbance of the unreacted MTS/PMS solution was defined as the blank (Abs_{blank}, 0 % cell viability), while the control was defined as cells incubated with hHBSS ($Abs_{control}$, 100 % cell viability). The relative cell viability was determined (Eq. (1)):

$$\text{Relative cell viability } (\%) = \frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}} \cdot 100\% \quad (1)$$

The osmolality of the solution after the remnants of the formulations were removed was determined on an Osmomat 3000 Freezing point osmometer (Genotec, Berlin, Germany) and the pH by a SenTix MIC pH electrode (VWR, Soeborg, Denmark).

2.11. Permeation of desmopressin through ex vivo porcine buccal mucosa

Cheeks from healthy experimental pigs (approximately 30–60 kg, Danish Landrace/Yorkshire/Duroc) were collected immediately after euthanization and kept in PBS on ice until use on the same day as harvesting the tissue. The cheeks were trimmed to remove the underlying tissue and cut to a thickness of 0.75 mm with an electric dermatome (Zimmer Biomet, Albertslund, Denmark) and mounted in Ussing sliders (diffusion area of 0.4 cm^2) and placed in EM-CSY-8 Ussing chambers (Physiologic Instruments, Santiago, CA, USA). NFF was placed on the buccal epithelium and mounted in the Ussing sliders with the tissue. A layer of Parafilm M® was applied to ensure contact between the NFF and the tissue. As a control, tissue was exposed to 2× MiniRin® (120 μg/dose) tablets in 2 mL hHBSS, (pH 6.8 in the donor chamber). The receiver chamber contained hHBSS (adjusted to pH 7.4). Aliquots of 100 μL were withdrawn from the receiver chamber over a 5 h period at 37 °C and replaced with warm (37 °C) hHBSS (adjusted to pH 7.4).

2.12. Quantification of desmopressin by liquid chromatography mass spectrometry (LC-MS)

100 μL samples were precipitated in 100 μL precipitation buffer (prepared by dissolving 2 g $ZnSO_4 \cdot 7H_2O$ in 55 mL ultrapure water and 50 mL acetonitrile) and centrifuged (20,000 ×*g*, 10 min, RT). The supernatant was analyzed by LC-MS on a Thermo Accela HPLC system coupled to a Thermo TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The injection volume was 30 μL on a Kinetex XB-C18 column (50 × 2.1 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA). Desmopressin was eluted using a mobile phase consisting of eluent A [0.1 % (v/v) formic acid in ultrapure water] and eluent B [0.1 % (v/v) formic acid in acetonitrile]. Samples were run with a gradient of 5 % → 28 % eluent B over 5 min at 0.8 mL/min at 40 °C. Samples were analyzed in single reaction monitoring (SRM) mode with electro-spray ionization in positive ion mode detecting desmopressin by monitoring the transition pairs *m/z* 535.37 precursor ion to *m/z* 328.4 product ion. Injection volume was 30 μL. LOD and LOQ were 2.3 ng/mL and 6.8 ng/mL, respectively. The data were processed using Skyline 20.1.0.155 (MacCoss Lab, Department of Genome Science, University of Washington, Seattle, WA, USA). For calculation of the average cumulative permeation across *ex vivo* porcine mucosa of desmopressin released from NFF, samples below LOQ were set to LOQ/2 *i.e.* 3.4 ng/mL.

2.13. Data and statistics

Statistical analysis was conducted in GraphPad Prism version 9.2.0. For statistical comparison of the mucoadhesion, a two-tailed unpaired *t*-test with unequal variances was employed. The variances in the groups were compared by statistical analysis by a F-test. For statistical comparison of the release of desmopressin, each point was compared by an unpaired t-test. Individual variances are assumed for each time point.

3. Results and discussion

3.1. Therapeutically relevant dose of desmopressin loaded in NFF

The overall aim was to explore a new DDS type for its ability to enhance the permeation of a therapeutic peptide across the oral mucosa

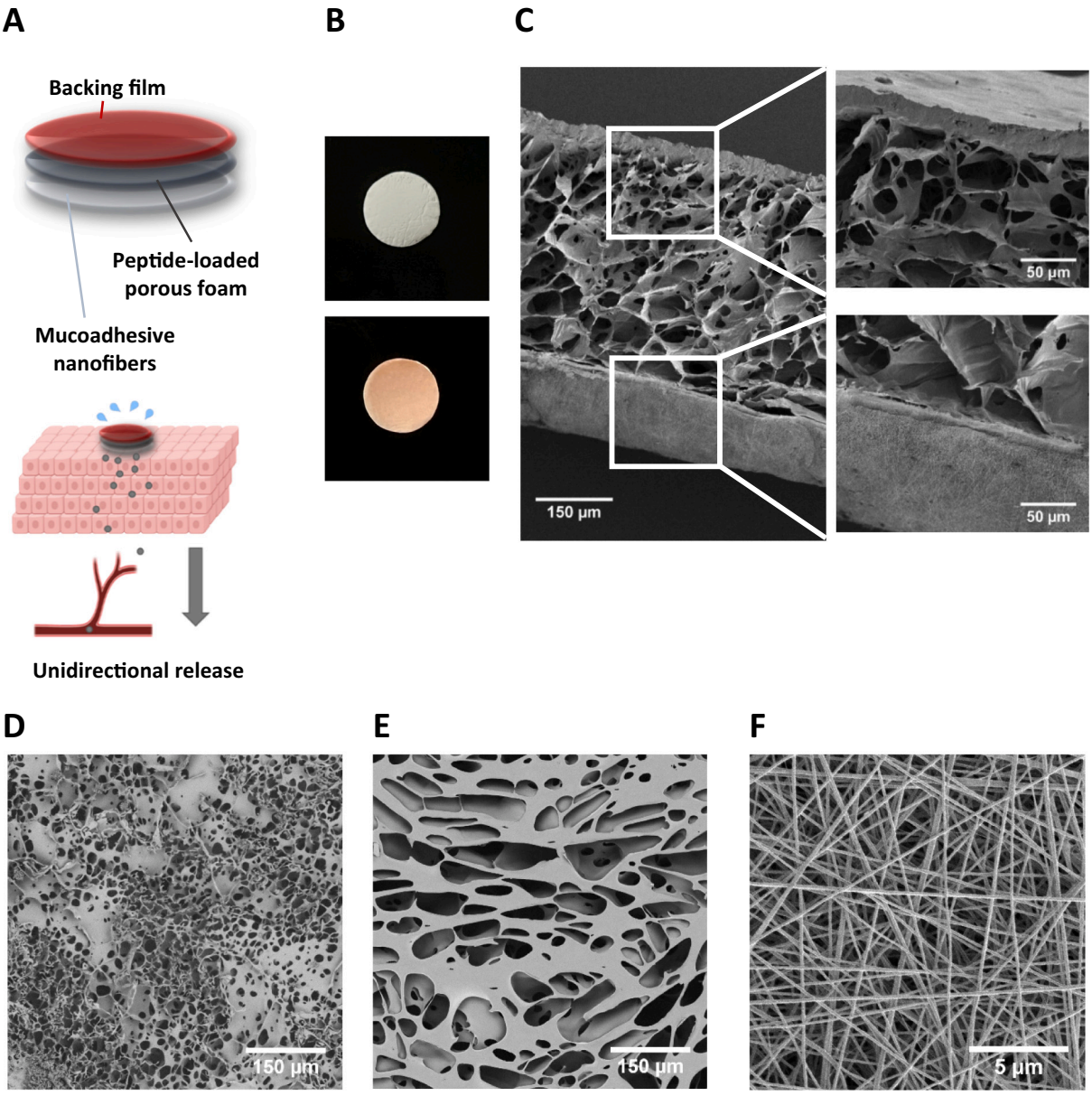

Fig. 1. Morphology of the multi-layered drug delivery system (DDS) composed of peptide-loaded foam, mucoadhesive electrospun nanofibers and water-repelling backing film – a nanofiber-on-foam-on-film (NFF) DDS with desmopressin. A) Schematic representation of the concept for the multi-layered NFF based on mucoadhesive electrospun nanofibers, peptide-loaded solid foam and a water-repelling backing film. B) Photo of a disc of 10 mm in diameter of NFF from the side of white nanofibers (top) or red water-repelling backing film (bottom). Representative scanning electron microscopy images of C) a cross-section of multi-layered NFF (the film-on-foam and nanofiber-on-foam interfaces are enlarged), D) the smooth surface of the peptide-loaded foam, E) the rough surface of the peptide-loaded foam, and F) the mucoadhesive electrospun chitosan/PEO nanofibers. The relative magnifications of the images are given by their respective scale bars. N = 2–3, where N is the number of individual samples visualized. The images are representative. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by retaining a high concentration of peptide at the site of application and by ensuring unidirectional drug release towards the mucosa for a prolonged period of time. Peptides are in general prone to instability issues, especially in liquid formulations, and to improve storage stability of desmopressin, a solid formulation, namely NFF, was prepared. The multi-layered NFF technology was based on i) mucoadhesive electrospun nanofibers, ii) a peptide-loaded foam, and iii) a water-repelling backing film (Fig. 1A–C). Each of the layers of the NFF served a specific purpose and different methods were applied to achieve the optimized properties of the three layers. The peptide-loaded foam was prepared by freeze-drying and served as a reservoir of the therapeutic peptide desmopressin. Desmopressin was loaded in the foam and the dose was 55.8 ± 4.6 µg (mean ± standard deviation (SD); N = 5, n = 3–4, where N is the number of individual batches and n is the number of samples per batch) desmopressin per dosage form of NFF (round patches of 10 mm in diameter) or 71.1 ± 5.9 µg/cm^2. The specific loading of desmopressin was 28.2 ± 0.2 µg per mg of foam (mean ± SD). The peptide-loaded freeze-dried foam showed a two-sided morphology: a smooth surface with small and uniformly distributed pores (oriented towards the petri dish during freeze-drying) (Fig. 1D), and a rough surface with larger pores (Fig. 1E). Mucoadhesive chitosan/PEO nanofibers were electrospun on the surface of the foam to ensure efficient adhesion of the multi-layered DDS to the oral mucosa (Fig. 1F). The chitosan/PEO nanofibers were electrospun in ultrapure water with minimum amounts of acetic acid (0.7 % (w/w)) as a solvent. The electrospun nanofibers were uniform without artifacts and had a mean

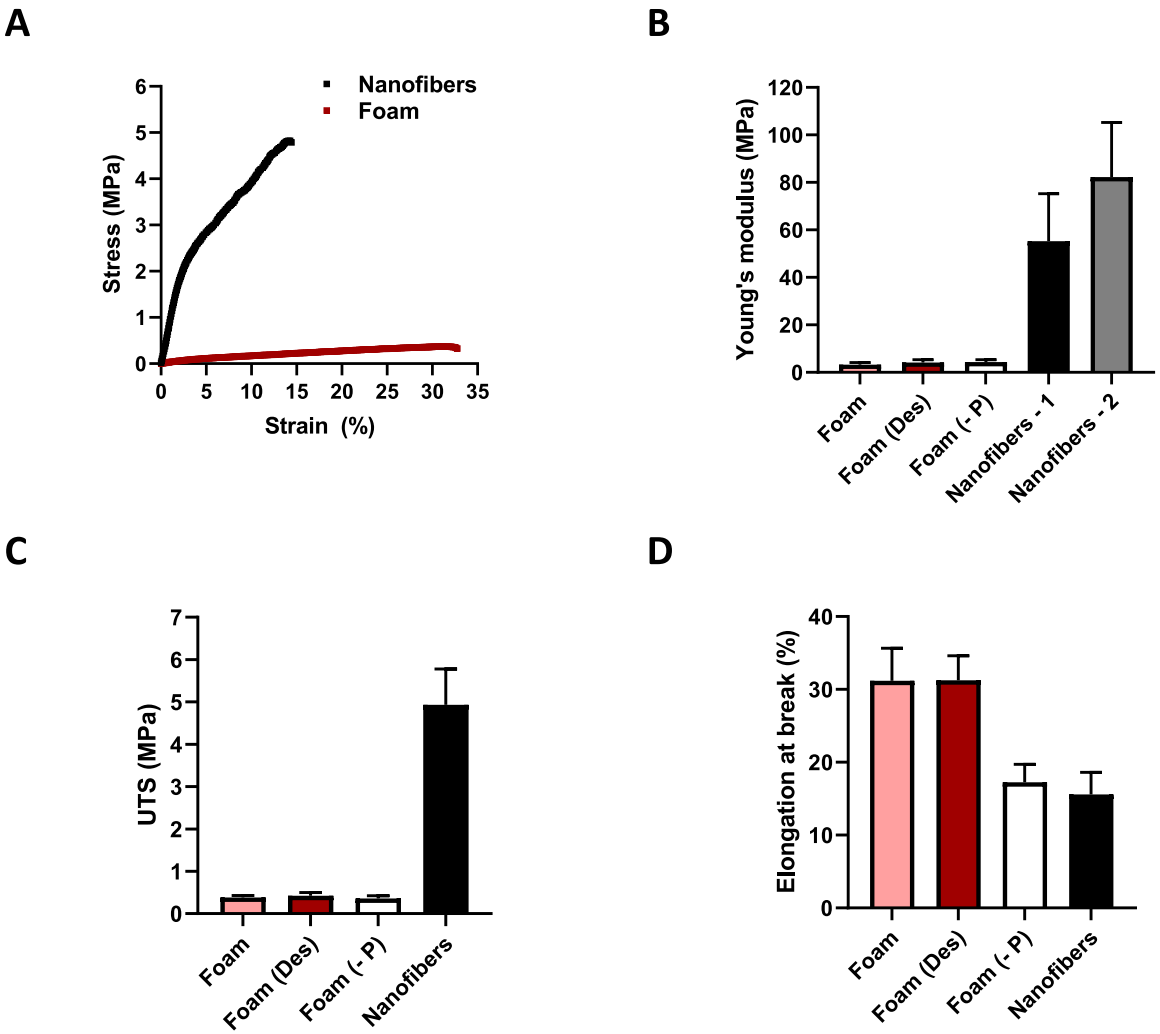

Fig. 2. Mechanical properties of neat solid foam, foam with desmopressin (Des), foam without plasticizers (—P) and electrospun nanofibers. A) Stress-strain curve for the aforementioned samples. B) Young's modulus. The Young's modulus was determined for the two distinct linear regions of the stress-strain curve for the nanofibers: Nanofibers-1 (strain from 0 to 0.4 %, SI) and Nanofibers-2 (0.6–1.0 %, SI). C) Ultimate tensile strength (UTS). D) Elongation at break. N = 2, n = 5–8, where N is the number of batches and n is the number of samples per batch analyzed. Data are presented as mean + SD.

diameter of 167 ± 27 nm (mean ± SD; N = 3, n = 100) comparable to previously described (Stie et al., 2020). A thin water-repelling backing film based on the hydrophobic polymer ethyl cellulose was applied to the porous foam to ensure unidirectional peptide release and to prevent peptide wash-out by saliva upon prolonged adhesion of the DDS to the oral mucosa (Fig. 1D). The SEM cross-sections of the NFF multi-layered system clearly indicated a tight and even connection between the distinctive layers of the NFF (Fig. 1C). From a technical point of view, it is worth noting that the multi-layered system demonstrates the possibility of electrospinning a separate layer of mucoadhesive nanofibers on a solid substrate; here the foam. This opens for the possibility of electrospinning nanofibers as mucoadhesive coatings on other types of substrates such as films, micro-tablets *etc.*

Desmopressin was previously successfully loaded in chitosan/PEO nanofibers by co-electrospinning the therapeutic peptide with the polymer blend (Stie et al., 2022). Although electrospinning is a very versatile technique, some drugs or excipients may have limited electrospinability in aqueous media because of low intermolecular entanglement as for *e.g.*, some proteins (Nieuwland et al., 2013) or due to high charge density as for *e.g.*, chitosan (Stie et al., 2019). Surfactants and organic solvents can be used to improve the electrospinability of dispersions by lowering the surface tension of the dispersion and to enhance evaporation of the solvent during spinning (Geng et al., 2005; Lancina et al., 2017; Ohkawa et al., 2004); however, the use of such potentially harsh conditions compromises the biocompatibility of the DDS and might furthermore reduce the stability of the peptide to be loaded. Inclusion of co-spinning polymers such as PEO is another strategy to facilitate water-borne electrospinning (Stie et al., 2019). As demonstrated, freeze-drying is an alternative technique to electrospinning for the production of solid peptide-loaded patches. Incorporation of the drug can be done in-process, but the foam is also suitable for loading of drugs by absorption or adsorption post preparation (Iftimi et al., 2019). The presented multi-layered NFF thus may also be used for loading of a variety of other drugs or excipients in the foam and/or in the electrospun nanofibers either by in-process or post-process incorporation.

3.2. *Mechanical properties of foam and nanofibers*

The optimal mechanical properties of the DDS, such as strength and flexibility, are crucial to allow for robust processing, transportation and for overall usability of the dosage form such as ease in removing the dosage form from the package and application to the site of drug absorption by a patient or caregiver. Furthermore, the NFF needs to be flexible to allow close adhesion to the curved surfaces of the oral mucosa. In light of this, the mechanical characteristics of the foam and

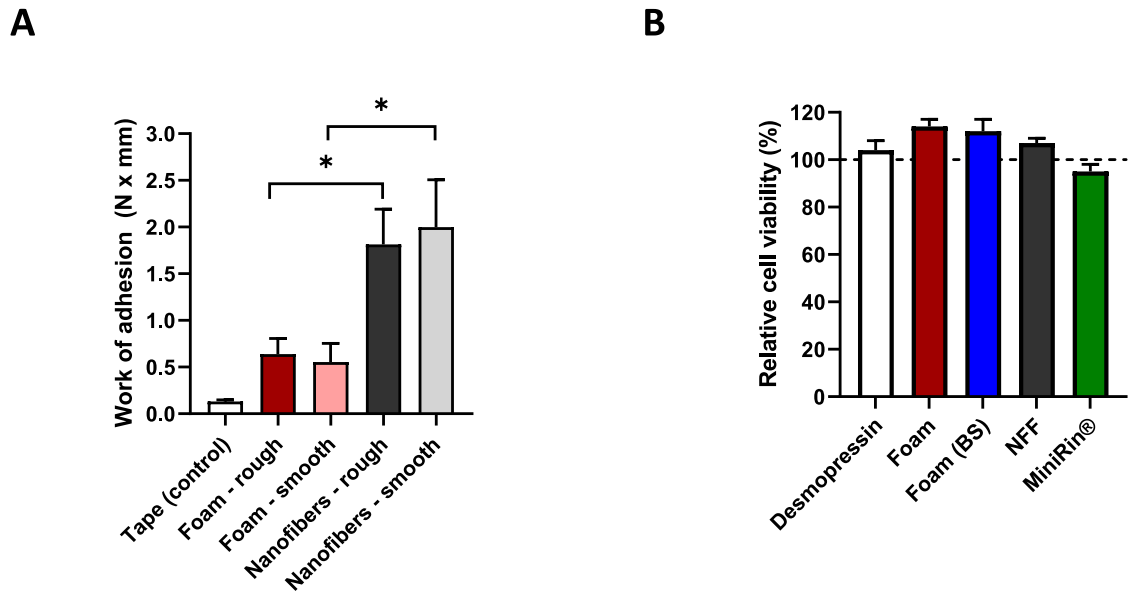

Fig. 3. Electrospun chitosan/PEO nanofibers improve mucoadhesion of biocompatible multi-layered DDS compared to the foam alone. A) Work of adhesion to *ex vivo* porcine buccal mucosa of tape used for mounting the samples on the probe (control), foam on the rough and smooth surface, respectively, and nanofibers electrospun on either the rough or the smooth surface of the foam. N = 3–7, where N is the number of repeats. Tissue samples obtained from at least two individual animals on two different days were included for each sample. $^*p < 0.05$. B) Evaluation of the biocompatibility of multi-layered NFF *in vitro*. The viability of human buccal TR146 cell monolayer after exposure to the foam, foam with backing film on the smooth surface (BS), multi-layered NFF and MiniRin® (60 μg) relative to the control (cells exposed to hHBSS, dashed line). Desmopressin (60 μg) was included as a control. N = 2, n = 3, where N is the number of cell passages and n is the number of samples tested per passage. The results are presented as mean + SD.

nanofibers were studied in tension mode. Both samples showed a behavior typical for ductile material (Fig. 2A). Interestingly, the stress-strain curves of nanofibers consisting of PEO and chitosan (1:1 (w/w)) possessed a linear region with a lower slope value (strain 0–0.4 %), following a linear region with a higher slope value (strain 0.6–1 %) (Figs. 2A & SI). It is speculated that this two-step behavior can be attributed first to the elastic modulus of PEO in the beginning of the strain-stress analysis, followed by a response related to the elastic modulus of chitosan. Most probably this can be due to the rigid and brittle chitosan properties (intra- and intermolecular hydrogen bonds in the pyranose backbone), in contrast to the flexible and elastic PEO chains (due to its linear structure). The foam appeared to possess superior flexibility as compared to mats of nanofibers that were more stiff (Fig. 2B). Inclusion of the peptide desmopressin (58 μg/dose) in the foam did not have a significant effect on the rigidity of the sample as the samples had similar Young's modulus values ($p > 0.05$) and in general did not affect the mechanical properties of the foam. None of the samples showed a well-defined yield point, which would have indicated the limit of elastic behavior and the beginning of plastic behavior. The nanofibers appeared to be much stronger than the foam samples (Fig. 2C). The latter had, however, superior ability to stretch when the foam formulation contained plasticizers (Fig. 2D). Importantly, it was observed while handling the samples that nanofibers, foam, and film were very flexible both alone and when combined, and thus could be handled without breaking.

3.3. Strong adhesion of multi-layered NFF to porcine buccal mucosa ex vivo

Mucoadhesion is an important property to ensure close contact between the DDS and the oral mucosa, to retain a high concentration of drug at the site intended for absorption, thereby enhancing drug diffusion across the mucosal barrier into the systemic circulation. The mucoadhesive properties of the NFF were evaluated by measuring the work of adhesion to *ex vivo* porcine buccal mucosa. The foam alone had limited adhesion to *ex vivo* porcine buccal mucosa (Fig. 3A) with no difference found between the more (rough) and less (smooth) porous surface of the peptide-loaded foam ($p > 0.05$). The presence of a layer of electrospun chitosan/PEO nanofibers on the foam significantly ($p < 0.05$) improved the mucoadhesive properties of the multi-layered DDS. Indeed, the work of adhesion was more than three times higher for the NFF without a backing film compared to the adhesion of the foam alone. By visual inspection, the NFF without the backing film appeared to swell and the underling tissue was dehydrated after detachment of the DDS from the buccal tissue, which indicates that the adhesion of the DDS to the mucosa was driven by the hygroscopic nature of the chitosan/PEO nanofibers. It was noted that the nanofibers did not separate from the foam during the mucoadhesion test. For reasons of comparison, an evaluation of the adhesion of MiniRin® to *ex vivo* porcine buccal mucosa was attempted, but the commercial tablets disintegrated instantaneously in the presence of the wetted tissue and the measurement could not be conducted.

Only biocompatible excipients were included in the formulation of the NFF. The biocompatibility of the NFF was evaluated *in vitro* by exposing a monolayer of human buccal TR146 cells to round patches of 10 mm in diameter of NFF, its individual components, *i.e.*, the foam with or without backing film and content of desmopressin, or in comparison to marketed a MiniRin® freeze-dried tablet. No changes in pH and osmolality of the test solution compared to the control (isotonic buffer on cell monolayer) were recorded in the presence of the NFF, whereas a slight increase in apical buffer osmolality from 300 mOsmol/kg to 337 ± 10 mOsmol/kg was observed for buffers on cell monolayer exposed to MiniRin®. All samples tested were equivalent to one dose of 58 μg desmopressin. As expected, none of the tested samples affected the viability of the buccal TR146 cell monolayer significantly compared to the control (Fig. 3B).

3.4. Controlled and unidirectional release of desmopressin from NFF

Controlled and unidirectional release is crucial to limit the loss of peptide drug by the salivary flow and to ensure a high concentration gradient of drug across the mucosa for a prolonged period of time. A

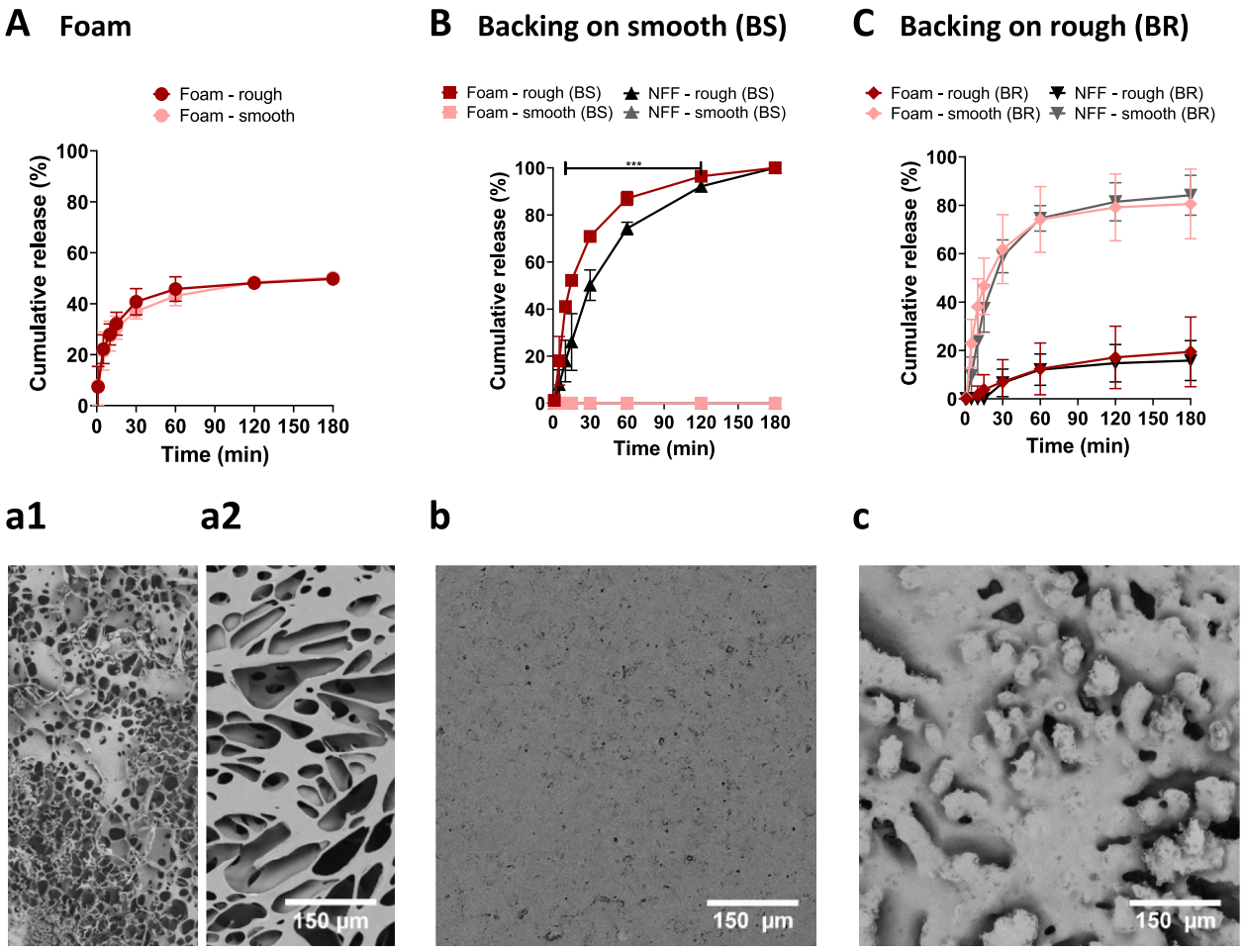

Fig. 4. Release of desmopressin from the foam and NFF. A) Release of desmopressin from either the smooth or the rough surface of the foam. Using a Ussing chamber setup, two release profiles were obtained simultaneously: The smooth surface of the sample was oriented towards the donor compartment and the rough surface of the samples towards the receiver compartment, and samples were drawn from each of the compartments over time. No water-repelling backing film was applied. SEM image of the smooth (SEM a1) and rough (SEM a2) surface of the foam. B) Release of desmopressin from the foam and multi-layered NFF with water-repelling film sprayed on the smooth surface (BS) of the foam (SEM b). Unidirectional release was achieved, and no peptide was detected for Foam – smooth (BS) and NFF – smooth (BS). Statistic significant difference (***$p < 0.001$) was found between Foam – rough (BS) and NFF – rough (BS) in the time interval 10–120 min. C) Release of desmopressin from the foam and multi-layered NFF with water-repelling film sprayed on the rough surface (BR) of the foam (SEM c). N = 5–9, where N is the number of individual samples analyzed. Results are presented as mean ± SD.

complete film with full coverage of the small pores in the foam was achieved after application of the hydrophobic water-repelling film matrix on the smooth surface of the foam (Fig. 4B). In contrast, the larger pores in the foam were still visible by SEM after application of the backing film to the rough surface of the foam, which indicates incomplete coverage of the pores on the surface of the foam (Fig. 4C).

The release of desmopressin from NFF was evaluated. For comparison, the release of desmopressin from the neat foam or from the foam with backing film was also assessed. The backing film or mucoadhesive nanofibers were applied either to the smooth or rough surface of the foam, respectively. The samples were placed between two diffusion chambers (Ussing chambers), and the release of peptide into each of the chambers was determined simultaneously over time. NFF is a mucoadhesive patch to be used in the oral cavity, *e.g.*, in the cheek and the physiological liquid available for release will therefore be saliva. According to Madsen et al. (2013), human saliva is ≥99 % water and the pH is 6.8 ± 0.4. Evaluation of the release of desmopressin from NFF was therefore conducted in aqueous-based medium at pH 6.8. The foam disintegrated rapidly in the aqueous test medium leading to rapid drug release into both chambers. In the absence of electrospun nanofibers and a water-repelling backing film, around 80 % of the total amount of desmopressin was released from the foam after 30 min, resulting in approximately 40 % peptide release into each of the diffusion chambers, respectively (Fig. 4A). In contrast, the layer of electrospun chitosan/PEO nanofibers and water-repelling backing film were still intact after 3 h in physiological buffer. Unidirectional release of desmopressin was achieved with spraying of the water-repelling backing film on the smooth surface of the foam (Fig. 4B). In contrast, unidirectional release was not fully achieved with application of the backing film on the rough surface of the foam as about 20 % of the total amount of released desmopressin was detected in the diffusion chamber fronting the backing film after 3 h (Fig. 4C). This is in good correlation with the visual appearance as observed with the SEM images, which showed insufficient coverage of the bigger pores and full coverage of the smaller pores of the rough and smooth surface of the foam, respectively. Furthermore, electrospun nanofibers on the rough surface of the foam significantly ($p < 0.001$) decreased the rate of desmopressin release (Fig. 4B). This indicates that the mucoadhesive electrospun chitosan/PEO nanofibers constitute a thin diffusion barrier for wetting of the desmopressin-loaded foam and thus decrease the release rate of the peptide.

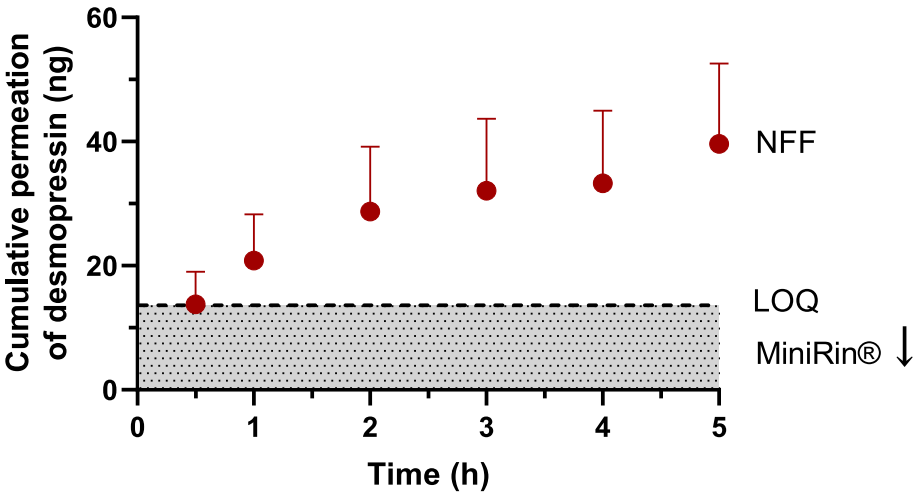

Fig. 5. Permeation of desmopressin from multi-layered NFF (203 ± 14 μg desmopressin/dose) and MiniRin® (240 μg desmopressin/dose) through *ex vivo* porcine buccal mucosa. The concentration of desmopressin in the receiver chamber was below the LOQ of the method of quantification (LC-MS) for all repetitions at all time points for mucosal tissue exposed to MiniRin® in 2 mL isotonic buffer. The cumulative amount of permeated desmopressin from MiniRin® tablets is therefore not displayed in the fig. N = 6–7, where N is the number of individual *ex vivo* porcine buccal mucosa. Results are presented as mean ± standard error of mean (SEM).

3.5. NFF improves permeation of desmopressin across buccal mucosa ex vivo

One of the major challenges for systemic delivery of therapeutic peptides is their low permeation across biological barriers including mucosal membranes because of the high molecular weight and hydrophilicity of peptides. It was hypothesized that the close adhesion of the NFF to the oral mucosa could increase the amount of permeated peptide. Mice and rats do not represent good models for the human buccal and sublingual mucosa as the epithelium of these regions, in contrast to that of the human, are keratinized (Kondo et al., 2014; Thirion-Delalande et al., 2017). Porcine buccal and sublingual mucosae are non-keratinized, have larger rete ridges and similar thicknesses as the human mucosa from these oral regions (Kondo et al., 2014; Thirion-Delalande et al., 2017). Accordingly, the permeation of desmopressin released from the NFF (203 ± 14 μg/dose or 259 ± 14 μg/cm^2, mean ± SD; N = 4, where N is the number of individual samples) across *ex vivo* porcine buccal mucosa was evaluated. The permeation of desmopressin from MiniRin® tablets (240 μg desmopressin) dissolved in 2 mL isotonic buffer across *ex vivo* porcine buccal mucosa was included for comparison. The permeated amount of desmopressin from commercial MiniRin® tablets was below the limit of quantification (LOQ) with the used quantification method (LC-MS) for all repeats at all time points (Fig. 5). In contrast, the permeated amount of desmopressin from NFF after one hour was on average higher than the LOQ for the LC-MS method applied and thus clearly on average higher than the permeation of desmopressin from MiniRin® tablets. This indicates that the NFF system indeed have the potential to improve the delivery of peptides across the oral mucosa compared to marketed formulations for oromucosal delivery, *e.g.*, freeze-dried tablets.

The exposed area of *ex vivo* porcine buccal mucosa was 0.4 cm^2. The average amount of desmopressin permeated after 5 h was ~40 ng equal to ~100 ng/cm^2, which corresponds to ~0.4 % of the initial dose of desmopressin loaded in the NFF system. As expected, the transmucosal permeation of desmopressin was significantly lower than that reported for small molecules across *ex vivo* porcine buccal mucosa when administered in electrospun patches (Clitherow et al., 2020; Kalouta et al., 2020). For example, the permeation of nicotine released from electrospun α-lactalbumin/PEO nanofibers across *ex vivo* porcine buccal mucosa after 5 h was ~3 % of the initial dose (Kalouta et al., 2020). However, the low permeation of therapeutic peptides challenging their delivery by non-invasive routes can be partly accounted for by applying mucoadhesive drug delivery technologies such as the NFF.

4. Conclusion

A novel DDS, specifically an NFF, was developed based on i) mucoadhesive electrospun chitosan-based nanofibers, ii) a freeze-dried foam for therapeutic peptide loading, and iii) a saliva-repelling backing film to ensure unidirectional release. The present study evaluated the morphological, mechanical and mucoadhesive properties of the NFF system and the release of the therapeutic peptide desmopressin from the NFF system as well as the resulting permeation of the peptide across porcine buccal mucosa *ex vivo*. Because of the unique properties of each of the layers of the NFF, *e.g.*, the flexibility, mucoadhesiveness and controlled peptide release, the NFF system is considered highly suitable for oromucosal administration. Interestingly, the *ex vivo* buccal permeation study suggests that the NFF can improve the permeation of desmopressin compared to that observed for desmopressin released from a commercial freeze-dried tablet for sublingual administration (MiniRin®). The NFF system shows potential as a biocompatible DDS for systemic delivery of therapeutic peptides.

CRediT authorship contribution statement

Mai Bay Stie: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Project administration. **Heidi Öblom:** Methodology, Investigation, Writing – review & editing. **Anders Christian Nørgaard Hansen:** Methodology, Investigation, Writing – review & editing. **Jette Jacobsen:** Conceptualization, Methodology, Validation, Funding acquisition, Writing – review & editing. **Ioannis S. Chronakis:** Conceptualization, Methodology, Validation, Funding acquisition, Writing – review & editing. **Jukka Rantanen:** Conceptualization, Methodology, Validation, Funding acquisition, Writing – review & editing. **Hanne Mørck Nielsen:** Conceptualization, Methodology, Validation, Funding acquisition, Project administration, Writing – review & editing. **Natalja Genina:** Conceptualization, Methodology, Investigation, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

Mai Bay Stie, Heidi Öblom, Jette Jacobsen, Jukka Rantanen, Hanne M. Nielsen, Natalja Genina are inventors of the NFF as covered by the submitted patent application PCT/EP2022/059128 entitled “Multilayered patch”.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.120429.

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