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Multi-layered nanofibrous mucoadhesive films for buccal and sublingual administration of drug-delivery and vaccination nanoparticles - important step towards effective mucosal vaccines

Running title: *Nanofibre-based mucoadhesive films for mucosal administration of nanoparticles*

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ABSTRACT

Nanofibre-based mucoadhesive films were invented for oromucosal administration of nanocarriers used for delivery of drugs and vaccines. The mucoadhesive film consists of an electrospun nanofibrous reservoir layer, a mucoadhesive film layer and a protective backing layer. The mucoadhesive layer is responsible for tight adhesion of the whole system to the oral mucosa after application. The electrospun nanofibrous reservoir layer is intended to act as a reservoir for polymeric and lipid-based nanoparticles, liposomes, virosomes, virus-like particles, dendrimers and the like, plus macromolecular drugs, antigens and/or allergens. The extremely large surface area of nanofibrous reservoir layers allows high levels of nanoparticle loading. Nanoparticles can either be reversibly adsorbed to the surface of nanofibres or they can be deposited in the pores between the nanofibres. After mucosal application, nanofibrous reservoir layers are intended to promote prolonged release of nanoparticles into the submucosal tissue. Reversible adsorption of model nanoparticles as well as sufficient mucoadhesive properties were demonstrated. This novel system appears appropriate for the use in oral mucosa, especially for sublingual and buccal tissues. To prove this concept, trans-/intramucosal and lymph-node delivery of PLGA-PEG nanoparticles was demonstrated in a porcine model. This system can mainly be used for sublingual immunization and the development of “printed vaccine technology”.

KEYWORDS

drug delivery; sublingual vaccines; mucoadhesive film; nanofibres; mucus penetrating nanoparticles; electrospinning

INTRODUCTION

The oromucosal route of administration is an alternative route for drug-delivery and vaccine delivery. This has been used successfully for local and systemic delivery of low-molecular-weight substances with a rapid onset of pharmacological effects, and also for drugs formulated into controlled-release systems (i.e. *Onsolis*[®], fentanyl comprising mucoadhesive film for buccal administration). Sublingual immunotherapy that makes use of allergens and antigens for the treatment of allergies and sublingual vaccination strategies, have also been explored. In addition, mucosal, especially sublingual delivery of macromolecular drug and antigen-delivery nanoparticles has become an important topic of recent research as well [1]. In general, high permeability, a lack of enzymatic barriers, mild pH values, easy access for self-administration, and opportunities to bypass first-pass metabolism, all make the non-keratinized oral regions attractive sites for the administration of drugs and/or vaccines [2]. Oral mucosae, especially its sublingual region, are densely populated with specialized dendritic cells. The adjacent submucosal tissue is drained with lymphatic vessels, through which free antigen as well as antigen-loaded dendritic cells move to reach regional lymph nodes. Thus, oral cavity and especially sublingual region is a potentially favourable site for inducing a specific immune response or tolerance towards given antigens and allergens. The sublingual mucosa, in particular, has been recognized as a suitable immuno-inductive area giving the opportunity for safe and efficient mucosal vaccination and immunomodulation [3].

However, although mucosal surfaces are the main route of pathogen entry, yet the induction of effective mucosal immunity is still a challenge for researchers in the field of vaccinology [4]. In order to induce an effective immune response not only is the vaccine system itself important but so too is the method of application [5]. Factors that limit successful administration and delivery of nano-based therapeutic systems include the mucus layer itself on the surface of oral mucosa, continuous saliva production, and epithelial absorption barriers [6]. Typically, administered drugs/antigens are efficiently removed by mucus clearance mechanisms and systemic absorption, precluding a prolonged local drug presence [7]. New approaches and formulations that utilize mucoadhesives, mucus penetrating particles, or absorption enhancers can enable effective transmucosal delivery of macromolecular therapeutics [8] and nanoparticle-based delivery systems [6]. In addition, a variety of dosage forms have been developed to face these obstacles for oral mucosal delivery – including mucoadhesive tablets, mucoadhesive oral films, fast

dissolving films as well as liquid formulations and sprays. Many nanoparticle-based delivery systems including polymeric and lipid-based nanoparticles have also been tested as mucosal drug-delivery and vaccination nanoparticle systems, especially for protein, peptide, and nucleic acid delivery [9].

Successful delivery of drug-delivery or vaccination nanoparticles through mucosal tissues represents a multidisciplinary problem that embraces aspects of mucosal physiology and mucosal barrier properties, nanotechnology and nanoparticle surface chemistries. This problem also embraces pharmaceutical sciences with particular reference to appropriate pharmaceutical formulations, dose regimens, drug-delivery devices, and proper elucidation of therapeutic approach [10]. For the first time, here we describe the combination of electrospun nanofibrous reservoir layers prepared by electrospinning, with mucoadhesive layers that together appear to represent an important new dosage platform for effective administration of drug-delivery and vaccination nanoparticles into the sublingual and buccal mucosa.

Electrospinning is a simple and efficient technique to produce nanofibers [11]. It utilizes a high electrostatic field to generate nanofibers from a fluid. Electrospun nanofibers often show large surface-to-weight (volume) ratio, high porosity, and excellent pore interconnectivity [12]. These unique features allow electrospun nanofibers have extensive applications in diverse areas including filtration, wound healing, cosmetic, drug delivery systems, and medicine [13,14]

Our nanofibrous mucoadhesive films are shown to avoid fast clearance of nanoparticles from sites of application, maintain a long-term concentration gradient of nanoparticles at the mucosal surface, and ensure unidirectional diffusion of nanoparticles towards mucosal surfaces by means of an impermeable surface layer(s) that faces the oral cavity.

MATERIALS AND METHODS

Preparation of nanofibrous mucoadhesive films

Each three-layered film prepared consisted of 1) a mucoadhesive layer, 2) a backing layer and 3) an electrospun nanofibrous reservoir layer. In the first step, the upper surface of a prepared mucoadhesive layer was coated with polymer to form a backing layer. In the second step the bottom side of each mucoadhesive layer was wetted with a vapour stream and a corresponding nanofibrous layer immediately pressed against this mucoadhesive layer.

Mucoadhesive layers were prepared as follows: initially Carbopol 934P (Lubrizol Advanced Materials, Cleveland, USA) and hydroxypropyl methylcellulose K4M (HPMC) (Colorcon Limited, UK) were combined in a 2:1 (w/w) ratio in water giving a viscous opaque solution that was supplemented with glycerol at 15% (w/w). The combination mixture was then treated by sonication to remove air bubbles and required volumes were poured out into plastic Petri dishes. Excess water was removed by evaporation at room temperature for 48 h leading to the formation of the desired mucoadhesive layers.

Non-adhesive backing layers were formed by the spraying of a 2% ethanolic solution of Eudragit[®] L 100-55 (soluble) directly onto the surface of a given mucoadhesive layer. During the spraying, each Petri dish with a given mucoadhesive layer was heated to 50°C to accelerate the evaporation of the solvent. The thickness of backing layer was measured using SEM pictures of cross-sectioned nanofibrous mucoadhesive films.

Nanofibrous reservoir layer

All nanofibrous reservoir layers were prepared using a roller electrospinning device (**see schema in Supporting information, section A**). The device contains a rotating cylinder, 145 mm in length and 20 mm in diameter, partially immersed in a blended polymer solution reservoir attached to a positive electrode. Blended polymer solutions were electrospun at a high voltage of 50 kV with the cylinder rotating at ~ 15 rpm in order to become rapidly coated with polymer solution followed by the electrospinning process itself and the creation of new materials. By this method each electrospun nanofibrous reservoir layer was collected on backing material (PEGATEX S 30 g/m²) that was moving along a negative collector electrode at a velocity of 30 mm/min. Electrospinning was carried out at a distance of 100 mm; air temperature 21°C, and air humidity 60 ± 2%.

Silk fibroin nanofibrous reservoir layer

Raw silk cocoons were degummed twice with 0.1 M of sodium carbonate and 0.5% of standard reference detergent at 100°C for 30 min, rinsed with warm water to remove the sericin from the surface of the fibre and then dried at room temperature. Silk fibroin solution (SF) was prepared by dissolving the degummed silk fibres in formic acid (98%). The formic acid solution used in the process contains 3 wt% calcium chloride. The SF concentration was fixed at 12 wt%. The SF solution was magnetically stirred at room temperature overnight, then electrospun at conditions described above.

The SF nanofibrous reservoir layers were immersed in ethanol for 30 min to induce crystallization of SF and reduce the water solubility of the nanofibrous reservoir layers. After drying at room temperature, the treated nanofibrous reservoir layers were immersed in distilled water overnight, which was followed by rinsing in distilled water to remove residual salts. Afterwards, the nanofibrous reservoir layers were air-dried.

Chitosan-PEO nanofibrous reservoir layer

Chitosan (viscosity 10 cP, 5 wt% in 1% acetic acid and a degree of deacetylation of 0.8) was purchased from Wako Pure Chemical Industries, polyethylene-oxide (PEO, average Mw ~ 400.000 g/mol by gel permeation chromatography) was obtained from Sigma-Aldrich and used as received. Deionized water with sodium chloride was used to prepare PEO polymer solution. Sodium chloride was added to an aqueous solution of 4 wt% PEO at 0.24 mol/L. Chitosan was then dissolved in 10 wt% citric acid to achieve a polymer concentration of 8 wt%. Chitosan and PEO solutions were afterwards blended and stirred at room temperature overnight at a volume ratio of 8/2 (chitosan-PEO). This blended chitosan-PEO polymer solution was electrospun at conditions described above.

Polycaprolactone nanofibrous reservoir layer

Polycaprolactone (PCL, Mw 80 000 g/mol, Sigma Aldrich) solution was prepared by dissolving PCL pellets in the solvent mixture acetone/ethanol (7/3 v/v) by means of overnight stirring at room temperature. The total polymer concentration was fixed at 16 wt%. Thereafter, the blended PCL polymer solution was electrospun at conditions described above.

Preparation of nanoparticles

Liposomes

Liposomes were prepared by the lipid film hydration method. The composition of liposomes was 10% DSPE-PEG, 89.5% EPC (egg phosphatidylcholine) (Avanti Polar Lipids, USA) and 0.5 molar % 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids, USA). After hydration by PBS, liposomes were extruded using a 200 nm polycarbonate filter (Millipore, USA).

Metallochelating nanoliposomes were prepared by the lipid film hydration method and were extruded using a 200 nm polycarbonate filter. The composition of liposomes was 5% DOGS-NTA-Ni-1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Avanti Polar Lipids, USA), 71% EPC, 19% POPG (1-palmitoyl-2-oleoyl-*sn*-

glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (Avanti Polar Lipids, USA). Prepared liposomes were mixed with His-tagged green fluorescent protein (GFP) (20ug GFP per 1mg of lipid). The preparation is described in detail in Masek et al [30].

PLGA-PEG nanoparticles

PLGA particles were prepared by the emulsifying method. Briefly, Poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide), Mw 55.000Da, PEG average 5.000Da (PLGA-PEG), Poly(D,L-lactide-co-glycolide) (lactide:glycolide (5:5), mol wt 30.000-60.000) (PLGA) (Sigma) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Avantilipids) were dissolved in 1ml chloroform and emulsified in 5 ml of 1% sodium deoxycholate solution using the ultrasound device SONOPULS HD 3100 (Bandelin, Germany). The particles were formed by evaporation from chloroform using a rotary evaporator. The excess of sodium deoxycholate was removed and PLGA-PEG particles were concentrated in one step using MikroKros hollow fibre modules (50 kDa) (Spectrum, USA).

Profilometry and macroscopic morphology analysis of nanofibrous mucoadhesive film

Scanning electron microscope (SEM) (Hitachi8010, Hitachi, Japan) was used to investigate the macroscopic morphology of nanofibrous mucoadhesive films, the surface texture of nanofibrous layers as well as nanoparticles adsorbed to the surfaces of fibres. A cross-section of a nanofibrous mucoadhesive film was prepared at -130°C. The sample was coated with Pt-Pd and observed by cryo-SEM at -130°C. Adsorption of fluorescent-labelled nanoparticles to nanofibres was observed by confocal microscopy (Leica SP2, Germany) and the adsorption of liposomes was observed by TEM (Philips Morgagni). Detailed measurements of the thicknesses of nanofibrous reservoir layers, texture analyses of these layers and of backing layers were performed using an optical surface metrology system (Leica DCM8, Germany) under confocal mode (50X objective).

Analysis of the diameter of nanofibrous reservoir layers

Nanofibrous reservoir layers were observed under a SEM Vega 3 (Tescan, Czech Republic) at an accelerated voltage of 20 kV. Prior to measurements, all the samples were sputter-coated (Q150R ES, Quorum Technologies Ltd., England) with gold at a thickness of 7 nm. The SEM images were analysed with NIS-Elements AR software. The average fibre diameter and its distribution were determined from 150 random fibres.

Measurement of water contact angle

The hydrophilicity of the nanofibrous reservoir layers was evaluated by water contact angle measurements using a See System E instrument (Advex Instruments, s. r. o., Czech Republic). A distilled water droplet of the size of 10 μL was placed carefully onto the surface of the nanofibres at room temperature. After a period of 20 s, the contact angle was recorded. The mean value and standard deviation (SD) were also calculated through testing at ten different positions on the same sample.

Determination of *in-vitro* release of nanoparticles from nanofibrous reservoir layer

PLGA-PEG or liposomal nanoparticles (20 mg/ml) were allowed to infiltrate nanofibrous reservoir layers (surface area of 1 cm^2) that were dipped into appropriate suspensions for 2 mins. Immediately after adsorption of particles, nanofibrous reservoir layers were cleansed of excess fluids and placed into PBS buffer (1 ml) for 30 mins. The final PBS solution of PLGA nanoparticles was diluted and the concentrations of released nanoparticles were calculated using a NanoSight 500 instrument (Malvern Instruments, UK). As an alternative method, the final PBS solution of fluorescence-labelled nanoparticles was diluted and the ratios of bound to released nanoparticles were calculated from differential fluorescence intensities measured at the emission wavelength of 560 nm using a spectrofluorimeter LS55B (Perkin Elmer).

Determination of *ex-vivo* release of nanoparticles from nanofibrous reservoir layer

The ability of the electrospun nanofibrous reservoir layer to release associated nanoparticles in real conditions (moistened mucosal tissue surfaces with limited water volumes, typical of viscous mucus layers etc.) was confirmed by 2 h incubation of nanofibrous mucoadhesive films with cryo-altered mucosa having reduced barrier functions at 37°C [45]. The release of nanoparticles from nanofibrous reservoir layers was demonstrated by observation of fluorescence signals on cross-sections of adjacent tissues by confocal microscopy. The surface of each mucosa was moistened (flow rate 0.1 ml/min) with PBS during experiments using a linear pump and tubing.

***Ex-vivo* study of penetration of nanoparticles into tissues**

Ex-vivo penetration of nanoparticles into adjacent mucosa was tested on freshly excised porcine sublingual or buccal tissues. Nanofibrous mucoadhesive films with fluorescently labelled nanoparticles were applied to mucosa and incubated for 2 h at 37°C. The surface of each mucosa was wetted as described above during the experiment.

Sublingual and buccal tissues were removed using surgical instruments immediately after slaughter of the animal. The excised tissues were transferred immediately to the laboratory in PBS buffer and were cut into cube-shaped pieces used for testing. The interval between slaughter of the animal and start of experiment did not exceed 20 mins.

***In-vivo* study of penetration of nanoparticles to adjacent mucosa and lymph node delivery**

The experiments on animals were approved by the Ethics Committee of the Veterinary Research Institute, Brno and by the Ethics Committee of the Ministry of Agriculture, Czech Republic.

Piglets (15 kg) were anesthetized with the short-term anaesthetic Zoletil (Virbac, France). Multi-layered mucoadhesive film loaded with fluorescently labelled PLGA-PEG nanoparticles in 2% sodium deoxycholate was applied to the porcine sublingual mucosa. After 2 h of incubation, animals were anesthetised and sacrificed by an *i.v.*-application of T61. Sublingual mucosa samples with nanofibrous mucoadhesive films attached and regional draining lymph nodes were excised, frozen and stored at -80°C. The tissues were sectioned using Leica Cryocut 1800 Cryostat (Leica, Germany). All sections were stained and observed by confocal microscope (Leica SP2). Nuclei were stained with SytoxBlue (Molecular Probes), Actin was stained with Alexa Fluor® 633 Phalloidin, phagocytic cells were stained with SLA Class II DR Antibody | 2E9/13 (Serotec) and goat anti-mouse secondary Alexa Fluor® 488 conjugate (Abcam). Nanoparticles were stained with lissamine-rhodamine phosphatidylethanolamine (Avanti Polar Lipids, USA).

Cryo-SEM of adhesion of nanofibrous layer onto mucosal surface

2 h post application of mucoadhesive films to piglets, cube-shaped samples of mucosal tissue with adhered mucoadhesive films were removed and cross-sectioned with Cryocut (Cryocut 1800, Leica, Austria) at -20°C. Subsequently, the samples were transferred under nitrogen atmosphere into the SEM (Hitachi SU8010, Hitachi Ltd., Japan). The tightness of adherence of the mucoadhesive layer to mucosa and the structure of nanofibrous mucoadhesive films were observed under cryo-mode by SEM at -130°C.

Cryo-alteration of porcine mucosa

Freshly excised porcine mucosa was rinsed twice with PBS buffer at room temperature. Subsequently, the rinsed sublingual mucosa samples were freeze-thawed in two cycles to -20°C. The sublingual mucosa samples were stored at -20°C until use. Cryo-altered mucosa was used as a positive control to confirm the in-situ release of nanoparticles from nanofibres.

Physicochemical analyses of nanoparticles

Nanoparticle sizes and zeta potentials were measured by dynamic light scattering (DLS) (ZetaSizer Nano ZS, Malvern, UK) at the wavelength of 633 nm. The sizes of nanoparticles and the rate of release of PLGA-PEG nanoparticles were measured using a Nanoparticle Tracking Analysis (NTA) (Nanosight 500, Malvern, UK). The samples were diluted appropriately prior to the measurement. During the NTA measurements, the camera level was set to 7. Captured videos were analysed with a detection threshold set to the value of 10. The morphology of nanoparticles was characterised using TEM. Specimens for TEM analysis were prepared by drop-casting particles on carbon coated copper grids stained with phosphomolybdic acid solution (2%) and dried at room temperature before observation. Bright field imaging was performed using TEM (Phillips 208 S, FEI, Czech Republic) operating at 80 kV.

RESULTS AND DISCUSSION

Nanofibrous mucoadhesive film

The main task for any mucoadhesive system used for mucosal delivery of drug-delivery or vaccination nanoparticles is maintaining effective nanoparticle concentrations at the given site of application for a sufficient time span to enable functional nanoparticle-mediated delivery to take place. This task can be accomplished by multi-layered nanofibrous mucoadhesive films for the controlled release and delivery of nanoparticles/macromolecules into the oral mucosa in a unidirectional fashion while avoiding nanoparticle losses from the site of the application due to wash-out by saliva (**Figure 1**).

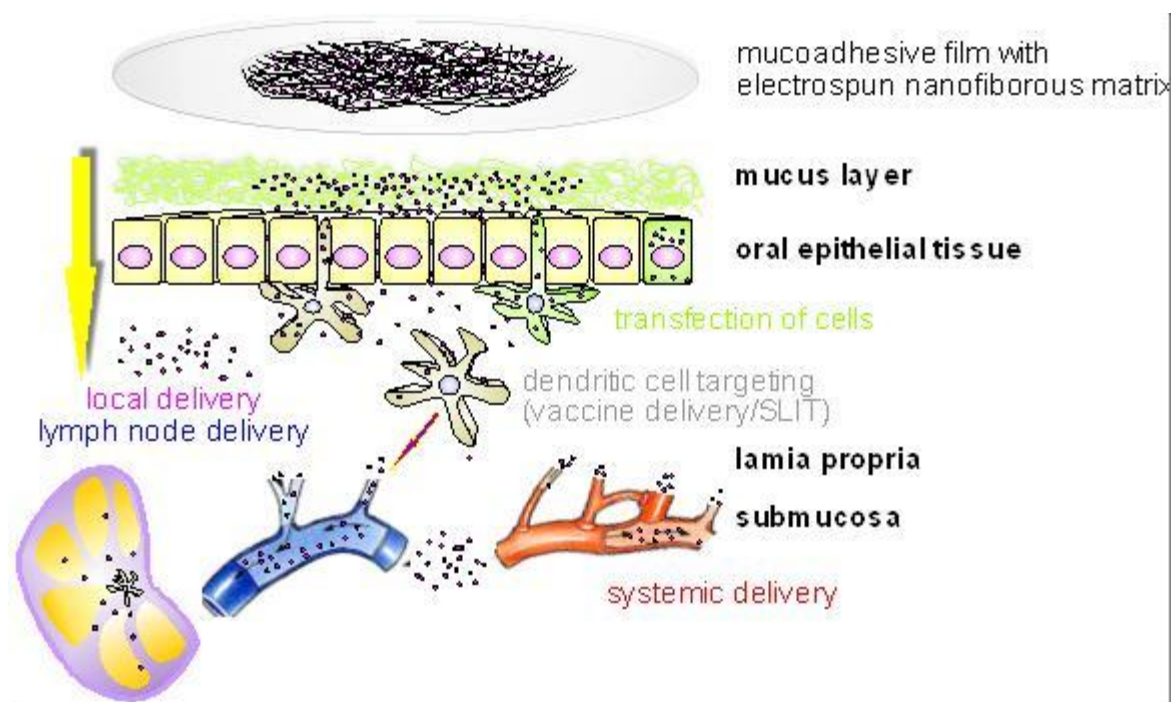


Figure 1. Schematic presentation of the principle for improving delivery of drug-delivery and vaccination nanoparticles by means of a nanofibrous mucoadhesive film

In our case, this objective was achieved by designing nanofibrous mucoadhesive films with three different layers (see **Figure 2**): a nanofibrous reservoir layer (1), a mucoadhesive layer (2), and a backing layer (3). Alternatively, an interlayer (4) could be added in between layers 2 and 3 (**Figure 2A**). The three-layered film is pictured in (**Figure 2B**) and the SEM image is shown in (**Figure 2C**). The nanofibrous layer serves as reservoir for nanoparticles attached to the surface of nanofibres or embedded into the pores between nanofibres. The mucoadhesive layer was introduced to keep the whole platform affixed at the site of its application for a prolonged time span adjusted by the degree of adhesiveness of the material used for the preparation of the mucoadhesive film layer. The backing layer was added to prevent diffusion of nanoparticles out of the site of application and to protect both the mucoadhesive layer and the nanofibrous reservoir layer from the effect of saliva and flow of mucosal fluid. Moreover, the backing layer facilitates the self-administration of the film to the oral mucosa by protecting the surface from sticky properties of the mucoadhesive film itself.

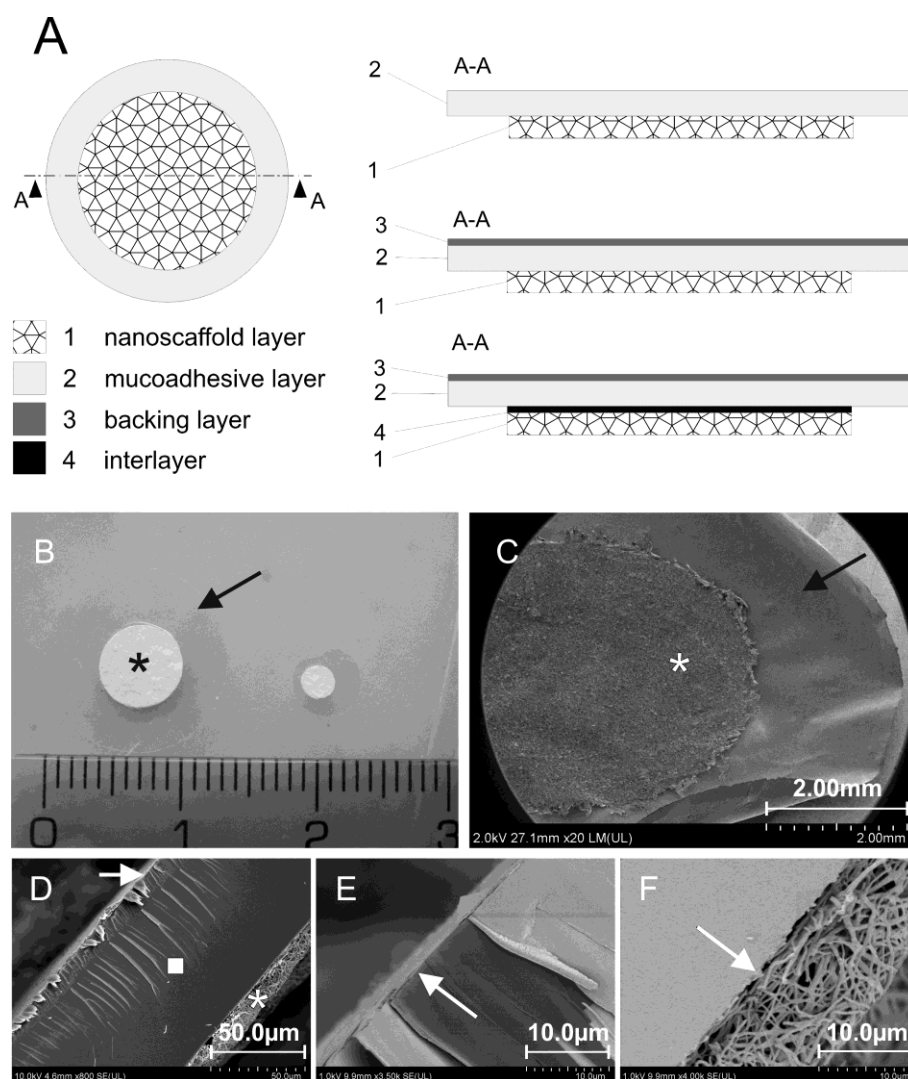


Figure 2. Schematic structure and real appearance of nanofibrous mucoadhesive film

Mucoadhesive film layer was prepared by a casting/solvent evaporation technique as described elsewhere [15] adapted for deposition of a backing layer onto the surface of a preformed mucoadhesive layer by spray-drying. In our case, we selected an oro-dissolving backing layer composed of Eudragit® L 100-55 that dissolves at pH 5.5 or higher (see Supporting information for dissolution kinetics of the backing layer). The oro-dissolving backing layer was introduced for better patient comfort since nanofibrous mucoadhesive films can dissolve or undergo erosion with time without the need for removal. By tuning the oro-dissolving properties of the backing layer, the time of adhesion of a given nanofibrous mucoadhesive film to the oral mucosa can be adjusted. Controlled thickness of 240 ± 86 nanometres can be achieved (**Figure 2D**). In contrast,

using a common solvent-casting method, the backing layer of several microns in depth is usually obtained [16-18].

The selected mucoadhesive layer was prepared with a specific composition for low swellability and a strong mucoadhesive strength, thereby ensuring sufficient mucoadhesive properties [19]. The thicknesses of our prepared mucoadhesive layers were observed to be approx. 80 μm , as measured by SEM in the cryo-mode (**Figure 2D**). Nanofibrous reservoir layers were then affixed onto these backing layer coated, pre-formed mucoadhesive layers. As observed by SEM in the cryo-mode, the structure of a given mucoadhesive layer remained essentially unchanged after coating with a backing layer by spray drying (see the arrow in **Figure 2E**), and also after attachment of a nanofibrous reservoir layer by means of a sticking procedure (see the arrow in **Figure 2F**). Profilometry was used for visualization of features of particular layers of the prepared nanofibrous mucoadhesive film (see supporting info, section B)

Electrospun nanofibrous reservoir layer

The internal features of electrospun nanofibrous reservoir layers are considered ideal for adsorption of nanoparticles and macromolecules due to their extremely high surface area and porosity, enabling a high nanoparticle to mass ratio in comparison with reservoir layers constructed from other materials [20]. For example, Jung et al. prepared a chitosan nanoparticle/polycaprone composite for sustained drug delivery intended as wound dressing material [21]. In general, well known flexibility of thin electrospun nanofibrous reservoir layers is also important for intimate contact to be established between nanofibrous mucoadhesive films and mucosal surfaces. Otherwise, adjusting the physical properties of electrospun nanofibrous reservoir layers is extremely important to achieve efficient adsorption of drug-delivery and/or vaccination nanoparticles, and their rapid/controlled release when required. Hydrophobic and ionic interactions are the dominant forces that influence binding and controlled release. Hence, we prepared nanofibrous reservoir layers with three different materials, PCL, chitosan/ PEO and SF (**Table 1, Supporting information, section C**). In addition, various treatment procedures were employed to change the surface properties of nanofibers. For example, mild sodium hydroxide or deoxycholate surfactant pre-treatment of PCL nanofibrous reservoir layers were both found to increase layer hydrophilicity (i.e. reduce the contact angle) that might otherwise be relatively hydrophobic in character (**Table 1**).

Material	Weight/m ² (g)	Contact angle (°)	Nanofibre diameter (nm)
PCL	5	118.77 ± 3.62	114.13 ± 17.87
SF	5	43.56 ± 5.07	670.22 ± 123.73
Chitosan-PEO	5	11.69 ± 3.18	169.3 ± 43.3
PCL pre-treated NaOH	5	54.04 ± 5.61	N/A*
PCL pre-treated sodium deoxycholate	5	15.1 ± 2.92	N/A*

Table 1: Physical properties of nanofibres

Application of nanofibrous mucoadhesive film to porcine mucosa

The nanofibrous mucoadhesive films used were able to adhere for at least 2 h to the sublingual region (**Figure 3A,B**). Neither adverse reactions such as local irritation observed in the course of testing, including the post-treatment period.

Tight adhesion of nanofibrous reservoir layer to oral mucosa

Our nanofibrous mucoadhesive films were also shown to adhere tightly to porcine sublingual mucosa (**Figure 3C-I**). The tight adherence of a nanofibrous mucoadhesive film to the porcine oral sublingual mucosa after 2 h incubation was confirmed by cryo-SEM on cube-shaped cross-section samples (**Figure 3C**). Mucoadhesive film is flexible enough to copy the surface of the sublingual mucosa (**Figure 3D**) and adhere firmly to it (**Figure 3E**). The benefits of such visible tight adhesion are that drug-delivery and/or vaccination nanoparticles located within the nanofibrous reservoir layer of nanofibrous mucoadhesive films are maintained in close contact with the surface of the mucosa and avoid being subject to mucosal self-cleaning effects caused by a continuous flow of saliva. Particular layers are clearly visible in (**Figure 3F**), (**Figure 3G**) showing mucoadhesive layer and SF nanofibrous reservoir layer. The layers can be well identified at the edges of the sample specimen where they were detached from the mucosal surface owing to cutting procedure during preparation of the sample. The upper layer of mucus is detached from mucosal tissue (detail in **Figure 3G**). Detailed structure of the SF nanofibrous reservoir layer adhered to the mucosal surface is shown at (**Figure 3H**). The mesh of mucin

fibers is visible in the bottom part. PLGA-PEG (poly(lactic-co-glycolic acid)-polyethylene glycol) nanoparticles are found adsorbed on the surface of nanofibers as well as permeating the mesh of mucin fibers. **Figure 3I** (detail from **Figure 3E**) represents a general view of SF nanofibrous mucoadhesive film and adjacent mucosa after 2 h incubation. Gel mucoadhesive layer can be seen below the nanofibrous layer. A mucin mesh is clearly distinguished on the surface of the mucosa in the middle part of the image. At the bottom of the image, particular epithelial cells are visible.

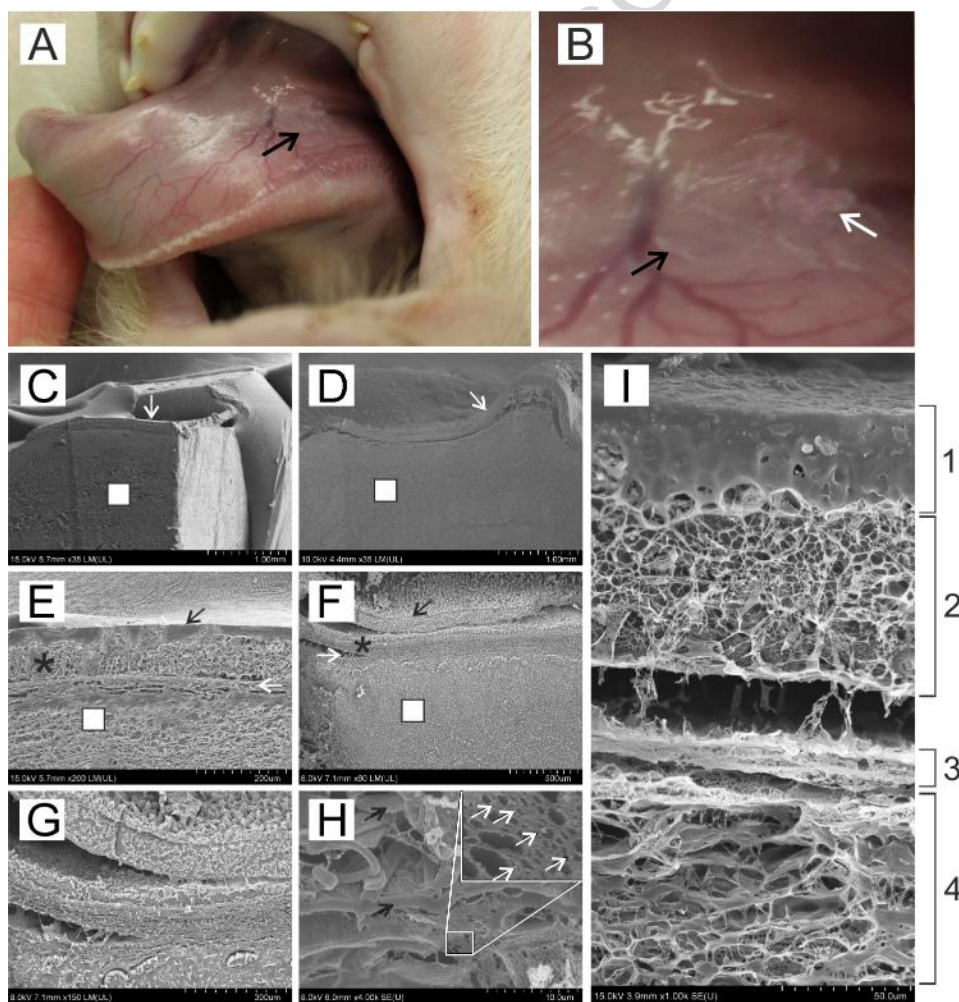


Figure 3. Scanning electron microscopy representation of tight adhesion of nanofibrous mucoadhesive film to the sublingual mucosa

Mucus penetration by drug-delivery or vaccination nanoparticles

Polymeric nanoparticles that possess a dense low-molecular weight polyethylene glycol (PEG) coating and are known as mucus penetrating particles (MPPs) with an enhanced ability to diffuse

through mucus layers [22]. MPPs penetrate through mucus by a mechanism of passive diffusion driven by concentration gradients. The dense, hydrophilic PEG coating prevents interactions of the hydrophobic nanoparticle core with mucin mesh and therefore minimises mucoadhesive interactions [23–25]. The inclusion of a PEG layer at the liposome surface also ensures mucus-penetrating properties [26]. PLGA-PEG nanoparticles and PEG containing liposomes were prepared for testing in this study. The morphology of both nanoparticles was studied using TEM. Both types of particles exhibited a regular spherical shape and were prepared as essentially monodisperse. The electron micrographs of model PLGA-PEG nanoparticles and liposomes are presented. The observed sizes of both nanoparticle types were in good agreement with DLS data as well as NTA data. Both liposomes and PLGA-PEG nanoparticles also demonstrated similar size distributions and zeta-potentials (electroneutral) (see **Supporting information, section D**).

Loading the nanofibrous reservoir layer with nanoparticles

SF nanofibrous reservoir layers were loaded with nanoparticle dispersions (liposomal or PLGA-PEG nanoparticles). Nanoparticle dispersion was applied directly to the surface of a given nanofibrous layer and were absorbed immediately and uniformly into the whole volumes of the given nanofibrous reservoir layers due to the capillary action. The process of loading affected neither physical nor morphological properties of the adjoining mucoadhesive film layer (**Figure 4A**). Strikingly, we observed a dense and homogenous covering of nanofibre surfaces by nanoparticles (PLGA-PEG or liposomes) as demonstrated by electron microscopy and confocal microscopy (**Figure 4B-F**). Furthermore, using GFP-proteoliposomes, a model for proteoliposomal vaccination nanoparticles [27–29], we were unable to observe any signs of nanoparticle aggregation, nor apparent disruption of GFP metallochelation binding to liposome surfaces [30], thereby suggesting that nanofibre adsorption is neither physically or chemically disruptive for lipid-based nanoparticle systems (**Figure 4D**).

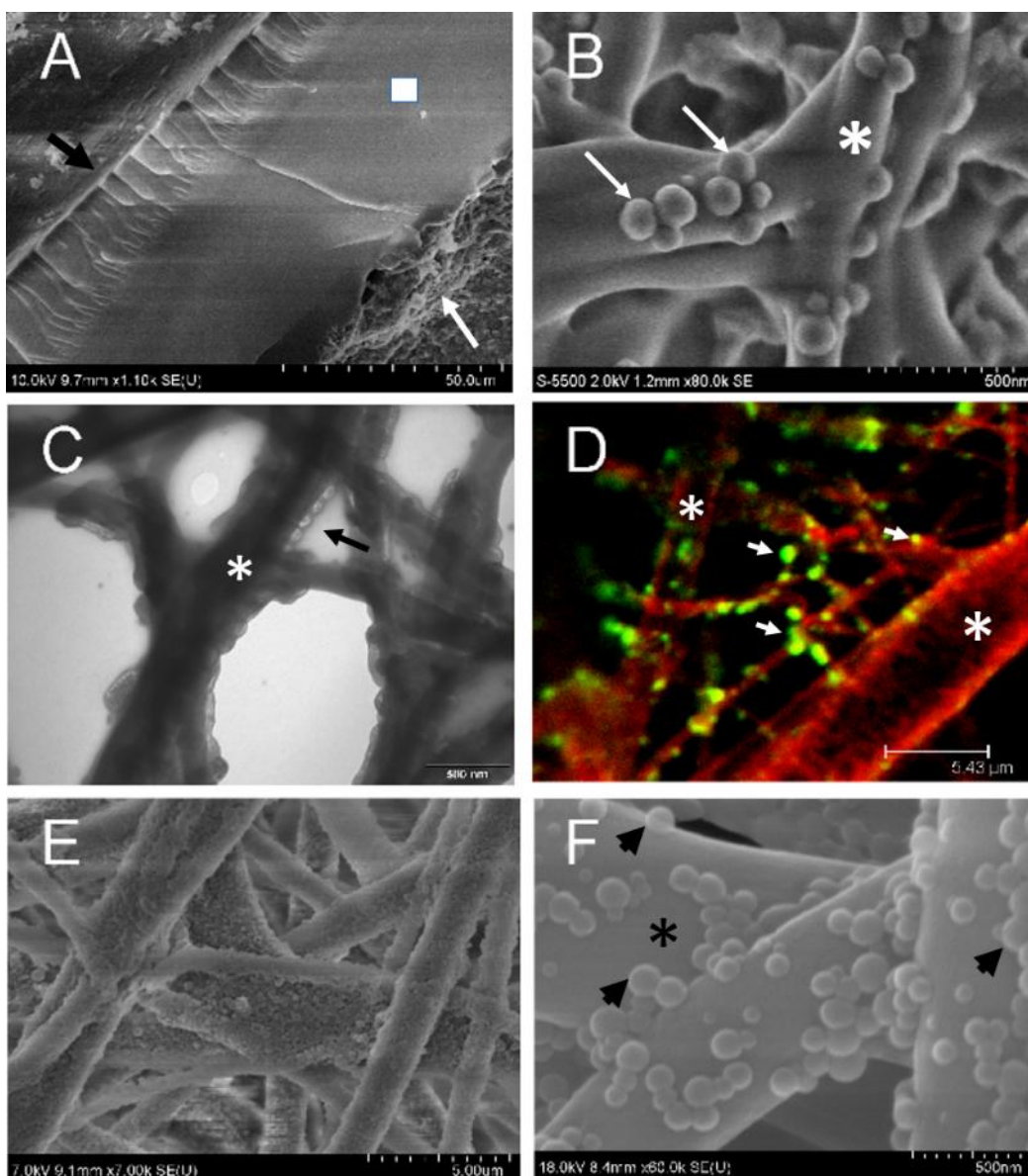


Figure 4. Liposomes and PLGA-PEG nanoparticles adsorbed to nanofibrous reservoir layer observed by cryo-SEM, SEM, TEM and Confocal Laser Scanning Microscopy

The release of nanoparticles from nanofibrous reservoir layer

The ability of nanofibrous reservoir layer to release adsorbed nanoparticles from nanofibrous reservoir layers into the surrounding milieu was demonstrated using different types of nanofibers. The tested nanofibrous layers were prepared with all three main biocompatible materials of interest, namely with PCL, chitosan-PEO, and SF polymers. In addition, PCL

nanofibrous reservoir layers were treated with 3M sodium hydroxide for 5 mins, or with sodium deoxycholate to improve the wettability of nanofibre surfaces. These procedures significantly improved wettability as reflected in a decrease in the contact angle (see **Table 1**). The extent of nanoparticle release from a given type of nanofibrous reservoir layer was determined by calculation of nanoparticle concentrations (measured by the NTA technique) after incubation of nanoparticle-loaded nanofibrous reservoir layers with PBS buffer (**Figure 5A**). Alternatively, *in-vitro* release was determined by monitoring changes in fluorescence intensity of lissamine rhodamine-labelled nanoparticles (PLGA-PEG or liposomes) after the incubation of nanoparticle-loaded nanofibrous layer in PBS buffer (see **Figure 5**). The NTA assay was found to be in good agreement with the fluorescence release assay data for both types of nanoparticles (data not shown). Moreover, cholate salts are widely used as penetration enhancers (see next paragraph), also sodium deoxycholate can perform dual role.

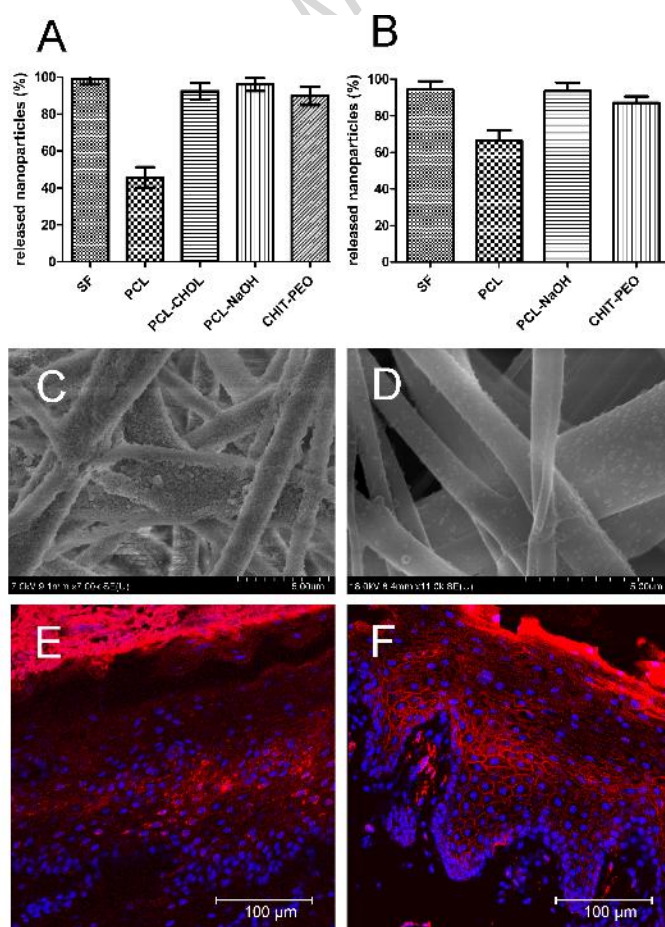


Figure 5. Determination of the quantity of released nanoparticles from reservoir nanofibrous layer *in-vitro/ex-vivo*

The extent of nanoparticle release was observed to be nanofibre dependent. Essentially quantitative release of nanoparticles was observed with both SF and chitosan-PEO nanofibers after 30 mins. On the other hand, when standard PCL nanofibers were used, then significantly lower levels of nanoparticle release were observed (approx. 50%). Thereafter, when PCL nanofibers were pre-treated with 3M sodium hydroxide solution or with 2% (w/v) sodium deoxycholate, then the levels of PLGA-PEG nanoparticle release were also near 100%. These data are in a good accordance with the reduction of a contact angle value caused by both pre-treatment procedures used (**Table 1**). The increase in hydrophilicity of the nanofiber surface (decrease of contact angle) results in weakening of hydrophobic interaction between relatively hydrophobic nanoparticles like PLGA-based nanoparticles. Therefore, such particles are deadhered more easily from the nanofiber surface and are released into the inter-fibre space and diffuse towards the mucosa. The almost complete release of PLGA-PEG nanoparticles from SF nanofibers *in-vitro* was confirmed by observation of nanoparticle-loaded nanofibers before and after the dissolution test using SEM (**Figure 5C, D**). In general, nanofibres with hydrophilic surfaces (chitosan-PEO, SF and PCL treated with NaOH) were all competent to release almost all adsorbed nanoparticles into the surrounding medium. By contrast, non-treated PCL nanofibers with rather more hydrophobic surface properties were not able to affect the quantitative release of PLGA-PEG nanoparticles or liposomes within the given incubation time (see **Figure 5A, B**).

However, the conditions occurring during an *in vitro* release test differ significantly from those occurring after the application of the nanofibrous film onto the mucosal surface *in vivo*. Therefore, the established cryo-altered mucosa test is used as an ideal model giving much less false negative results with regard to the assessment of the potential of mucosal delivery system to release the nanoparticles directly onto the site of application. Cryo-altered mucosa has limited barrier functions while maintaining its general physical properties and anatomical structure [31]. This model was used to confirm the data from *in vitro* release experiments and for testing the release of PLGA-PEG nanoparticles from electrospun nanofibrous reservoir (SF) adhered on cryo-altered mucosa *ex vivo*. The *ex-vivo* release test using cryo-altered porcine oral mucosa testified to the ability of released nanoparticles to reach the mucosal surface and penetrate into deep mucosal tissue (**Figure 5E, F**).

The penetration of fluorescently labelled PLGA-PEG nanoparticles into the epithelium of cryo-altered mucosa was investigated. Whereas the fluorescence signal comes predominantly from the upper part of the epithelial tissue in case of PLGA-PEG particles without sodium deoxycholate, incubation of cryo-altered mucosa with sodium deoxycholate makes the fluorescence signal from nanoparticles more uniform within the tissue (see **Figure 5E, F**). This observation is in good accordance with the fact that a barrier of extracellular lipids is located in the upper third of epithelium [32]. It is important to note that the mechanism of action of cholate salts lies in solubilisation of the extracellular lipids. So, this type of pharmaceutical excipient acts only if its local concentration is maintained above the critical micellar concentration [33]. This implies that the dosage form should release the nanoparticles as well as excipients in unidirectional manner, it should be in the intimate contact with oral mucosa for a prolonged period of time, and it should prevent dilution of the substances by saliva.

For subsequent *ex vivo* and *in vivo* experiments, our preference was to use SF nanofibrous mucoadhesive films.

Mucus penetration of nanoparticles and interactions with mucosal tissue – ex vivo and in vivo models

Dramatic anatomical differences are observed in buccal and sublingual mucosa among species. In general, large animals possess a non-keratinized stratified buccal mucosa, which is more similar to the anatomy of the human mucosa. In terms of availability, thickness, and permeation properties, the porcine buccal mucosa appears to be the most appropriate animal model due to the highest similarity to human mucosa [34]. By contrast, rodents possess keratinised mucosa in the sublingual region. In spite of this fact, many studies evaluating biological effects *in vivo*, including sublingual vaccination nanoparticle formulations, are performed on more accessible small animal models (mice, rat) having poorly permeable, keratinised oral mucosa [35]. In our studies we used both pig and mouse models with our nanofibrous mucoadhesive films applied to buccal and sublingual mucosa. In our case, nanofibrous mucoadhesive films were also designed to have properties appropriate for application to the oral region of mice, piglets, and even man.

Due to availability and simplicity, the evaluation of the effectiveness of transmucosal delivery of nanoparticles is often tested on *ex-vivo* tissues. As discussed above, crucial consideration for the permeation test must be adequate tissue storage and isolation before the experiment [31]. Freshly

excised mucosal tissues were used for all *ex-vivo* penetration tests. The penetration tests were started approximately 30 mins after excision and were conducted under physiological conditions in terms of temperature and with continuous moistening of the surface as described in the section Materials and Methods.

Ex-vivo study of penetration of nanoparticles to porcine oral mucosa

Ex-vivo penetration of nanoparticles to oral mucosa was tested on freshly excised porcine sublingual and buccal tissues (see the schema in Supporting information, section E). A given nanofibrous mucoadhesive film with pre-loaded nanoparticles was applied to excised mucosa and incubated for 2 h at 37°C. During the whole experiment, the surface of the mucosa was moistened with PBS buffer to simulate conditions in the oral mucosa. For control purposes, free nanoparticles were applied to excised mucosa. The difference between both routes of application was clearly distinguishable by means of the intensity of pink colouration at the site of application. Whereas nanofibrous mucoadhesive films maintained their fluorescent nanoparticles in the middle of a mucoadhesive ring during the incubation period, free nanoparticles were washed out from mucosal surface after a short time of the test and did not penetrate into mucosa at all (Supporting information, section E).

The penetration of nanoparticles into oral mucosa was confirmed by cross-sectioning of adjacent mucosa and observation with confocal microscopy (see **Figure 6**). Freshly excised sublingual and buccal oral mucosa with model PLGA-PEG nanoparticles and DSPE-PEG (distearoyl phosphatidylethanolamine-polyethylene glycol) liposomes were tested. The penetration to the epithelium of the oral mucosa was observed in all tested samples. The intensity of fluorescence reflects the concentration gradient of nanoparticles diffusing deeper into submucosal tissue. The transport of model nanoparticles in epithelium undergoes the paracellular pathway as clearly demonstrated by confocal microscopy in tissue slices. **Figure 6A** shows the paracellular transport of nanoparticles in detail.

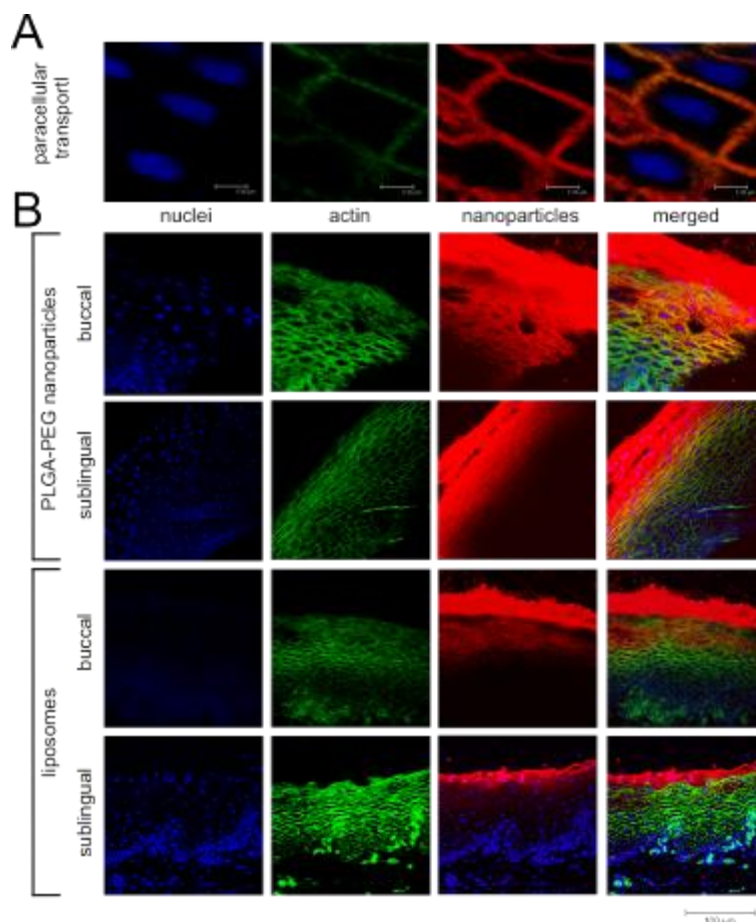


Figure 6. Oral mucosa after 2 h *ex-vivo* incubation at 37°C with nanofibrous mucoadhesive films containing rhodamine labelled nanoparticles

However, cleaning of mucosal surface by continuous production of saliva, presence of viscous mucus layer and the existence of epithelial barriers are main factors limiting the extrapolating of the results from *in vitro* dissolution test and *ex-vivo* tests to real *in vivo* conditions. Therefore, *in vivo* study of penetration of nanoparticles into adjacent mucosa was carried out using mouse and pig models.

In-vivo study of penetration of nanoparticles into adjacent mucosa in mouse model

In the case of the mouse model, administration of nanoparticles by means of a given nanofibrous mucoadhesive film resulted only rarely in specific cell-associated delivery even if a high concentration of nanoparticles was maintained on the surface of sublingual mucosa for a 2 h period without washing out by saliva (**Figure 7B, C**). This is in good agreement with the fact that rodent sublingual mucosa is highly keratinised and thus the permeability for nanoparticles is low. The presence of high concentration of specific immune cells (determined as MHC II

positive cells) in this area is clearly visible in **Figure 7A**. Accordingly, the porcine model is more appropriate for sublingual delivery experiments given the lower levels of keratinisation and the greater anatomical similarity of porcine sublingual to the human equivalent mucosa [34]. Cells responsible for the transport of nanoparticles and antigens across mouse sublingual mucosa, have been defined by Nagai and coworkers [36].

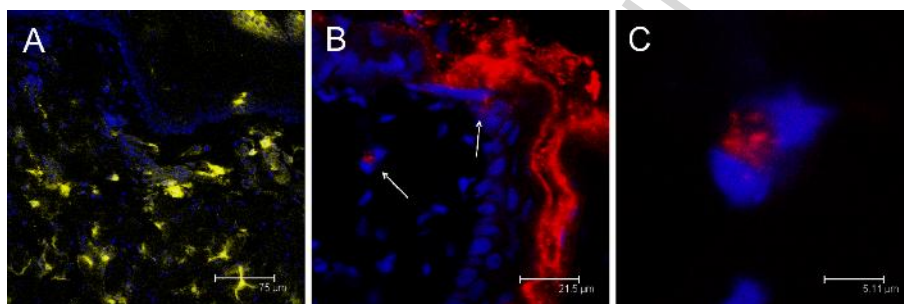


Figure 7. *In-vivo* delivery of PLGA-PEG nanoparticles to mouse sublingual mucosa

Mucosal and transmucosal transport of nanoparticles to regional lymph nodes in piglets

Owing to strong keratinisation of sublingual mucosa in mice, the pig model is more appropriate for sublingual delivery experiments because of similarity with the anatomy of human sublingual mucosa [34]. We believe that the described system is especially suitable for sublingual immunotherapy and vaccine applications. Thus, finally, the piglet model was used to evaluate the delivery of PLGA-PEG nanoparticles into lymph nodes. Sodium deoxycholate was tested in this experiment as a penetration enhancer. Endocytosis of fluorescent NPs by antigen presenting cells (APC) was demonstrated by histochemical staining of tissue sections and their observation by confocal microscopy.

Cross-sectioned slices from histological samples excised 2 h after the application of nanoparticles demonstrated penetration of nanoparticles into mucosa (**Figure 8A**) as well as into regional lymph nodes (**Figure 8B, C**). Some portion was recognised and endocytosed by Swine Leucocyte Antigens (SLA) type II positive cells (porcine antigen presenting cells, pAPC) and detected in draining regional lymph nodes (**Figure 8D, E**). Also pAPC cells with fluorescent nanoparticles were found in both B and T-cell area in lymph nodes (**Figure 8D, E**). This observation is well confirmed by a detail picture of pAPC with endocytosed fluorescent nanoparticles (**Figure 8F**). The interaction of human DCs with PLGA-PEG or liposome nanoparticles was studied *in vitro* to demonstrate their ability to endocytose them and to see their

subcellular distribution. Accumulation of fluorescent nanoparticles in the cytoplasm of human DCs was found to be similar to that in porcine DCs (see **Supporting information, section F**).

In general, successful delivery by the oromucosal route of administration is opposed by mucus and mucosal secretions (diffusion barrier) [37]. The mucosal epithelium also represents another main barrier for transport of nanoparticles and macromolecules into the submucosa (absorption barrier) [2,37]. Several strategies were introduced for prolonged or enhanced oromucosal delivery of pharmaceutical substances: mucoadhesive formulations, mucus penetrating particle formulations, fast dissolving films and co-application of penetration enhancers.

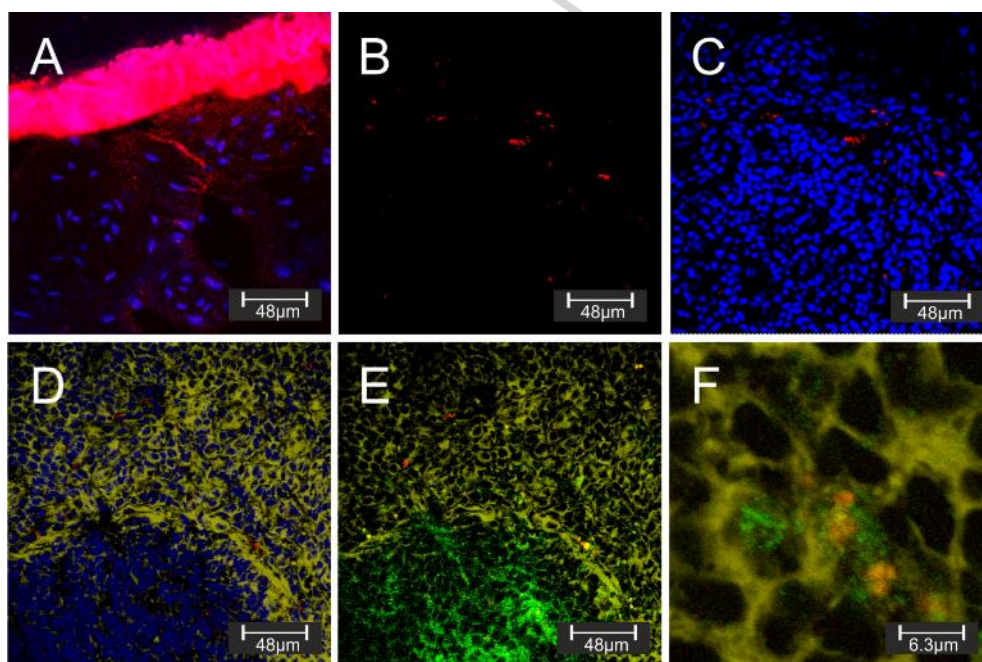


Figure 8. Lymph node delivery of PLGA-PEG nanoparticle applied onto sublingual mucosa via nanofibrous mucoadhesive films.

Thus far, PLGA-PEG nanoparticles are the most frequently used polymer nanoparticle options used for transmucosal delivery. The density of PEG coating is crucial for its mucus penetrating properties [38]. Nevertheless, MMPs have been widely used for mucosal delivery of a broad range of macromolecules, for example peptides, proteins, and nucleic acids [26]. The presence of PEG also appears to enhance the lymph node delivery of nanoparticles [23]. Such nanoparticles appear useful as vaccination nanoparticles since they can promote the transport of an

encapsulated molecular adjuvant and antigen to the draining lymph nodes. Nanoparticle-mediated accumulation of molecular adjuvants to lymph nodes should enable a significant decrease in dose of molecular adjuvants. This approach has been reported with a number of toll-like receptor (TLR) agonists, including monophosphoryl lipid A (MPLA), CpG DNA, poly (I:C), and small-molecule TLR7/8 compounds [39]. However, while PEGylation may promote tissue penetration of nanoparticles, functional delivery of molecular adjuvants and antigens to APCs can be impaired suggesting the need for nanoparticle triggerability [40] and/or targetability. In general, ideal nanoparticle for mucosal delivery of vaccines/immunotherapeutics may comprise a paradigm known as ABCD nanoparticle structure as described by A.D. Miller: antigen/immunomodulator/API payload of nanoparticle (A-component) surrounded by associated lipidic/polymeric material (B-component) forming the AB-core. The AB-core requires shielding coatings protecting from interactions of nanoparticle with mucus (C-component) and, finally, targeting layer (D-components—specific ligands e.g. mannan, anti-DEC 205, TLR agonists, etc.) might be added to target/activate specific immune dendritic cells [41].

The above mentioned paradigm is perfectly suited for liposomes, the first nanoparticles which received FDA (Food and Drug Administration) approval for human application and have appeared as marketed products, also represent versatile biocompatible systems composed of biological lipids. Besides albumin-based nanoparticles and monoclonal antibodies (also included in nanoparticles), liposomes remain only drug nanocarriers approved by FDA for human applications. Factors such as the size, the surface charge, and surface modification, lamellarity, presence of targeting moieties etc. influence particles biodistribution and their fate in an organism. Liposomes are unique concerning to their ability to co-encapsulate drugs and antigens of various physical-chemical properties (*e.g.*, molecular weight, hydrophobicity, charge and shape) in one self-assembly system. The inclusion of a PEG layer in the liposome surface also ensures mucus-penetrating properties [26]. Effective delivery of nanoparticles across the epithelium is a great challenge, too, and in fact the transfer of nanoparticles across the epithelium requires paracellular pathways involving the penetration through extracellular matrix. This process limits the velocity of diffusion across the epithelium. A frequent strategy to overcome the epithelium barrier is co-administration of nanoparticles with absorption enhancers [33,42]. Cholate salts are examples of the most commonly used enhancers [34]. Accordingly, we tested the mucosal penetration of PLGA-PEG nanoparticles after loading into our nanofibrous

mucoadhesive films containing a mixture of 2% deoxycholate sodium. Subsequent data demonstrated unidirectional penetration of nanoparticles into porcine submucosa *in vivo* and their internalisation into immune cells (see **Figure 6, 7, 8**).

Nanofibrous mucoadhesive films seem to be an ideal dosage form for nanoparticle-based vaccine delivery systems. As reviewed by Dukhin and Labib, the pathway of antigens and nanoparticles to a lymph node can be divided into particular steps. The transport across mucus layer (1), across epithelium and its barriers (2), transport through the interstitial fluids of submucosa (3), and transport through lymphatic capillaries to a lymph node (4). As the first three steps are driven by passive diffusion, the transport through lymphatic ways is driven by convective diffusion. This explains why nanoparticles were observed in the lymph nodes after only a few hours post mucosal administration [32]. With respect to vaccines, the endocytosis of nanoparticles by APCs and their active translocation to draining lymph nodes also represents another important mechanism involved in the penetration of nanoparticles through mucosal tissue. Accordingly, this high adsorption loading capacity ensures high concentration of nanoparticles to be reached after the rapid release from reservoir layer to the limited volume of the fluids at the application site. This concentration gradient then exerts a “pressure” on the mucosal layer so rapidly enabling the formation of a nanoparticle diffusion potential across the mucosal surface into the submucosa (**Figure 1, 8A, supporting information, section E**).

Dendritic cells present in the submucosa are then free to capture nanoparticles for delivery to the local lymphatic nodes that drain the submucosal zone of application [44]. Nanoparticles not captured by DCs, are otherwise free to diffuse through the submucosa reaching lymphatic capillaries by means of which they drift to the local lymph nodes for capture by professional APCs [32].

The surface adhesive properties of the nanofibres in our nanofibrous reservoir layers can be modified by virtue of the very materials used to prepare nanofibrous reservoir layers or by downstream treatment [20,21] (**Figure 5**). Such variations of surface properties, either inherent or chemically induced, ensure that the nanoparticle binding and release characteristics of nanofibrous reservoir layers can in principle be tailor-made or adapted according to the requirements of the corresponding nanoparticle delivery system being used for high concentration binding and chemical potential driven release into the submucosa.

Taking the nanofibrous mucoadhesive film as a whole, if a nanofibrous reservoir layer is central to nanoparticle binding and release, then the properties of the other components should also be optimal. Thus the swelling properties of the neighbouring mucoadhesive film layer should have no direct effect on the binding and release of nanoparticles directed to the mucosal surface. In contrast to our own observations, others have reported that nanoparticles can be incorporated into mucoadhesive film layer and experience a delay in release through the mucosa caused by gradual swelling of the mucoadhesive film and diffusion-limitations [18]. Otherwise, the mucoadhesive film layer needs to be prepared to resist movements of the tongue and the extensive production of saliva by sublingual glands. Furthermore, motional stress is more pronounced in the sublingual region as compared to the buccal mucosal region. Thus, nanofibrous mucoadhesive films need to be particularly flexible and thin with sufficient mucoadhesive properties to maintain adherence. The protective backing layer can be modified in the mean of solubility according to specific demands without any influence on the release rate of nanoparticles, too. The shape, composition, surface area and thickness of mucoadhesive layers all need to be optimized to achieve the desired mucoadhesive properties and time span of adhesion. As demonstrated in our study, mechanical and physical-chemical properties of nanofibrous materials predetermine their application for development and production of mucosal drug delivery systems. Especially, nanoparticle drug delivery systems and vaccines are applications of great potential with respect to non-invasive mucosal applications. Such systems can be superior to gel materials with respect to compatibility with a broad range of material intended for incorporation, technology of production and storage conditions.

CONCLUSION

Overcoming the barriers to successful carriage of drug-delivery and vaccination nanoparticles into submucosal tissues and draining lymphatic nodes is a prerequisite for many new drug and vaccination opportunities. Here we demonstrate on an *ex vivo* and *in vivo* pig model how electrospun nanofibrous mucoadhesive films can serve as protective nanoparticle reservoirs for the controlled and sustained delivery of nanoparticles into submucosal tissue and draining lymphatic node sites.

Furthermore, our electrospun nanofibrous mucoadhesive films can in principle be variously prepared from different polymeric materials and surface functionalised for use with many

different types of nanoparticles. Liposomes, viruses and virus-like particles, polymeric and lipid-based nanoparticles, biopolymers (e.g. protein and peptide antigens, plasmid DNA, polysaccharides), molecular adjuvants and pharmaceutical excipients (e.g. enhancers of penetration, mucolytics and cryoprotectants) can be combined with nanofibrous materials to develop appropriate product for non-invasive mucosal application.

Owing to the potential versatility of our presented systems, we anticipate future applications in the development of non-invasive sublingual vaccines and immunotherapeutics as well as in enabling the use of therapeutic drug-delivery nanoparticles in the oromucosal environment. The development of industrial-scale production of our nanofibrous mucoadhesive films is now in progress. We propose to describe this technology going forward as “printed vaccines technology” and testing of this system with some vaccine formulations like influenza and papilloma virus are being in progress.

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FIGURE CAPTIONS AND TABLES

Figure 1. Schematic presentation of the principle for improving delivery of drug-delivery and vaccination nanoparticles by means of a nanofibrous mucoadhesive film

High adsorption loading capacity of nanofibrous material ensures a high concentration of nanoparticles to be reached after the rapid release from reservoir layer to the limited volume of the fluids at the application site. Protective backing layer prevents removal of nanoparticles from the site of administration by flow of mucosal secretions and saliva. A concentration gradient is formed, which then exerts a “pressure” on the mucosal layer, thus rapidly enabling the formation of a nanoparticle diffusion potential across the mucosal surface into the submucosa. The different fate of nanoparticles (local/systemic delivery) is based on their physicochemical properties and presence of targeting moieties. Dendritic cells (DCs) present in the submucosa are then free to capture vaccination nanoparticles for delivery to the local lymph nodes that drain the submucosal zone of application. Vaccination nanoparticles not captured by DCs, are otherwise free to diffuse through the submucosa reaching lymphatic capillaries by means of which they drift to the local lymph nodes for capture by professional antigen-presenting cells.

Figure 2. Schematic structure and real appearance of nanofibrous mucoadhesive film

A: The scheme shows the bottom view (left) and cross-section (right) of possible variants for construction of nanofibrous mucoadhesive film; B: Photograph of nanofibrous mucoadhesive film, left – design for large animal experiments (pig), and right – design for small animal experiments (mice) (centimetre scale). Nanofibrous reservoir layer (asterisk), mucoadhesive layer (arrow); C: Scanning electron microscopy (SEM) picture showing individual layers of a three-layered nanofibrous mucoadhesive film. The mucoadhesive layer creates a peripheral adhesive ring surrounding the central part on which the electrospun nanofibrous reservoir layer is fixed. Nanofibrous reservoir layer (asterisk), mucoadhesive layer (arrow); D: Cross-section of mucoadhesive layer observed in its native state after freezing (cryo-SEM). Nanofibrous reservoir layer (asterisk), mucoadhesive layer (square) and backing layer (arrow); E: Detail of cross-section of nanofibrous mucoadhesive film, arrow indicates the Eudragit® L 100-55 backing layer (arrow); F: Detail of cross-section of nanofibrous mucoadhesive film, arrow indicates the connection of the mucoadhesive layer and the nanofibrous reservoir layer.

Figure 3. Scanning electron microscopy representation of tight adhesion of nanofibrous mucoadhesive film to the sublingual mucosa

A: Application of nanofibrous mucoadhesive film with SF nanofibrous reservoir layer to porcine sublingual mucosa. Black arrow indicates mucoadhesive layer 2h after the administration. B: Detail of mucoadhesive film. Black arrow indicates mucoadhesive layer, white arrow indicates PLGA-PEG loaded nanofibrous reservoir layer. C-I: Adhered film after 2 h of contact incubation with porcine sublingual mucosa as observed by Cryo-SEM. C: A cube-like sample of sublingual tissue with a nanofibrous mucoadhesive film in a cryo-SEM chamber at -130°C; D: tight adhesion of a nanofibrous reservoir layer to sublingual mucosa; E: A detailed picture of tight adhesion; F & G; Illustrative pictures with clearly visible mucoadhesive layer (upper) and SF nanofibrous reservoir layer (lower) detached from the mucosal surface during the sample preparation; H: detailed picture of SF nanofibrous reservoir layer adhered to the mucosal surface (nanofibres marked by black arrows). The mesh of mucin fibres is visible in the bottom part. PLGA-PEG (poly(lactic-co-glycolic acid)-polyethylene glycol) nanoparticles are found adsorbed on the surface of nanofibres as well as permeating the mesh of mucin fibres (white arrows in the detailed inset); I: A general picture of SF nanofibrous mucoadhesive film and adjacent mucosa after 2 h incubation. A residue of mucoadhesive gel layer is seen above the nanofibrous layer. The gap between the nanofibrous reservoir layer and the mucosa is an artefact generated during sample preparation. A mucin mesh is clearly distinguished on the surface of the mucosa in the middle part of the image. At the bottom of the image, particular epithelial cells are visible. 1) Residue of removed backing and mucoadhesive layers; 2) nanofibre reservoir layer; 3) layer of mucin; 4) surface layer of mucosal epithelium tissue.

Figure 4. Liposomes and PLGA-PEG nanoparticles adsorbed to nanofibrous reservoir layer observed by cryo-SEM, SEM, TEM and Confocal Laser Scanning Microscopy

A: Cross-section of an SF nanofibrous mucoadhesive film with liposome-loaded reservoir layer (cryo-SEM). Backing layer (black arrow), mucoadhesive layer (white square) and the nanofibrous reservoir layer (white arrow); B: Detail of SF nanofibrous reservoir layer loaded with liposomes (SEM, deceleration mode). Liposomes (white arrow), nanofiber (asterisk). Note the spherical shape of liposomes adsorbed on nanofibres and the fact that their aqueous internal cavities are intact in spite of being in vacuum for 10 mins or more; C: Detail of liposomes

adsorbed to the PCL nanofibrous reservoir layer (TEM). Liposomes (white arrow) nanofiber (asterisk). Note the appearance of liposomes as hollow spheres demonstrating their preserved vesicular structure. The shell-like structures of liposomes with aqueous interiors are clearly visible; D: GFP-proteoliposomes (green) adsorbed to PCL nanofibrous layer (red) (nanofibres were counterstained with fluorescent lipid lissamine rhodamine-PE). Liposomes (white arrow), nanofiber (asterisk); E: SF nanofibrous reservoir layer with adsorbed PLGA-PEG nanoparticles (SEM); F: Detailed picture of PLGA-PEG nanoparticles adsorbed onto the surface of the SF nanofibrous reservoir layer (SEM). PLGA-PEG nanoparticles (black arrowheads) and nanofiber (black asterisk).

Figure 5. Determination of the quantity of released nanoparticles from reservoir nanofibrous layer *in-vitro/ex-vivo*

A: Extent of liposome release from different types of nanofibrous reservoir layer determined by means of the modified dissolution test; B: Extent of PLGA-PEG nanoparticle release from different types of nanofibrous reservoir layer by modified dissolution test; C: Representative picture of PLGA-PEG loaded onto an SF nanofibrous reservoir layer as observed by SEM before the dissolution test; D: Representative image of SF nanofibrous layer after the dissolution test; E: Cross-section of cryo-altered porcine sublingual mucosa with PLGA-PEG nanoparticles (red) penetrated into mucosal tissue after 2 h incubation with a nanofibrous mucoadhesive film; F: Cross-section of cryo-altered sublingual mucosa penetrated with PLGA-PEG nanoparticles (red) with 2% sodium deoxycholate after 2 h exposition.

Figure 6. Oral mucosa after 2 h *ex-vivo* incubation at 37°C with nanofibrous mucoadhesive films containing rhodamine labelled nanoparticles; A: Images demonstrating the paracellular pathway of nanoparticle penetration through porcine sublingual epithelium (PLGA-PEG nanoparticles were used throughout); B. Penetration of PLGA-PEG nanoparticles and liposomes into porcine oral mucosa tissue after release from nanofibrous mucoadhesive films.

Figure 7. *In-vivo* delivery of PLGA-PEG nanoparticles to mouse sublingual mucosa; A: MHC II positive cells (yellow-green stained cells) in sublingual region of mice. Note a dense population of sublingual mucosa by APC (bar 75µm); B: The deposit of nanoparticles (red) on the surface of sublingual mucosa after 2 h. The arrows indicate nanoparticles internalised in

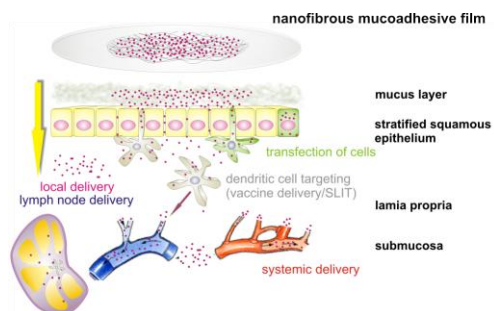
specialised APC cells. No free diffusion of nanoparticles across the keratinised sublingual mucosa in mice was observed (bar 21.5µm); C: Detail of internalised nanoparticles (bar 5.11µm).

Figure 8. Lymph node delivery of PLGA-PEG nanoparticle applied onto sublingual mucosa via nanofibrous mucoadhesive films; A: Cross-section of porcine sublingual mucosa after 2 h incubation *in-vivo*. PLGA-PEG loaded nanofibrous mucoadhesive film (red layer), PLGA-PEG nanoparticles (red dots) penetrating through sublingual tissue, nuclei of epithelial cell (blue); B: Regional lymph node. PLGA-PEG nanoparticles (red) on the cross-section of a regional lymph node (cortex); C: PLGA-PEG nanoparticles (red) on the cross-section of a regional lymph node (cortex), cell nuclei were counterstained by Sytox Blue; D: Regional lymph node. PLGA-PEG particles (red) in the cortex (T-cell area) of a regional lymph node. In the bottom part of the picture, a primary lymph nodule (folliculi lymphatici corticales) is clearly distinguished. Yellow-actin, blue-nuclei; E: Regional lymph node. PLGA-PEG particles (red) in the cortex of a regional lymph node. A high number of SLA II-positive B-cells is typical of a primary lymph nodule. pAPC in cortex containing fluorescent nanoparticles (red); F: Detailed picture of pAPC (green) transporting endocytosed fluorescent PLGA-PEG nanoparticles (red) into T-cell region in a lymph node. pAPC was stained by monoclonal antibody against SLA. Stromal cells (yellow-green) in the cortex are stained by antibodies against actin.

Material	Weight/m ² (g)	Contact angle (°)	Nanofibre diameter (nm)
PCL	5	118.77 ± 3.62	114.13 ± 17.87
SF	5	43.56 ± 5.07	670.22 ± 123.73
Chitosan-PEO	5	11.69 ± 3.18	169.3 ± 43.3
PCL pre-treated NaOH	5	54.04 ± 5.61	N/A*
PCL pre-treated sodium deoxycholate	5	15.1 ± 2.92	N/A*

Table 1: Physical properties of nanofibres

* refers to diameter of untreated PCL nanofibres.



Graphical abstract