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Methods to determine the interactions of micro- and nanoparticles with mucus

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Abstract

The present review provides an overview of methods and techniques for studying interactions of micro- and nanoparticulate drug delivery system with mucus. Nanocarriers trapped by mucus are featuring a change in particle size and zeta potential that can be utilized to predict their mucus permeation behavior. Furthermore, interactions between nanoparticulate drug delivery systems and mucus layer modify the viscoelasticity of mucus which can be detected via rheological studies and quartz crystal microbalance with dissipation monitoring (QCM-D) analysis. To have a closer look at molecular interactions between drug carrier and mucus small-angle neutron scattering (SANS) is an appropriate analysis technique. Moreover, different methods to determine particle diffusion in mucus such as the newly established Transwell diffusion system, rotating silicone tube technique, multiple-particle tracking (MPT) and diffusion NMR are summarized within this review. The explanations and discussed pros and cons of collated methods and techniques should provide a good starting point for all those looking forward to move in this interesting field.

Key words: mucus purification, mucus interaction, mucus penetration, in vitro systems

1. Introduction

Drug delivery systems have in many cases to cross several biological barriers in order to reach their site of action. One of these biological barriers is the dynamic semipermeable mucus layer. Mucus is a complex aqueous mixture of glycoproteins, lipids and salts covering many epithelial surfaces like the gastrointestinal tract (GI-tract), the vagina, the lung, the eye and various others in the human body. The viscous, elastic and sticky mucus layer that lines all these mucosal tissues protects the underlying epithelia by rapidly trapping and removing foreign excipients [1]. Drug delivery systems including micro- and nanoparticles are trapped in mucus layers by steric hindrances or adhesion processes. The limited permeability of particles through the mucus barrier leads in case of a rapid mucus turnover such as it is the case in the GI-tract to their rapid clearance from the delivery site and in many cases particles do not reach their target at all. In contrast, on mucosal tissues with a slow mucus turn over even mucoadhesive particles seem advantageous [2]. A profound knowledge about methods and techniques allowing a precise and accurate evaluation of mucus particle interactions is consequently substantial for the design and development of more efficient drug delivery systems.

According to our knowledge different methods and techniques are used by various research groups to analyze the behavior of drug carriers in the mucus network. This review provides an overview about currently available as well as novel analytical methods for the evaluation of the fate of particulate delivery systems in mucus layers. Furthermore, it shall contribute to a harmonization of different techniques allowing a better comparison of results obtained by various research groups. During the last two years experts in the field of micro- and nanoparticulate drug delivery systems have intensively collaborated with experts in the field of mucus within the EU-project ALEXANDER [3]. They have utilized various mucus permeation techniques, have compared results obtained by different methods and techniques

with each other and improved these techniques due to their gained knowledge. Within this review their know-how and experience about various methods and techniques to study the mucus permeation and mucoadhesion behavior of micro- and nanoparticulate drug carrier systems is summarized.

2. In vitro methods

2.1 Intestinal mucus collection and purification

For all particle mucus interaction studies the type of mucus and its preparation is crucial. Several sources of mucus have been used for permeation / transport studies ranging from native mucus gels, scraped gently from the surface of gastrointestinal mucosa of the pig [4, 5] to horse bronchial mucus [6]. Mucins are the major gel forming components of mucus gels which have a major role in governing the pore size in a mucus gel so many permeation studies have used mucins to mimic the mucus gel barrier [7, 8]. Unfortunately several groups have used mucin from porcine stomach type II from Sigma Aldrich. This preparation has been exposed to proteolytic action during isolation and has not been fully separated from co-isolated contaminated proteins, lipids etc. The mucin structure has been irreversibly altered and will not form a gel when concentrated to levels x4 that present in the native mucus gel. Attempts have been made to purify sigma mucin [9] by ethanol precipitation, however, this will not restore its gel forming properties as much of the mucin polymeric structure (essential for gel formation) was lost by the action of the proteases. Due to the lack of rheological properties, attempts have been made to generate a biosimilar mucus using sigma pig mucin [10] by adding lipid, bovine serum albumin and poly acrylic acid. Although this results in similar rheological moduli (e.g. G' and G'') to porcine intestinal mucus, the charge on the gel, its interactions and size filtering properties will be different. In order to isolate native mucins to use in permeation studies several purification methods have been used Li et al. [11] have

partially purified the mucin from pig stomach however, they used a method without steps taken to prevent proteolysis which would degrade the mucin leading to loss of gel forming properties.

The most effective method for isolating native mucin involves solubilization in a proteolytic inhibitor cocktail followed by fractionation in a CsCl equilibrium density gradient [12, 13]. This method produces mucins which are still gel forming [14]. Other groups have included 4 – 6 M guanidinium hydrochloride in the extraction solution and in the CsCl gradient [15, 16]. The problem with this is that it is a chaotropic agent and will denature the mucins and reduce their ability to form a gel. Studies by Georgiades et al. have shown that GuHCl disassembles the semi dilute network structure of porcine gastric mucin solutions by unfolding proteins and disrupting non-covalent interactions [17]. In studies where it is necessary to visualize particles in a scraped mucus gel or the gel contains food particles and other debris, the gel can be cleaned up by gently stirring for one hour at 4 °C in sodium chloride (0.1M) (1:5 w/v mucus to sodium chloride) and then centrifuged (10,400g at 10 °C) for 2 hours. This procedure can be repeated several times to aid in the collection of visually clean mucus.

Finally, studies can be carried out using mucus producing cell models e.g. HT29-MTX cell line [14] which allow measurement of permeation and uptake however there is a note of caution as these are cancer cell lines and therefore absorption properties may be different from normal and it is well documented that mucins produced by cancer cells have shorter carbohydrate chains [18] and this could alter the gel structure.

2.2 Mucus nanoparticle interaction studies via zeta potential and particle size measurements

Mucus main components independently of its origin are glycoproteins, lipids, water, detached epithelial cells, electrolytes and bacteria, however, the exact composition may differ regulated

by its physiological or mechanical role [19]. The contact between negatively charged mucus and nanoparticulate drug delivery systems can influence the physico-chemical properties of such nanoparticles. To analyze these interactions different methods are available. One of these methods is based on an assay developed within the ALEXANDER project, where surface properties of charged nanoparticles are tested after incubation with intestinal mucus [13]. In brief, a nanoparticle suspension is mixed with diluted native mucus following an incubation period over 4 hour at 37 °C and 300 rpm. At predefined time points samples are analyzed relating to their change in particle size and zeta potential. These alterations in surface properties of nanoparticles after incubation in mucus should give information about a possible adsorption of mucus to the particles indicated by a change in zeta potential as well as an increase in particle size. Results should help to clarify transport phenomena [20].

Pereira et al. could prove with this method that negatively charged nanoparticles exhibit in contrast to positively charged particles no physico-chemical modifications after incubation with mucus [13]. The positively charged chondroitin sulfate-chitosan nanoparticles instead showed an increase in particles size as well as a decrease of the zeta-potential over the experimental period. The negatively charged mucus might coat the positively charged particles with a layer owing to electrostatic interactions resulting in alterations of these particle physico-chemical properties.

A study by Dawson et al. observed, after incubation of carboxylated polystyrene particles and cationic poly(D,L-lactic-co-glycolic)acid nanoparticles in pig gastric mucin, similar negative zeta-potentials for these two nanoparticle formulations [20]. The authors concluded that mucus components adsorb easily positively as well as negatively charged nanoparticles.

In a study from Nafee et al. the interaction between solid lipid nanoparticles (SLNs) and mucin were investigated related to binding SLNs to superficial mucus [21]. If the particle size of SLNs did not change after incubation the research group concluded a lack of interaction

and therefore the possibility for the particles to penetrate through mucus. The negatively charged SLNs retained in particle size over the experimental period.

Evaluating the mucoadhesion of polymers, Takeuchi et al. used the described technique the other way around [22]. Commercial available porcine mucin particles were suspended in buffer and mixed with various polymer solutions. The zeta potential of the mucin particles was evaluated relating to mucoadhesion of the polymers. They could demonstrate that the addition of several types of chitosan and Carbopol changed the zeta potential of the mucin particles; whereby the addition of hydroxypropylmethylcellulose had no effect on the zeta potential. The incorporation of cationic chitosan to the negatively charged mucin particles converted the zeta potential of the mixture to less negative values according to the chitosan concentration. They suggested that chitosan as well as Carbopol polymers have a high affinity to the mucin particles [22]. Furthermore, this method can be used to assess the stability of nanoparticles in biological fluids containing various proteins and enzymes [23].

2.3 Mucus nanoparticle interaction studies via rheological measurements

An integrated structure of biopolymers describes the mucus at the chemical level [24]. The physical behavior of this structure is a complex non-Newtonian, thixotropic gel. According to shear stress mucus acts as a viscous liquid or an elastic solid. The rheological properties of mucus differ as a function of shear stress, time rate of shearing and length scale [24].

Rheological synergism has been proposed as an *in vitro* parameter to determine the mucoadhesive properties of polymers: the higher the rheological synergism, the stronger the particle interactions with mucin [25].

Barthelmes et al. for instance tested the interactions of thiolated and unmodified poly(acrylic acid) as well as chitosan nanoparticles with vesical mucin on a plate-plate combination rheometer [2]. After the addition of mucus to the particles, an immediate increase in viscosity

especially for cationic thiolated chitosan nanoparticles was observed. The increase in viscosity was based on disulfide bond formation between the free thiol groups on the particle surface and cysteine-rich subdomains of glycoproteins within the intravesical mucus layer.

Müller et al. used rheological investigations to determine the capacity of proteolytic excipients to cleave mucoglycoproteins being responsible for the integrity of the mucus [26].

Mucolytic enzymes, such as papain, disintegrate the mucus resulting in a decrease of the viscoelastic properties of the mucus layer, which can be determined by rheological experiments. As illustrated in Fig. 1, they have proven that after 6 hours of incubation with mucus, nanoparticles containing papain on their surface led to a liquefaction of the mucus, whereas nanoparticles without papain retained a relative mucus viscosity of 70% [26].

2.4 Mucus permeation studies

2.4.1 Transwell-Snapwell system

Permeation studies across freshly excised tissue are commonly performed with Ussing chambers [27]. Norris et al. adapted the Ussing chamber system with Snapwell rings and filters obtaining a vertical layer for mucus between the donor and acceptor chambers as is illustrated in Fig. 2 [28]. The three compartment diffusion system is thereby based on a donor and acceptor compartment separated by a central compartment containing a vertical layer of mucus. The mucus layer is surrounded with two permeable filters attached to the Snapwell ring to allow particles to permeate through the mucus. Modified Transwell-Snapwell diffusion chambers were used from Müller et al. [26]. Both chambers containing a volume of 2 ml and a diffusion area of 2.14 cm^2 are used to analyze the transport of labeled nanoparticles in mucus. The central compartment surrounded with two permeable polycarbonate filters is filled with 100 μl of freshly prepared natural mucus, attached to the Snapwell ring located between the donor and acceptor compartment. The diffusion chamber system is kept in an

incubator at 37°C for the experimental period and the diffused content of particles or drugs can be analyzed by spectrophotometry or chromatography.

A study performed by Müller et al. utilizing this system demonstrated that the immobilization of the mucolytic enzyme papain on the poly(acrylic acid) nanoparticle surface had a significant influence on the particle transport across porcine mucus by cleavage of mucoglycoprotein substructures [26, 29]. The mucolytic enzyme modification of poly(acrylic acid) nanoparticles led to a 3.0-fold increased diffusion rate in comparison to unmodified poly(acrylic acid) nanoparticles.

Furthermore, Laffleur et al. could demonstrate with this system that ‘slippery’ neutral nanoparticles prepared via ionic interaction between the anionic polymer poly(acrylic acid) and the cationic polymer poly(allylamine) exhibited a high diffusion efficiency [30]. The neutral nanoparticles were compared with poly(allylamine) nanoparticles and poly(acrylic acid) nanoparticles prepared by ionic gelation with tripolyphosphate and calcium ions, respectively.

However, the central compartment of the Transwell-Snapwell system containing the vertical mucus layer is separated from the donor and the acceptor compartment by filters [28]. Due to the separation of the mucus layer from the donor chamber the Transwell Snapwell diffusion system has an additional barrier for particles compared to the human gastrointestinal passage. This circumstance is a main drawback of this method as a direct contact of particles with the mucus layer is avoided via the permeable filter and therefore the simulation of the in vivo situation is limited.

2.4.2 Transwell diffusion system

One system to determine mucus diffusion is the Side-by-Side® diffusion apparatus consisting one donor and one receiver as well as a custom membrane holder [31]. The membrane holder

is placed between the two compartments containing the mucus separated between two drug-permeable membranes [19]. In contrast to the Side-by-Side® diffusion apparatus and the Transwell Snapwell system, a novel established method to study the diffusion in mucus is the Transwell diffusion system, a two compartment system [32]. A 24-well plate represents the acceptor compartment and additional 24 inserts the donor compartments, which are separated from each other by a membrane covered with 50 mg of fresh mucus as it is illustrated in Fig. 3. This membrane, occupying a surface of 33.6 mm², is suitable to spread the mucus on its surface, but impermeable for the mucus and allows drugs or particles to permeate. Samples can be taken from the acceptor chamber at predetermined time points and the diffused content of particles or drugs is analyzed by spectrophotometry or chromatography.

Friedl et al. have already established this system with self-emulsifying drug delivery systems (SNEDDS) within a sufficient experimental runtime over 4 hours [32]. They demonstrated that the particle size of nanoemulsion correlates with the permeation rate. Furthermore, they demonstrated a significant influence of single excipients such as Cremophor RH 40 and Triacetin on the mucus diffusion of SNEDDS. As shown in Fig. 4, formulations consisting of 10, 20 and 40% of Triacetin resulted in a continuous improvement of particle permeation according to the increasing amounts of the excipient. A 2-fold enhancement could be observed for SNEDDS consisting of 40% of Triacetin compared to the Triacetin-free control.

Groo et al. tested the diffusion of different formulations of paclitaxel loaded lipid nanocapsules (LNCs) through pig gastrointestinal mucus using a similar Transwell diffusion system [33]. This system contains Snapwell inserts with a membrane area of 1.12 cm² and 50 µl of mucus were used. Hence, the Snapwell inserts have a higher surface area as the explained Transwell system above. Groo et al. revealed that paclitaxel LNCs containing a positive charge or neutral surface and additional short propylene glycol chains on their surface were suitable to diffuse through the mucus [33].

One advantage of using this newly established two compartment system is the application of samples directly on the surface of the mucus, which simulates much closer the in vivo situation. Thus, the direct interaction between mucus and drug delivery systems can be investigated. A second advantage of this system is the time saving process. Friedl et al. demonstrated a sufficient experimental runtime over 4 hours with a thin mucus layer [32]. In contrast, previous systems such as the three chamber Side-by-Side diffusion apparatus have long experimental durations of 15-20 hours owing to a comparatively large amount of mucus, fixed between donor and acceptor compartment [19, 31]. The use of a 24 Transwell plate and the short experimental duration established a fast comparison of different samples.

Beyond these advantages, the system allows the usage of any kind of mucus as well as native, purified or synthetic mucus. As mucus layers cover various epithelial surfaces in humans their functionality greatly differs [19]. Lieleg et al., for instance, showed that the mucus according to the location in the body varies in pH [34]. Crater et al. in contrast, observed differences in the particle transport through purified mucus in comparison to the particle transport in crude mucus [35]. They assumed that the differences may be occurred through different mesh structures of the native and the purified mucus or a different composition of the two mucus layers and therefore results are highly depending on the purification protocol.

Utilizing the described Transwell system, permeation of particles can be examined within different purified mucus samples, different located mucus layers or at different pH values at the same time. Especially for high-priced drugs an decreased volume of the two compartments is convenient as in the Transwell® diffusion system only 250 μl test solution in the donor chamber and 500 μl buffer solution in the acceptor chamber are sufficient [32]. A smaller acceptor compartment offers the opportunity to test lower drug amounts without using high sensitive and expensive analytic apparatus. This possibility to use the Transwell diffusion system with smaller or bigger inlets is another main advantage of this novel developed system.

2.5 Mucus diffusion studies: rotating silicone tube

For a better understanding of particle transport through the mucus layer, a diffusion system relating to the depth of diffusion was investigated [36]. In brief, clean mucus was filled in silicon tubes of 60 mm length and 4 mm in diameter and closed on one end with a silicon cap. At the open end of the tube labeled particle suspensions were added and the silicone tube closed securely with a second silicon cap. All the tubes were kept under horizontal rotation (≈ 50 rpm) in an incubator at 37°C . At the end of each experiment silicone tubes were frozen. For analyzing the depth of diffusion into the mucus layer, the frozen tubes were cut into slices of 2 mm in length, beginning with the end, where the particle suspension was added.

Dünnhaupt et al. introduced this diffusion model to compare the penetration behavior of fluorescein diacetate (FDA) labeled unmodified as well as thiolated anionic and cationic polymeric nanoparticles. A deeper mucus diffusion behavior of anionic poly(acrylic acid) particles (PAA) in comparison to positively charged chitosan nanoparticles (CS) was confirmed with this diffusion model. The repulsion between the anionic moieties of the mucus layer and the negatively charged unmodified or modified poly(acrylic acid) nanoparticles led to deeper diffusion of the nanoparticles as illustrated in Fig. 5 [36].

One advantage of developed diffusion method might be the possibility to obtain more detailed information about the diffusion rate of particles within the mucus. The segmentation of the mucus-filled silicone tubes clarifies the depth and quantifies the amount of diffused particles in the mucus layer. Therewith achieved results about the diffusion behavior of particles can be combined with other methods, where for example the amount of permeated particles is determined as mentioned above. Additionally, the developed diffusion system is suitable for testing nanoparticles in different kinds of mucus or gel layers [37]. The diffusion behavior of particles can be compared with mucus from different compartments of the body such as

gastrointestinal or cervical mucosa in the same experiment. Furthermore, the small size of these silicon tubes facilitates the assessment of 20 or more tubes simultaneously. The low material costs and the use of basic laboratory instruments such as a rotator or an incubator are further advantages of this method. Nevertheless, besides all these advantages one disadvantage of this system is the accurate filling of the silicone tubes to avoid the incorporation of air bubbles into the mucus layer, which influences the outcoming results. Furthermore, possible changes in the mucus structure caused by the rotating movement must be taken into account.

2.6 Multiple particle tracking

Multiple particle tracking (MPT) involves the video microscopy and post-acquisition analysis of time-resolved particle trajectories within the particular matrix under study, e.g. cell cytoplasm, mucus, etc. As a technique it allows for the dynamic measurement of the movement of individual particles within a heterogeneous matrix, and as such contrasts to static techniques which quantify only the bulk movement of particles. MPT is not only capable of the accurate individual assessment of the diffusion of hundreds of particles [38] but also provides ‘behavioral’ or qualitative information on the environment in which the particles are moving. In particular it can reveal information on particle-matrix interactions e.g. interactions of particles with mucin fibers or the steric trapping of particles within the mucus mesh network [39]. Such qualitative information is obtained through the more in-depth analysis of the time-resolved particle trajectories to determine the different modes of particle diffusion [40] (see also below). Hanes’ group have been prominent early pioneers in using MPT to assess particle diffusion through heterogeneous matrices [41]. The group subsequently applied the technique to understand and quantify particle, including nanoparticle (NP), transport through a range of heterogeneous biological samples [42, 43]. For example,

the group used MPT to study the transport of amine and carboxylated modified polystyrene NPs through the sputum of cystic fibrosis patients and relating the respected transport to the micro-viscosity and macro-viscosity characteristics of the sputum [44]. The group has also used MPT to quantify, for example, the intracellular transport of non-viral polyethylene-imine/DNA nano-sized polyplexes [45].

MPT involves the simultaneous capture of the movement of hundreds of individual particles within a particular matrix, the basic principles of which include:

- Labeling of the particles with a fluorescent dye whose physico-chemical properties are appropriate for the particle under study, and which will provide for a high signal to noise ratio in the biological matrix under investigation. Issues such as high background auto-fluorescence of the matrix itself can compromise image quality and particle tracking [46]. Although not essential, a confocal microscopy platform may allow for improved signal to noise data capture as more defined excitation and emission sources can be selected (see below).
- Inoculation of particles within the matrix. Clearly the pre-experimental preparation of the matrix will impact the efficiency and effective distribution of particle inoculation. Similarly the concentration of the particle inoculum itself can cause particle aggregation within the matrix [47]. In mucus samples (0.5 g) we have found an inoculum of a 25 μ l 0.002% NP suspension to consistently lack aggregation problems. Particle distribution within the matrix also requires a period of equilibration following inoculation and prior to video capture of the experiment proper, e.g. typically mucus samples are equilibrated with particles at 37°C for 2 hours prior to experimentation.
- Video-microscopy capture of particle movement within the matrix. This will involve maintenance of the matrix under appropriate physiological conditions, e.g. 37°C, and the use of a high speed camera to capture particle movements. Typically, camera speeds of at least 30 frames per second are used with videos generally 10-20 seconds in length; the

short collection periods are sufficient to collect robust data and help minimize sample degradation issues, e.g. as discussed for mucus [19].

- The microscope itself maybe either standard wide-field epifluorescence or confocal. Nevertheless, for mucus samples particle movement is most commonly captured in 2-dimensions (X-Y), principally due to the isotropic nature of the matrix, i.e. particle movement in X=Y=Z. Further, 2-dimensional capture avoids inaccuracies in subsequent data and trajectory analysis that may be introduced by the process of Z-sectioning through the sample.

The basic principles involved the post-acquisition analysis of particle movement and particle trajectories include:

- Use of appropriate software, e.g. Fiji ImageJ, which can track the video movements of the particles at resolutions as high as 5 nm [48]. The software converts the movement of each particle as captured by high-speed video microscopy into individual particle trajectories. Appropriate rules need to be applied to the analyses e.g. videos capturing particle movement in 2-dimensions should provide that any single particle eligible for analysis must remain within same X-Y plane throughout all frames of the entire video capture period.
- The movement of particles is translated into individual particle trajectories which are initially represented as numeric pixel data. This data is then converted from pixels into metric distance based on the microscope and video capture settings. From this the inter-frame distances moved by each particle in the X-Y plane are expressed as a squared displacement (SD). The mean square displacement (MSD) of one particle represents the geometric mean of the sum of all of that particle's square displacements throughout its transport trajectory [49]. Typically in a single experiment MSD data is calculated for at least 120 particles of each NP species under study, and the average of these MSD values (ca. 120 values) represents an "ensemble mean square displacement" defined by $\langle \text{MSD} \rangle$.

The Effective Diffusion Coefficient (D_{eff}) of a particular NP species can then be determined by $D_{eff} = \langle MSD \rangle / (4 * \text{frame rate})$, where 4 is a constant relating to a 2-dimensional mode of video capture and the frame rate relates to the speed of video capture [50].

A schematic presentation of the principle of particle tracking is given in Fig. 6.

The MPT technique tracks the change in movement of each single particle as a function of time. Unlike particle diffusion in water, which is non-restrictive and unchangeable with time, particles will undergo varying degrees of hindrance during their diffusion through a polymeric gel matrix such as mucus. Here the mucin fibres are undergoing continuous association and disassociation and the network as a whole undergoes elastic behavior resulting in the formation and collapse of aqueous cages surrounded by mucin fibres [51]. As such there will be significant potential for particle-gel interactions within mucus the probability of which increases as a function of time [52]. Accordingly, how the diffusion of individual particle changes with time not only provides information on the kinetics of movement but also the nature of movement, e.g. changes in time from unrestricted to restricted movement, e.g. “pearl on string trajectory”, may be indicative of increased particle interactions with, or trapping within, the mucin network. Such interactions captured by MPT have helped reveal information on the structure of the mucus mesh network [39] and on mucus micro-rheology [24].

The analysis underpinning studies on the nature or mode of particle diffusion can be undertaken by calculating the $\langle MSD \rangle$ for each particle over successive time scales, with a change $\langle MSD \rangle$ over this time period revealing the mode of particle motion which is represented in the literature by an exponential anomalous (α) [53]. Particle transport in water is described as ‘freely diffusive’ and α is equal to 1, while in an accelerated mixing environment α can be greater than 1 with the movement described then as ‘active diffusion’. Particle diffusion in mucus is often restricted and the α value is less than 1 with diffusion then

generally described as 'sub-diffusive' [54] and where α values between 0.2 and 0.9 reflecting varying degrees of hindrance to particle movement [47]; particles with an α value of less than 0.2 are considered to be completely immobilized. A nuance building of individual particle behavior within this analysis technique is established to draw conclusions concerning the distribution of diffusive properties of the entire particle population. Such information can provide a unique assessment the heterogeneity of particle movement and the presence of outlier sub-populations indicative of distinctive pathways of diffusion through the matrix [55]. In summary MPT allows for the dynamic measurement of the movement of individual particles and sub-populations within heterogeneous biological and non-biological matrices. The information it provides complements that obtained by static approaches to assess particle diffusion. The main technical challenge for any investigator wishing to use the approach will be their ability to undertake high-speed fluorescent video microscopy.

2.7 QCM-D method

QCM-D (quartz crystal microbalance with dissipation monitoring) is in effect a high resolution weighing device that senses mass deposition of less than 1 ng/cm^2 [56]. The induced change of resonance frequency (f) due to mass adsorption on the quartz crystal is measured together with energy dissipation (D) in real time, which is then used to convert to other parameter output such as the adsorbed mass, thickness, and viscoelastic properties of the layer [57]. The experimental set up consists of a quartz disc sandwiched between a pair of electrodes and can be made to oscillate by applying an alternating current (AC) voltage. When a thin film is applied to the sensor the frequency (f) will decrease and if the film is rigid the decrease in frequency is proportional to the mass of the film. However, if it is a soft film like mucus/mucin it will not be in phase with the oscillation of the crystal (i.e. energy dissipation D) which is also a measure of viscoelasticity. If a polymer/nanoparticle is then applied and an

interaction takes place a large change in frequency will occur and the variation in viscoelasticity of the complex will cause a variation in D . The principle of this technique is shown in Fig 7.

This technique can be adapted to study the interaction of polymers and nanoparticles with mucine. In detail, the QCM-D instrument is able to measure two things.

First, the variation in quartz crystal resonance frequency (Δf), which is related with the mass uptake and release at the sensor surface can be measured. There are different approaches both for rigid (Sauerbrey equation) and flexible films to correlate the variation of frequency with the mass change [58]. Thus, the instrument can be calibrated for calculating the mass absorbed on the sensor.

Secondly, the energy dissipation (ΔD), which is related with the structure and viscoelasticity of the film, can be determined. Briefly, QCM-D measures the dissipation of energy by the system, which is the part of accumulated energy lost at each oscillation, after switching off the exciting electric field. When the film is rigid (air in Fig. 8), the oscillation decays very slowly. When the viscoelasticity of the film increases (for instance in an hydrogel water absorption) the decay is much faster (liquid in Fig. 8). In mucin-polymer nanoparticle interactions, it should be noted that the polymer and the nanoparticles when interact with the mucin modify its viscoelasticity, the monitoring of the dissipation (ΔD) gives insights about the nature of this interaction.

Moreover, sequential multi-frequency measurement (the frequencies corresponding to the harmonic frequencies of the crystal) is done in order to record the different overtones of the oscillating system. It is important to point out that each overtone has a specific penetration depth, and they are measuring the behavior at different depths of the attached film. From a practical point of view, the decay distance is the penetration depth of the frequency measurements in QCM-D experiments. It is the maximum recording distance. For a 5 MHz crystal in water, the maximum penetration depth is $\delta \approx 250$ nm. The sensor's overtones have

higher frequencies, so the penetration depth will be shorter. This means that, as they are measuring at different depths of the deposited film, they can be used to record the film response in different points at the same time. This characteristic has been used to study how the polymer or the nanoparticles are interacting with the mucin film. If the polymer were only absorbed on mucin surface, the overtones response would be different. On the other hand a penetration that affects the whole mucin leads to similar overtones behavior.

Chayed and Winnik studied that the interaction between porcine mucin (bovine submaxillary mucin) and mucoadhesive polymers-based nanoparticles by means of QCM-D, and demonstrated that QCM-D is a promising technique for studying the mucoadhesive properties of biopolymers [59]. Recently, Wiecinski et al. studied QCM-D can be used as a screening method of biodurability of toxic nanoparticles [60]. They suggested that the interaction of nanoparticles with commercial porcine gastric mucin (CPGM) could be a good model to understand the impact of nanoparticles in the body.

Recently, in the frame work of the European Project Alexander a QCM-D based method has been optimized to study the interaction of polymers and nanocarriers with fresh porcine gastric mucin. More than 50 samples of nanocarriers designed for mucus permeation have been analyzed with this method. It is interesting to highlight that the method developed is able to discriminate between the ionic interaction of charged nanoparticles and polymers with the negative charged mucin, and the penetration of the samples through the mucin. Second, as it could be expected the cationic polymers are easily absorbed on the mucin surface, and can be quantified following the frequency decrease as stated. Moreover, the variation of Dissipation (D) allows discriminating between pure adsorption (e.g. chitosan), with real mucin permeation (e.g. thiolated cationic polymers). It should be noted that when the permeation is produced, the dissipation rapidly decreases and increases in a moment, and then remains constant. The dissipation overtones get closer but the frequency plots have no changes.

2.8 Small-angle neutron scattering method

When investigating relationships between physical properties and molecular structure, the determination of molecular organization within complex systems is fundamental; small-angle neutron scattering (SANS) - a powerful, non-destructive technique that probes organizational structure on a 1-200 nm lengthscale - can be easily applied for the study of delicate biological materials, although most studies published to date have focused on mucin [61-65].

The first studies using SANS to investigate the structure of pig gastric mucin were published in 2002 by Hong et al. [61]. Hong et al. found that individual mucin chains in unbuffered aqueous solvents adopted a cylindrical structure with a cross-sectional radius of c.a. 23 nm and a length of 96 nm. They also observed that a core/shell structure was adopted, where the core was formed by the protein backbone and the shell by the radial sugar chains. The evolution of the chain structure with changes in pH was also investigated; at pH 7, the mucin chains were swollen whereas, at pH 2 the mucin behaved like polymer chains dispersed in a “theta” solvent confirming an increase in hydrophobic interactions.

Waigh et al. found evidence of a highly organized structure, discussed in terms of polydomain nematic phases above some critical semi-dilute concentration, and polyglycan orientation under magnetic field at lower concentration [62]. They also determined the cross-sectional radius and length of the mucin, obtaining results in agreement with Hong et al.

SANS was also used in combination with small-angle X-ray scattering (SAXS) to determine the structure of pig gastric mucin [63], the chain dimensions determined from this study were significantly different to previous studies, i.e. radius c.a. 3.6 nm and length c.a. 12.5 nm.

Griffiths et al. used SANS, combined with diffusion NMR to study the structure and dynamics of pig gastric mucin and the effect of addition of synthetic polymers often used as drug carriers [65]. From SANS, they observed that pig gastric mucin contains hydrophobic domains of approximately 9 nm spread apart by ca. 80 nm as shown in Fig. 9.

Charged polymers such as polyamidoamine dendrimers (PAMAM) or polyethyleneimine had an effect on the spacing of these hydrophobic groups depending on the pH investigated as shown in Fig. 10, whereas the neutral poly(ethylene oxide) had little effect on the structure of mucin, regardless of pH.

Georgiades et al. [64] recently focused their studies on purified gastric and duodenal mucin using SANS and SAXS techniques. They confirmed that mucin forms a gel network above the semi-dilute concentration, studying the gel formation as a function of pH - a decrease in pH leads to microphase separation of the gels. They also found the persistence length of both mucin types to be c.a. 8 nm.

The structure of intestinal mucus (I_{mucus}) rather than mucin, and the changes arising from the incubation of enzyme-conjugated nanoparticles has been recently investigated within the ALEXANDER project [66]. SANS revealed that the scattering from I_{mucus} exhibited a strong $Q^{-2.6}$ dependency, characteristic of a fractal-like structure of a dense gel network. The samples incubated with particles or enzymes surprisingly showed little change in scattering pattern revealing that the mucus network was unaffected by these enzymes or enzyme conjugated nanoparticles, at least on the lengthscale probed by the SANS technique i.e. 1- 400 nm.

2.9 Diffusion NMR

Diffusion NMR – formally termed pulsed-gradient spin-echo NMR (PGSE-NMR) – is perhaps a unique experimental approach applicable to multi-component or heterogeneous systems. PGSE-NMR - non-invasive, non-destructive and retaining the chemical specificity of NMR - enables the behavior the several components within such multi-component systems to be simultaneously extracted from a single experiment. Structural information is encoded into the diffusion coefficient, which decrease with an increase in molecular size and are a sensitive reflection of any binding or obstruction the diffusing specie experiences within the solution.

There have been relatively few studies of diffusion in mucin/mucus systems [67, 68]. Li et al. investigated the diffusion of bile salt and phospholipid aggregates (of varying concentration from 1-7g/dL) in mucin (2 wt %) [67, 68], interpreting the changes in aggregate diffusion in terms of the transport of dietary lipid and solubilized drug through the aqueous boundary layer of the intestinal tract.

Lafitte et al. utilized the diffusion of poly(ethylene glycol) polymers (PEGs) spanning a molecular weight range $1020 \text{ g mol}^{-1} < M_w < 716,500 \text{ g mol}^{-1}$ through pig gastric mucin (PGM) (5 wt %) as a probe to understand the relative importance of pH, ionic strength and temperature on defining mucin gel structure [69]. They concluded that the structure of the mucin gel – as perceived by the stronger dependency on the PEG diffusion - was dominated by pH effects, compared with ionic strength and temperature effects. For example, it was found that intermediate molecular weight PEG displayed a slower diffusion at pH 4 compared to both pH 1 and 7. The increase in diffusion between pH 1 and 4 was due to the mucin network being less homogeneous at pH 1 compared with pH 4. More surprising was the fact that the diffusion of the PEGs was faster at pH 7 than at pH 4. Associated with the drop in the pH, is a change in the physicochemical characteristics of the mucin molecule, and therefore the dynamics of the gel, due to the evolving balance of electrostatic and hydrophobic interactions. This is manifest as a decrease in the flexibility of the PGM molecules within the network, and the emergence of stronger hydrophobic interactions due to the decreasing net charge on the mucin molecule. These interactions facilitate the diffusion of PEG of intermediate molecular weight but not the largest PEGs which are too sterically hindered.

In a similar vein, the same research group investigated the transport in mucin and mucus systems of polysorbate 80 (PS-80), a non-ionic surfactant commonly used as an excipient in many pharmaceutical formulations [70]. The study highlighted the existence of two populations of PS-80 molecules within a single sample: a non-surface-active fraction that can

move freely in the system ($\text{PS-80}_{\text{free}}$) and a surface-active fraction that forms micellar structures resulting in a slower diffusion in the bio-gel ($\text{PS-80}_{\text{mic}}$). However, the reduced transport rate of PS-80 in mucin is not due to a specific interaction with mucin but to a simple interaction of the PS-80 with the mucin network. PS-80 contains fatty acid chains and long ethylene oxide chains and as such, they are expected to behave in a similar fashion to PEG, showing steric hindrance on diffusion processes in the mucin network.

Upon addition of the cationic surfactant tetradecyltrimethylammonium chloride (TTAC) to the mixture of mucin and PS-80, the formation of mixed micelles between PS-80 and TTAC occurred [71]. Depending on the composition of the mixture, complex-formation led to precipitation. Diffusion NMR measurements demonstrated that at low concentrations of PS-80, mixed PS-80, TTAC and mucin aggregates were formed, but an increase in PS-80 concentration caused the dissolution of the precipitate, limiting the interactions between the mixed micelles and the mucin.

Griffiths et al. examined the diffusion coefficient of a series of candidate drug carrier polymers as a function of mucin concentration by diffusion NMR [65]. Recasting their data as a relative diffusion coefficient (the diffusive rate of the candidate polymer in mucin solution normalized to the diffusive rate of the same polymer observed in the absence of mucin) was shown to be a useful method to screen polymer/mucin interactions. For “interacting” polymers, the relative diffusion coefficient was substantially slower in the presence of physiological amounts of mucin. These data and this novel presentation method, showed a complex (and polymer-specific) relationship with molecular weight – for non-interacting polymers, there was the expected “obstruction” inducing decrease in diffusion rate for the two PEGs (10 K and 100 K g mol^{-1}) but for “interacting” polymers such as the PAMAM dendrimers, G2 and G4 (3,256 g mol^{-1} and 14,215 g mol^{-1} respectively), there was a negligible difference in the relative diffusion coefficient. Further and again reflecting the nature of the interaction, the mucin retards the diffusion of the two PAMAMs to a degree

comparable to the larger PEG, notwithstanding the latter's much greater size. The greatest reduction is shown by the highly branched poly(ethylene imine) 25 K g mol⁻¹ sample. These data reflect the complex interplay between obstruction-only behavior (PEG, e.g. the basis of PEGylation [72]) and obstruction *plus* binding to the mucin (PAMAM, PEI) where additional retardation arises due to the molecular characteristics of the polymer and the mucin.

The effect of enzyme decorated nanoparticles as well as the equivalent bare particles on the self-diffusion coefficient of pig intestinal mucin has been investigated recently within the ALEXANDER project [66] to compliment the SANS study discussed earlier. The results demonstrated that the presence of (enzyme and) enzyme decorated particles increased the diffusion coefficient of mucin, indicating an enhanced mobility due to the perturbation of the stiff gel network, whereas bare nanoparticles did not affect the diffusion of the mucin.

3. In vivo methods

In order to forecast the behavior of drug delivery systems in vivo, experimental set-ups in vitro have to be close to the in vivo reality.

Several research groups are performing in vivo studies to analyze the mucoadhesive and mucus penetration properties of nanoparticles in different mucus layers [2, 26, 73-80]. They focused on the fate of particulate drug delivery systems in mucus especially on their uptake into the epithelium of the GI-tract after oral administration [43]. Basset and Carne' showed in early studies that the intestinal epithelium is a barrier which cannot be permeated by particulate drug delivery systems [81]. In cats, for instance, intestinal mucus surrounds particles of India ink to prevent direct contact with epithelial cells [75]. Furthermore, Gruber et al. have observed the effect of several particles on gastrointestinal mucus in dogs and demonstrated that particles were transformed into mucus-covered 'slugs' independently of their size, density or composition [76]. Additionally, comparable results were observed after

administration of two different types of microparticles to rats. Particles remained for an equivalent time in the rat intestine, but covered with mucus and withdrawn by the animals [77].

Lamprecht et al. furthermore, investigated the bioadhesion in reference to particle sizes specific to inflamed colonic mucosa [78]. They examined the particle deposition in the mucus as well as the particle uptake into the tissue from rats after oral administration. They could verify a size dependency of particle deposition in the thicker mucus layer next to the inflammation. Examined particles with the smallest size (0.1 μm) had the highest binding capacity into the mucus. Submicron-sized carriers showed a promising target for treating inflammatory bowel disease due to a high deposition rate next to the inflamed colonic mucosal areas.

Studies on the transport of various fluorescence labeled PEGylated nanoparticles (PEG-NPs) were examined by Yoncheva et al. utilizing optical microscopy of isolated tissue segments after oral administration to rats [79]. The received micrographs showed the presence of marker within the cells of intestinal villi. They assumed that a PEGylation of NPs can improve their uptake into the epithelial cells. Moreover, they could demonstrate that PEG-NPs rather interacted and bind directly with the cell surface than with mucus components.

In several studies the ligated intestinal loop model is used to evaluate the uptake of nanocarriers [82-85]. After exposing the abdomen of anaesthetized animals, usually rats, loop of ileum was prepared via ligation. Samples containing a fluorescence labeled drug or carrier are administered via injection into these loops. At preassigned time points the animals were sacrificed and the loops treated for following investigation. The drug carriers may (i) remain in the intestinal lumen; (ii) trapped in mucus; (iii) or penetrate through the mucus layer reaching the epithelium [83].

Jin et al. for instance used the ligated intestinal loops in vivo technique to investigate the transport of epithelium-targeting nanoparticles as an oral insulin carrier [83]. NPs prepared of

trimethyl chitosan chloride (TMC), conjugated and non-conjugated with globlet cell-targeting ligand (CSKSSDYQC peptide) showed different penetration profiles. Results demonstrated that unmodified TMC-NPs remained in lumen and were trapped in mucus. This circumstance was 2.2-fold higher than for conjugated TMC-NPs. Furthermore, the working group could demonstrate within this study that the CSKSSDYQC peptide conjugated TMC-NPs could improve the absorption of insulin.

A study by Li et al. showed that the detained effect of mucus on the absorption of a drug incorporated into chitosan nanoparticles (CNP) was more pronounced than on the absorption of a drug from core shell corona nanolipoparticles (CSC) via ligated intestinal loops technique [85]. CSC, compared with CNP, penetrated deeply into villi after 2 hours experimental time observed via confocal microscope. Pretreated removal of mucus in the loops confirmed the trapping efficacy of the mucus to the CNP.

Several in vivo studies were arranged in terms of mucoadhesion of drug carriers with a longer resistance time near the active side [2, 26, 80]. The turnover of mucus layers at various surfaces differs, whereby mucoadhesive drug carriers are powerful target-orientated systems.

Müller et al., for instance, performed in vivo studies, which focused on the distribution of particles in the gastrointestinal tract. These studies should give an overview of promising mucoadhesive and mucus penetrating particles and were closer to the human system than an in vitro experiments [26]. Briefly, the mucoadhesive properties of papain-functionalized nanoparticles were observed within the gastrointestinal tract of 24 Sprague-Dawley rats. It could be demonstrated that the penetration into the mucus layer could be improved via the immobilization of the mucolytic enzyme papain on the surface of poly(acrylic acid) nanoparticles. This result is attributed to the content of remaining nanoparticles in the anatomical segments as shown in Fig. 11. For comparison reason a mixture of fluorescein diacetate (FDA) and mannitol was utilized.

Albrecht et al., for instance, evaluated the remaining FDA amount incorporated in polycarboxyl-cysteine microparticles on rat intestinal mucosa after oral application [80]. Segments of the intestine were analyzed regarding the fluorescence marker on the mucosa after administration of FDA labeled microparticles encapsulated in newly developed Eutex (Eudragit L100-55 and latex) capsules as well as in conventional enteric-coated capsules. It could be demonstrated that microparticles administered in Eutex capsules adhered significantly longer to the intestinal mucosa than administered in conventional enteric-coated capsules.

Within in vivo experiments performed by Barthelmes et al., the mucoadhesive properties of thiolated nanoparticles on intravesical mucosa were estimated [2]. For this purpose fluorescein diacetate loaded thiolated chitosan nanoparticles were administered to the urinary bladder of rats. After different incubation periods, the content of remained nanoparticles was detected. Barthelmes et al. could prove that thiolated chitosan nanoparticles had a 4-fold increased mucoadhesion compared to their unmodified ones.

Takeuchi et al. evaluated the mucoadhesive properties of chitosan-loaded liposomes after oral administration to rats via confocal laser scanning microscopy [74]. Based on the higher detected chitosan-coated liposome amount in the mucus layer in contrast to non-coated liposomes they could confirm the mucoadhesive function of the coating process.

4. Concluding remarks

The mucus barrier is an effective barrier towards micro- and nanoparticulate drug carrier systems owing to its lower adhesive clearing layer. The diffusion through this mucus barrier is therefore a complex phenomenon. Within this review various methods and techniques to hurdle this barrier utilizing micro- and nanoparticulate drug carrier systems were summarized.

Mucus nanoparticle interaction studies via zeta potential, particle size and rheological measurements as well as QCM-D method and SANS technique are useful methods to investigate the interaction of nanoparticulate drug delivery systems with mucus layers. Furthermore, several techniques presented within this article are available to measure the diffusion of particles through mucosal systems. For instance, the Transwell-Snapwell method, the Transwell diffusion method, rotating silicone tube technique, multiple particle tracking and diffusion NMR are promising diffusion systems. An overview of all different methods and techniques presented within this review is provided in Tab. 1.

A simple technique to verify particle-mucus interaction is given by particle size and zeta potential measurements. Not only for this technique, also for permeation studies via Snapwell and Transwell systems as well as the rotating tube method no expensive equipment is required. In contrast, the purchase of a diffractometer, pulsed-gradient spin-echo NMR spectrometer, rheometer as well as a high-speed fluorescent video microscope as primary apparatus represents a costly investigation.

In particular, it is not necessary to label nanocarriers if measurements are performed via diffusion NMR, QCM-D method, SANS and rheology. For diffusion/movement experiments via the Transwell-Snapwell system, the Transwell diffusion system, rotating silicone tube technique and multiple-particle tracking technique a labeling step of particles is required in order to determine particles in the mucus.

With regard to a short experimental time, the MPT is convenience to measure the microscopic motion of hundreds of particles within seconds. Penetration studies using Transwell-Snapwell system, Transwell diffusion system or rotating silicone tube technique require a longer experimental time. In addition, nanoparticle mucus interaction studies via particle size and rheological measurements are as well more time consuming. Differences between methods utilized to study particle diffusion in the mucus gel layer are based on the static and dynamic process. The MPT and the diffusion NMR allow dynamic measurement of the movement of

particles within a matrix, whereas Transwell-Snapwell system, Transwell diffusion system or rotating silicone tube technique are static measurements. For the detection of viscoelastic changes in mucus after addition of nanocarriers bearing mucolytic enzymes on their surface the rheometer and the QCM-D model are advantageous. Furthermore, the QCM-D model has the benefit to separate between particle mucus interaction and particle mucus penetration. The use of SANS measurements can give an insight of the relationship between physical properties and molecular structures of complex systems. MPT, as one of the presented diffusion methods, is suitable for the dynamic measurement of the movement of individual particles and sub-populations within heterogeneous biological and non-biological matrices. Additionally, the MPT provides also information on interactions between particulate drug delivery systems and matrices such as particle trapping within mucus. The diffusion behavior of multi-component unlabeled systems in mucus can be analyzed in a single experiment with the use of diffusion NMR technique. By use of the rotating tube technique the depth of diffused particles in the mucus layer can be clarified. Within this technique, but also for the Transwell diffusion system, different kinds of mucus can be tested in one experiment

Overall, it could be shown that different convenient methods and techniques were already established to evaluate the interactions between mucus layers and particulate drug delivery systems, whereby each technique has its benefits and drawbacks. Nevertheless, it has to be considered that not every technique and method already used by various research groups is presented within this review. Therefore, the authors of this review make no claim to be complete. In conclusion, the behavior of drug carriers within mucus can be explained far better using several techniques and methods simultaneously. The combination of presented techniques and methods might help to improve the evaluation of novel drug delivery systems in mucus layers.

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Figure captions

Fig. 1: Comparison of proteolytic properties of enzyme formulations by determination of the decrease of mucus viscosity at pH 6.5. Fresh porcine intestinal mucus (x) was incubated with 0.5% (w/v) PAA particles (○), papain (□), papain-g-PAA particles (■), papain-g-PAA + CYS-OET particles (●). Indicated values represent mean \pm SD of at least three experiments (adopted from Müller et al. [26])

Fig. 2: Modification of the Ussing chamber system with Snapwell rings. The mucus layer is surrounded with two permeable filters attached to the Snapwell ring and incorporated between the donor and the acceptor compartment (adopted from Norris et al. [28])

Fig. 3: A schematic presentation of the Transwell diffusion system; A: acceptor compartment; B: semipermeable membrane; C: mucus gel layer; D: donor compartment (adopted from Friedl et al. [32])

Fig. 4: Overview of diffused amount of model drug through mucus layer related to the impact of Triacetin. Indicated values are means of at least three experiments \pm SD. (adopted from Friedl et al. [32])

Fig. 5: Distribution of FDA labeled particles in natural porcine intestinal mucus pH 6.5 tested in the silicone tubes. left diagram: unmodified poly(acrylic acid) particles (grey bars) and thiolated poly(acrylic acid) particles (black bars); right diagram: unmodified chitosan particles (grey bars) and thiolated chitosan particles (black bars) (adopted from Dünnhaupt et al. [36])

Fig 6: The movement of each particle (P) was video captured as individual particle trajectories (Figure 6A) over a minimum of 30 consecutive frames with the criterion that any individual particle tracked must display a continuous presence in the X-Y plane throughout the respective 30 sequential frames. For example P1 in (6A) above tracked from (Δt_1) to (Δt_{30}) . The distance moved in the X-Y trajectory by each particle over a time interval (Δt) , i.e. camera frame rate, is expressed as a squared displacement (SD) (Figure 6B), $SD = (X_{\Delta t})^2 + (Y_{\Delta t})^2$. A mean square displacement (MSD) for any single particle can then be calculated by $MSD = [(X_{\Delta t})^2 + (Y_{\Delta t})^2]_{(\Delta t n=1-30)}$

Fig. 7: Scheme of the experiment described to study by QCM-D mucin interaction with nanoparticles. Only one overtone is represented

Fig. 8: Scheme of the Quartz Crystal dissipation in two media, air and water (adopted from Rodahl et al.[58])

Fig. 9: Illustration of mucin consisting hydrophobic globules linked by a glycosylated Spacer (reprinted with permission from Griffiths et al. [65]. Copyright 2010 American Chemical Society)

Fig. 10: Illustration of 5 wt% mucin scattering at pH 7 and 37 °C in absence of 0.5 wt% PAMAM dendrimer G2.0 (closed circles) and in presence of 0.5 wt% PAMAM dendrimer G2.0 (open circles); (reprinted with permission from Griffiths et al. [65]. Copyright 2010 American Chemical Society)

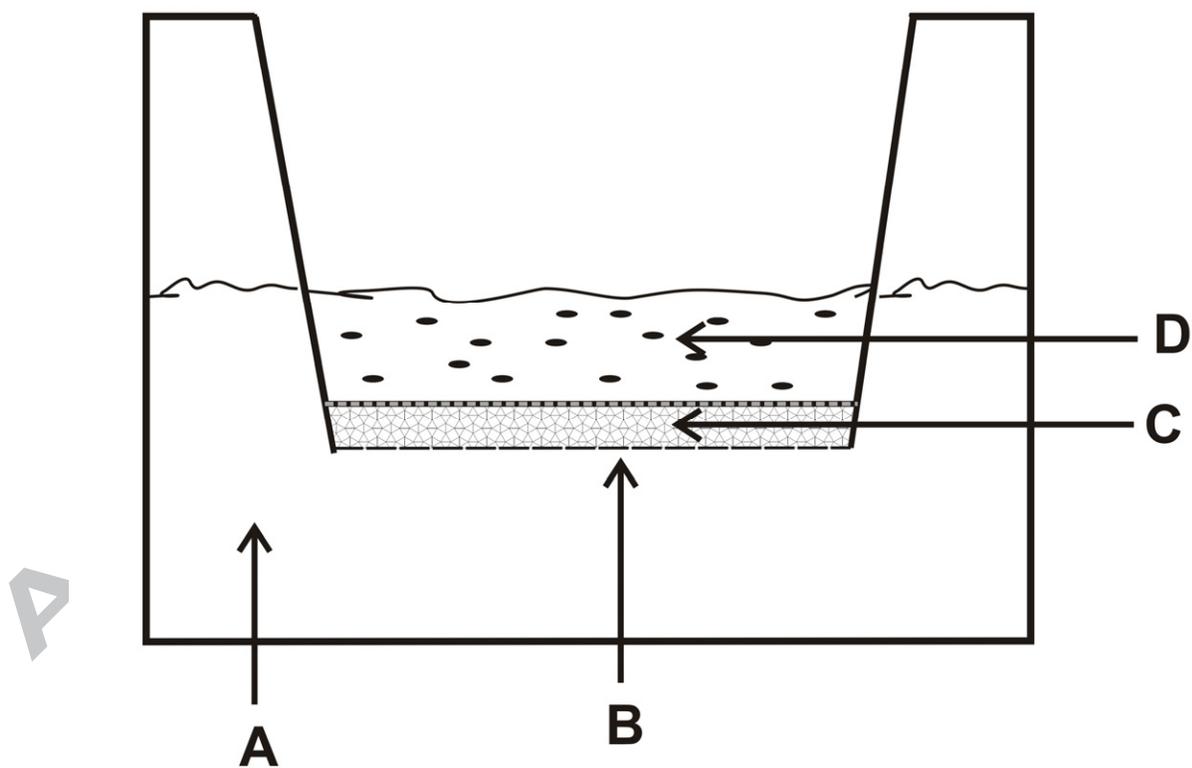
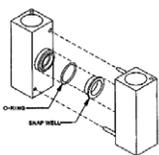
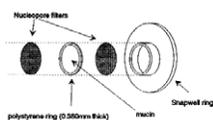
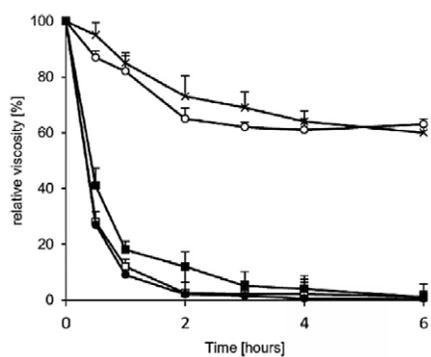
Fig. 11: Illustration of the remained nanoparticles content in the mucosa of removed rat gastrointestinal tract segments after oral administration and 3 h of incubation. (adopted from Müller et al. [26])

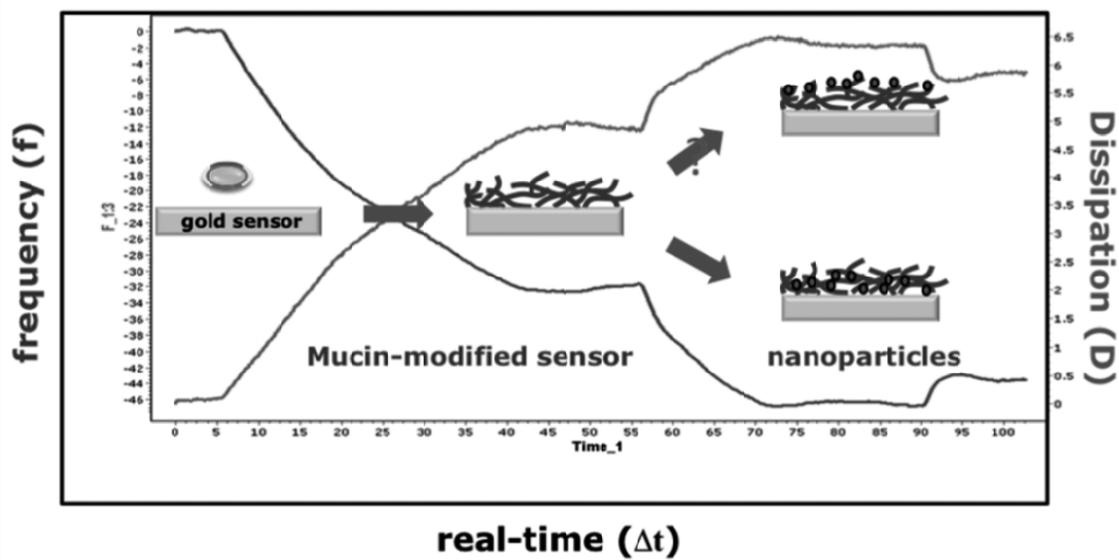
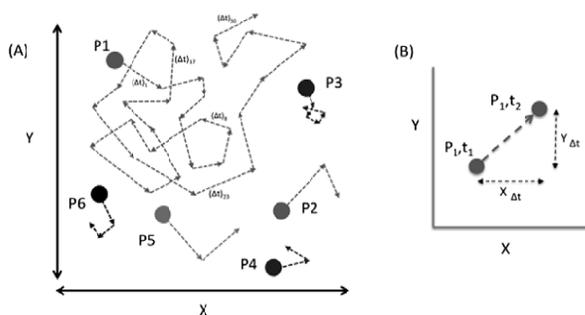
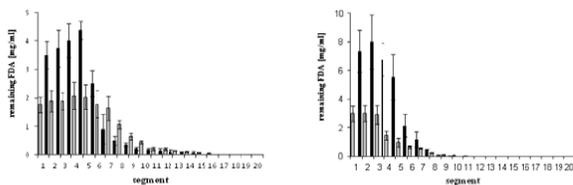
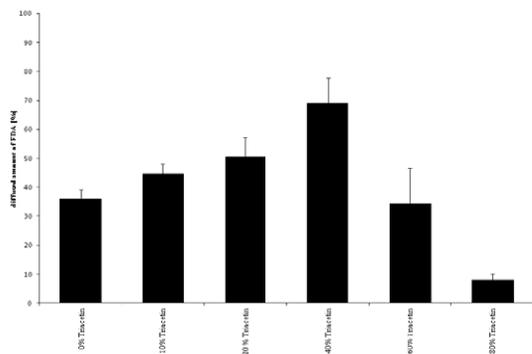
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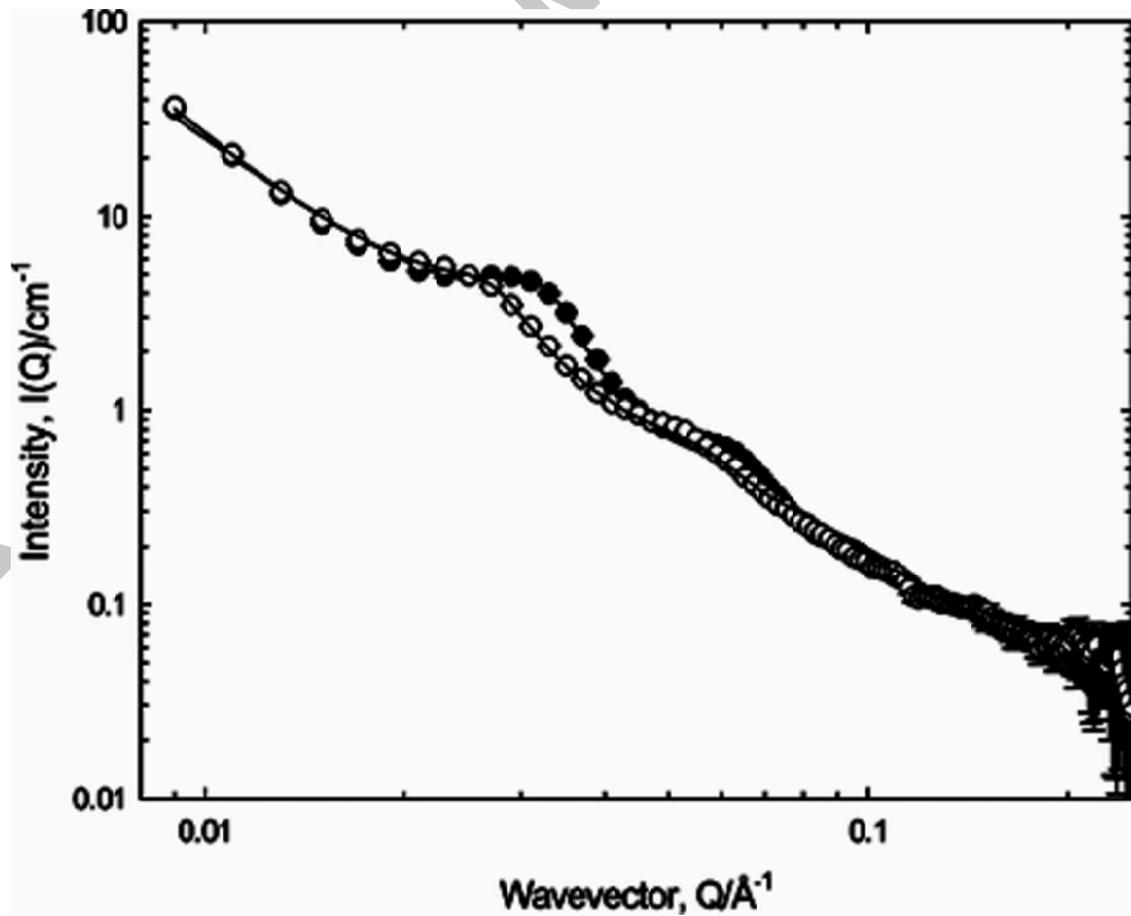
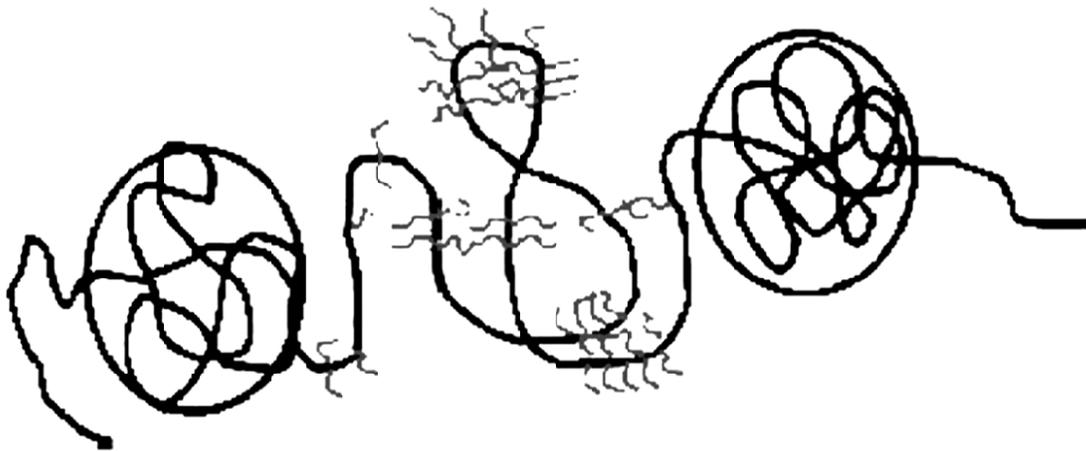
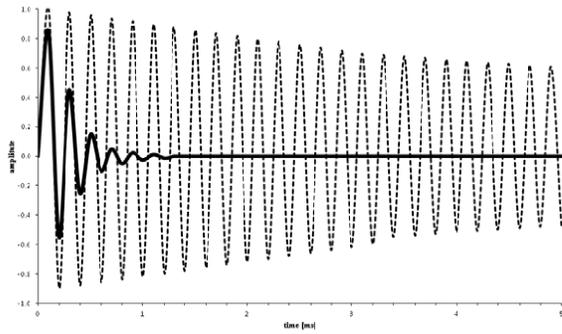
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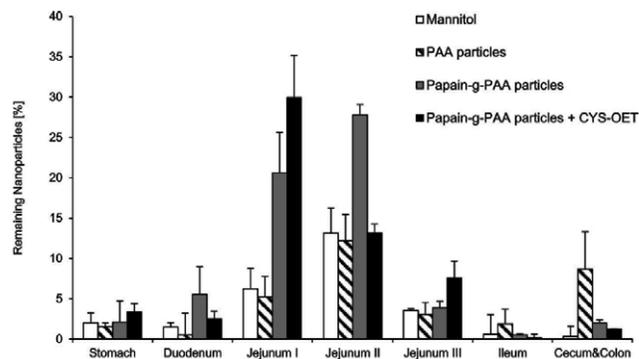
Tab. 1: Comparison of presented methods and techniques among each other (I.S. – particle-mucus interaction studies; P.S. – particle permeation studies; D.S. particle diffusion studies)

	I.S. via particle size and zeta potential measurement	I.S. via rheological measurement	QCM-D method	SANS method	P.S. via Transwell-Snapwell system	P.S. via Transwell diffusion system	D.S. via rotating tube	MPT method	Diffusion NMR method
Used technique within this review	particle-mucus interaction study	particle-mucus interaction study	particle-mucus interaction study	particle-mucus interaction study	particle permeation study	particle permeation study	particle diffusion study	particle diffusion study	particle diffusion study
Mainframe required	no	yes:rheometer	yes: weighing device	yes: diffractometer	no	no	no	yes: fluorescence video microscope	yes: NMR spectrometer
Additional apparatus required	no	no	no	yes: particle sizer	yes: fluorometer	yes: fluorometer	yes: fluorometer	no	no
Labeling of particles required	no	no	no	no	yes	yes	yes	yes	no
Experimental time	long	long	medium		long	long	long	short	
Kind of measurement					static	static	static	dynamic	dynamic
Required sample amount	high	high	high		high	low	low	low	

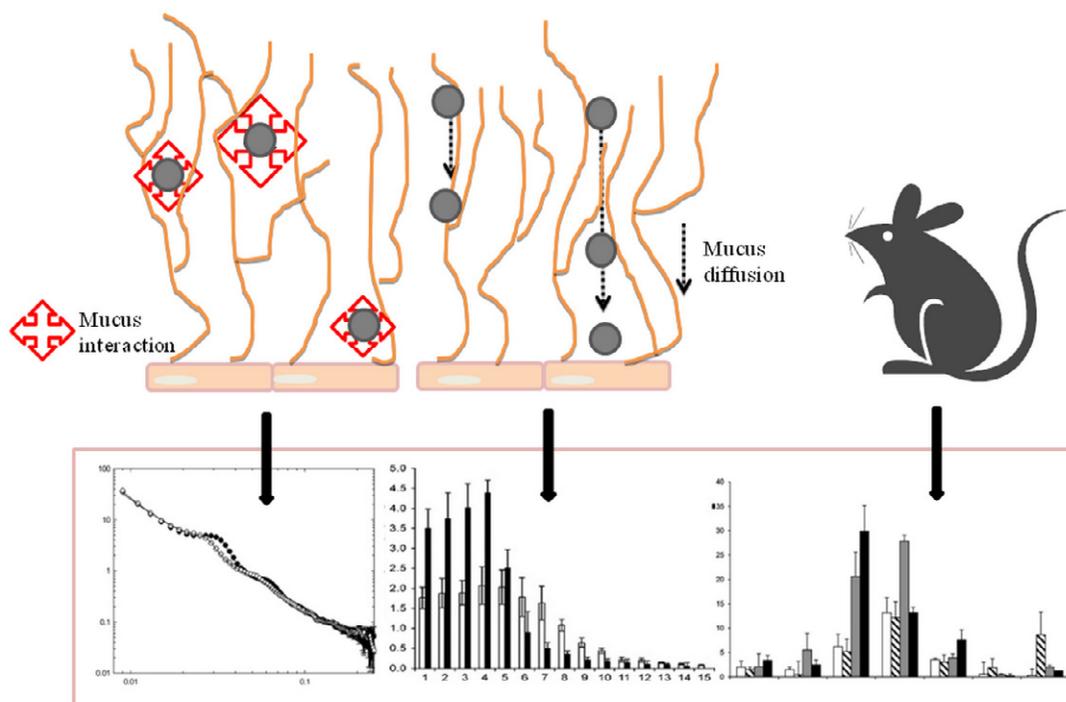








Graphical abstract



Highlights

- Methods to examine interactions between mucus layer and nanocarriers were depicted.
- Methods to analyze particle diffusion in mucus were summarized.
- The explanations allow a better comparison of obtained results.
- The discussed pros and cons provide a good starting point in this research field.