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In the fast-growing field of nanomedicine, mucus is often the first barrier encountered by drug products in the body, and can be the only barrier if it is not overcome by the drug delivery system. Thus, there is a need to design new nanomedicines that are able to diffuse easily across mucus to reach their pharmacological targets. In this design process, mucus diffusion studies are mandatory and have an important role in the selection of the best drug candidates. However, there is currently no standard procedure for diffusion studies across mucus. In this Foundation Review, we discuss the differences observed within mucus models and experimental protocols in diffusion studies, with an emphasis on nanomedicine diffusion.

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Frederic Lagarce received his PhD in 2004 and, since 2012, has been a professor of pharmaceutical technology and biopharmaceutics in the University of Angers, France. He is also a hospital pharmacist, and so his research has a translational focus (from bench to bedside). His main interest is in cancer therapy, especially bioavailability enhancement by exploiting the interactions between drug products (mainly nanosystems) and living tissues. This field involves not only biological barrier-crossing studies, but also
stability assessments of active moieties. Finding new answers to medical needs using innovative drug formulations is what drives him to work every day.

Introduction

Colloids are being increasingly developed and used to enhance the efficacy, and reduce the toxicity, of drugs. In this promising area of so-called 'nanomedicine', some new drug formulations have already reached the market [1] and there is a substantial amount of research underway into new colloidal formulations to enhance their use as drug delivery systems. For example, encapsulation in nanodevices such as liposomes or nanocapsules can help the drug to have the desired distribution in the body, thus enabling it to reach its pharmacological target in sufficient concentration and avoiding other tissues where it can be toxic. Encapsulation also helps the drug to overcome biological barriers, such as the intestinal epithelium or the blood–brain barrier. The journey of a colloidal carrier in the body is complex and has been reviewed recently [2]; however, it often starts with an encounter with mucus. In fact, mucus is a complex biological material that lubricates and protects many tissues. Given that mucus is ubiquitous, colloid systems are in contact with it in many areas of the body, including lungs, gastrointestinal tract, vagina, eyes and nasal tract. Thus, it is necessary to characterize nanoparticle behavior in mucus during the process of formulation design and optimization.

Irrespective of its origin, mucus comprises water (approximately 95%), glycoproteins (i.e. mucins), lipids (0.5–5%), mineral salts (0.51%) and free proteins (1%) [3]; however, mucus displays different properties and fine composition depending on its location in the body. It inhibits penetration by numerous viruses [4] and is a useful barrier against other pathogens. However, mucus also constitutes a potentially efficient barrier to the delivery of nano-sized drug

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delivery systems. Thus, it is of primary importance to design nanocarriers that are able to cross mucus, therefore, mucus diffusion studies are needed. Despite this need, there is currently no standard protocol available for mucus diffusion studies; here, we determine whether the different models used to study nanocarrier diffusion in mucus are similar enough to each other to provide approximately the same results or if there is a need for standardization. We first evaluate differences and similitudes of mucus models described in the literature. We then go on to compare diffusion models and evaluate the impact of experimental conditions on diffusion. Finally, we highlight how physicochemical properties of nanoparticles influence their diffusion through mucus.

Mucus models

The different models

There are various models of mucus described in the literature (Table 1), from the simplest *ex vivo* model to the closest *in vivo* models, and from simple mucin, artificial mucus, to natural mucus from horse, pig or human. It is also possible to use pathologic mucus and mucus produced by specialized cells for *in vitro* transcellular crossing or uptake experiments. *Ex vivo* or *in vivo* models have also been described.

The simplest models include only mucin solutions reconstituted with different solutes. Norris and Sinko prepared reconstituted mucin by mixing mucin with sodium phosphate and a sodium carbonate buffer, adjusted to pH 6.5 [7]. By contrast, Dawson *et al.* prepared reconstituted artificial pig gastric mucus by mixing pig gastric mucin (PGM) 60 mg/mL, dipalmitoylphosphatidylcholine (DPPC), bovine serum albumin (BSA) and Hepes buffer (pH 7.4) [12]. Bhat *et al.* prepared a model of cystic fibrosis mucus (CFM) by adding calf thymus DNA and BSA to reconstituted pig gastric mucus solution [16], whereas Bhat prepared a reconstituted pig gastric mucus solution by mixing PGM (40 mg/mL) and isotonic phosphate buffer (pH 7.4) containing sodium azide, followed by two rounds of centrifugation and then dialysis [58]. Larhed *et al.* prepared an artificial mucus model comprising purified PGM (0.4%), a lipid mixture (3%), pig serum albumin (3.1%), DNA (0.5%), Tween 80 (0.75%) and 10 mM phosphate buffer [13].

Thus, these different preparation protocols resulted in different mucus models of differing pH and with different physicochemical properties. In fact, mucus models prepared from diluted mucin using different methodologies differed not only from each other, but also from crude mucus models extracted from animals.

Advantages and limits of the models

Crude mucus is the most ideal model but it has some disadvantages. First, it is difficult to access a source of mucus from an individual. Second, the composition and, thus, chemical and

TABLE 1

Mucus models found in the literature

Mucus model	Origin		Ref
Mucin	Type II/pig gastric purified mucin		[5–8]
	Type III/pig gastric unpurified mucin		[9,10]
	Semi purified		[11]
Artificial mucus	Reconstituted pig gastric mucus (BSA + DPPC + buffer)		[12]
	MLPD		[13]
	Reconstituted pig gastric mucus (mucin typeII + lecithin + BSA + HEPES buffer, *etc.*)		[9]
Natural mucus	Rat intestinal mucus		[14]
	Pig gastric mucus		[15,16]
	Pig intestinal mucus (PIM)		[5,8,13]
	Horse respiratory mucus		[17]
	Human cervico-vaginal mucus (CVM)		[18–26]
	Human airway mucus (HAM)		[27]
Natural but pathologic mucus	Cystic fibrosis sputum (CFS) or cystic fibrosis mucus (CFM)		[16,23,28,29]
	Chronic rhinosinusitis mucus (CRSM)		[30]
***In vitro* model**	HT29-MTX		[31–37]
	HT29-FU		[34]
	HT29GlucH		[38]
	HT29-H		[39,40]
	Calu-3		[41]
	Co-culture	90/10	[37]
	Caco-2/HT29-MTX	75/25	[37]
	(relative % of each cell line given in the next column)	76/24	[35]
		70/30	[42]
		50/50	[37,43]
	Co-culture Caco-2/HT29-H	50/50	[44]
Tissues *ex vivo*	Porcine nasal mucosa		[45]
	Rat jejunum portion		[46–48]
	Rat intestine		[49]
	Rat ileum		[50,51]
***In vivo* model**	Rat		[32,46,47,50,52,53]
	Mice		[54–57]

physical properties can vary between batches because of interindividual variability; for example, mucin concentrations varied from 10 ± 2 to 47 ± 3 mg/mL among six different mucus samples [29]. To limit interindividual variation, some batches can be collected and mixed. Mucus samples can be stored at −20°C without significant change. For example, no effect of freezing and thawing on viscoelasticity [29] was observed and storage at −20°C did not influence the diffusion coefficients of drugs [5]. However, a little evaporative loss from cervicovaginal mucus (CVM) was observed at room temperature and storage at −20°C reduced evaporative loss [59].

Pig is a relatively large animal and so it is possible to obtain sufficient mucus from only few animals to perform several experiments. Moreover, pig mucus and human mucus are similar in structure and molecular weight [60], which is important given that it is also possible to observe differences in mucus properties for different animals of the same species [61]. As a result, commercial pig mucin has been used as a mucus substitute to prepare mucin solutions. Two forms of PGM are currently available commercially: purified mucin (type II) and unpurified mucin (type III). The main advantage is that the composition is more stable, although this is not always relevant given that mucus contains various other components, such as lipids, proteins, or salts. Thus, some researchers have focused on reconstituted mucus; for example, Dawson *et al.* [12] mixed PGM with BSA, DPPC and buffer, whereas McGill and Smyth [9] mixed mucin with lecithin, BSA and HEPES buffer; an artificial mucus model was also proposed by Larhed [13]. Interestingly, reconstitution of mucus with mucin did not suppress its variability compared with crude mucus, in that McGill and Smyth observed some heterogeneity in composition and nonuniformity of the rehydrated mucin polymers used in *in vitro* prepared mucus models [9].

Griffiths *et al.* demonstrated that the extraction process modifies commercial PGM samples. It disrupts the disulfide bridges, leading to a weaker sol–gel transition at around pH 4 and a lack of gel formation [10]. The structural perturbation in mucin was confirmed by the lack of interactions between mucin and poly(-ethylene glycol) (PEG). Bhat *et al.* compared drug diffusion through CFM and through gel and sol fractions generated by a separation process. Unfortunately, this process altered the mucin structure, as evidenced by the highly branched structures observed by transmission electron microscopy (TEM) [16].

In vivo and *ex vivo* assays can be consuming in terms of the number of animals required. However, working with slaughterhouses enables large amounts of animal mucosa to be obtained from only a few animals. For example, Wadell *et al.* studied diffusion through porcine nasal mucosa [45]. This mucosa was large enough and only one individual was used to achieve tissue specimens for a full six-chamber experiment, whereas a higher number of smaller animals were needed to get the same area. However, for studies involving intestinal mucosa, fewer animals are needed.

Model comparisons

Studies compared molecule diffusion through different mucus models and showed different apparent permeability according to the mucus model. Mucus composition depends on the origin of the mucus (species and organ source) and its composition influences its properties and reactivity against others molecules.

The weight-average molecular mass of mucin comprising mucus is different depending on the function of its original location [62]. For example, the molecular mass of pig colonic mucin is 5.5×10^6 Da, of human cervical mucin is 11×10^6 Da and of PGM is 44×10^6 Da. The influence of mucus model composition on mucus properties has largely been studied over the past few years, with obvious differences reported between pathogenic and nonpathogenic models. For example, chronic rhinosinusitis mucus (CRSM) and cystic fibrosis sputum (CFS) have similar barrier properties because the viscoelasticity of mucus gel is exacerbated in both cases by pathogenic inflections and chronic inflammation. As a consequence, results of Lai *et al.* suggested that CRSM has greater adhesivity compared with healthy CVM [30]. The solution environment also has an impact on mucus properties, particularly in the case of disease. For example, in CFM, the high extracellular Ca^{2+} concentration leads to thick mucus over the long term [63]. The poor bicarbonate availability in this mucus can explain its high viscosity and mucin aggregation, because of the ability of bicarbonate to sequester Ca^{2+} [64]. The high levels of soluble proteins on the mucosa partially explain the characteristically thick mucus in asthma and other bronchial inflammatory diseases [65].

Purified PGM solution did not provide an accurate model of native mucus because it did not exactly reflect mucus constituents such as water, mucin and lipids, mineral salts and free proteins in either their quality or quantity, which increased the possible interactions between particles and mucus. Larhed *et al.* studied the diffusion of different drugs through native pig intestinal mucus (PIM) and purified pig gastric mucin (PPGM) [5]. They demonstrated that the diffusion coefficient of lipophilic drugs was reduced in a native PIM model but less so in PPGM, compared with the diffusion coefficient in buffer. For example, 36% of cyclosporine A diffused in PPGM and 16% in PIM. The same phenomenon was observed with another drug: 78% of 1-deamino-8-D-arginine-vasopressin (dDAVP) diffused in PPGM and only 17% in PIM. Thus, a substantial part of the mucus barrier was likely to be formed by other components of the native mucus besides mucin. Moreover, a relation between lipophilicity (i.e. log *P*) and diffusions in PIM was observed but no relation was found in PPGM. Thus, the native PIM was a more realistic model of gastrointestinal mucus and provided more information regarding the barrier properties of mucus *in vivo*. Similarly, McGill and Smyth's study showed that rhodamine B permeation was significantly different in mucin solution and in an artificial CFS model because of the differences in the composition of the mucus model [9].

Components other than mucin have also been found to be responsible for the reduced diffusion of lipophilic drugs in PIM compared with PPGM. Larhed *et al.* identified lipids and proteins as components with an important impact on drug diffusion [13], with the drugs interacting with the lipids and proteins. The same authors tried to reproduce artificial mucus with a composition mimicking PIM. However, the diffusion obtained in their artificial mucus was similar but not identical to that in PIM, highlighting the fact that it is difficult to reconstitute the complete structure of native mucus.

Bhat *et al.* compared the diffusion of three drugs through buffer, native mucus and synthetic mucus models [16]. For the drugs

tested, the observed permeability was always in the following rank order: buffer > reconstituted pig gastric mucus (i.e. mucin solution) > whole CFM > CFM sol fraction > synthetic CFM > CFM gel fraction. Synthetic CFM solutions were prepared by adding BSA and DNA to pig gastric mucus solutions. Lieleg *et al.* observed that mucin concentration was an essential parameter for diffusion: if mucin concentration increased, the impact of the barrier became more pronounced [66].

Mucin concentration can vary with the location of mucus in the body, as well as with various other physiological and pathological parameters [67]. Griffiths *et al.* studied the diffusion of polymers in mucin solutions that ranged in concentration from 0 to 5% (w/w). A decrease in the diffusion rate was shown with increasing mucin concentration [10]. Therefore, diffusion depends largely on mucus components and its proportions, which can be explained by the fact that interfiber spacing depends only weakly on hydration but more on the concentration of mucin [21].

In addition to observed differences between mucus model compositions, differences in mucus model structure have also been observed and have a role in particle and drug diffusion. The average pore size of human CVM, determined by fitting the measured diffusion rates of particles to Amsden's obstruction-scaling model, was 340 ± 70 nm [18]. The average mesh spacing of human CFS was 140 ± 50 nm, as shown by the dynamics of mucus-resistant particles [28]. However, comparison is difficult because results depend on the source of mucus as well as on the method of sample preparation, which can disrupt mucus structure. Ensign *et al.* observed variations in the mucus mesh at different anatomical locations [68].

The sampling method used is also important, given that mucus comprises two layers, the firmly adherent mucus and the loosely adherent mucus [69]. If the extraction method is too energetic, the sample will contain remnants of mucosa, such as cells and DNA, whereas, if the sample is too superficial, it might contain only the free mucus layer.

The increased hydration of ovulatory endocervical mucus (OCM) compared with other mucus secretions (during nonovulatory periods and at other mucosal tissues) increased the pore size of mucin. Tang *et al.* performed studies of PLGA nanoparticle diffusion in OCM and CVM [23]. Differences between OCM and CVM led to a difference in the penetration improvement resulting from the PEG coating. A modest increase in penetration rate was observed in OCM, whereas the same modification improved the penetration rate in CVM 400-fold.

As discussed above, mucin is often used to prepare mucin solutions or artificial mucus, although the preparation methods used to obtain mucin can disrupt its structure. Therefore, even if mucin is used at the same concentration as in natural mucus and with the other components of mucus, differences can be observed compared with crude mucus. For instance, anionic particle mobility was significantly higher in purified PGM than in native intestinal mucus [8]. The difference in mesh structure of native mucus compared with that of purified mucin and/or differences in composition between the two media were related to this difference in mobility. The degradation occurring during the purification procedure has been related to one or other of these differences. Mucin was present at high concentrations in both media, and cationic particle mobility was similar, owing to the adhesion of the cationic particles to negatively charged mucin fibers. Particle transport rates were more heterogeneous in native mucus, because of the higher heterogeneity of the porosity of the mucus mesh. The more homogeneous nature of the purified mucin solution versus native mucus was supported by microscopic observation.

Nanoparticle transport behavior was not significantly different in colonic mucus on the surface of freshly excised mouse colon tissues compared with mucus scraped from the tissue surface. This suggests that the collected mucus layer was, in this case, not disturbed [68], and enabled the researchers to study the mucus barrier effect only.

Diffusion systems

The different models

Various protocols have been developed to evaluate interactions between particles and mucus (Table 2), such as the mucoadhesion assay [48,70,71], *in vivo* experimentations [32,55] with radioactivity studies [53] or pharmacokinetic (PK) studies [46,54,72], binding proprieties [73] and diffusion studies. Here, we focus on diffusion protocols and briefly discuss mucoadhesion, which is often the first step of diffusion.

To study drug and particle diffusion, numerous systems have been used, including multiple particle tracking (MPT) [8,12,18–20,22,23,25,28,74–76]; two samples tubes that are then filtered and centrifuged [11]; side-by-side systems [6,16]; side-on-three compartment diffusion [15]; diffusion chambers [45] including Ussing chambers [46,47,49,77]; modified Franz diffusion cells [14]; modified Transwell-Snapwell® diffusion chambers [7,29]; modified diffusion cell setups [9]; fluorescence recovery after photobleaching (FRAP) [21]; radioactivity with two syringes [5,13]; Transwell® covered by cells [24,41,78]; Transwell® diffusion [32,33,35–39,42,43,50]; and cell association [79].

Side-on-three compartment diffusion is one of the most commonly used methods. The diffusion cell comprises one donor compartment, one acceptor compartment and one central compartment containing the mucus model. Drugs or particles are placed in the donor compartment and their arrival in the acceptor compartment is evaluated over time. Given that mucus is placed between two compartments, the amount of drug or particles in this compartment determines their permeability or diffusion coefficient through the mucus.

Membranes between compartments are impermeable to mucus but not to drugs or particles; thus, their capacity to retain the drug or particles must be well known to distinguish the effects of the membrane versus the mucus diffusion. Therefore, different side-on-three compartments have been developed, in the form of diffusion chambers (i.e. side-by-side® diffusion cells) customized with a membrane holder (Fig. 1) [6,16].

Numerous research teams have developed in-house manufactured side-on-three compartment diffusion cells. For example, Shaw *et al.* added a polycarbonate filter membrane and metal gauze filters to a diffusion cell [15]. Norris and Sinko modified Transwell-Snapwell® chambers, comprising two compartments and a tissue between them, by adding filters and a ring on which to place mucus [7], as did Sanders *et al.* [29]. Similarly, Grubel and Cave modified a microfiltration device to obtain a permeability device [11]. When studies focus on mucosal tissue, the experimental systems used are simpler because tissue is more easily

maintained between compartments. For example, Bravo-Osuna *et al.* used a Ussing cell comprising two compartments separated by rat intestinal epithelium [77], whereas Wadell *et al.* placed porcine nasal mucosa in diffusion chambers [45].

Another approach was used by Lai *et al.* [19]. Particle transport rates were measured by analyzing trajectories of fluorescent particles in mucus bulk, by MPT. In this context, only one compartment is needed, thus avoiding the membrane affecting diffusion. The microscopic motion of hundreds of fluorescent particles is recorded by video microscopy and, thus, particle detection is performed in mucus without disturbing the system. In the same way, FRAP [21] has been used to investigate the mobility of labeled molecules in mucus and biogels. The sample is placed on a microscope and a high-intensity laser beam is used to bleach the fluorescence of the molecules, which causes a drop in the fluorescence intensity. The diffusion coefficient is obtained by the recovery profile of the fluorescence intensity, which results following diffusion of the nonbleached molecules into this area (Fig. 2) [80].

As a noninvasive method, pulsed-gradient spin-echo NMR (PGSE-NMR) was used by Griffiths *et al.* [10] to quantify the diffusion of a probe polymer. The central feature of this technique is the application of a magnetic field gradient that encodes the position of the molecule into the NMR signal.

Other original models can be used to predict particle diffusion through mucus. For example, a 2D model was developed for studying the interaction of surface-modified lipid nanocapsules (LNC) with mucus. This 2D model, based on surface balance measurements at a constant pressure or area, can be used as a screening method for choosing suitable surface-modified LNC formulations for assessing diffusion using 3D models [81].

Advantages and limits of diffusion systems

The limits of diffusion protocols are related to the quantification or detection methods used. Some teams have used radiolabeled drugs and detected resulting radioactivity by using a liquid scintillation counter. For example, Bravo-Osuna *et al.* used ^{14}C mannitol [77] and Wadell *et al.* used ^{14}C mannitol and D-(2-3H) glucose [45], whereas Larhed *et al.* used ^{14}C mannitol, ^{3}H propranolol, ^{14}C hydrocortisone, ^{3}H testosterone and other radiolabeled drugs [5,13]. By contrast, radiolabeled ^{14}C ibuprofen was used by Shaw *et al.* [15]. In addition, Saltzman *et al.* labeled molecules with fluorescein and measured their diffusion coefficients by using computer imaging of fluorescence profiles and by FRAP [21]. However, working with radioactivity is expensive and is not easily accessed because of the need for specific equipment; in addition, agreement and safety rules are both necessary and stringent.

Much equipment is required for MPT, including a silicon-intensified target camera mounted on an inverted epifluorescence microscope equipped with a 100× oil-immersion objective lens; the appropriate filters; glass chambers; specific software; and

TABLE 2

Evaluation of colloid diffusion with different models related to their *in vivo* efficacy (predictability)

Route	*In vitro* model	*Ex vivo* model	*In vivo* model	Ref	Drug nanocarrier	Predictability/conclusions
Oral	Caco-2/HT29-M6		Efficiency	[82]	Encapsulation of calcitonin into chitosan nanocapsules	*In vitro* model revealed that the mucoadhesive properties of chitosan nanoparticles may represent a key factor for their ability to improve peptide absorption after oral administration
		Ussing chambers (rat jejunum)	*In vivo* bioadhesive study and pharmacokinetic (PK)	[47]	Paclitaxel (PTX)-loaded pegylated nanoparticles (NP)	Similar improvement of bioavailability was observed for PEG PTX-NP *in vitro* and *in vivo*
	Mucin adhesion (mucin type III)		PK study	[83]	PTX-loaded chitosan–vitamin E succinate (CV) nanomicelles (chitosan thiolated or not)	Thiolation improve fourfold AUC for CV nanomicelles and lead only to a twofold increase in mucin adhesion
	Muco-adhesion		PK study	[84]	Enoxaparin loaded nanocomplexes (chitosan grafted glyceryl monostearate copolymers)	Mucoadhesion results are not showed (only: mucoadhesion significantly increased with modification of chitosan with GM compared with that of chitosan, GM graft ratio: 3.7% = 11.1% > 18.6% and chitosan 100 kDa > 20 kDa) Vivo: C < 3.7% < 11% > 18% and 100 kDa > 50 kDa Same conclusion: maximal bioavailability for nanocomplexes prepared using CS100-GM11.4% copolymers
Colon	MPT in mucus	Tracking on freshly excised mucosal tissues		[68]		Displacement of particle were not significantly different in collected colonic mucus and in *ex vivo* colon tissue
Pulmonary	MPT in CFS		*In vivo* airway gene transfer	[85]	DNA nanoparticles composed of plasmid DNA compacted with block copolymer of poly-L-lysine and PEG (2, 5, and 10 kDa)	All DNA nanoparticles were immobilized in freshly CFS. Mice receiving $CK_{30}PEG_{10k}$ or $CK_{30}PEG_{5k}$ DNA nanoparticles exhibited higher luciferase expression than $CK_{30}PEG_{2k}$ (due to higher nuclease attack of $CK_{30}PEG_{2k}$)

fluorescent particles. Lai *et al.* [18,19] and Suk *et al.* [28] also used fluorescent polystyrene (PS) particles obtained from molecular probes. Using the same method, Crater and Carrier used fluorescent FluoSpheres® obtained from Invitrogen molecular probes [8]. Tang *et al.* [23] prepared fluorescent PLGA nanoparticles. Unfortunately, fluorescent labeling can modify carrier properties and, thus, their diffusion ability.

In addition to quantification or detection methods, the diffusion system has a role in diffusion evaluation. Experimental variability was observed by Bhat *et al.* [16] using a modified side-by-side diffusion cell and mucus placed in an approximately 3-mm thick chamber. This thickness is higher than is found *in vivo*. With a similar system, Norris and Sinko observed the same variability in the measurements with a smaller mucus thickness of 0.38 mm [7]. The variability was not only related to the thickness of mucus, but also to the complexity of the diffusion system. In fact, given that the membrane is a supplementary barrier to diffusion, its choice is important. The goal should be to select a membrane with the lowest effect on diffusion to observe phenomena purely related to mucus. In their study, Bhat *et al.* showed that drug diffusion was under membrane control for the three drugs that they tested [6]. In the case of diffusion across compartments, when the donor solution is placed in the donor compartment at the start of the experiment, the empty membrane becomes soaked by solution, corresponding to the time of flow establishment [86]. Steady-state conditions are then established. Membrane thickness should be the lowest and experimentation duration should be high enough to overlook the time of flow establishment. Experimentation duration should also be long enough to enable membrane equilibration, but also short enough to avoid significant concentration variations in the donor solution. In such a steady-state condition, equation of permeability can be simplified. Using these particular conditions, Bhat *et al.* determined permeation at steady state for drug diffusion across CFM [16].

The apparent permeability P_{app}, expressed in cm s^{-1}, is classically calculated using Eq. (I) [7,14,45,51,77]:

$$P_{app} = \frac{dQ}{AC_0\,dt} \quad \text{(I)}$$

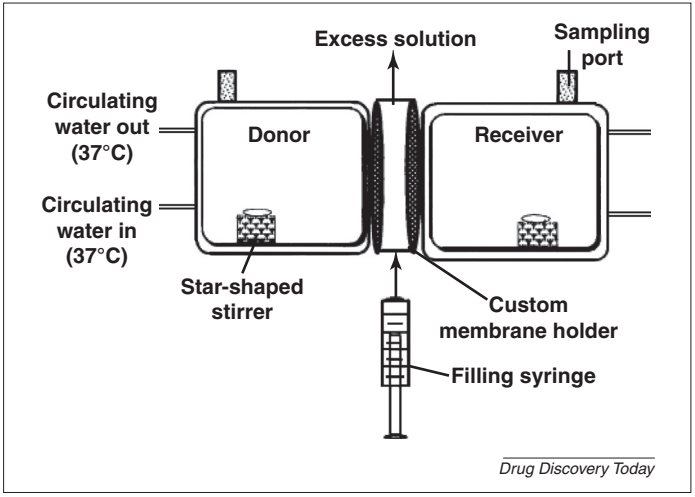

FIGURE 1

Side-by-side diffusion cell with a customized membrane holder.
Figure reproduced, with permission, from Bhat *et al.* [6].

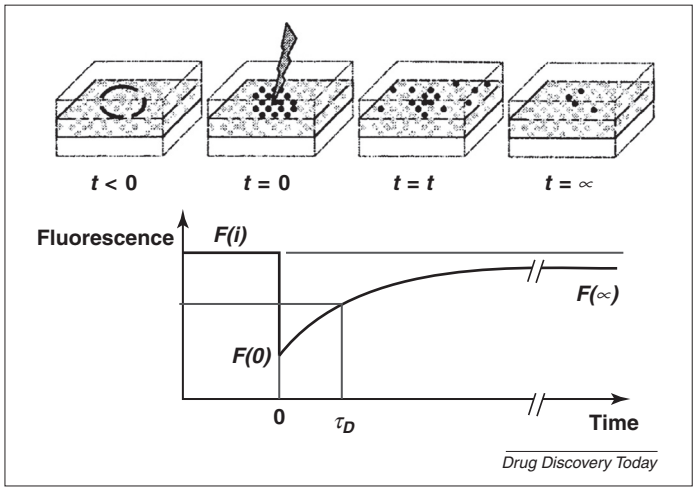

FIGURE 2

Schematic representation of a fluorescence recovery after photobleaching experiment. The initial fluorescence before bleaching is recorded as $F(i)$. At $t = 0$, a drop in fluorescence to $F(0)$ is caused by a high-intensity light beam bleaching the molecules. The bleached molecules exchange their position in the bleached area with non-bleached fluorescent molecules from the surrounding, due to the random motion/diffusion. This results in a recovery of the observed fluorescence.
Figure reproduced, with permission, from Occhipinti and Griffiths [80].

where dQ/dt is the rate of drug appearance on the received side (μg s^{-1}), C_0 is the initial concentration over the donor side (μg mL^{-1}) and A is the surface area (cm^2).

It is important to note that Eq. (I) is valid and can be used only if the membrane volume is negligible versus the volume of the compartments; that is, $\lambda < 0.02$. However, if either the length of membrane equilibration or membrane volume is too high, Eq. (I) should be modified using λ [87].

Some researchers have used Eq. (II), which is based on this previous formula, but that takes into account the contribution of each barrier [6,16]. In case of multilayer systems, membrane permeability, P, is given by Eq. (II):

$$P = \frac{D}{\delta}$$

$$\frac{1}{P_{total}} = \sum\frac{1}{P} = \sum\frac{\delta_i}{D_i} \quad \text{(II)}$$

where D is the diffusion coefficient of the molecule, expressed in $cm^2\ s^{-1}$. The thickness of the layer δ is expressed in cm. A side-on-three compartment system contains two membranes and a mucus layer, the former of which can constitute an important barrier to diffusion [88]. In this case the permeability can be expressed as:

$$\frac{1}{P_{total}} = \frac{1}{P_{mb1}} + \frac{1}{P_{mb2}} + \frac{1}{P_{mucus}}, \quad \text{(III)}$$

where P_{mb1} and P_{mb2} represent the permeability across each of the membranes, P_{mucus} is the permeability across the mucus layer and P_{total} is the permeability across the entire system.

In the case of a lack of system uniformity, a concentration gradient appears and constitutes a pseudo-membrane [89]. The existence of unstirred layers implies that, in any phenomenon depending on the difference between the two surface concentrations, possible serious errors can be made by using the difference between the bulk concentrations. The effect of the unstirred layer

is more complicated than the case of stirred layer, and is given by Eq. (IV):

$$\frac{1}{P_{total}} = \sum \frac{1}{P_{mb}} + \frac{1}{P_{mucus}} + \sum \frac{1}{P_{conc}}, \quad \text{(IV)}$$

where P_{conc} is the permeability across the pseudo-membrane together with the concentration gradient. For example, Korjamo *et al.* observed that, by varying stirring speed, the concentration gradient had a different effect on drug permeability across Caco-2 cells on Transwell® [90].

The method becomes very inaccurate when diffusion is almost entirely rate controlled by the unstirred layers [91]. This is why, if the concentration in each compartment varies substantially between the start and end of the experiment, stirring is necessary to homogenize the media.

Parameters influencing diffusion and comparisons

Diffusion in mucus depends on its composition [13], such as the mucin concentration [66], which, as discussed above, depends on the mucus model used [8]. Thus, the choice of mucus model is crucial. The mucus model used needs to be the closest to the type of physiological mucus encountered by the drug delivery system *in vivo*. Particle diffusion also depends on the surface chemistry of the particle [8] and the particle size [22]. However, other parameters can also influence diffusion and should be checked.

Grubel and Cave observed that the effect of the formulation on mucus viscosity appeared to determine the movement of clarithromycin through mucus [11]. For example, the greater proportion of inactive polymeric ingredients in Biaxin® granules, the higher the increase in the viscosity and the more enhanced the barrier properties of gastric mucin. Moreover, Sanders *et al.* showed that the elastic modulus of mucus influenced the percentage of transported nanospheres [29].

When Griffiths *et al.* added dendrimers to mucin solutions, changes in the mucin scattering were induced, indicating an interaction between these polymers and mucin [10], a change that was pH dependent. As a consequence, the diffusion of polymers showed a complex dependency on both pH and mucin concentration. Cao *et al.* demonstrated that mucus undergoes a pH-induced conformation change [92], whereas Lieleg *et al.* showed that increasing the mucus pH from 3 to 7 resulted in a general increase in particle mobility because acidic mucus formed a higher and more selective barrier compared with neutral mucus [66]. Given that mucus pH varies with its function and, therefore, localization in the body (Fig. 3), the choice of mucus source to set up a model has to be made carefully.

Shaw *et al.* demonstrated that the diffusion of ibuprofen increased for higher pH values, as a result of changing the electrostatic repulsion interaction and lowering the viscosity of mucus [15]. Interactions were related to the ionized state of the mucus and of ibuprofen. No change was observed for paracetamol, which is a unionized drug. Therefore, the effect on the charge interaction between the drug and mucus is more important than the effect of viscosity on diffusion in this study. Lieleg *et al.* found that electrostatic interactions were sensitive to the ion content of a solution. Given that the surface charges of synthetic particles or polymers were partially shielded by solubilized ions in buffer, the strength of the attractive or repulsive forces between diffusing particles and mucus depended on the salt content [66].

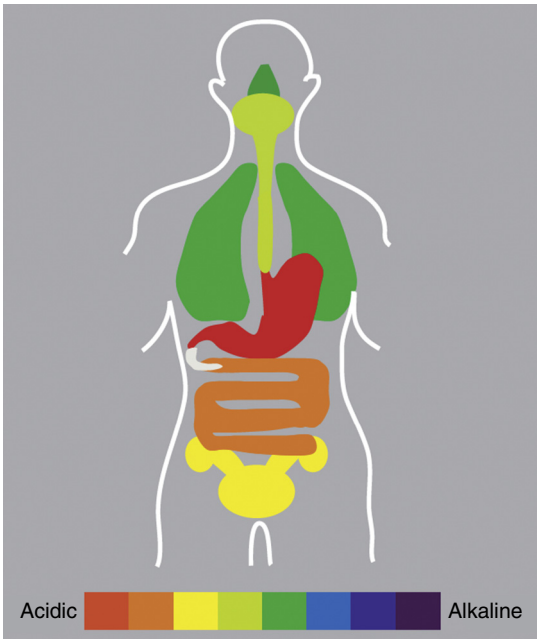

FIGURE 3

Mucus pH related to its localization in the body.
Figure adapted, with permission from Lieleg *et al.* [66].

The concentration of the studied particles is also important. For example, Lai *et al.* demonstrated that the addition of a high concentration of particles to CVM prevented their transport and caused the collapse of the mucus fibers, whereas a low concentration of particles did not cause bundling and allowed particle movements [19]. At high PS particle concentration (i.e. 10%, v/w), hydrophobic interactions can cause the aggregation of mucin fibers in human cervical mucus (HCM) [26]. Similarly, Wang *et al.* observed that the effect of mucoadhesive nanoparticles on mucus depended on the particle concentration [76].

Therefore, drug and particle diffusion are sensitive to pH, ionic force, viscosity, particle concentration, and the experimental conditions of diffusion studies (i.e. when and how long diffusion was observed). Given that experimental conditions can change between researchers and teams, comparisons between studies must therefore be made with caution.

The specific case of colloid diffusion through mucus

Knowing the relation between colloidal carrier properties and their ability to diffuse in mucus enables better design of these drug delivery systems. Here, we discuss each parameter that should be optimized to gain better colloid diffusion in mucus.

Colloid design: size choice

Most studies conclude that smaller particles move faster in mucus [52]. For example, Sanders *et al.* observed a difference in the transport of PS nanospheres through CFS, depending on particle size. Increasing particle size from 124 nm to 270 nm or 560 nm decreased the mean percent of nanospheres transported after 150 min, caused mainly by stronger steric obstruction [29]. Similarly, Norris and Sinko observed the limited ability of particles >0.5 μm to diffuse through mucus [7]. By contrast, for Hosseinzadeh *et al.*, a larger

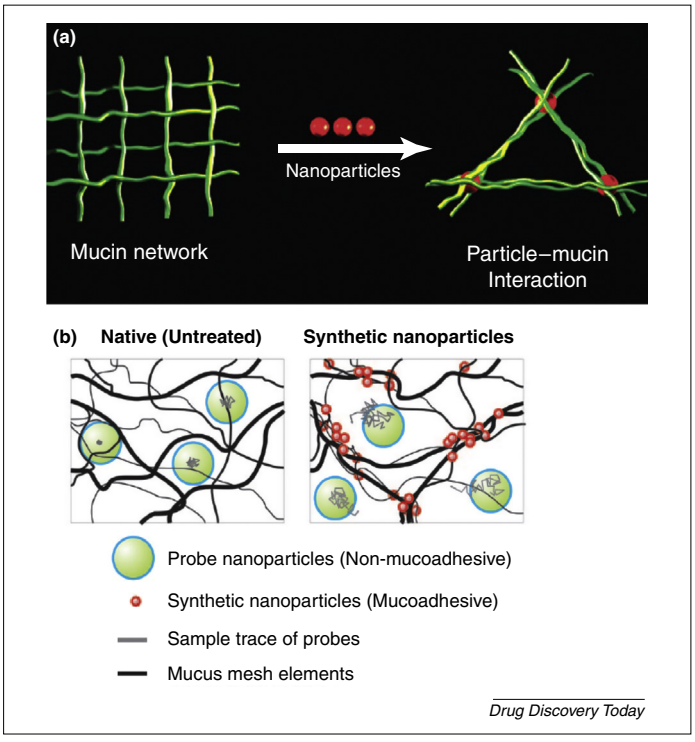

FIGURE 4

(a) A mucus disruption induced by particles. A mucus fiber network is depicted on the left with the introduction of particles leading to their entanglement with mucus resulting in a change of the fiber network. (Figure reproduced, with permission, from McGill and Smyth [9].) **(b)** The potential effects of mucoadhesive particles on the mucus structure. Mucoadhesive particles can increase mucus pore sizes by bundling mucin fibers with adhesive interactions. (Figure reproduced, with permission, from Wang *et al.* [76].)

surface area was provided by smaller nanoparticle sizes, and increased adsorption in mucin, which led to higher mucoadhesive properties for mucoadhesive nanoparticles [70]. Other studies [9,51] confirmed the relation between size and mucoadhesion, which influences particle diffusion.

Size effect might be the result of a steric obstruction of mucin mesh spacing that is linked to mucin interfiber spacing size. This size depends on the mucus model and the method used for its determination. For example, Saltzman *et al.* determined the geometric characteristic of HCM gels by scanning electron microscopy (SEM) and diffusion studies [21]. The probable interfiber spacing, assuming a random fiber arrangement, was 170 nm and the inferred interfiber spacing obtained by measuring diffusion coefficient was 150 nm. Olmsted *et al.* predicted a mesh spacing of 100 nm, by applying Amsdem's obstruction-scaling model to HCM and by electron microscopy [26]. Yudin *et al.* revealed that mucus has a fibrous structure with a 500-nm interfiber spacing between the primary elements and an additional finer structure with a spacing of approximately 100 nm [93]. Similarly, Kirch *et al.* observed by cryogenic SEM that horse respiratory mucus had large pores heterogeneously combined with very small pores [17], which is in accordance with findings for CVM [18]. In SEM images, normal HAM pores ranged from tens to hundreds of nanometers in diameter, with many pores <100 nm [27].

Colloid design: considerations of surface properties

As we discuss below, surface properties such as electric charge, chemical moieties, hydrophilicity and/or lipophilicity have been showed to have a significant role in the ability of colloidal carriers for mucus diffusion. Thus, a negative relation between the diffusion of peptides and their lipophilic properties (i.e. log *P*) in PIM has been observed [5]. Mistry *et al.* also determined that polysorbate-coated PS nanoparticles increased transport in mucus by increasing hydrophilicity [55]. A surface modification of the PS particle with pegylated polysorbate, increased transport through mucus not only by increasing the hydrophilicity, but also by reducing the negative charge of the PS particle. Wang *et al.* observed hydrophobic interactions between hydrophobic domains on the particles and the mucin fibers [20]. These interactions led to an attraction between particles and mucin. Similarly, Norris and Sinko observed that the amidine PS microspheres, which have the lowest hydrophobicity, also had the highest permeability through gastrointestinal mucin solution [7]. Thus, to avoid lipophilic interactions, the particle surface must be hydrophilic, although other properties have been shown to have a role in diffusion and different types of interaction are balance each other out. In the same study, the zeta potential was also shown to be a valuable indicator of the diffusion ability of PS particles, with a lower zeta potential favoring a higher diffusion ability. Repulsive electrostatic interactions were also observed between negatively charged particles and negatively charged mucin. However, if a particle was too attracted by mucin because of lipophilic or electrostatic interactions, the particle became entangled in mucus. By contrast, if a particle was too repulsed by electrostatic interaction, it was unable to diffuse through mucus. In the case of hydrophilic particles, diffusion is easier for particles that have no charge (i.e. neutral particles).

Dawson *et al.* prepared cationic particles by adding a cationic surfactant (PLGA-DDAB/DNA) to COOH PS-particles to enhance their hydrophilicity. They observed that cationic particles aggregated with mucus, which might have led to larger mucus pores and promoted more rapid transport for a fraction of particles [12]. Mura *et al.* modified PLGA nanoparticle surface charge with chitosan (CS), pluronic F68 (PF68), and poly(vinyl alcohol) (PVA) [24]. A positive zeta potential was obtained with PLGA/CS nanoparticles, whereas it remained almost neutral for PLGA/PVA nanoparticles, and was negative for PLGA/PF68 nanoparticles. PLGA nanoparticles exhibited a hydrophobic surface, which interacted with the hydrophobic domains of the mucin chains. PLGA/CS and PLGA/PVA nanoparticles became entrapped by mucus, whereas PLGA/PF68 nanoparticles diffused unimpeded between the mucin networks. Hydrophobic interactions were balanced by electrostatic repulsions. The coating with Pluronic® F127 (PF127) on PLGA particles led to a near-neutral surface charge, whereas coated particles diffused more freely in CRSM compared with uncoated particles [30]. The modification of liposomes with PF127 improved diffusion through native rat intestinal mucus, because of the distribution of the hydrophilic polyoxyethylene part of PF127 on the liposome surface [14]. As a consequence, hydrophobic and electrostatic interactions of the liposome with mucin were reduced.

Crater and Carrier showed significant differences between anionic and cationic particle mucus-penetrating capacities [8]. Particle

mobility was inversely related to surface zeta potential. Sufficient surface coverage of anionic functionalities obtained with anionic (carboxylate or sulfate) particles suppressed the attractive interactions between the hydrophobic PS particle cores and mucin, whereas inadequate surface coverage with cationic (amine) particles resulted in increased hydrophobic interactions and led to the formation of particle aggregate. As a consequence, negative charge covering resulted in a significantly higher transport rate for particles exhibiting a hydrophobic core.

Mucoadhesive molecules

As discussed above, the literature has demonstrated evidence for a link between mucoadhesion and mucodiffusion. To some extent, mucoadhesive nano- or microparticles have more possibilities by which to diffuse, if they manage to avoid becoming entrapped in mucus because of interactions that are too strong. Thus, some studies have used mucoadhesive molecules associated with colloidal carriers to improve particle diffusion through mucus. Various novel mucoadhesive polymers have been developed, including lectins, thiolated polymers, bioadhesive nanopolymers, pluronics, alginate-polyethylene glycol acrylate and poloxomer [94]. For example, Ezpeleta *et al.* showed that lectin conjugates used for the delivery of hydrophobic drugs had an important affinity for mucin [73]. CS is another well-known mucoadhesive polysaccharide that forms disulfide bonds with cysteine-rich domains of mucus and also displays electrostatic interactions. Mucoadhesion effectiveness depends on polymer chemical features, such as MW, chain length, spatial arrangement, flexibility, hydration of polymer, hydrogen bonding, charge, and polymer concentration. Moghaddam *et al.* found better mucoadhesion with smaller nanoparticles with medium MW CS [95]. This polymer was modified by the addition of thiol group to obtain thiolated polymers called 'thiomers', capable of forming a thiolsulfide exchange reaction. Bravo-Osuna *et al.* developed a modified CS, called thiolated CS, which left particles still able to diffuse through the mucus [77]. Gradauer *et al.* also found that thiolated CS-coating doubled liposome mucoadhesion compared with uncoated liposomes [96]. The design of nanomicelles based on the acetylcysteine (NAC) functionalized CS-vitamin E succinate copolymer exhibited an ability to penetrate mucus [83]. As a consequence, NAC molecules increased the bioavailability of CS-vitamin E succinate nanomicelles, because of its good thiol activity. Petit *et al.* also found that CS enhanced nanoparticle mucoadhesion. Moreover, the mucoadhesion was enhanced twofold by the introduction of thiol groups on the surface of the CS nanoparticles [48]. A novel preactivated thiolated CS improved mucoadhesion compared with thiolated CS because of more active sulfhydryl moieties being available that protected thiol against early oxidation [71]. A novel amphiphilic copolymer was developed by Wang *et al.* by grafting glyceryl monostearate on CS [84]. This hydrophobic modification increased the mucoadhesion of the CS nanoparticles significantly ($P < 0.05$). Chen *et al.* compared the mucus penetration of liposomes modified with PF127 or CS [97]. They demonstrated that PF127-liposomes were inclined to penetrate the mucus and then to accumulate more effectively in intestinal tissue, owing to their more neutral and hydrophilic surface compared with CS liposomes and non-modified liposomes. Most CS liposomes were trapped in the mucus, resulting in limited mucus penetration. Moreover, CS use is not without risk to the administration site, because CS has a tendency to form complexes with mucin and other proteins, which could cause major disturbances to the epithelium membrane [98].

Coating particle with PEG

To avoid hydrophobic and electrostatic interactions, mucus-penetrating particles (MPPs) were coated with PEG, a hydrophilic and uncharged polymer. This coating minimized efficiently particle adhesion to mucus constituents [30]. Griffiths *et al.* observed that non-ionic polymers, such as 10-kDa MW or 100-kDA PEG did not interact with mucin, whereas dendrimers and polyethylenimine (PEI) exposed strong electrostatic (pH-dependent) interactions. Therefore, by designing polymer-based drug delivery systems, electrostatic interactions can be modulated to obtain good diffusion through mucus [10]. Similarly, the results of Tang *et al.* suggested that the sufficient PEG density of poly(sebacic acid) (PSA)–PEG particles provided rapid nanoparticle penetration of CVM and CFS [23]. PSA particles were strongly trapped by CVM, whereas PSA–PEG particles diffused unimpeded. Lai *et al.* showed that coating with 2-kDA PEG chains increased not only PS nanoparticle transport rates in CVM, but also the homogeneity of transport [19].

In agreement with findings in CVM [19], a dense covalent coating of low MW PEG led to particles penetrating more easily in other types of mucus, such as CFM [28], CRSM [30], and HAM [27]. Similarly, PEG-coated particles of 100, 200 and 500 nm penetrated mucus more rapidly than did uncoated particles of the same size [18]. Zabaleta *et al.* compared the apparent permeability through intestinal rat tissues of particles coated with different size of PEG: 2, 6 or 10 kDa MW [47]. The apparent permeability of particles pegylated with PEG of 2 kDa MW or 6 kDa MW was 2.5 times higher than nanoparticles pegylated with PEG of 10 kDa MW. A lower interaction between mucus layer and nanoparticles pegylated with PEG of lower MW explained the findings. Wang *et al.* also increased the coated particle displacements in CVM by a reduction in PEG from 10 kDa MW to 2 kDa MW [20]. However, particles coated with 5 kDa MW displayed rapid mucus-penetrating properties. These results indicated that a crucial MW threshold exists between 5 and 10 kDa. A small difference in the surface PEG coverage led to a 700-times decrease in the transport rate of PEG 2 kDA-PS particles with 40% PEG coverage, compared with the same particle covered with 65–70% PEG. Mert *et al.* observed that PLGA–vitamin E-PEG 1 kDa nanoparticles were as strongly trapped in CVM as uncoated PS nanoparticles, despite the coating, whereas PLGA/vitamin E-PEG 5 kDa nanoparticles rapidly penetrated CVM. This was the result of inadequate surface coverage of 1-kDA PEG [25]. Similarly, mucoadhesion of DNA particles was not reduced by low MW PEG coatings, probably because of inadequate PEG surface coverage [85]. Thus, inadequate PEG surface density appears to be a crucial limiting factor for the development of MPP. These results completed the design requirement of PEG-coated MPP. In conclusion, suitable particles must exhibit: (i) PEG of sufficiently low MW and (ii) a sufficiently high density of PEG surface coverage.

The hydrophobic core of PS particles formed polyvalent adhesive interactions with hydrophobic domains along mucin fibers and possibly with other mucus constituents. Coating particles

with PEG might reduce these particle–mucus adhesive interactions if the MW of PEG is too low to support adhesion by polymer interpenetration and hydrogen bonding [99–101]. PEG with low MW adopted a brush conformation that could facilitate the diffusion of particles in mucus by hindering the hydrophobic interactions [99,100], as, for example, is the case for PEG of 2 kDa MW or 6 kDa MW. By contrast, the disposition of longer PEG chains (i.e. 10 kDa) was different at the nanoparticle surface and favored the interpenetration and interaction with the mucus fibers [101]. PEGs with a too low MW, for example 1 kDa, were distributed inside or physically adsorbed on the nanoparticle surface. The pegylated nanoparticles obtained had a conserved high affinity for the mucus [99]. Regardless of these considerations, biodegradable MPPs have been developed and tested *in vitro* [102] and *in vivo* [57]. However, rapid penetration of CVM by these MPPs with PEG MW from 1 kDa to 10 kDa showed that a large range of PEG MW can allow the preparation of muco-inert nanoparticles. The determination of PEG MW range can be affected by various factors, such as the particle size, core material, type of mucus and surface PEG density. MPPs improved mucus diffusion, vaginal drug distribution and retention without causing inflammation, and PEG improved the mucus penetration of other carriers, such as solid lipid nanoparticles [103].

Diffusion enhancer

McGill and Smyth treated mucus with functionalized PS nano- and microparticles to disrupt the mucus before molecule diffusion [9]. The disruption was significant, increasing permeation of fluorescein and rhodamine through different mucus models. Similarly, Wang *et al.* from the team of J. Hanes used high concentrations of mucoadhesive particles (MAP) (Fig. 4), which enlarged mucus mesh pores to increase muco-inert PEG-coated particle diffusion by tenfold [76]. The mucoadhesive properties of amine-modified PS particles sized 200 nm resulted from the hydrophobic core and positive charges. Exposure to MAP can be a dangerous strategy because it can significantly increase the risk of infection or toxicity by enhancing penetration by pathogens or other foreign particles with muco-inert surfaces. Ensign showed that hypotonic formulations improved epithelial surface distribution and retention of MPP [56].

Another strategy to disturb the mucus layer is to use mucolytic molecules, such as DNase and N-acetylcysteine, to enhance nanoparticle diffusion through CFS [104]. Müller *et al.* functionalized particles with papain, a highly mucolytic enzyme, to reduce mucin crosslinks [105]. As a consequence, the application of these particles on PIM decreased mucus viscosity and improved particle diffusion.

Concluding remarks

Diffusion is a complex phenomenon that is sensitive to mucus composition and experimental parameters. To predict *in vivo* reality, the mucus selected for *in vitro* studies must be similar in composition and structure to the *in vivo* targeted mucus and the experimental parameters must be controlled carefully.

Mucus is an efficient barrier for particle diffusion because of physicochemical (hydrophobic, electrostatic, and hydrogen) interactions and steric occlusion related to its structure. Three strategies to improve diffusion through mucus have been described in the literature: (i) disruption of the mucus barrier; (ii) adhesive particles; and (iii) MPP [69], the importance of which has increased over the past few years [106].

In this Foundation Review, we have shown that the results for diffusion studies are often linked to the model used; therefore, a standard experimental protocol is needed to enable cross comparison of the colloids in terms of their ability to diffuse across mucus. The set-up of predictive models is also mandatory to enable the design of effective colloidal carriers that will diffuse easily through mucus, thus improving the performance of these new drug delivery systems.

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