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Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale

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Introduction: The benefits of mucoadhesive systems are related to the increased *in situ* residence and intimate contact of the delivery vehicle with the mucosa. The recent emergence of nanomedicine and the properties of nanoparticulate systems have created new challenges in understanding the nature and mechanisms of nanoscale mucoadhesion and in the development of methodologies for measuring its mucoadhesive potential. Even when usually regarded as an advantageous property, mucoadhesion can be an inconvenience for nanosystems, and strategies have been developed for minimizing interactions with the mucosal tissues/fluids.

Areas covered: This article summarizes the basic concepts of mucoadhesion at the nanoscale, different techniques used for measuring the mucoadhesive potential of nanosystems and strategies for increasing/decreasing mucoadhesive interactions.

Expert opinion: The mucoadhesion behavior of materials in bulk and at the nanoscale can significantly differ. Advances in the methodology used for studying the mucoadhesion phenomenon have contributed to its better understanding and, more importantly, the development of strategies to increase/decrease mucoadhesion. However, development of new methodologies for studying mucoadhesion at the nanoscale and the refinement of existing methodologies are still required. Also, a substantial amount of information is still lacking, particularly related to formulation issues, on how to translate lessons learnt at the bench top to the bed side.

Keywords: cytoadhesion, mucosal surfaces, mucus, nanotechnology, polymeric nanoparticles

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1. Introduction

The adhesive phenomenon has been long studied in a multitude of fields and has been defined as the prolonged binding between two materials when in contact with each other [1]. In the particular field of biological sciences, it is usually referred to as bioadhesion when at least one of the materials involved in the adhesive process is biologic in nature or, even more specifically, as mucoadhesion if the biologic material is a mucosal surface [2]. The nature and strength of the interfacial forces involved in the establishment and duration of mucoadhesion are diverse and have been the focus of thorough investigations over at least the last 30 years. The amount of knowledge, as well as the practical applications thereby resulting, ascertains a valuable role to mucoadhesion when addressing mucosal drug delivery. In general, the importance of mucoadhesive drug delivery systems can be translated into increased drug availability, protection of labile molecules and/or the ability to provide controlled/prolonged drug release, properties which have been shown to be directly or indirectly

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Article highlights.

- The mucoadhesion phenomenon is widely recognized as important in the design of mucosal drug delivery systems. Extensive data from bulk mucoadhesive experiments are limited when considering the nanoscale and, in some cases, misleading; thus, study of the mucoadhesion behavior of nanosystems is justified.
- The interaction of drug delivery systems with mucus modulates their mucoadhesion behavior. Understanding how nanosystems interact with individual mucin chains and the 3D structure of mucus is paramount.
- Different techniques can be used to study mucoadhesion at the nanoscale, including mucin particle method, microgravimetric methods (quartz crystal microbalance), atomic force microscopy, optical techniques (ellipsometry, surface plasmon resonance), diffusion/particle tracking methods, cytoadhesion methods, *ex vivo* methods, *in vivo* administration/*ex vivo* analysis and *in vivo* imaging.
- Different approaches have been used to increase the mucoadhesion of drug nanocarriers, the use of mucoadhesive polymers being the most popular, either as building blocks of nanosystems or by surface modification. Also, modulation of the surface charge of nanosystems can substantially influence the interaction with mucosal fluids, in particular with mucin.
- Size can substantially influence the diffusion of drug carriers through the mucin mesh that composes mucus fluids. Sizes in the range of 200 – 500 nm seem preferential for enhanced diffusion, while higher or smaller diameters may decrease transport through the mucus layer.
- Although mucoadhesion is usually sought for drug nanocarriers, recent research indicates that this may not always be desirable. Dense PEGylation of nanosystems has been shown effective in decreasing the interaction with mucus fluids and, when combined with adequate size, allow for nearly unhindered diffusion through these fluids.

This box summarizes key points contained in the article.

related with increased *in situ* residence and intimate contact of these systems with mucosal surfaces [3].

With the recent upsurge of interest in nanotechnology-based drug delivery systems and the development of available nanotools, new perspectives and challenges were introduced in the field of mucoadhesion. Particularly, traditional approaches towards the evaluation of mucoadhesives are inadequate or may present limitations when considering the nanoscale [4]. Hereby, we go through the latest developments in the design of mucoadhesive nanoparticulate systems, mainly for drug delivery, and development of evaluation techniques for its characterization.

2. Implications of mucoadhesion for nanomedicines

Generally, the main advantages advocated for designing mucoadhesive nanoparticulate systems are similar to the ones sought for other types of mucoadhesive formulations. The prolonged retention and intimate contact of drug delivery systems with the mucosa allow these to better deliver its payload to the surface and underling tissues, allowing higher permeation and consequently increased bioavailability and pharmacological effect [5-8]. This fact seems to be not only advantageous for systemically delivered drugs but also for those intended to exert its effects topically [9]. Moreover, it can be attributed to the lower exposure of the released active molecules, particularly labile ones (e.g., most proteins), to harsh environments such as in the gastrointestinal tract (GIT) before being transported through the epithelium. However, in the case of nanosystems, there is more than just releasing drugs in close proximity of the epithelial lining. Due to its nanometric sizing, these systems may also be taken up by epithelial cells or other cell types present at the mucosal surface, or cross the mucosal barrier and continue its migration through the surrounding tissues [10]. The slower transit and higher resistance to self-cleaning mechanisms of mucosal tissues provided by mucoadhesion increases the chance for uptake of drug-loaded nanoparticles.

Although mucoadhesion is usually regarded as a helpful phenomenon for drug delivery, this may not be always the case. Mucoadhesion of nanosystems to off-target tissues has negative consequences and may be hard to modulate in particular cases. For instance, pre-lung retention of particles during inhalation is usually a huge drawback for pulmonary delivery of drugs, mechanisms such as inertial impaction, settling or electrostatic precipitation, or impaired diffusion being usually involved [11]. Even if not designed with mucoadhesive purposes, the mucus of the upper respiratory tract finds in colloidal systems excellent targets for retention and rapid elimination. Moreover, in cases where nanoparticles are retained in the mucus at the target site and in order to reach the epithelial lining, there is still the need to tackle the mucin mesh of the mucus fluids, which recent reports showed to be an important hurdle for rapid nanoparticle transport, particularly in pathological situations in which the mucus presents increased viscosity (e.g., cystic fibrosis) [12,13]. The necessity of nanosystems to rapidly reach epithelial cells is particularly important for therapeutic agents requiring intracellular delivery. Further in this manuscript, we detail strategies used to circumvent these problems.

3. Important concepts in mucoadhesion

In the following sub-sections, a brief but imperative synopsis of relevant aspects of mucoadhesion is presented, with emphasis being focused on issues particularly relevant to nanosized systems.

3.1 Properties of mucosal tissues and fluids

One of the main common features of mucosal surfaces is the presence of an adherent sheet of a highly hydrated fluid (mucus) acting primarily as a protection layer of the underlying tissue. However, differences on the histochemistry (e.g.,

presence and distribution of carbohydrate residues) or morphology (e.g., surface roughness or folding) of mucosal surfaces from different body sites may influence mucoadhesion of polymers [14]. Also, temperature at these sites varies over a relatively wide range, with values as low as 30°C in the nasal mucosa [15], about 34°C in the cornea [16,17] or around 36 – 37°C along the GIT [18]. A factor of utmost importance is pH. Values differ significantly between different mucosal tissues, different sites of the same mucosal tissue and even with health/disease status. For instance, pH in the GIT can vary from as low as 1 in the stomach to as high as around 7.5 in the terminal ileum [19]. This fact can influence dramatically both the behavior of mucosal fluids and mucoadhesives. Mucosal tissues are presented to a wide range of shear stress values that can influence mucoadhesion. Moderately high values can potentiate polymer/mucus interaction and thus adhesion; however, if shear stress is too high, time for interaction and adhesive consolidation may not be sufficient, which adds to the rapid mucus/polymer mixture washout caused by shear-thinning effect and physical removal.

Mucus can be seen as the first barrier for drug absorption but also as a target for delivery systems docking as prolonged retention will increase the likelihood of delivering its payload(s) to the underlying mucosa. Mucus is produced by mucosal cells and continuously secreted into the lumen of the cavities it coats, being shed or digested subsequently. It is essentially composed of water (90% or more) and mucins (≈ 5%), these glycoprotein being responsible for the viscoelastic gel-like properties of this fluid, although other components such as electrolytes, lipids, proteins, enzymes, immunoglobulins, nucleic acids, and cells or cell debris are also present [20,21]. Mucus layer thickness, viscosity, composition and turnover (a few seconds to hours) are variable on location and can be altered in response to disease or physiological changes (e.g., higher viscosity and secretion of pulmonary mucus in cystic fibrosis patients or lack of production of cervicovaginal mucus during menopause) and external stimuli (e.g., increased production after contact with toxic or irritating materials) [22-24]. These differences are able to significantly influence the mucoadhesion behavior of nanosystems as previously shown [25]. Interaction of polymers or other entities (e.g., bacteria) with mucin is known to be responsible by mucoadhesion. The span of possible interactions with mucin is wide and includes electrostatic (mucin is highly negatively charged at most physiological pH values, with an isoelectric point value around 2 – 3 [26]), hydrophobic (related with the presence of hydrophobic domains in the molecule structure) and hydrogen bonding with mucoadhesive molecules [21]. Besides secretory mucins, membrane mucins (or cell-associated mucins) are also present at epithelial cells surface composing the glycocalyx [20]. This last structure is responsible for docking mucus to the subjacent mucosa but can also interact with mucoadhesive molecules. Moreover, specific adhesion to epithelial cell surface is also a possibility, being this particular case of bioadhesion refereed by some authors as cytoadhesion [3].

Also important, particularly when considering particles from some nanometers to a few micrometers diameter, is the structure of the mucus fluids. Even if not completely elucidated and difficult to observe, microscopy imaging reveals that mucus comprises a heterogeneous tridimensional network, being essentially composed of a framework of mucin fibers randomly entangled, creating an endless system of canals in which particles can diffuse and/or be retained [27,28]. The diameter of the formed mesh spaces is not yet clear but has been previously estimated around 20 – 200 nm [28]. According to these observations, particles with diameters higher than around 60 nm would be captured or at least slowed when traveling through mucus. However, recent particle diffusion studies shed additional light on the subject, demonstrating that larger nanoparticles (up to at least 500 nm) can also diffuse through the mucus in an almost unhindered fashion as long as adhesive interaction with mucus could be minimized (see further in the text) [29]. Indeed, further studies using cervicovaginal mucus showed increased values for mesh spaces (around 340 nm) and higher heterogeneity (range 50 – 1800 nm) [30]. Also, mucus structure is not rigid; instead, it changes continuously in a dynamic fashion. For instance, dramatic changes such as shrinkage or swelling can be observed when environmental conditions change, namely ionic strength, pH and osmotic pressure [23], or when drugs/excipients are added [31]. In particular, polymeric nanoparticles have been shown able to alter the barrier properties of mucus (e.g., enhanced drug diffusion) or even originate the collapse of mucin fibers, these facts being related with the use of relatively high concentrations of particles and their ability to establish mucoadhesive bonds [29,32].

3.2 Mucoadhesive interactions and materials

Understanding the interaction between mucoadhesive materials, usually polymeric in nature, and mucosal tissues/fluids is essential for the development of mucoadhesive systems. Several general theories of adhesion have been used to describe mucoadhesion as previously reviewed [2,33] and summarized in **Table 1**. Even if *per se* these theories present flaws and are insufficient in explaining mucoadhesion, the combination between them seems to be able to explain quite well most of the cases.

Generally, the mucoadhesive phenomenon can be divided into two major steps: first, there is the need to create an intimate contact between the pharmaceutical system and the mucosal tissue/fluid; second, interpenetration of the components of both systems occurs, leading to the establishment of intermolecular interactions (predominantly attractive in nature) and thus the consolidation of the adhesive bonding [2,33-34]. Adhesion can be established by either nonspecific (mainly electrostatic interaction, hydrophobic interaction, hydrogen bonding and/or van der Waals forces) or specific (e.g., cell surface glycoconjugates–lectins interaction [35]) intermolecular interactions [36]. This step is dependent on multiple variables intrinsically related with the mucosal tissue/fluid, used mucoadhesive polymer(s) and surrounding environment (e.g., pH value,

Table 1. Adhesion theories commonly used to describe mucoadhesion (reviewed in [2] and [33]).

Adhesive theories	Brief description
Adsorption theory	Hydrogen bonding and van der Waals interactions are the driving forces of the adhesion phenomenon
Diffusion theory (Interpenetration theory)	Mostly used for polymer adhesion; inter-diffusion of polymer chains across a surface or material is responsible for the establishment of adhesive forces
Electronic theory	Attractive forces are established by electron transfer between adhesive surfaces with different electronic structures (e.g., between cationic polymers such as chitosan and negatively-charged mucin)
Fracture theory	More related to the detachment phenomena (rupture of adhesive bonding); it assumes that rupture occurs at the interface between the two adhered systems
Mechanical theory	Interlocking between a shapeable systems (e.g., a liquid) and the irregular surfaces of a solid result in adhesion
Wetting theory	Primarily used for liquids or low viscosity gels; the ability of a liquid to spread through a surface is related to the work of adhesion

temperature, water content, presence of specific molecules and ionic strength).

Another important aspect of the adhesion of nanoparticulates to a substrate seems to be particle shape and deformation on adsorption, particularly when soft, elastic polymeric structures are considered. Contrary to classical theories of larger particle adhesion, in which only particle adhesion energy at the contact surface is considered [37], adhesion of nanoparticles appears to be better described by the interplay between its elastic (which varies with deformation), interfacial and adhesion energies [38]. This fact alone may justify the need for studying mucoadhesion at the nanoscale, even when bulk adhesion properties of the materials used for producing nanostructures are well known.

After the mucoadhesion is established and consolidated, equilibrium between new adsorptive and disruptive bonds determines the maintenance of the adhesive binding. Complete detachment of a polymer chain implies that all bond points are disrupted simultaneously, this phenomenon being unlikely when considering submicron particles as previously demonstrated for latex particles [39]. Therefore, and unlike conventional dosage forms, it seems that washing out of nanoparticles from the adhesive interface is most likely to occur by natural mucus turnover than because of desorption phenomena that lead to the rupture of the mucoadhesive bonding.

Mucoadhesive substances are usually polymeric in nature. Their ability of adhering to mucin is often regarded in terms of specificity: substances are classified as first generation when nonspecific bonding is established or as second generation if specific bonding with particular moieties of mucin or the glycocalyx of epithelial cells (in this case also referred to as cytoadhesive) is observed. Most of the commonly used hydrophilic polymers in mucoadhesive pharmaceutical dosage forms/delivery systems are first generation (e.g., poly(acrylic acid) derivatives, cellulose derivatives, several natural polysaccharides and derivatives) [40], while a few other substances such as N-(2-hydroxypropyl)methacrylamide copolymers [41] or lectins [42,43] are classified as second generation. Of particular interest is the modification of different polymers with thiol groups, which has been shown to significantly improve the mucoadhesive potential over its non-thiolated matching parts [44,45].

Physical–chemical properties of polymers are an important aspect concerning mucoadhesion [2,46]. Generally, the presence of groups that can establish covalent (e.g., thiol groups) and/or non-covalent (e.g., hydroxyl groups) bonding with mucus favor the phenomenon; on the other hand, polymer architecture and spatial conformation are also important, being long (optimum range estimated around 10 – 4000 kDa), linear and flexible polymeric chains recognized as potentially advantageous for mucoadhesion, facilitating in particular interpenetration with mucins. Hydration state and swelling of a polymer can also drastically change its behavior or even govern the mechanism of adhesion [47].

4. Evaluation of mucoadhesive properties of nanoparticulate systems

The mucoadhesive potential of conventional dosage forms or materials has been preferentially measured by methods that directly assess the necessary force or time to detach the mucoadhesive in bulk from a model membrane (*ex vivo* mucosa or synthetic membranes) [48-50]. These methods may involve different detachment techniques but overall they are easy and fast to perform, and seem to be more closely related to what happens *in vivo* than other *in vitro* methodologies when considering the administration of conventional drug dosage forms [51]. It is interesting to notice that the majority of the available data on the mucoadhesive properties of most polymers was determined this way. However, limitations induced by the bulky nature of these methods can substantially influence mucoadhesion results, namely, because it is not always easy to ascertain whether detachment is observed at the mucoadhesive interface or is simply a loss of cohesion between mucoadhesive polymer molecules or between mucosa/mucosal fluid components [52]. In the particular case

of mucoadhesive nanosystems, the lack of relevance of data obtained by these methods can be even larger. The closer proximity to single molecular interactions between polymer and biomolecules of the mucosal environment, as well as the surface properties of nanosystems (morphology and curvature, polymer disposition or anchoring, use of other surface modifiers), justifies the necessity of developing adequate techniques to fully understand mucoadhesion at the nanoscale. Indeed, analytical tools that can evaluate mucoadhesive interaction at the molecular level are considered particularly advantageous for predicting nanosystems interaction with the mucosa or its components and have been adapted in some cases to the specific evaluation of nanomedicines as these seem to be more powerful than bulk techniques. In most cases, these methods provide an indirect evaluation of mucoadhesion in the sense that classical forces are not evaluated as such; rather, they provide valuable information on the positive and negative interactions that may contribute to adhesion.

In the following sub-sections, we provide an overview of the various techniques and methodologies that have been developed to assess mucoadhesion of nanosystems. For the purpose of this manuscript, these have been classified as indirect methods, where mucoadhesion is inferred from the balance between contributing and detrimental interactions between nanosystems and components of the mucosa or mucosal fluids (usually mucins), or as direct methods, meaning that mucoadhesion is evaluated *in vivo* or in close proximity to what happens *in vivo* (e.g., using tissue explants). A comparison among several features of the different methods is presented in **Table 2**.

4.1 Indirect methods

The most common way for measuring the mucoadhesive potential of nanoparticulate systems *in vitro* is assessing its interaction with mucin. Although these experiments fail to mimic the *in vivo* setting, they provide important insights to the underlying mechanisms of mucoadhesion of nanoparticulate systems. In general, experiments can be performed using mucin in solution, film or particulate according to the requirements of the techniques involved in the measurement of the interaction. Although the basic concept behind various methods is common, different techniques can be used for the measurement of mucin–nanoparticle interaction, presenting particular advantages and inconveniencies for each.

4.1.1 *Mucin particle method*

Some of the first techniques described for measuring nanoparticle–mucin interaction were based on the direct determination of the amount of nanoparticle or mucin that interact with each other once dispersed in aqueous media. Originally developed for determining the mucoadhesive potential of raw polymeric materials, these tests are commonly referred to as 'mucin particle method' because, once in solution, mucins are arranged as nanosized structures (usually around 200 – 600 nm) [21,53]. It involves measuring the amount of mucin in solution involved in the adsorption of nanosystems on simple mixture and incubation during a predefined amount of time. The degree of adsorption of nanoparticles/mucin particles can be determined by the variations in size [54], ζ potential [55] or electrophoretic mobility [56] of formed complexes. In particular cases where the adsorption leads to the formation of large complexes, this interaction can also be assessed by separating the adsorbed fraction of nanoparticles by controlled centrifugation and dosing the remaining mucin in the supernatant (e.g., by size exclusion chromatography or colorimetry) [57-59]. However, mucin can also be deposited alongside nanoparticle–mucin complexes if these last are of reduced diameter and require the use of high centrifugation forces. Another disadvantage is related with the possibility of other types of interactions to occur, namely bridging and flocculation of nanoparticle–mucin complexes [57]. The interaction of mucin in solution with nanoparticles has also been assessed by measuring the transmittance of the dispersion after an adequate incubation time [60]. The decrease of the transmittance values is then correlated with mucoadhesiveness assuming that bonding between nanoparticles and mucin will originate macro-aggregates that decrease the passage of the incident light. Another possibility is to produce fluorescent nanoparticles and determine the amount of non-adhering nanoparticles in the supernatant after centrifugation of nanoparticle–mucin complexes [61]. One general potential disadvantage of these mucin particle methods is that the interaction between nanoparticles and higher concentrations of mucin than those observed *in vivo* (mucin particles are composed of highly packed chains of this glycoprotein) might influence significantly mucoadhesive behavior.

4.1.2 *Microgravimetric methods*

Mucoadhesion of nanoparticles has been evaluated by evaluating the amount of material adsorbed onto mucin with a quartz crystal microbalance [62]. The technique is based on the variation of the resonance frequency of a quartz crystal as a function of changes to its mass (**Figure 1**). Briefly, the quartz crystal resonator is covered by a layer of mucin and, after a resonance frequency baseline is established, is incubated with a dispersion of the nanoparticles of interest. Changes to the baseline resonance frequency are recorded based on the piezoelectric effect, and converted into adsorbed mass of nanoparticles by means of the Sauerbrey equation [63]. This powerful technique (sensitivity up to the nanogram) allows the performance of dynamic studies in liquid medium, providing valuable kinetic information of the mucoadhesion process of nanosystems. However, there is a lack of published data on mucoadhesion of nanoparticulates using this technique, making it difficult to assess its real value.

4.1.3 *Atomic force microscopy*

Atomic force microscopy (AFM) is a powerful technique which has been conventionally used for topographical studies of different samples. The principle is simple: a probe scans at a very close distance the surface of a sample while measuring attractive and

Table 2. Comparison of key features of different techniques for the evaluation of mucoadhesion of nanosystems.

Methods for measuring mucoadhesion		Insight on mucoadhesion mechanism	Dynamic/ real-time measurement	*In vivo* relevance	Feasibility	Cost
Indirect methods	Mucin particle method	Low	No	Low	High	Low
	Microgravimetric methods	Medium	Yes	Low	Medium	Medium
	Atomic force microscopy	High	Yes	Low	Low	High
	Optical techniques	Medium	Yes	Low/medium	Medium	High
	Diffusion/particle tracking methods	High	Yes	Medium	High	Medium
Direct methods	Cytoadhesion methods	Medium	Optional	Medium	Medium	Medium
	Ex vivo methods	Low	Optional	Medium	Medium	Medium
	In vivo administration/*ex vivo* analysis	Low	No	High	Medium	Medium
	In vivo imaging	Low	Yes	High	Low	High

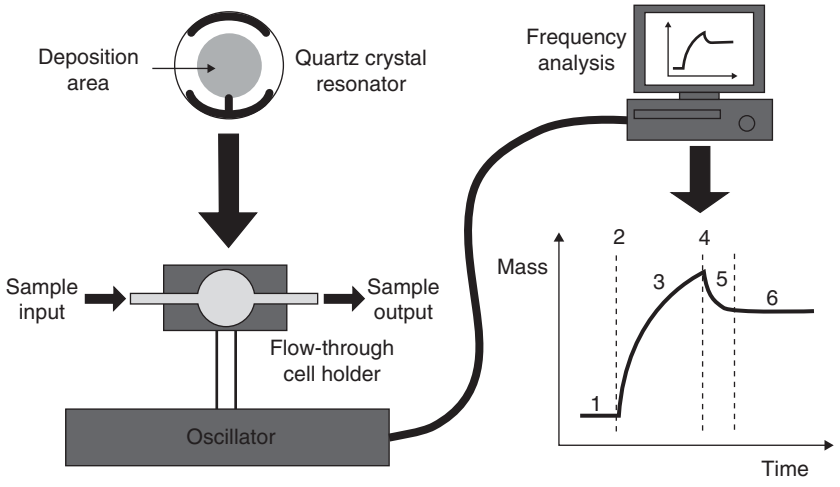

Figure 1. Example of a QCM set up for determining the mucoadhesive potential of nanosystems. The quartz crystal resonator is placed in a flow-through holder where the gold-covered deposition area is bathed by a mucin solution in order to create a sheet of mucin. After equilibration, the nanoparticle dispersion and/or different washing solutions are injected in the flow-through cell. Oscillation of the quartz crystal is induced by applying an alternating current and the frequency of oscillation (dependent on the amount of mass adsorbed at the surface of the quartz crystal resonator) is registered by an oscillator and analyzed by using the Sauerbrey equation. A plot of a simple mass uptake experiment (nanoparticle adsorption/desorption) as a function of time is depicted: (1) establishment of a steady baseline after the QCM has been covered by mucin; (2) addition of nanoparticle dispersion; (3) real-time adsorption of nanoparticles; (4) washing of non-adherent nanoparticles and (5) persistence of adhered nanoparticles.

QCM: Quartz crystal microbalance.

repulsive forces exerted at the molecular level [64]. Usually, the result is a 3D surface image. The differences between topographical images of mucosal cells incubated with diverse polymers can give an idea of mucoadhesion potential by evaluating its roughness [65]. However, the tip of the probe can be modified to hold different molecules of interest (e.g., a polymer or mucin) and used for measuring its interaction with different samples (e.g., mucin/polymer covered surfaces or cell layers). The outcome of the attraction/repulsion force balance is able to deflect the cantilever attached to the probe and the results are transduced into force–distance measurements [66]. For instance, this technique has been successfully used to measure the interaction between core-shell nanogel particles – composed of a poly (*N*-isopropylacrylamide) (p(NIPAAm)) core and a poly(methacrylic acid)-grafted-poly(ethylene glycol) monomethacrylate (p(MMA-*g*-EG)) shell – and mucin layers [67]. Dried granules of the nanogel were adhered to the top of an AFM probe tip by a micromanipulation system and the interaction forces (both repulsive and attractive) with a mucin layer were evaluated during the approach and the detachment of the probe with the

mucin. A schematic of the basic principles of AFM as used for determining the mucoadhesive behavior of nanosystems and a typical example of a measurement are presented in **Figure 2**.

A major interesting feature of AFM is that measurements can be carried out in aqueous solutions, which allows mimicking better biological systems. Also, the ability of directly measuring adhesion at the molecular level with high resolution (measures force in the range of a few nanoNewtons) is one of its main advantages. On the disadvantage side, the cost and complexity of the methods used are high; also, reproducibility is not always easily achievable.

4.1.4 *Optical techniques*

Several optical techniques have been used for monitoring the adhesion between mucin and nanosystems. Although presenting significant differences, these techniques are based on the changes of the properties of incident light on a surface immobilized substrate on binding with an analyte (in this particular case, usually a mucin film and nanoparticles, respectively). Of particular interest is the ability to provide real-time and quantitative data. For instance, ellipsometry has been used by Svensson *et al.* to measure the mucoadhesive interaction between different nanosystems (e.g., chitosan-modified particles or cubosomes) and mucin-coated surfaces [56,68]. The calculation of the adsorbed weight per surface area of nanoparticles is based on the refractive index of the mucin film. Also, this technique was successfully used in measuring increments of the mucin film thickness with nanometric resolution, allowing the study of the deposition profile of the nanoparticles. Of particular interest is the dynamic nature of the determinations, allowing obtaining readings of the same sample at different time points as well as performing applications of nanoparticle samples and surface rinsing during the course of one measurement.

Another optical method which has also been successfully used is surface plasmon resonance [62]. In one study, Lamprecht *et al.* used a resonant mirror biosensor to study the interaction of different polymer-based nanoparticles with mucin [69]. In this case, changes in the refractive index at the mucoadhesive surface are probed based on evanescent wave intensity [70]. Mathematical modeling of the results allows obtaining objective quantitative data about the association and dissociation rate, and affinity of nanoparticles to mucin [69]. Other optical techniques also present the potential for measuring the adsorption of nanoparticles to mucin-coated layers, therefore, justifying future research.

4.1.5 *Diffusion/particle tracking methods*

Different techniques for measuring diffusion have been used to evaluate the mobility of different types of particles and deduce its interaction with components of various biological aqueous media (e.g., cell cytoplasm [71]). In the cases where the used fluid is natural mucus or a simulant containing mucin, mucoadhesion of nanosystems can be inferred from the impediment to its unhindered diffusive movements. Among the various techniques available and tested for evaluating diffusion of molecules and particles [72-77], real-time optical tracking methods, and in particular multiple particle tracking (MPT), have been extensively used to measure the interaction between different nanosystems (including polymeric particles, viruses and virus-like particles) and mucus/mucus simulating fluids [28,30,78-80]. Even if not developed with the primary purpose of measuring mucoadhesion, these studies provide enormous insights to the underlying mechanisms of nanoparticle–mucus interaction and binding. Briefly, MPT involves the analysis of tens or hundreds of particle 2D trajectories by means of video microscopy. Usually, nanoparticulate systems present fluorescence or are made fluorescent in order to be tracked, and incubated with mucus or a simulant fluid [80,81]. The samples are then placed under a fluorescent microscope for video capture. After imaging processing, high-resolution time-resolved trajectories can be used to obtain important quantitative parameters such as diffusivity/immobility and transport mode of tracked nanosystems (see [81] for an excellent detailed description of MPT technology). Mucoadhesion can simply be inferred from the degree of immobility of these systems.

Among the advantages of MPT is the ability to provide both individual and ensemble analysis of particle transport/immobility in real-time. Also, the use of mucus or other biologically relevant media makes it more predicting of *in vivo* events. Even if data analysis may be time consuming and, depending on the setting used, complex, the actual experiments are very rapidly and easily executed. Therefore, MPT constitutes an interesting high-throughput method for the analysis of mucoadhesion of nanosystems.

4.2 Direct methods

4.2.1 *Cytoadhesion methods*

This type of studies is usually conducted by using epithelial cell monolayers of the mucosal tissue of interest. These studies evaluate in particular cytoadhesion and are usually performed by incubating fluorescently labeled nanoparticles with cells in culture; the degree of adhesion can then be evaluated qualitatively by fluorescent microscopy [69,82]. Although simple and rapid to execute, the lack of tissue architecture, function and other important cell types (e.g., mucus producing cells) limits the value of the obtained results. For instance, in order to minimize the absence of normal levels of mucus, some authors suggested the addition of mucin at the surface of the cell monolayer [69]. Others proposed the use of a more refined model where intestinal epithelial cell monolayers (Caco-2 cells) are co-cultured with mucus-producing cells (HT-29 cells), with the intention of better mimicking the physiology and architecture of the mucosa present in the small intestine [83]. Indeed, this modification demonstrated to be influential in the pattern of chitosan nanocapsule interaction with the monolayers: there was generally a higher degree of association with monolayers at the apical surface when HT-29 cells were used (compared to Caco-2 monoculture) and, more remarkable, the chitosan nanocapsules showed preferential association with mucus-producing cells. These

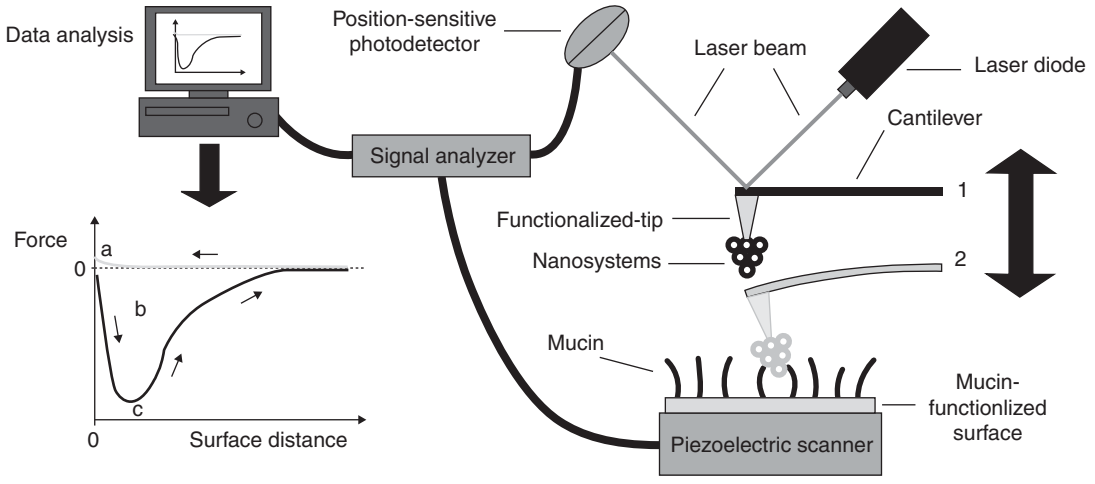

Figure 2. Basic components of an atomic force microscope and typical result of a mucoadhesion experiment. The position of a cantilever with a tip functionalized with the nanosystems is tracked by a laser beam. The laser is deflected according to the bending behavior of the cantilever as a result of attractive (typical bending represented in position (2)) or repulsive forces between the nanosystems and the mucin surface. Combined with the compression/distention signal from the piezoelectric scanner, a signal analyzer is able to convert data into force values. For the practical purpose of mucoadhesion experiments, the cantilever is moved downward from position (1) until it contacts the mucin-functionalized surface (position (2)) and returns to position (1). The signal from the downward path (from 1 to 2) is represented by a gray line in the graph. After a steady, near-to-zero signal, positive force values (a) are observed when the functionalized-tip reaches and starts compressing the mucin layer. The return path (from 2 to 1) is represented by the black line in the graph, where the negative force values are due to detachment phenomena, with the negative peak (c) representing the maximum force required to detach the nanosystems from the mucin layer. The force values then return to near zero values when attractive and repulsive forces cease to act between the functionalized-tip and the mucin layer. The AUC (b) represents the work necessary for complete detachment.

results seem to highlight the limitations of simple cell models but, at the same time, also provide evidence that *in vitro* co-culture models could be a good tool for the assessment of mucoadhesive behavior of nanosystems.

Another important phenomenon to be considered when using this type of methodology is nanoparticle uptake by cells, as discerning this from cytoadhesion is not always straightforward or even possible [84]. Multi-angle and cross-section visualization, as well as low temperature incubation of nanosystems with cells (in order to decrease cell membrane fluidity and, consequently, uptake), seem to be interesting strategies to abbreviate this problem (**Figure 3**) [85]. Thus, in general, cell adhesion methods have been proved to provide important insights into the mechanism and dynamics of mucoadhesion, while having a more or less rough representative structure of the target mucosa.

4.2.2 *Ex vivo* methods

These methods have been long described for the evaluation of the mucoadhesive properties of nanoparticles [86,87]. Usually, experiments are performed with radio-labeled or fluorescent-labeled nanoparticles and the retention in mucosal tissue explants is assessed by continuous or discrete washing with aqueous fluids, therefore, mimicking to some degree the physiological washing processes that occur *in vivo*. The experimental apparatus can be more or less complex, but generally it attempts to imitate the major anatomical–physiological features of the mucosal surface (e.g., target mucosal tissue, trajectory steep, temperature, humidity). The amount of collected radioactivity or fluorescence in washing fluids is correlated with the ability of nanoparticles to reside *in loco* and is indicative of its mucoadhesive potential. Alternatively, the mucosal tissue can be further processed for label extraction and assay [88]. Simpler methods such as determining the balance between the weight of initially applied and remaining nanoparticles have also been described, particularly when using larger particles [89]; however, this last methodology seems to be limited due to the difficulty in separating the remaining nanoparticles from the tissue.

In one particular case, Sandri *et al.* [90] used an in-house apparatus by mounting portions of rat jejunum in between the donor and the receptor chambers of a Franz diffusion cell, maintained at 37°C, and placing the nanoparticle samples in the donor chamber. The receptor chamber was physically separated from the tissue by means of an impermeable membrane. The natural intestinal washing mechanism was simulated by an incoming flux of an adequate buffer solution at a precise rate of 0.7 ml/min by means of an HPLC pump. The outcoming buffer was collected and assayed for the fluorescein isothiocyanate dextran loaded in nanoparticles, mucoadhesion

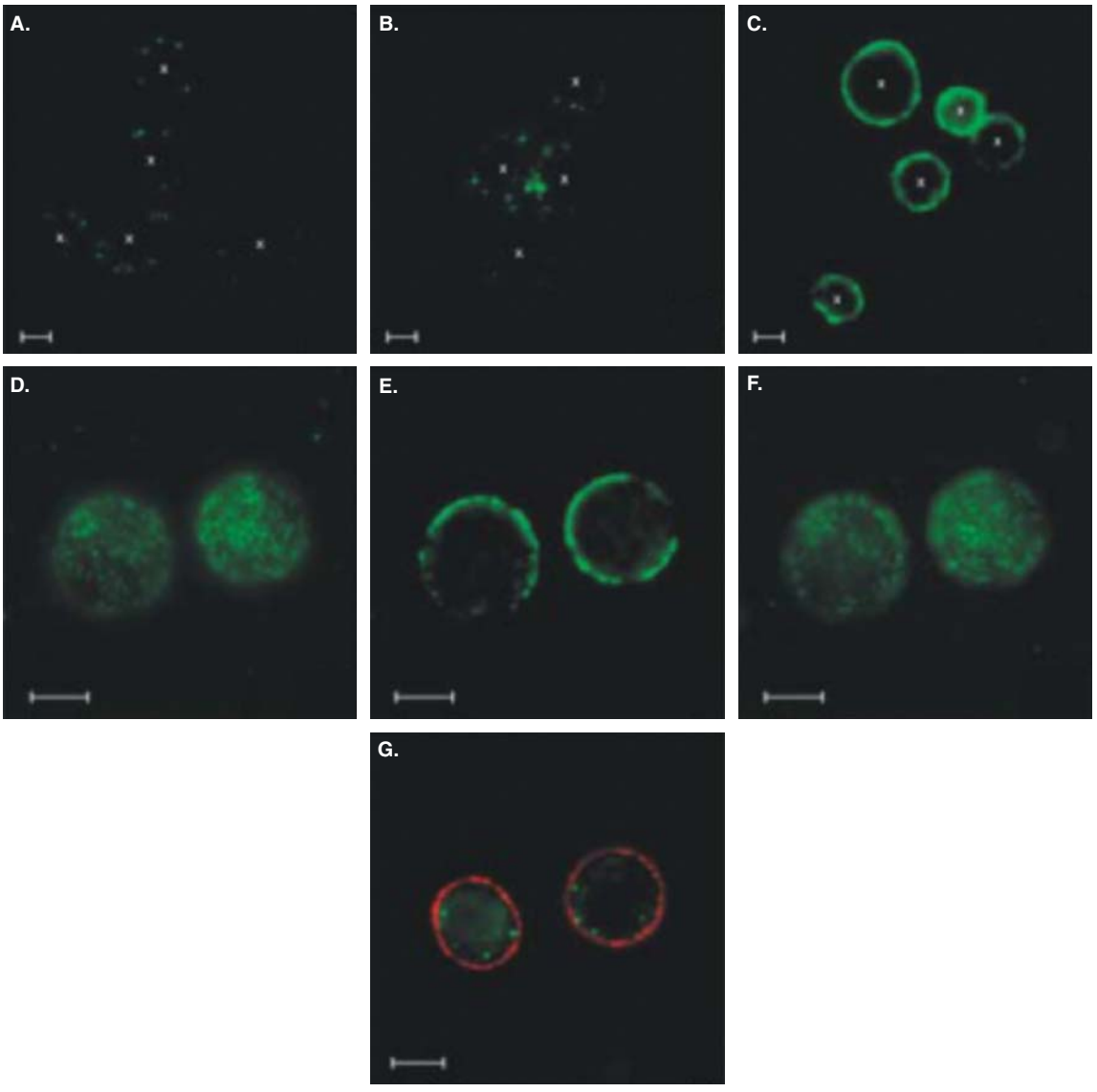

Figure 3. Fluorescence microscopy images of Caco-2 cells incubated with plain BOD-PLGA (A), human serum albumin-modified BOD-PLGA **(B)** and wheat germ agglutinin-modified BOD-PLGA **(C)** nanoparticles for 60 min at 4°C (BOD is responsible for the green signal). The center of the cell is marked with an "x" (A,B,C) for orientation.Optical cross-sections of Caco-2 cells incubated with wheat germ agglutinin-modified BOD-PLGA nanoparticles at 4°C taken at the top **(D)**, center **(E)** and bottom **(F)** of the cell, evidencing membrane attachment. Incubation for a further 30 min at 37°C and staining of the cell membrane with FM® 4 – 64 (red signal) **(G)** evidenced cell uptake. Scale bar represents 10 μm.

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BOD-PLGA: BODIPY 493/503-loaded poly(lactic-*co*-glycolic acid).

being measured indirectly by calculating the amount of remaining drug in the donor chamber [90]. Of particular interest, this method uses widely available standardized equipment and requires only minimal amounts of tissue per experiment.

Static methods have also been described in which mucosal explants are immersed in nanoparticle dispersions or a nanoparticle dispersion sample is placed on top of the mucosa, and the amount of fluorescent-labeled nanoparticles retained is afterwards determined by extracting the dye from the tissue with an organic solvent [91,92]. Another possibility is to use confocal laser scanning microscopy [93]. In specific cases, the polymer itself can be quantified; for instance, latex nanoparticle mucoadhesion to *ex vivo* rat intestinal mucosa has been assessed by turbidimetric analysis after scraping the tissue/nanoparticle contact area or by FTIR-ATR analysis of the dried mucosa after incubation with nanoparticle dispersions [94]. Results from these techniques were shown to be complementary as they provide desorption (turbidimetry)

and adsorption (FTIR-ADR) kinetic analysis, being also consistent with each other [39].

The use of a natural mucosa for nanoparticle adhesion, and thus more physiologically relevant, can be advocated as one of the most interesting and advantageous feature of these methods. However, the absence of the natural mechanisms that contribute to retention/washing of particles is also able to limit the analysis of the obtained results. Other main disadvantages of these techniques are its variability related with mucosa preparation and sampling, and the use of animals. This last can be reduced by using tissues from slaughter houses that would otherwise be discarded, or by maximizing the number of experiments that can be performed with the tissue from one single animal. Additionally, poor insight on mucoadhesion phenomena is obtained in most of the described methods but the combination of real-time and dynamic analytical tools could provide additional power to these techniques. For instance, Henning *et al.* recently assessed the mucociliary clearance by using a particle tracking technique in *ex vivo* embryonic chicken trachea [95,96]. The setting used seems to be quite interesting as it combines the analytical power of particle tracking techniques with the relevance of an *ex vivo* setting.

4.2.3 *In vivo* administration/*ex vivo* analysis

These techniques involve the administration of nanoparticulate systems to living animals, mucoadhesion being evaluated following sacrifice. In a typical setting, fluorescent- or radioisotope-labeled nanosystems are administered directly (e.g., in the vagina or oral cavity) or indirectly (e.g., inhalation for pulmonary delivery or by mouth for GIT mucosal delivery) to the desired mucosal tissue. Animals are then sacrificed after a preset amount of time and qualitative and/or quantitative examination of retained labeled-nanosystems in the mucosal tissue is performed. Several techniques can be used depending on the type of label used to trace the nanosystems and mucosa processing. For instance, confocal microscopy of mucosal sections or fluorometric assay after tissue extraction of fluorescent dye has been successfully used for quantifying the retention of fluorescently-labeled nanosystems in the stomach and small intestine after oral administration (with or without the use of a gastric cannula) to rodents [97-102]. Also, autoradiographic detection of polyhexyl-[3-^{14}C]-cyanoacrylate nanoparticles on the intestinal mucosa of rats after oral administration has been described as a feasible technique, with the particular advantage of being able to detect and quantify very small amounts of material [103]. Direct observation of nanoparticle aggregates on site has also been performed, for instance after oral administration [104], but this methodology appears very subjective to be of true value in the evaluation of the mucoadhesive nature of nanoparticles.

This type of techniques is able to circumvent the difficulty of mimicking the physiological washing mechanisms that influence nanoparticle adhesion and retention, this fact being an important advantage over *ex vivo* testing. However, the poor insight into the phenomena involved in mucoadhesion is still present but, above all, the number of required animals usually limits the number of possible experimental settings to be performed. Another limitation of these techniques is related to the possibility of nanoparticle binding to non-target tissues during indirect administration to mucosal sites (e.g., the lung or intestine). With this in mind and in order to study the mucoadhesion of nanoparticles to the intestinal mucosa, Yin *et al.* developed a rat *in vivo* alternative method to the oral administration of the formulation that allowed these researchers to minimize its pre-intestinal interaction with the GIT tract [105]. More specifically, an ileal loop was made after anesthesia and abdominal incision, by which the fluorescent-labeled nanoparticles were directly syringed; after a 2 h period, the loop was flushed with saline and the amount of non-adhered particles was measured by fluorimetry.

4.2.4 *In vivo* imaging

These techniques can be regarded as the most relevant in terms of real life use of drug dosage forms and delivery systems [106-109]. In cases where an active agent is included, the mucoadhesive behavior can also be directly correlated with the pharmacological effect. However, its use for evaluating the mucoadhesive performance of nanoparticulate systems has been diminutive. *In vivo* imaging techniques allow tracking of the natural trafficking of nanoparticles without minimal interference by using adequately labeled nanoparticles. For instance, Ramteke *et al.* used X-ray photographs to track the stomach retention of gliadin-based nanoparticles containing barium sulfate as a contrast agent [61]. Although limited resolution was obtained, images seem to show accumulation of nanoparticles in the stomach at least up to 3 h, allegedly because of the mucoadhesive nature of gliadin. In another study, poly(methyl vinyl ether-*co*-maleic anhydride) (P(MVE-*co*-MA)) nanoparticles modified with different types of cyclodextrin (CD) derivatives and labeled with Technetium-99m (^{99m}Tc) were tracked during its transit in the GIT of rats by using γ scintigraphy [102]. The animals were fed by mouth, anesthetized and placed in prone position on a γ camera for imaging at different time points. In particular, scintigraphic images showed that the CD-modified nanoparticles (140 – 180 nm diameter) were retained in the GIT due to its mucoadhesive nature, without any observable systemic absorption (**Figure 4**). Also, these mucoadhesive properties have been correlated, at least partially, in a posterior study with enhanced biovailability of paclitaxel after encapsulation in CD-modified P(MVE-*co*-MA) nanoparticles [110].

Although extremely relevant in terms of real life use, these techniques present some drawbacks. Beside the typical limitations of *in vivo* experimenting, such as ethical issues, high cost and variability, the amount of information on the mucoadhesive phenomena of studied systems is also virtually inexistent. Also, manipulation and pharmacological intervention for animal immobilization (i.e., anesthetics) can modify the natural physiology of the mucosal site of interest, namely the GIT. Finally, image interpretation may be flawed, particularly because of difficulties in accurate anatomical localization of the nanoparticle signal in 2D images.

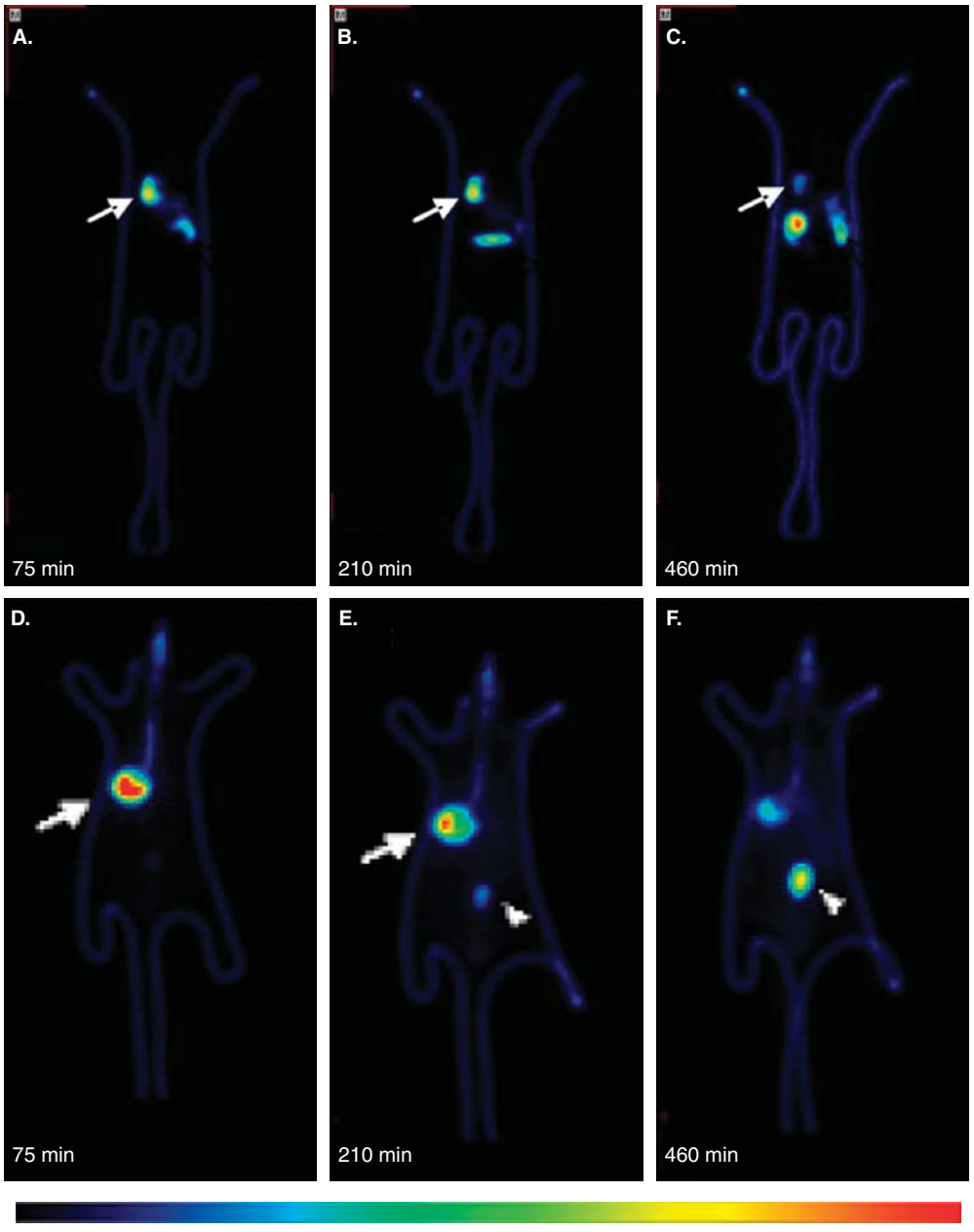

Figure 4. Comparison of the biodistribution of ^{99m}Tc-labeled, HPCD-NP (panels A – C) and a solution of ^{99m}Tc tetraoxygen ($^{99m}TcO_4^-$) used as control (panels **D – F**). Panels **A – C** show scintigraphic images after oral administration of 10 mg of ^{99m}Tc-labeled HPCD-NP at 75, 210 and 480 min post-injection of anesthesia, respectively (arrow: stomach). Panels **D – F** show scintigraphic images after oral administration of 1 mCi of $^{99m}TcO_4^-$ solution at 75, 210 and 480 min post-injection of anesthesia, respectively (arrow: stomach; arrow head: bladder).

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HPCD-NP: Hydroxypropyl-β-cyclodextrin-modified nanoparticles.

5. Strategies for increasing/decreasing mucoadhesion of nanoparticulate systems

5.1 Use of mucoadhesive materials

Clearly, the easiest way to develop mucoadhesive nanosystems is by using mucoadhesive materials (mostly, but not exclusively [97], polymeric in nature) for its production [111]. However, this may not be always completely straightforward as not all mucoadhesive materials can readily originate nanoparticles or it simply cannot originate nanoparticles with adequate properties to be used as drug carriers, such as enough drug loading or stability. An alternative option is

the use of mixture of mucoadhesive polymers with matrix-forming materials or produce new copolymers combining the advantages of both matrix-forming and mucoadhesive polymers [112]; however, total mucoadhesive potential may be expected to be lower due to reduced density of the mucoadhesive polymer at the particle surface. Also, nanoparticle formation or/and its original size, drug release or stability properties may be compromised by the inclusion of mucoadhesive polymers.

Chemical modification of already mucoadhesive polymers has been a popular strategy for increasing nanoparticle adhesion. For instance, chitosan and its derivatives, particularly thiomers, are highly regarded as good mucoadhesive polymers and the production of nanoparticles from these materials is well established. The major benefit of chitosan is the ease by which various chemical groups may be added, in particular to the C-2 position, allowing for tailoring of its functional properties [113]. In one study, Bernkop-Schnürch *et al.* developed chitosan-based nanoparticles and showed their ability to increase the retention of a fluorescent model compound (fluorescein diacetate) in *ex vivo* intestinal mucosa [88]. Particularly, this group showed that thiolation of the used chitosan (chitosan-4-thiobutylamidine) for producing nanoparticles was able to substantially enhance retention when compared to equivalent chitosan nanoparticles (up to more than twofold); further, there seemed to be a direct correlation between available free thiol groups at the nanoparticle surface and mucoadhesion enhancement. In another study, Yin *et al.* also found an increase in intestinal mucoadhesion of thiolated chitosan nanoparticles over non-thiolated chitosan nanoparticles (around 2.1- to 4.7-fold increase) and a trend for higher mucoadhesive potential with increasing molecular mass (MM) of chitosan or thiolated-chitosan [105]. Moreover, these authors were able to find a direct correlation between enhanced pharmacological effects (decrease in blood glucose levels) and increased mucoadhesive properties of insulin-loaded nanoparticles when administered to rats by the oral route or directly into the ileum. Other examples of well-studied derivatives of chitosan, such as N-trimethylchitosan, have also been shown to significantly enhance *in vitro* and *ex vivo* mucoadhesion of nanoparticulates when compared to the unmodified polymer [90,114].

In a similar strategy to chitosan, other positively-charged copolymers have also been used to increase the mucoadhesiveness of nanoparticles. For example, Henning *et al.* showed that diethylaminopropylamine-poly(vinylalcohol)-*graft*-poly(lactic-*co*-glycolic acid) (DEAPA-PVA-g-PLGA) presents increased mucoadhesive properties over PVA-*g*-PLGA nanoparticles (expressed in terms of reduced mucociliary transport rates in an embryonic chicken trachea *ex vivo* model) [96]. The increased mucoadhesive properties are thought to be due to electrostatic interaction of the amine groups present in DEAA with the negatively-charged mucin. However, and even if we might be led to think that, in general, positively-charged nanocarriers would present higher mucoadhesiveness, the truth is that particle charge seems to be only a small piece of the puzzle which is the overall properties of the polymers. The lack of electrostatic attraction may be compensated by other types of attractive forces. For instance, it was also found that negatively-charged poly(vinylsulfonate-covinyl alcohol)-graft-poly(lactic-*co*-glycolic acid) (P(VS-VA)-g-PLGA) nanoparticles presented similar mucoadhesiveness to DEAPA-PVA-g-PLGA [96]. In the case of P(VS-VA)-g-PLGA, vinylsulfonate groups are believed to strongly interact with mucin bonding due to its H-bond acceptor qualities, thus explaining the observed results.

5.2 Surface modification

The use of mucoadhesive polymers as matrix forming material is frequently limited by several reasons with cost, poor encapsulation of the desired drug, or toxicological and biodegradability issues being the most frequent ones. Therefore, coating of drug-loaded nanosystems with mucoadhesive materials has been the most popular strategy for enhancing or decreasing mucoadhesive properties. Surface modifiers used for enhancing mucoadhesion of nanoparticulate systems include polyacrylates [115], lectins [116,117] or pectin [101], to name some examples. Of particular notice, chitosan has deserved particular focus as surface modifier of nanosystems. For instance, Kawashima and collaborators found significantly longer lower plasma calcium levels after oral administration of chitosan-coated calcitonin-loaded liposomes to rats, when compared to plain calcitonin-loaded liposomes [100]. Also, similar pharmacological effect was observed by the same group for chitosan-coated PLGA nanoparticles containing the same peptide after inhalation in a rat model [118]. It is advocated that the observed improved pharmacological effect is related, at least in part, to the higher retention and epithelial uptake of chitosan-modified nanosystems in the small intestine or lungs of rats, allegedly due to the mucoadhesive properties conferred by chitosan. Others have also reached comparable results with pectin-coated calcitonin-loaded liposomes after oral administration in a rat model [101]. In all three cases, chitosan or pectin were associated at the surface of nanosystems by adsorption.

Although convenient, coating nanosystems by simple adsorption of mucoadhesive polymers at the surface is not always able to provide effective mucoadhesive properties as surface shedding is rapid, particularly on dilution with biological fluids. To circumvent this problem, covalent attachment of mucoadhesives moieties to pre-formed nanoparticles is commonly preferred [117]. In particular, covalent attachment has been widely used for coating nanosystems with chitosan and derivatives. For instance, in order to improve the mucoadhesion properties of PLGA nanoparticles, the group of Bernkop-Schnürch attached covalently chitosan or thiolated chitosan to the surface of the preformed PLGA particles [119]. Both systems provided increased mucoadhesion when tested by a porcine intestinal *ex vivo* washing method. In particular, thiolation allowed for prolonged residence time of nanoparticles when compared to chitosan modification alone, a strategy which has also previously provided good results when testing the bulk polymer [49]. Indeed, the

use of thiomers derived from chitosan as the coating moiety has been another popular strategy for increasing mucoadhesion. Several studies showed an enhancement of this type of systems over their non-coated or chitosan-coated equivalents. For example, *ex vivo* studies performed by Bravo-Osuna *et al.* with thiolated chitosan-coated poly(isobutyl cyanoacrylate) (PIBCA) nanoparticles on rat intestinal mucosa appear to support that the mucoadhesive behavior is governed by the transport through the mucus layer and by the subsequent bond establishment with mucin chains [92]. In detail, nanoparticles coated with higher MM thiolated chitosan showed improved mucoadhesion, presumably by facilitated interpenetration with the mucin network due to longer chitosan chains. This increase in mucoadhesion with higher MM has also been reported by others [105,120].

Charge properties of surface bounded polymers *per se* play an important role on mucoadhesive behavior. The negative charge of mucin at most physiological pH values, as well as of the cell membrane, makes positively-charged polymers of particular interest when mucoadhesion is desired. For instance, chitosan seems to be advantageous over other negatively-charged polymers (polyacrylates, alginate) [91], contrasting with the results observed for bulk mucoadhesion experiments performed by Grabovac *et al.* where this difference was not significant or even opposed [49]. However, physiological changes can counter balance charged nanosystems ability to adhere to mucus/mucosa, as previously seen for liposomes: cationic liposomes showed higher mucoadhesion to normal rat intestine, while anionic liposomes seem to prevail over the previous when colitis was induced [25]. Chiefly, differences in electrolyte composition of mucus, as induced by colitis, seem to be responsible by this shift.

In both cases, the hindered diffusion of charged particles through mucus can be easily understood by electronic adhesive interaction with mucin fibers. Positively-charged nanoparticles are attracted by the opposite charge of mucin and stick to it rendering immobilization; on the other hand, negatively-charged nanoparticles suffer electrostatic repulsion by mucin fibers, therefore, reducing its partition across mucus. This also explains how some virus (e.g., Rhinovirus) can easily permeate mucus by maintaining a neutral net surface charge as well as hydrophilic character to avoid hydrophobic adhesive interaction with hydrophobic domains of mucin [23,24].

As stated above, decoration of nanosystems surface with hydrophilic polymer chains that can establish attractive interaction with mucin has been generally shown to increase mucoadhesion [87]. However, several different properties can be related with different mucoadhesive profiles at the nanoscale level which are not always in agreement with what is known for mucoadhesive bulk behavior of these polymers. For instance, MM of polymer chains seem to be of importance as it has been shown that low molecular chains of non-charged hydrophilic polymers such as PEG (MM 2 – 10 kDa) can indeed dramatically decrease the mucoadhesion of nanoparticles [75,96,121-122]. These observations can be explained in view of the diffusion theory of mucoadhesion, where polymer chains must have sufficient length to allow interpenetration which leads to nanoparticle/mucin anchoring [123]. Below this critical length point, it seems that PEGylation does not contribute to mucoadhesion; rather, it creates a stealth hydrophilic, electrically neutral shelter that inhibits the establishment of other potential mucoadhesive forces such as attraction between the hydrophobic nanoparticle core and hydrophobic domains of mucin, or electrostatic attraction between the charged particle and mucin and/or epithelial cell surface. Of course, an adequate PEGylation density is required to camouflage the adhesive properties of non-PEGylated particles [75,121]. Investigations of Svensson *et al.* [56] on the interaction between mucin and cubosomes modified with poly(ethylene oxide) (PEO) by means of triblock copolymer of PEO and poly(propylene-oxide) (PPO) (PEO-PPO-PEO) also support the observations of Hanes *et al.* [29,121,122]. This group used electrophoretic mobility and ellipsometry to study mucoadhesion and concluded that, although moderately influenced by environmental pH, PEO modification conferred these systems with a weak ability to interact with mucin chains.

5.3 Size modulation

Even if the chemical nature of nanoparticles, particularly of its surface, seems to be the most influential factor for mucoadhesion to occur, changing the size of nanocarriers can by itself significantly influence mucoadhesion. However, size influence in the mucoadhesive behavior of nanosystems has shown to be tricky and dependent in multiple factors (**Figure 5**). A simplistic analysis would immediately consider that one of the important factors is the change in surface area related with changing diameter of nanoparticles. This means that, in general, more or less possible mucoadhesive interactions could be established once the particles size decreases or increases, respectively. For instance, it has been shown that around 300 nm nanoparticles coated with thiolated chitosan presented better retention than nanoparticles with higher diameters [92]. This last study also suggests that smaller sizes could increase diffusion and convention of nanoparticles through mucus, thus, highlighting the importance of enhanced transport for subsequent attachment by mean of adhesive interactions [92]. However, this analysis does not seem to be that straightforward, as increasing size is also known to be able to increase retention. Indeed, steric impediment may be observed due to the limited dimensions of the liquid pores available for diffusion within the 3D mucin network. For instance, Sanders *et al.* showed that nanoparticles with around 120 nm diameter were only moderately retained in cystic fibrosis sputum, while larger ones (around 560 nm) were almost completely immobilized [73]. Similar results were also observed by Norris and Sinko for polystyrene (PS) particles diffusion through mucin in solution [72]. Moreover, when size increases to the micrometric range, there seems to be inability of particles to migrate through the mucus after initial adhesion to the surface of this fluid as shown by Ponchel *et al.* in a series of adsorption isotherms and kinetics

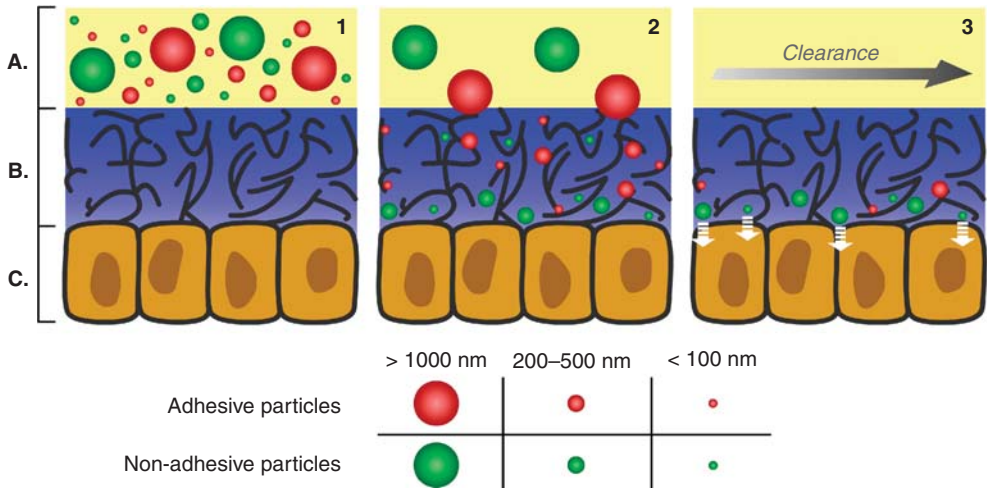

Figure 5. Schematic representation of the interaction of several types of particles with the mucus layer. (1) After initial administration/arrival to the mucosal site, particles will interact and diffuse through the mucus differently according to its properties (see text for further details). (2) Larger particles are not able to diffuse through the mucus layer due to steric hindrance, but can interact with the luminal layers in cases where adhesive bonding with mucin chains (in dark grey) can be established. As for smaller particles, these can diffuse differently through the mucus layer depending on adhesive properties and specific size. (3) On the action of natural mechanisms of clearance, particles are progressively removed from the mucosal site (particularly at the luminal side) while nanoparticles that have reached the epithelial cell lining can further undergo cell uptake or tissue penetration (represented as white dashed arrows). Legend: A – mucosal tissue lumen/external environment; **B** – mucus layer and **C** – epithelial cell lining.

studies using an *ex vivo* methodology [39,124]. These observations were recently corroborated by MPT experiments by Lai *et al.*, which showed that the transport through mucus of 1 µm or larger particles was highly hindered, probably due to steric obstruction, contrasting with 100 – 500 nm nanoparticles, which were made non-adhesive by dense surface PEGylation (PEG MM ranging from 2 to 3.4 kDa) [30,125]. Still, in the studies by Ponchel *et al.* [39,124], and considering particles in the range of 200 – 700 nm composed of either PS or PIBCA, after initial adsorption the particles were able to permeate the porous mucus layer, allowing for new particles to attach to the now free locations on the mucus surface. This fact can be seen as beneficial as it promotes further possibility of adsorption interactions at even deeper layers of the mucus, and not only at the surface of this biological fluid. These results have been, at least in part, supported by confocal laser scanning microscopy studies of rat intestine after oral administration of different types of liposomes ranging from around 180 nm to 4.6 µm [100].

In another interesting experiment, Hanes and co-workers compared the diffusion of PS nanoparticles with 100, 200 and 500 nm in human cervical mucus by MPT [29]. Surprisingly, the 100 nm particles, either PEGylated or COOH-modified, showed higher retention than larger ones. These observations have been explained based on the heterogeneity of the mucus mesh: smaller nanoparticles (100 nm) can diffuse by smaller, tortuous pores and be retained in 'dead end' pockets; on the other hand, larger nanoparticles (200 – 500 nm) will only diffuse by less constrained channels, with larger mesh spacing, which means that its hindrance will only be conditioned by possible direct interaction with mucin fibers [126]. However, in cases where mucus mesh is tighter, such as in the case of sputum of cystic fibrosis patients, diffusion of nanoparticles with 500 nm diameter can be substantially reduced [127].

Another phenomenon that can influence diffusion/immobilization of nanosystems in mucus is the tendency to form aggregates. Even if individual size of nanoparticulates favors diffusion, self-binding will impair the transport rate by originating aggregates with diameters too high for unhindered diffusion through the mucus mesh [75]. Also, because aggregation of nanosystems is known to be influenced by concentration, this last factor can lead to the hypothesis that the same nanoparticles have different mucoadhesive behavior over a gradient of concentration. There is also the possibility that higher concentrations of nanoparticulates affect the normal structure of mucus by collapsing mucin fibers, thus rendering complete immobilization [29]. Finally, generalization that modulation of size can be used to increase/decrease mucoadhesion is not true. For instance, Henning *et al.* studied the mucociliary clearance of PS particles ranging from 50 nm to 6 µm and found no differences [96]. Overall, it is advisable to study the influence of size on a case-to-case basis as multiple factors (e.g., surface chemistry and charge, mucus properties) with different influence levels seem to be involved in the final mucoadhesive behavior of nanosystems.

5.4 Other strategies

Other options for enhancing or complementing the mucoadhesion properties of nanosystems have been suggested. For instance, modulation of the pH at the microenvironment level may be an advantageous strategy in those cases where the mucoadhesiveness of nanoparticles is significantly influenced by H^+ activity [57,62,68]. With this purpose, a buffer system could be included in the nanoparticle formulation. The same principle may also be applied to other types of stimuli-sensitiveness (e.g., shear, temperature). Indeed, the advantages provided by mucoadhesive nanosystems can be also enhanced by conferring other types of functional properties. For example, stimuli-sensitive polymers can be used in the formulation of nanosystems to modulate adhesion at different regions of a mucosal tissue tract [128]. Also, the design of 'intelligent' microcarriers or dosage forms for drug-loaded nanosystems can also provide an interesting way of avoiding its mucoadhesion until it reaches the region of interest, for example, the colon in the case of GIT administration [129,130]. As for decreasing mucoadhesiveness, it has been suggested that direct disruption of mucus with mucolytic agents co-administered with nanosystems could be an interesting approach [126]. This would be particularly important for pathological conditions in which the high viscosity of mucus impairs its transposition by particles (e.g., increased viscosity of pulmonary mucus in cystic fibrosis patients).

6. Expert opinion

In recent years, modulation of the mucoadhesive properties of nanosystems has captured an increasing substantial interest from researchers enrolled in nanomedicine. This fact led to the development of specific techniques to study mucoadhesion at the nanoscale and the increased understanding of the phenomenon. In particular, the knowledge about mucoadhesives as obtained from previous studies performed with bulk techniques may not fit the purpose of predicting the behavior of nanosystems produced with the same materials when interacting with mucosal sites or its components. The importance of fine tuning the mucoadhesive properties of nanomedicines for mucosal delivery seems to be vital in developing effective therapeutics.

Besides significant advances in the field, other issues still remain poorly studied. For instance, a recent paper by Sakloetsakun *et al.* [131] highlighted the importance of testing the influence of components that will be used in the formulation of the pharmaceutical dosage forms for nanoparticulates in the mucoadhesive behavior of these last. Indeed, reported results from this study showed significant variation in mucoadhesion of chitosan-based nanoparticles in the presence or absence of commonly used excipients. It is noteworthy that this type of interaction has been systematically disregarded in the up-to-date literature. Another interesting topic of study has to deal with the shape of nanosystems because the majority of the studied systems possess a spheroid shape. Recent technological advances allow for precise control at the nanoscale of particle shape and the obtaining of multiple geometrical forms [132]. Indeed, the latest studies using differently shaped silicon or PS particles showed that these are able to present diverse functional behavior and interact differently with cells [133,134]. Therefore, it is expectable that mucoadhesive interaction can also be influenced by nanoparticle shape. As for the methods of mucoadhesion evaluation, other techniques previously used for measuring the mucoadhesion potential of different polymeric materials and variations of those already used for nanosystems surely deserve thoughtful investigation. Among different conventional techniques, our literature search found only one study involving rheological measurements [9], its use being limited to basic viscosity measurements. Indeed, these techniques have been quite useful in the evaluation of mucoadhesive properties of different polymers and other molecules [135-137] and might be an interesting approach for complementing the information on nanoparticulates mucoadhesion. Others, such as AFM, have not been fully explored in the nanomedicine field. However, its use in other scientific fields has been extensive, either by using similar techniques as described above or variations (see [138] for a thorough review), thus granting high value to AFM and assuring further use of this powerful technique in understanding mucoadhesion of nanosystems for drug delivery. Also, the mucoadhesion phenomenon of nanosystems has been mostly studied towards application to the oral administration of drugs. Consequently, there is the need to refine and increase the amount of available techniques for testing systems intended to be administered by other important mucosal routes, because all of these may benefit from increasing knowledge on how mucoadhesion affects nanoparticulate drug delivery.

Despite the substantial efforts in the field, it is still required to further complement and strengthen basic knowledge of how molecular and nanoscale properties of nanosystems can influence mucoadhesion. More than a simple question of which mucoadhesive materials should be used, research must also strongly focus on how size, shape and surface charge and morphology of nanosized systems can influence adhesion to mucosal tissues/fluids. Currently available technology seems to have enough potential in order to allow developing techniques to conduct such investigations, even if further refinements (as discussed previously in this paper) would be helpful. The span of available materials (particularly those with confirmed strong mucoadhesive properties at the nanoscale, such as chitosan or thiolated polymers) and techniques to produce nanosystems grant flexibility in obtaining adequately tailored mucoadhesive (or non-mucoadhesive) nanomedicines with potential use in well-defined clinical practice settings.

Declaration of interest

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