EX VIVO Mucosa Rheology as a Novel Approach to Investigate Mucoadhesion

Cristiana F. B. Cunha[1,2], Mai Bay Stie[1,2], Vito Foderà[1,2], Hanne Mørck Nielsen[1,2] and Stine Rønholt[1,3,*]

[1]Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
[2]Center for Biopharmaceuticals and Biobarriers in Drug Delivery (BioDelivery), University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.
[3]LEO Foundation Center for Cutaneous Drug Delivery, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.
*Corresponding author (e-mail address: stine.roenholt@sund.ku.dk)

ABSTRACT

This work presents a novel approach to assess mucoadhesion of pharmaceutical excipient liquid dispersions onto *ex vivo* mucosae. This was done by small deformation rheology. The viscosity of aqueous dispersions of two pharmaceutically relevant excipients, chitosan and polyethylene oxide, was investigated when applied onto *ex vivo* intestinal, buccal, and sublingual porcine mucosal tissue. Our results demonstrate promising insight obtained by using a new *in vivo*-relevant approach to evaluate local and region-specific mucoadhesion of dispersions intended for mucosal application, although further optimisation of the method is required to achieve its full potential.

INTRODUCTION

Mucosal drug delivery holds great potential to improve both efficacy and safety of numerous drugs. Moreover, it is a more patient-friendly alternative to invasive drug administration by injections. However, the mucosal epithelium also presents a great permeation barrier that must be overcome to ensure sufficient drug absorption. A common strategy to improve drug permeation across mucosal barriers is to include mucoadhesive excipients in drug delivery systems[1]. This strategy ensures longer residence time at the mucosae, consequently maintaining a high concentration of drug at the site of absorption, hereby improving drug permeation across the barrier.

Various biophysical methods, including small deformation rheology, can be used to evaluate and explore the potential of mucoadhesive excipients and formulations. Previously, Hassan and Gallo (1990) successfully developed a rheological method for *in vitro* assessment of mucin-polymer bioadhesive strength by measuring the viscosity of polymer-mucin combinations and subtracting the contributions of the individual components. This method has been widely used for assessing the interaction of mucoadhesive excipients with mucin dispersions[2], biosimilar mucus[3], and isolated native mucus[4,5]. However, *ex vivo* mucosae were not applied in such investigations. Methods that involve *ex vivo* mucosal tissue often rely on indirect evaluations of mucoadhesion by quantification of drug loss after washout by liquid flow[6,7].

Mucus is a complex viscoelastic barrier, and its properties depend on the anatomical origin. For example, throughout the gastrointestinal tract, mucus is generally divided into a firmly membrane-bound layer and a more loosely adherent matrix composed of amongst other secreted mucins; and the rheological properties of the mucus and its composition have been shown to depend on its interregional localization[8]. Furthermore, the complexity of the mucosae barrier, this being the mucosal epithelium, the mucus layer, and the highly complex interplay occurring between these, is obviously only partly represented when only using isolated mucus and/or mucin solutions *in vitro*. More representative models and novel approaches are therefore needed to achieve a better understanding on the interaction of therapeutic drugs, excipients, and drug delivery systems with the mucosae.

Inspired by the simpler rheological approach first developed by Hassan and Gallo (1990), the aim of this study was to develop a rheology-based method to evaluate mucoadhesion of dissolved or dispersed excipients on *ex vivo* mucosal tissue from different anatomical sites. Such an *ex vivo* method would benefit from more closely representing the full complexity of the mucosae *in vivo*, especially considering the regional differences of its multiple layers. It was hypothesized that mucoadhesion of excipients is not only attributed to interactions with mucus and its different components, but also to interactions with the underlying mucosal epithelium.

METHODS

Materials

Chitoceuticals chitosan 95/100 (degree of deacetylation 96%, Mw 100–250 kDa) was purchased from Heppe Medical Chitosan (Halle, Germany). Polyethylene oxide (Mw 900 kDa, PEO), Dulbecco's phosphate buffered saline (PBS) and acetic acid anhydride (Ph.Eur. ≥ 99 %), was from Sigma Aldrich (St. Louis, MO, USA). Loctite® Power Flex gel was purchased from Henkel (Ballerup, Denmark). Sandpaper (38728, K120, 93 mm) was purchased from Millarco A/S (Lystrup, Denmark). Ultrapure water (18.2 MΩ × cm) purified by a PURELAB flex 4 (ELGA LabWater, High Wycombe, UK) was used.

Sample preparation

Aqueous dispersions of 2 % (w/w) chitosan with 0.7 % (w/w) acetic acid in ultrapure water and 2 % (w/w) PEO were prepared in ultrapure water and stirred at room temperature for two days prior to the rheology experiment to ensure that the polymers were completely dispersed.

Isolation of porcine tissue

The isolation of porcine tissue was conducted according to the authorization for the use of animal by-products and derived products for research and diagnosis approved by the Danish Veterinary and Food Administration (license number DK-13-oth-931833). Surplus intestines from healthy pigs, 40 – 70 kg (Danish Landrace/Yorkshire/Duroc) were obtained immediately after euthanization: 1 - 3 m jejunum was isolated distal to the ligament of Treitz, both ends of the isolated intestine were clamped and the tissue was kept on ice until further use (up to 8 h after isolation). Sections of isolated jejunum (approximately 5 cm) were cut and carefully opened along the mesentery line of the intestine, without affecting the mucosae (**Fig. 1-B**). Portions of the jejunum with food debris were discarded. For experiments performed on intestinal tissue without mucus layer, the mucus was very gently scraped of the intestinal surface with a glass slide. From the same pig, surplus tongue and cheeks were isolated and kept hydrated in PBS on ice until use (up to 8 h after isolation). Porcine tongues and cheeks were trimmed

using surgical scissors, hereby obtaining a flat mucosal surface; this was important to maximize the mucosal area available (**Fig. 1-A**). Finally, thin sections of 0.75 mm in thickness of buccal and sublingual mucosae were cut with an electric dermatome (Zimmer Biomet, Albertslund, Denmark) and kept in PBS on ice until use.

Small deformation rheology

Sandpaper was used as a rough surface to keep the tissue in place during measurements. The mucosal tissue was immediately glued (Loctite® Power Flex gel) to the centre of a disc of sandpaper approximately 10 cm in diameter (38728, K120, 93 mm, Millarco, Denmark) as shown in the **Fig. 1-C**. For the buccal and sublingual mucosa, the same sample setup was followed, however the excess of PBS was removed carefully before gluing the tissue to the sandpaper. Immediately after, the tissue on sandpaper was placed on the Peltier plate and immobilized by a custom-made accessory fixating the sandpaper (**Fig. 1-D**) during the experiment. 255 µL of test solution (polymer dispersion) was carefully added to the centre of the tissue, and the viscosity was evaluated using an ARES-G2 Rheometer (TA Instruments, New Castle, DE, USA) fitted with a sandblasted plate (SST ST SB, 20 mm SM-SW, from TA Instruments, New Castle, DE, USA). A conditioning step was applied with a pre-shear set to 100 s^{-1} followed by an equilibrium step of 2 min at 37 °C. Then, a steady state flow step was conducted using a shear rate of 0.1 to 3000 s^{-1} with three consecutive measurements of 10 s each, allowing a maximum of 5 % variance, collecting 10 points per decade. A broad range of shear rates was chosen for proof-of-concept.

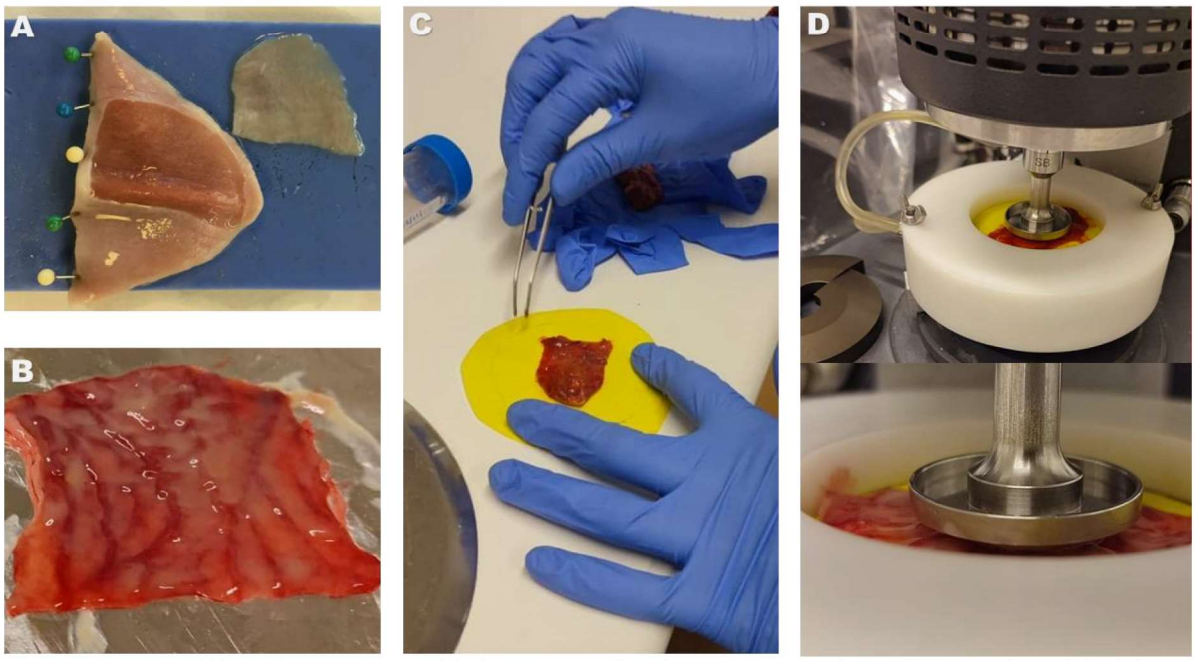

FIGURE 1: A) Sublingual mucosa cut with dermatome after the tongue was trimmed. **B)** Section of the jejunum opened along the mesentery line. **C)** Sample of jejunum glued to the middle of sandpaper. **D)** Custom-made accessory to fixate sandpaper with the tissue during the experiment and below a zoom-in.

Data analysis

The experiments using jejunum and buccal mucosae were run in quadruplicate (N=4, n=1-2), the tests of jejunum without mucus were run in triplicate (N=3, n=1) and the experiments with only the test solutions (polymer dispersions) were run in triplicates, twice per batch (N=3, n=2). The experiments using sublingual mucosae were run in triplicate when evaluating chitosan (N=3), but only in duplicate when evaluating PEO (N=2) due to limited tissue availability. The average viscosity was plotted with error bars as standard error of the mean (SEM). Except in case of PEO on sublingual mucosae for which only the average viscosity was plotted, without error bars.

RESULTS

The mucoadhesion of chitosan and PEO, two pharmaceutically relevant excipients, was evaluated by measuring the viscosity of the excipients in the presence of *ex vivo* porcine mucosae. Chitosan is positively charged under acidic conditions due to the protonation of the amino groups of this polysaccharide, which provides a strong electrostatic interaction with the negatively charged components of the mucus[8,9]. In addition, hydrophobic interactions and hydrogen bonds also contribute to the strong mucoadhesion of chitosan[10]. PEO is a synthetic neutral polymer, and in contrast to chitosan, it is highly water-soluble at neutral pH and only binds weakly to mucins[11,12]. Chitosan and PEO were chosen for proof-of-concept of the novel *ex vivo* rheology-based method, as they represent different degrees of mucoadhesion i.e., as a positive (chitosan) and negative (PEO) control of mucoadhesion.

The viscosity of chitosan and PEO was measured on *ex vivo* mucosae from different anatomical sites, i.e., the small intestine, cheek, and tongue. Interactions of the polymers with the mucosae are expected to result in an increase in viscosity because of restriction in fluid flow due to the binding of the polymers to the mucosae. The viscosity was recorded by applying a broad range of shear rates, as the methodology was in the first step of development. Furthermore, the oral cavity shear rate, in general, is considered to be between 1 and 1000 s^{-1}, but it is also very contested and variable[13,14] and in the jejunum the physiologically relevant range is between 1 and 10 s^{-1} [15]. Different tendencies were seen from viscosity measurements from different anatomical sites. When evaluated on jejunum mucosa, chitosan presents higher viscosity than PEO, as expected, since chitosan is known to display stronger mucoadhesive properties **(Fig. 2-A)**. It should be noted that a difference in viscosity over the entire range of shear rate applied is also observed for the control samples, chitosan, and PEO liquid dispersions (no mucosae), but this difference is much less pronounced (**Fig. 2-D**), indicating that the presence of the intestinal mucosae influences the mucoadhesion of the polymers. Surprisingly, a similar difference between the viscosity of the same two polymers is not observed on the sublingual (**Fig. 2-B**) and the buccal epithelium (**Fig. 2-C**). The viscosity of PEO is slightly lower than the viscosity of chitosan on sublingual mucosa, but no difference in the viscosity of chitosan and PEO was measured when lower shear rates were applied, i.e., shear rates in the intestinal physiological relevant range (1 to 10 s^{-1}).

The role of mucus was briefly investigated due to the differences observed between the mucosae. Sublingual and buccal mucosal sections were kept submerged in PBS until use to maintain the tissue viability, hydration, and integrity during handling and cutting. This causes a significant washout of the saliva from the mucosal surface and thus also likely removes the mucus layer to a great extent. Excess buffer was removed before the addition of the polymer samples, but even small differences in the level of hydration and handling of the sublingual and buccal mucosae could affect the viscosity measurements. In contrast, handling the jejunum was

gentler, meaning that the mucus layer was preserved to a much larger extent, keeping both loosely and firmly adherent mucus. In general, chitosan and PEO show the same trend both in the presence and absence of the mucus layer on the intestinal tissue (**Fig. 2-A**), yet with a higher recorded viscosity for chitosan compared to that of PEO. Furthermore, when the mucus layer of the jejunum was removed, the viscosity of PEO decreased slightly more than in the case of chitosan, especially in the low shear rate range, which, as mentioned previously, represents a more physiologically relevant shear rate for this anatomical origin. Thus, it is suggested that the mucoadhesion of chitosan is related not only to interactions of the polymers with the mucus matrix, but also with the underlying epithelial layer. This is in accordance with previous investigations, as chitosan binds to integrins present on the cell membranes of the epithelial layer [16,17], which also contributes to greater mucoadhesion.

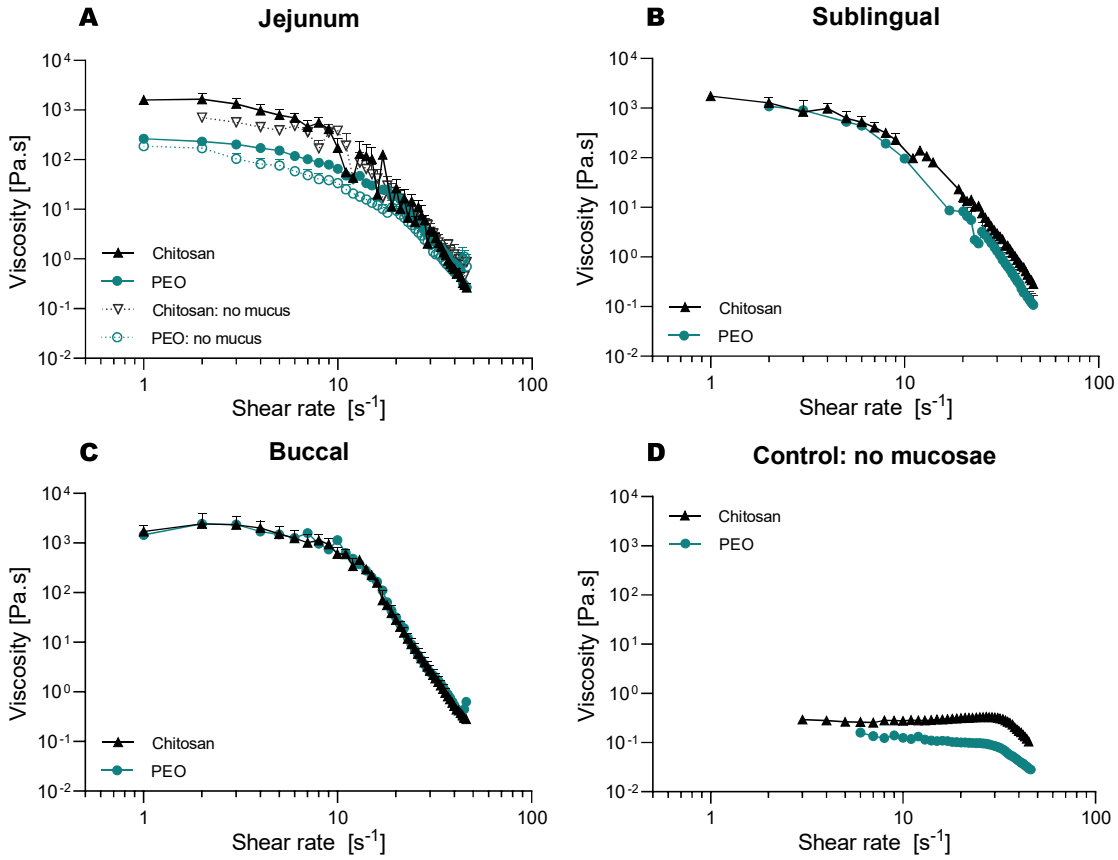

FIGURE 2: Viscosity of chitosan and PEO as a function of shear rate on jejunum with mucus layer (solid line) and without mucus layer (no mucus, dashed line) **(A)**, on sublingual **(B)** and on the buccal epithelium **(C)**. Viscosity of dispersions of chitosan and PEO (no mucosae) **(D)**.

Drug delivery across the mucosae is generally very specific to the exact anatomical region, especially when the delivery systems include mucoadhesive excipients with the aim of maintaining a drug concentration gradient at the absorption site. Furthermore, drug delivery systems can be tailored and tuned to enhance permeation across mucosae, but to do so, it is important to rely on *in vivo*-relevant models comprising all interregional variability of and within the mucosae. This novel *ex vivo* rheology-based approach is promising for investigating

the mucoadhesion of excipients to a specific region of the gastrointestinal tract without disturbing the mucosae. There is minimal handling and perturbance of the mucosae during the isolation of the gastrointestinal tissue and sample preparation. Although this rheology-based approach still requires optimization, the method can represent a sensitive way to investigate the interactions of excipients or drug delivery systems with mucosae, considering its complexity throughout the entire gastrointestinal tract. This is a key aspect in the field of drug delivery when investigating biological responses to excipients, especially when taking into consideration the great heterogenicity of mucosae *in vivo*.

CONCLUSION

A novel rheology-based approach was developed to investigate the interactions of liquid dispersions with mucosae, using *ex vivo* porcine tissue as a representative of *in vivo* tissue. In these proof-of-concept studies, the viscosity of chitosan and PEO was shown to be dependent on the anatomical origin of the mucosae and the handling of the mucosae. Interestingly, the presence of the entire mucosae, constituting of the epithelium and mucus layers, influenced the viscosity of chitosan and PEO differently. This novel *ex vivo* rheological methodology has the potential to be very useful for evaluating mucoadhesion to a specific region of undisturbed mucosae under more *in vivo* relevant conditions compared to traditional methods, but optimization of this state-of-the-art method is still required to achieve its full potential.

ACKNOWLEDGEMENTS

The authors acknowledge the Novo Nordisk Foundation (BioDelivery; Grand Challenge Programme: NNF16OC0021948), Lundbeck Foundation (project no. R303-2018-2968), and VILLUM FONDEN (project number: 19175) for funding this research. Department of Experimental Medicine at the University of Copenhagen is acknowledged for providing access to the porcine tissue used for the *ex vivo* studies. The authors thank LEO Pharma for availability of the dermatome equipment.

BIBLIOGRAPHY

(1) Mortensen, J. S.; Stie, M. B.; Harloff-Helleberg, S.; Nielsen, H. M. Overcoming the Mucus Barrier. *Organelle and Molecular Targeting* 2021, 355–379. https://doi.org/10.1201/9781003092773.

(2) Hassan, E. E.; Gallo, J. M. A Simple Rheological Method for the *in Vitro* Assessment of Mucin-Polymer Bioadhesive Bond Strength. *Pharmaceutical Research* 1990, *7* (5), 491–495. https://doi.org/10.1023/A:1015812615635.

(3) Rønholt, S.; Vissing, K. J.; Nielsen, H. M. Assessment of Mucoadhesion Using Small Deformation Rheology Revisited. *Annual Transactions of the Nordic rheology society,* 2017, *25*, 63–70.

(4) Barmpatsalou, V.; Rodler, A.; Jacobson, M.; Karlsson, E. M. L.; Pedersen, B. L.; Bergström, C. A. S. Development and Validation of a Porcine Artificial Colonic Mucus Model Reflecting the Properties of Native Colonic Mucus in Pigs. *European Journal of Pharmaceutical Sciences* 2023, *181*, 106361. https://doi.org/10.1016/j.ejps.2022.106361.

(5) Tollemeto, M.; Huang, Z.; Christensen, J. B.; Nielsen, H. M.; Rønholt, S. Mucoadhesive Dendrons Conjugated to Mesoporous Silica Nanoparticles as a Drug Delivery Approach for Orally Administered Biopharmaceuticals. *ACS Applied Materials & Interfaces* 2023, *15*, 8810. https://doi.org/10.1021/acsami.2c16502.

(6) Madsen, K. D.; Sander, C.; Baldursdottir, S.; Pedersen, A. M. L.; Jacobsen, J. Development of an *Ex Vivo* Retention Model Simulating Bioadhesion in the Oral Cavity Using Human Saliva

and Physiologically Relevant Irrigation Media. *International Journal of Pharmaceitics* 2013, *448* (2), 373–381. https://doi.org/10.1016/j.ijpharm.2013.03.031.

(7) Mosgaard, M. D.; Strindberg, S.; Abid, Z.; Petersen, R. S.; Thamdrup, L. H. E.; Andersen, A. J.; Keller, S. S.; Müllertz, A.; Hagner Nielsen, L.; Boisen, A. *Ex Vivo* Intestinal Perfusion Model for Investigating Mucoadhesion of Microcontainers. *International Journal of Pharmaceutics* 2019, *570*, 118658. https://doi.org/10.1016/j.ijpharm.2019.118658.

(8) Sandri, G.; Rossi, S; Ferrari, F; Bonferoni, M. C.; Caramella, C. M.; Mucoadhesive Polymers as Enabling Excipients for Oral Mucosal Drug Delivery. *Oral Mucosal Drug Delivery and Therapy,* 2015, 53–88. https://doi.org/10.1007/978-1-4899-7558-4_4.

(9) Ways, T. M. M.; Lau, W. M.; Khutoryanskiy, V. V. Chitosan and Its Derivatives for Application in Mucoadhesive Drug Delivery Systems. *Polymers,* 2018, 10(3), 267. https://doi.org/10.3390/polym10030267

(10) Sogias, I. A.; Williams, A. C.; Khutoryanskiy, V. V. Why Is Chitosan Mucoadhesive? *Biomacromolecules*, 2008, 9(7), 1837–1842. https://doi.org/10.1021/bm800276d.

(11) Albarkah, Y. A.; Green, R. J.; Khutoryanskiy, V. V. Probing the Mucoadhesive Interactions Between Porcine Gastric Mucin and Some Water-Soluble Polymers. *Macromolecular Bioscience,* 2015, *15* (11), 1546–1553. https://doi.org/10.1002/mabi.201500158.

(12) Amiji, M.; Park, K. Surface Modification of Polymeric Biomaterials with Poly(Ethylene Oxide), Albumin, and Heparin for Reduced Thrombogenicity. *Journal of Biomaterials Science, Polymer Edition,* 1993, *4* (3), 217–234. https://doi.org/10.1163/156856293X00537.

(13) Gallegos, C.; Quinchia, L.; Ascanio, G.; Salinas-Vásquez, M.; Brito-de la Fuente, E. Rheology and Dysphagia: An Overview. Annual Transactions of the Nordic rheology society, 2012, *20*(8).

(14) Ong, J. J. X.; Steele, C. M.; Duizer, L. M. Challenges to Assumptions Regarding Oral Shear Rate during Oral Processing and Swallowing Based on Sensory Testing with Thickened Liquids. *Food Hydrocolloids* 2018, *84*, 173–180. https://doi.org/10.1016/j.foodhyd.2018.05.043.

(15) Barmpatsalou, V.; Dubbelboer, I. R.; Rodler, A.; Jacobson, M.; Karlsson, E.; Pedersen, B. L.; Bergström, C. A. S. Physiological Properties, Composition and Structural Profiling of Porcine Gastrointestinal Mucus. *European Journal of Pharmaceutics and Biopharmaceutics* 2021, *169*, 156–167. https://doi.org/10.1016/j.ejpb.2021.10.008

(16) Hsu, L. W.; Ho, Y. C.; Chuang, E. Y.; Chen, C. T.; Juang, J. H.; Su, F. Y.; Hwang, S. M.; Sung, H. W. Effects of PH on Molecular Mechanisms of Chitosan–Integrin Interactions and Resulting Tight-Junction Disruptions. *Biomaterials* 2013, *34* (3), 784–793. https://doi.org/10.1016/j.biomaterials.2012.09.082.

(17) Liu, M.; Zhang, J.; Zhu, X.; Shan, W.; Li, L.; Zhong, J.; Zhang, Z.; Huang, Y. Efficient Mucus Permeation and Tight Junction Opening by Dissociable “Mucus-Inert” Agent Coated Trimethyl Chitosan Nanoparticles for Oral Insulin Delivery. *Journal of Controlled Release* 2016, *222*, 67–77. https://doi.org/10.1016/j.jconrel.2015.12.008.