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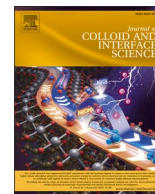
On the mucoadhesive properties of synthetic and natural polyampholytes

Manfei Fu [a], Sergey K. Filippov [b], Adrian C. Williams [a], Vitaliy V. Khutoryanskiy [a,*]

[a] School of Pharmacy, University of Reading, Whiteknights, Post Office Box 224, Reading RG6 6AD, United Kingdom
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GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

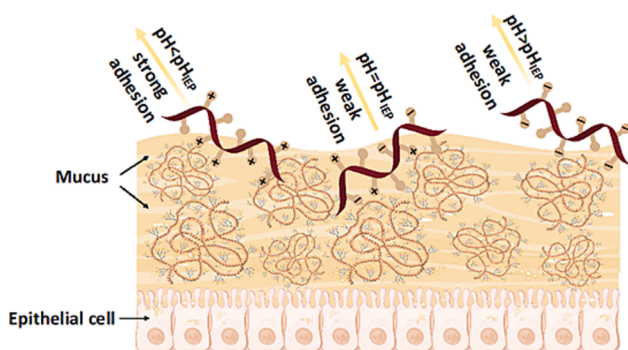*Hypothesis:* The mucoadhesive characteristics of amphoteric polymers (also known as polyampholytes) can vary and are influenced by factors such as the solution's pH and its relative position against their isoelectric point (pH_{IEP}). Whilst the literature contains numerous reports on mucoadhesive properties of either cationic or anionic polymers, very little is known about these characteristics for polyampholytes

Experiments: Here, two amphoteric polymers were synthesized by reaction of linear polyethylene imine (L-PEI) with succinic or phthalic anhydride and their mucoadhesive properties were compared to bovine serum albumin (BSA), selected as a natural polyampholyte. Interactions between these polymers and porcine gastric mucin were studied using turbidimetric titration and isothermal titration calorimetry across a wide range of pHs. Model tablets were designed, coated with these polymers and tested to evaluate their adhesion to porcine gastric mucosa at different pHs. Moreover, a retention study using fluorescein isothiocyanate (FITC)-labelled polyampholytes deposited onto mucosal surfaces was also conducted

Findings: All these studies indicated the importance of solution pH and its relative position against pH_{IEP} in the mucoadhesive properties of polyampholytes. Both synthetic and natural polyampholytes exhibited strong interactions with mucin and good mucoadhesive properties at $pH < pH_{IEP}$.

1. Introduction

Mucoadhesion is defined as attractive interactions between materials of a dosage form and a mucosal surface [1–4]. All water-soluble and weakly cross-linked hydrophilic polymers exhibit some mucoadhesive properties due to their interactions with mucin via hydrogen bonding or electrostatic effects or/and their ability to penetrate into the mucus gel to form an interpenetrating layer [5]. Mucoadhesive polymers are commonly used in the design of dosage forms including tablets [6], films [7], patches [8] and gels [9] for transmucosal drug delivery. Mucoadhesion also plays important role in taste perception for some food formulations [10,11] and in dental care [12].

Anionic polymers such as poly(acrylic acid), carboxymethylcellulose, alginate and pectin [13] exhibit strong mucoadhesive properties due to the ability of their carboxylic groups to form hydrogen bonds with hydroxyl groups present in oligosaccharide fragments of mucins [14,15]. Strong mucoadhesive properties of cationic polymers are usually due to the electrostatic attractive interactions with negatively charged carboxylic and sulphate groups present in mucins. Non-ionic polymers typically exhibit poorer mucoadhesive properties compared to polyelectrolytes [16] although their ability to adhere to mucosal tissues can be substantially enhanced by introducing functional groups capable of forming covalent bonds with mucins [17].

Polyampholytes are macromolecules containing both anionic and cationic groups in their structure and can be synthetic or of natural origin. Polyampholytes have some unique physicochemical properties, including the existence of an isoelectric point (pH_{IEP}), which is defined as the pH at which the macromolecules have a net zero electrical charge [18–20]. At pHs below pH_{IEP} the macromolecules of polyampholytes carry an overall positive charge and at pHs above this point they become net negatively charged. Consequently, the behavior of polyampholytes is strongly dependent on solution pH. Proteins are natural polymers that have amphoteric properties due to the presence of both acidic (e.g. aspartic and glutamic acids) and basic (e.g. lysine and arginine) amino acid residues in their structure.

There are very few reports in the literature evaluating mucoadhesive properties of synthetic or natural polyampholytes. Some authors reported that mucoadhesive properties of gelatine, a denatured protein derived from collagen, are poor and comparable to non-ionic polymers [21]. However, gelatine derivatised through its additional amination was shown to exhibit considerable mucoadhesive performance both in vitro and in vivo in rats [22]. More recently, Nishio et al. [23] reported strong mucoadhesive properties for polyampholyte hydrogels synthesised from acrylic acid and N,*N*-dimethylaminopropyl acrylamide. Adhesion of milk proteins to the oral mucosa was demonstrated by Withers et al. [24] and was related to the drying sensation observed following consumption of protein rich dairy beverages. The current literature demonstrates that a detailed mechanistic understanding of the factors affecting the mucoadhesive properties of amphoteric polymers is lacking, which is especially important for proteins.

This study investigates the factors affecting the mucoadhesive properties of both synthetic and natural polyampholytes. Two polyampholytes were synthesized by reacting L-PEI with either succinic anhydride (SA) or phthalic anhydride (PA). Bovine serum albumin (BSA) was selected as a natural polyampholyte due to its extensive use and characterisation, excellent solubility in water over a broad range of pHs, and good stability in solutions. The polyampholytes were fully characterized by proton nuclear magnetic resonance (^{1}H NMR) and Fourier transformed infrared spectroscopies (FTIR), turbidity-pH measurements and electrophoretic mobility at various pHs. Turbidimetric titration and isothermal titration calorimetry (ITC) characterized the interactions of the polyampholytes with porcine gastric mucin in solutions. Model mucoadhesive tablets, coated with polymers and the protein, were used to evaluate adhesion of the dosage form to porcine gastric mucosal surface. The retention of the polyampholytes in solutions on porcine gastric mucosal surfaces was evaluated by fluorescent labelling the polymers and using fluorescence microscopy-based flow-through assay. To the best of our knowledge, this is the first study to systematically evaluate the mucoadhesive properties of polyampholytes to elucidate the factors affecting their ability to adhere to mucosal surfaces.

2. Materials and methods

2.1. Materials

Poly(2-ethyl-2-oxazoline) (PEOZ, MW ~ 50 kDa, Đ = 3–4), succinic anhydride, phthalic anhydride, dimethyl sulfoxide (DMSO), triethylamine (TEA), deuterium oxide (D_2O), deuterated methanol (MeOD-d4), sodium fluorescein, fluorescein isothiocyanate (FITC), fluorescein isothiocyanate-dextran (FITC-dextran, average MW 10,000), bovine serum albumin (BSA) and mucin from porcine stomach (type II) (PGM) were obtained from Sigma-Aldrich (Gillingham, U.K.). Urea, hydrochloric acid (37 %), sodium hydroxide, hydroxypropyl methylcellulose, microcrystalline cellulose, barium sulfate, magnesium stearate and phosphate-buffered saline (PBS) tablets were obtained from Fisher Scientific (Loughborough, U.K.). Dialysis membrane (MWCO 3.5 kDa) was purchased from Medicell Membranes Ltd. (U.K.). Fresh porcine gastric tissue was provided by P.C. Turner Abattoirs (Farnborough, UK). All other chemicals were of analytical grade and used without further purification.

2.2. Synthesis of succinylated L-PEI and phthaylated L-PEI

L-PEI was synthesized by acidic hydrolysis of poly(2-ethyl-2-oxazoline) (PEOZ) following the protocol of Shan et al. [25] Succinic anhydride (0.50 eq, 1.16 g) or phthalic anhydride (1.00 eq, 3.47 g) were dissolved in 15.00 mL DMSO and then mixed with 45 mL of the L-PEI (1.00 g, 1.00 eq) solution in DMSO, before triethanolamine (1.50 eq, 3.25 mL) was added (ESI). The mixture was stirred for 12 h at 40 °C and then diluted with deionized water and dialyzed against deionized water for 72 h. All polymers were recovered by freeze-drying (1.88 g (87.03 %) and 3.88 g (86.80 %) yield for succinylated L-PEI and phthaylated L-PEI, respectively).

2.3. Characterization of succinylated L-PEI and phthaylated L-PEI

2.3.1. ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H NMR)

10 mg succinylated L-PEI or phthaylated L-PEI was dissolved in 1 mL D_2O, whereas L-PEI was dissolved in 1 mL MeOD-d4. Spectra were recorded as the average of 128 scans using a 400 MHz Bruker spectrometer. Further details can be found in ESI.

2.3.2. Fourier transform infrared (FTIR) spectroscopy

Polymers were analysed between 4000 and 950 cm^{-1} at a resolution of 4 cm^{-1} as an average of 64 scans using a diamond sampling accessory. Data were recorded by a Nicolet iS5 spectrometer (Thermo Scientific, U. K.).

2.3.3. Turbidity measurements

The effects of pH on turbidity of succinylated L-PEI, phthaylated L-PEI and BSA solutions were studied using a JENWAY 7315 spectrophotometer (Bibby Scientific Ltd, UK) at 400 nm at different pHs. The pH was adjusted by adding 0.1 M NaOH or HCl. Every titration was repeated in triplicate and the turbidity values are reported as mean ± standard deviation.

2.3.4. Electrophoretic mobility measurements

The effects of pH on electrophoretic mobility of succinylated L-PEI, phthaylated L-PEI and BSA solutions were studied in folded DTS-1070 capillary cells by using a Malvern Zetasizer Nano-S (Malvern Instruments). All samples were dissolved in deionized water (1 mg/mL) and pH was adjusted by adding 0.1 M NaOH or HCl. All measurements

were conducted at 25 °C and repeated in triplicate; the values are reported as mean ± standard deviation.

2.4. Mucin interaction studies

All experiments were performed with 1 mg/mL polyampholyte aqueous solutions and mucin dispersions, freshly prepared prior to each experiment. Porcine gastric mucin (PGM) was dispersed in deionized water, sonicated for 15 min and centrifuged at 1000 rpm for 5 min. The pH of the supernatant was adjusted using 0.1 M NaOH or HCl. Mixtures of mucin dispersions with polymer solutions were prepared at a wide range of ratios before turbidity was measured at 400 nm using a JENWAY 7315 spectrophotometer (Bibby Scientific Ltd, UK). To verify the role of hydrogen bonding and hydrophobic effects on the mucoadhesive interactions [26], in a separate set of experiments PGM dispersions and polymer solutions were additionally prepared in 8 M urea, also at 1 mg/mL. All measurements were repeated in triplicate, and values reported as mean ± standard deviation.

2.5. Isothermal titration calorimetry (ITC)

Binding interactions between PGM and polymers [27] were studied using a TA NANO ITC 2G Isothermal Titration Calorimeter (Calorimetry Science Crop., USA). In each titration experiment, 100 µL of a polymer solution at a defined pH was loaded into the syringe and titrated into mucin dispersions with the same pH within a 950 µL calorimeter sample cell. The control experiments of titrating polymer solutions or mucin into a buffer were conducted using solutions at the same pH. Titrations were performed automatically with 5.05 µL aliquots from the syringe injected into the sample cell every 300 s. All ITC measurements were conducted at 25 °C. Titrations of mucin solution into a buffer were also performed as a negative control with buffer to buffer titrations used as a reference. The experimental change in enthalpy (ΔH) was obtained by integrating the raw data with results analysed using Origin Lab® version 9.0 and NanoAnalyze software. "One-set-of-sites" model was used for the fitting when applicable. [28] $\Delta H_{change} = \Delta H_{end} - \Delta H_{start}$ was taken as a measure of enthalpy change during titration for non-sigmoidal processes. In light of mucin molecular weight uncertainty and rather broad synthesized polymer molecular weight, weight concentration were used for ITC data analysis instead of molar values. This feature makes the determination of a binding constant, and hence entropy change, ΔS, unreliable, and so we focus on the ΔH_{change} analysis.

2.6. Ex vivo gastric mucoadhesive studies

2.6.1. Mucoadhesive studies of tablets on porcine gastric mucosa

Blank tablets were prepared by compression of a blend of hydroxypropyl methylcellulose (40 %), microcrystalline cellulose (40 %), barium sulphate (19 %) and magnesium stearate (1 %) using a single punch tableting machine (RIVA G.B. Ltd, UK). The obtained tablets were coated with 2 % (w/v) polymer aqueous solutions containing 5 % (w/v) sodium fluorescein using a mini spray coater / drier (Caleva Process Solution Ltd, UK). The average tablet weight, thickness, diameter, hardness and coating efficiency were determined for 10 tablets in every batch (data in ESI).

Mucoadhesion of the polymer coated tablets was determined using a tensile test with a TA.XT Plus Texture Analyser (Stable Micro Systems Ltd., Godalming, UK) with freshly excised porcine gastric mucosal tissue. Before each test, dissected gastric tissue (4.0 × 4.0 cm²) was mounted on a glass slide with the mucosal side upward and pre-rinsed with 2.5 mL of simulated gastric fluid (SGF), prepared by dissolving 2.0 g NaCl and 3.0 mL HCl in 1 L deionized water before adjusting pH with 0.1 M HCl or NaOH. The pH of each mucosal tissue was adjusted with 0.1 M NaOH or HCl and measured with a FiveEasy F20 pH meter (Mettler-Toledo GmbH, Switzerland). The tissue was placed in a temperature- and humidity-controlled chamber of a Stable Micro Systems

texture analyser (Stable Micro Systems Ltd, UK) and equilibrated at 37.0 ± 0.1 °C. The texture analyser was used in the 'adhesive test' mode with a pre-speed of 2.0 (mm/s) and test-speed of 2.0 mm/s. Each model tablet was attached to the probe by two-sided sticky tape and moved downward to the mucosal tissue surface with an applied force of 2 N and remained in contact for 15 s. The probe was subsequently withdrawn at 10.0 mm/s. Twenty tablets coated with each polymer were measured at each different pH and the work of adhesion was calculated from the area under the detachment curve; all values are reported as mean ± standard deviation.

2.6.2. Retention studies on porcine gastric mucosa

The flow through method followed our previous report [29]. FITC and polymers at a weight ratio of 1:20 were dissolved in 30 mL DMSO at 40 °C and the mixture was stirred overnight. The resultant solution was diluted with deionized water and dialyzed against deionized water for 72 h. All FITC-labelled polymers were recovered by freeze-drying. Porcine gastric mucosal tissue (1.5 × 2.0 cm²) was mounted on an inclined glass slide with the mucosal side upward and pre-rinsed with 1 mL freshly prepared solutions with different pHs, adjusted with 0.1 M NaOH or HCl. All experiments were conducted at 37.0 ± 0.1 °C in an incubator. Briefly, tissue background fluorescence intensity ($I_{background}$) was collected from each blank tissue. Then, 20 µL of FITC-succinylated L-PEI, FITC-phthaylated L-PEI, FITC-BSA or FITC-dextran (negative control) solution was dosed onto the mucosal surface and fluorescence images were recorded as initial fluorescence intensity (I_0). After 3 min of dosing, the mucosal surface was washed with solutions of different pH using a syringe pump (Harvard Apparatus model 981074, Holliston, MA, USA) at 0.43 mL/min. Fluorescence images of the mucosal tissue (I_t) were acquired periodically using a Leica MZ10F stereo-microscope (Leica Microsystems, Wetzlar, Germany) with the GFP filter-fitted Leica DFC3000G digital camera at 2.0 × magnification, 735 ms of exposure time, 2.0 × gain, 1.0 × gamma and pseudo color at 520 nm. The microscopy images from each time point were analysed using ImageJ software (Version 1.53 t, 2022) and fluorescence intensity calculated according to Equation (1):

$$\text{Fluorescence intensity } (\%) = \frac{I_t - I_{background}}{I_0 - I_{background}} \times 100\% \qquad (1)$$

where the 0 min point was set as 100 %. Results are presented as the fluorescence intensity of the FITC-labelled polymers (after subtracting the background fluorescence from each wash image) at different wash-time points as a function of irrigation time (0–60 min). Triplicate experiments were performed for each polymer and values are reported as mean ± standard deviation.

2.7. Statistical analysis

All experiments were conducted with a minimum of 3 replicates and data are presented as mean ± standard deviations. GraphPad Prism software (version 9.5.1; GraphPad Software Inc., San Diego, CA, USA) was used to analyse data using a one-way analysis of variance (ANOVA) and two-tailed Student's t-tests where $p < 0.05$ was set as the statistical significance criterion.

3. Results and discussion

3.1. Synthesis and characterization of succinylated L-PEI and phthaylated L-PEI

Commercially available PEOZ (50 kDa) was fully hydrolysed to form L-PEI, which was subsequently reacted with succinic or phthalic anhydrides to synthesize succinylated L-PEI and phthaylated L-PEI (Scheme 1).

The resultant polymers were characterized using ¹H NMR and FTIR
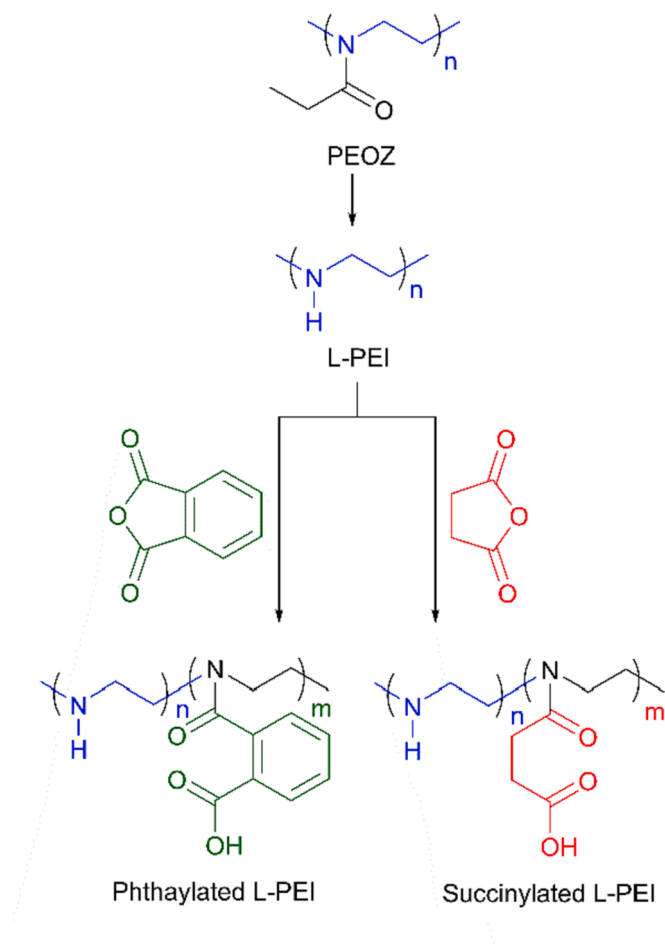
Scheme 1. Synthesis of succinylated and phthaylated L-PEI from PEOZ.

spectroscopies. These results are presented and discussed in ESI. The physicochemical properties of novel polyampholytes as well as BSA in solutions were evaluated using turbidity and electrophoretic mobility measurements at different pHs.

It is well known that polyampholyte macromolecules undergo contraction and in some cases aggregation, when their solution pH is approaching pH$_{IEP}$ [30]. Enhanced aggregation usually manifests as

onset of turbidity. This makes turbidimetric studies of their solutions at different pHs a suitable technique for determining their pH$_{IEP}$ value. Fig. 1 shows turbidity–pH and electrophoretic mobility-pH profiles for solutions of our novel synthetic polyampholytes and for BSA. Both succinylated and phthaylated L-PEI exhibit a very pronounced increase in solution turbidity with the maximal values at 4.85 ± 0.05 and 7.71 ± 0.08, respectively. A further increase in solution pH results in substantial reduction of turbidity values. These turbidity – pH profiles are typical for solutions of polyampholytes, which undergo aggregation near pH$_{IEP}$ [31]. When pH < pH$_{IEP}$ or pH > pH$_{IEP}$, due to the presence of excess positively or negatively charged groups, the polymer is fully hydrated and water soluble. Aggregation of BSA when solution pH approaches pH$_{IEP}$ was not observed; its solutions remain transparent across pH 2–12, potentially because it is insufficiently hydrophobic to aggregate. Therefore, it was not possible to determine pH$_{IEP}$ of BSA using the turbidimetric technique.

Another useful method to determine pH$_{IEP}$ for polyampholytes is to assess electrophoretic mobility at different solution pHs [32]. These profiles are typical for amphoteric polymers and colloidal particles, where increases in solution pH results in a gradual transition from positively charged values to negatively charged ones. The point on this profile, where electrophoretic mobility values cross zero corresponds to their pH$_{IEP}$.

The pH$_{IEP}$ values determined using electrophoretic mobility measurements for succinylated L-PEI, phthaylated L-PEI and BSA were 4.30 ± 0.04 and 6.86 ± 0.16 and 5.09 ± 0.08, respectively. The pH$_{IEP}$ of BSA has been reported at 4.7–4.9 [33–35], which is broadly in agreement with the value determined in our work.

Table 1 summarises the pH$_{IEP}$ values determined by the different methods. The IEP values from the two techniques are within a pH unit of each other with variance attributable to the different properties evaluated. For example, turbidity is detected only when relatively large aggregates are present, but the aggregation process may start at slightly different pH, and electrophoretic mobility is dependent on the

Table 1
Characteristics of polyampholytes.

Polymer	[a]DS	pH$_{IEP}$ (Turbidity)	pH$_{IEP}$ (Electrophoretic mobility)
Succinylated L-PEI	46 %	pH 4.85 ± 0.05	pH 4.30 ± 0.04
Phthaylated L-PEI	86 %	pH 7.71 ± 0.08	pH 6.86 ± 0.16
BSA	n.a.	-**	pH 5.09 ± 0.08

[a] Degree of substitution; ** not possible to determine using the turbidimetric technique.
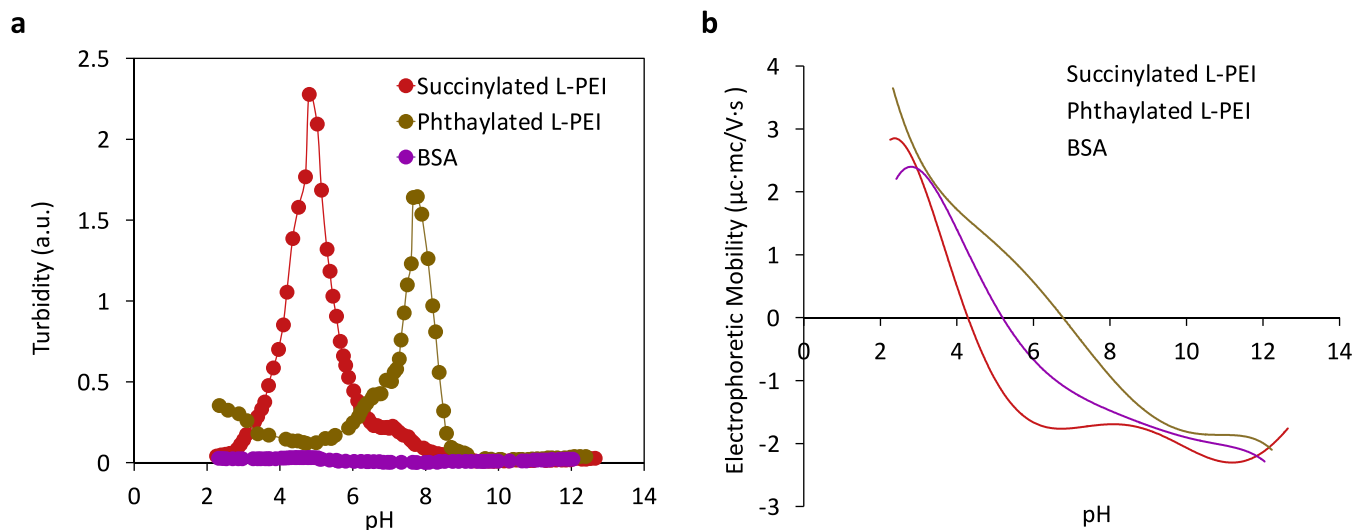

Fig. 1. Effect of pH on solution turbidity (a) and electrophoretic mobility (b) of 1 mg/mL succinylated L-PEI, phthaylated L-PEI and BSA aqueous solution.

conformation of macromolecules and shape of the aggregates. The hydrophobic group of phthaylated L-PEI, has weaker ionic content than succinylated L-PEI, and consequently displays a higher pH_{IEP} than the succinylated derivative. The substantial difference between the pH_{IEP} values of these three polyampholytes provided an opportunity to study their mucoadhesive interactions and properties over a broad range of pHs, below and above their pH_{IEP}.

3.2. Mucin interaction studies

The major role of mucus is protection and lubrication of epithelial cells [36,37]. Mucins are glycoproteins with a high molecular weight (0.5–40 MDa) [22,24] and are the major component of mucus. They bear a negative charge due to the presence of carboxylate groups and ester sulfates. Porcine gastric mucin (PGM) has good storage stability and relatively reproducible properties [38]. When dispersed in aqueous solutions it forms a colloidal system with polydisperse particles, whose

size depend on pH. Fefelova et al. [39] reported that when PGM is dispersed in deionised water (pH 6.8) it forms colloidal system with a bimodal size distribution, with mean particle sizes around 100 nm and 500 nm present. Under acidic conditions (pH 2.0) PGM undergoes further aggregation and forms larger particles up to 3000 nm. Similar observations were also reported by Sogias et al. [5]. Albarkah et al. [40] additionally reported that the particle size and size distributions of PGM is strongly dependent on the use of sonication. Despite the polydispersity, pH- and sonication-dependent nature of the particles of PGM it is commonly used as a model system to study mucoadhesive interactions with polymers [41–43].

Turbidimetric titration is a very common and simple technique to study mucoadhesive interactions [41,44,45], and assesses aggregation of mucin particles when they bind to macromolecules of a mucoadhesive polymer. Here, turbidimetric titrations of PGM were conducted with solutions of polyampholytes at five different pHs, selected to represent conditions below, near and above pH_{IEP} of each polyampholyte. It
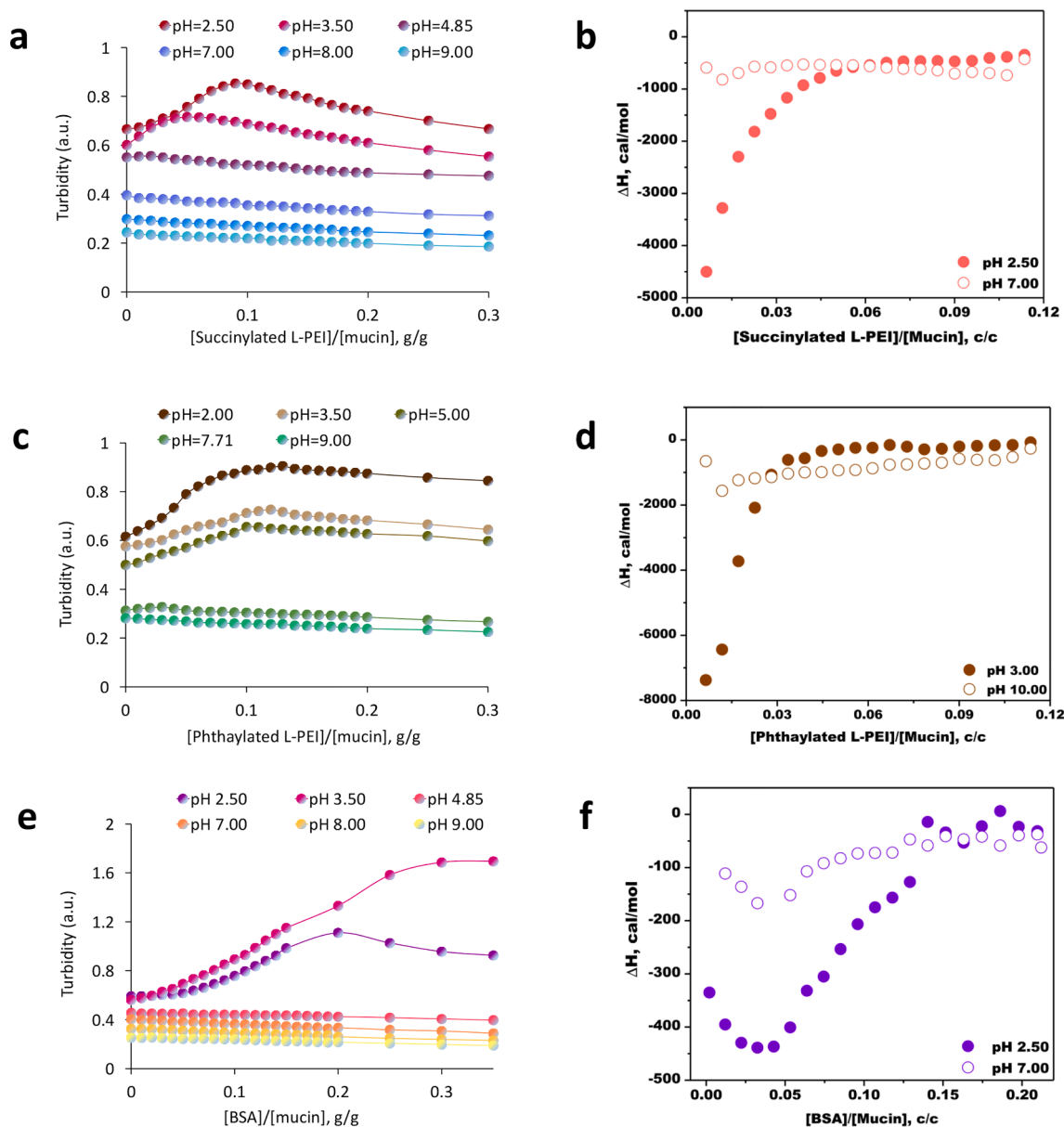
Fig. 2. Turbidimetric titration curves of 1 mg/mL porcine gastric mucin with 1 mg/mL succinylated L-PEI (a), phthaylated L-*EPI* (c) and BSA (e) in aqueous solution; effect of the pH on the interaction between polyampholytes and mucin using isothermal titration calorimetry: 1 mg/mL PGM was titrated with 1 mg/mL succinylated L-PEI at pH 2.50 and pH 7.00 (b), 1 mg/mL PGM was titrated with 1 mg/mL phthylated L-PEI at pH 3.00 and pH 10.00 (d) and 1 mg/mL PGM was titrated with 1 mg/mL BSA at pH 2.50 and pH 7.00 (f). Mean average ± standard deviation, n = 3.

should be noted that the ionic strength of the solutions was not controlled in these experiments to better mimic physiological conditions, as it is known that this varies considerably throughout the GI tract.

Fig. 2 presents the results of these experiments. The initial turbidity values observed for PGM solutions were higher when solution pH was lower and this was consistent with the previous reports [5,39] attributed to deprotonation of mucin's carboxylic groups and further aggregation of its particles. The titration of PGM with all three polyampholytes in solutions with pH < pH$_{IEP}$ clearly showed the presence of strong mucoadhesive interactions leading to increased solution turbidity and aggregation of mucin particles. The maximal turbidity values occur when the surface of the mucin particles is fully saturated with macromolecules of polyampholyte; these values are presented in Table S1 (ESI). The ratios of [polyampholyte] / [mucin], at which the maximal turbidity values are observed are dependent on the nature of amphoteric polymers and solution pH.

It is interesting to note that when the solution pH was equal to or above pH$_{IEP}$ of each polyampholyte, turbidity linearly decreased with addition of further portions of the polymer. This linear decrease is typically associated with simple dilution effects and indicates the absence of attractive interactions. Therefore, we can conclude that mucoadhesive interactions between polyampholyte and mucin are observed only at pHs < pH$_{IEP}$, where the macromolecules positively charged, and mucin remains negatively charged. This indicates that the nature of these interactions is primarily electrostatic. It is important to note that the interactions between a dosage form and a mucosal surface will be more complex than interactions between macromolecules of a polymer and mucin dispersions in solutions. On a surface, a dosage form will deposit and wet (or spread) before adhesion of the macromolecules to the mucosal surface, followed by their deeper penetration into the mucus gel and formation of an interpenetrating layer with mucin biomacromolecules [4].

To further explore the nature of mucoadhesive interactions, additional turbidimetric titration experiments were conducted in solutions containing 8 M urea (Figures 5S–7S, ESI). Urea is known to disrupt hydrogen bonding and hydrophobic effects; it also has some mucolytic properties [5]. The initial turbidity of mucin in urea solutions was around half of the values observed without any additive in aqueous solutions demonstrating the mucolytic properties of urea.

It is clearly seen from the titration curves that the presence of 8 M urea in solution does not prevent polyampholytes from interacting with mucin. However, there is a clear shift in [polyampholyte] / [mucin] ratios at which maximal turbidity was detected, with higher values in the case of synthetic polyampholytes (Table S1, ESI). This indicates that more macromolecules of synthetic polyampholytes are required to saturate the surface of mucin. Partially this is related to greater surface area of mucin, disrupted by mucolytic effect of urea but may also be attributable to the involvement of hydrogen bonding and hydrophobic effects in the mucoadhesive interactions. In the case of BSA, the shift in the position of turbidity maximum on the titration curve is not observed at pH 2.0; however, at pH 3.5 saturation of mucin with BSA is seen at a lower [BSA] / [mucin] ratio. This could be related to the effects of urea on the conformation of BSA in contrast to minimal effects on the synthetic polyampholytes. Overall, the titration experiments conducted in the presence of 8 M urea confirm that the primary nature of mucoadhesive interactions between polyampholytes and mucin is electrostatic attraction.

Isothermal titration calorimetry (ITC) is a powerful technique that can be used to study thermodynamics of specific interactions between compounds present in solutions. [46–49] Previously, ITC has been reported in the studies of mucoadhesive interactions between mucins and chitosan [50,51], epigallocatechin [52] and poly(carboxylic acids) [40]. ITC data showed an exothermic interaction between polyampholytes and mucin at pH < pH$_{IEP}$. As enthalpy change is directly proportional to a number of broken and formed bonds, it can be used to monitor the binding efficiency between species. The interaction between

phthaylated L-PEI (Fig. 2d) and mucin at pH 3.00 showed a strong binding effect with ΔH_{change} of −7.5 cal/mol and a high binding constant with a classical sigmoidal binding curve, whereas at pH = 10.00 the data show weak non-specific interactions (ΔH_{change} of −1.5 cal/mol). Such difference confirms the previous findings from the turbidity experiments evidencing the role of electrostatic interactions between polymers and mucin. The profile of succinylated L-PEI interacting with mucins is similar to that of the phthaylated material but with a lower enthalpy change ΔH_{change} = -4.5 cal/mol (Fig. 2b). For BSA (Fig. 2f), there is an initial dip in enthalpy change with low levels of mucin added, potentially reflecting changes in the BSA rather than interactions between the protein and the mucin. However, for succinylated L-PEI and BSA, at pH = 2.50, both displayed stronger interactions with mucin than at pH = 7.00 where only weak non-specific interactions were recorded. In summary, the ITC results confirmed the findings from the turbidimetric titrations that polymer: mucin interactions at pH < pH$_{IEP}$ are more pronounced than when pH > pH$_{IEP}$.

3.3. Ex vivo gastric mucoadhesion studies

Tensile testing is commonly used to quantify mucoadhesive properties of pharmaceutical formulations. In such tests, a pharmaceutical dosage form is usually attached to a mobile probe of a mechanical testing instrument, such as a texture analyser, and is then brought into contact with animal mucosal tissue. After a defined period of contact, the dosage form is withdrawn from the mucosal tissue and a withdrawal force versus distance curve is recorded. This withdrawal curve is then used to calculate two mucoadhesion parameters, the peak force of detachment and the work of adhesion (calculated as the area under the curve). To conduct tensile tests, tablets were first formulated to ensure that they would not swell or rapidly disintegrate upon exposure to moisture. These tablets were subsequently spray-coated with polyampholytes mixed with sodium fluorescein.

The inclusion of sodium fluorescein allowed visualisation of the coating (Fig. 3a) and the coating weight gain was 1.6 %, 1.4 % and 1.3 %, for succinylated L-PEI, phthaylated L-PEI and BSA, respectively. Testing evaluated mucoadhesion of tablets coated with succinylated L-PEI, phthaylated L-PEI and BSA, applied to pig gastric mucosa and the peak force of detachment (Fig. 3b) and the work of adhesion (Fig. 3c) determined. The pH of the mucosal tissues was varied in these experiments by adding small portions of 0.1 M NaOH or HCl directly on their surface, controlled with a pH meter; the pHs of the solutions were again selected to where pH was either below, above or at pH$_{IEP}$ of each polyampholyte. Blank tablets without polyampholyte coating were used as a negative control.

The blank tablets did not show any significant pH-dependent differences in their detachment characteristics (Fig. 3b and c). In all cases, the polymer coated tablets showed stronger mucoadhesive properties than uncoated blank tablets. However, the tablets coated with polyampholytes exhibited strong adhesion dependence on the tissue pH. When the pH was below pH$_{IEP}$ of each polyampholyte, the coated tablets exhibited significantly stronger mucoadhesive performance, both in terms of the peak force of detachment and the work of adhesion values (p < 0.0001). Under these conditions the polyampholyte is positively charged and so can interact with mucus predominantly electrostatically, resulting in strong mucoadhesion. However, other factors such as the effect of water transport or capillary forces may also play a considerable role in mucoadhesion [53,54]. In contrast, a significant reduction in mucoadhesive properties was observed in experiments conducted at pH ≥ pH$_{IEP}$. This can be attributed to a lack of electrostatic attraction between the dosage form and mucosal tissue when the surface of tablets is either non-charged or negatively charged. The adhesion in this case can only be achieved either through weak hydrogen bonds or physical entanglements between the macromolecules of polyampholyte and mucins. There were no significant differences between adhesion at pH = pH$_{IEP}$ with adhesion when pH > pH$_{IEP}$.
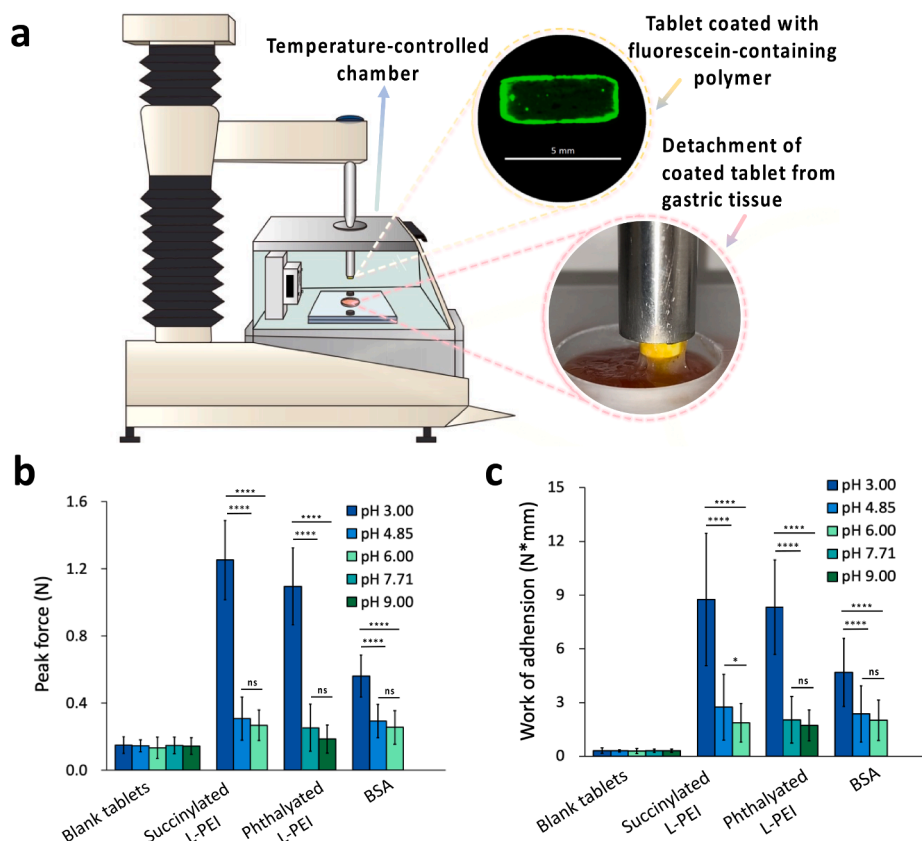
Fig. 3. Illustration of tensile test methodology (a); effect of pH on peak force of detachment (b) and work of adhesion (c) of model tablets coated with succinylated L-PEI, phthaylated L-PEI and BSA on porcine gastric mucosa at 37 ± 0.1 °C. Mean $\pm$ standard deviation, n = 20. Mean $\pm$ standard deviation, n = 20. The statistically significant differences are represented as: **** p < 0.0001; *ns* is not significant.

The tablet adhesion studies show a strong correlation exists between the mucoadhesive properties of polyampholytes assessed in solutions (e. g. turbidimetry) and when applied in a solid state (as a tablet coating). In both cases, strong mucoadhesive properties were observed when pH was below the pH_{IEP} of the polyampholyte.

The above studies explored adhesion in essentially "static" systems which poorly reflect fluid dynamics encountered on clinical use. Previously, we have developed a fluorescence microscopy-based flow-through test that provides information on the retention mucoadhesive formulations on animal mucosal tissues [29,55].

The two synthetic polyampholytes and BSA were labelled fluorescently and prepared in solutions for flow through experiments. Retention of FITC-labelled succinylated L-PEI, phthaylated L-PEI and BSA was evaluated on pig gastric mucosa washed with solutions of different pHs. FITC-labelled dextran was used as a negative control due to its poor mucoadhesive properties.

Fluorescence images (Fig. 4e, Figures 8S–9S) were analysed using Image J software and converted into numerical values (Fig. 4a-d) taking fluorescence at time zero as 100 % (with tissue background auto-fluorescence removed).

Throughout 60 min of washing with solutions at pH < pH_{IEP}, there was a statistically significant greater retention of all the polyampholytes compared to FITC-dextran. This is attributed to electrostatic attraction between the positively charged polyampholytes and negatively charged mucins. [56] After 5 min washing, the retention of FITC-succinylated L-PEI, FITC-phthaylated L-PEI and FITC-BSA was 57.4 %, 44.5 % and 72.3 %, respectively, whereas the retention of FITC-dextran was 17.9 % (Table 2S, ESI). Further, 33.6 %, 30.6 % and 43.9 % of FITC-succinylated L-PEI, FITC-phthaylated L-PEI and FITC-BSA, respectively, remained on the tissue after 60 min washing. In contrast, FITC-dextran poorly interacted with the gastric mucosa and, resulting in only 7.3 % of initial

fluorescence remaining after 60 min washing through. It should be noted that it is feasible that this remaining fluorescence may derive from FITC-dextran penetrating into the gastric tissue rather than adhering to the surface. For our new synthetic polyampholytes, these results are in agreement with the peak force of detachment data (Fig. 3b) with the succinylated material showing slightly greater peak force and retention values than the phthaylated derivative. This may be associated with the differences in the degrees of substitution (DS) of the synthetic polyampholytes (see ESI). According to DS data, succinylated L-PEI (DS = 46 %) retains more cationic secondary amine groups than phthaylated L-PEI (DS = 86 %), leading to increased electrostatic interactions with carboxylate groups and ester sulfates within negatively charged mucins.

At pH $\geq$ pH_{IEP}, polyampholytes illustrated significantly better retention compared to FITC-dextran. It could be explained by their polyelectrolyte nature and higher viscosity. However, FITC-BSA showed greater retention throughout the washing study than either FITC-succinylated L-PEI and FITC-phthaylated L-PEI. It is likely that the smaller molecular weight and compact conformation of BSA as a globular protein allows its greater penetration into the gastric mucosa which allows better retention.

It is interesting to note that retention of the negative control FITC-dextran over a wide range of pHs also was found to be pH-dependent with statistically significant differences between the wash-off profiles observed at pHs 4.85 and 6.00 when evaluated at 20 mins (p < 0.05) and at pH 6.00 and 7.71 after 60 min (p < 0.01). Dextran is a non-ionic polysaccharide and so does not carry a pH dependent charge. However, the gastric mucus itself is also affected by pH as gastric mucin undergoes a pH-dependent sol–gel transition, existing in a gel state at acidic pHs, and in a "solution phase" at neutral pH [57]. Physiologically, at lower pH (e.g. pH ~ 2 as *in vivo*), the gel phase mucin acts as a barrier to prevent diffusion of materials back to the tissue surface and indeed
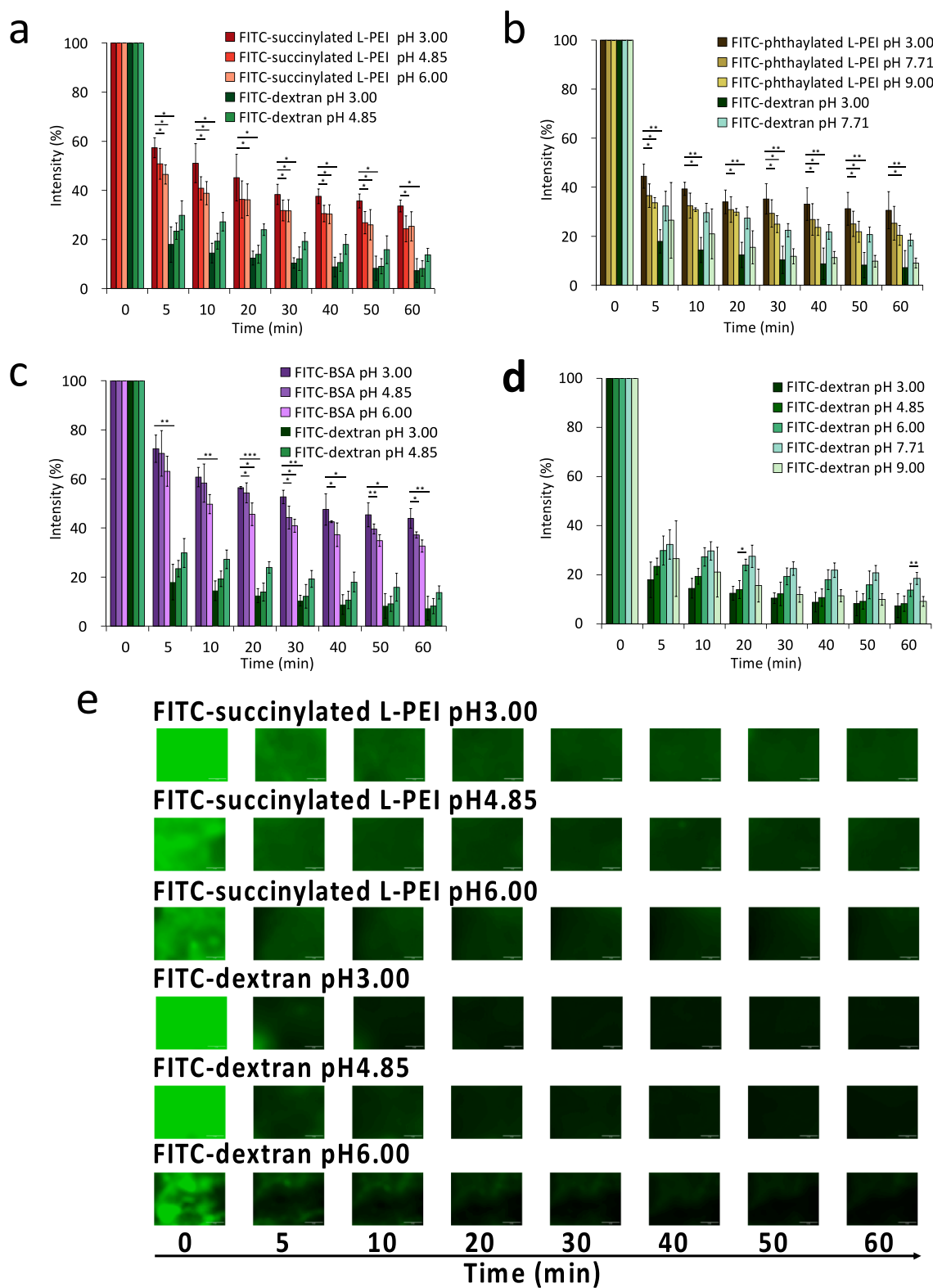
Fig. 4. Effect of pH on mucosal retention of 1 mg/mL FITC-succinylated L-PEI (a), FITC-phthaylated L-PEI (b), FITC-BSA (c) and FITC-dextran (d) on porcine gastric mucosa washed with different volumes of SGF (0.43 mL/min) for 60 min and FITC-dextran as negative control at $37 \pm 0.1\ °C$; exemplar fluorescence images (e) with retention of FITC labelled succinylated L-PEI and FITC-dextran after irrigation with different volumes of SGF under different pH at a flow rate of 0.43 mL/min; FITC-dextran was used as negative control. Scale bars are 2 mm. Mean $\pm$ standard deviation, n = 3. The statistically significant differences are represented as: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns: no significance.

the high viscosity of the gastric mucus gel on the luminal side can prevent stomach HCl from reaching the mucosal tissue. [58] At neutral pH, when the mucin is in a solution state, then FITC-dextran could penetrate into the mucus layer resulting in the highest retention at pH = 7.71.

4. Conclusion

Polyampholytes have attracted lots of attention in the last decades due to their unique physicochemical properties. However, research exploring mucoadhesive properties and mechanisms of polyampholytes is limited. This is the first study that systematically explores the mucoadhesive properties of synthetic and natural polyampholytes. Two new polyampholytes were synthesised by reacting linear polyethylene imine with succinic or phthalic anhydrides. Bovine serum albumin was chosen as a representative of natural polyampholytes. These polyampholytes were used to study mucoadhesive interactions with mucin at different pHs. These materials were also evaluated as model dosage forms (coated tablets and solutions) in terms of their adhesion to and retention on porcine gastric mucosa at different pHs. It was established that pH of solution plays a major role in determining the extend of mucoadhesive interactions and ability of these materials to adhere to mucosal tissue. When the solution pH is below the pH_{IEP} of each polyampholyte, they exhibit strong attractive interactions with mucin and very good mucoadhesive ability towards mucosal tissues, driven predominantly by electrostatic forces with some contributions from chain entanglements and other weak attractions. When the solution pH is around or above the pH_{IEP} the polyampholytes exhibit modest mucoadhesive properties and is attributable to chain entanglement or penetration into the mucus layer. The use of three dissimilar polyampholytes, a broad range of pHs and experimental techniques to evaluate mucoadhesive properties in this study provides confidence that the relationship between solution pH, pH_{IEP} and mucoadhesive performance will be common for all amphoteric polymers. This information is important as it will allow predicting mucoadhesive performance of many amphoteric systems, including commonly used gelatin, many other proteins and also synthetic polymers.

CRediT authorship contribution statement

Manfei Fu: Data curation, Formal analysis, Investigation, Methodology, Visualization. **Sergey K. Filippov:** Formal analysis, Investigation, Writing – review & editing. **Adrian C. Williams:** Supervision, Writing – review & editing. **Vitaliy V. Khutoryanskiy:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vitaliy Khutoryanskiy reports financial support was provided by The Royal Society.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2023.12.176.

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