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Supplementary Information

Title Novel glycopolymer hydrogels as mucosa-mimetic materials to reduce animal testing

Michael T. Cook, Sarah L. Smith, and Vitaliy V. Khutoryanskiy

Materials: D-glucosamine hydrochloride and N-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Alfa Aesar (U.K.). Acryloyl chloride, sodium nitrite, potassium carbonate, 3-(mercaptopropyl)trimethoxysilane (MPTES), 1,4-dioxane, N,N'-methylenebisacrylamide (MBA), 2,2'-azobis(2-methylpropionitrile) (AIBN), fluorescein isothiocyanate (FITC), fluoresceinamine (isomer I), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), phosphate buffered saline tablets (PBS) and low molecular weight chitosan (103 kDa, 85.6 % degree of deacetlyation) were all purchased from Sigma-Aldrich (U.K.). Pectin (54 kDa, 36 % degree of esterification) was a kind gift from Herbstreith and Fox (Germany). 2-Hydroxyethyl methacrylate was a gift from Vista Optics Ltd (Widnes, U.K.), and was purified by vacuum distillation. 2 x 2 cm^2 squares of glass were cut in-house from glass microscopy slides purchased from VWR (U.K.). Dialysis membranes with a 12-14 kDa molecular weight cut-off were purchased from Visking (U.K.). Unless specified, all reagents were used without further purification. Porcine gastric mucosa and bovine corneas were sourced from a local abbatoir (PC Turner, U.K.) on the day of slaughter.

Synthesis of N-acryloyl-D-glucosamine (AGA): The AGA synthesis was modified from Hall et al.[1] Firstly, potassium carbonate (5.53 g) was dissolved in deionised water (20 mL). To this was added D-glucosamine hydrochloride (8.6 g, 40 mmol, 1 eq), with stirring. Once dissolved, sodium nitrite (0.14 g) was added, and the solution cooled to -5 °C using an ice-salt bath.

Acryloyl chloride (6.5 mL, 80 mmol, 2 eq) was then added dropwise, with stirring, ensuring that the temperature of the reaction never exceeded 5 °C. The reaction was maintained at < 5 °C for 3 h, at which point the reaction was allowed to slowly warm to room temperature, and left to proceed overnight. After 24 h, the reaction mixture was precipitated into cold ethanol (200 mL) and filtered, to remove salts. The filtrate was then added slowly into cold diethyl ether (1 L), resulting in the formation of a creamy precipitate. The mixture was kept cool for 48 h, then filtered to yield crude AGA. In order to further purify AGA, the product was redissolved and eluted through Amberlite®, then lyophilised to yield a white solid (5.93 g, 63 % yield). ^{1}H NMR (400 MHz, DMSO-d6, δ): 7.94 (d, 1H, NH), 6.49 (d, 1H, CH), 6.40 (dd, 1H, $R\mathit{H}C{=}CH_2$), 6.08 (dd, 1H, RHC=C*H*H), 5.57 (dd, 1H, RHC=CH*H*), 4.97 (t, 1H, CH), 4.94 (d, 1H, OH), 4.69 (d, 1H, OH), 4.45 (d, 1H, OH), 3.04-3.70 (m, 7H, 5 x CH and 1 x CH_2). ^{13}C NMR (100 MHz, DMSO-d6, δ): 168.74 (C=O), 129.82 (C=C), 129.54 (C=C), 90.79 (C1), 71.53 (C5), 70.64 (C4), 69.99 (C3), 60.52 (C2), 54.12 (C6). FTIR (ATR): ν = 3326 (OH), 3276 (NH). 2911 (CH stretch), 1651 (C=O), 1608 (C=C), 1555 (NH) cm^{-1}; MS (ESI) m/z: [M + H]+ calcd for $C_9H_{15}NO_6$, 234.0899; found, 234.0973.

Synthesis of MPTES-modified glass: 2 x 2 cm^2 squares of glass were cleaned by immersion in NaOH (100 mL, 4 M) for 48 h, followed by rinsing with deionised water, and immersion in HCl (100 mL, 4 M) for a further 48 h. The slides were then rinsed with deionised water, then ethanol, and dried under reduced pressure for 2 h. The clean glass sheets were then placed into a flask containing 1,4-dioxane (200 mL), and MPTES added (4 mL). The reaction was then heated to reflux. After 24 h, the glass sheets were removed from solution, washed with ethanol, then water, and dried under vacuum.

General procedure for the synthesis of glass-bound hydrogels: Monomers (totalling 5.0 mmol) were dissolved in deionised water (2.5 mL), and added to a 20 mL beaker containing MPTES-modified glass. The flask was then sealed with a septum and degassed with nitrogen bubbling (20 min). AIBN (13.5 mg, 82 μmol) and MBA (0.8 mg, 5.2 μmol) were then dissolved in ethanol (2 mL), and degassed with nitrogen bubbling (20 min). The ethanolic solution of AIBN and MBA were then transferred to the solution of monomers and MPTES-modified glass via syringe. The resulting polymerisation mixture was then heated to 60 °C under N_2 and allowed to proceed for 6 h. Glass-bound hydrogels were then removed from the flask and purified by immersion into ethanol (200 mL), which was regularly replenished over the course of 48 h. The glass-bound hydrogels were then placed into deionised water (200 mL), which was regularly replaced over a further 48 h.

Synthesis of glass-bound 100 % HEMA hydrogels: Using the general procedure described above, with the monomer feed mixture comprised solely of HEMA (0.61 mL, 5.0 mmol). FTIR (ATR): ν = 3420 (OH), 2960 (CH stretch), 1715 (C=O, ester), 1450 (CH_2 bend), 1396 (CH_3 bend), 1270, 1247, 1055, 1069, 1020, 949, 943, 899, 871, 854, 749 (fingerprint) cm^{-1}.

Synthesis of glass-bound 20 % AGA hydrogels: Using the general procedure described above, with the monomer feed mixture comprised of HEMA (0.49 mL, 4.0 mmol) and AGA (236 mg, 1.0 mmol). FTIR (ATR): ν = 3420 (OH), 2960 (CH stretch), 1715 (C=O, ester), 1646 (C=O, amide), 1544 (NH), 1450 (CH_2 bend), 1396 (CH_3 bend), 1270, 1247, 1055, 1069, 1020, 949, 943, 899, 871, 854, 749 (fingerprint) cm^{-1}.

Synthesis of glass-bound 30 % AGA hydrogels: Using the general procedure described above, with the monomer feed mixture comprised of HEMA (0.43 mL, 3.5 mmol) and AGA (354 mg, 1.5 mmol). FTIR (ATR): ν = 3420 (OH), 2960 (CH stretch), 1715 (C=O, ester), 1646 (C=O, amide), 1544 (NH), 1450 (CH_2 bend), 1396 (CH_3 bend), 1270, 1247, 1055, 1069, 1020, 949, 943, 899, 871, 854, 749 (fingerprint) cm^{-1}.

Equlibrium swelling degree measurements: Post-purification, a section of hydrogel was removed by scalpel and weighed. These samples were then freeze-dried and reweighed. The equilibrium swelling degree was then calculated according to equation 1.

Equation 1: equilibrium swelling degree = m_{eq}/m_d

Where m_{eq} is the mass at equilbrium swelling, and m_d is the dry mass.

Solid-state NMR: Solid state NMR measurements were conducted on a Bruker Avance III 700 MHz NMR spectrometer. High Resolution Magic Angle Spinning (HR-MAS) ^{1}HNMR experiments were conducted on 20 % and 30 % AGA hydrogels which had been cut from the surface of the MPTES-modified glass, freeze-dried, then reswollen in D_2O. Cross Polarization Magic Angle Spinning (CP-MAS) ^{13}C NMR was conducted on dry, powdered forms of the hydrogels.

Mechanical testing of glass-bound hydrogels: Mechanical testing was conducted on a TA.XTplus texture analyser (Stable Microsystems) with a P\1K steel probe at a rate of 1 mms^{-1}. Hydrogels were compressed by 1 mm, which was converted to a deformation ratio using

the height of the hydrogel, measured using a micrometer. The elastic modulus of the materials was then calculated according to the method described by Guilherme et al.[2]

Synthesis of fluorescein-labelled chitosan:[3] Chitosan (1 g) was dissolved in acetic acid solution (0.1 M, 100 mL). Then, FITC (19 mg) was dissolved in methanol (10 mL). Methanol (100 mL) was then slowly added to the solution of chitosan, with stirring. The methanolic FITC solution prepared previously was then added to the solution of chitosan, dropwise. The reaction was allowed to proceed in the dark for 3 h. The fluorescein-labelled chitosan was then precipitated into a large volume of NaOH (0.1 M, 1 L), filtered, and extensively dialysed until fluorescein was not detectable in the dialysate.

Synthesis of fluorescein-labelled pectin: Pectin (200 mg) was added slowly to PBS (20 mL), with vigorous stirring. After dissolution, EDC (17 mg, 89 µmol, 1 eq) and sulfo-NHS (38 mg, 175 µmol, 2 eq) were added, and the reaction allowed to proceed for 15 min. The pH was then adjusted to 7.0 using NaOH (1 M), and a methanolic solution of fluoresceinamine (31 mg/mL, 1 mL) added dropwise. The reaction was allowed to proceed at room temperature in the dark, for 24 h. Fluorescein-labelled pectin was subsequently purified by extensive dialysis. The purity was confirmed by TLC, using an eluent of ethyl acetate: hexane: acetic acid (40:10:5).

Retention testing: Retention was studied using a flow-through system developed in-house.[4] The system consists of a channel on which a testing substrate (either *ex vivo* mucosa, 'mucosa-mimetic' or PTFE) is placed, over which an eluent can be flowed via a syringe-pump, all of which is contained within an incubator, maintained at 37 °C. Either fluorescein-labelled

chitosan or pectin (1 % (w/v), 20 μL) was then pipetted onto the testing substrate. The testing substrate was then imaged using a Leica MZ10F fluorescence stereomicroscope, equipped with a GFP filter set and monochrome camera, using an exposure time of either 20 or 80 μs for chitosan or pectin, respectively. The eluent was then flowed over the testing substrate (6 mL/min), and subsequent images were taken at 1, 2, 3, 5, 10, and 20 mL elution volume. The quantity of polymer remaining on the surface of the testing substrate was then assessed using ImageJ. Briefly, the region on which the fluorescent polymers were pipetted was selected, and the brightness of the pixels measured. This brightness was then measured at the remaining time points, and the % fluorescence calculated with respect to the starting brightness value. Eluents used were PBS, simulated gastric juice (USP), and simulated tear fluid (0.2 % (w/w) $NaHCO_3$, 0.008 % (w/w) $CaCl_2.2H_2O$, 0.67 % (w/w) NaCl, pH 7.4; described by Marques et al.)[5] Retention data was treated with two-way ANOVA (multiple comparisons), using Bonferroni post-hoc test. $P < 0.05$ was taken to be statistically significant.

Supplementary Figures:

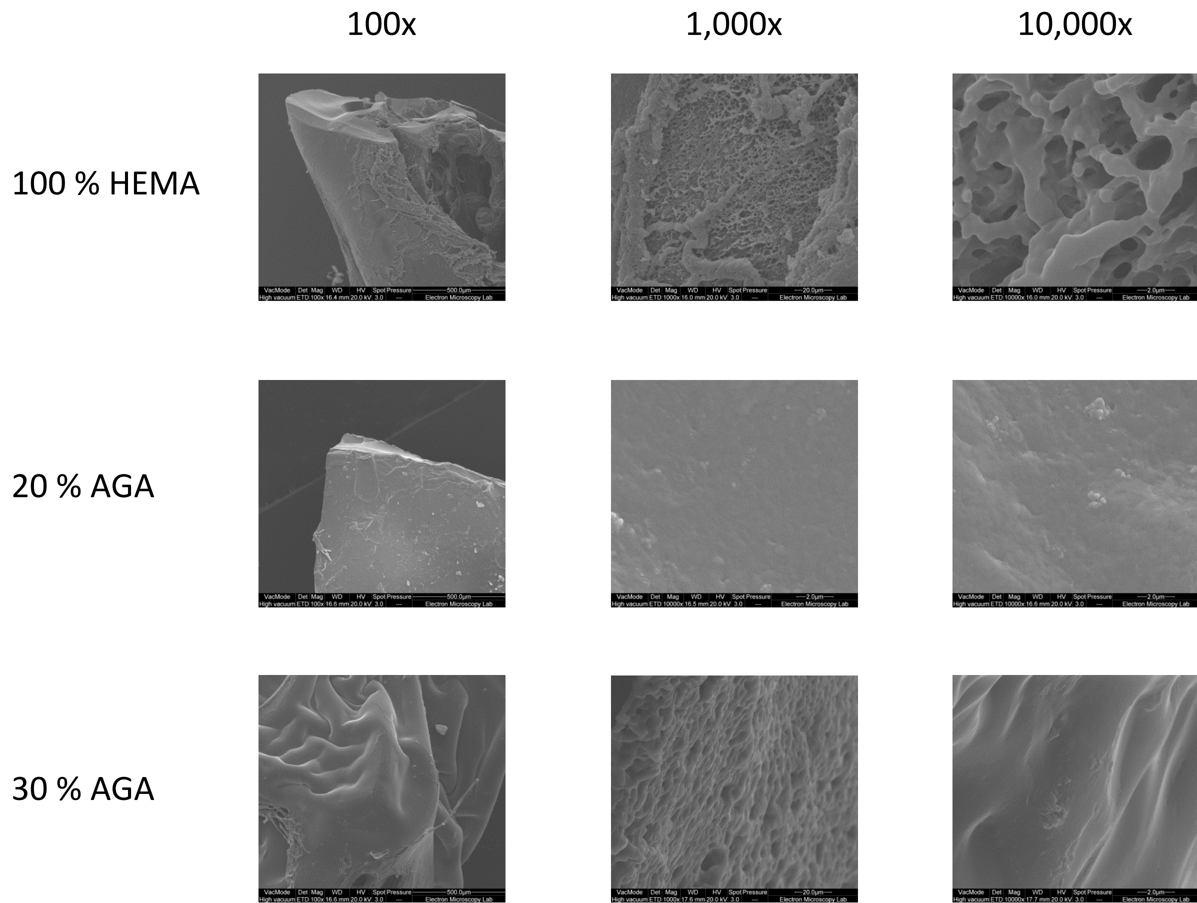

Figure S1. SEM images of glass-bound hydrogels

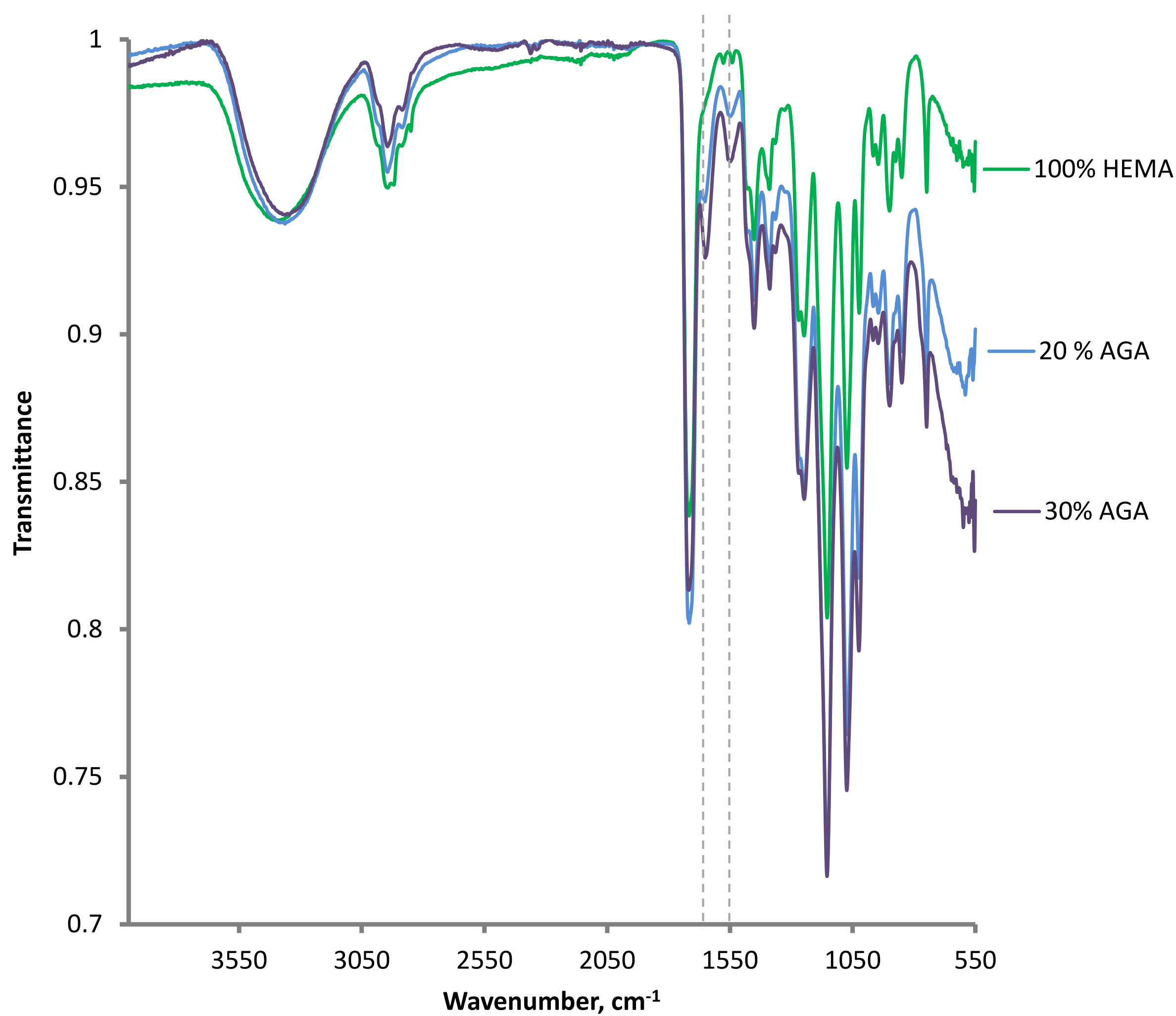

Figure S2: ATR-FTIR of glass-bound hydrogels, confirming incorporation of AGA into the copolymer hydrogels.

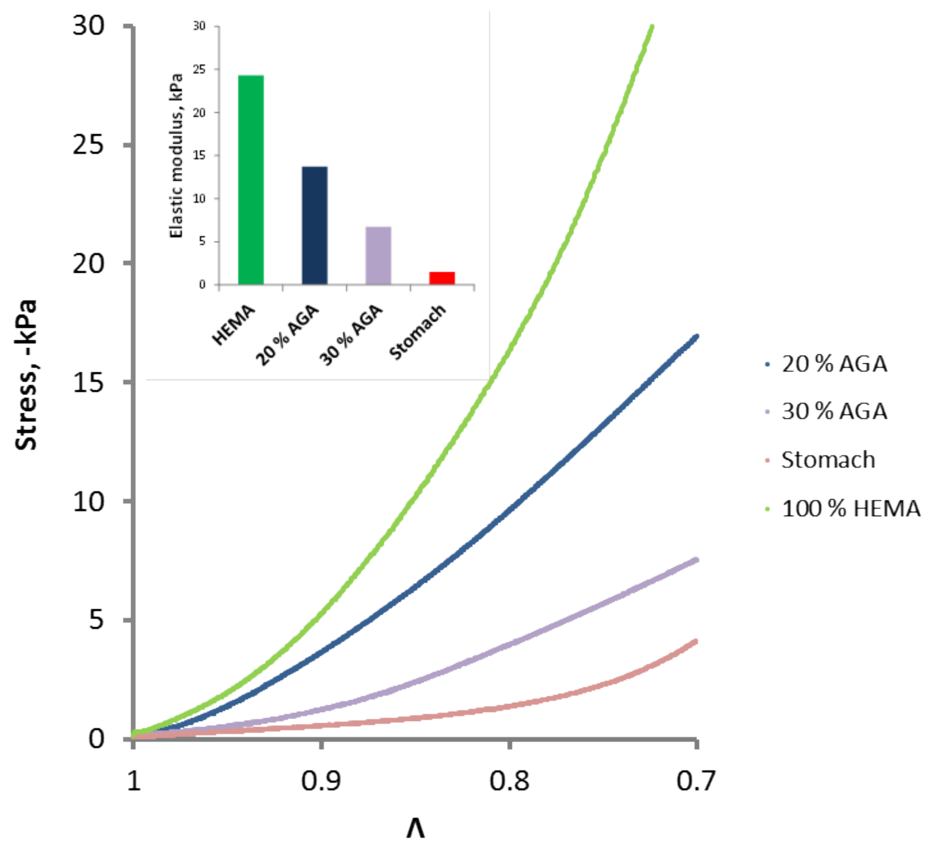

Figure S3. Compression testing of hydrogels and stomach mucosa. Λ is the macroscopic deformation ratio, previously defined.[6]

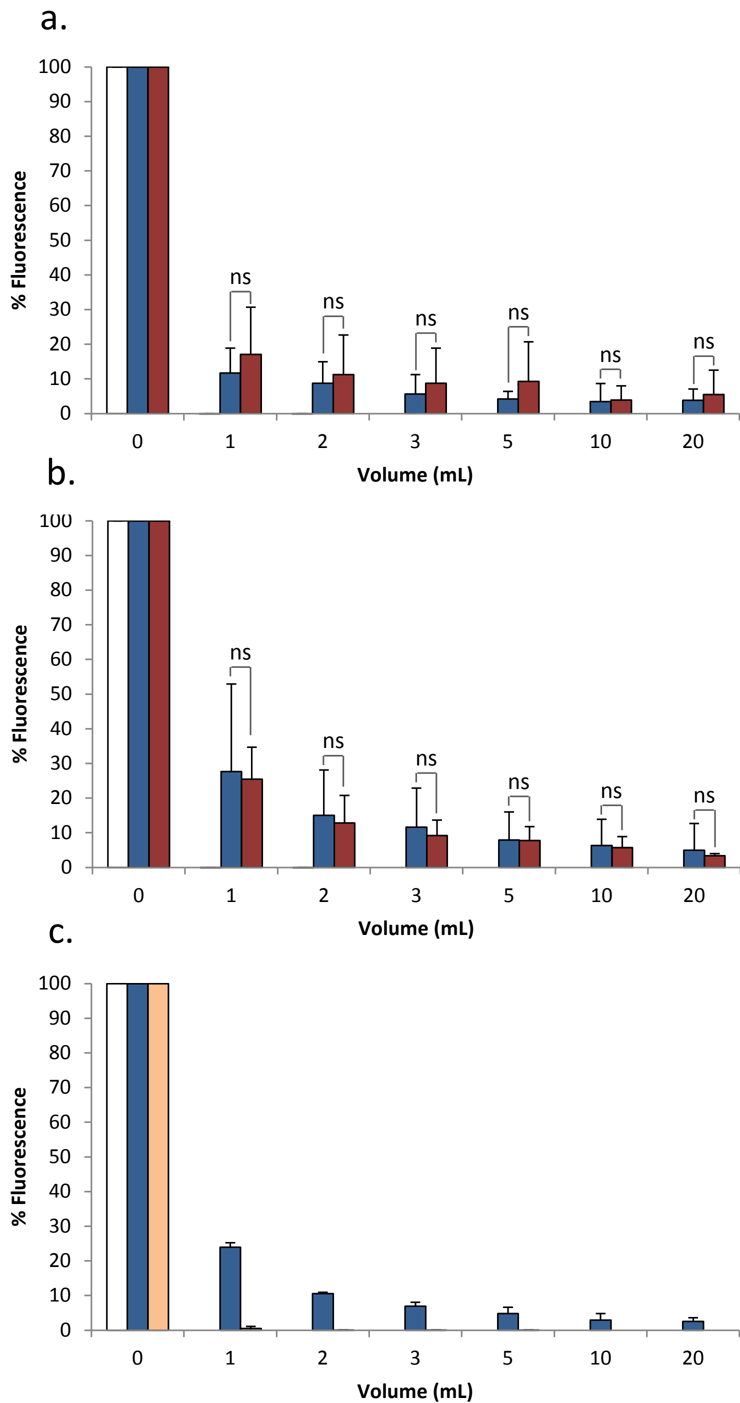

Figure S4. Retention of FITC-dextran (1 mg/mL) on PTFE (white), 20 mol% AGA (blue), porcine gastric mucosa (red), and bovine cornea (orange) during washing with PBS (a), simulated gastric juice (b), and simulated tears (c). 20 mol% HEMA is statistically insignificant from gastric mucosa in both cases ($p < 0.05$), as determined by two-way ANOVA with Bonferroni post-hoc test.

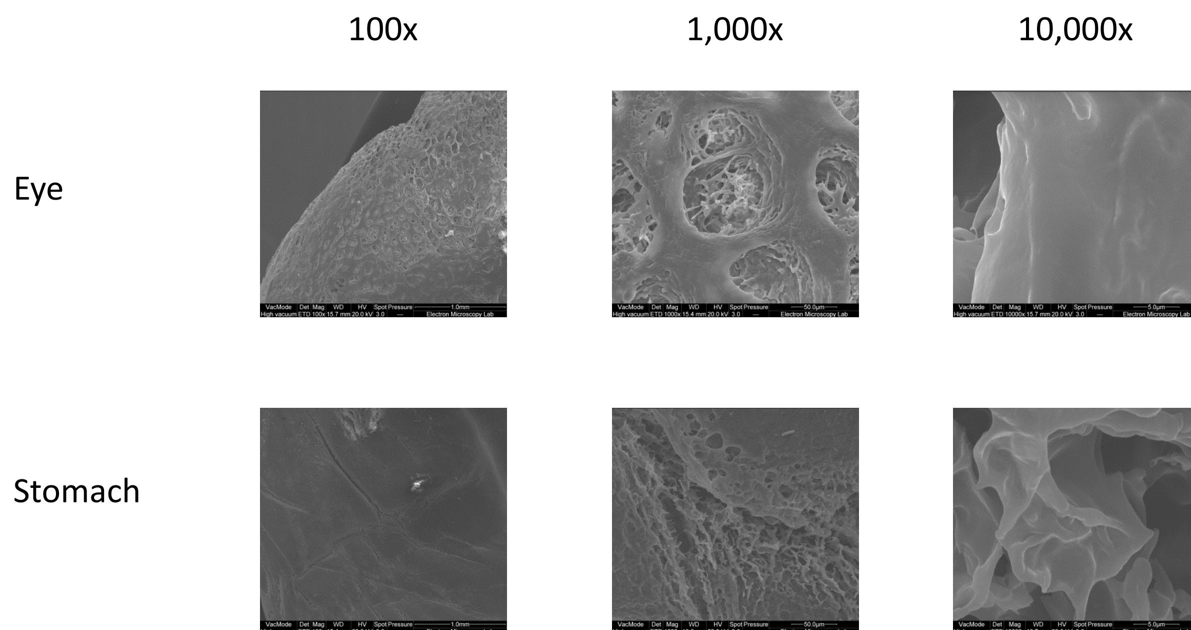

Figure S5. SEM images of mucosal membranes studied in mucoadhesion testing.

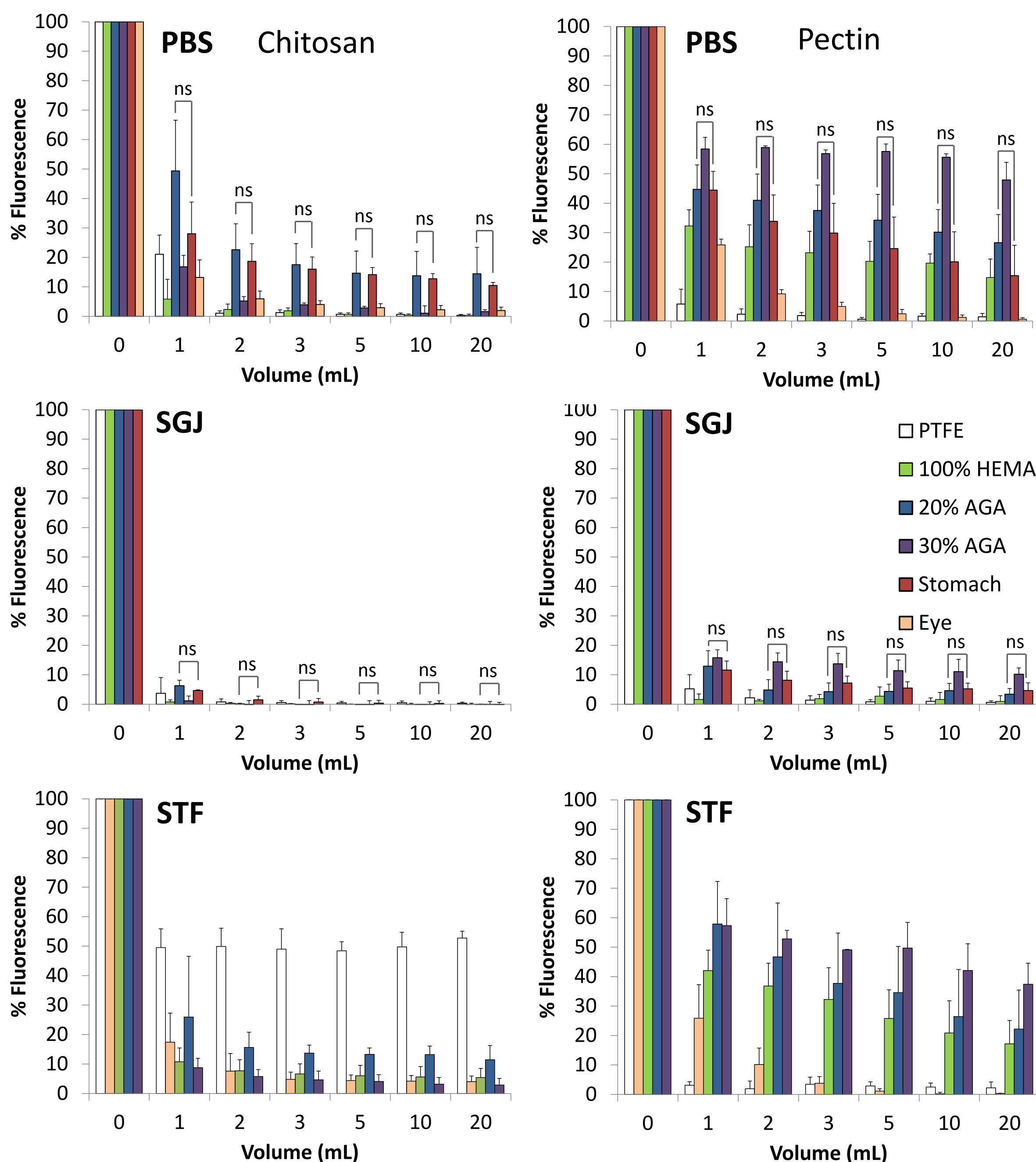

Figure S6: Enlarged version of retention data shown in Figure 3 (main text)

References

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