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Development of maleimide-modified poly(*N*-(2-hydroxylpropyl) methacrylamide) as a novel mucoadhesive polymer for nasal drug delivery

Xiaoning Shan [a,b], Robert Pola [c], Daulet B. Kaldybekov [a,d], Sam Aspinall [e], Fhataheya Buang [f], Adrian C. Williams [a], Tomáš Etrych [c], Vitaliy V. Khutoryanskiy [a,g,*]

[a] *Reading School of Pharmacy, University of Reading, Whiteknights, PO Box 224, Reading RG6 6DX, United Kingdom*
[b] *Small Molecules CMC Drug Product, BeOne Ltd., 30 Science Park Road, Zhongguancun Life Science Park, Changping District, Beijing 102206, China*
[c] *Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Heyrovského nám. 2, Prague 6, Czech Republic*
[d] *Department of Chemistry and Chemical Technology, Al-Farabi Kazakh National University, 050040 Almaty, Kazakhstan*
[e] *Department of Clinical, Pharmaceutical & Biological Sciences, School of Life and Medical Sciences, University of Hertfordshire, Hatfield AL10 9AB, United Kingdom*
[f] *Centre for Drug Delivery Technology and Vaccine (CENTRIC), Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[g] *Physicochemical, Ex Vivo and Invertebrate Tests and Analysis Centre (PEVITAC), University of Reading, Whiteknights, PO Box 224, Reading RG6 6DX, United Kingdom*

A R T I C L E I N F O

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A B S T R A C T

Nasal drug administration is an attractive option for systemic drug delivery, but it is often limited by inadequate nasal drug absorption. In this study, we investigated novel mucoadhesive poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA)-based copolymers functionalised with maleimide moieties as formulations to enhance the retention of dosage forms on the nasal mucosa. PHPMA and its maleimide derivatives were synthesised using controlled radical polymerisation, resulting in polymers with well-defined structures, low dispersity, and variable maleimide functionality. Functionalisation of PHPMA with maleimide groups substantially improved its mucoadhesive properties. Importantly, copolymers with low maleimide content, which were nevertheless sufficient for mucoadhesion, showed no significant cytotoxicity against HEK293 cells and no mucosal irritation in a slug mucosal irritation assay. These PHPMA derivatives demonstrate excellent potential as mucoadhesive materials for developing dosage forms for nasal drug delivery.

1. Introduction

Nasal drug administration has been established as an alternative route for systemic delivery of drugs, restricted to intravenous administration, due to the large nasal surface area, porous endothelial membrane, high total blood flow, the avoidance of first-pass metabolism, and ease of delivery site accessibility [1]. However, drug absorption through the nasal mucosa is generally affected by the physicochemical properties of the drug [2], the nasal mucus layer and mucociliary clearance [3], and the use of absorption enhancers [4]. A major limitation to widespread adoption of nasal drug delivery is inadequate nasal drug absorption.

Several drug delivery systems (DDS), such as microspheres [5–7], liposomes [8–10] and gels [11,12] have demonstrated good bioadhesive characteristics and are able to control the rate of drug clearance from the nasal cavity whilst concomitantly protecting the drug from enzymatic degradation in nasal secretions.

Polymeric mucoadhesive DDS have also been investigated to increase the residence of drug formulations in the nasal cavity, resulting in improved nasal drug absorption. The mucoadhesive properties of polymers can be further enhanced through chemical derivatisation by introducing functional groups capable of rapidly forming covalent bonds with mucins under physiological conditions. Examples of such groups include thiols, which can form disulfide bonds with the thiol groups naturally present in mucins [13,14], and various unsaturated moieties such as acryloyl, methacryloyl, crotonoyl, maleoyl and itaconoyl groups, which can undergo Michael-addition reactions with mucin thiols resulting in covalent binding [15–17]. Further, maleimide groups have been shown to have a significant positive effect on the mucoadhesion performance of some hydrophilic polymers. For example, liposomes

* Corresponding author.
E-mail address: v.khutoryanskiy@reading.ac.uk (V.V. Khutoryanskiy).

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decorated with maleimide-functionalised PEG exhibited superior *in vitro* retention on urinary bladder tissue, due to their ability to form covalent bonds with thiols present on mucosal surface [18]; maleimide-functionalised nanogels exhibited excellent mucoadhesive properties on *ex vivo* conjunctival tissue when compared to the well-known mucoadhesive polymer chitosan [19–21]; maleimide-functionalised chitosan demonstrated superior mucoadhesive properties to the parent chitosan [22]. More recently, Armengol et al [23] reported the synthesis of Eudragit S100 modified with maleimide groups and demonstrated its enhanced mucoadhesive properties, highlighting its potential application as a denture base material.

(*N*-(2-hydroxylpropyl)methacrylamide)-based copolymers (PHPMA) are hydrophilic biocompatible copolymers that have been widely explored as carriers for chemotherapeutic agents, and at least six of PHPMA-based therapeutics have progressed into phase I or phase II clinical trials or compassionate clinical trials [24–26]. In addition, PHPMA is a "mucus-inert" coating material that enhances mucus permeation of nanoparticles [27]. Liu et al. [28] developed PHPMA-coated trimethyl chitosan-based nanoparticles and demonstrated that the PHPMA coating significantly improved the diffusion of these nanocarriers through both human cervicovaginal mucus and the epithelial layer. In contrast, non-coated trimethyl chitosan nanoparticles were found to be less diffusive in both mucus and E12 cells. In another study [29], Liu et al. investigated the effect of molar mass of PHPMA as a ''mucus-inert'' material for overcoming the intestinal absorption barrier and found that the trimethyl chitosan-based nanoparticles coated with PHPMA of lower weight-average molar mass M_w (17 kDa) exhibited the highest stability and excellent permeability across mucus while a coating with high M_w polymer (120 kDa) resulted in premature dissociation of the PHPMA shell and hindrance in mucus. Liu et al. [30] developed PHPMA-coated wheat germ agglutinin-modified lipid-polymer hybrid nanoparticles, co-loaded with silibinin and cryptotanshinone, and showed that PHPMA enhanced nanoparticle mucus penetration through the mucus *in vitro*. Lu et al. [31] modified the surface of mesoporous carbon nanoparticles coated with chitosan and an overlying PHPMA layer and concluded that the mucus-permeable nanocarrier could effectively overcome multiple gastrointestinal absorption barriers; oral bioavailability of drug-loaded nanoparticles was 2.76-fold greater than that of a commercial preparation. Despite these findings, research on the mucoadhesive properties of PHPMA and its derivatives remains limited, with most studies focusing primarily on mucus penetration. It is anticipated that PHPMA will exhibit poor mucoadhesive properties due to its non-ionic nature and ability to permeate through mucus. Although PHPMA is not inherently biodegradable under physiological conditions, it is suitable for nasal administration and will ultimately be eliminated through the gastrointestinal tract.

Here, we employed a novel approach to enhance the mucoadhesive properties of PHPMA by introducing maleimide groups into the polymer's side chains. Liquid formulations based on PHPMA and its maleimide derivatives with sodium fluorescein as a model compound were prepared and their retention on freshly excised sheep nasal mucosa were evaluated using fluorescent microscopy. Additionally, tensile testing was conducted to assess the mucoadhesive properties of these novel materials. The toxicological properties of parent PHPMA and its maleimide derivatives was studied in a HEK293 cell line and through a slug mucosal irritation assay.

2. Materials and methods

2.1. Materials

The monomers *N*-(2-hydroxypropyl)methacrylamide (HPMA) and 3-(3-methacrylamidopropanoyl)thiazolidine-2-thione (Ma-β-Ala-TT) were prepared as described previously [32]. 2,2′-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V70) was obtained from FUJIFILM Wako Pure Chemical Corporation (Japan). (1-cyano-1-methyl-ethyl) benzenecarbodithioate (CTA), *tert*-butyl alcohol, *N,N*-dimethylacetamide (DMA), dimethyl sulfoxide (DMSO), azobisisobutyronitrile (AIBN) and 2-aminoethyl maleimide trifluoroacetate (AEMI) were from Merck (Czech Republic). Acetone and diethyl ether were from Lach-Ner (Czech Republic). *N,N*-diisopropylethylamine (DIPEA) was from Iris Biotech GmbH (Germany). Deuterium oxide (D_2O), sodium fluorescein, glycol chitosan, calcium chloride dihydrate, sodium chloride, potassium chloride and benzalkonium chloride (BAC) were from Sigma-Aldrich (UK). DMEM High Glucose was from Capricorn Scientific (Germany). 10 % fetal calf serum was from GE Healthcare Life Sciences (USA). 1 % penicillin/streptomycin was from Nacalai Tesque Inc (Japan). CellTiter 96 aqueous MTS reagent powder was from Promega Corporation (USA). Phenazine methosulfate was from Thermo Fisher Scientific (USA). Phosphate buffered saline (PBS) tablets were purchased from Fisher Scientific (UK).

2.2. Synthesis of PHPMA functionalised with maleimide groups (PHPMA-Mi)

The copolymer poly(HPMA-*co*-Ma-β-Ala-TT) (PHPMA-TT) was synthesised *via* reversible addition − fragmentation chain transfer (RAFT) copolymerization of HPMA (2 g, 13.97 mmol, 87.5 mol.%) and Ma-β-Ala-TT (515 mg, 2.0 mmol, 12.5 mol.%) using V70 (0.013 mmol, 4.1 mg) as an azo initiator and CTA (0.027 mmol, 5.9 mg) as a chain transfer agent. The molar ratio of monomers/CTA/initiator was 1200:2:1. The polymerisation mixture was dissolved in *tert*-butyl alcohol with 15 % of DMA (22.8 mL, 0.7 M solution of monomers), transferred into a glass ampule, purged with argon and sealed. After 16 h of reaction at 40 °C, the product was diluted with DMSO and isolated by precipitation in acetone/diethyl ether, then washed with diethyl ether and dried under vacuum. To remove dithiobenzoate (DTB) ω-end groups, the resulting copolymer was reacted with AIBN (10 M excess) in DMSO (15 % w/w solution of polymer) under bubbling with argon for 3 h at 70 °C in a sealed ampule. The reaction mixture was then precipitated with acetone/diethyl ether; the precipitate was washed with diethyl ether and dried under vacuum to yield copolymer PHPMA-TT-1. The copolymer PHPMA-TT-2 was synthesized similarly, but with 25 mol.% Ma-β-Ala-TT and with the molar ratio of monomers/CTA/initiator 1600:2:1.

The obtained reactive polymer precursors (PHPMA-TT) were dissolved in DMA, and a 1.1 M excess of 2-aminoethyl maleimide trifluoroacetate (AEMI) relative to the TT (thiazolidine-2-thione) groups was added to the solution; while DIPEA (*N,N*-diisopropylethylamine) was used as the base in a 1.1 M amount. The progress of the reaction was monitored using HPLC and after removing all TT groups from the polymer, the reaction mixture was precipitated in acetone/diethyl ether. The precipitate was then washed with diethyl ether and dried under vacuum to form PHPMA-Mi conjugates.

2.3. Characterization of polymers

2.3.1. Proton nuclear magnetic resonance (^1H NMR)

^1H NMR spectra of the polymers were recorded using a Bruker spectrometer operating at 250 MHz using DMSO or D_2O (15 mg/mL) as the solvent. All chemical shifts are given in ppm. MestReNova software was used for the analysis of spectra.

2.3.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on a Nicolet iS5 spectrometer equipped with a diamond attenuated total reflection (ATR) accessory. After a background scan was collected, samples were placed on the ATR crystal and scanned from between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1} and an average of 64 scans. The OMNIC software was used for spectral analysis.

2.3.3. Size exclusion chromatography (SEC) and UV–vis

The molecular weights and polydispersity of polymers were measured by size exclusion chromatography (SEC) on a HPLC system (Shimadzu, Japan) equipped with UV, differential refractive index and multi-angle light scattering detectors (Wyatt Technology Corp., USA) using TSKgel G4000 SWXL, (Tosoh Bioscience, Japan) (80 % methanol, 20 % 0.3 M acetate buffer pH 6.5) at a flow rate of 0.5 mL/min. The calculation of molecular weights from the light-scattering intensity was based on the known injected mass, assuming 100 % mass recovery. The content of the thiazolidine-2-thione (TT) groups was determined spectrophotometrically on a Helios Alpha UV–vis spectrophotometer (Thermospectronic, UK) using the absorption coefficients in methanol for TT (ε_{305} = 10 300 L mol^{-1} cm^{-1}).

2.3.4. Calculation of the degree of PHPMA modification with maleimide groups

The degree of polymer modification was calculated from ^{1}H NMR spectra of PHPMA-Mi in D_2O based on the integrated areas (*I*) of –CH signals of the HPMA monomer and the –CH═CH– signals of maleimide moieties, as displayed in the following equations:

$$\frac{I[\mathrm{CH=CH}]}{2y} = \frac{I[\mathrm{CH}]}{x} \quad (1)$$

$$Maleimide(\%) = \frac{y}{x+y} \quad (2)$$

where $I_{[CH=CH]}$ is the integrated area of –CH═CH– of maleimide moieties, $I_{[CH]}$ is the integrated area of –CH of HPMA moieties, *x* is the number of repeating units of HPMA moieties, *y* is the number of repeating units of maleimide moieties.

Then the equation (2) was modified to:

$$Maleimide(\%) = \frac{I[\mathrm{CH=CH}]}{2{*}I[\mathrm{CH}] + I[\mathrm{CH=CH}]} \times 100 \quad (3)$$

2.4. In vitro nasal mucoadhesion studies

2.4.1. Preparation of polymer/fluorescein sodium mixtures and artificial nasal fluid

Sodium fluorescein solutions (0.05 mg/mL) were prepared in deionised water into which polymer samples were dissolved; 10 mg of either PHPMA, PHPMA-Mi, or glycol chitosan were dispersed in 10 mL of the sodium fluorescein solution and pH was adjusted to 5.70. The mixtures were stirred at room temperature for 24 h until complete dissolution, and were covered with aluminum foil to protect from the light.

Artificial nasal fluid (ANF) was prepared according to the established protocol [33,34] by dissolving 7.45 g NaCl, 1.29 g KCl and 0.32 g $CaCl_2{\cdot}2H_2O$ in 1000 mL of deionised water. The solution was left stirring overnight at room temperature. The artificial nasal fluid was kept at 37 °C in a water bath throughout the experiments.

2.4.2. Fluorescence retention studies on nasal mucosa

Sheep heads were obtained from P.C. Turner Abattoir (Farnborough, UK) and transported to the laboratory in a cold box (3–4 °C). The nasal septum tissue with mucosal lining (1.5 × 1 cm) was carefully dissected and extracted from each head with scissors, washed with 1 mL of ANF and placed on a microscope slide. All tissues were used within 24 h of animal slaughter.

All experiments assessing retention of formulations on nasal mucosa were conducted at 37 °C in an incubator. Images of mucosal surfaces were taken using a fluorescence stereo microscope (MZ10F, Leica Microsystems, UK), equipped with an GFP filter and a Zeiss Imager A1/AxioCam MRm camera. All images were at 0.8 × magnification with a 211 ms exposure time. Initially, fluorescence images of mucosal tissues were recorded for each sample to collect background fluorescence intensity. Then, 20 µL solution of 1 mg/mL either PHPMA, PHPMA-Mi or glycol chitosan containing 0.05 mg/mL sodium fluorescein was placed on the mucosal surface and fluorescence images were recorded. After 3 min of dosing, the mucosal tissues were transferred to the incubator and irrigated with ANF using a syringe pump at 0.43 mL/min. Fluorescence images of the mucosal tissue were collected periodically and analyzed using ImageJ software to measure pixel intensity after each wash. Results are presented as fluorescence intensity versus the time of irrigation after subtracting the background fluorescence from each wash image. Sodium fluorescein solution (0.05 mg/mL) in deionised water was used as a negative control and glycol chitosan solution (1 mg/mL) was used as a positive mucoadhesive control. The experiments were conducted in triplicate.

2.4.3. Mucoadhesive properties studied using tensile testing

Tensile testing was performed on a TA.XT plus texture analyser (Stable Mirco Systems, UK) where nasal tissue (4 cm^2) was incubated to 37 °C before being placed on a platform (24 mm opening) that was surrounded by water at 37 °C to maintain the tissue temperature during the test. Each sample was prepared by soaking a filter paper (Ø 15 mm) in polymer solution (3 mg/mL) using ANF as the solvent for 30 s before drying in a vacuum oven at 25 °C for 20 mins to obtain a dry polymer coated filter paper, which was then attached to the probe *via* a carbon tab (12 mm). The contact time between the probe and the tissue was 30 s with 0.98 N (100 g) of force before pulling apart with a removal speed of 1 mm/s. All samples were tested in triplicate. T.A. Exponent software was used to record the area under the force versus distance curves (work of adhesion) as well as the force of adhesion/adhesive strength which is the maximum force needed to detach tissue from the polymer coated filter paper.

2.5. Biocompatibility studies

2.5.1. Cytotoxicity studies

HEK293 was cultured in DMEM High Glucose supplemented with 10 % fetal calf serum and 1 % penicillin/streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2. Cell viability was assessed using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS assay). Cells were seeded in 96-well plates at 3 × 10^3 cells/well and incubated overnight at 37 °C in 5 % CO_2 with humidified air for cell attachment. The cells were then treated with various concentrations of the polymers (25, 50, 75, 100, 125 and 150 µg/mL) for 72 h. The negative control group consisted of untreated cells and was considered as 100 % viable cells. After 72 h, treatment media were replaced with new growth media and 20 µL MTS solution (prepared in phosphate buffered saline) containing 2 mg/mL of CellTiter 96 Aqueous MTS reagent powder and 0.92 mg/mL of phenazine methosulfate. The cells were incubated for a further 4 h before absorbance (Abs) was measured at 490 nm using an Infinite 200 PRO microplate reader (Tecan Group Ltd, Switzerland). The results are expressed as percentage of cell viability compared to the negative control group using the following equation:

$$Cell\ viability(\%) = \frac{(\mathrm{Abs_{Treatment}} - \mathrm{Abs_{Blank}})}{(\mathrm{Abs_{Control}} - \mathrm{Abs_{Blank}})} \times 100 \quad (4)$$

2.5.2. Slug mucosal irritation assay

The slug mucosal irritation (SMI) assay was performed according to our previously published reports [35,36]. *Arion lusitanicus* slugs were collected locally in the Harris Garden (Reading, UK) and were housed in plastic containers and fed with lettuce, cabbage, and cucumber. Each slug's body lining was carefully inspected and only slugs showing no evidence of macroscopic injuries with clear tubercles and foot surface were used for testing purposes. Slugs weighing between 6 and 20 g were isolated from the colony and placed individually in 1 L glass beakers lined with a paper towel moistened with 20 mL of phosphate buffered

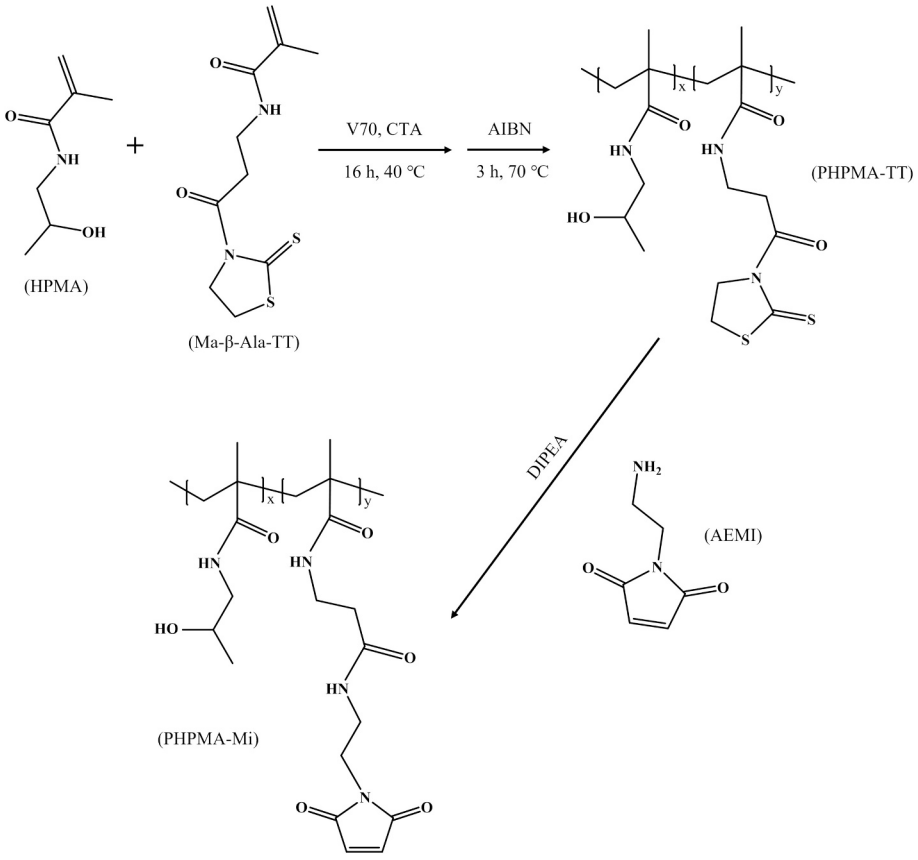


Fig. 1. Synthesis of PHPMA-Mi conjugates.

saline (PBS, pH 7.40). They were kept at room temperature for 48 h before the start of an experiment. All beakers were covered with cling film pierced with small holes to allow air exchange. Each slug was individually weighed before the experiment and then placed in 90 mm plastic Petri dishes lined with Whatman filter paper moistened with either positive/negative controls (2 mL of 1 % BAC prepared in PBS and 2 mL of PBS solution, respectively) or 2 mL of each test materials (PHPMA, PHPMA-Mi) prepared in PBS at 1, 2 or 3 mg/mL. After a 60-min contact period, slugs were taken out, rinsed with 10 mL of PBS, gently wiped with a paper towel, and then reweighed. The mucus production (MP) was estimated as a slug body weight loss and calculated using the following equation:

$$MP = \frac{(m_b - m_a)}{m_b} \times 100\% \quad (5)$$

where m_b and m_a are the weights of a slug before and after experiment, respectively. Each experiment was repeated 5 times using different slugs and the results are presented as mean ± standard deviation.

2.6. Statistical analysis

All experiments were conducted at least in triplicate and data expressed as mean ± standard deviation with the probability of $p \leq 0.05$ considered as significant. GraphPad Prism statistical analysis software (version 7.0) was used to analyze data using one-way analysis of variance ANOVA and paired *t*-tests.

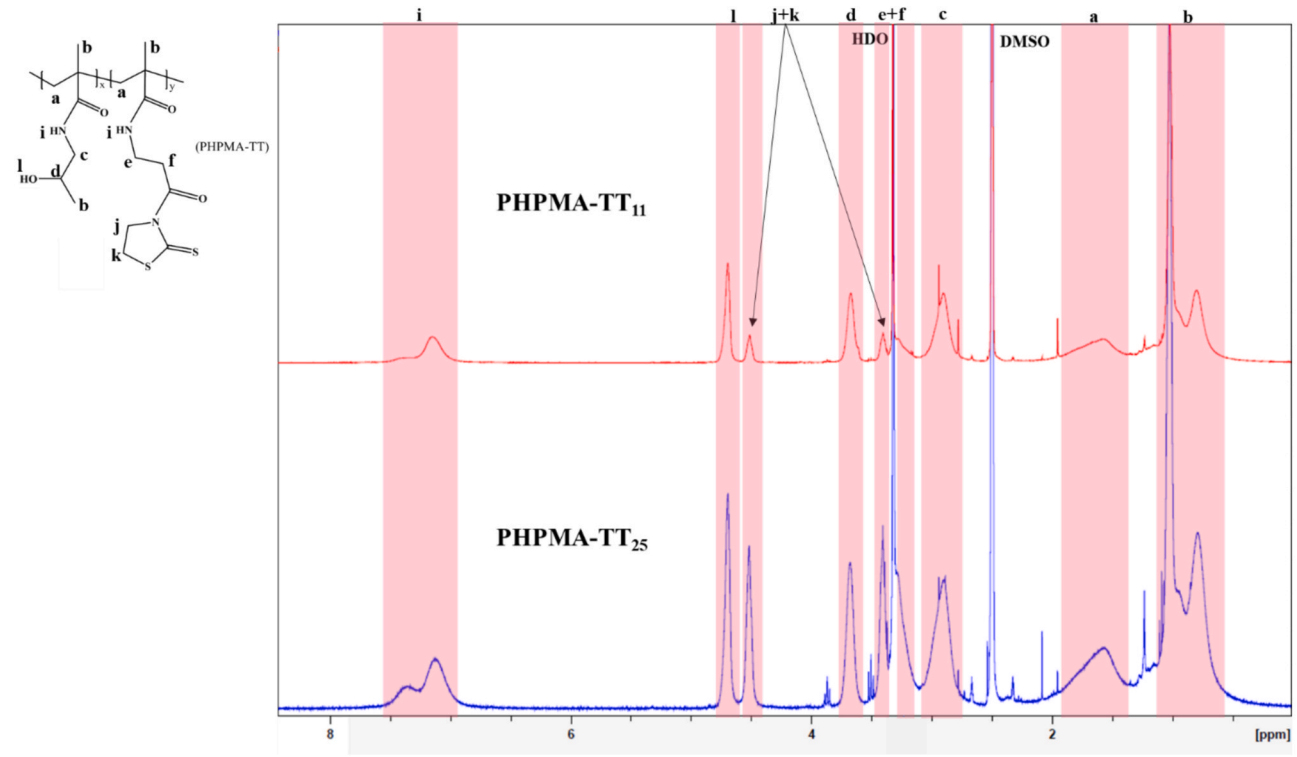
3. Results and discussion

3.1. Synthesis and characterisation of PHPMA-Mi conjugates

PHPMA-Mi conjugates were synthesised by a two-step procedure. First, RAFT copolymerisation of HPMA with Ma-β-Ala-TT was performed to prepare PHPMA-TT copolymers. Ma-β-Ala-TT was selected as a comonomer because it contains a thiazolidine-2-thione group, which acts as an efficient reactive handle for post-polymerisation modification. This allows for facile and quantitative substitution of the thiazolidine-2-thione groups with nucleophilic compounds such as AEMI to introduce maleimide functionalities in the second step (Fig. 1). Moreover, the main chain end terminating CTA were removed by the reaction with AIBN to avoid any side reaction both in the following synthetic steps or polymer evaluation. The removal of the CTA had no effect on the molecular weight and dispersity. Both polymer precursors were characterized by SEC and ^{1}H NMR in DMSO due to insolubility of the polymer precursors with high amount of TT groups in water. The resultant PHPMA-Mi copolymers were characterised using ^{1}H NMR, FTIR and SEC.

The ^{1}H NMR spectrum of PHPMA showed signals which were in agreement with the published literature [37]: δ (ppm) 3.82 (peak *d*, CH of PHPMA side chain), 3.19–2.90 (peak *c*, CH_2 of PHPMA side chain), 1.99–1.51 (peak *a*, CH_2 of PHPMA backbone), 1.17–0.74 (peak *b*, CH_3 of PHPMA backbone and CH_3 of PHPMA side chain). Following the introduction of maleimide groups, new signals appeared at δ (ppm) 3.70–3.18 (peak *e* and *g*), 2.30 (peak *f*) and 6.81 (peak *h*) corresponding to $–CH_2$ attached to amines, $–CH_2$ attached to the carbonyl groups, and –CH═CH– of maleimide moieties, respectively. The signal at δ (ppm) 7.56 (peak *i*) was attributed to the NH group in the copolymer side chain (Fig. 2). This signal was too weak to be discerned in the PHPMA spectrum, likely due to the lower number of NH groups in the PHPMA homopolymer compared to the PHPMA-Mi copolymers. The ^{1}H NMR spectra of PHPMA-Mi also confirmed the absence of TT group signals, indicating complete substitution of TT groups by maleimide groups, which were further analysed by HPLC equipped with UV detection (**Fig. S1**).

The degree of functionalisation of the polymers with maleimide



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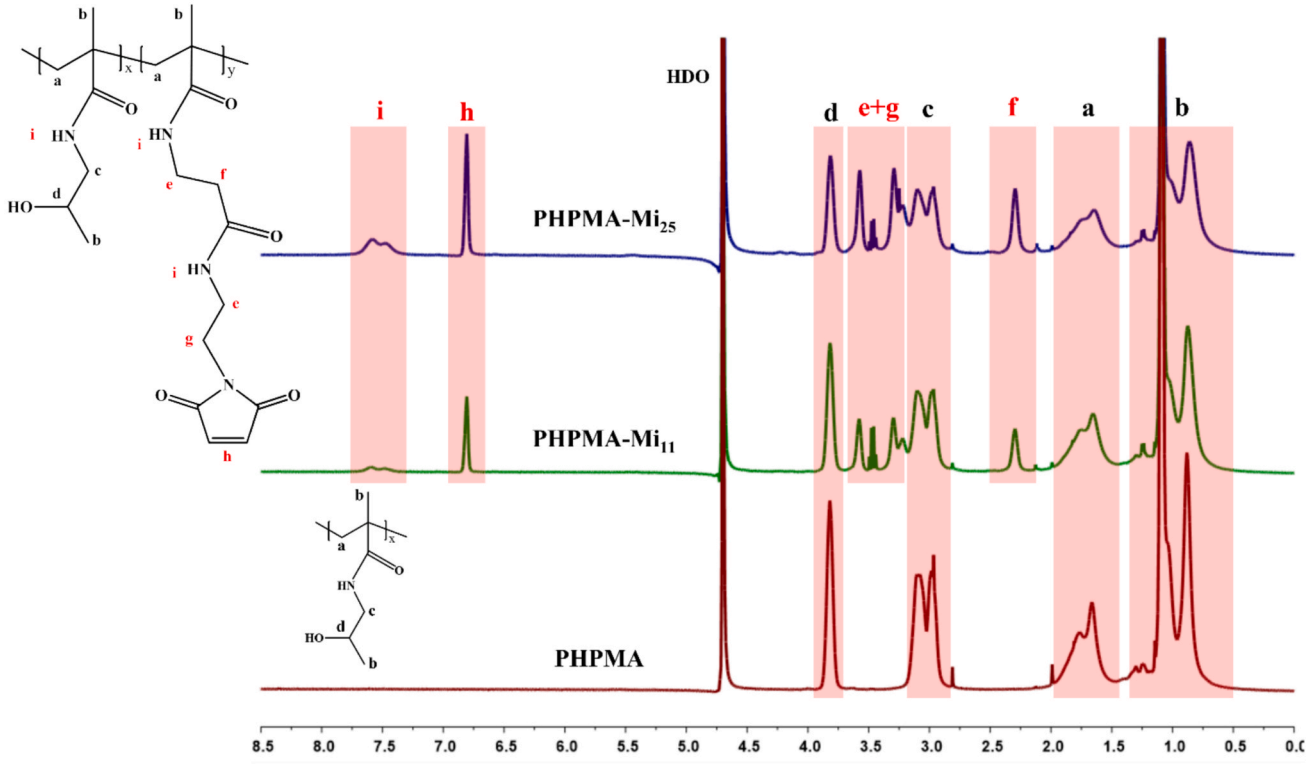

Fig. 2. ^{1}H NMR spectra of A – polymer precursors PHPMA-TT recorded in DMSO and B – PHPMA and PHPMA-Mi recorded in D_2O.

Table 1
Characterisation of polymers.

Products	M_n^a, kDa	M_w/M_n^a	Content of TT[b]/maleimide[c] groups (mol.%)
PHPMA	69.2	1.08	0
PHPMA-TT_{11}	67.4	1.06	11.5[b]
PHPMA-TT_{25}	65.6	1.08	25.2[b]
PHPMA-Mi_{11}	67.6	1.10	11[c]
PHPMA-Mi_{25}	63.2	1.14	25[c]

[a]Molecular weights and polydispersity were determined using SEC with RI and LS detection.
[b] TT groups content was determined by UV–vis spectrophotometer.
[c] Maleimide content was determined using ^{1}H NMR.

groups was evaluated from ^{1}H NMR spectra of PHPMA-Mi in D_2O based on the integral values (I) of –CH signals of HPMA monomer and –CH═CH– signals from the maleimide moieties, as detailed in Table 1. The maleimide substitution levels were found to be 11 % and 25 % following the reaction with the reactive polymer precursors PHPMA-TT-1 and PHPMA-TT-2, respectively. For clarity, the maleimide-functionalised PHPMA samples are annotated according to their Mi content (i.e., PHPMA-Mi_{11} contains 11 % maleimide groups and was synthesised from PHPMA-TT_{11}). The polymers have low dispersity (1.10 for PHPMA-Mi_{11} and 1.14 for PHPMA-Mi_{25}) as demonstrated by SEC (**Fig. S2**).

The FTIR spectrum of PHPMA shows the following peaks: 3337 cm^{-1} (N–H and O–H stretch); 2970 and 2923 cm^{-1} (alkyl C–H stretch); 1640 cm^{-1} (amide C═O stretch); 1528 cm^{-1} (N–H bend); 1443 cm^{-1} (alkane); and 1200 cm^{-1} (C–O stretch). The successful modification of PHPMA with maleimide groups to form PHPMA-Mi is evidenced not only by the enhanced N–H and C–H stretch but also by strong features appearing at 1705 cm^{-1} (C═C stretch), 831 cm^{-1} (═C–H bend) and 696 cm^{-1} (═C–H bend), and the signals were significantly strengthened with the higher content of maleimide groups (Fig. 3).

3.2. In vitro nasal mucoadhesion studies

The mucoadhesive properties of PHPMA, PHPMA-Mi_{11} and PHPMA-Mi_{25} solutions containing sodium fluorescein were studied on freshly excised sheep nasal mucosa, irrigated with ANF. In these experiments, sodium fluoresceine was used as a fluorescent model drug to assess the ability of the polymers to enhance its retention on the nasal mucosa. Glycol chitosan was included as a mucoadhesive positive control, while sodium fluorescein alone served as the negative control. Fig. 4 shows the retention of sodium fluorescein mediated with glycol chitosan, PHPMA, PHPMA-Mi_{11} and PHPMA-Mi_{25} on sheep nasal mucosa. Numerical values from these experiments are summarised in **Table S1**.

As expected, parent PHPMA exhibited relatively poor mucoadhesive properties as only ~7.1 % of fluorescence remained on nasal mucosa after 60 min washing which was similar to that for sodium fluorescein (Fig. 4b). PHPMA conjugates with the greater amount of maleimide groups resulted in better retention after each wash. For example, the retention values of PHPMA, PHPMA-Mi_{11} and PHPMA-Mi_{25} after 5 min washing were approximately 20.5 %, 34.6 % and 42.2 %, respectively, calculated based on the fluorescence intensity. No significant difference in fluorescence retention was observed between PHPMA-Mi_{25} and glycol chitosan, indicating the excellent mucoadhesive performance of PHPMA-Mi_{25}. Additionally, PHPMA-Mi_{11} demonstrated significantly better mucoadhesive properties compared to the parent PHPMA at all time points ($p < 0.005$). The superior mucoadhesive properties of PHPMA-Mi are likely due to the high reactivity of maleimide groups towards thiol groups present in cysteine residues on the mucosal membrane and the formation of covalent bonds *via* Michael-type addition reactions [22,38]. Consequently, the polymers with higher maleimide content (PHPMA-Mi_{25}) were anticipated to show greater adhesion compared to the polymers with a lower maleimide content (PHPMA-Mi_{11}).

A comparison of these retention data with previously reported results for similarly designed formulations shows broad agreement. For instance, Porfiryeva *et al.* [34] investigated the retention of sodium fluorescein formulated with cationic Eudragit EPO and its acryloylated

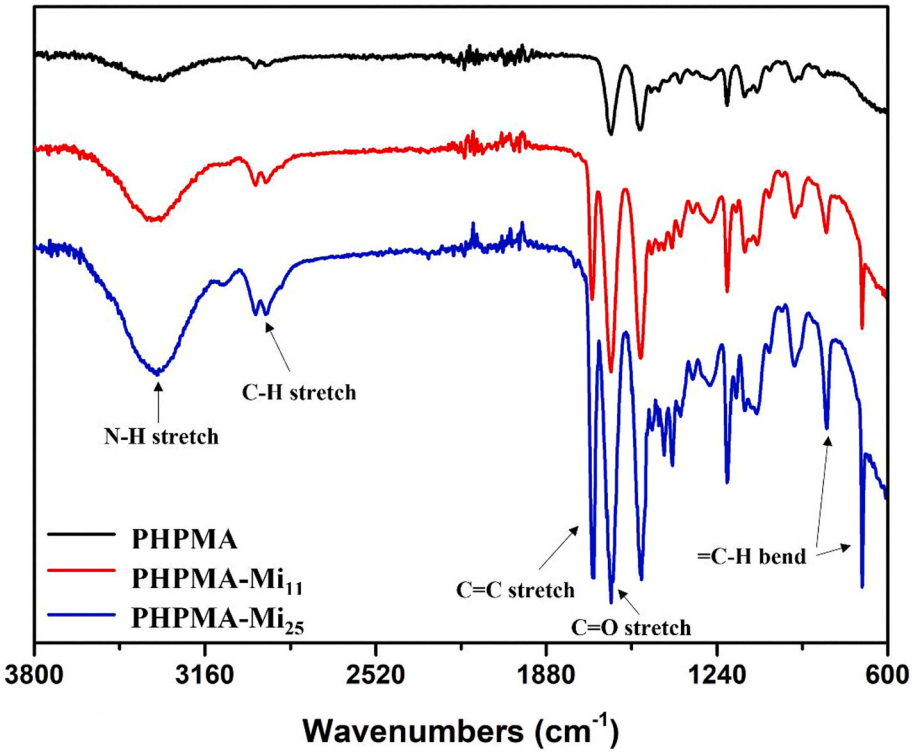

Fig. 3. FTIR spectra of PHPMA and PHPMA-Mi.

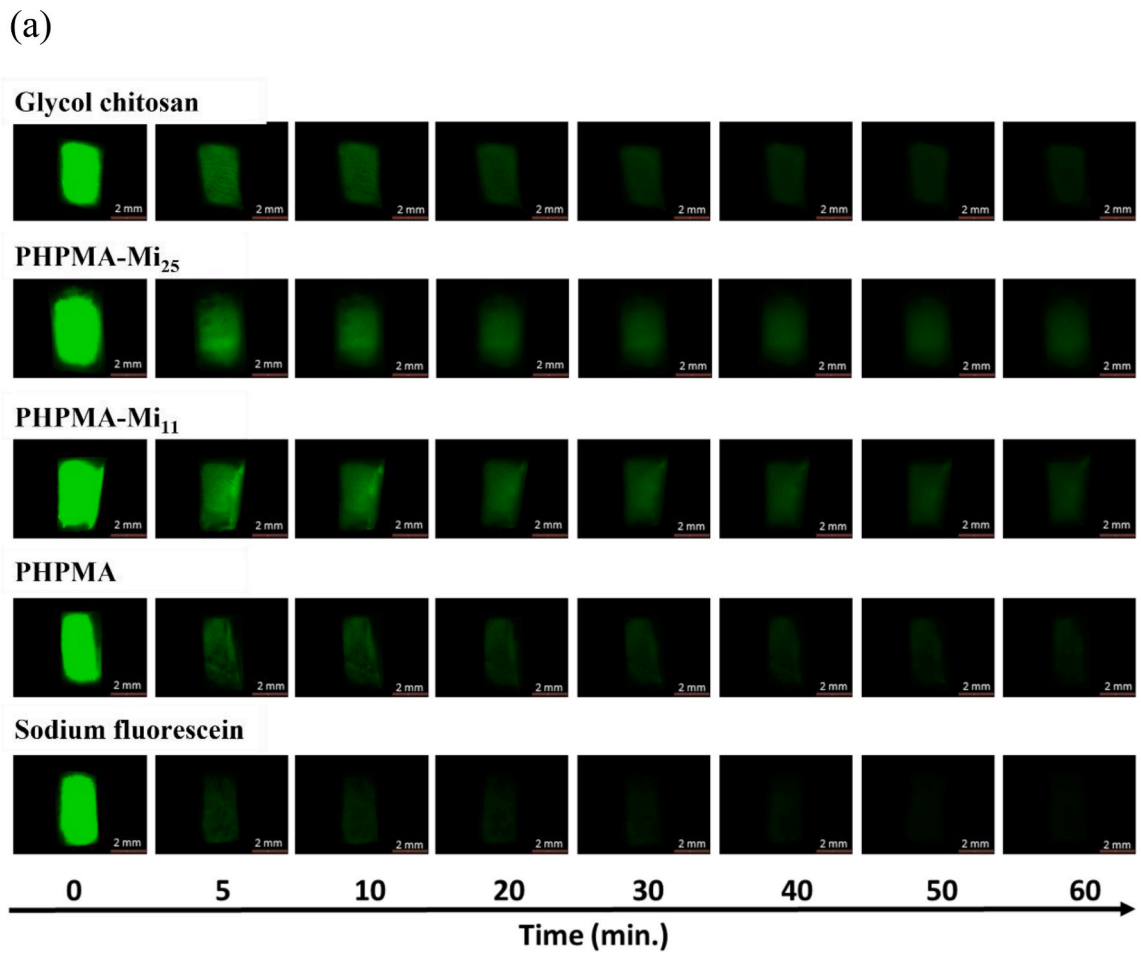

Fig. 4. (a) Fluorescence images showing retention of 1 mg/mL glycol chitosan, PHPMA, PHPMA-Mi$_{25}$, PHPMA-Mi$_{11}$ solutions containing 0.05 mg/mL sodium fluorescein, and pure 0.05 mg/mL sodium fluorescein solution on sheep nasal mucosa and washed with ANF. Scale bar is 2 mm. (b) Retention of 1 mg/mL glycol chitosan, PHPMA, PHPMA-Mi$_{11}$, PHPMA-Mi$_{25}$ solutions containing 0.05 mg/mL sodium fluorescein and pure 0.05 mg/mL sodium fluorescein solution on sheep nasal mucosa as washed with different volumes of ANF (pH = 5.7, n = 3, mean ± SD, "*" represents $p < 0.05$).

derivatives, using a comparable fluorescence-based flow-through method on *ex vivo* sheep nasal mucosa. The retention profiles of formulations incorporating maleimide-functionalised PHPMA were similar to those observed for formulations containing acryloylated Eudragit EPO.

Tensile testing was also used to investigate the mucoadhesive properties of polymer samples. The force of detachment or adhesive strength indicates the force required to overcome the adhesive bonds formed between the sample and nasal mucosa, while the work of adhesion is the area under the force-distance curves. Dextran was used as a negative control due to its poor mucoadhesive properties [39]. The work of adhesion values showed that PHPMA-Mi$_{25}$ was statistically more mucoadhesive than PHPMA ($p < 0.05$) and PHPMA-Mi$_{11}$ ($p < 0.05$) (Fig. 5b), albeit PHPMA-Mi$_{11}$ and PHPMA-Mi$_{25}$ displayed similar force of detachment (Fig. 5a). Overall, the adhesive strength of the polymers correlated well with their work of adhesion as M$_{25}$PHPMA exhibited greater force of detachment and work of adhesion relative to the parent PHPMA. This is in good agreement with the fluorescence retention studies on nasal mucosa.

3.3. Toxicological studies

The HEK293 cell growth inhibitory effect of the copolymers was studied over 72 h (Fig. 6). Cytotoxicity was expressed as mean values and are summarised in **Table S2**. Previously, the toxicity of PHPMA has been tested in other cell lines such as HeLa, L-cells and WI-38, and none of the tested cell lines showed any cytotoxic effects [40]. Similarly, in this study, PHPMA had no cytotoxic effect on the viability of HEK293 cells even at high concentrations (>100 μg/mL). It could be seen that 11 mol.% maleimide-functionalised copolymer (PHPMA-Mi$_{11}$) showed no significant toxicity even at high concentrations (150 μg/mL). In contrast, 25 mol.% maleimide-functionalised copolymer (PHPMA-Mi$_{25}$) was more toxic compared to parent PHPMA, probably due to the maleimide groups acting as a reactive oxygen species (ROS)-scavenging inhibitor. The maleimide groups are able to deactivate intracellular glutathione and cysteine, thus increasing cytotoxicity [41,42]. Nevertheless, cell viability remained above 70 % when exposed to PHPMA-Mi$_{25}$, meeting the threshold set by the International Organisation for Standardisation (2009), which considers viability above this level to be non-toxic. It should also be noted that when these formulations are applied *in vivo* these will not remain in contact with the nasal mucosa for extended times such as the 72 h exposure used in these studies, hence mitigating potential toxic effects.

The mucosal irritancy of PHPMA and PHPMA-Mi conjugates was tested in *Arion lusitanicus* slugs. Fig. 7 presents the results of mucus production by slugs exposed to a filter paper soaked in solutions of PHPMA and PHPMA-Mi conjugates prepared in PBS as well as the positive and negative controls. In experiments with 1 % solution of BAC in PBS (pH 7.35), used as a positive control, slugs experienced a severe irritation, producing approximately 36 ± 5 % of yellow mucus, whereas

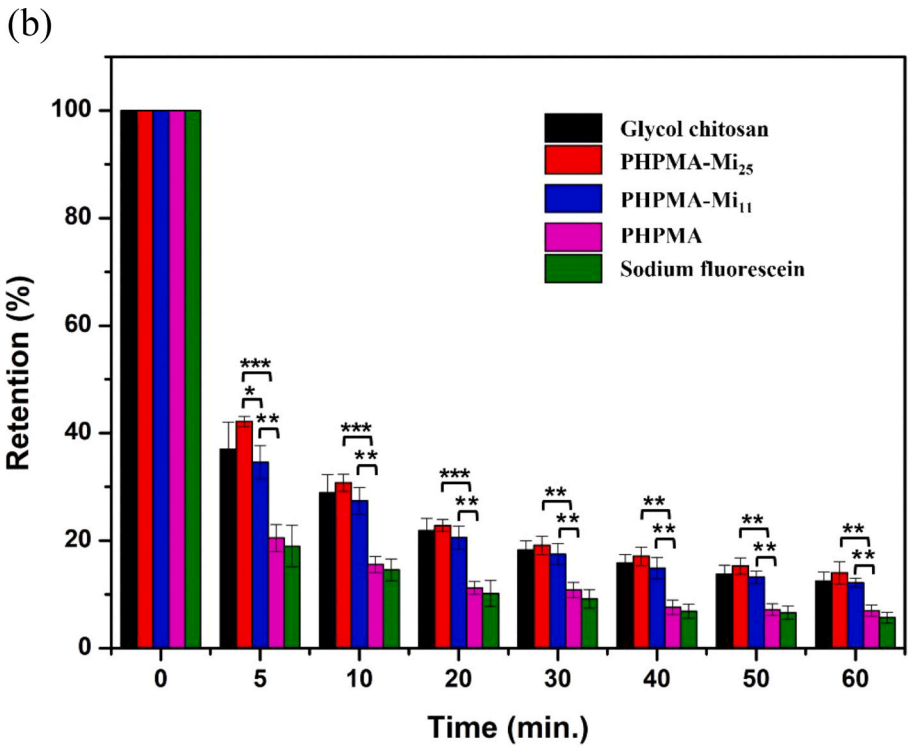

Fig. 4. (*continued*).

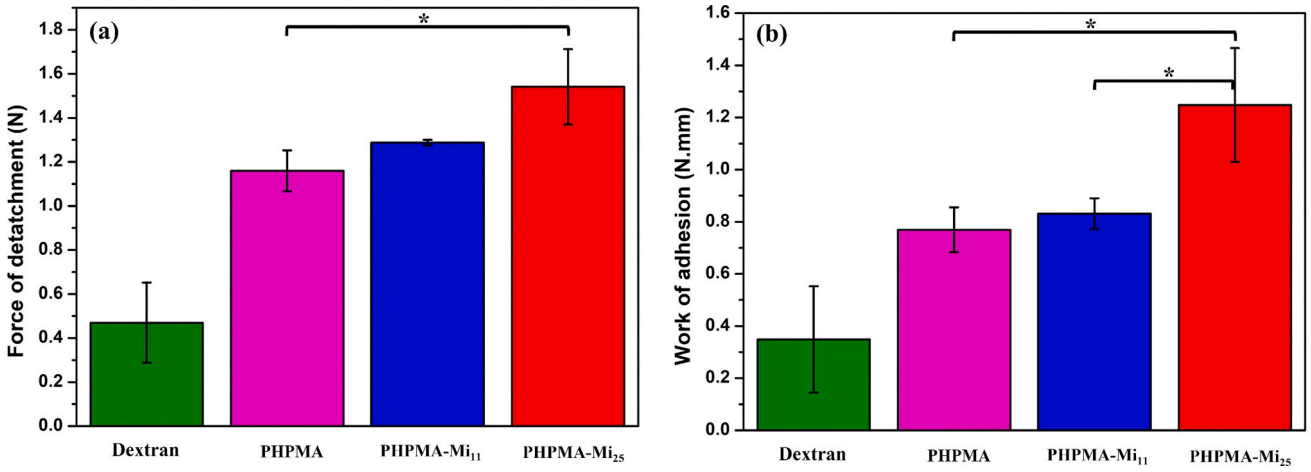

Fig. 5. (a) Force of detachment and (b) work of adhesion of dextran, PHPMA, PHPMA-Mi_{11} and PHPMA-Mi_{25} to sheep nasal mucosa measured using tensile testing (n = 3, mean ± SD, "*" represents $p < 0.05$).

slugs exposed to PBS (used as a negative control, pH 7.40) provided a low level of mucus production of 4 ± 1 % (**Fig. S3** in Supporting information for the images of slugs exposed to various test materials). A significant variability of the data observed in these experiments with the positive control is explained by slugs' increased activity and tendency to escape contact with an irritant chemical. In all experiments with negative control and PHPMA-based biomaterials, slugs secreted colourless mucus, indicating a lack of irritant properties of the materials.

4. Conclusions

In this study, we have successfully synthesised and tested maleimide-functionalised PHPMA copolymers as potential mucoadhesive excipients. *In vitro* nasal mucoadhesion studies revealed that PHPMA-Mi copolymers exhibited superior mucoadhesive properties on nasal mucosa tissue compared to the parent PHPMA due to the binding of maleimide groups to thiols present on mucosal surfaces. Cytotoxicity studies and slug mucosal irritation assay further demonstrated non-toxic nature of these polymers. The results indicate that highly biocompatible polymer systems based on PHPMA functionalised with maleimide groups hold promise as materials for advanced nasal delivery applications in the future. In addition to nasal administration, these materials may also be of interest for other routes of mucosal delivery, such as ocular, buccal, or vaginal applications.

CRediT authorship contribution statement

Xiaoning Shan: Writing – original draft, Methodology, Investigation, Formal analysis. **Robert Pola:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Daulet B. Kaldybekov:**

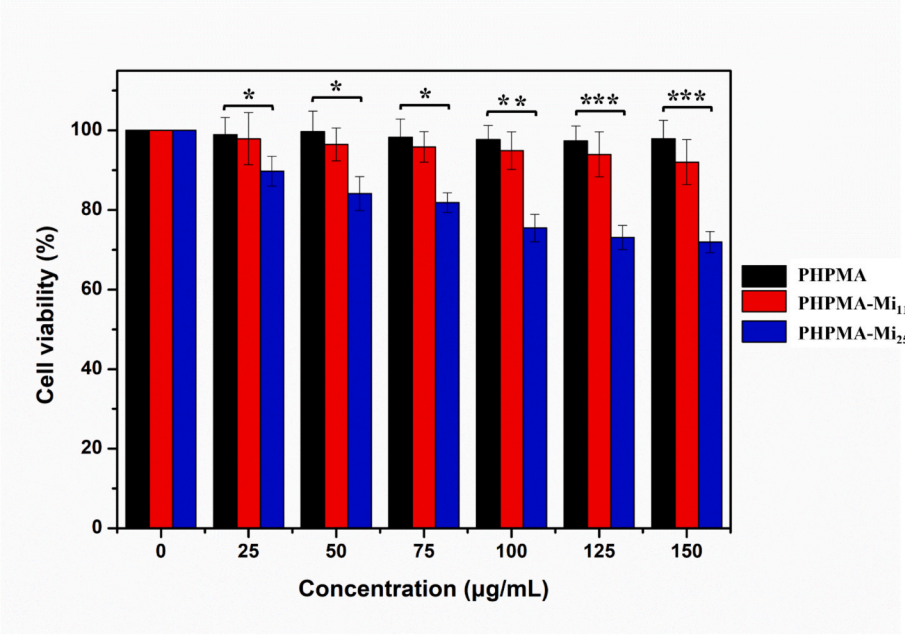

Fig. 6. Viability of HEK 293 cells determined after treatment with the polymers at different concentrations (25, 50, 75, 100, 125 and 150 μg/mL) for 72 h. The untreated cells served as the control. Values were expressed as means ± SD (n = 3), "*" represents $p < 0.05$, "**" represents $p < 0.005$, "***" represents $p < 0.001$.

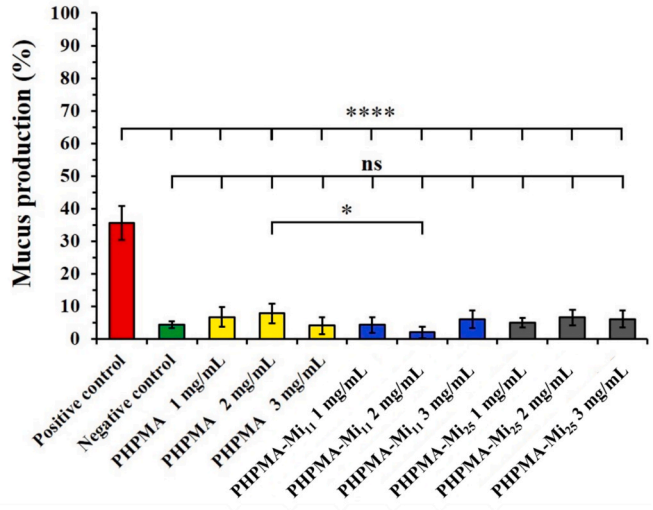

Fig. 7. Mucus production by *Arion lusitanicus* slugs in response to 60 min exposure to PHPMA, PHPMA-Mi_{11} and PHPMA-Mi_{25} as well as positive and negative controls. Statistically significant differences are given as: "****" represents $p < 0.0001$; "*" represents $p < 0.05$; "ns" represents no significance.

Visualization, Validation, Investigation, Formal analysis, Data curation. **Sam Aspinall:** Methodology, Investigation, Formal analysis, Data curation. **Fhataheya Buang:** Methodology, Investigation, Formal analysis, Data curation. **Adrian C. Williams:** Writing – review & editing, Supervision. **Tomáš Etrych:** Writing – review & editing, Resources, Methodology. **Vitaliy V. Khutoryanskiy:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Appendix A. Supplementary data

HPLC traces and UV–Vis spectra of PHPMA and its derivatives, SEC profiles of polymers, retention on nasal mucosa values, values of viability of HEK 293 cells, images of slugs exposed to solutions of polymers. Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2025.114193.

Data availability

All raw data supporting the findings of this study are available in the Supplementary Information or from the corresponding author upon reasonable request.

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