AUTHOR(S): IBIE, C.O., THOMPSON, C.J. and KNOTT, R.

TITLE: Synthesis, characterisation and in vitro evaluation of novel thiolated derivatives of polyallylamine and quaternised polyallylamine.

YEAR: 2015

Publisher citation: IBIE, C.O., THOMPSON, C.J. and KNOTT, R. 2015. Synthesis, characterisation and in vitro evaluation of novel thiolated derivatives of polyallylamine and quaternised polyallylamine. Colloid and polymer science [online], 293(6), pages 1737-1748. Available from: https://doi.org/10.1007/s00396-015-3561-4

OpenAIR citation: IBIE, C.O., THOMPSON, C.J. and KNOTT, R. 2015. Synthesis, characterisation and in vitro evaluation of novel thiolated derivatives of polyallylamine and quaternised polyallylamine. Colloid and polymer science, 293(6), pages 1737-1748. Held on OpenAIR [online]. Available from: https://openair.rgu.ac.uk

Synthesis, Characterisation and in-vitro Evaluation of Novel Thiolated derivatives of Polyallylamine and Quaternised Polyallylamine

Journal:	*Colloid and Polymer Science*
Manuscript ID:	CPS-2014-0354
Manuscript Type:	Original Contribution
Date Submitted by the Author:	22-May-2014
Complete List of Authors:	Ibie, Chidinma; The Robert Gordon University, School of Pharmacy and Life Sciences Thompson, Colin; The Robert Gordon University, School of Pharmacy and Life Sciences; Knott, Rachel; The Robert Gordon University, School of Pharmacy and Life Sciences
Keywords:	mucoadhesion, thiolation, quaternisation, thiomer, cross-linking, amide, amidine

Synthesis, Characterisation and *In-vitro* Evaluation of Novel Thiolated Derivatives of Polyallylamine and Quaternised Polyallylamine

C.O. Ibie, C.J. Thompson, R. Knott

School of Pharmacy and Life Sciences,

The Robert Gordon University,

Riverside East

Garthdee Road

Aberdeen AB10 7GJ, UK

e-mail: c.o.ibie@rgu.ac.uk

Telephone no - 01224262561

ABSTRACT

Polyallylamine (Paa) was quaternised by methylation of its primary amines using methyl iodide to yield quaternised Paa (QPaa). Average level of polymer quaternisation was determined by elemental analysis and was found to be 72 ± 2mol%. Subsequent thiolation of Paa (15kDa) and QPaa using two different thiolation procedures involving carbodiimide mediated conjugation to N-acetylcysteine (NAC) and modification of the polymers using 2-iminothiolane hydrochloride yielded their respective NAC and 4-thiobutylamidine (TBA) conjugates: Paa-NAC/QPaa-NAC and Paa-TBA/QPaa-TBA.

Estimation of the free thiol content of thiomers by iodometric titration showed that Paa-NAC and QPaa-NAC displayed 60 ± 1.2 and 60 ± 4.3µmol free thiol groups per gram polymer respectively, while Paa-TBA and QPaa-TBA conjugates displayed 490 ± 18 and 440 ± 21µmol free thiol groups per gram polymer respectively.

Assessment of polymer mucoadhesion using the mucin adsorption assay method revealed that Paa-NAC and Paa-TBA had the best mucoadhesive profile both adsorbing >20% more mucin than the parent polymer Paa. However, thiolation of QPaa was not observed to result in a marked improvement in adsorption of mucin. Also, while other thiolated derivatives were stable in tris/phosphate buffer pH 8 with no change in free thiol content, Paa-TBA solutions displayed *in-situ* crosslinking of free thiol groups to a disulphide network which was evident after 2 hours. These results show that thiolation of Paa enhanced its mucoadhesive properties, with Paa-TBA also exhibiting *in-situ* gelling properties. Hence, these novel thiolated Paa derivatives exhibit properties which may be useful in facilitating transmucosal drug delivery and controlled drug release.

Key words: mucodhesion; thiolation; quaternisation; thiomer, *in-situ* crosslinking; amide; amidine.

1. INTRODUCTION

Mucoadhesive polymers have gained increasing importance in the systemic delivery of drugs through mucosal routes due to their unique ability to adhere to the mucus layer via chain interpenetration and entanglements, covalent and non-covalent (charge-based, hydrogen bonding, hydrophobic) interactions with components of mucus [1, 2, 3]. This mucoadhesive process extends the residence time of the dosage form at the application site allowing adequate time for polymers that enhance mucosal wall permeation to exert their effect while also creating a steep concentration gradient which facilitates the process of passive diffusion necessary for optimal drug absorption [4, 5]. Polymers which have been used successfully in mucoadhesive dosage form design include chitosan, polyacrylic acid and alginate [2, 6, 7, 8]. Their distinctive mucoadhesive property has been attributed to the availability of charged functional groups within their structure which are able to foster ionic/electrostatic interaction with components of mucin . Cationic polymers like chitosan have however been found to have better mucoadhesive properties that anionic ones as they exert their mucoadhesive effect by interaction of their positively charged (primary amine) groups with negatively charged terminal sialic acid or sulphonated residues on the caborhydrate residues of mucin [9, 10].

Optimisation of the structure of these polymers for mucosal drug delivery often necessitates modification of the polymer backbone. This may involve processes like trimethylation of primary amine groups (quaternisation) which addresses the pH-dependent solubility of polymers like chitosan while also stabilising the positive surface charge of polycations [11, 12, 13]. Thiolation of these polymers, which involves the immobilisation of reactive thiol groups on the primary amine groups of the polymer has also been reported to enhance mucoadhesion; thiol groups can oxidise to the reactive thiolate anion S^- above pH 5 initiating thiol-disulphide interactions with cysteine-rich subdomains of mucin glycoproteins [14, 15, 16]. Other desirable properties that may be associated with thiomers include their ability to chelate metal ions of endogenous proteases thereby offering increased enzymatic protection to their proteinous substrates [17, 18] and their tendency to form in-situ crosslinked networks/gels above pH 5 which can be useful in controlled drug release [19, 20].

The work carried out by our group focuses on progressive alteration of the structure of Paa aimed at improving its function in the areas of transmucosal/transepithelial transport of drugs and macromolecules, enzymatic protection of proteins and peptides intended for oral delivery and controlled drug release. Previous research carried out with Paa includes modification of Paa by quaternisation of its primary amine groups imparting a permanent pH-independent positive charge to the polymer and the attachment of hydrophobic pendant groups (palmitoyl, cholesteryl and cetyl chains) to the Paa backbone yielding amphiphillic polyelectrolytes (AP) with improved potential for hydrophobic interaction and increased enzymatic protection [21, 22, 23].

This paper reports further work to expand the application of Paa by assessing the impact of thiolation on mucoadhesion while also evaluating the potential of using the obtained thiomers in other drug delivery applications. Thiolation of QPaa was also carried out in order to ascertain if a synergistic mucoadhesive effect based on both electrostatic and thiol-disulphide interactions with mucin can be obtained. Thiolation of Paa and QPaa was possible either through carbodiimide mediated coupling of the primary amine groups of the polymer to N-acetylcysteine creating a stable amide bond or by reacting the polymers with 2-iminothiolane which yields the 4-thiobutylamidine derivatives of the parent polymer. The thiomers obtained were subsequently characterised and their mucoadhesive profile evaluated in comparison to that of their parent polymers.

2. MATERIALS AND METHOD

2.1. MATERIALS

Poly(allylamine hydrochloride) (average Mw = 15kDa), tris(hydroxymethyl)aminomethane (Tris base) (≥ 99%), iodomethane, amberlite IRA-96 resin (20-50 mesh), sodium iodide, N-(3-Dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDAC), sodium hydroxide, N-hydroxysuccinimide (NHS), N-acetylcysteine, 2-iminothiolane hydrochloride, sodium borohydride, phosphate buffer saline(PBS), iodine solution(0.5M), starch solution(2%) and Porcine gastric mucin (crude type II) were all purchased from Sigma-Aldrich UK.

2.2. SYNTHESIS OF POLYMERS

2.2.1. Quaternisation of polyallylamine

The methods used for the purification of the polymers polyallylamine from poly(allylamine hydrochloride) and subsequent quaternisation of Paa to yield QPaa have been previously described in earlier reports published by our group [21]. The degree of quaternisation of the product was estimated by elemental analysis and results were obtained in triplicate.

2.2.2. Synthesis of Paa and QPaa N-acetylcysteine conjugates

Thiolation of Paa/QPaa by conjugation to N-acetylcysteine via an amide bond was carried out separately using a similar method to Yin et al. [25] (figure 1). N-acetylcysteine (250mg; 1.53mmol) was dissolved in 100ml of deionised water into which EDAC and NHS were added consecutively up to a final concentration of 200mM to activate the carboxylic acid groups of N-acetylcysteine. The mixture was adjusted to pH 4-5 using 2M HCl and left stirring at room temperature for 1 hour, after which Paa/QPaa (250mg) was added into the reaction mixture and the pH of the mixture readjusted to between pH 4-5. The reaction was carried out under nitrogen at room temperature for 5 hours without exposure to light. A control experiment containing equivalent concentrations of N-acetylcysteine and Paa without EDAC/NHS was also set up in the same way and allowed to run simultaneously.

The reaction mixtures for the test and control experiments were then dialysed (molecular weight cut-off - 7kDa) in the dark at 4°C, once against 5mm HCl, twice against 5mm HCl containing 1% NaCl, once again against 5mm HCl and finally against 0.4mM HCl. The polymer conjugates were isolated by dialysis and then freeze dried (VirTis advantage freeze drier, Biopharma Process Systems, UK). The lyophilised product obtained was characterised and stored at -20°C.

Where '**R**' represents Paa/QPaa

Fig. 1 Thiolation reaction for EDAC/NHS mediated coupling of N-acetylcysteine to Paa/QPaa

2.2.3. Modification of Paa and QPaa using 2-iminothiolane

The thiolation of Paa and QPaa using amidine linkages was carried out separately following the method previously described by Bernkop Schnurch et al. [26] (figure 2). Paa/QPaa (500mg) was dissolved in 50ml deionised water and the pH adjusted to 6.5 using 5M HCl. 2-iminothiolane hydrochloride (400mg) was added into the flask, and the reaction left stirring under nitrogen. The experiment was conducted at in the dark at room temperature for 14 hours. The polymer conjugates were then isolated by dialysis and freeze dried as described in 2.2.2, after which they were also characterised and stored at -20°C.

Where '**R**' represents Paa/QPaa

Fig. 2 Reaction scheme for Paa/QPaa thiolation using 2-iminothiolane

2.3. CHARACTERISATION OF POLYMERS

2.3.1. Elemental analysis

The elemental analysis protocol used for estimating the degree of quaternisation of QPaa was carried out as reported previously by our group [21]. QPaa samples (1mg) were analysed for the abundance of carbon, hydrogen, nitrogen and halogens using a Perkin Elmer series 2 elemental analyser (Perkin Elmer, UK). While samples of each thiomer were analysed as described for QPaa but also for the presence of sulphur.

2.3.2. Determination of free thiol content

The amount of free thiol groups immobilised on each thiolated conjugate was estimated by iodometric titration using a 2% starch solution as indicator. Each thiomer (10mg) was dissolved in 1ml of deionised water acidified with a drop of 2M HCl. 1% starch indicator (300µl) was added into the polymer solution before titrating the solution with a 1mM iodine solution until a permanent blue colour characteristic of the iodine-starch complex was observed [27]. The amount of thiol groups (in mols) per gram polymer was estimated from a calibration plot prepared from titrating iodine against increasing concentrations (2-100mgml^{-1}) of an N-acetylcysteine reference standard (R^2= 0.99). Iodometric titrations for each polymer as well as the controls were carried out in triplicate.

2.3.3. Estimation of total thiol substitution and disulphide bond content

The total amount of thiol substituents per gram polymer was obtained by reducing the disulphide bonds formed during the thiolation reaction using sodium borohydride ($NaBH_4$) followed by determination of free thiol content as described in section 2.3.1. [27]. A 1ml solution (1mgml^{-1}) of each thiomer in tris buffer pH 7.4 was prepared in a glass vial and mixed with 4% sodium borohydride solution (2ml) and the reaction incubated at 37°C for 1hour in a shaking water bath. The reaction was then stopped by slowly adding 400µl of 5M HCl with gentle stirring. Each reaction mixture was subsequently subjected to iodometric titration as described above and the free thiol content obtained used to obtain the total thiol substitution. The disulphide bond content of each thiomer was estimated by subtracting free thiol content obtained for each polymer prior to the reduction process from the total thiol content which was obtained after treatment with the reducing agent. This was done in triplicate.

2.3.4. Zeta potential

The zeta potential (mV) of 1mgml^{-1} solutions of each polymer in tris buffer was determined at 25°C by photon correlation spectroscopy (PCS) (Zetasizer Nano-ZS, Malvern Instruments, UK).

2.3.5. Differential scanning calorimetry (DSC)

Polymer samples (2-3mg) were placed in hermetic aluminium pans and subjected to DSC analysis within -90°C to 370°C at a heating rate of 20°Cmin^{-1} under nitrogen using a Q100 differential scanning calorimeter (TA instruments, UK) precalibrated with indium [15, 22].

2.4. *IN-VITRO* EVALUATION OF MUCOADHESIVE CAPACITY OF POLYMERS

Serial dilutions (0.1-1mgml^{-1}) of mucin in tris buffer were prepared from a 1mgml^{-1} stock solution of porcine mucin in tris buffer pH 7.4 obtained by probe sonication. The absorbance of each diluted mucin sample at 251nm was obtained by UV spectrometry (Agilent G1103A photo diode array, Agilent Technology, China) and the values plotted against the equivalent sample concentration to obtain a standard calibration curve (R^2= 0.99).

Assessment of the mucoadhesive capacity of each polymer was determined by measurement of the amount of mucin adsorbed by each polymer using a similar method to that described by Modi. et al. [28]; 0.25ml of a 0.5mgml^{-1} solution of each polymer in tris buffer pH 7.4 was mixed with 1mgml^{-1} mucin in tris buffer pH 7.4 and the mixture incubated at 37°C in a shaking water bath for 5 hours. Control samples were also prepared by mixing the aforementioned mucin in tris buffer solution with only 0.25ml tris buffer pH 7.4 and then incubated as described above. All control and test samples were subsequently transferred into separate eppendorf tubes and centrifuged at 10,000rpm for 30minutes, and the concentration of mucin in each supernatant measured by UV spectrometry at 251nm as described earlier.

Percentage (%) of total mucin adsorbed to each sample of polymer was calculated as shown below:

% mucin adsorption (M_{ad}) = [M_o – M_s] / M_o x 100

Where, M_o = concentration of free mucin in control supernatant

M_s = concentration of free mucin in the sample supernatant

2.5. *IN-SITU* CROSSLINKING AND REDUCTION IN FREE THIOL CONTENT OF THIOMERS

Samples (4mg) of each thiomer were hydrated in 1ml of tris buffer and buffered to pH 8 using 0.1M tris base, after which 3ml of PBS was added into each thiomer solution. The samples were incubated at 37°C in a shaking water bath and the change in the free thiol content of each sample with time estimated over 8 hours, by withdrawing 1ml of each sample every 2hours and titrating with iodine solution as described in 2.3.2. (any solids formed were separated out by centrifuging the sample at 10,000rpm for 10minutes, prior to titration).

3. RESULTS AND DISCUSSION

3.1. POLYMER SYNTHESIS AND CHARACTERISATION

3.1.1. Validation of polymer synthesis

The average degree of quaternisation of QPaa as estimated by elemental analysis was found to be 72 ± 2% and average yield of the process was found to be 76.2 ± 5% . Immobilisation of reactive thiol groups on primary

amino groups on the Paa/QPaa backbone was carried out using two types of covalent bonds. Paa and QPaa were coupled via a stable amide bond to N-acetylcysteine using a water-soluble carbodiimide cross-linker (EDAC) and NHS to form Paa/QPaa-N-acetylcysteine conjugates (presumptive structures shown in figure 3 below).

Fig. 3 Presumptive structure of repeating units of N-acetylcysteine conjugates of a) Paa: Paa-NAC b) QPaa: QPaa-NAC

Paa and QPaa were also coupled to 4-thiobutylamidine via a reaction with 2-iminothiolane hydrochloride, a thiol-containing imidoester forming Paa/QPaa-4-thiobutylamidine conjugates. These TBA conjugates have a protonated amidine bond which bears an extra positive charge on the thiol constituent at pH 7.4 as can be seen in figure 4 below [19, 29].

Fig. 4 Presumptive structure of repeating units of thiobutylamidine conjugates of a) Paa: Paa-TBA b) QPaa: QPaa-TBA

Optimisation of the coupling reaction between the polymers and N-acetylcysteine necessitated the inclusion of NHS in the cross-linking reaction as shown in Fig. 1 to stabilise the O-acylisourea intermediate product of the EDAC-carboxylic acid reaction which is susceptible to hydrolysis and consequently has a short life span in aqueous media [30]. The reaction was also carried out under nitrogen and at pH 4.5 to limit air or pH-induced oxidation of thiol groups to the reactive thiolate anion S^- resulting in the formation of intramolecular disulphide bond formation [31]. An N-acylated amino acid was used during the reaction to prevent the occurrence of unwanted side reactions resulting in the formation of oligo/poly cysteine conjugates [27]. After lyophilisation, all polymer conjugates appeared as white, powders of fibrous structure which were readily soluble over a wide pH range (3-8). The mean percentage yield (n=3) of Paa-NAC and QPaa-NAC conjugates were estimated at 68.8 ± 2.8% and 73.6 ± 2.3 % respectively, while the percentage yield of the Paa-TBA and QPaa-TBA conjugates was estimated at 73.1 ± 4.4% and 83.6 ± 7.7 %, respectively. The total sulphydryl group content of each conjugate as well as the amount available as free thiols (SH) and disulphide (S-S) bonds was estimated by iodometric titration as described in section 2.2.4 and the results shown in Table 1.

Table 1: Total thiol content, free thiol and disulphide bond content of thiomers (µmolg^{-1} polymer). Indicated values are mean ± S.D. (n = 3)

Polymer	Free SH content (µmolg^{-1})	S-S bond content (µmolg^{-1})	Total thiol Substitution (µmolg^{-1})
Paa-NAC	60 ± 1.2	280	340 ± 4.1
QPaa-NAC	60 ± 4.3	220	280 ± 3.3
Paa-TBA	490 ± 18	590	1080 ± 28
QPaa-TBA	440 ± 21	560	1000 ± 31

Covalent attachment of N-acetylcysteine to Paa and QPaa was confirmed by the negligible amount of thiol groups ($0.2 \pm 0.06 \mu molg^{-1}$ polymer) detected in the control samples prepared without EDAC/NHS after the dialysis process. The coupling efficiency of the EDAC/NHS mediated thiolation process was relatively low resulting in the attachment of fewer molecules of the sulphydryl-containing moiety on the polymer backbone than polymer conjugates obtained using 2-iminothiolane. This contributed to the relatively low levels of thiolation observed in Paa/QPaa-NAC conjugates as can be seen from Table 1. The relatively low coupling efficiency of the EDAC-mediated thiolation process has previously been reported by other research groups [25, 27] working on the thiolation of similar polycations using EDAC concentrations ranging between 25-200mM and has been attributed to a side reaction of EDAC with the nucleophilic thiolate anion that results in the formation of an adduct that is subsequently hydrolysed to one of the reaction by-products, urea [32]. In contrast, the reaction of Paa/QPaa with 2-iminothiolane was observed to proceed with greater efficiency considering the relatively high levels of sulphydryl groups substitution obtained for TBA conjugates (table 1) .

3.1.2. Zeta potential

The surface charge of each polymer in tris buffer pH 7.4 as analysed by zeta potential measurement is detailed in table 2 below.

Table 2: Zeta potential (mV) of $1 mgml^{-1}$ solutions of polymers in tris buffer pH 7.4. Values indicated are mean ± S.D. (n=3)

Polymer	**Paa**	**QPaa**	**Paa-NAC**	**QPaa-NAC**	**Paa-TBA**	**QPaa-TBA**
Zeta potential	41.9 ± 2	45.0 ± 3	35.7 ± 1	37.4 ± 1	46.9 ± 1	48.4 ± 1

Results show that the surface charge of the polymers was found to vary with the nature of the substituting group. Quaternisation enhanced the cationic charge of both Paa and thiolated Paa derivatives, while conjugation of Paa/QPaa to NAC resulted in a reduction of cationic surface charge. On the other hand, thiolation using 2-iminothiolane resulted in retention of cationic charge of both parent polymers (Paa and QPaa).This difference is probably because while the cationic substructure of the amidine group (figure 4) facilitates the retention of cationic charge in TBA-based thiomers, the substitution of protonable primary amine groups with uncharged amide bonds reduces the cationic charge in NAC-based thiomers. This marked variation in the surface charge of the different thiomers obtained could have significant implications on their capacity to complex with insulin and influence processes like tight junction opening and mucoadhesion that benefit from charge-based interactions [33]. Polymer surface charge could also influence the biodistribution and cellular uptake of insulin PECS formed from the polymers [33].

3.1.3. DSC

Thermal analysis carried out on the various polymers and conjugates by DSC also indicated that the synthesis process resulted in novel derivatives of Paa/QPaa.

Fig. 5 DSC thermograms of Paa and Thiolated Paa derivatives

The impact of variations in the structure of different Paa derivatives on their physical and mechanical properties was reflected by DSC. Paa has no bulky side groups attached to it and hence has a relatively streamlined shape. This facilitates packing of the polymer molecules into crystallites increasing T_m [34, 35]. The DSC thermogram of Paa (figure 5) showed a sharp T_m occurring at about 138°C suggesting a semi-crystalline structure. The T_m appeared to occur simultaneously with decomposition of the polymer chains and may imply that the temperature required to disrupt the polymer crystallites also led to degradation of polymer chains. The T_g of Paa was found to be -12°C which implies that the polymer is rubbery at ambient or body temperature (consistent with experimental observations). Thiolation of Paa was found to be associated with a slight increase in T_m from approximately 138 to about 150°C. However, while Paa-TBA which contains the amidine bond appears to have retained a semi-crystalline structure exhibiting a sharp T_m at about 140°C -150°C, Paa-NAC exhibited a shallow, broad endotherm at about the same temperature. This difference could likely be due to the fact that the amidine group exhibits relatively less branching than N-acetyl cysteine and the protonated amidine bond is also more likely to partake in intermolecular bonding strengthening the crystal lattice. Although both thiolated Paa samples showed no T_g on their DSC thermograms, Paa-TBA samples were observed to change from glassy to rubbery at room temperature, indicating that this polymer may have a T_g which was too subtle to be observed in the DSC thermograms.

Fig. 6 DSC thermograms of QPaa and thiolated QPaa derivatives

The attachment of bulky side groups to polymers increases the stiffness of the chain raising T_g [34]. Quaternisation which involves the attachment of bulky quaternary groups to the Paa/thiolated Paa backbone may hinder close packing of the crystallites and limit intermolecular hydrogen bonding thereby increasing system disorder [36]. This could be seen by the broad endothermic peaks exhibited by quaternised samples (figure 6). The quaternary group also creates steric bulk limiting chain flexibility and mobility of the polymer molecules. Hence, all quaternised polymers remained partially amorphous and brittle at ambient temperature except for QPaa-NAC which was soft but not rubbery. Quaternised derivatives exhibited no T_g and the T_m of quaternised samples was much higher than their non-quaternised counterparts (290-300°C). This is similar to the thermal profile of QPaa-based AP, which showed similarly higher T_m than their non-quaternised Paa-based AP and no detectable T_g [21].

The relatively higher T_m exhibited by quaternised polymers may be because these polymers are already in an extended conformation in their crystallite as a result of the increased stiffness of the polymer chain caused by the bulky quaternary group [36, 37]. This reduces their entropy of melting (ΔSm) or "gain in randomness" during their melting transition. According to the equation which defines T_m as ΔHm/ΔSm (where ΔHm represents the enthalpy of melting), this reduction in ΔSm will lead to an increase in T_m [36, 37]. The increase in T_m of quaternised polymers has also been attributed to a possible ionic interaction between $CH_2N^+(CH_3)_3$ and Cl^- facilitating packing of the chains into a crystal structure restoring some degree of order to the polymer structure [21]. QPaa also appeared to show decomposition of polymer chains occurring alongside its T_m. The

disappearance of the sharp endothermic peaks seen in Paa and Paa-TBA suggested substitution of constituent primary amine groups.

3.2. *IN-VITRO* EVALUATION OF MUCOADHESIVE PROPERTIES

Evaluation of the mucoadhesive capacity of each polymer based on their in-vitro mucin adsorption profile indicated that both quaternised and thiolated polymers showed better mucoadhesive properties than the unmodified Paa backbone as can be seen in figure 7.

Thiolated Paa (Paa-NAC and Paa-TBA) exhibited the highest level of mucin adsorption amongst the different polymers tested performing better than their quaternised counterparts exhibiting similar levels of thiolation. This highlights the fact that mucoadhesive interactions are dependent on the ability of the functional groups present on the backbone of the carrier polymer to access and efficiently interact with compatible components of the mucin glycoproteins [3]. Thus polymer-mucin interactions are governed by multi-factorial mechanisms which determine the nature and strength of the mucoadhesive bonds and consequently, the mucoadhesive performance of the polymer [3]. A high level of polymer charge density and substitution (quaternisation, hydrophobic or thiolation) could result in a greater degree of interchain repulsion resulting in conformational changes which may decrease chain flexibility and limit interpenetration/entanglements between polymer-mucin molecules [38, 39]. Also, steric hindrance created by the presence of a high proportion of attached groups on the polymer backbone shielding charged groups may limit access to compatible groups thereby reducing mucoadhesive interaction [40].

Fig. 7 Mucoadhesive capacity (% mucin adsorption) of Paa, QPaa and their thiolated derivatives

Hence in consideration of the aforementioned facts, assessing the mucoadhesive performance of the various thiomers as a function of level of thiol substitution indicated that although the thiol content of Paa-TBA greatly outnumbers that of Paa-NAC, Paa-TBA was slightly less mucoadhesive than Paa-NAC.

This could be associated with the high level of thiol substitution of Paa-TBA influencing polymer conformation and affecting mucin interaction or could also be as a result from the polymer thiol groups being more reactive with themselves (intra-chain thiol-disulphide crosslinking) than with those of the mucin glycoproteins . However, it appears steric effect becomes more pronounced with the QPaa-based thiomers which were already substituted with quaternary groups as can be seen from figure 7, only QPaa-NAC which had a low level of thiolation exhibited better mucoadhesive properties than QPaa as a result of thiolation. On the contrary, QPaa-TBA showed reduced mucoadhesive properties, as this thiomer exhibited similar levels of mucoadhesion with the unmodified backbone which signifies a noticeable loss in the mucin-interaction facilitating effects of both quaternisation and thiolation. This was probably caused by steric hindrance as well as reduced chain flexibility as a result of the high degree of both quaternary and thiol substitution present in QPaa-TBA, therefore resulting in a cumulative inhibition of effective polymer-mucin interactions realised with both Paa-TBA and QPaa. This effect has been observed by other groups working with similar quaternised thiomers.

Therefore, although the mucoadhesion facilitating effects of polymer thiolation can be clearly seen by the marked increase in mucin adsorption of Paa due to thiolation, the results also highlight the need to optimise levels of substitution/quaternisation of the parent polymer to obtain the beneficial effects of these alterations on mucoadhesion.

3.3 *IN-SITU* CROSSLINKING PROPERTIES

Oxidation of free thiol groups above pH 5 results in the formation of intermolecular and intramolecular disulphide bonds [14]. This means that thiolated polymers are capable of forming *in-situ* crosslinked gel networks above pH 5. The change in free thiol content of the different thiomers in phosphate buffer pH 8 was monitored by iodometric titration. It was observed that with the exception of Paa-TBA all other thiomers did not show any drop in level of free thiol content over the 8 hour incubation period and their solutions remained clear. Paa-TBA solutions on the other hand became cloudy in 1 hour and at the 2 hour time period, a visibly crosslinked network was observed within the vial as shown in figure 8. Centrifugation of this sample and analysis of the supernatant for the presence of free thiol by iodometric titration indicated that no free thiols could be detected. This confirms the crosslinking of free thiols to disulphides at pH 7.4 occurred simultaneously with the formation of this network structure, a characteristic exhibited by thiolated polymers.

The crosslinking process was observed to be initiated by the addition of PBS into the solution of Paa-TBA in Tris buffer, implying that the process of thiol oxidation to disulphides may have been catalysed by the metal ions present in the buffer solution. This ability of metal ions to catalyse such oxidative processes has been previously documented by other research group [41]. The presence of tris in the buffer mixture was also observed to play a role in the formation of the swollen or expanded crosslinked network shown in figure 8. Hydration of the dry polymer sample (Paa-TBA) with PBS was observed to lead to the formation of a collapsed gel, therefore the expanded network observed when PBS is added into a solution of Paa-TBA in tris buffer could be a direct effect of the increase in cationic charge of the polymer in tris buffer pH 7.4 (protonation of the primary amine group of tris base by HCl creates more positive charges within the system) resulting in increased interpolymer chain repulsion and consequent swelling [42]. Such crosslinked polymer networks that undergo volume phase transitions like shrinking/swelling in response to external environmental conditions have been used in the design of hydrogels and bioadhesive systems that control the release of incorporated drugs based on changes in the porosity of the dosage form in response to different stimuli.

The disparity in the crosslinking behaviour of Paa-TBA and Paa-NAC could be associated with the difference in the nature (charge) of the linkage bearing the thiol substitutents. Paa-NAC thiol groups are attached via an uncharged amide bond while the amidine linkage through which the thiol groups of Paa-TBA are attached to the Paa backbone is cationic, hence creating a significant electrostatic difference in the local environment of the respective thiol moieties of Paa-TBA and Paa-NAC. Such differences in the charge density of neighbouring attached groups have been shown to greatly influence thiol-disulphide exchange reactions and are consistent with the findings of other groups working with similar thiolated chitosans [18, 43]. The formation of disulphide crosslinks by thiolated groups attached to the quaternised thiomers could have been impeded by the bulky quaternary ammonium groups present on the polymer backbone sterically limiting inter-chain thiol-disulphide

interactions, as the close proximity of interacting thiol groups has been shown to improve the crosslinking process [44]. The tendency of Paa-TBA to form an *in-situ* crosslinked network at physiological pH would be considered advantageous as such crosslinked systems have been used in various drug delivery applications due to their ability to offer controlled (pH-dependent) release of incorporated materials as well as extend the residence of dosage forms at the site of application [19, 20, 45, 46].

Fig. 8 *In-situ* crosslinked network formed by Paa-TBA after 2 hours incubation in tris/phosphate buffer solution

4. CONCLUSION

The report has shown that thiolation of Paa and QPaa was possible either through EDAC/NHS mediated coupling of the primary amine groups of the polymer to N-acetylcysteine or by modifying the polymers with 2-iminothiolane yielding the N-acetyl cysteine and 4-thiobutylamidine derivatives respectively. Thiolated Paa derivatives were shown to have improved mucoadhesive qualities, with Paa-TBA also exhibiting *in-situ* crosslinking properties. However, thiolation of QPaa did not yield thiomers with significant improvements in mucoadhesive properties when compared to the parent polymer, QPaa. Therefore implying that these polymers would need further optimisation of their structure in order to satisfy the rational for their development.

ACKNOWLEDGEMENTS

The authors acknowledge the Institute for Health and Wellbeing Research, Robert Gordon University and the Scottish Overseas Research Scholarship Awards Scheme for providing the funding for this work.

REFERENCES

1. Kwashima, Y., Yamamoto, H., Takeuchi, H. and Kuno,Y. (2000) Mucoadhesive $_{DL}$-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. Pharmaceutical Development Technology, 5, 77-85.
2. Jintapattanakit, A., Junyaprasert, V.B. and Kissel, T. (2009) The role of mucoadhesion of trimethyl chitosan and PEGylated trimethyl chitosan nanocomplexes in insulin uptake. Journal of Pharmaceutical Sciences, 98(12), 4818-4830.
3. Longer, M.A and Robinson, J. R. (1986) Fundamental aspects of bioadhesion. Pharmacy Int.5, 114-117.
4. Junginger, H.E. (1990) Bioadhesive polymer systems for peptide delivery. Advanced Drug Delivery Reviews, 53, 321.
5. Dodou, D., Breedveld, P. and Wieringa, P.A. (2005) Mucoadhesives in the gastrointestinal tract: revisiting the literature for novel applications. European Journal of Pharmaceutics, 60, 1-16.
6. Bernkop Schnurch, A., Kast, C.E. and Richter, M.F. (2001) Improvement in the mucoadhesive properties of alginate by the covalent attachment of cysteine. Journal of Controlled Release, 71(3), 277-285.
7. Rathnam, G., Narayanan, N. and Ilavarasan, R. (2008) Carbopol-based gels for nasal delivery of progesterone. AAPS Pharmaceutical Science Technology, 9, 1078-1082.

8. He, P., Davis, S.S. and Illum, L. (1998) In vitro evaluation of the mucoadhesive properties of chitosan microspheres. International Journal of Pharmaceutics, 166, 75-88.
9. Bernkop-Schnurch, A., Arkhipova, V., Sakloetsakun, D., Hombach, J., Barthelmes, J. and Dunnhaupt, S. (2011) Distribution of thiolated mucoadhesive nanoparticles on intestinal mucosa. International Journal of Pharmaceutics, 408, 191-199.
10. Hassan, E.E. and Gallo, J.M. (1990) A simple rheological method for the in-vitro assessment of mucin-polymer bioadhesive strength. Pharmaceutical Research, 7, 491-495.
11. Jintapattanakit, A., Junyaprasert, V.B., Mao, S. and Kissel, T. (2008) Physicochemical properties and biocompatibility of N-trimethyl chitosan: effect of quaternisation and dimethylation. European Journal of Pharmaceutics and Biopharmaceutics. 70(2), 563-571.
12. Sadeghi, A.M.M., Dorkoosh, F.A., Avadi, M.R., Saadat, P., Rafiee-Tehrani, M. and Junginger, H.E. (2008) Preparation, characterisation and antibacterial activities of chitosan, N-trimethyl chitosan (TMC) and N-diethylmethyl chitosan (DMEC) loaded with insulin using both ionotropic gelation and polyelectrolyte complexation methods. International Journal of Pharmaceutics, 355, 299-306.
13. Sadeghi, A.M.M., Dorkoosh, F.A., Avadi, M.R., Bayat, F., Weinhold, M., Delie et al. (2008) Permeation enhancer effect of chitosan and chitosan derivatives: comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells. European Journal of Pharmaceutics and Biopharmaceutics, 70, 270-278.
14. Leitner, V.M., Walker, G.F. and Bernkop-Schnurch, A. (2003) Thiolated polymers: evidence for the formation of disulphide bonds with mucus glycoproteins. European Journal of Pharmaceutics and Biopharmaceutics, 56, 207-214.
15. Roldo, M., Hornof, M., Caliceti, P. and Bernkop-Schnurch, A. (2004) Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. European Journal of Pharmaceutics and Biopharmaceutics, 57, 115-121.
16. Kafedjilski, K., Krauland, A.H., Hoffer, M.H. and Bernkop-Schnurch, A. (2005) Synthesis and in vitro evaluation of a novel thiolated chitosan. Biomaterials, 26, 819-826.
17. Bernkop-Schnurch, A., Walker, G. and Zarti, H. (2001) Thiolation of polycarbophil enhances its inhibition of intestinal brush border membrane bound aminopeptidase N. Journal of Pharmaceutical Science, 90, 1907-1914.
18. Bernkop-Schnurch, A. and Thaler, C.S. (2000) Polycarbophil-cysteine conjugates as platforms for oral polypeptide delivery systems. Journal of Pharmaceutical Science, 89(7), 901-909.
19. Bacalocostantis, I., Mane, V.P., Kang, M.S., Goodley, A.S., Muro, S. and Kofinas, P. (2012) Effect of thiol pendant conjugates on plasmid DNA binding, release and stability of polymeric delivery vectors. Biomacromolecules, 13, 1331-1339.
20. Verheul, R.J., van der Wal, S. and Hennink, E.W. (2010) Tailorable thiolated trimethyl chitoisans for covalently stabilised nanoparticles. Biomacromolecules, 11, 1965-1971.
21. Thompson, C.J., Tetley, L., Uchegbu, I.F., Cheng, W.P., Qu. X., Ding, X. and Yang, Z. (2008) The effect of polymer architecture on the nano self-assemblies based on novel comb-shaped amphiphilic poly(allylamine). Colloid Polymer Science, 286, 1511-1526.

22. Thompson, C.J., Tetley, L. and Cheng, W.P. (2010) The influence of polymer architecture on the protective effect of novel comb-shaped amphiphillic poly(allylamine) against in vitro enzymatic degradation of insulin- Towards oral insulin delivery. International Journal of Pharmaceutics, 260(2), 229-237.

23. Thompson, C.J., Tetley, L., Uchegbu, I.F. and Cheng, W.P. (2009B) The complexation between novel comb shaped amphiphillic polyallyamine and insulin –Towards oral insulin delivery. International Journal of Pharmaceutics, 376, 216-227.

24. Thompson, C. J. and Cheng, W.P. (2011) Chemically modified polyelectrolytes for intestinal protein and peptide delivery. In C.F. Van der Walle [Ed] Peptide and Protein Delivery. Elsevier Inc., London, UK, 123-158.

25. Yin, L., Ding, J., He, C., Cui, L., Tang, C. and Yin, C. (2009) Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. Biomaterials, 30, 5691-5700.

26. Bernkop-Schnurch, A., Krauland, A.H. and Guggi, D. (2004) Oral insulin delivery: the potential of thiolated chitosan-insulin tablets on non-diabetic rats. Journal of Controlled Release, 95, 547-555.

27. Vigl, C., Leitner, K., Albrecht, K. and Bernkop-Schnurch, A. (2009) The efflux pump inhibitory properties of (thiolated) polyallylamines. Journal of Drug Delivery Science and Technology, 19 (6), 405-411.

28. Modi, J., Joshi, G. and Sawant, K. (2012) Chitosan based mucoadhesive nanoparticles of ketoconazole for bioavailability enhancement:formulation, optimisation, *in vitro* and *ex vivo* evaluation. Drug Development and Industrial Pharmacy, 10, 1-8.

29. Bernkop-Schnurch, A., Hornof, M. and Zoidl, T. (2003) Thiolated polymers-thiomers: synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates. International Journal of Pharmaceutics, 260(2), 229-237.

30. Damink, L.H.H.O., Dijkstra, P.J., Vanluyn, M.J.A., Vanwachem, P.B., Nieuwenhuis, P. and Feijen, J. (1996) Cross-linking of dermal sheep collagen using a water soluble carbodiimide. Biomaterials, 17, 765-773.

31. Bernkop-Schnurch, A., Krauland, A.H., Leitner, K. and Palmberger, T. (2004) Thiomers: potential excipients for non-invasive peptide delivery systems. European Journal of Pharmaceutics and Biopharmaceutics, 58, 253-263.

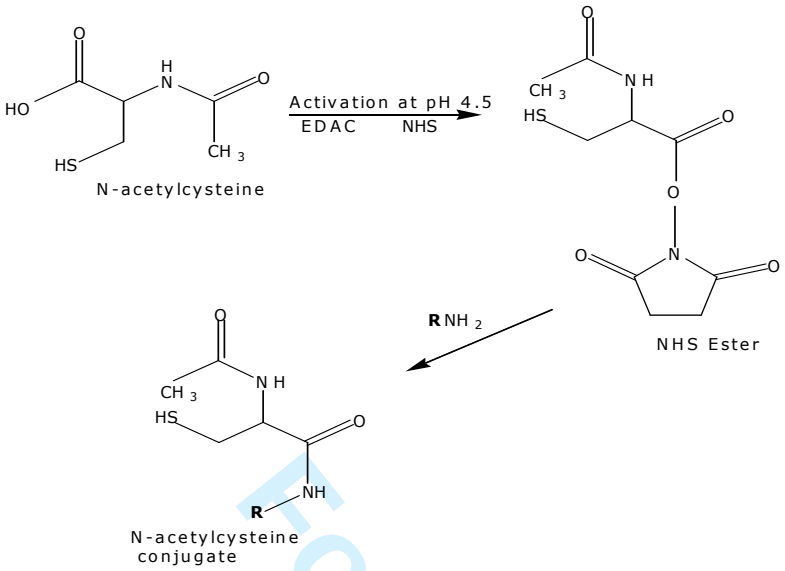
32. Millotti, G., Samberger, C., Frohlich, E. and Bernkop-Schnurch, A. (2009) Chitosan-graft-6-mercaptonicotinic acid: synthesis, characterisation and biocompatibility. Biomacromolecules, 10, 3023-3027.

33. He. C, Hu, Y., Yin, L., Tang, C. and Yin, C. (2010) Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials, 31, 3657-3666.

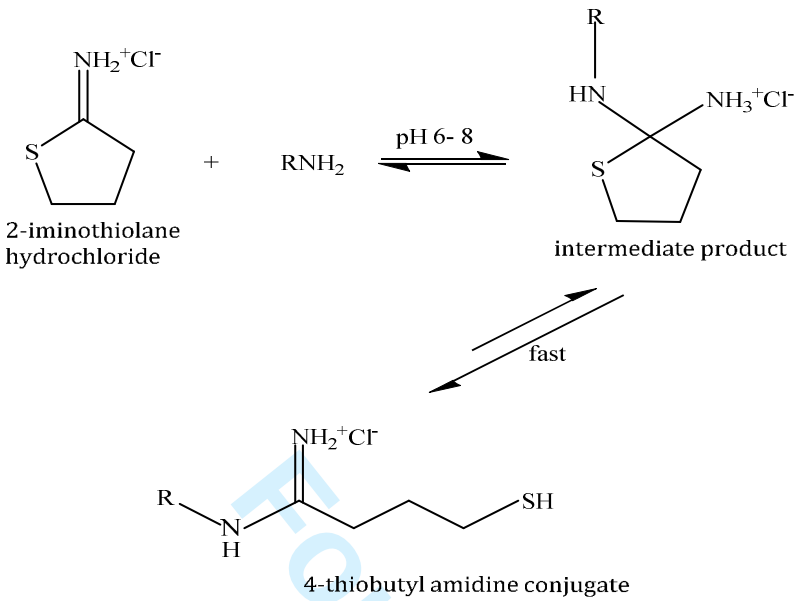
34. Hoffman, S. A. (2006) Selecting the right polymer for biomaterial application. In I. F. Uchegbu and A.G. Schatzlein [Ed] Polymers in drug delivery. Taylor and Francis group, Boca Raton, 7-22.

35. Buckton, G. (2002) Solid-state properties In: M.E. Aulton [2nd Ed]. Pharmaceutics: The science of dosage form design. Churchill Livingstone, Edinburgh, 141-151.

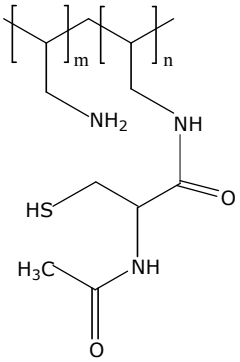
36. Billmeyer, F. W. Jr. (1984) *Textbook of Polymer Science*, 3rd Edition. Wiley-Interscience, New York.
37. Rodriguez, F. (1996) *Principles of Polymer Systems*. 4th Edition. Hemisphere, New York.
38. Jonker, C., Hamman, J.H. and Kotze, A.F. (2002) Intestinal paracellular permeation enhancement with quaternized chitosan: *in situ* and *in vitro* evaluation. International Journal of Pharmaceutics, 238, 205-213.
39. Hamman, J.H., Kotze, A.F. and Schultz, C.M. (2003) N-trimethyl chitosan chloride: optimum degree of quaternisation for drug absorption enhancement across epithelial cells. Drug Development and Industrial Pharmacy, 29, 161-172.
40. Hamman, J.H., Kotze, A.F. and Synman, D. (2003) Evaluation of the mucoadhesive properties of N-trimethyl chitosan chloride. Drug Development and Industrial Pharmacy, 29, 59-67.
41. Cullis, C.F. and Trimm, D.L. (1968) Homogenous catalysis of the oxidation of thiols by metal ions. Discussions of the Faraday Society, 46, 144-149.
42. Eisley, L. (2010) Water: The medium of Life. In: R. Garrett and C.M. Grisham [Ed] Biochemistry. Brooks/Cole, Boston, USA, 28-47
43. Snyder, G.H., Reddy, M. K., Cennerazzo, M. J. and Field, D. (1983) Use of local electrostatic environments of cysteines to enhance formation of a desired species in a reversible disulfide exchange reaction. Biochim Biophys Acta 749, 219–226.
44. Bernkop-Schnurch, A. and Marschutz, M.K. (2002) Thiolated polymers: self-crosslinking properties of thiolated 45-kDa poly(acrylic acid) and their influence on mucoadhesion. European Journal of Pharmaceutical Science, 15, 387-394.
45. Shu, S., Zhang, X., Teng, D., Wang Z. and Li, C. (2009) Polyelectrolyte nanoparticles based on water-soluble chitosan-poly(L-aspartic acid)-polyethylene glycol for controlled protein release. Carborhydrate Research, 344, 1197-1204.
46. Shu, S., Zhang, X., Wang. X, D., Wang Z., Li, C. and Zhang, X. (2009) Disulphide cross-linked biodegradable polyelectrolyte nanoparticles for the oral delivery of protein drugs. New Journal of Chemistry, 33, 1882-1887.



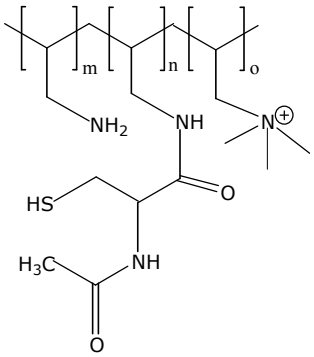
Definition: Black and white diagrammatic illustration created from chemdraw



Definition: Black and white diagrammatic illustration created from chemdraw

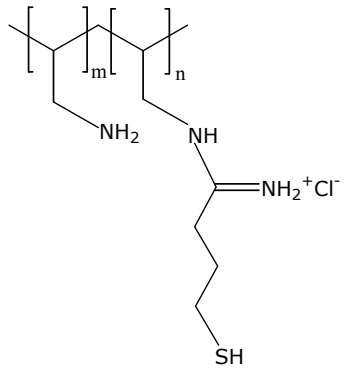


a)

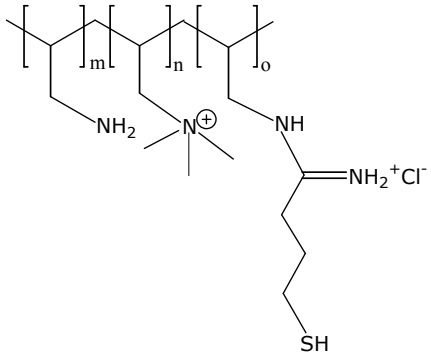


b)

Definition: Black and white diagrams created from chemdraw

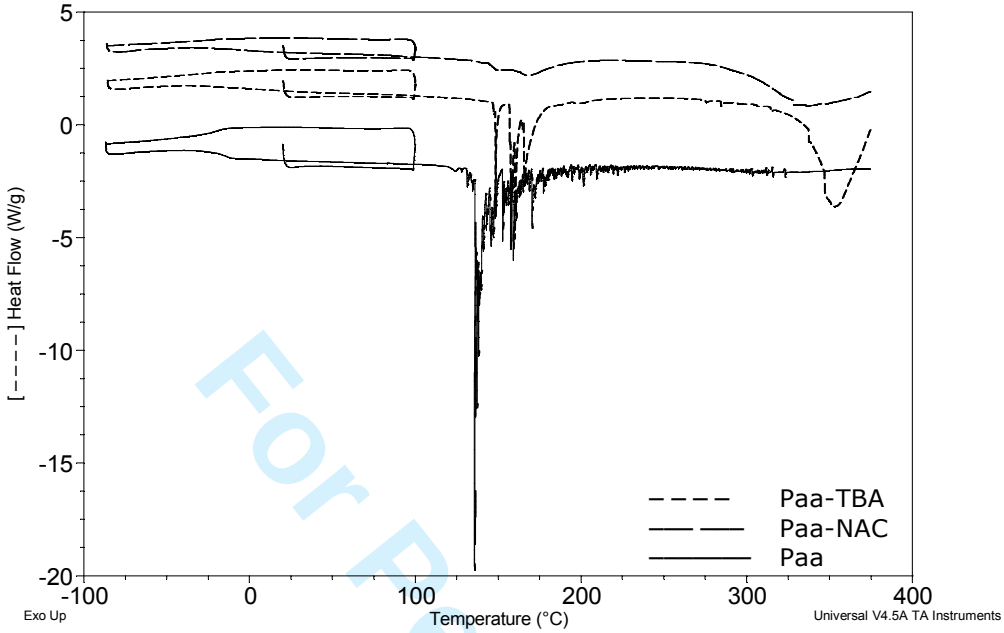


a)

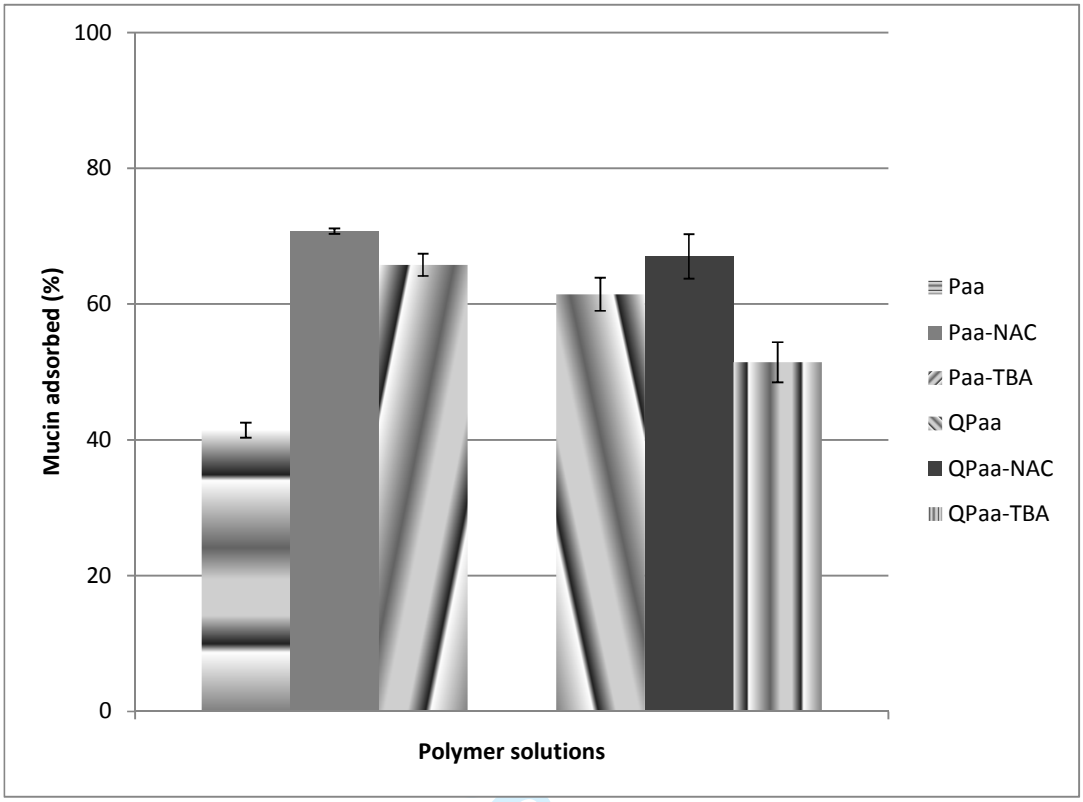


b)

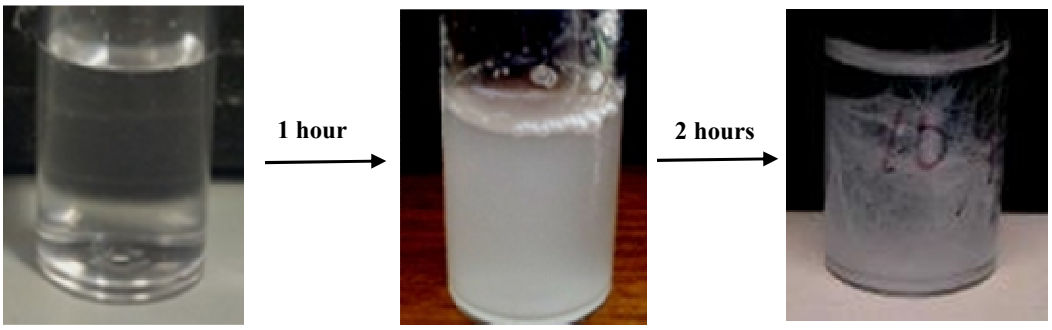
Definition: Black and white diagrams created from chemdraw



Definition: Black and white graphic with no shading



Definition: Black and white graphic/line art with shading created with microsoft excel



Definition: Photographs with lettering.