The effect of the antioxidant on the properties of thiolated poly(aspartic acid) polymers in aqueous ocular formulations

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Abstract

Thiolated polymers are a promising new group of excipients, but their stability against atmospheric oxidation has not been investigated in detail, and only a few efforts have been made to improve their stability. The oxidation of the thiol groups in solutions of thiolated polymers may result in a decrease of mucoadhesion and unpredictable *in situ* gelation. The aims of our work were to study the stability of aqueous solutions of thiolated polymers and the effects of stabilizing agents.

We investigated thiolated poly(aspartic acid) polymers stabilized with dithiothreitol, glutathione or acetylcysteine. The effects of these antioxidants on the gel structure, mucoadhesion and drug release were determined by means of scanning electron microscopy, swelling, rheology, adhesion and drug release tests. It was concluded that the stability of polymer solutions containing antioxidants is sufficient for one day. Polymers stabilized with dithiotreitol demonstrated fast swelling and drug release, but weaker mucoadhesion as compared with the other samples. Polymers stabilized with glutathione displayed the weakest cohesive properties, resulting in fast and uncontrolled drug release and moderate mucoadhesion. Acetylcysteine-stabilized polymers exhibited an optimum cross-linked structure, with free thiol groups ensuring polymer-mucin interactions, resulting in the best mucoadhesive properties.

Key words: thiolated polymer, mucoadhesion, drug release, stability, rheology

1. Introduction

In 1995, the International Pharmaceutical Excipient Council defined excipients as substances other than the active drug substance or finished dosage form, which are appropriately evaluated for safety and are included in a drug delivery system to aid the processing of the drug delivery system during its manufacture. They can protect, support and enhance the stability, and bioavailability of the drug, or they can improve the patient acceptability, assist in product identification, or enhance any other attributes of the overall safety and effectiveness of the drug delivery system during storage or use [1]. However, excipients are neither inert nor inactive substances, and they can also cause adverse reactions [2, 3].

Thiolated polymers are second-generation mucoadhesive polymers, which can be potentially applied as excipients in sustained drug delivery. They are able to form covalent bonds with the cysteine-rich sub-domains of the mucus glycoproteins, and also hydrogen-bonds and van der

Waals' forces. Thiolated polymers can increase the residence time on the mucosal surface and improve the bioavailability of the drug [4]. Additionally they have several other advantageous properties, such as their *in situ* gelling, enzyme-inhibitory capabilities, permeation-enhancing effect and efflux pump inhibition [5-7]. All these advantageous properties have already been described, but little information is available about the stability of thiolated polymers, which is an important factor in drug delivery formulation.

Earlier studies [8, 9] revealed the lower stability of thiolated polymers in solutions as a result of thiol oxidation. In the oxidation process, inter- and intramolecular disulfide bonds are formed, limiting the permeation enhancement and mucoadhesive character of the polymers. The oxidation of thiol groups is faster at pH ≥ 6 because of the increase in the concentration of negative thiolate anions, $-S^-$, which are more capable of oxidation.

There are two possibilities for the stabilization of thiolated polymers in solution: 1) the use of reducing agents (antioxidants) or 2) the protection of the thiol groups by the formation of disulfide bonds in a controlled way [8-10].

The addition of a reducing agent during or after the polymer synthesis ensures the stability of the thiol groups in solution, providing free thiol groups for better mucoadhesion and permeation. In earlier studies, 2-mercaptoethanol [8], dithiothreitol (DTT), sodium borohydride [11]. hydroxylamine [12], EDTA [13] and sodium cyanoborohydride [14] have been used to avoid the oxidation of thiol groups.

In thiol group protection, the thiol groups are shielded from oxidative effects by disulfide bonds. In earlier studies, this type of protection was performed with 6-mercaptonicotinamide [10,15] 2-mercaptonicotinamide [16,17] or 3-methyl-1-phenylpyrazole-5-thiol [18]. Thanks to the addition of these protective agents, the thiolated polymers have improved stability and maintain their mucoadhesive and other properties [10]. A disadvantage of this method is the longer synthetic pathway.

In our work, thiolated poly(aspartic acid) (ThioPASP) was used. PASP is a biocompatible, biodegradable, non-toxic polymer and does not generate immunogenicity [19, 20]. ThioPASP polymers are designed to be used in ophthalmic therapy as liquid formulations which display a solution-to-gel transition [21] due to disulfide formation at the ocular surface. This type of *in situ* gelation permits the development of a formulation of ThioPASP that is easy to administer as solution and has a prolonged residence time thanks to the mucoadhesive gel formation. However,

the undesired formation of disulfides under atmospheric conditions might deteriorate the swelling, adhesive and related properties of the polymer. Accordingly, in this study the solutions of ThioPASP were stabilized by the addition of three types of reducing agents: DTT, glutathione (GSH) and acetylcysteine (ACC). DTT is a strong, while GSH and ACC are considered to be weaker reducing agents [22].

DTT has been a preferred reagent for the reduction of disulfide bonds for decades, even though it is expensive and unstable in solution [22, 23]. GSH can be found in all parts of cells, where its main function is to provide the main redox buffer and to protect cells from oxidative stress. Thanks to the high concentration of GSH in the cytosol, protein disulfide bonds are rarely formed [24] ACC has low toxicity and mild side-effects when administered intravenously, orally or by inhalation [25]. It is used in ophthalmic therapy as an excipient and also as an active pharmaceutical ingredient (API). It has favourable effects in corneal diseases such as keratoconjuctivitis sicca, filamentary keratitis or corneal ulcers [26]. DTT was chosen as a reference antioxidant, and GSH and ACC as compounds present in cells or used pharmaceutically; they are considered to be safe and non-toxic, especially in ocular therapy.

The aim of our work was to determine the effects of the antioxidant agent on the polymer stability in aqueous solution, the final gel structure and properties such as their swelling capability, rheology, mucoadhesion and drug release. In possession of the results, we planned to find the optimal stabilizing agent for the ThioPASP polymers, which will provide an appropriate structure for mucoadhesion and drug release to ensure a potential *in situ* gelling ocular drug delivery system.

2. Materials and methods

2.1. Materials

Previously synthetized ThioPASP polymers were used [21, 27] and the molar ratio of thiolated repeating units to the total number of repeating units was calculated to be 10% n/n.. Polymer samples were prepared with different stabilizing agents. The following reducing agents were added to the samples before the lyophilization procedure: DTT (Merck, Darmstadt, Germany), GSH (Merck, Darmstadt, Germany) and ACC (Reanal, Budapest, Hungary). The amount of antioxidant was 1% w/w of the polymer in each case. The polymer samples stabilized with these reducing agents are referred to below as ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC,

respectively. Chemical structures of the polymer and the antioxidants are included in supporting information for better understanding.

As a model oxidant, 1 M $NaBrO_3$ solution was used. A phosphate-buffered saline (PBS) solution of pH-= 7.4 was prepared by dissolving 8 g dm^{-3} NaCl, 0.2 g dm^{-3} KCl, 1.44 g dm^{-3} $Na_2HPO_4 \cdot 2H_2O$ and 0.12 g dm^{-3} KH_2PO_4 in distilled water; the pH was adjusted with 0.1 M HCl. Lachrymal fluid of pH = 7.4 was prepared by dissolving 2.2 g dm^{-3} $NaHCO_3$, 6.26 g dm^{-3} NaCl, 1.79 g dm^{-3} KCl, 96.4 mg dm^{-3} $MgCl_2 \cdot 6H_2O$ and 73.5 mg dm^{-3} $CaCl_2 \cdot H_2O$ in distilled water; the pH was adjusted with 1 M HCl [27]

Mucin (porcine gastric mucin type II, for mucoadhesion measurements), and sodium diclofenac (SD, for drug release measurements) were purchased from Sigma Aldrich (USA, St Louis, MO).

2.2. Determination of the concentration of free thiol groups

The stability of polymer in presence of antioxidant was investigated in aqueous solution (pH = 7.4 PBS solution), which were stored at -20 °C, 4 °C and 25 °C. The stability of polymer was indicated by the free thiol content. The percentage of free thiol groups was determined on 0, 1, 7, 14 and 28 days. For each measuring point, the solutions containing 10% w/w of polymer with different polymer-antioxidant combinations were prepared in triplicate. The thiol content of the polymer solutions containing stabilizing agent was determined by Ellman's assay similarly to the method described earlier for non-stabilized ThioPASP [28]. At pre-determined time intervals, one polymer solution (10% w/w of polymer; PBS pH = 7.4) of each composition (ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC) was diluted with buffer solution (pH = 8) to yield a final polymer concentration of approximately 0.05% w/v at room temperature and analysed immediately after dilution. 180 µl of each sample solution was diluted in 1800 µl aqueous buffer solution (pH = 8 containing 1 mM ethylenediaminetetraacetic acid disodium salt) and 20 µl of 10 mM Ellman's solution was added to the mixture [29]. The absorption spectrum of the solution was recorded with a UV-VIS spectrophotometer (Specord 200, Analytic Jena, Jena, Germany) after a reaction time of 20 min (T = 37 °C during the reaction). The thiol content of the solution was calculated from the absorption peak at 405 nm, using the calibration curve determined for ACC (ε = 12622 M^{-1} cm^{-1}). The thiol content determined includes the number of thiol groups both on the polymer and on the stabilizing agent.

2.3. Kinetics of swelling

The water absorption capacity of the ThioPASP gels was determined gravimetrically. The ThioPASP gels were prepared in a syringe. The ThioPASP polymer was dissolved in PBS and 1 M oxidant solution was than added. The final concentration of both the polymer and the 1 M oxidant solution was 20% w/w. After gelation, the gel was pushed out of the syringe, cut into four equal parts (for the four parallel measurements) and dried in vacuum at 30 °C to constant weight. The weights of the discs were measured and they were placed into distilled water at room temperature. The discs were taken out of the water after defined time periods, the surplus water was removed by blotting and the weights of the gels were measured gravimetrically [27].

2.4. Morphology/internal structure

The hydrogels containing 10% w/w ThioPASP and antioxidant swollen in PBS were freeze-dried and sputter-coated with gold (Bio-Rad SC 502, VG Microtech, Uckfield, United Kingdom). The internal structure of the ThioPASP networks was determined by scanning electron microscopy (SEM) (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan).

2.5. Rheology

The rheological properties were studied with a Physica MCR101 rheometer (Anton Paar, Graz, Austria). The measuring device was of cone and plate type (diameter 25 mm, gap height in the middle of the cone 0.046 mm, and cone angle 1°). To find a standard, reproducible oxidant circumstance, in our tests we used sodium bromate as model oxidant to simulate the oxidative state of the eye, which can help to study the gelation properties of our polymer, and compare the effect of the applied antioxidant on it. ThioPASP polymers containing different antioxidants were dissolved in PBS at three concentrations and the polymer solutions were mixed with 1 M oxidant on the plate of the rheometer to simulate the contact with the eye and the investigation was started immediately to follow the gelation from the beginning of the contact. The final polymer concentrations were 5, 7 or 10% w/w and the final concentration of the oxidant was 20% w/w. When the mucoadhesion was studied, the polymer solutions were mixed with the mucin dispersion in PBS before the addition of the oxidant (the final mucin concentration in the mixtures was 5% w/w and both the final polymer and oxidant concentrations were identical to those of the samples prepared without mucin). Each measurement was carried out on a freshly-made sample

and was started immediately after mixing of the compositions. The gelation of the ThioPASP polymer was followed at a constant frequency of 1.0 Hz at a constant strain of 1% at 25 °C. In our investigation, the gelation time was defined as the time at which a maximum could be observed in the curve of the differential with respect to time [27] (differential curves are not presented in the paper). Viscoelastic character was determined by frequency sweep tests after the gelation, with a strain of 1% at 25 °C. Storage modulus (*G'*) was determined over the angular frequency range from 0.1 to 100 s^{-1}. The strain value (1%) used in the measurements was in the range of the linear viscoelasticity of the gels [27].

2.6. Adhesion test

Adhesion tests were performed with a TA-XT Plus (Texture analyzer, ENCO, Spinea, Italy) instrument equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm. Samples containing different amounts of ThioPASP were dissolved in PBS and 20% w/w of 1 M oxidant solution. 20 mg of the sample was attached to the cylinder probe and was gelated for 10 min. The sample was then placed in contact with a filter paper disc wetted with 50 µl mucin dispersion (8% w/w, prepared in simulated lachrymal fluid (pH = 7.4)) or with simulated lachrymal fluid, and a 2500 mN preload was applied for 3 min (the compression speed was 2.5 mm min^{-1}). The cylinder probe was then moved upwards to separate the sample from the substrate at a prefixed speed of 2.5 mm min^{-1}. 10 parallel measurements were carried out [30]. The work of adhesion (*A*, mN mm) was calculated as the area under the "force versus distance" curve (AUC) [27, 31, 32].

2.7. Drug release

The drug release profile of SD was determined with a vertical Franz diffusion cell system (Hanson Microette Plus TM, Hanson Microette TM Topical &Transdermal Diffusion Cell System; Hanson Research Corporation, Chatsworth, CA). 300 µl of formulation containing 7% w/w polymer solution with 0.1% w/w SD and 20% w/w oxidant solution in final concentration was placed on a Porafilm membrane filter (pore size 0.45 µm, Porafil; Machenerey-Nagel, Germany, and Pall Life Sciences, Washington, NY) impregnated with pH = 7.4 buffer solution. The two solutions were mixed on the membrane filter as a donor phase. 7 ml of PBS (pH = 7.4) was used as acceptor phase and thermostated at 35 °C. Measurements were performed for 24 h and five parallel measurements were carried out. 0.8 ml samples were taken from the acceptor phase by the

autosampler and replaced with fresh PBS. The released SD was quantified by UV spectrophotometry (Helios α Thermospectronic UV-spectrophotometer v4.55, Unicam; Thermo Fisher Scientific, Waltham, MA) at 275 nm [27, 33].

2.8. Statistical analysis

The results of stability, swelling, mucoadhesion, and drug diffusion were also analyzed statistically with GraphPad Prism version 5 software. Two-way ANOVA analysis was applied with Bonferroni post-tests. A level of $p \leq 0.05$ was taken as significant, $p \leq 0.01$ as very significant,and $p \leq 0.001$ as highly significant.

3. Results and discussion

3.1. Stability of polymers

The stability of components in a dosage form is a crucial issue, and must therefore be investigated during formulation development. As earlier studies [8, 9] showed, thiolated polymers have poor stability in aqueous solutions at $pH > 6$ because of the atmospheric oxidation of thiol groups resulting in the formation of inter- and intramolecular disulfide bonds. In ocular therapy, aqueous formulations are preferred, and the stability of the components in this medium is therefore a very critical factor.

In our work, different reducing agents were used to stabilize aqueous ThioPASP formulations and the effects of additives and the storage conditions of the formulations were investigated. The concentration of the antioxidant were set at a minimum, 1% w/w reducing agent referring to the polymer amount was applied. In case of ophthalmic formulation, the low concentration of additives is important to avoid the irritation and other side effects.

The results can predict the time within which the dosage form can be safely used after the preparation of the solution. Aqueous polymer solutions were stored frozen (-20 °C), in a refrigerator (4 °C), and at room temperature (25 °C), and the percentage of free thiol groups was determined at given times (0, 1, 7, 14 and 28 days). The decrease in the number of free thiol groups during storage is shown as a function of time in Fig 1.

The changes in the free thiol content during storage indicate that the polymer solutions are stable within 24 h (the free thiol content is > 90%), but the thiol content decreases significantly after the first day, independently of the storage temperature. The most marked fall can be seen at 25 °C (There were no significant differences between the formulations at this storage temperature, $p > 0.05$ in each cases).

Although the thiol groups include those of small molecular antioxidants and of the polymer, the results can give a guideline for choosing the proper additive. ThioPASP/ACC and ThioPASP/GSH can preserve the free thiol content at above 90% for 7 days while thiol content of formulations containing DTT highly decreases. Without a deeper analysis of the mechanism of the antioxidant effect, ACC and GSH are recommended for the stabilization of ThioPASP because the major part of the thiol groups remain reduced (free thiol content of ThioPASP/GSH is significantly, and very significantly higher than that of ThioPASP/DTT on 14 and 28 days respectively, at 4 °C). In ThioPASP/DTT samples, oxidation of thiol groups occurs in a large extent and we can assume that the thiol groups either of the antioxidant or the polymer, or even of both components are strongly oxidized after one day.

3.2. Swelling

The swelling properties of a dosage form must be taken into consideration in each process where water uptake can take place, such as drug release and mucoadhesion (where the sample meets a hydrated surface such as a mucin dispersion or the mucosa). The swelling of non-hydrated or partially-hydrated samples can affect the interpenetration, the entanglement of the polymer chains and the formation of chemical interactions.

Gravimetry was used to determine the swelling properties of ThioPASP gels. Figure 1 shows the water-uptake capacity (*%S*), calculated from the following equation [34]

$$\%S = \frac{M_t - M_0}{M_0} \times 100 \quad (1)$$

where M_0 is the mass of the dry gel (g) and M_t is the mass of the swollen gel (g). This value (*% S*) gives information about the water-uptake capacity of the polymer.

During the 6 h measurements, the swollen ThioPASP/DTT and ThioPASP/ACC polymer discs remained mechanically stable due to the formation of disulfide linkages between the polymer chains.

Figure 2 indicates that ThioPASP/DTT has a lower water-uptake capacity than that of ThioPASP/ACC. The ThioPASP/GSH polymer disintegrated after 1 h, and therefore the final water uptake of these gels cannot be taken into consideration. The initial parts of the curves of ThioPASP/ACC and ThioPASP/GSH reveal the similar swelling properties of these hydrogels, although the final values cannot be compared because of the partial disintegration of the latter. The tentative explanation of these results that during the cross-linking of ThioPASP/ACC and ThioPASP/GSH samples polymer-polymer disulfides, dimers of the stabilizing agent and asymmetrical disulfides consisting of polymer chains and ACC or GSH can also be formed simultaneously. In the case of ThioPASP/DTT, intramolecular disulfide formation of DTT is preferred leading to smaller amount of asymmetrical polymer-DTT disulfides. This results in a lower cross-linking density of ThioPASP/ACC and ThioPASP/GSH samples as asymmetrical disulfides do not act as netpoints, while in the case of ThioPASP/DTT samples mostly polymer–polymer disulfides are formed. Accordingly, the swelling results for the first 60 minutes are similar for ACC and GSH containing samples (no significant differences up to 60 min), while ThioPASP/DTT had a higher number of netpoints, resulting in a smaller degree of swelling (there are highly significant difference between ThioPASP/ACC and ThioPASP/DTT after 30 min).

3.3. Morphology

The ThioPASP/GSH polymer gel fell apart during the swelling measurement, while ThioPASP/DTT and ThioPASP/ACC remained coherent. The difference in the internal structures of the gels can be clearly seen in the SEM pictures (Fig. 2). ThioPASP/DTT and ThioPASP/ACC have a continuous structure with rather large pores, while ThioPASP/GSH shows a more porous structure. The poor mechanical stability is not preferred at the application site, because it can result in unpredictable drug release, residence time and bioavailability.

3.4. Rheology

The gelation time can be critical in case of an *in situ* gelling ophthalmic formulation: too fast gelation can lead to inadequate spreading on the surface and foreign body sensation to the patient, while an elongated gelation results in a too fast elimination by tear drainage and blinking. ThioPASPs are redox-sensitive systems, therefore they display a solution-to-gel transition in the presence of oxidizing agent. The eye is highly exposed to oxidative stress, the most important

oxidants are reactive oxygen species (ROS) and reactive nitrogen species (RNS). The concentration of the ROS/RNS in the eye is very high due to its exposition to several environmental factors like high pressure of oxygen, light exposure, ultraviolet and ionizing radiation, foreign chemicals and pathogenic microbes, which can also produce oxidant species [35]. In *in vivo* circumstance, ThioPASP solutions can probably gelify thanks to this oxidative effect of the eye, but in our test we used sodium bromate as model oxidant to simulate this state.

The gelling process can be traced by changes of the storage modulus. The storage modulus (G') represents the elastic behaviour of the sample, therefore shows the changes in the gel state. In previous work [27], we proved that mucin plays an important role in gelation, by decreasing the gelation time and increasing the final value of the storage modulus. We therefore determined the gelation time of the ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC polymers at different polymer concentrations (5, 7 and 10% w/w) to assign the effects of the antioxidants to the gelation time and gel strength (Fig. 3).

At low polymer concentration (5% w/w), no gelation was observed in the case of ThioPASP/DTT or ThioPASP/ACC, while ThioPASP/GSH showed moderate gelation after 5 min in mucin free-samples. After the addition of mucin, a weak gel structure evolved in ThioPASP/GSH and ThioPASP/ACC samples at the beginning of the measurement, but in the case of ThioPASP/DTT only after 15 min. At medium polymer concentration (7% w/w), ThioPASP/DTT and ThioPASP/GSH were able to form a gel structure without mucin, but ThioPASP/ACC did not start to gelify. In the presence of mucin ThioPASP/GSH and ThioPASP/DTT samples showed a transformation from a sol to a gel within 10 min. At high ThioPASP concentration (10% w/w), in both the mucin-containing and the mucin-free samples, ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC formed a gel structure within 5 min.

The nature of the reducing agent influences the gelation time in the mucin-containing and mucin-free samples at low (5% w/w) and medium (7% w/w) polymer concentration. Increase of the ThioPASP concentration decreased the gelation time. The ThioPASP/DTT had the longest gelation time at all concentrations, which was decreased by the addition of mucin, suggesting the strongest antioxidant (reducing) effect against the oxidizing agent. In the mucin-free samples, the ThioPASP/ACC sample at low polymer concentrations (5 and 7% w/w) did not gelify, while at 10% w/w ThioPASP concentration the gelation was as fast as in the case of ThioPASP/GSH. In the mucin-containing samples, immediate gelation could be measured at low polymer

concentrations (5 and 7% w/w), which is indicated by the elevated *G'* values. Our results demonstrate that the gelation process is effected by the type of the oxidizing agent, the polymer concentration, and the presence of mucin. At 10% w/w polymer concentration, the gelation occurred within 5 min and did not immediately. In our previous work we presented wash away and adhesion test as well where this type of polymers showed high resistance against the simulated elimination mechanism even in sol state of the system [27]. In view of this earlier statement, probably a thin layer of the polymer solution can remain on the ocular surface which does not cause foreign body sensation to the patient, and after the total gelation provide a long-lasting effect.

The frequency sweep test was started after full gelation. Figure 5 depicts the variation in *G'* with angular frequency for the ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC formulations with mucin-containing and mucin-free samples.

At all polymer concentrations and in all cases (mucin-containing and mucin-free), each sample showed a frequency-independent storage modulus. At low polymer concentration (5% w/w), there was no significant difference between the mucin-containing and mucin free-samples in the ThioPASP/DTT samples. In the cases of ThioPASP/GSH and ThioPASP/ACC a significant increases in *G'* could be seen after the addition of mucin. At medium polymer concentration (7% w/w), a significant difference could be observed only in the case of ThioPASP/ACC between the mucin-containing and mucin-free samples. The values of *G'* of ThioPASP/DTT and ThioPASP/GSH samples were not higher in the presence of mucin. The increase of the *G'* values in the presence of mucin suggests an interaction between the polymer and the mucin chains. At the highest polymer concentration, there was a negligible difference between the *G'* values with and without mucin, which indicates the formation of a stiff gel structure independently of the presence of mucin. Furthermore, a very slight decrease was observed in the case of the mucin-containing samples; we believe that mucin-polymer interactions are still present at this concentration, but the gel structure weakens due to the heterogeneity of the mucin dispersion.

The rheological mucoadhesion measurements demonstrated that the ThioPASP/DTT sample does not have strong mucoadhesive properties and possibly does not favour the formation of covalent bonds between thiolated polymers and mucin glycoproteins via thiol/disulfide exchange reactions. ThioPASP/GSH and ThioPASP/ACC are mucoadhesive up to 7% w/w polymer concentration, suggesting that ThioPASP/DTT has a strong reducing capability which hinders the chemical interaction of ThioPASP and mucin, while GSH and ACC do not impede disulfide

formation between the polymers, while providing sufficient atmospheric stability of ThioPASP at the same time.

3.5. Adhesion tests

The mucoadhesion of the ThioPASP samples was characterized by tensile tests, which involved the measurement of the force of detachment (*F*, mN) and the total work of adhesion (*A*, mN.mm), the latter calculated from the area under the force–distance curve [36]. The measured values of *F* and *A* are depicted in Fig. 6.

The values of *F* in the case of the ThioPASP/DTT sample increased continuously as the concentration was elevated until 7% w/w polymer concentration, above which a plateau was observed. At 3, 5 and 7% w/w polymer concentrations, an increase in polymer concentration was accompanied by an increase in the number of free thiol groups at the interface, which are able to form disulfide bonds with mucin. At high polymer concentration, the ThioPASP gel is strongly cross-linked by both chemical and physical bonds and entanglements. The number of accessible free thiol groups on the polymer is therefore decreased and the interpenetration of the polymer and the mucin is limited. Similar observations can be seen in the case of the *A* values. At high polymer concentration (10% w/w), the low number of free thiol groups and the highly cross-linked structure limited the interpenetration of the gel into the mucin layer, resulting in a plateau in the *A* curve. As we described previously (swelling), in the presence of DTT as reducing agent, the higher number of polymer–polymer disulfide linkages and the shorter free chain length decrease the interpenetration of the polymer chains of mucin and the sample.

The *F* curve of ThioPASP/GSH increased continuously with increasing polymer concentration. At 7% w/w polymer concentration, a weak gel formed, resulting in an easier rupture during the tensile test, and thus the cessation of adhesion is mainly due to the cohesive failure of the gel. In spite of the weak structure, the number of free thiol groups increased as the ThioPASP concentration was elevated, resulting in increases in the *F* and *A* values.

The ThioPASP/ACC sample at low polymer concentrations (3 and 5% w/w) displayed a constant *F* value because at those concentrations the sample is in a sol state and the lack of cohesion results in a poor mucoadhesive character. At 7% w/w polymer concentration, gelation occurred, as shown in the rheological experiments, resulting in the significant increase in *F* values, but at higher polymer concentration (10% w/w) the number of free thiol groups did not increase at the

surface, which resulted in a plateau in the *F* curve. In the case of *A* at high polymer concentration a continuous increase in the values can be seen thanks to the increasing interpenetration.

The results suggest the existence of an optimum polymer concentration for interpenetration and the formation of mucoadhesive interactions. Our results show that ACC can be a potential stabilizing agent (in most cases *F* and *A* values are significantly higher compared with those of ThioPASP/GSH and ThioPASP/DTT); the cross-linked structure formed provides considerable mucoadhesion, because a sufficient amount of free thiol groups is available to establish a strong polymer–mucin interaction, and the atmospheric stability of the ThioPASP in solution is also sufficient when this antioxidant is used.

3.6 Comparison of results of the rheology and adhesion tests

The results of rheological measurements and adhesion tests can be correlated easily by the calculation of synergism parameters of rheology ($\Delta G'$) can be determined by dynamic oscillatory rheometry, calculated as follows [27, 37]:

$$\Delta G' = G'_{(mix)} - (G'_{(polymer)} + G'_{(mucin)}) \quad (2)$$

where *G'* is the storage modulus of the systems.

If the calculated synergism parameters are negligible, it is reasonable to use the relative rheological synergism parameters ($\Delta G'_{rel}$), which express the relative increments in viscoelasticity with regard to the polymer and mucin solutions alone [27, 37]:

$$\Delta G'_{rel} = \Delta G'/(G'_{(polymer)} + G'_{(mucin)}) \quad (3)$$

In the case of adhesion tests, the normalized mucoadhesion parameters (ΔAUC/AUC) can be used, calculated as follows [38]:

$$\Delta AUC/AUC = (AUC_m\text{-}AUC_b)/AUC_b \quad (4)$$

where AUC_m is the work of adhesion in the presence of mucin and AUC_b is the work of adhesion of blank measurements (with simulated lachrymal fluid).

The rheological measurement of mucoadhesion is a bulk method, where the polymer and the mucin are mixed, while in the case of adhesion tests mucoadhesion is measured at the interface between the polymer and the mucin. The mucoadhesion occurs at the interfacial layer, the thickness of which depends on the interdiffusion between the mucin and the polymer molecules.

Figure 7 presents the calculated absolute and relative synergism parameters of *G'* at an angular frequency of 1 s^{-1}. The calculated normalized mucoadhesion parameters are depicted in Fig. 8.

The ThioPASP/DTT formulations in the bulk rheological method did not show synergism with mucin; moreover, increase of the polymer concentration further decreased the synergism, resulting in more and more negative $\Delta G'$ and very low $\Delta G'_{rel}$ values. However in the adhesion tests as surface method, mucoadhesion could be observed which increased as the concentration was elevated, but the normalized ΔAUC/AUC values indicate that the higher netpoint density of the ThioPASP/DTT samples hinders the formation of new polymer–mucin interactions as the concentration increases.

ThioPASP/GSH at low polymer concentration was mucoadhesive in the bulk method, but with increase of the polymer concentration its mucoadhesivity decreased. As compared with the DTT-stabilized samples, the adhesion tests indicated weak synergism with mucin, which can be explained by its weak cohesivity and resistance against the tensile load.

Up to 7% w/w polymer concentration, the ACC-stabilized formulation displayed marked synergism as compared with the other two types of formulation. At 7% w/w ThioPASP/ACC, the gel structure was formed, which can provide optimum mucoadhesion proved by both rheological experiments and adhesion tests (high values of $\Delta G'$, $\Delta G'_{rel}$ and ΔAUC/AUC).

3.7. Drug release

SD release from the ThioPASP/DTT, ThioPASP/GSH and ThioPASP/ACC gels (as optimum, 7% w/w polymer concentration) was determined with a vertical Franz diffusion cell system for 24 h (Fig. 9).

The results indicate that the ThioPASP/GSH gel releases the highest amount of SD (~80%) after 24 h, while ThioPASP/DTT and ThioPASP/ACC release lower amounts of SD and there is no significant difference between the latter two. These results correspond with the results of the swelling and SEM measurements. The very porous ThioPASP/GSH fell apart after 1 h, and therefore SD is able to diffuse easier out from the gel. ThioPASP/DTT and ThioPASP/ACC have a more cohesive structures than that of ThioPASP/GSH, resulting in a slower diffusion of the active component.

The kinetics of the drug release was determined with the Korsmeyer-Peppas equation as follows:

$$\frac{M_t}{M_\infty} = kt^n \quad (5)$$

where M_t and M_∞ are the amounts of drug released at time t and at equilibrium, respectively, while M_t/M_∞ is the fraction of drug released, k is the kinetic constant and n is the release exponent describing the mechanism of the release [39]. These values were determined from the equation of the power law fitted to the curve of the amount of drug released (% w/w) against time (min) and are showed in Table 1 [27].

The fitting results reveal that all the samples undergo non-Fickian diffusion, because the n values are between 0.5 and 1. These results correspond to those of the swelling measurements, where non-Fickian swelling of the gels was also found. The non-Fickian release mechanism is a swelling-controlled mechanism, where simultaneous water uptake and active ingredient desorption occur.

It can be concluded that ThioPASP/GSH has the fastest release (significantly higher values after 5 hours compared with ThioPASP/DTT and ThioPASP/ACC), but this type of formulation cannot be used in controlled release because of the disruption of the gel. Although ThioPASP/DTT and ThioPASP/ACC release lower amounts of SD, their release profile can be designed thanks to their stable structures and these formulations can provide sustained, continuous release.

4. Conclusions

In the present work, three types of reducing agents were used to ThioPASP. The aim was to find an optimum stabilizing agent which can increase the stability of aqueous formulations and ensure an appropriate gel structure for mucoadhesion and drug release. ThioPASP gels were first characterized by swelling and rheological methods, followed by the determination of their mucoadhesion (rheological and tensile tests) and drug release.

The results suggest that the stability of the polymer solution containing antioxidants is sufficient for one day. ThioPASP stabilized with DTT showed fast swelling and drug release, but as compared with the other samples its mucoadhesion is rather weak. ThioPASP stabilized with GSH has the weakest cohesive properties, resulting in fast, but rather uncontrolled drug release and its mucoadhesion is only slightly stronger than that of ThioPASP/DTT. ACC stabilized ThioPASP has an optimum cross-linked structure with free thiol groups ensuring polymer–mucin interactions, resulting in the best mucoadhesive properties. Its swelling properties are than those of the other two formulations, which also plays an important role in mucoadhesion and drug release.

It can be concluded that the optimum stabilizing agent is ACC. At 7% w/w ThioPASP/ACC concentration gel structure can be formed which can be base for a controlled released system and this structure can provide optimum mucoadhesion.

Acknowledgements

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Figure captions

Fig. 1 Changes in free thiol content of ThioPASP polymer solutions during storage with various temperature of storage: a) -20 °C, b) 4°C and c) 25 °C. The stabilizing agents were (■)ThioPASP/DTT; (●) ThioPASP/GSH; (▲) ThioPASP/ACC.

Fig. 2 Swelling kinetics of ThioPASP gels: (■) ThioPASP/DTT; (●) ThioPASP/GSH; (▲) ThioPASP/ACC.

Fig. 3. SEM pictures of lyophilized swollen ThioPASP gels: a) ThioPASP/DTT, b) ThioPASP/GSH and c) ThioPASP/ACC.

Fig. 4. Increase in the storage modulus of the ThioPASP solutions upon oxidation: (■) ThioPASP/DTT; (●) ThioPASP/GSH and (▲) ThioPASP/ACC; with and without (open symbol) mucin at (a) 5, (b) 7 and (c) 10% w/w polymer concentrations.

Fig. 5. Frequency sweep test of ThioPASP gels: (■)ThioPASP/DTT; (●) ThioPASP/GSH and (▲) ThioPASP/ACC; with and without (open symbol) mucin at different polymer concentrations (a) 5, (b) 7 and (c) 10% w/w.

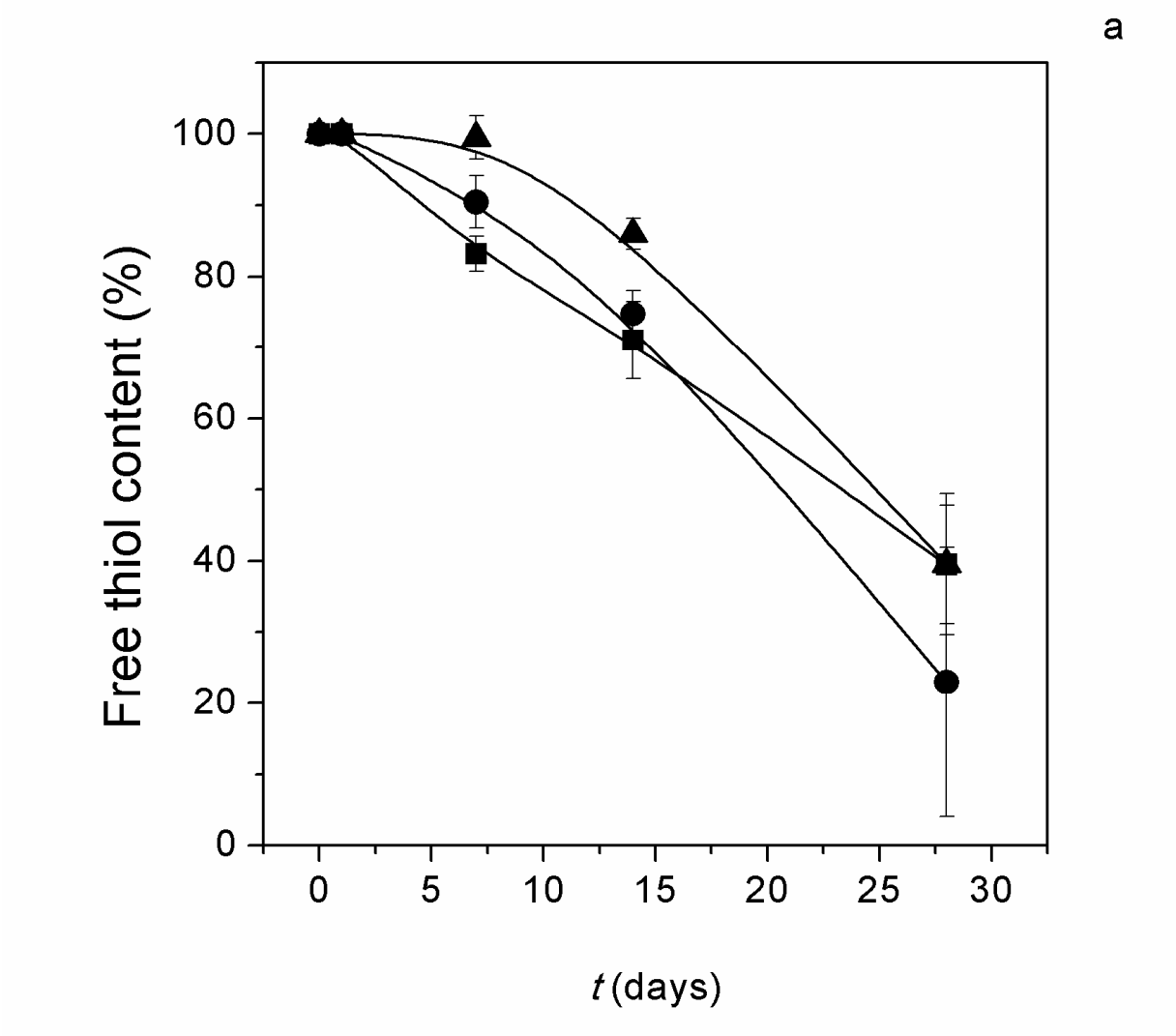
Fig. 6. a) Adhesive force (*F*) and b) adhesive work (*A*) as functions of ThioPASP concentrations: (■)ThioPASP/DTT; (●)ThioPASP/GSH; (▲)ThioPASP/ACC.

Fig. 7. The calculated a) absolute and b) relative synergism parameters of *G'* in rheological measurements.

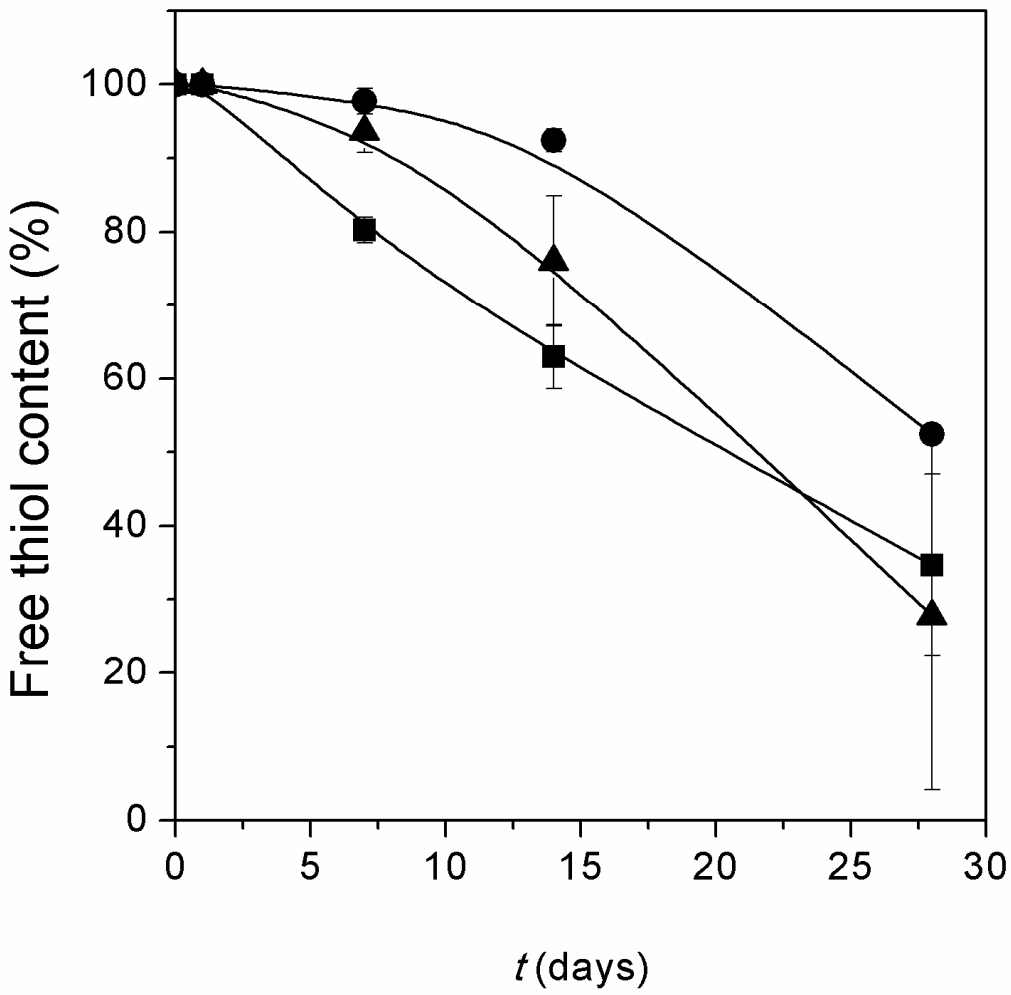
Fig. 8. The calculated normalized mucoadhesion parameters of work of adhesion in tensile test measurements.

Fig. 9. Release of SD from ThioPASP gels at 7% w/w polymer concentration: (■) ThioPASP/DTT; (●) ThioPASP/GSH; (▲) ThioPASP/ACC.

Fig 1



b



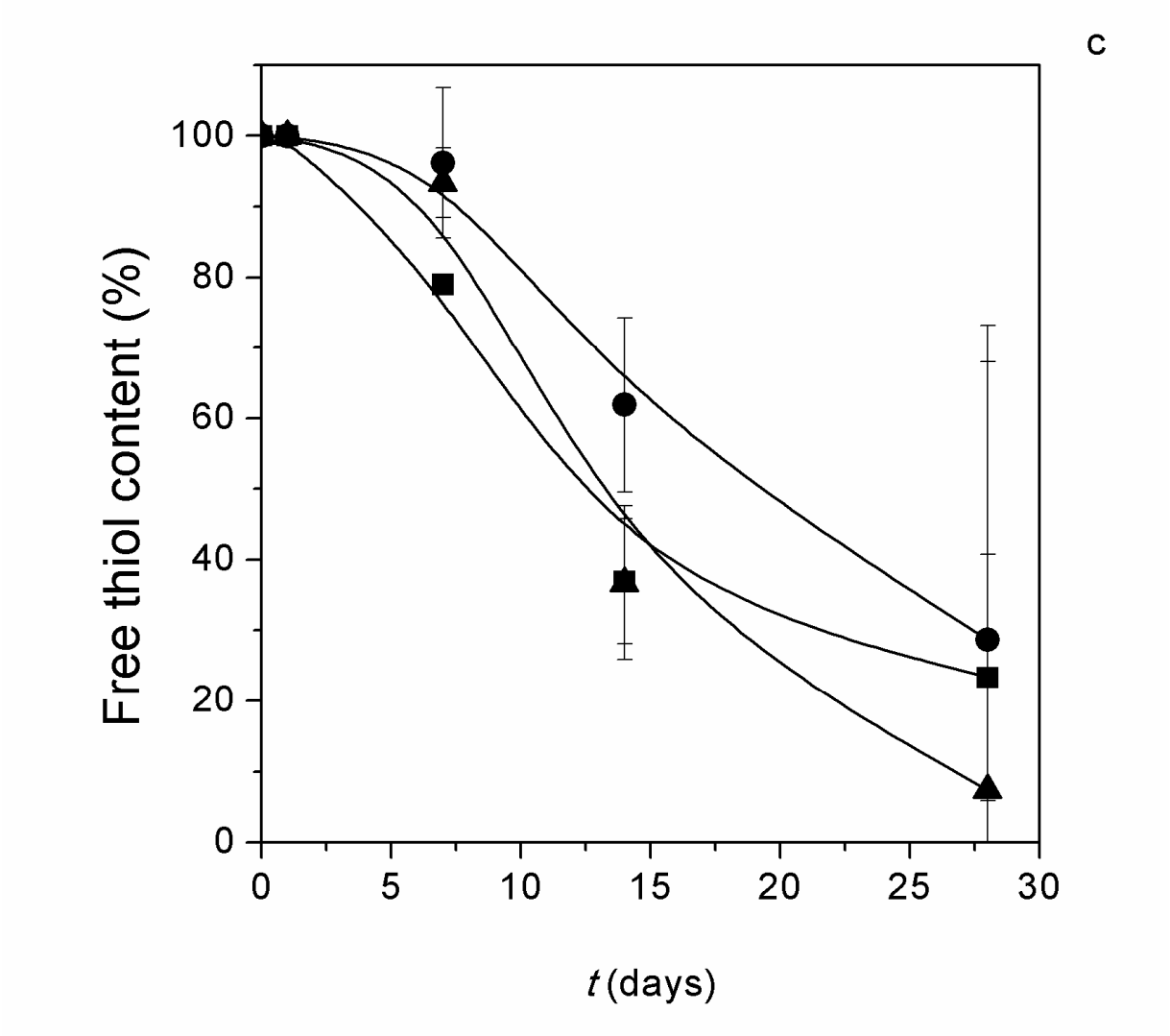


Fig 2.

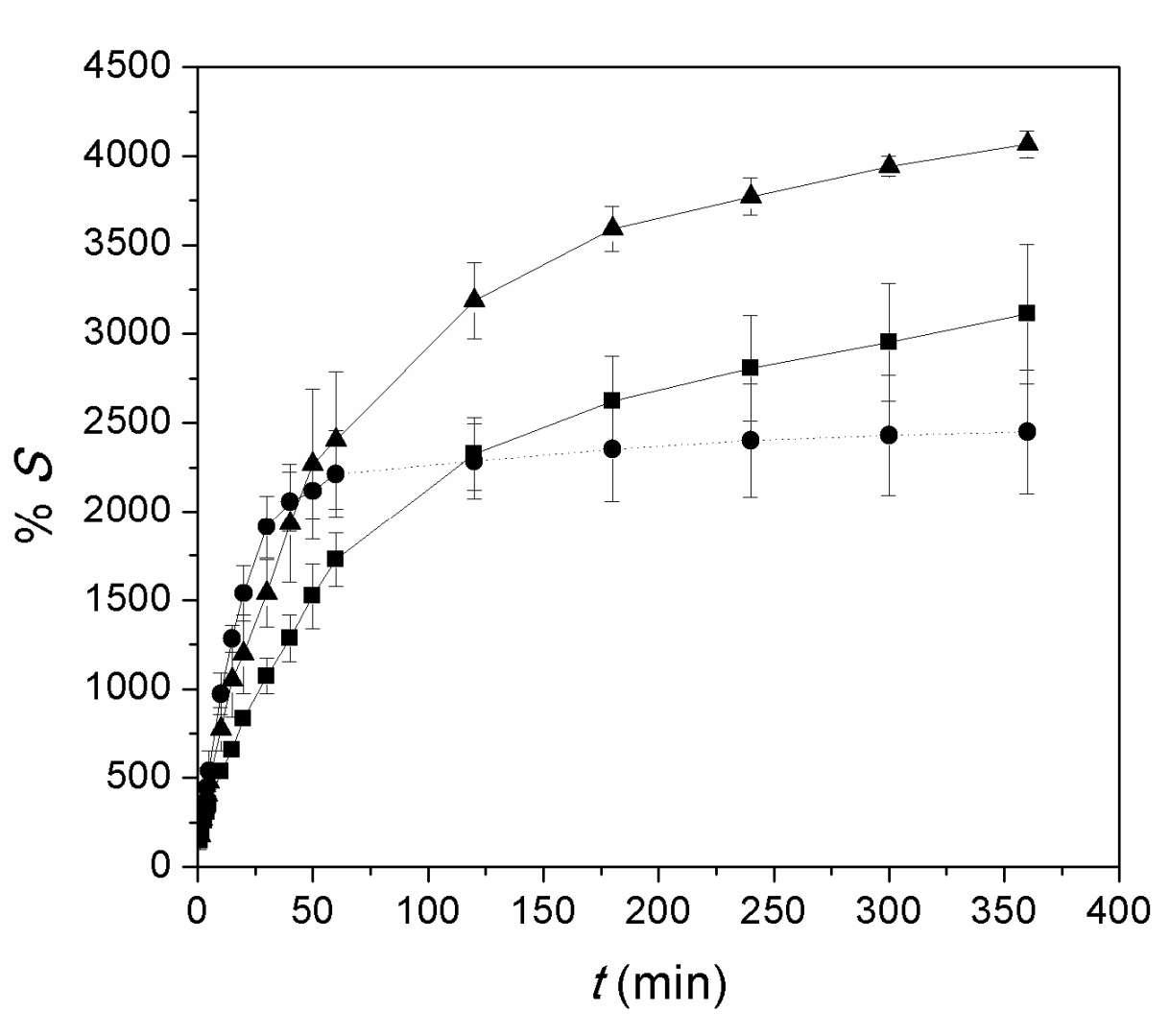
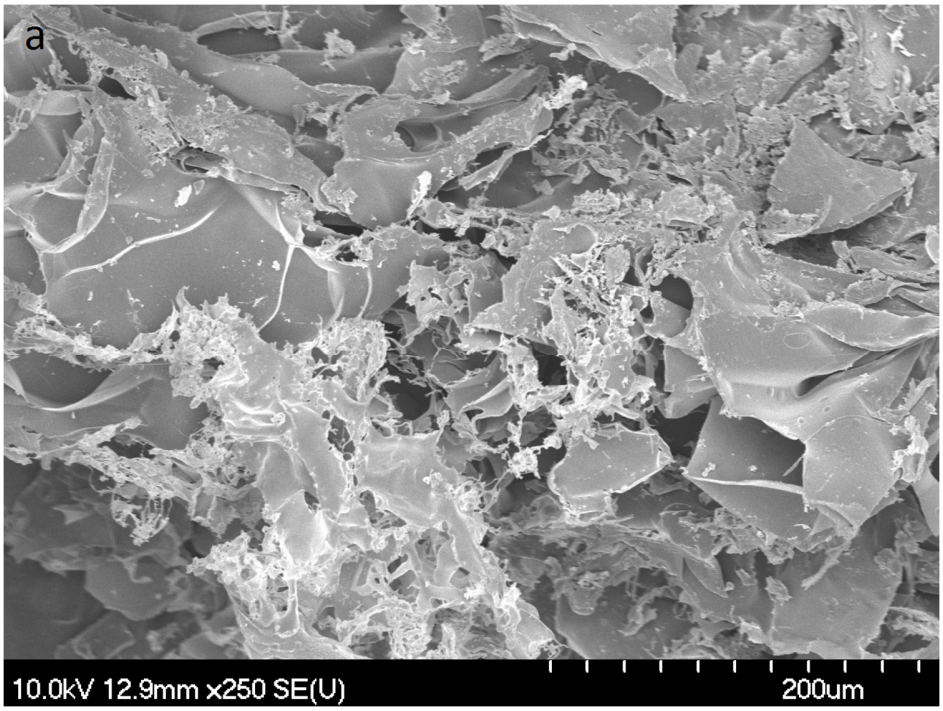
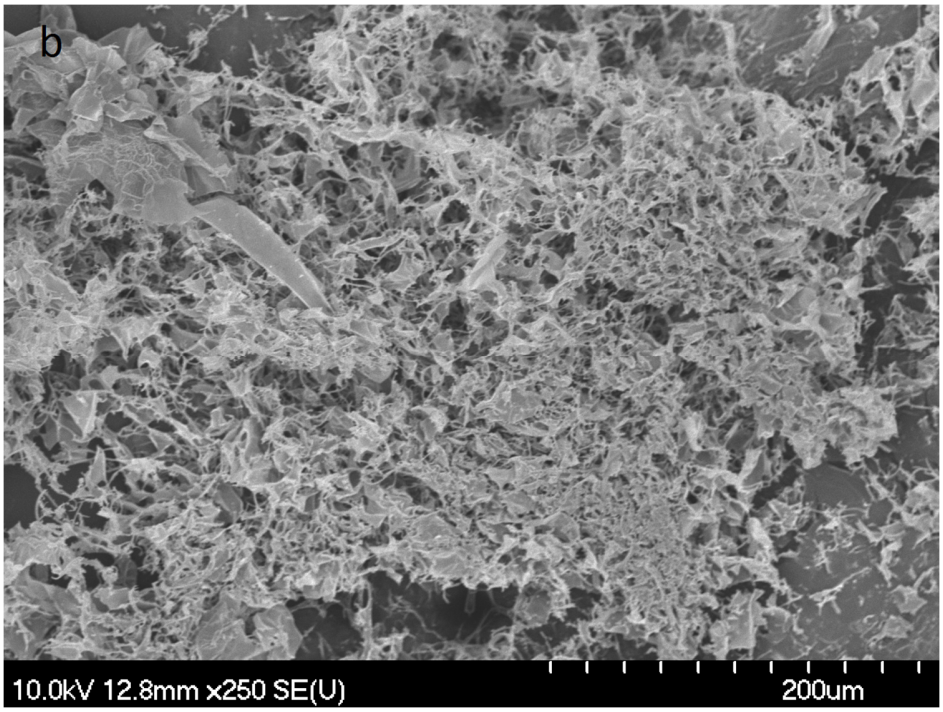

4500
4000
3500
3000
2500
2000
1500
1000
500
0
% S
0
50
100
150
200
250
300
350
400
t (min)

Fig 3.


a
10.0kV 12.9mm x250 SE(U)
200um


b
10.0kV 12.8mm x250 SE(U)
200um

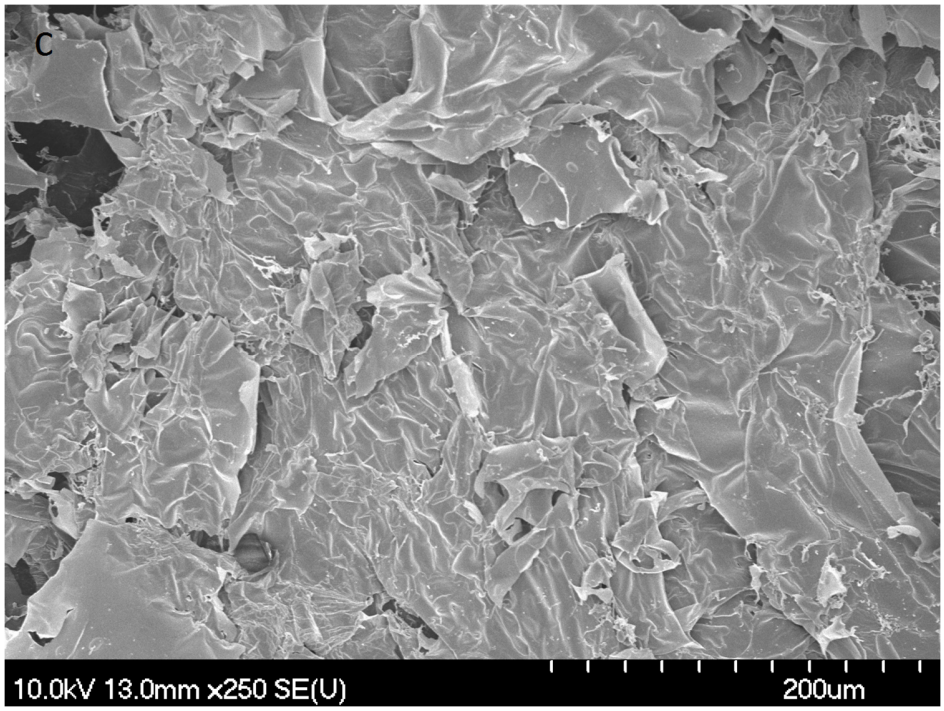
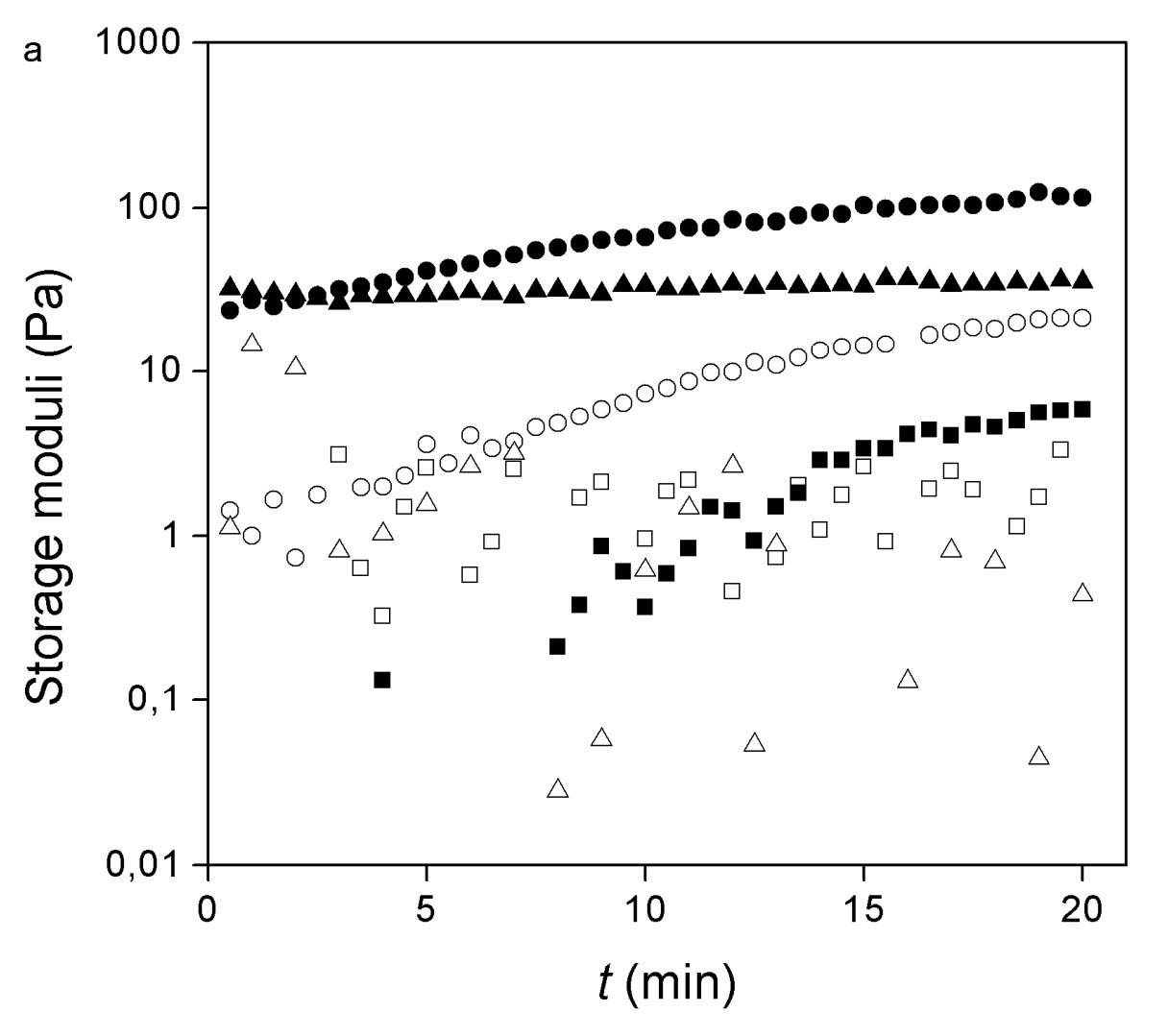
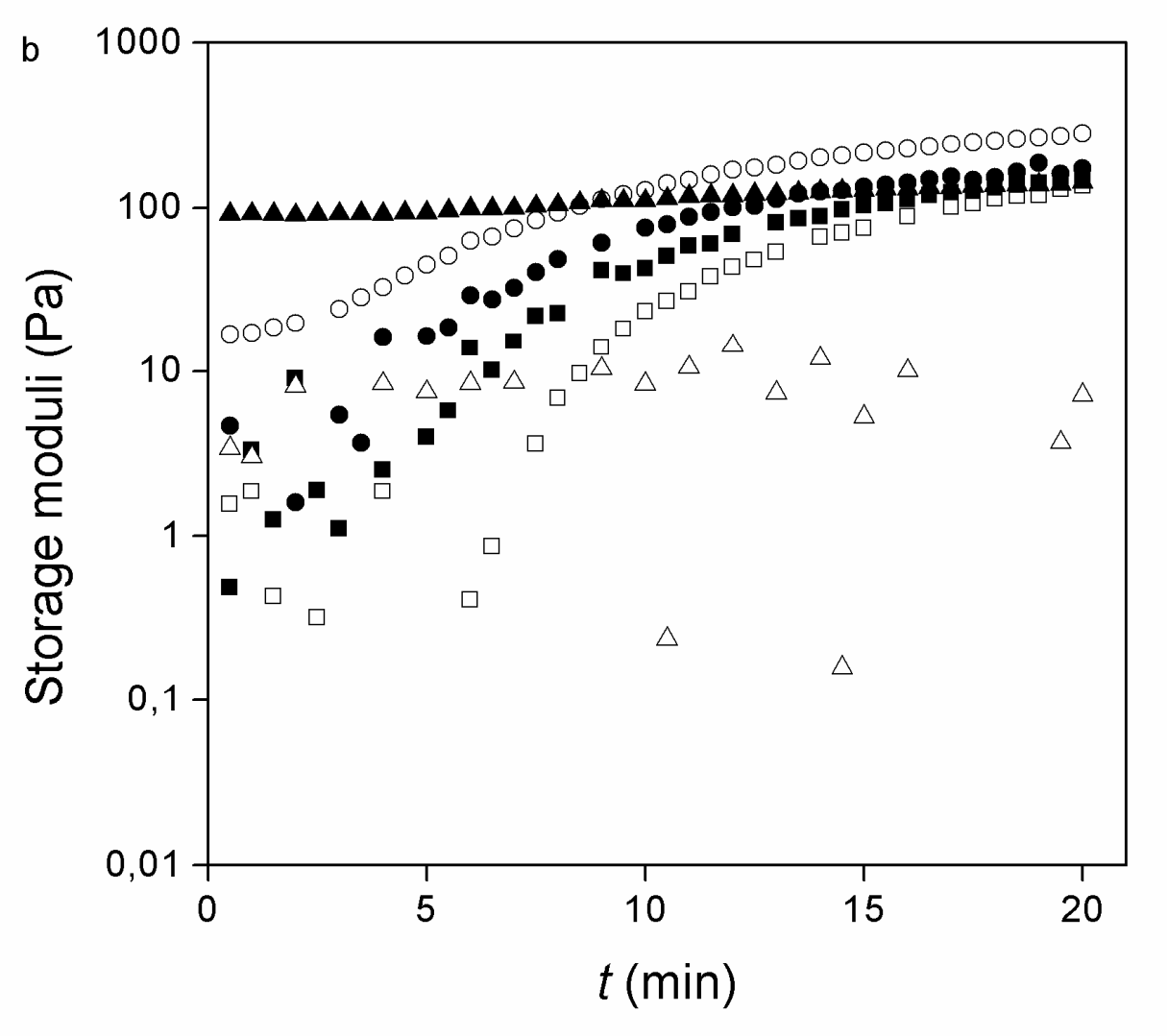


Fig 4.


a
1000
100
10
1
0,1
0,01
Storage moduli (Pa)
0
5
10
15
20
t (min)


b
1000
100
10
1
0,1
0,01
Storage moduli (Pa)
0
5
10
15
20
t (min)

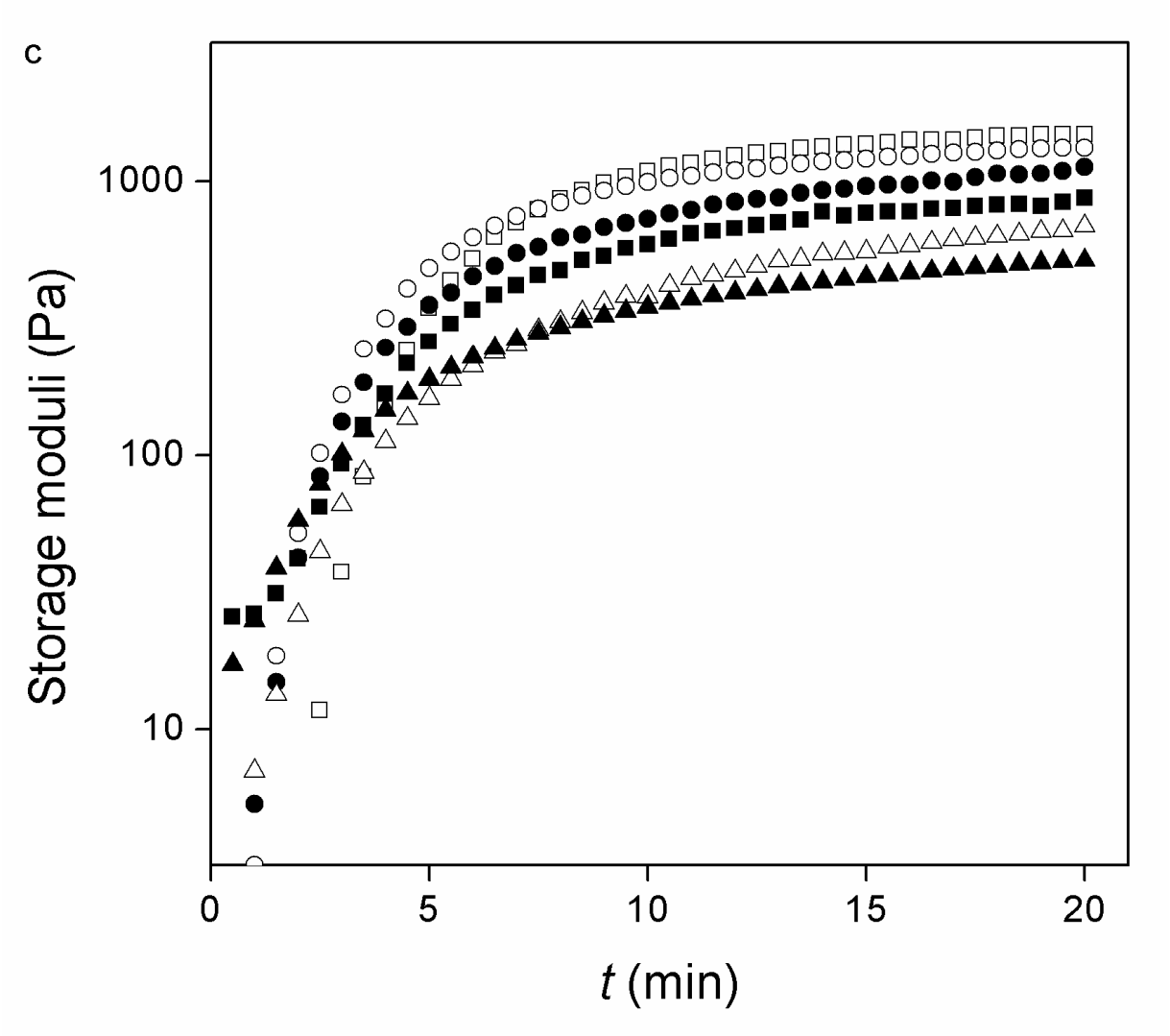
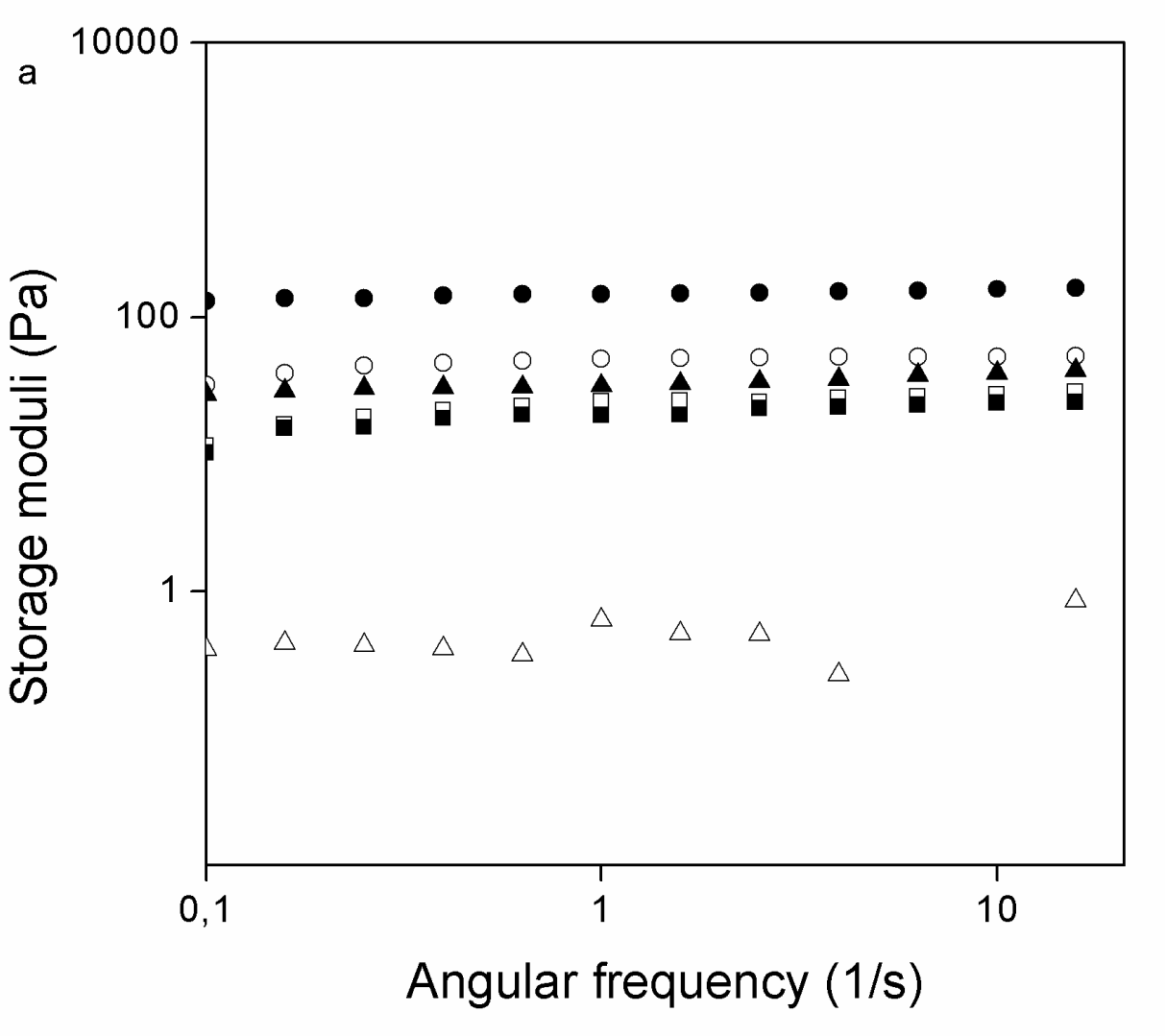
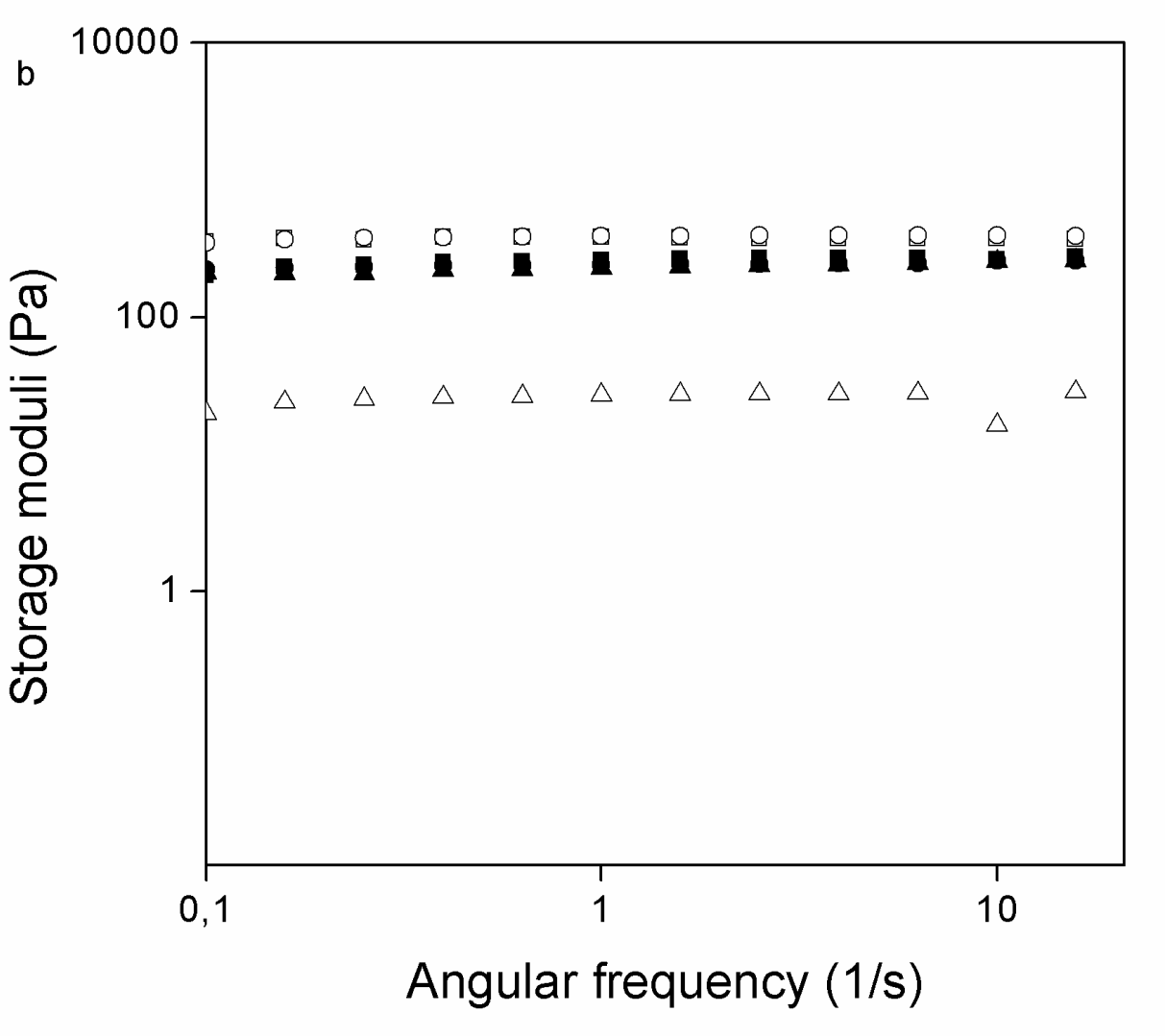


Fig 5.


a
10000
100
1
Storage moduli (Pa)
0,1
1
10
Angular frequency (1/s)


b
10000
100
1
Storage moduli (Pa)
0,1
1
10
Angular frequency (1/s)

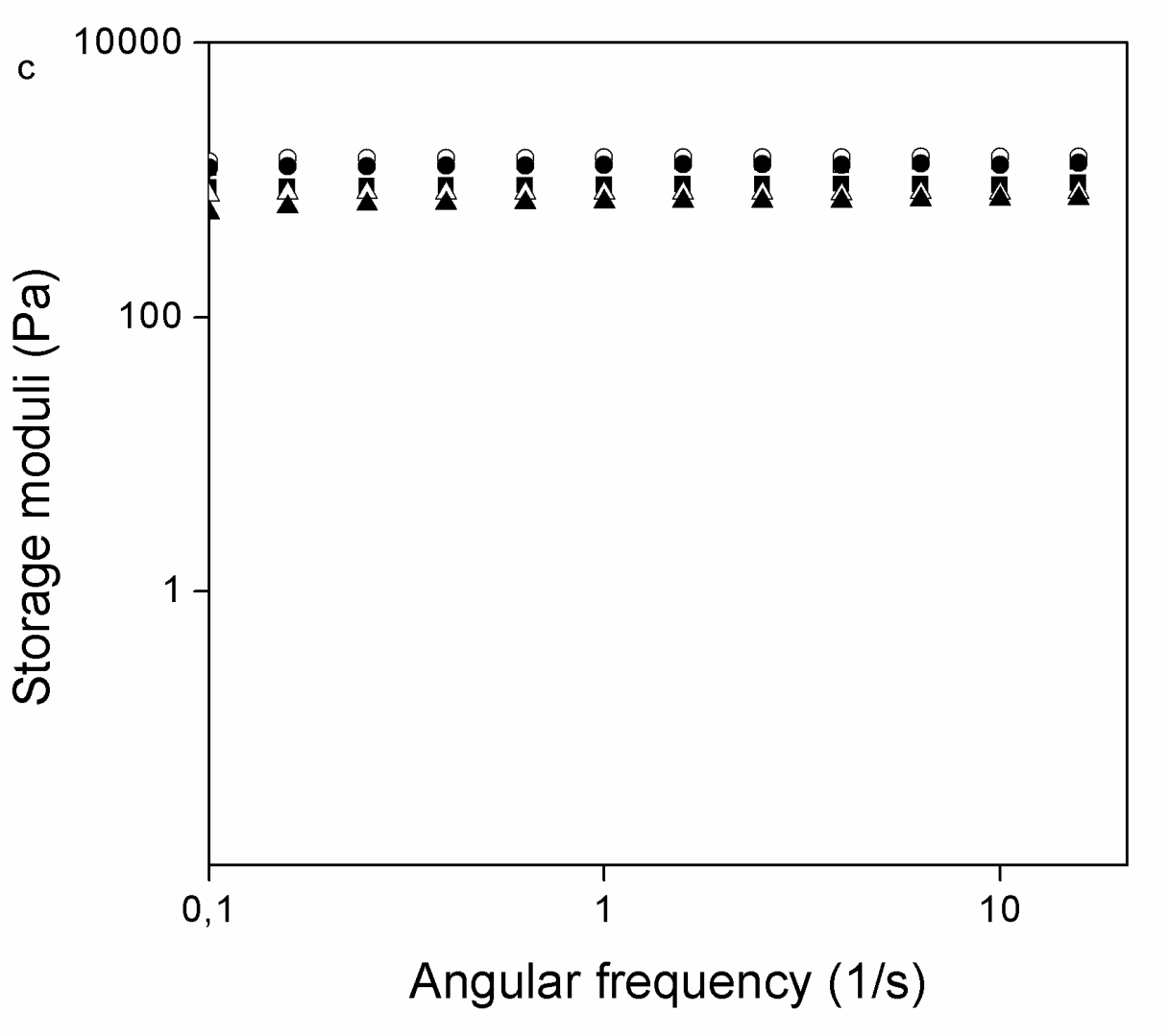
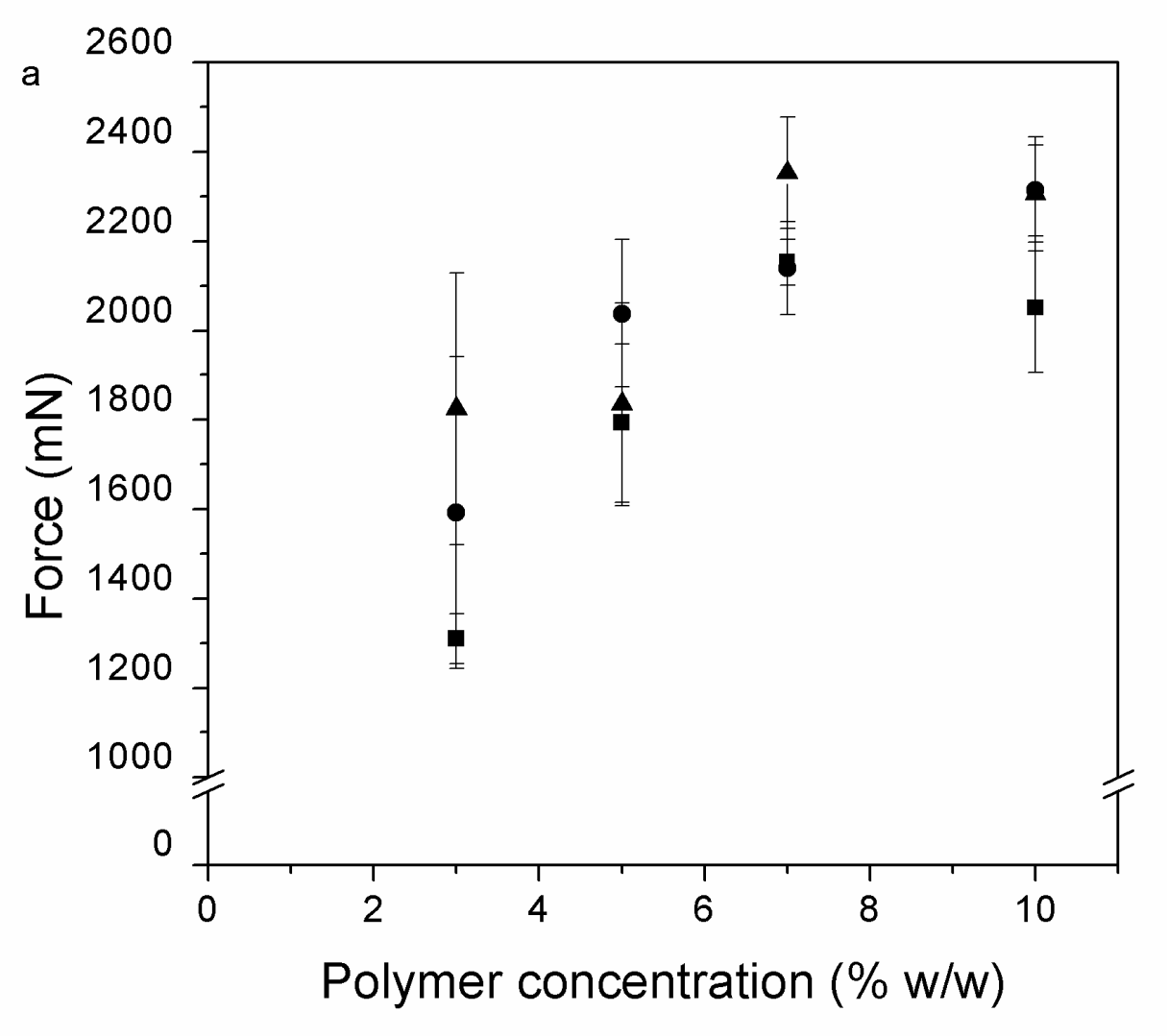


Fig 6.


a
2600
2400
2200
2000
1800
1600
1400
1200
1000
0
Force (mN)
0
2
4
6
8
10
Polymer concentration (% w/w)

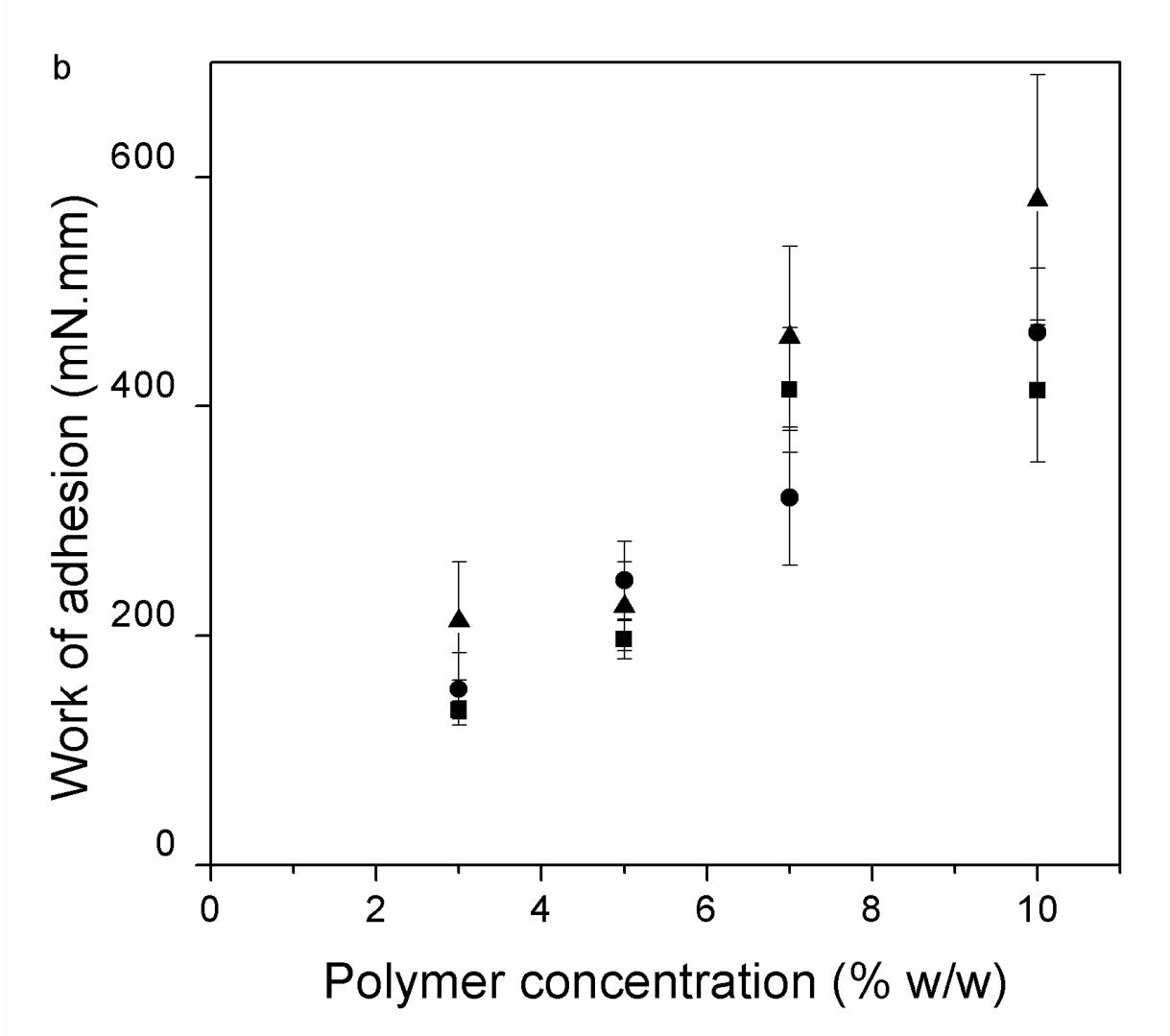
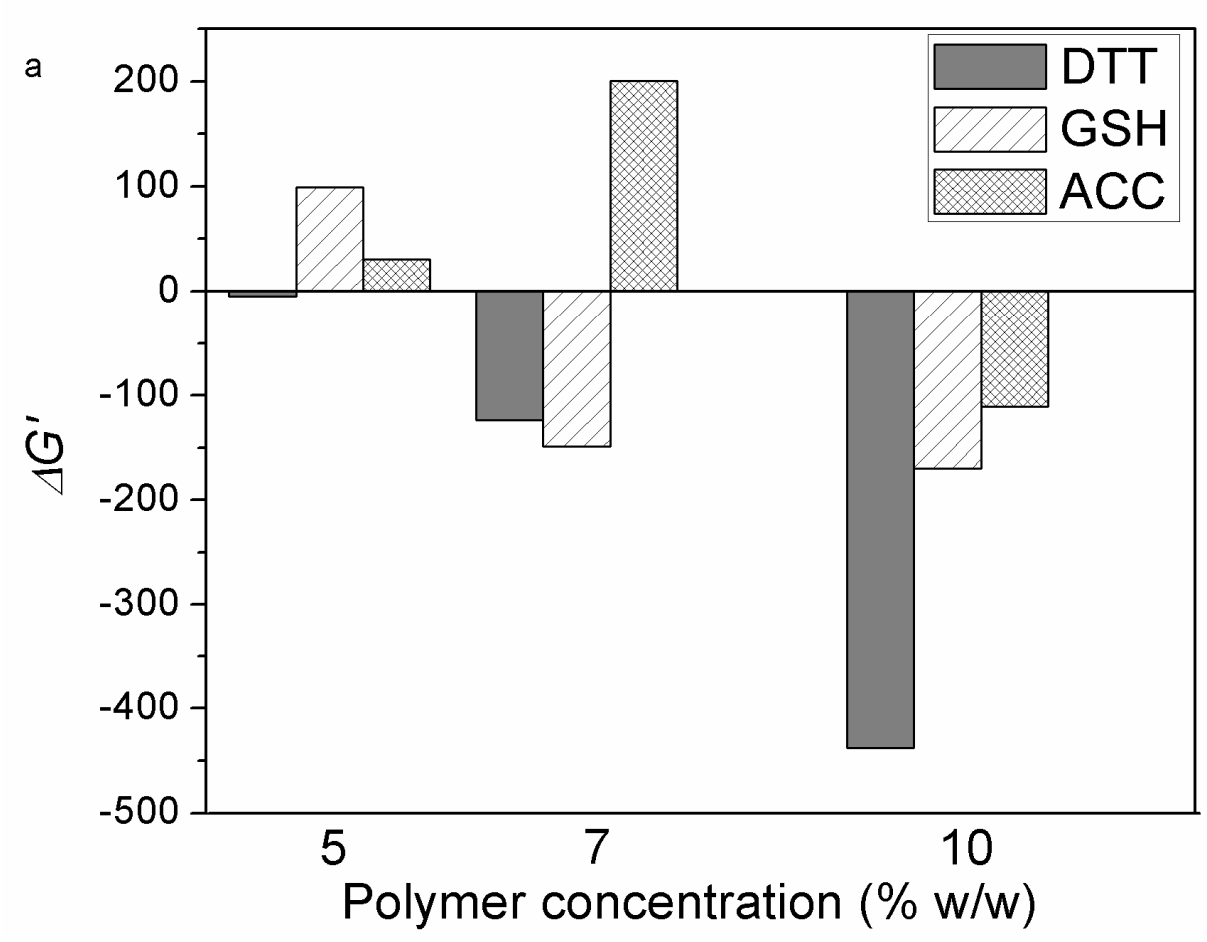

b
Work of adhesion (mN.mm)
600
400
200
0
0
2
4
6
8
10
Polymer concentration (% w/w)

Fig 7.


a
200
100
0
-100
-200
-300
-400
-500
ΔG'
DTT
GSH
ACC
5
7
10
Polymer concentration (% w/w)

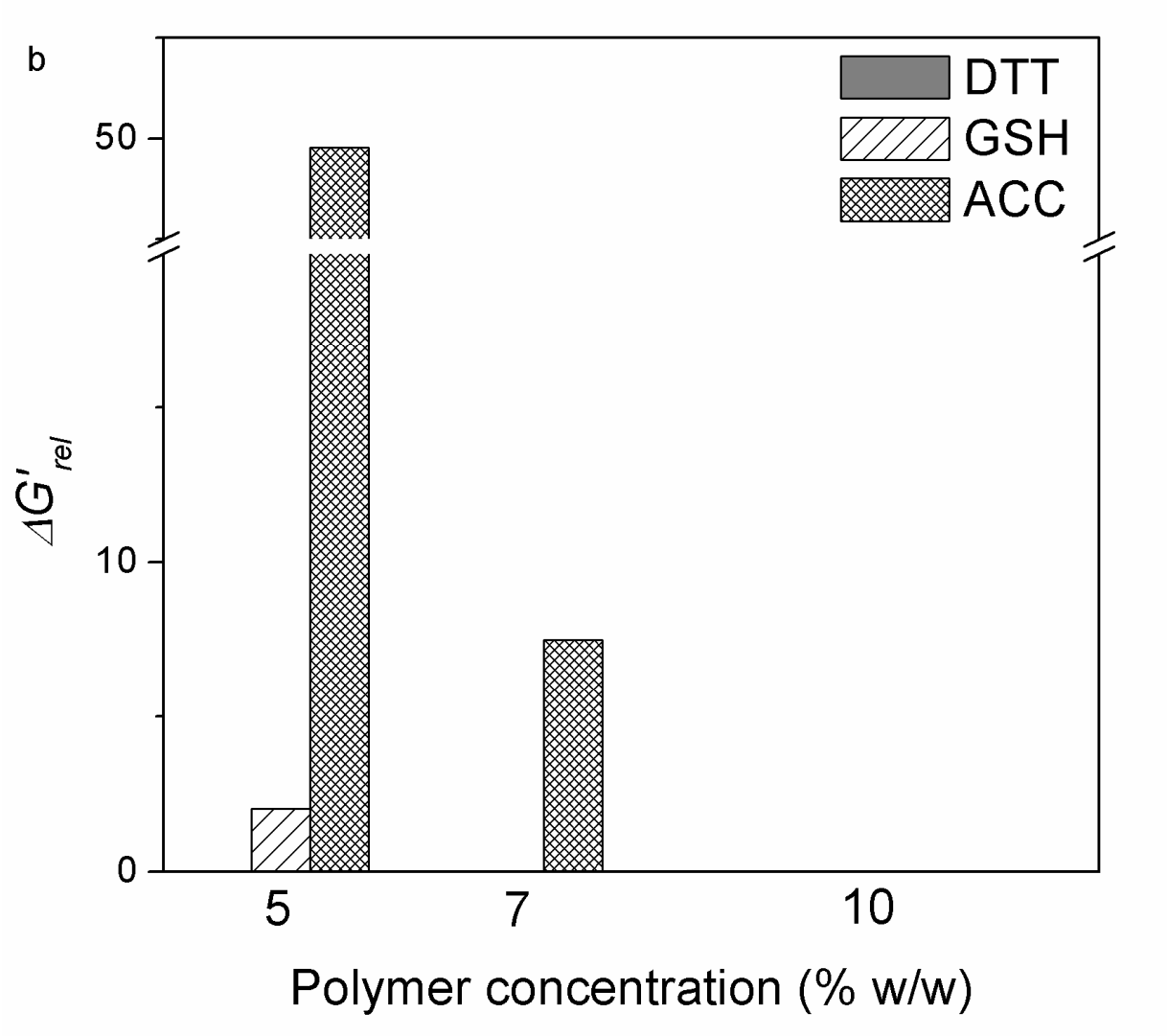


Fig 8.

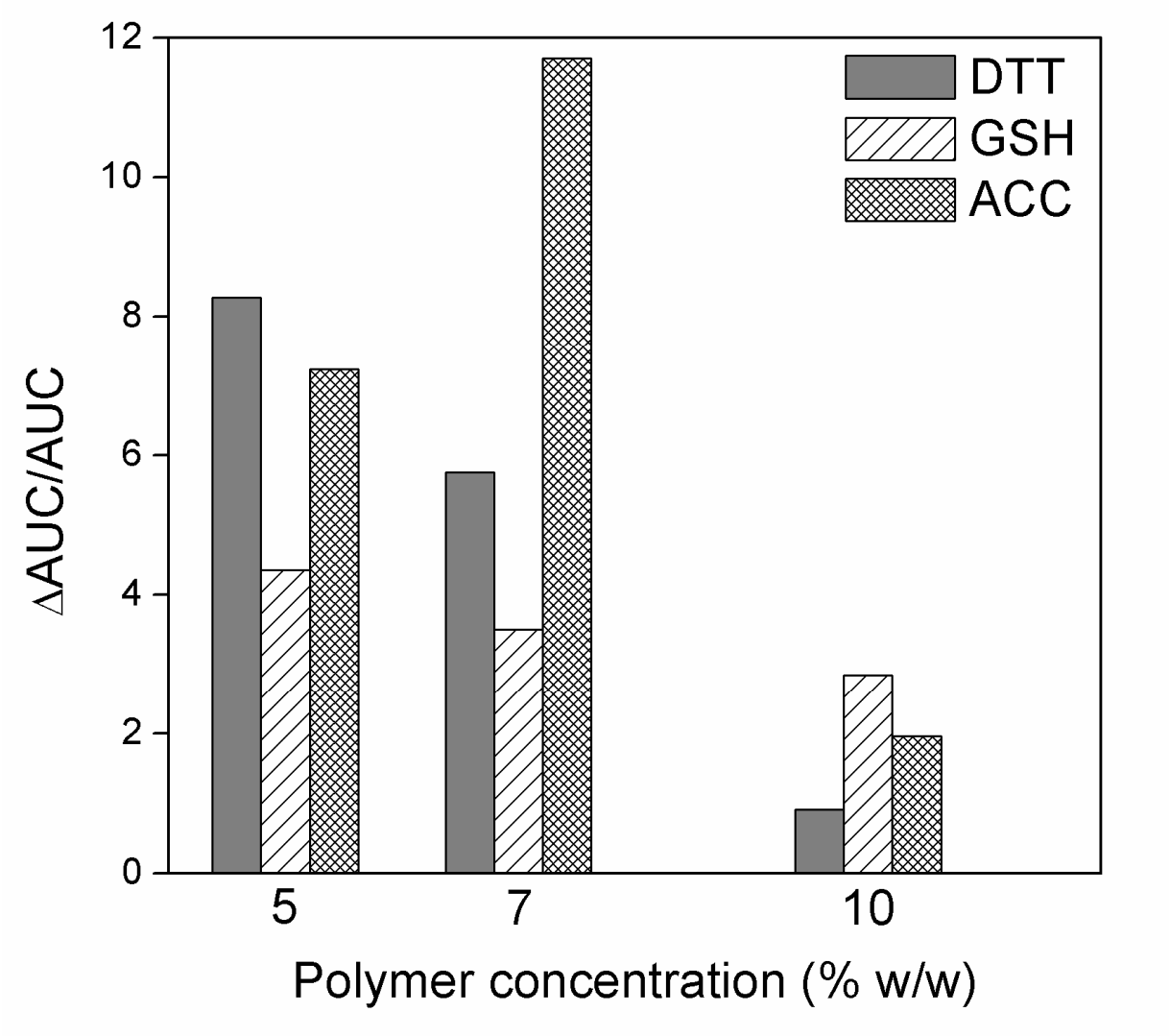
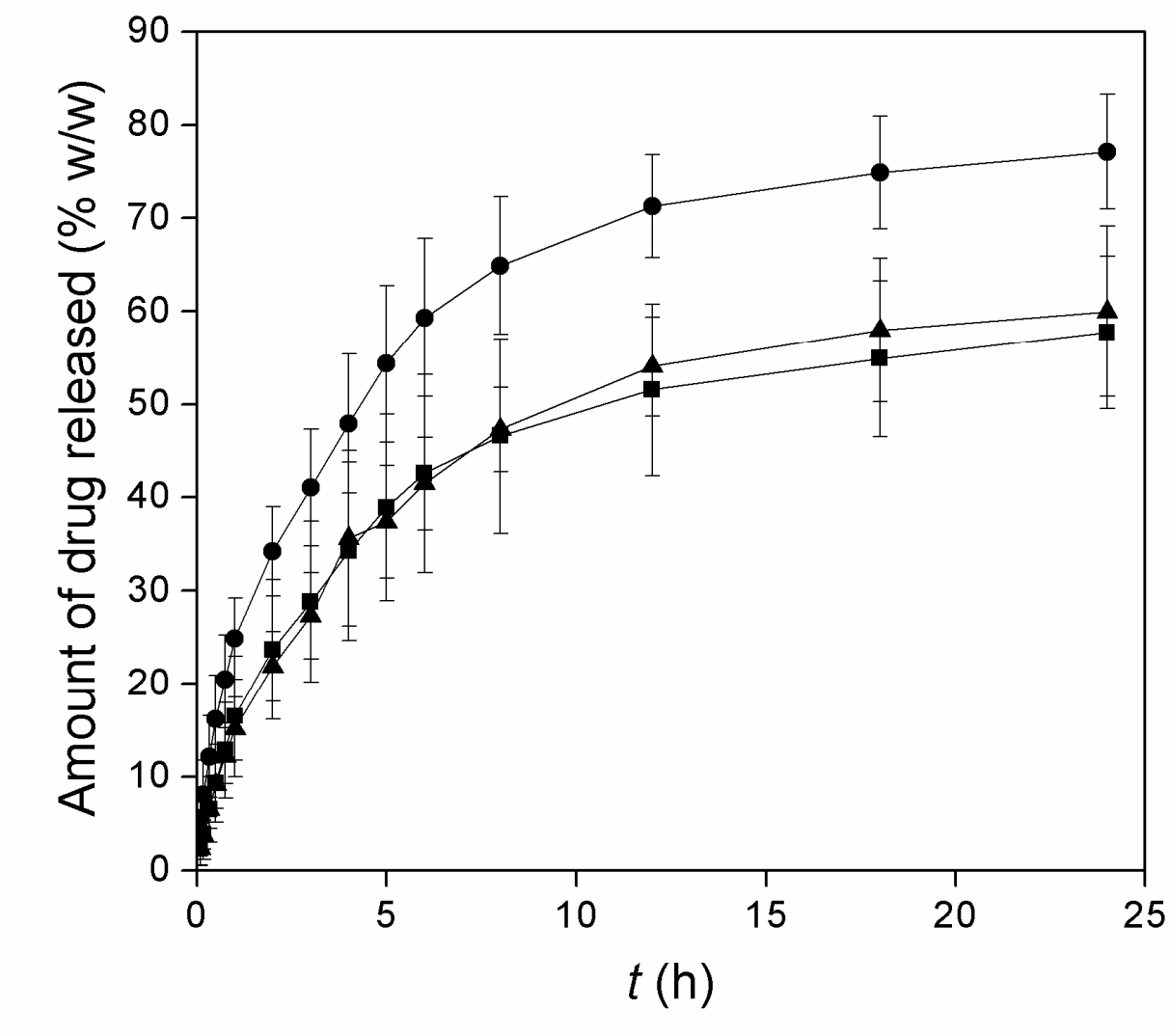
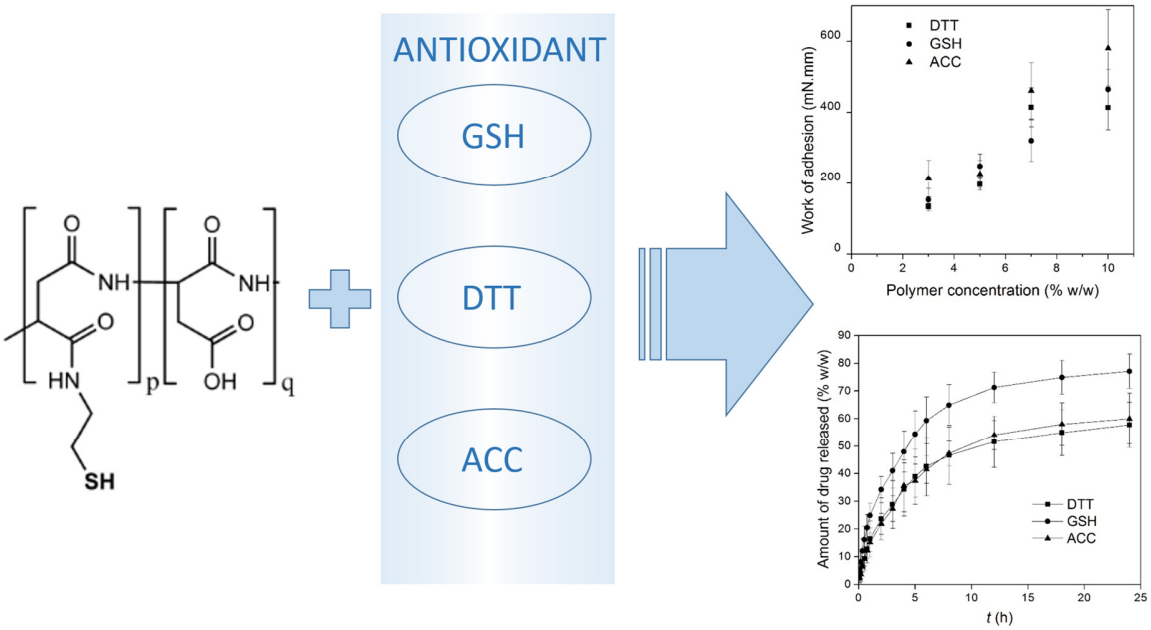

12
10
8
6
4
2
0
ΔAUC/AUC
DTT
GSH
ACC
5
7
10
Polymer concentration (% w/w)

Fig 9.



Graphical abstract


ANTIOXIDANT
GSH
DTT
ACC
NH
O
HN
SH
OH
p
q
Work of adhesion (mN.mm)
Polymer concentration (% w/w)
DTT
GSH
ACC
Amount of drug released (% w/w)
t (h)