Binding and inhibition of protease enzymes, including MMPs, by a superabsorbent dressing *in vitro*

• **Objective:** To demonstrate the binding and inactivation action of a superabsorbent dressing on proteolytic enzymes MMP-2, MMP-9 and collagenase using an established methodology.
• **Method:** An *in vitro* assay of MMP binding and collagenase inactivation has been conducted using the superabsorbent wound dressing (Eclypse; Advancis Medical UK). Dressings in this category, and other absorbents, have been claimed to possess MMP-binding characteristics; however, for most there is no published evidence as yet. In this series of experiments, we have used validated experimental techniques to evaluate such activity.
• **Results:** Results show that the superabsorbent dressing does have a statistically-significant effect in binding two of the most important MMPs, MMP-2 and MMP-9, as well as inhibiting collagenase.
• **Conclusion:** These results support this activity for the superabsorbent dressing and indicate a probable beneficial clinical action in reducing the influence of these enzymes in delayed wound healing.
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superabsorbent wound dressing; exudate management; matrix metalloproteases; collagenase

Epidermal wound healing is a complex, highly coordinated process, where different cell types and biologically-active molecules, such as cytokines, growth factors and extracellular matrix (ECM) components, play an integral role.

Essential for tissue repair is the metalloproteinase (MMP) enzyme family. MMPs are a group of over 20 zinc-dependent proteolytic enzymes, which share a similar structure to each other and functionally are capable of degrading almost every component of the extracellular matrix (ECM). They are multifunctional, being important in many physiological processes including tumorigenesis, normal ECM remodelling in wound repair, and have been implicated in many pathological processes, which involve the inflammatory response. Numerous MMPs and their inhibitors have been developed for possible therapeutic uses.[1] MMPs are but one group of proteases present at the wound site, which can act on ECM and non-ECM components, affecting degradation and modulation of the ECM, growth-factor activation and cell–cell and cell–matrix signalling.[2]

MMPs, zinc-dependent endopeptidases, are secreted by different cell types such as keratinocytes, fibroblasts and inflammatory cells during wound healing, thereby regulating this process in a very coordinated and controlled way.[3] Their role in delayed wound healing has been the topic of considerable research and discussion in recent years. Historically, MMP-2 and MMP-9 were known as gelatinases A and B, respectively; they were first identified in association with wound 'chronicity' by Wysocki et al.,[4] being evident in wound fluid, with a concomitant reduction in the natural inhibitors or tissue inhibitor of metalloproteinase (TIMPs).

More recently, MMPS of both endogenous and exogenous (bacterial) origin have been linked with pathogenesis in wound healing.[5–7] MMP-mediated defects in the interaction between cells and the ECM have been incorporated into the theory of dynamic reciprocity,[8] in an attempt to describe the complex series of defects, which manifest as delayed healing.

MMP-2 and MMP-9 are recognised as the most important MMPs in chronic wounds. Therefore, they have been used in this study. It is also known that dressings containing SAPs are able to bind and inhibit other proteases, such PMN elastase.[9] Hence, it is most likely that this dressing will also be able to reduce the levels of other MMPs and proteases in

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Table 1. Statistical results of MMP and collagensae binding studies

Sample ID	Incubation time (hours)			
	0	1	8	24
MMP-2 binding				
Control (pg/ml)*	4504.9 ± 83.0	4513.7 ± 104.8	4512.0 ± 97.8	4550.7 ± 119.6
Dressing (pg/ml)*	3646.6 ± 68.0	2387.8 ± 96.6	2185.8 ± 83.6	2164.6 ± 74.7
• Binding (%)	19.1	47.1	51.6	52.4
p-value	0.0001	0.0001	0.0001	0.0001
MMP-2 elution				
Control (pg/ml)*	0.0 ± 0.0	0.0 ± 0.0	59.6 ± 55.8	0.0 ± 0.0
Dressing (pg/ml)*	0.0 ± 0.0	0.0 ± 0.0	79.6 ± 48.7	59.6 ± 55.8
p-value	—	—	0.1489	0.3343
MMP-9 binding				
Control (pg/ml)*	1772.4 ± 28.6	1697.8 ± 42.0	1853.4 ± 19.1	1793.1 ± 68.9
Dressing (pg/ml)*	1370.7 ± 16.2	1372.9 ± 41.9	1452.1 ± 10.7	1459.9 ± 46.2
• Binding (%)	22.7	19.1	21.7	18.6
p-value	0.0001	0.0001	0.0001	0.0001
MMP-9 elution				
Control (pg/ml)*	499.8 ± 13.4	531.5 ± 10.4	539.9 ± 9.6	531.6 ± 7.7
Dressing (pg/ml)*	399.8 ± 14.8	443.8 ± 25.9	479.5 ± 20.5	499.6 ± 13.8
p-value	0.0003	0.0938	0.4505	0.9922
Collagenase binding				
Control (U/ml)*	0.174 ± 0.005	0.166 ± 0.003	0.157 ± 0.004	0.164 ± 0.005
Dressing (U/ml)*	0.157 ± 0.010	0.118 ± 0.009	0.103 ± 0.007	0.114 ± 0.004
• Reduction (%)	9.8	28.8	34.2	30.4
p-value	0.1644	0.0002	0.0001	0.0001
Collagenase elution				
Control (U/ml)*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SE [U/mL]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Dressing (U/ml)*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SE [U/mL]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
p-value	—	—	—	—

* Results presented as mean ± SEM

general. This should have a beneficial effect during the wound cleansing phase, where proteolytic activity is high and likely to cause tissue destruction. It has been reported that modulation of MMPs is beneficial in the treatment of chronic wounds.[10]

The Eclypse (Advancis Medical) range of wound dressings are highly absorbent, high capacity wound dressings designed to absorb and retain fluid, reduce potential leaks and minimise the risk of maceration. The dressing has a rapid wicking face combined with a highly absorbent moisture locking system. The absorbent crystals form a gel on contact with fluid, which is locked away and retained in the dressing, even under pressure. In this respect, MMPs in exudate are absorbed and cease to be metabolically active. Eclypse is similar in construct and mode of action to other superabsorbent dressings.[11,12]

The backing features a bacteria proof, water resistant barrier film to prevent strikethrough with a high moisture vapour transfer rate to prolong wear time. Eclypse is indicated for moderate- to heavily-exuding wounds.[13] The available clinical evidence comprises a cohort clinical study on pressure ulcers,[14] a quantitative comparative study on the effect on sub-bandage pressure,[15] and a study on cellulitis management.[16]

A variety of approaches have been taken in an attempt to reduce inflammation by down-regulating MMP activity in wounds. The provision of a sacrificial substrate, such as exogenous collagen, is one way to achieve this.[17] Another is to sequester co-factors, such as zinc, so reducing enzyme activity. In the Eclypse dressing, MMP-2 and MMP-9 regulation, and inactivation of collagenase is achieved by enzyme binding through electrostatic interactions, which renders the enzyme inactive. The purpose of this study is to demonstrate the binding and inactivation action of the superabsorbent on these proteolytic enzymes using an established methodology.[18]

Method

Binding of MMP-2 and MMP-9

Human MMP-2 and MMP-9 protein was obtained from R&D Systems GmbH (Wiesbaden-Nordenstadt). The lyophilised proteins were reconstituted as recommended in the manufacturer's instructions. For experiments, working solutions of MMP-2 5000pg/ml and of MMP-9 2000pg/ml were fabricated.

Wound dressing samples were cut using 8mm punch biopsies (0.5cm^2). Glass cover slips (0.5cm^2) were used as controls. Both were placed into 24-well cell culture plates, with 1ml protease working solution added to each well and incubated for up to 24 hours at 37°C. After incubation, supernatants were collected, immediately frozen and stored at –20°C until testing. Subsequently, bound protein was eluted from the individual wound dressing samples by shaking in 1ml phosphate buffered saline (PBS) for 1 hour at 37°C.

For determination of both the MMP-2 and the MMP-9 concentrations, the specific enzyme-linked immuno sorbent assays (ELISAs) were purchased (R&D Systems GmbH) and run, as recommended by the manufacturer. Optical density was measured at

Fig 1. Reduction of the MMP-2 concentration in the supernatant by the superabsorbent

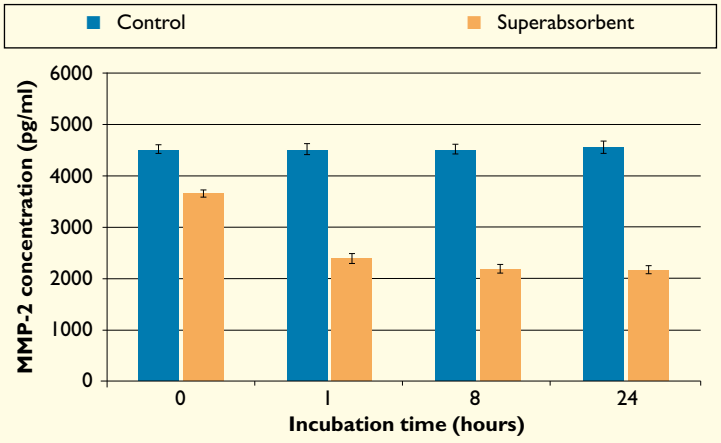
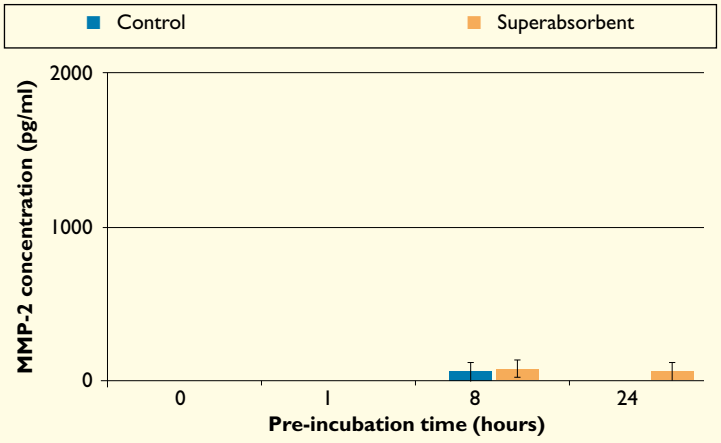


Fig 2. MMP-2 release from the dressing samples into the eluate



References

1 Fingleton, B. Matrix metalloproteinases as valid clinical targets. Curr Pharmacol Design. 2007; 13: 333–346.

2 McCarty, S.M., Cochrane, C.A., Clegg, P.D., Percival, S.L. The role of endogenous and exogenous enzymes in chronic wounds: a focus on the implications of aberrant levels of both host and bacterial proteases in wound healing. Wound Repair Regen. 2012; 20: 125–136.

3 Martins, V.L., Caley, M., O'Toole, E.A. Matrix metalloproteinases and epidermal wound repair. Cell Tissue Res. 2013; 351: 255–268.

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450nm with a reference measurement at 620nm using a plate photometer. Subsequently, the MMP concentrations were evaluated according to a four-parameter fit with coordinates for optical density (linear scale) and concentration (logarithmic scale).

Inhibition of collagenase activity:
Collagenase from the EnzChek Collagenase/Gelatinase Assay Kit (Invitrogen) was reconstituted, as recommended in the manufacturers' instructions. For experiments, a collagenase solution of 0.2U/ml was prepared in reaction buffer (0.1M Tris–HCl, pH 8.0, containing 0.2mM sodium azide and 0.5% bovine serum albumin). Wound dressing samples were cut using 8mm punch biopsies, corresponding to 0.5cm^2. Glass cover slips (0.5cm^2) were used as controls. Both were placed into 24-well cell culture plates, with 1ml collagenase solution was added to each well and incubated for up to 24 hours at 37°C.

After incubation, supernatants were collected, immediately frozen and stored at –20°C until testing. Subsequently, bond protein was eluted from the individual wound dressing samples by shaking in 1ml PBS for 1 hour at 37°C.

Collagenase activity was determined using the Collagenase/Gelatinase Assay Kit (Invitrogen). The assay was run as recommended in the instructions. Briefly, 80µl reaction buffer and 20µl DQ gelatin substrate were added followed by the injection of a 100µl sample. The fluorescence was measured continuously for 1 hour, at room temperature (excitation wavelength: 495nm, emission wavelength: 538nm), using a fluorescence plate reader.

Additionally, eluted dressing samples were incubated for 1 hour with 800µl reaction buffer and 200µl DQ gelatin. Afterwards, 200µl samples were transferred to a black 96-well plate and substrate turnover was measured by fluorescence intensity (excitation wavelength: 495nm, emission wavelength: 538nm) using a fluorescence plate.

Statistical analyses
Experiments were carried out four times and measurements were performed in duplicate each. All values are expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA)was carried out to determine statistical significances. Differences were considered statistically significant for $p<0.05$.

Results
The full tabulation of statistical analysis of results is presented in Table 1.

MMP-2 binding
The superabsorbent dressing exhibited a significant binding capacity for MMP-2 *in vitro* (Fig 1). On contact, the dressing samples quickly reduced the amount of MMP-2 in the supernatant. In addition, only marginal protein residues were detected in the eluate (Fig 2). The protease was so tightly bound by the superabsorbent that even disruption of the samples and aggressive elution techniques (vortexing or ultrasonic bath) did not lead to the release of MMP-2 *in vitro*.

MMP-9 binding
The superabsorbent also demonstrated a highly significant binding capacity for MMP-9 (Fig 3). Only a minor quantity of the protease was released from the dressings in the subsequent elution step compared with the control (Fig 4).

Collagenase inhibition
The superabsorbent dressing significantly reduced the activity of collagenase in the supernatant (Fig 5). No enzyme activity was detectable in the eluate

(data not shown). Additionally, it could be shown that the collagenase is bound so tightly to the dressing samples, that no substrate turn-over was measured in solution (data not shown).

Discussion

While numerous dressings and wound treatments are claimed to possess MMP-binding capacity, very few have robust scientific support. In this study we have subjected a superabsorbent dressing, which is frequently used in the management of exuding chronic wounds, to *in vitro* assessment of MMP-binding.[9,19,20] In this series of experiments, the superabsorbent polymer dressing tested statistically significant binding of MMP-2 and MMP-9, and of collagenase. In this respect, it performs equal to, or better than, other similar dressings

The superabsorbent exhibited a significant binding capacity for the clinically important MMPs-2 and MMP-9 *in vitro*. The proteases were so tightly bound by the superabsorbent that even disruption of the samples and aggressive elution techniques did not lead to the release of MMP-2 *in vitro*. Furthermore, it also demonstrated a highly significant binding capacity for MMP-9. A minor quantity of the protease was released from the dressings in the subsequent elution step compared to the control.

While only MMP-2 and MMP-9, and collagenase were studied in these experiments, it is reasonable to conjecture that the binding capacity of the superabsorbent polymers will have a similar effect on other enzymes in this category. However, as these are the current focus of ECM degradation in wound chronicity no other enzymes were studied.

Superabsorbent polymers consist of flexible chains of the polymer polyacrylate that possesses a high density of carboxylate groups.[21] After coming into contact with water, the sodium ions joined to the carboxylate groups detach and go in solution, which leaves negative charged groups behind. As they repel each other, they cause the polymer to unwind and absorb more water. The hydrogen in water is bound to polyacrylate due to electrostatic forces but it can be easily replaced by other molecules with positively charged groups like proteins which are then attached instead.[22] It is most likely that MMP-2 and MMP-9 are bound by this mechanism. Moreover, the superabsorbent dressing significantly reduced the activity of collagenase in the supernatant ($p<0.01$ at all time points; Fig 5). Additionally, there was no detectable activity of collagenase released from the wound dressing samples into the eluate at any time point and no substrate-turnover was observed by the dressing samples. This suggests that the collagenase is bound so tightly that enzyme activity seems to be completely abolished.

For this experiment, we wanted the 'controls' to show that (a) the proteases are stable under the

Fig 3. Reduction of the MMP-9 concentration in the supernatant by the superabsorbent

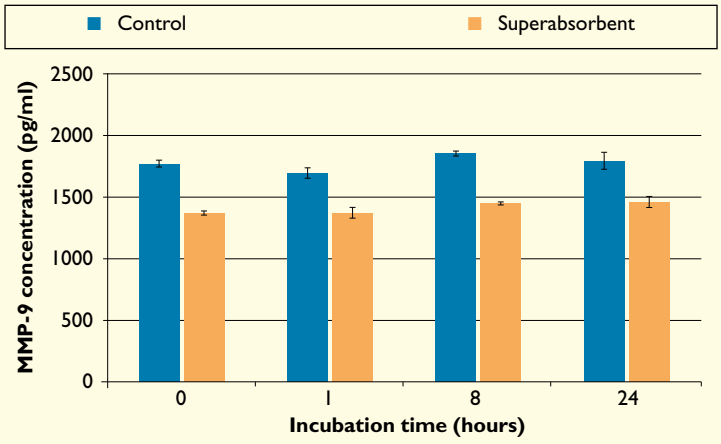


Fig 4. Release of MMP-9 into the eluate by the superabsorbent

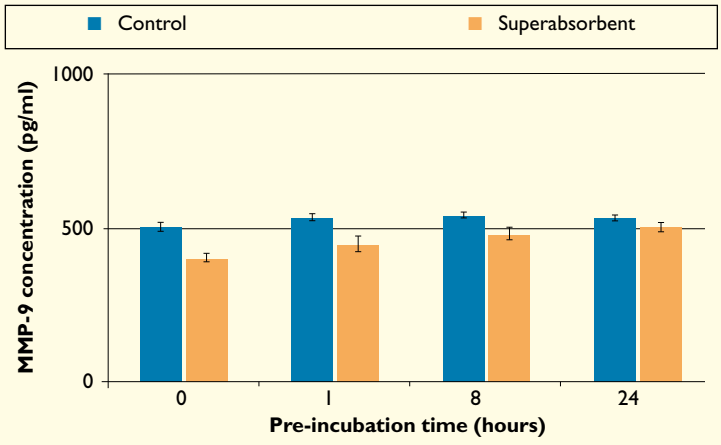
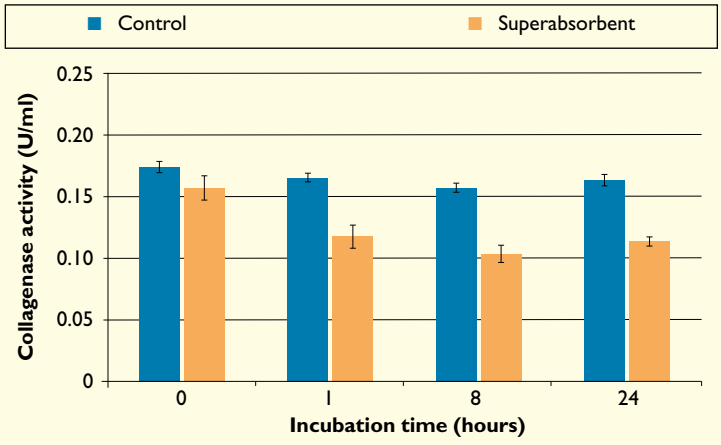


Fig 5. Reduction of the collagenase activity in the supernatant by the superabsorbent



chosen test conditions and (b) no unspecific binding to the surrounding environment occurs (or at least very little). Both would lead to measurement of a reduced concentration in the test samples and consequently one would conclude a false binding. Hence, in the very first tests that we did many years ago, control wells were left empty. Of course, this setup features a draw back that could be criticised, as no control material could then be transferred for the elution step. Therefore, we included glass cover slips as controls because they proved to be inert in respective validation tests. They can be handled similar to the test samples and provide good controls in the binding assays.

Conclusion

The superabsorbent displayed statistically significant binding properties for both MMP-2 and MMP-9 in this *in vitro* assay. Furthermore, the protease enzyme collagenase is completely inhibited. It may therefore, be expected that the superabsorbent will be effective in the treatment of 'chronic' wounds when the proteolytic enzymes are active and likely to cause tissue destruction and provoke a chronic inflammatory reaction. ■

4 Wysocki, A.B., Staiano-Coico, L., Grinnell, F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. J Invest Dermatol. 1993; 101: 64–68.

5 Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P.Ø. et al. Why chronic wounds will not heal: a novel hypothesis. Wound Rep Regen. 2008; 16: 2–10.

6 Rayment, E.A., Upton, Z., Shooter, G.K. Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer. Br J Dermatol. 2008; 158: 951–961.

7 Trengove, N.J., Stacey, M.C., MacAuley, S. et al. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen. 1999; 7: 442–452.

8 Schultz, G.S., Davidson, J.M., Kirsner, R.S. et al. Dynamic reciprocity in the wound microenvironment. Wound Repair Regen. 2011; 19: 134–148.

9 Wiegand, C., Abel, M., Ruth, P., Hipler, U.C. Superabsorbent polymer-containing wound dressings have a beneficial effect on wound healing by reducing PMN elastase concentration and inhibiting microbial growth. J Mater Sci Mater Med. 2011; 22: 2583–2590.

10 Motzkau, M., Tautenhahn, J., Lehnert, H., Lobmann, R. Expression of matrix metalloproteases in the fluid of chronic diabetic foot wounds treated with a protease absorbent dressing. Exp Clin Endochrinol Diabets. 2011; 119; 286–290.

11 Cutting, K.F. Managing exudate using a superabsorbent polymer dressing: a 53 patient clinical evaluation. J Wound Care. 2009; 18: 200–205.

12 Guy, H., Godar, S. Managing highly exuding wounds with Eclypse dressing. Brit J Nurs. 2010; 19: 6(Suppl.), S24–29.

13 Gardner, S. How to guide: managing high exudate wounds. Wounds. 2012; 7: 1.

14 Lloyd-Jones, M. The role of Eclypse adherent sacral in managing sacral pressure ulcers. Br J Comm Nurs. 2011; 16: 9 (Suppl.), S38–S42.

15 Cook, L. Effect of super-absorbent dressings on compression sub-bandage pressure. Br J Comm Nurs. 2011; 16: 3 (Suppl.), S38–S43.

16 Rafter, L. Management of acute cellulite with Eclypse. Wounds. 2011; 7: 4, 62–68.

17 Cullen, B., Smith, R., Mcculloch, E. et al. Mechanism of action of PROMOGRAN, a protease modulating matrix, for the treatment of diabetic foot ulcers. Wound Repair Regen. 2002; 10: 16–25.

18 Wiegand, C., Hipler, U.C. Polymer-based biomaterials as dressings for chronic stagnating wounds, Macromolecular Symposia. 2010; 294: 2, 1–13.

19 Eming, S., Smola, H., Hartmann, B. et al The inhibition of matrix metalloproteinase activity in chronic wounds by a polyacrylate superabsorber. Biomats. 2008; 29: 2932–2940.

20 Wiegand, C., Heinze, T., Hipler, U.C. Comparative *in vitro* study on cytotoxicity, antimicrobial activity, and binding capacity for pathophysiological factors in chronic wounds of alginate and silver-containing alginate. Wound Repair Regen. 2009; 17: 511–521.

21 Pytlik, E., Molino, D., Moritz, J. Superabsorbent polymers (SAP). In: Introduction to Polymers. UB Engineering—University at Buffalo, 2005. Available from: http://tinyurl.com/crodmmn [Accessed April 2013].

22 Paustian, C., Stegman, M.R. Preparing the wound for healing: The effect of activated polyacrylate dressing on debridement. Ostomy Wound Manage. 2003; 49: 9, 34–42.