Genipin-Crosslinked Gelatin Hydrogel for the Detection and Treatment of Active Metalloprotease Releasing *Pseudomonas aeruginosa.*

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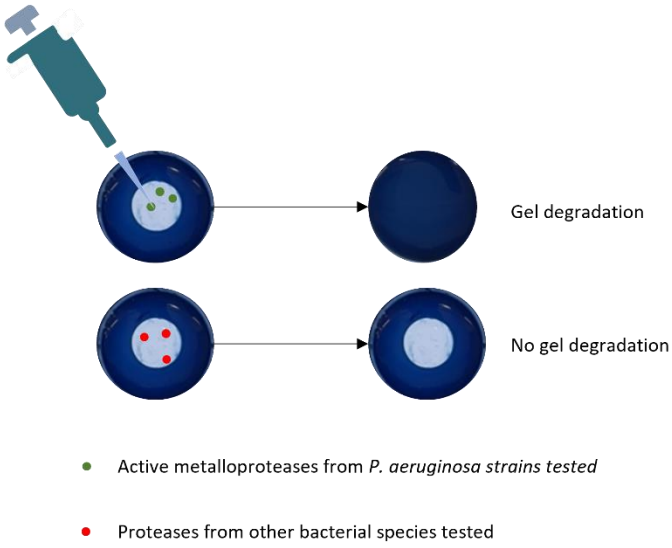
ABSTRACT

We report a genipin-crosslinked gelatin hydrogel for the detection of the pathogenic strains of *Pseudomonas aeruginosa* releasing active metalloproteases. Enzymatic degradation of the blue gel gives a clear visual signal that indicates the presence of *P. aeruginosa*. A total of 36 strains of both Gram-positive and Gram-negative bacteria were tested and only *P. aeruginosa* strains caused degradation of the gels within 24 h. Gene sequencing revealed that *P. aeruginosa* strains with mutations in their quorum sensing operon were not able to degrade the hydrogels even though the genes coding for metalloproteases were present. Finally, we created a gel multilayer that can both detect and treat active strains of *P. aeruginosa* through the release of bacteriophage. Our system provides a simple and reproducible assay for *P. aeruginosa* metalloproteases and showcases the feasibility of responsive wound dressings able to both detect and treat *P. aeruginosa* infection.



INTRODUCTION

Healthcare Associated Infections (HAIs) represent 4 million cases in Europe annually.[1] These infections result from direct or indirect healthcare interventions, and among these cases, 147,000 patients die annually. In the European Union, the treatments associated with HAIs are estimated to cost around €7 billion yearly to healthcare systems.[2] Accordingly, there is an urgent need for new methods of early detection and treatment of HAIs which include urinary tract infections (UTI's), wound and surgical site infections and respiratory tract infections.[3] The current gold standard for specific pathogenic bacterial identification is based on bacterial culture but this technique is time consuming, typically 48 hours for a result.[4] This time delay means that antibiotics are often used prophylactically on suspicion of infection. Indeed, in wounds, evidence of wound heat, peri-wound redness and patient reported pain are symptoms that can be confused with inflammation rather than infection, leading to potential misuse and overuse of antibiotics, which can contribute to the evolution of antibiotic resistance.[5]

HAIs are mostly caused by pathogenic bacteria of the ESKAPEE group, composed of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter species and Escherichia coli*.[6] These bacteria can be highly virulent and exhibit antibiotic resistance.[7] Therefore, rapid detection

methods of these bacteria could have utility in improving patient care. Here we report a genipin-crosslinked gelatin hydrogel sensing specifically the presence of *Pseudomonas* strains releasing active metalloproteases.

Proteases are a class of enzymes targeting peptide bonds, which are often released by bacteria in the ESKAPEE group. Protein-based hydrogels are therefore good candidates for protease susceptible sensing systems.[8–10] Proteases are organized into different classes, namely aspartic, glutamic, metallo, cysteine, serine, asparagine, and threonine proteases, based on their different catalytic sites. They have also been grouped according to similarities in the active sites and substrate affinities, for example in the MEROPS database.[11]

Different types of collagen are known to be degraded by bacterial collagenolytic proteases such as metalloproteases of the M9 family, as well as serine proteases from S1, S8 and S53 families.[12] Among the common pathogenic bacteria found in wound infections, *S. aureus*, *P. aeruginosa and E. faecalis* secrete serine proteases and metalloproteases, some from the same family.[13–15] Metalloproteases possess one or two metal ions in their catalytic site, Zn^{2+} being the most frequent one.[16] In the M4 thermolysin family, the metal centre is tetrahedrally coordinated by two Glu residues, one His residue and a water molecule. During catalysis, the water molecule gets activated and acts as a nucleophile for protein cleavage.[17,18]

In this study we set out to design a simple gelatin hydrogel film that would degrade in the presence of the 'gelatinases' which could in principle include serine and metalloproteases released by *S. aureus, E. faecalis and P. aeruginosa.* Surprisingly, in our study the hydrogels were only degraded by *P. aeruginosa* and remained stable when exposed to proteases from *S. aureus and E. faecalis*. This was unexpected given that all three bacterial species can express S1 proteases and/or M4 gelatinase (**Table 1**). It should be noted that the presence of a protease gene does not necessary correlate with enzymatic activity. Several reports show that the *gelE* gene coding for gelatinase in *E. faecalis* does not always lead to expression of the active protein.[19,20] However, out of the 36 strains tested only *P. aeruginosa* was capable of degrading the gel in less than 48 hours. Among the *P. aeruginosa strains* tested, 69.2% caused degradation.

Table 1 – Examples of proteases expressed by ESKAPEE pathogens		
Bacteria	**Protease**	**Family[11]**
Pseudomonas aeruginosa[21]*	LasA/staphylolysin	Metalloprotease M23
	LasB/pseudolysin	Metalloprotease M4
	AprA/alkaline protease	Metalloprotease M10
	PIV/protease IV	Serine S1
	PASP/*Pseudomonas aeruginosa* small protease	Serine S85
	LepA/large exoprotease	-
	MucD	Serine S1
	PAAP/*Pseudomonas aeruginosa* aminopeptidase	-
Staphylococcus aureus[22]*	Aur	Metalloprotease M4
	V8/SspA/GluV8	Serine S1
	ScpA	Cysteine C47
	SspB	Cysteine C47
Enterococcus faecalis[13]*	GelE	Metalloprotease M4
	SprE	Serine S1
Acinetobacter baumannii[23]	PKF	Serine S1 unassigned
	CpaA**	Metalloprotease M72
*Streptococcus agalactiae (GBS)**	CspA	Serine -
Escherichia coli	Protease I	Serine -
	Protease II	
* Shown to degrade some collagen types.[15,24–27] **Not present in *A. baumannii* ATCC17978.[23]		

Gelatin has potential advantages as a matrix for sensing bacterial proteases due to its biocompatibility, biodegradability, and low cost. It is already used as a key component of commercial wound dressings in brands such as DuoDerm® and Granuflex®. One disadvantage of gelatin is its animal origin leading to usage restriction in some countries as well as not having a well-defined amino-acid sequence. Gelatin is a polypeptide generated by partial hydrolysis and denaturation of collagen[28], which is the major protein in tissues and bones. Alkaline or acidic hydrolysis are used in industrial production to give type A (from pigs, poultry and fish) or type B (from cows) gelatin respectively.[29] Gelatin has a molecular weight between 40 kDa and 90 kDa[30] and is composed of 18 amino acids, with glycine, proline, and hydroxyproline being the major ones.[31] However, gelatin is not robust enough for direct hydrogel applications and usually requires additional chemical crosslinking. Genipin is a glycone, a two membered heterocycle with hydroxy and methacrylate functionality (**Figure 1**

(a)). Genipin is extracted from *Gardenis jasminoides Ellis* fruits and can be used to crosslink gelatin. Its low toxicity compared to other synthetic crosslinking agents, about 10^4 times less cytotoxic than glutaraldehyde makes it potentially attractive for biomedical applications.[32]

Gelatin-genipin hydrogels have been investigated previously for different applications. Kirchmajer et *al*.[33] synthesized and characterized genipin crosslinked gelatin hydrogels and found that they could be used as vascularisable soft tissue scaffolds having pro-angiogenic effects when used in combination with PMA (phorbol-12-myristate-13-acetate), a stimulator of angiogenesis. Gattazzo et *al.*[34] found that gelatin-genipin hydrogel could be used in skeletal muscle tissue engineering. Gelatin-genipin hydrogels have also been used in combination with other materials to generate composite hydrogels. It is the case in Xiao et *al.*[35] where gelatin, known for its bioactivity, was combined with silk fibroin which has higher mechanical integrity and slower degradation. This composite was shown to be a potential candidate for cartilage, non-load-bearing bone tissue engineering and regeneration. Cui et *al*.[36] mixed gelatin with chitosan before crosslinking to obtain interpenetrating polymer networks. It was found that this hydrogel was pH-sensitive and could then be used in drug delivery or sorbent applications. Wound dressing and healing applications have also been investigated. For instance, Ilkar Erdagi et *al.*[37] synthesized genipin-crosslinked gelatin interpenetrated diosgenin-modified nanocellulose hydrogels and loaded neomycin drug. The hydrogel then showed good antibacterial activity against Gram-positive and Gram-negative bacteria suggesting it might have utility in wound dressing applications. Other gelatin-genipin hydrogel systems have been loaded with drugs and exhibited anti-bacterial activity.[38,39]

Here we show that genipin-crosslinked gelatin hydrogels can be used to detect the presence of *Pseudomonas* strains releasing active metalloproteases. Addition of a therapeutic layer also demonstrated the potential use of this hydrogel for the treatment of these bacteria.

RESULTS AND DISCUSSION

Preparation of the hydrogels

Genipin-crosslinked type A gelatin hydrogels were prepared with different crosslinker concentrations before being tested in the presence of proteases. Crosslinking of the hydrogels

induces a blue colour due to increased conjugation (see **Figure 1**). The change of colour allows the crosslinking reaction to be followed by UV/Vis and fluorescence spectroscopies. In **Figure S2 (a)**, absorbance of hydrogels with different concentrations of genipin was measured at 590 nm over 18 h. In **Figure S2 (b)**, fluorescence experiments were carried out at λ_{ex}=590 nm and λ_{em}=610 nm for 2 h on 7.5wt% G hydrogels with different concentrations of genipin. Absorbance and fluorescence emission intensity both increased with increased crosslinking.

Oscillation strain experiments were performed for 7.5wt% G hydrogels with different crosslinker concentrations to determine the suitable strain used in further experiments. 0.1% strain was selected as no decrease of storage modulus was observed at this level (**Figure S3 (a)**). Storage modulus was then monitored for 20 min at 0.1% strain and 1 rad/s frequency for 7.5wt% G hydrogels with different crosslinker concentrations. The higher the crosslinker concentration, the higher the storage modulus which can be associated with the strength of the hydrogel (**Figure S3 (b)**). Swelling results are provided in **Table S3**. All genipin-crosslinked hydrogels showed similar equilibrium swelling ratios in water, likely due to the additional physical crosslinking **(Table S3)**. Nevertheless, a small reduction in swelling ratio (from c.a. 13 to 10) while increasing genipin loading from 0.1 to 1wt% was observed. The hydrogel prepared in the absence of genipin did not swell but fully dissolved in water.

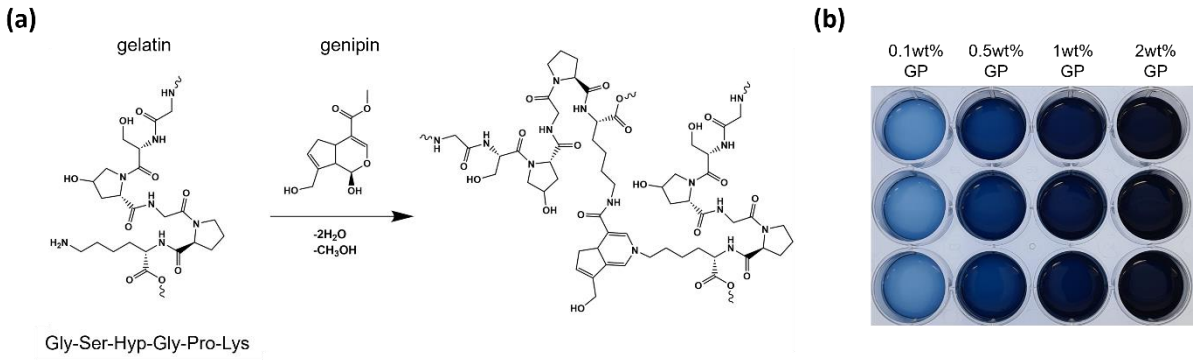


Figure 1 (a) Structure of crosslinked gelatin-genipin hydrogels (G-GP hydrogels). **(b)** 7.5wt% G hydrogels with different concentrations of genipin (GP) crosslinker after 24 h crosslinking.

Enzymatic degradation studies

Once stable crosslinked hydrogels were produced, their susceptibility to protease was investigated. Initially, the degradation of the crosslinked hydrogels in the presence of the isolated serine protease trypsin was investigated using rheology. In solution with 0.5% trypsin, all the 7.5wt% G hydrogels showed a significant decrease in their storage modulus as they were degraded (**Figure S5 (a)** and **Table S4**). In contrast, the storage modulus of hydrogels in tris-buffer did not change significantly over the same timescale. (**Figure S5 (b)** and **Table S4**). The loss in weight of 7.5wt% gelatin hydrogels with different crosslinker concentrations in the presence of 0.1% trypsin solution is shown in **Figure 2 (a)**. The hydrogel with the highest concentration of genipin, i.e. 1wt% GP, showed little to no degradation, while the hydrogel without crosslinker was completely degraded between 8 and 24 h. Scanning electron microscope (SEM) confirmed degradation of the hydrogel with a higher pore formation after exposure to the enzyme (**Figure 2 (b)-(d)**).

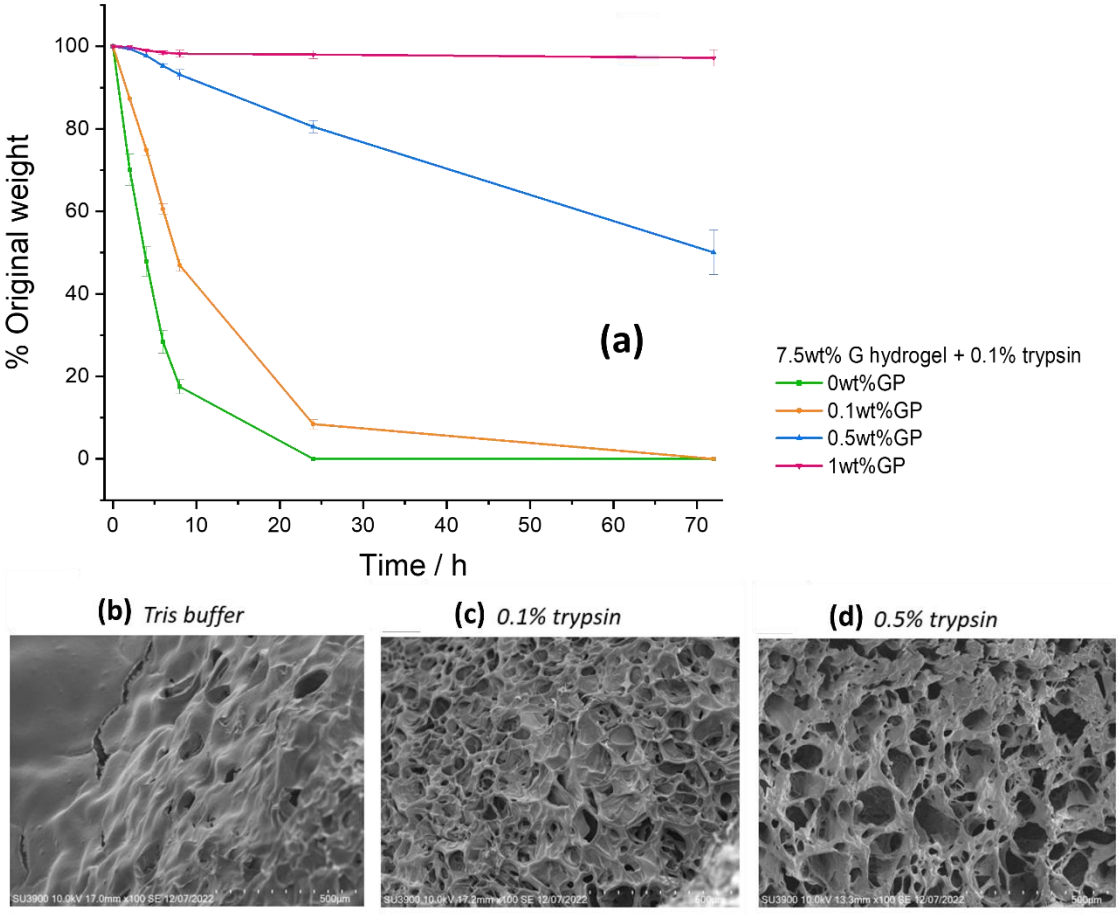


Figure 2 (a) Weight monitoring of 7.5wt% G hydrogels with different concentrations of genipin in 0.1% trypsin solution and SEM images of 7.5wt% G – 0.1wt% GP hydrogels after soaking in **(b)** tris buffer, **(c)** 0.1% trypsin and **(d)** 0.5% trypsin (scale bar: 500 μm). Error bars correspond to one standard deviation.

Microbiological testing of crosslinked hydrogels

Based on these pre-screening results, 7.5wt% gelatin hydrogels crosslinked with 0.1wt% genipin were chosen for further investigation with bacterial supernatant. Bacterial strains were grown in Brain Heart Infusion (BHI) or Tryptic Soy Broth broths for 18 h at 37 °C and were then centrifuged at 4000 rpm for 10 min to collect cell-free supernatant containing proteases. Supernatants from 36 bacterial strains were tested, comprising both Gram-positive and Gram-negative human pathogens. A simple assay was designed where crosslinked sterilized hydrogels were prepared in the bottom of 12-well plates. A disc of gel was cut and removed from the centre of the plate and the supernatant was then pipetted into the circular space. If the gel degraded, the central well was filled with solubilized fragments of the blue gel and turned blue (**Figure 3**). It was shown the autoclaving process to obtain sterile hydrogels did not impact the degradation result (**Figure S4** and **Table S2**). This assay gave a clear visual signal of gel degradation, which could be monitored in real time using a UV-Vis absorbance plate. Supernatant degradation tests were performed thrice in triplicate (**Figures S6-S8**). **Figure 4** summarizes the results of this test, showing the bacterial strains tested and whether they degraded the hydrogel. The tests show that only some *P. aeruginosa* strains were able to degrade the gels in under 24 hours, even though many of the other strains tested are known to release proteases. For example, *S. aureus* strains express proteases as can be seen with the degradation of the un-crosslinked gelatin hydrogels (**Figure S1**). However, they do not degrade the crosslinked hydrogels. It might be these proteases are not able to access their degradation sites because of the crosslinking.

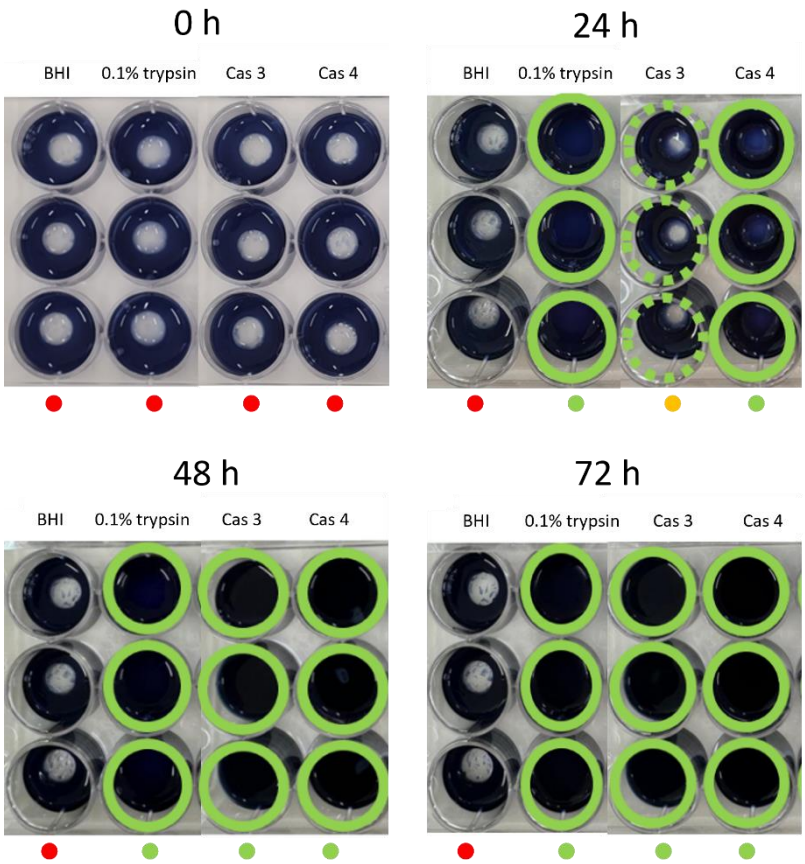


Figure 3 – Bacterial strain test on 7.5wt% G-0.1wt% GP hydrogels with *Pseudomonas aeruginosa* Cas3 and Cas4 strains supernatants – Pictures at 0, 24, 48 and 72 h showing the clear visual representation of no degradation (●) (the central well is empty) partial degradation (●) (the central well starts to fill) and degradation (●) (the central well is filled).

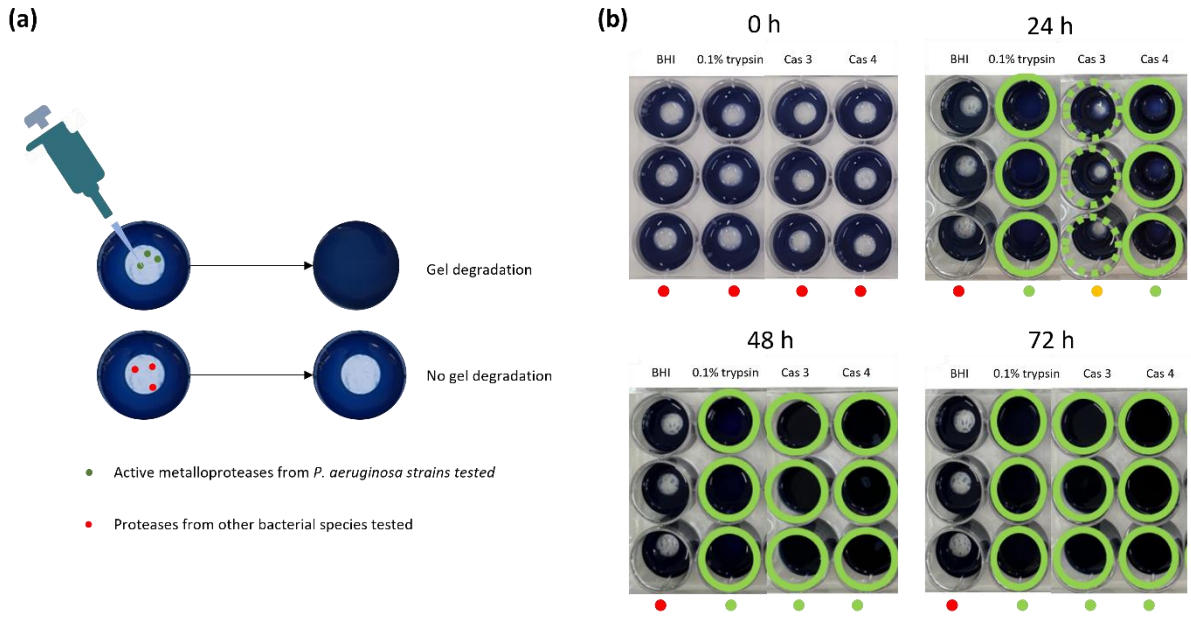


Figure 3 (a) Summary of the genipin-crosslinked gelatin hydrogel assay and **(b)** Bacterial strain test on 7.5wt% G-0.1wt% GP hydrogels with *Pseudomonas aeruginosa* Cas3 and Cas4 strains supernatants – Pictures at 0, 24, 48 and 72 h showing the clear visual representation of no degradation (●) (the central well is empty) partial degradation (●) (the central well starts to fill) and degradation (●) (the central well is filled).

			Degradation								
Bacteria & Solutions	Bacterial strains	Gram	24 h			48 h			72 h		
			1	2	3	1	2	3	1	2	3
0.1% trypsin											
BHI											
Pseudomonas aeruginosa	PAO1	-									
	GH12	-									
	Cas2	-									
	Cas3	-									
	Cas4	-									
	372261	-									
	NCTC 10662	-									
	PA10 1990	-									
	PAE45321	-									
	RP73	-									
	NCTC 13437	-									
	CF1-004-CRCN	-									
	ATCC 27853	-									
Enterococcus faecalis	NCTC 775	+									
	NCTC 12201	+									
	ATCC 29112	+									
Enterococcus faecium	NCTC 12204	+									
Staphylococcus aureus	ATCC 9144	+									
	1199B	+									
	19x19643	+									
	NCTC 13616	+									
	USA300	+									
	H560	+									
Staphylococcus epidermidis	RP62A	+									
Klebsiella pneumoniae	NCTC 13368	-									
	M6	-									
Acinetobacter spp	AYE (ATCC BAA1710)	-									
	ATCC 17978	-									
Escherichia coli	NCTC 12923	-									
Group B Streptococcus	NCTC 818	+									
	2603V/R	+									
	ATCC 12386	+									
	515	+									
	H36B	+									
	COH1	+									
	18RS21	+									

Figure 4 – Summary of the results of the strain tests on 7.5wt% G – 0.1wt% GP hydrogels ● no degradation ● partial degradation ● degradation.

To confirm that the degradation was due to enzyme activity, supernatant from *P. aeruginosa* PAO1 was heated to 80 °C before being tested. The heat-treated supernatant did not degrade the gel (**Figure S9**).

An effective sensor for protease activity in a real wound environment needs to be able to show a result in as short a time as possible, i.e., within ideally one hour. In **Figure 5**, the change in absorbance at the centre of the well plate is shown as a function of time. A rapid increase in absorbance was measured when 0.1% trypsin and *P. aeruginosa* PAO1 supernatant were added. The absorbance showed a clear difference to the control (BHI) after one hour for 0.1% trypsin and PAO1 supernatant.

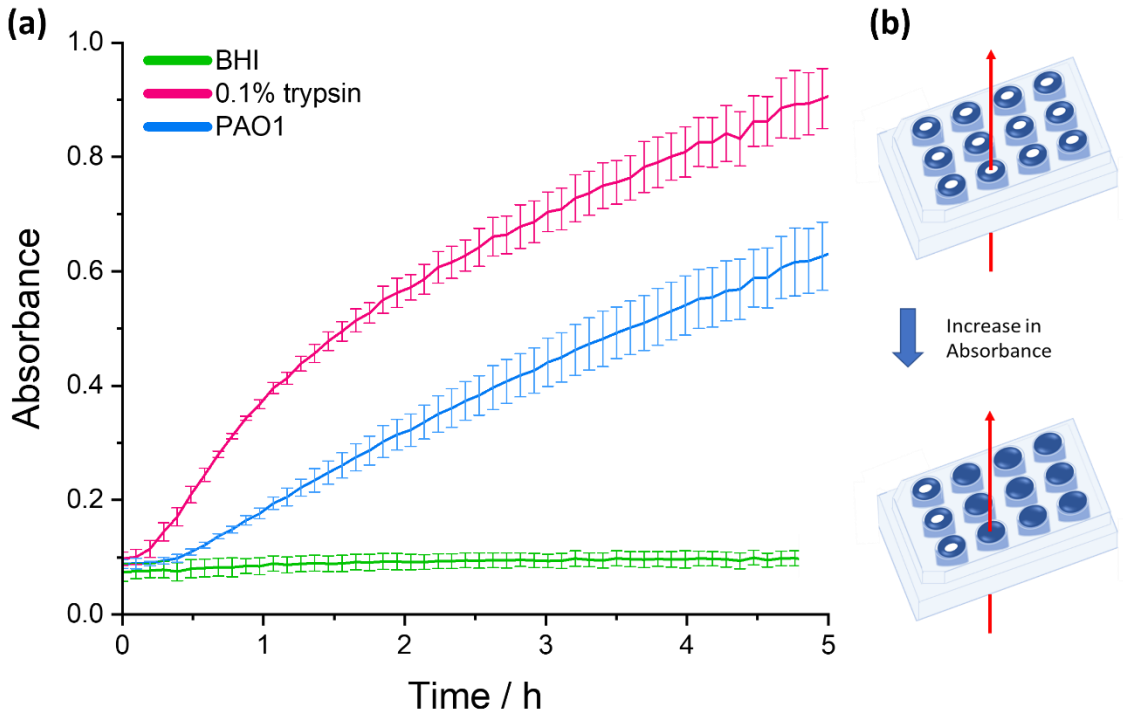


Figure 5 (a) Absorbance changes at 590 nm of 7.5wt% G-0.1wt% GP with BHI, 0.1% trypsin, PAO1 for 5 h. **(b)** Set up of the experiment. Error bars correspond to one standard deviation.

Protease inhibitors to identify specificity

To determine which class of proteases are responsible for the degradation of our gelatin-genipin hydrogels, further tests were performed adding specific protease inhibitors to the

supernatant of the *P. aeruginosa* PAO1 strain. 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was used as a serine protease inhibitor and ethylenediaminetetraacetic acid (EDTA), as an inhibitor of zinc-metalloproteases. The inhibiting action is summarized in **Figure S10**.

As expected, 24 hours after adding trypsin and inhibitors to the gel, AEBSF mixed with 0.1% trypsin prevents the degradation of the hydrogel (**Figure S11-S13**). At 24 hours after adding PAO1 supernatant with inhibitors, a slight degradation can be seen when AEBSF is used but not for EDTA. This observation suggests that metalloproteases could be responsible for the degradation of the hydrogels in the presence of PAO1 supernatant. The decrease in degradation rate in the presence of AEBSF can be explained by metalloproteases being pro enzymes and needing activation from other enzymes. If serine proteases are inhibited, they would not be able to activate the metalloproteases and secondary weaker activators must intervene.[40,41]

After 48 hours, *P. aeruginosa* PAO1 combined with AEBSF shows total degradation of the gel while PAO1 with EDTA does not, thus confirming that metalloenzymes are responsible for the degradation. A summary of the results from the protease inhibitors is shown in **Figure 6**.

			Degradation					
Bacteria & Solutions	Bacterial strains	Gram	24 h			48 h		
			1	2	3	1	2	3
BHI								
BHI + AEBSF								
BHI + EDTA								
0.1% trypsin								
0.1% trypsin + AEBSF								
0.1% trypsin + EDTA								
Pseudomonas aeruginosa	PAO1	-						
	PAO1 + AEBSF	-						
	PAO1 + EDTA	-						

Figure 6 – Summary of the results of the protease inhibitors tests on 7.5wt% G – 0.1wt% GP hydrogels ● no degradation ● partial degradation ● degradation.

Genomics investigation

Elastase A (LasA), elastase B (LasB) and the alkaline protease (AprA) are three major metalloproteases expressed by *Pseudomonas aureus* strains. Our hydrogels were then tested with mutant strains which did not express either *lasB, aprA,* or *lasRI*. These strains were used in Bonchi *et. al*[42]. *LasR* and *lasI* are part of the *las* quorum sensing system of *P. aeruginosa* strains and regulate the expression and secretion of virulence factors. This *las* system was shown to activate the expression of *lasA*, *lasB* and *aprA*.[43] Hence *lasRI* mutants result in quorum sensing deficient strains.[44,45] A strain deficient in all three genes coding for LasA, LasB and AprA proteases was also tested.

Figure S14-S16 show that supernatant harvested from the triple mutant, lacking LasA, LasB and AprA proteases, did not degrade gels, indicating that LasA, LasB or AprA protease is likely to be responsible for degradation. However, when supernatant from mutant PAO1 lacking the *lasB* or *aprA* genes was used the gel did degrade. Furthermore, the hydrogels were not degraded by the supernatant of mutant lacking *lasR* which controls the expression of *lasB*, *lasA* and *aprA*. When *lasR* is inactivated, *lasB*, *lasA* and *aprA* activity is disrupted and metalloproteases may not be released, thus explaining no degradation of the hydrogels.[45,46] It was shown in literature that LasB activates protease IV which then activates LasA which could explain the delayed degradation observed in the case of PAO1 ΔlasB.[45] A *lasA* mutant would be needed to further understand the action of these metalloproteases. A summary of the results from testing several *P. aeruginosa* PAO1 mutants is shown in **Figure 7**.

Bacteria & Solutions	Bacterial strains	Gram	Degradation											
			24 h			48 h			72 h			144 h		
			1	2	3	1	2	3	1	2	3	1	2	3
0.1% trypsin														
BHI														
BHI + 50µg/mL T														
BHI + 200µg/mL G														
Pseudomonas aeruginosa	PAO1 Lausanne	-												
	PAO1 Cowell	-												
Pseudomonas aeruginosa mutants	PAO1 ΔlasRI (PA06330)	-												
	PAO1 ΔlasB (PA3274)	-												
	PAO1 ΔaprA (PA1249)	-												
	PAO1 ΔlasA ΔlasB ΔaprA	-												

Figure 7 - Summary of the results of the mutant tests on 7.5wt% G – 0.1wt% GP hydrogels ● no degradation ● partial degradation ● degradation.

Genomics data was available for eight of the *P. aeruginosa* strains tested (see **Figure 8**). It was found that RP73, NCTC 13437, CFI_004_CRCN strains did not degrade the hydrogels. One significant difference identified from the genome sequences of these strains compared to genomes of strains that caused degradation was observed in the sequence of the *lasR* gene.

Changes in this gene can also be observed in the NCTC 10662 strain which only started to degrade the hydrogels after 48 h and totally after 72 h. This suggests that *lasR* plays an important role in the regulation of expression of active metalloproteases and thus influences hydrogel degradation. Indeed, it has been shown in literature that deletion or mutation in *lasR* sequences had a direct impact on virulence factors gene activity.[47]

		Gene							
Pseudomonas. aeruginosa strains	Degradation of gels	ST	AprA	LasA	LasB	LasI	LasR	RhlR	QslA
Cas3		253	F432C, K445Q		Q102R				
372261		244	K445Q	L9M, P12S, A19V, E61D, V111A, S340G	N244D, N282K, S471R				A100D
GH12		235	K445Q	T262S	Q102R				
PAO1		549		L9M, A19V, E61D, A192V, R223S, S340G	G241S, N244D, N282K, S471R				
NCTC 10662		1203	K445Q	V111A, A194T			V76A		
RP73		198	D352H	L9M, A19V, E61D, A166E, S340G	G241S, N244D, N282K, S471R		1 bp del near C-terminus		A100D
NCTC 13437		357		T262S	Q102R		V226I		
CFI_004_CRCN		357		T262S	Q102R		V226I	Q19STOP	

Figure 8– Genomics of several *P. aeruginosa* strains and comparison of the protein sequence of potentially important metalloproteases and regulating factors.

Specific treatment with multilayered hydrogel containing phage INV-03

Bacterial strains releasing active metalloproteases prevent effective wound healing.[48,49] Thus, detection and treatment of these strains is attractive.

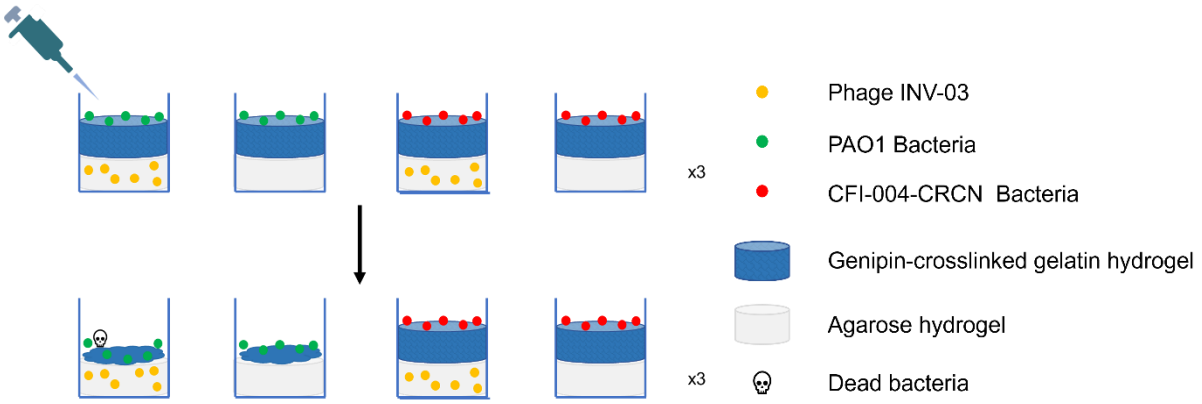


Figure 9 – Set-up of the multilayer experiment and expected results for the treatment of *Pseudomonas aeruginosa* PAO1.

Triggered release of *Pseudomonas* specific phage INV-03 was tested from multilayer hydrogels with the *P. aeruginosa* PAO1 strain. Phage INV-03 was incorporated into a lower agarose gel layer, giving a final concentration of 10^9 PFU.mL^{-1}. PAO1 strains release metalloproteases as evidenced above which should degrade the upper gelatin layer. Following degradation, PAO1 is exposed to the phage incorporated in the lower agarose layer. The *P. aeruginosa* CFI-004-CRCN strain, which does not degrade the crosslinked gelatin layer was tested as a negative control (**Figure 9**).

P. aeruginosa strains PAO1 and CFI-004-CRCN are susceptible to phage INV-03 as tested by their growth analysis by using various dilutions of the phage (**Figure 10**). Indeed, the growth of both strains is limited and then completely inhibited with increasing concentrations of phage INV-03.

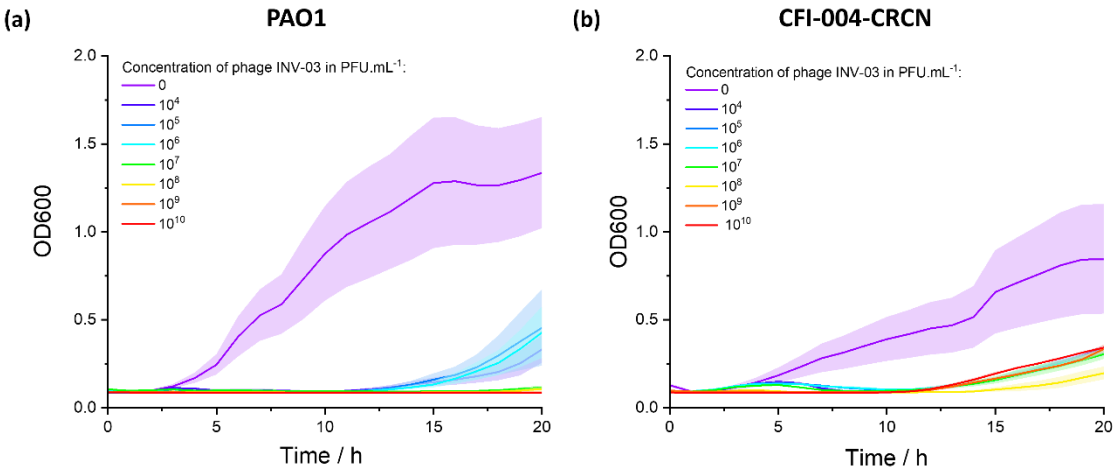


Figure 10 – Growth analysis of *Pseudomonas* **(a)** PAO1 and **(b)** CFI-004-CRCN strains in presence of phage INV-03. Error bars are standard errors.

After confirmation that both strains were susceptible to phage INV-03, triggered release experiment was performed. **Figure 11** shows the colony forming unit (CFU) count for both strains, with and without phage in the underlying gel layer. It can clearly be seen that there is reduction of PAO1 growth in phage INV-03 exposed hydrogels while no effect on growth can be observed for CFI-004-CRCN.

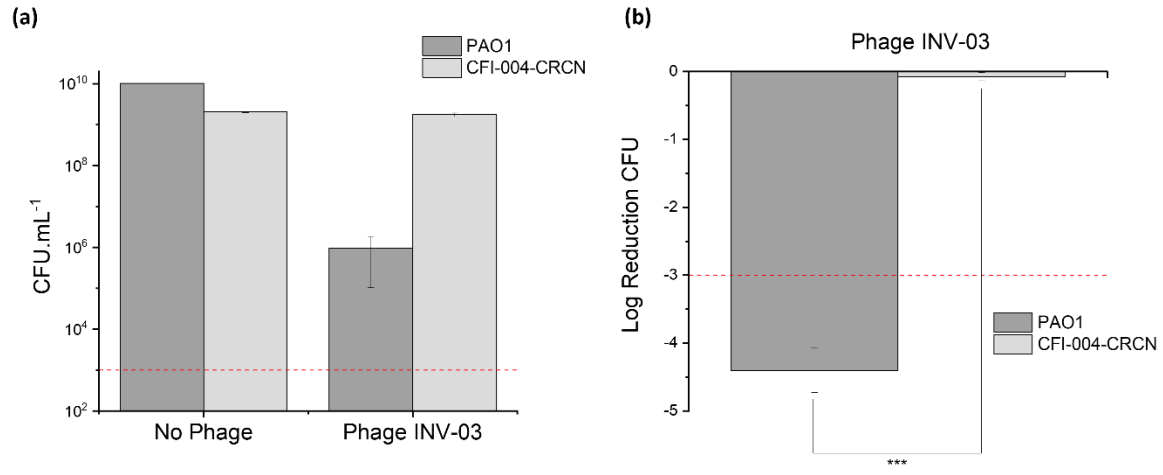


Figure 11 (a) Viable cell counts of *P. aeruginosa* PAO1 and CFI-004-CRCN strains with and without phage in multilayer experiments. **(b)** Log reduction CFU of *P. aeruginosa* PAO1 and CFI-004-CRCN strains after multilayer experiment with phage INV-03 in the lower agarose layer. Red line represents the lower limit of detection. Error bars are standard errors.

Colony counting data can be found in **Figure S17** as well as the log reduction data in **Figure S18**.

This successful addition of a therapeutic layer containing bacteria specific phages demonstrates a potential architecture of multilayered smart wound dressings. In a context of antibiotic resistance, this system thus sounds promising.

CONCLUSIONS

Genipin-crosslinked gelatin hydrogels were successfully synthesized, and their optical and mechanical properties characterized. Enzymatic tests showed good sensitivity of the hydrogels to proteases. Bacterial tests and genomic investigation then confirmed that the hydrogels could potentially be used to detect *P. aeruginosa* strains releasing active metalloproteases. We have used the gelatin hydrogel in a multi-layer system containing phage INV-03 and showed a reduction in the CFU of *P. aeruginosa* PAO1 strain after degradation of the gelatin layer. We believe this system has potential as a future wound

dressing candidate having all the beneficial properties of a hydrogel along with the ability to detect and treat certain *Pseudomonas* strains. The system could also be used to differentiate acute from chronic *P. aeruginosa* infections. For instance, chronic airway infections of cystic fibrosis are associated with LasR mutated strains which is not the case for acute infections.[50,51] For this sensing system to be viable in a clinical setting, it should then be tested against biofilms and also against matrix-metalloproteases, a class of human cells metalloproteases, which are commonly found in wound infections.

ASSOCIATED CONTENT

Supporting Information:

Materials and methods, hydrogel absorbance and fluorescence graphs, rheology studies, swelling ratio, pictures of bacteria test on hydrogel, pictures of inhibitor test on hydrogel, pictures of mutant test on hydrogel, colony counting and log reduction data for multilayer experiment.

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