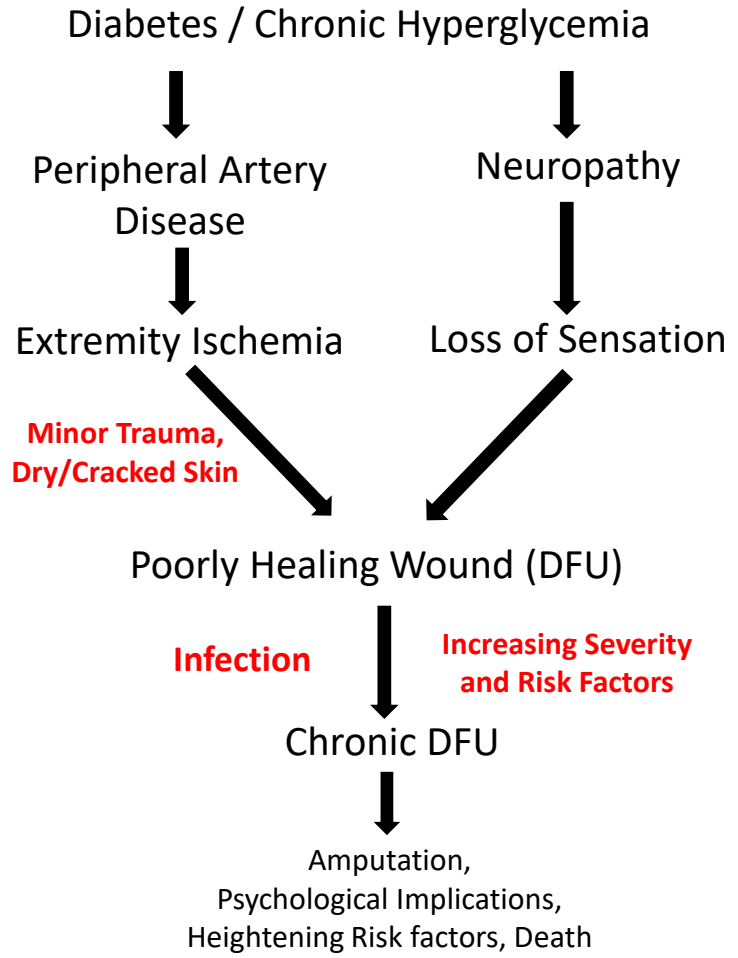
Characterization of Plastically Compressed Collagen Hydrogels With and Without Silver Nanoparticles for Tissue Repair

Luka Vukovic, Christopher McTiernan, Emilio Alarcon
The University of Ottawa Heart Institute and Department of Chemistry and Biomolecular Sciences

Introduction

- Diabetics are 10-20 times more likely to experience lower extremity amputations due to Diabetic Foot Ulcers (DFUs)[1] which are poorly healing wounds extending through the dermis.
- DFUs are reported to have a 5 year mortality rate of 42%.[2]
- DFU care represents a massive 33% of all the costs within diabetes care.[3]
- Current treatments include:
  - longterm antibiotic regiments that can be both topical and systemic
  - wound debridement to remove dead and/or infected tissue
  - wound off loading to reduce pressure on the wound
  - moist wound dressings to promote healing
- However, current wound dressings do not consistently provide symptom alleviation, wound protection, and healing.[4]
- Tissue-like wound dressings may offer improved rates of re-epithelialization but can also be designed to reduce the risk for severe infections.[5,6]



Engineering Tissue-Like Wound Dressings

Project Aim: Develop collagen hydrogels with
(1) antiseptic properties by functionalizing them with biocompatible silver nanoparticles
(2) improved mechanical properties by plastically compressing them.

- Advantages to other wound dressings:
  - Provides a 3D extra-cellular matrix that allows cells to proliferate into
  - Has very low antigenicity [7]
  - Holds large quantities water [5]
- Disadvantages:
  - Effectiveness is still unclear due to limited studies and research bias [8,9]
  - Collagen alone does not carry **antiseptic properties**
  - Has weak **mechanical properties** relative to epithelial tissue [5]

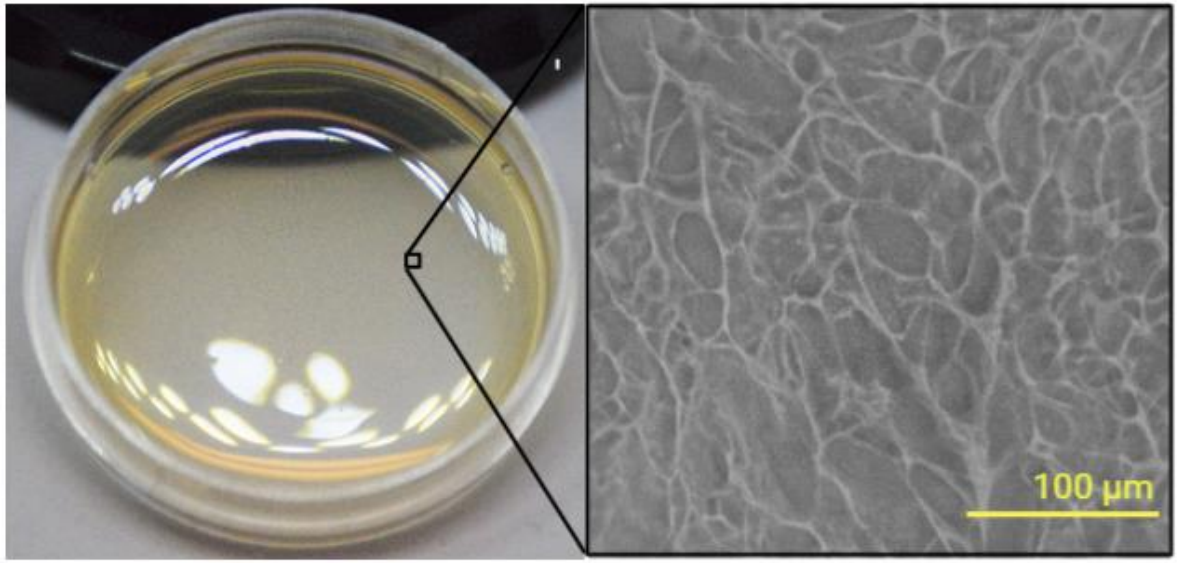


Figure 1. Collagen hydrogel biomaterial (left) prepared from medical grade Type I porcine collagen and cryogenic scanning electron microscopy image (right) of the 3D hydrophilic collagen polymer network.

Improving Collagen Hydrogels

- **Antiseptic properties** can be improved by functionalizing hydrogels with silver nanoparticles (AgNPs) [6]
- Hypothesis I
  - The developed *in situ* method for photochemical functionalization of AgNPs can provide antibacterial properties without human cytotoxicity.

- **Mechanical properties** can be improved by plastically (irreversibly) compressing hydrogels [10,11,12]
- Hypothesis II
  - The developed hydrogel compression method can improve mechanical properties by increasing collagen network density.

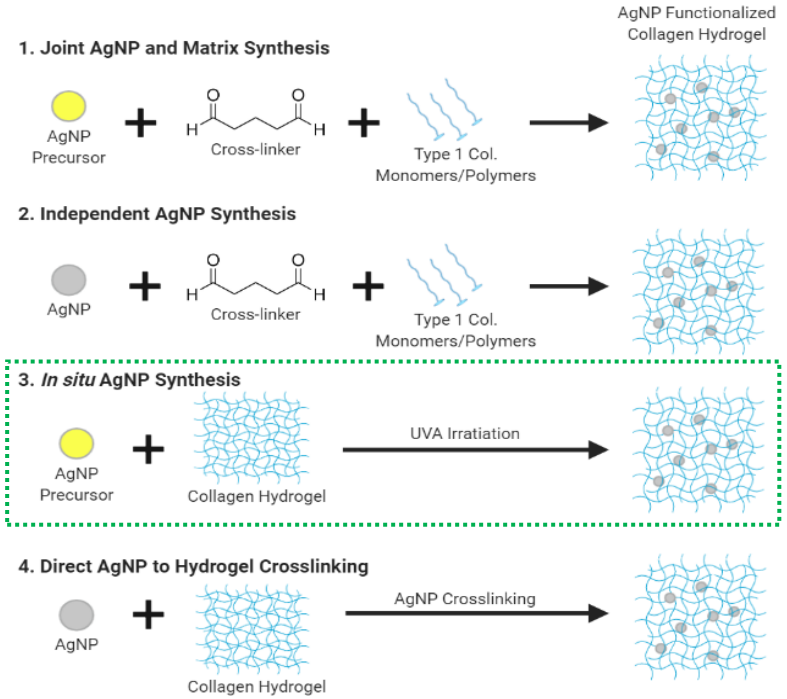


Figure 2. Different methods for functionalizing of collagen hydrogels with AgNPs. Green box was the selected approach in this project. Figure was adapted from a similar summary produced by Hui-Li *et al.* (2019).[13]

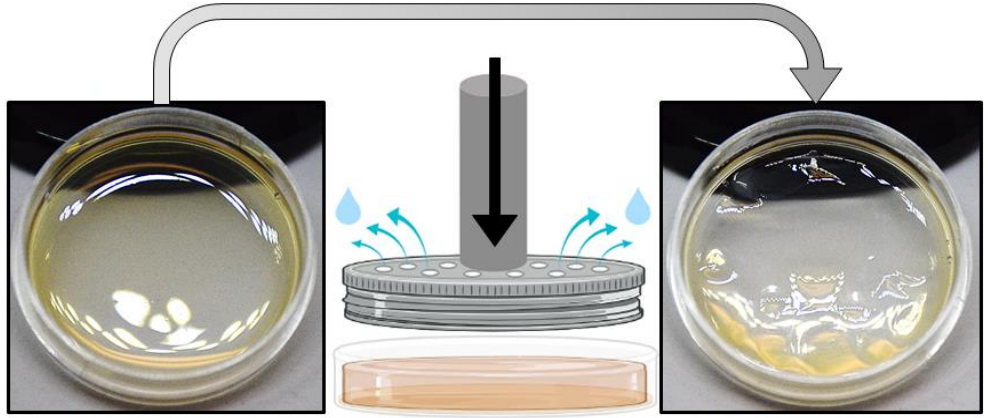
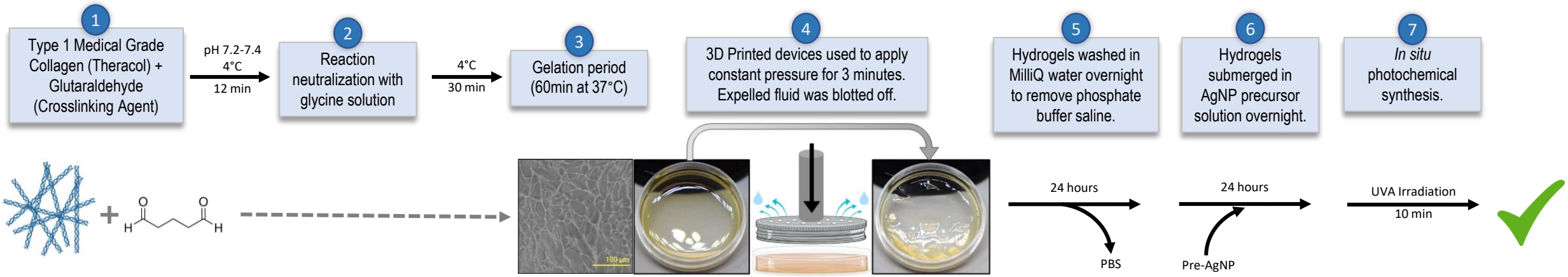


Figure 3. Illustration of plastic compression procedure using the 3D printed plastic compression device. Collagen hydrogel before (left) and after (right) plastic compression. Pores passing through the device allow water to escape as the hydrogel is compressed for three minutes.

Preparation of Collagen Hydrogels



Solid Mass Content of Collagen Hydrogels

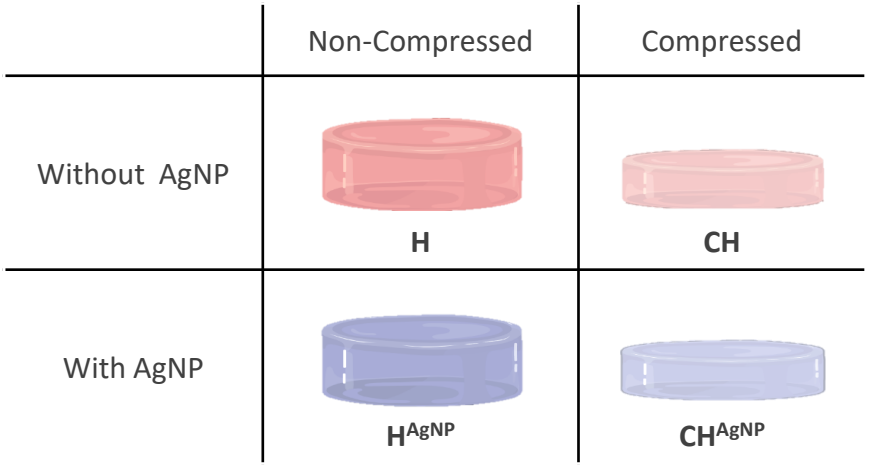


Figure 4. The four categories of developed experimental collagen hydrogels. Hydrogel (H), Compressed Hydrogel (CH), Hydrogel with AgNP (H^{AgNP}), Compressed Hydrogel with AgNP (CH^{AgNP})

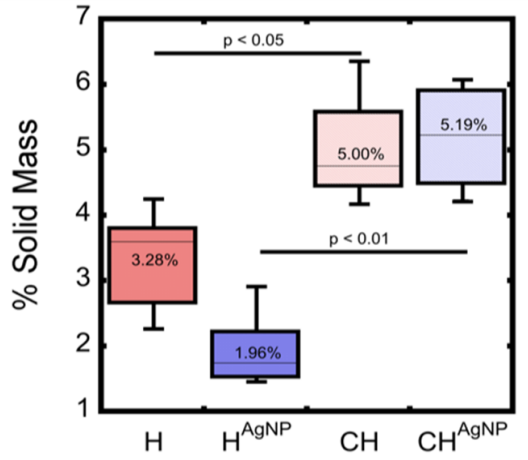


Figure 5. Solid mass content (SMC) of the four types of hydrogel. H (n=7); CH (n=4); H^{AgNP} (n=6); CH^{AgNP} (n=4). Sample means labeled in each boxplot.

Do reported changes in SMC reflect a permanent change in physical properties?

Confirming Compression is Plastic (Irreversible)

- Swelling tests inform us on the hydrogel's ability to decompress (reabsorbed water).
- Compressed Hydrogels (CH) do not swell to pre-compression weights.
- CH gels maintain properties even after dehydration ($p<0.001$).
- Swelling was only significant in CH gels ($p<0.01$) despite the change being small (21% increase).

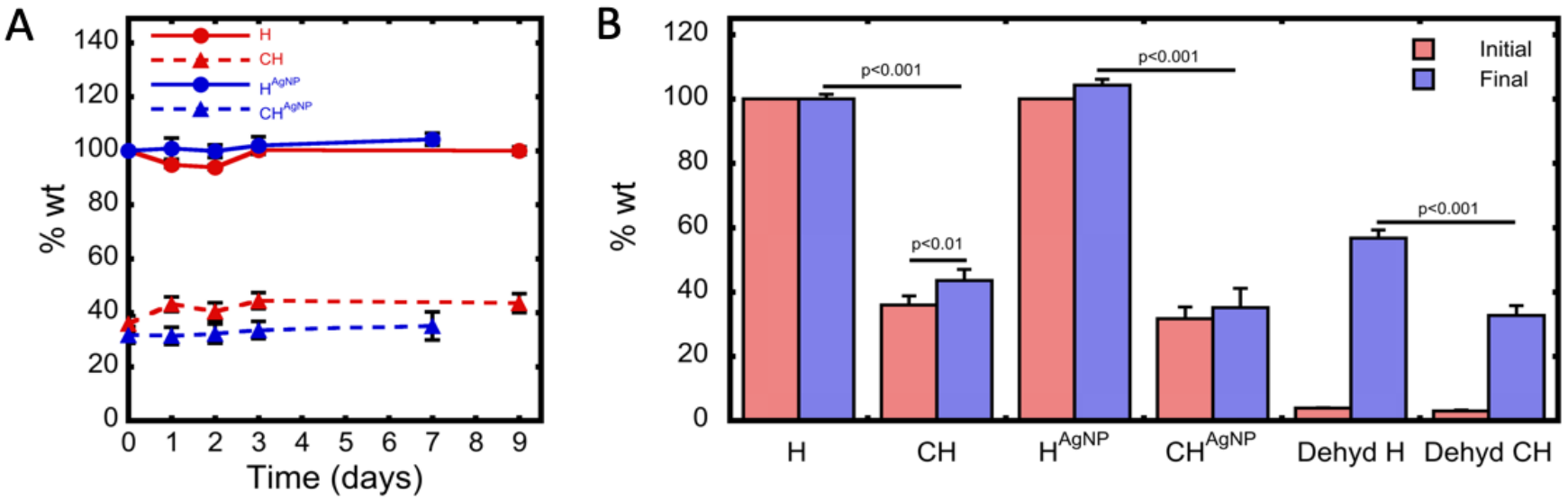


Figure 6. Swelling capacities of the four types of hydrogel. **(A)** Hydrogel weight as percentage of the pre-compression weight while submerged in 1x PBS or Milli-Q water if the hydrogel contained AgNPs. **(B)** Initial and final hydrogel weights during the swelling period. H (n=3); CH (n=3); H^{AgNP} (n=2); CH^{AgNP} (n=4); Dehyd H (n=3); Dehyd CH (n=4).

Compression's Effect on Viscosity and Collagen Network Density

- The average pore size increased after compression ($p<0.001$), thus collagen network density decreased in H/CH gels.
- This may be due to microscopic tearing of pores to create larger pores, an insufficient applied pressure during compression, or from computational bias in pore analysis which groups smaller pores into larger pores.
- Thus compression did not change viscosity (resistance to deformation) between H/CH ($p>0.6$) and H^{AgNP}/CH^{AgNP} gels ($p>0.1$).
- Small sample sizes and lack of Cryo-SEM images limited validity in the doubling viscosity from H^{AgNP} to CH^{AgNP} gels.

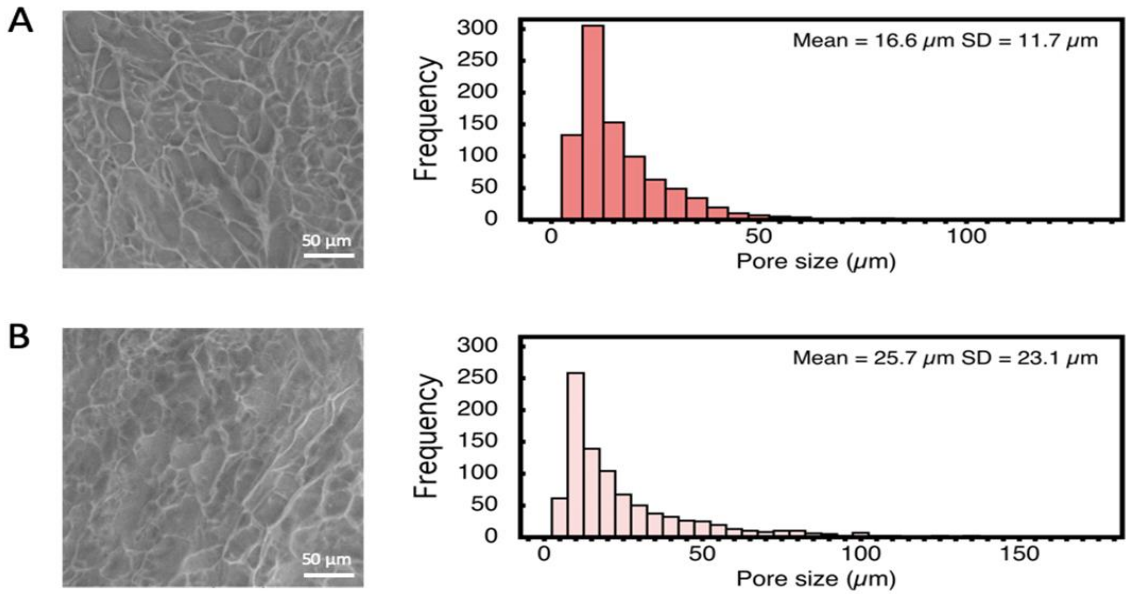


Figure 9. Frequency distribution of collagen pore sizes as a diameter based on representative Cryo-SEM images (left). **(A)** Non-compressed hydrogel. **(B)** Compressed hydrogel. The mean pore diameter of compressed hydrogels is significantly greater than that of non-compressed hydrogels ($p < 0.001$) based on a student t-test and Kolmogorov-Smirnov cumulative distribution test.

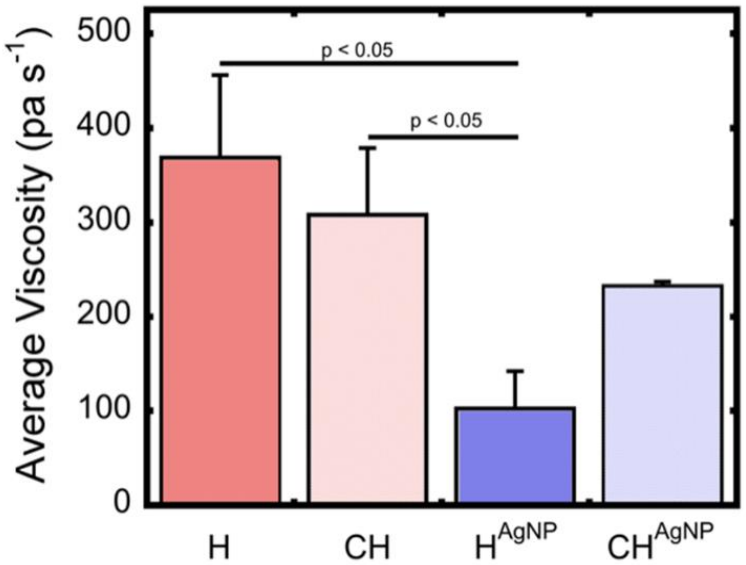


Figure 8. Average viscosities of the four types of hydrogel as measured on a rheometer at 37°C. Error bars represent the standard error. H (n=6); CH (n=7); H^{AgNP} (n=2); CH^{AgNP} (n=2).

Evaluating Degradability: Collagenase Assay

- CH^{AgNP} gels had the lowest 24-hour degradation rate; 33% lower ($p<0.001$); relative to other groups.
- AgNPs or Ag^{+} oxidation products may inhibit collagenases.[15]
- Compression alone had no effect on degradation.
- However, compression and AgNPs may work synergistically to provide the most robust properties.
- Experimental error from small sampling weights may have confounded the initial CH^{AgNP} degradation weights in (A).

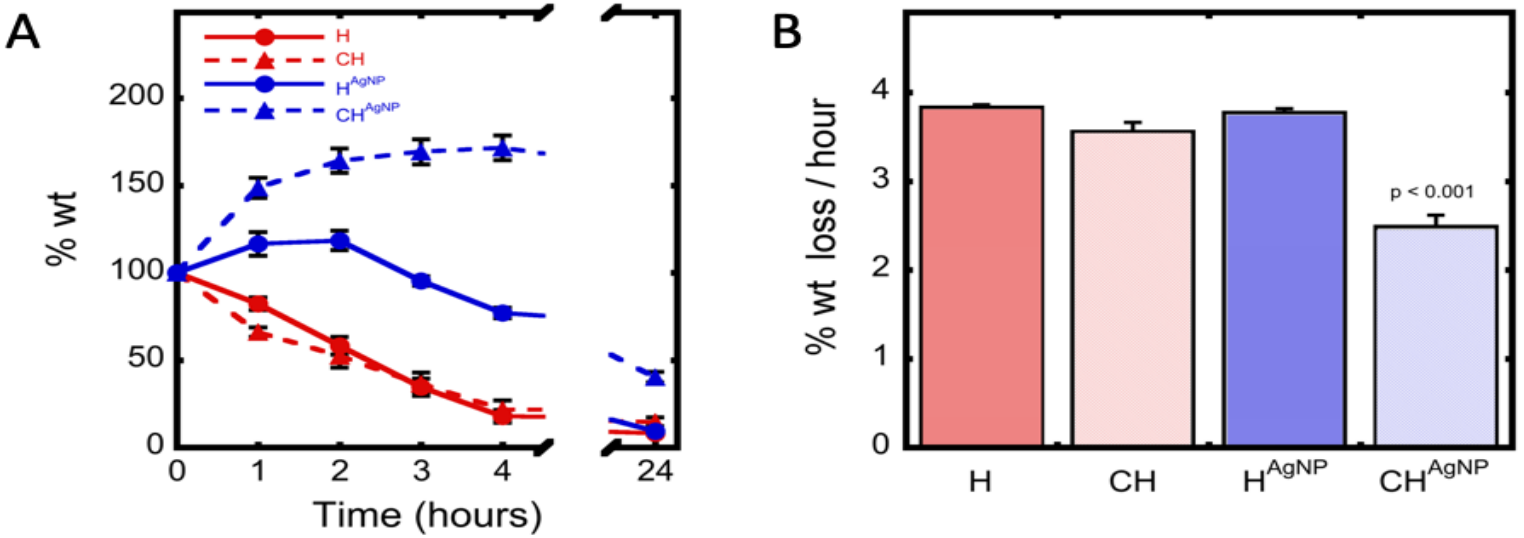


Figure 7. (A) Collagenase assay illustrating hydrogel weight loss (degradation) as a function of time. The % weight of hydrogel remaining was measured over 24 hours while submerged in 1mL of 5U type I collagenase solution. H (n=9); CH (n=9); H^{AgNP} (n=6); CH^{AgNP} (n=6). Error bars represent standard error. **(B)** 24-hour average hydrogel degradation rate. Sample sizes are the same as in A and error bars represent standard error. Significance was assessed by comparing CH^{AgNP} to each other hydrogel via a two-sample t-Test assuming unequal variances.

Confirming Hydrogel Functionalization with AgNPs

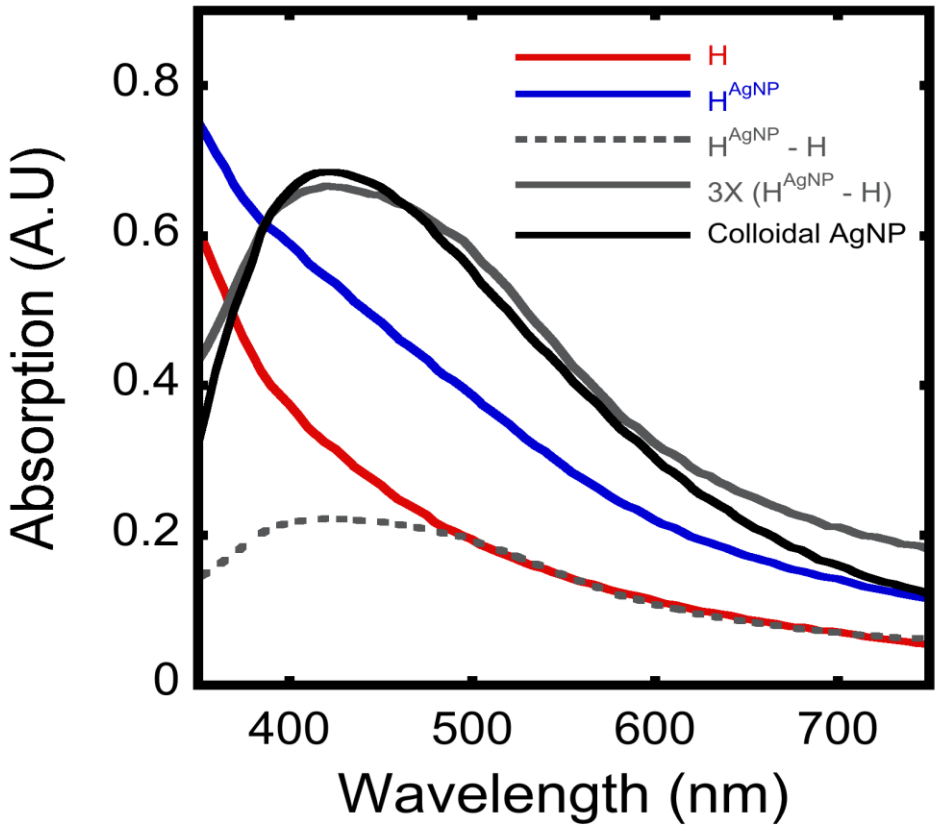


Figure 8. UV-Vis absorption spectra of unmodified and AgNP functionalized hydrogels. (H^{AgNP} – H) was produced from the difference between hydrogels before (H) and after (H^{AgNP}) UVA irradiation. The difference spectrum was amplified 3x and overlaid on the spectrum of colloidal citrate protected AgNPs. Prior to UV-Vis measurements, hydrogels were washed to remove any weakly bound AgNPs. Spectra correspond to the average of n=4 samples. Water was used as a blank in all measurements.

- Absorption spectrum of AgNP functionalized hydrogels display a shoulder around 400 nm, characteristic of AgNPs.
- An unwashable colour change (from light yellow to yellow/red) was observed after photochemical synthesis of AgNPs.
- 420nm peak corresponds to an average particle size of 50nm based on prior characterizations of wavelength maxima.[14]
- Light scattering from the gel may exaggerate the particle polydispersity (width of the absorbance peak).

Fibroblast Viability on Hydrogels

- AgNPs severely limited fibroblast viability ($p < 0.001$).
- Compression seems to have a small beneficial effect on cell viability.
- CH had 5.3% more Live cells than control ($p < 0.001$; unmarked), but was not significant in comparison to the H group ($p = 0.07$).
- AgNP containing gels induced apoptosis, an indicator for irreparable DNA damage.

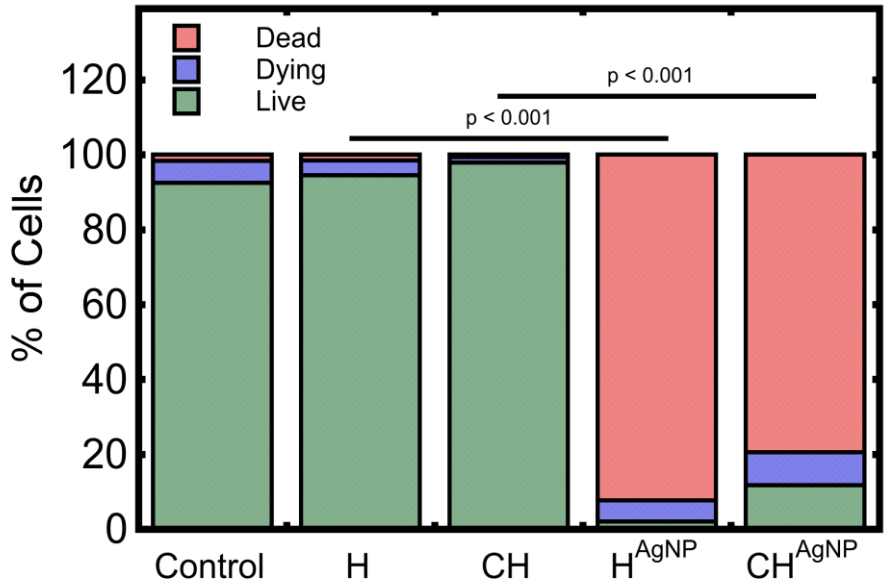


Figure 11. Cell viability assay on immortalized human fibroblasts grown on the four types of hydrogel. Four wells were seeded per group (three for CH) at 2500 cells/well (0.75 cm^2). Total cell count including Live, Dying, and Dead cells: control (n=7857), H (n=9075), CH (n=6919), HAgNP (n=1900), CHAgNP (n=668).

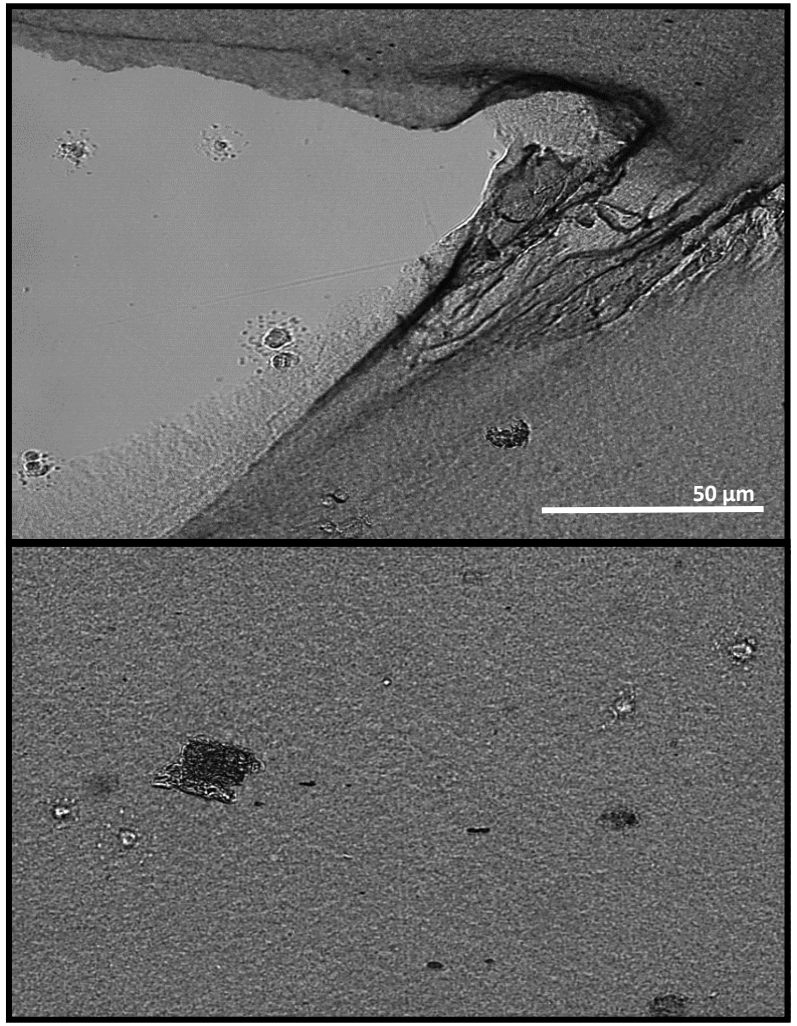
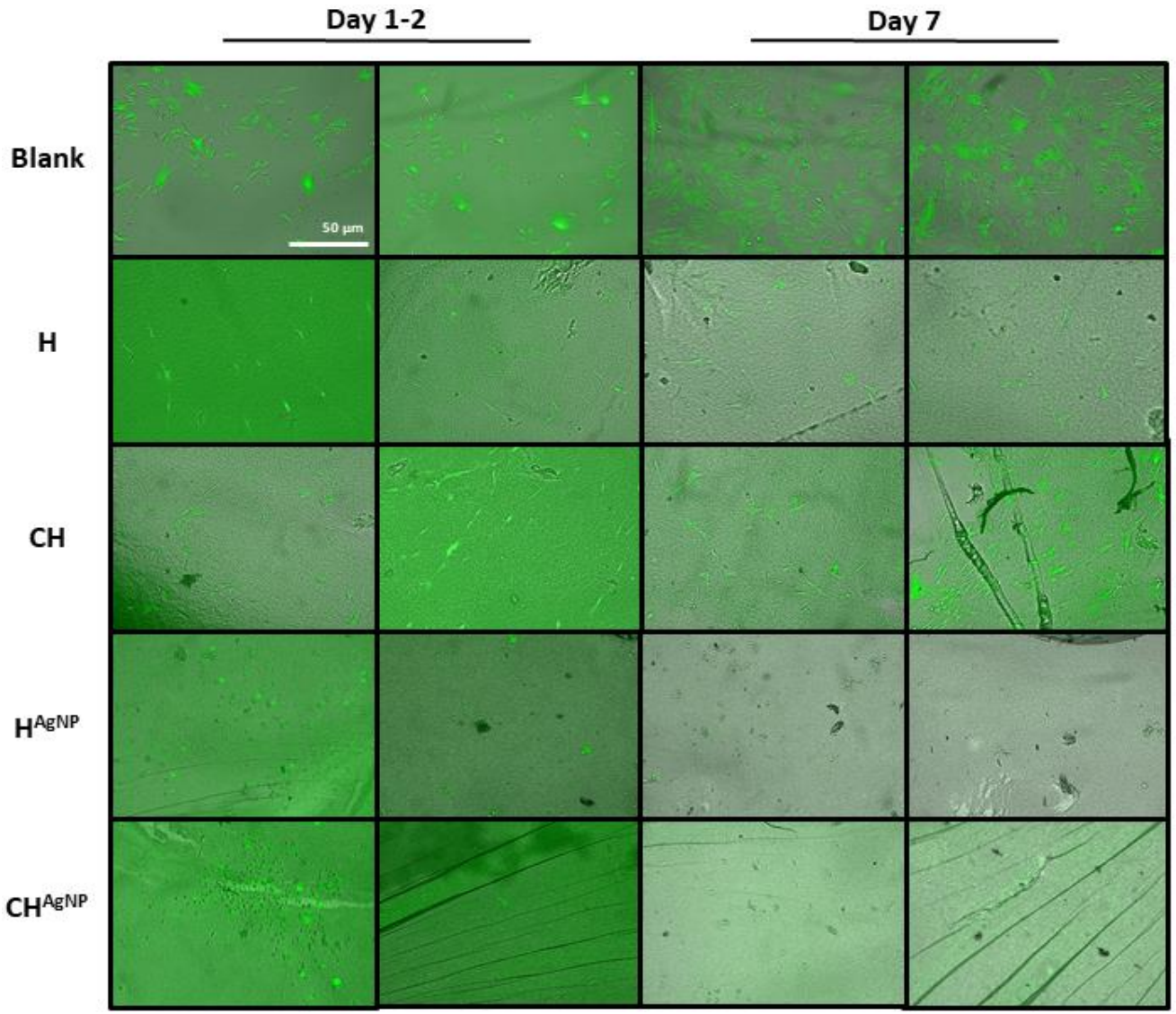


Figure 12. Brightfield (top) and darkfield (bottom) microscopic images of apoptotic human fibroblasts 1 day after seeding on non-compressed (top) and compressed (bottom) AgNP functionalized hydrogels.

Fibroblast Proliferative Capacity on Hydrogels



- H and CH gels did not support proliferation rates similar to the control group despite having similar Day 5 cell densities in the Live/Dead assay.
- Lower than expected proliferation rates may be because an over-confluent cell population was lifted and applied during seeding.
- CH gels had more proliferation compared to H gels.
- AgNP Functionalized Gels:
  - No proliferation was observed likely due to their cytotoxicity.
  - Small, circular cell shapes observed on day 1 suggest that no cell attachment took place.

Figure 13. Multichannel (fluorescent/brightfield) images of human fibroblast proliferation on four types of collagen hydrogel. Fibroblasts used were immortalized and transfected with green fluorescence protein (GFP). N = 4 wells per group. Control wells which contained no hydrogel approached confluency at day 7. Three random images per well were taken daily for 7 days.

Conclusions *Relative to Unmodified Hydrogels (H)*

CH	Prediction	Result	
Degradability	↑	-	Mechanical properties may not have changed appropriately to improve degradability.
Viscosity	↑	-	(1)
Avg Pore Size	↓	↑	(1)
Viability	Viable	Viable	Compression may provide a modest improvement in fibroblast survival and proliferation.
Proliferation	-	↑	
CH^{AgNP}			
Degradability	↑	↑	May be due to silver's inhibition of collagenase.
Viscosity	↑	-	(1)
Avg Pore Size	↓	?	No Cryo-SEM images were available for analysis.
Viability	Viable	Not viable	(2)

(1) May be the result of an ineffective compression method.
(2) Speculative reasons for cytotoxicity:
- Leftover AgNP precursor ($AgNO_3$) after *in situ* functionalization.
- Particle oxidation and leaching of Ag^+ ions.

To improve mechanical properties, modifications in compression should be considered.

Characterizing AgNP stability and residual $AgNO_3$ post functionalization is required to elucidate the reasons for gel cytotoxicity.

The developed CH^{AgNP} gels showed a substantial improvement in their resistance to degradation. Further work should build on these gels with the aim of rendering them biocompatible via variations in AgNP functionalization.

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Acknowledgements

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