

Developing novel plant waste-based hydrogels for skin regeneration and infection detection in diabetic wounds

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SUMMARY

More than 70,000 diabetic patients have amputations annually due to wound care complications. Poor circulation and hyperglycemia-related nerve damage, which are complications of diabetes, prevent natural wound-healing processes. The purpose of this investigation is to develop a hydrogel to aid skin regeneration by creating an extracellular matrix for fibroblast growth with antibacterial and infection-detection properties. We developed two natural hydrogels based on pectin and potato peels and characterized the gels for fibroblast compatibility through rheology, scanning electron microscopy, swelling, degradation, and cell cytotoxicity assays. All five assays indicated that potato peel starch and citrus pectin were capable of mimicking natural skin, with a relevant pore diameter and stiffness. Both groups were able to absorb 40 times their weight before naturally degrading and supported upwards of 500% NIH 3T3 fibroblast proliferation. Additionally, anthocyanin, a pigmented flavonoid, was encapsulated in hydrogels and used as an infection biomarker and preventative agent. We conducted a pH responsivity test and time-to-kill antibacterial assay, validating the ability of anthocyanin to noticeably change colors in hydrogels and assert bactericidal effects even at low concentrations. A concentration of 200 milligrams could prevent bacteria growth and showed no effect on fibroblast growth. Overall, this experiment fabricated various hydrogels capable of acting as skin substitutes and counteracting infections to facilitate wound healing. Following further testing and validation, these hydrogels could help alleviate the 13-billion-dollar financial burden of foot ulcer treatment.

INTRODUCTION

Diabetic wounds are often debilitating, costly, and potentially life-threatening. Causes of diabetic wounds can range from the smallest scratch to ulceration of whole skin layers. The most common diabetic wound is a foot ulcer, a prolonged sore on a patient's foot that often recurs (1). Patients with high glucose levels, such as those with diabetes, experience substantially lower rates of healing due to impairment of fibroblast migration and a slowed immune response (2). Fibroblasts are necessary to create new extracellular matrices, break down fibrin clots, and produce collagen to close the wound (3). Additionally, increased

glucose levels cause scaring and swelling of nerves, leading to weak neuropathy in the foot (4). This has further implications; for instance, patients may not realize they have a small contusion and keep walking, which places the pressure of the whole body directly on the foot. The extended duration of such pressure opens the wound and allows more infections. Complications due to infections including gangrene result in lower limb amputations. The serious implications of diabetic wounds are reflected in recently uncovered data: 80% of non-traumatic amputations were related to infected ulcers in diabetic patients, 1 in 10 diabetic patients have had foot ulcers, upwards of 13 billion dollars are lost annually to diabetic ulcer care, and more than 70,000 diabetic patients have amputations each year (5). This impact is further exacerbated in developing countries, where diabetes and related complications are significantly higher (6).

Pitfalls of current treatments have led researchers to investigate an alternative treatment: a partial skin substitute. Examples of mainstream skin substitutes include Dermagraft®, Integra®, Alloderm®, and Apilgraf®. These substitutes mimic natural skin extracellular matrix, absorb wound exudates, avoid infections, and provide water to the wound (7). However, a majority of commercially available skin substitutes are essentially decellularized skin grafts that are implanted at the site of the wound. Using decellularized grafts entails its own problems: there is a chance of cellular rejection, scars and discoloration are all prevalent side effects, and it takes considerable time and money for implantation followed by treatment (8). This prevents all people, regardless of money and the severity of their wounds, from finding a personalized product. A new skin regenerative hydrogel, that would not require implantation decreasing the chance of rejection, that is both accessible and effective is an urgent necessity in the wound regeneration field and commercial market.

We aimed to address issues related to current skin substitutes by comparing the effectiveness of hydrogels made of inexpensive and naturally occurring everyday waste. Hydrogels are a 3D network of crosslinked polymers that can absorb significant amounts of liquid, which means hydrogels are able to act as skin substitutes for wound healing (9). We focused on two promising biomaterials: potato peel starch and pectin. Each biomaterial chosen has specific characteristics necessary for wound regeneration as well as the removal of infectious agents.

Potato peels have antibacterial properties due to surface glycoalkaloids, which have membrane-disrupting effects in both gram-positive and gram-negative bacteria (10).

Antibacterial properties are important facets of extracellular matrices due to the development of antibiotic-resistant bacteria (11). Potato peel extracts, specifically starch, also exhibit collagen stimulation, which further recreates hospitable conditions for cell proliferation. Potato peel extracts can increase the expression of type 1 collagen and secretion through the pathway controlled by transforming growth factor, TGF- β receptor (12). Natural antioxidants, metabolites, and fibers are cytotoxic to microbial pathogens and related to faster healing.

The second biomaterial tested was pectin, a structural polysaccharide found in plant cells, and functions in cell binding and adhesion (13). Pectin is rich in galacturonic acid, which serves a structural role as the monomer and a functional role as an acidifying agent (14). Galacturonic acid aids in pectin solubility and elasticity. Pectin mimics other polymers used for scaffolding, such as gelatin, but has the advantage of being easily extractable in larger quantities and accessible (15). Supplemented with chitosan, these hydrogels have the capability to drastically increase fibroblast production *in vitro* as well as in real wound areas (16).

One of the most challenging issues in diabetic wounds is controlling and treating infections. To detect these infections in a timely and readily noticeable manner, anthocyanin food colorant was incorporated into the hydrogel. Anthocyanin reacts strongly to sudden pH increases, which can signal an infection. Anthocyanin's ionic molecular structure causes it to be more stable in acidic solutions, changing to red in acidic pH, purple in neutral pH, and a bluish green in increasing pH levels. Anthocyanin also has anti-cancer, anti-inflammatory, antimicrobial, anti-oxidization, and anti-diabetic properties (17). As anthocyanin is a strong antibacterial chemical in plants, it was hypothesized the same behavior would be observed in the fabricated hydrogels. The concentration of anthocyanin has been shown to be inversely related to both gram-positive and gram-negative biofilm biomass and adhesion in crops (18). Research has also shown that anthocyanin can negatively affect bonding such as mental ionic bonding and methylation in bacterial cell membranes (19). The hydroxyl groups in anthocyanin make it primarily hydrophobic despite its ionic structure, allowing for passive diffusion into bacterial membranes. Anthocyanin then causes leakage of sodium and potassium into the cell of the bacteria, which disrupts homeostasis and causes widespread cell death (20). Furthermore, preliminary research suggests the anthocyanin is not cytotoxic to human cells, but its effect on skin cells like fibroblasts has not been studied (21). Anthocyanin will, in theory, cause a noticeable color change in the hydrogel and allow patients to reach out to caregivers and notify them about a potential infection before amputation becomes the only option.

The purpose of this investigation was to develop a hydrogel to aid in diabetic wound healing by addressing the lack of an enhanced extracellular matrix for fibroblast growth and high rates of wound infections. The three phases of

this project involved the creation and comparison of novel hydrogels and antibacterial agents for a comprehensive approach to diabetic wound regeneration. In phase 1, we hypothesized cross-linking potato peel to chitosan as well as pectin to chitosan would create a hydrogel capable of exhibiting physiological stuffiness, porosity, swelling, and degradation to healthy skin. Structural characterization was conducted through rheological frequency sweep, scanning electron microscopy, and biodegradation studies. In phase 2, we hypothesized anthocyanin would cause a noticeable color change in hydrogels soaked in different pH solutions and exhibit *Escherichia coli* K12 growth in solution. In phase 3, we hypothesized the hydrogels and biomaterials used, including anthocyanin, would not be cytotoxic to fibroblast proliferation and support cell viability. MTT assays were conducted to quantify proliferation. Overall, the novel potato peel and pectin hydrogels could support four-fold fibroblast proliferation, respond colorimetrically to pH fluctuations, and inhibit bacterial growth. This will be able to support hospitals in developing countries dealing with high rates of diabetic wounds. Patients can be given simple instructions and be given proper care to treat their wounds.

RESULTS

Recreation of skin elasticity

Using a rheometer, we measured the following frequency-dependent parameters to ensure that each sample demonstrated the structural characteristics of hydrogels and skin substitutes: G' , G'' , and complex moduli (22). The complex modulus is a combinative measure of the elasticity (G' modulus) and viscosity (G'' modulus) of the material. We used chitosan (CS), alginate (AG), potato peel starch plus chitosan (PS+CS), citrus peel pectin plus chitosan (PE+CS), and anthocyanin (CS+PS+AC and CS+PE+AC) were tested in all assays. The positive control was the Wound Dimora

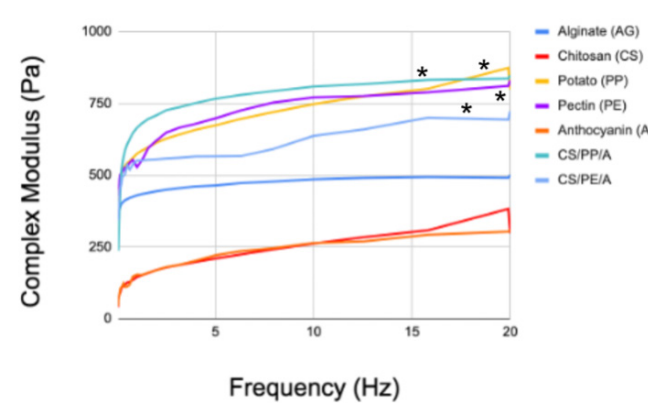


Figure 1. Potato peel and pectin hydrogels mimic skin complex modulus. Samples were tested under a rheometer frequency sweep to determine complex modulus at various frequency (n=6). The complex modulus is a measure of the elasticity (G') and viscosity (G'') of the material. All complex moduli of potato peel and pectin hydrogel formulations were significantly different than the controls, alginate and chitosan (single factor ANOVA, $p < 0.05$). * $p < 0.05$

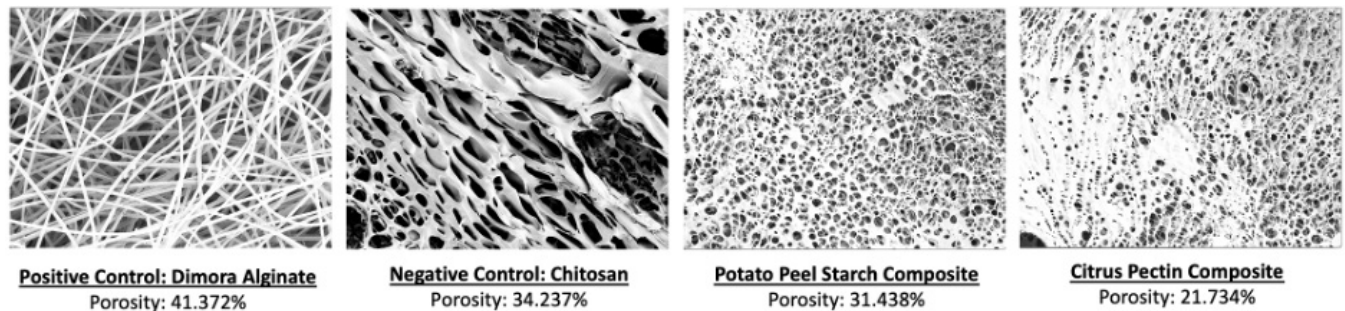


Figure 2. Potato peel and pectin hydrogels have open pore geometry and skin matrix morphology. Each sample's surface topology was viewed under a scanning electron microscope at 100X magnification and analyzed under ImageJ software (n=6).

alginate hydrogel as it is widely used as a treatment to diabetic wounds, and the negative control was the chitosan hydrogel which is not intended to mimic skin microstructure. In the rheology assay, the chitosan and potato peel starch composite hydrogel with encapsulated anthocyanin had the highest elasticity and complex modulus, while the chitosan hydrogel had the lowest (**Figure 1**). To be a hydrogel, a material must exhibit a G' modulus higher than G'' , and this characteristic was observed in all samples (23). The experimental groups could bear more pressure than the control groups (single-factor ANOVA, $p < 0.05$) and we found that anthocyanin had no structural effect on the hydrogel (two-tail t-test, $p > 0.05$).

Determining surface porosity of hydrogels

To compare the average pore diameter and porosity of each hydrogel to one another and to those present in skin's natural extracellular matrix, randomized scanning electron micrographs of surfaces were taken at 100X magnification and analyzed using ImageJ software (**Figure 2**). Skin fibroblasts tend to settle in pores with a diameter ranging from 30 to 90 micrometers. All samples had pores that were within this range: AG: 41.37 ± 6.41 , CS: 57.74 ± 1.11 , CS+PS: 82.10 ± 12.57 , and CS+PE: 48.30 ± 24.32 (**Figure 3**). A one-

factor ANOVA test revealed statistically significant pore size differences ($p < 0.05$).

Swelling and biodegradability properties of hydrogels

Hydrogels were soaked in cell culture medium for 24 hours, with their soaked weight and dry weight being measured over time. The hydrogels were all able to absorb over 40,000%, or 40 times, their weight in water. Each sample was able to maintain this absorbance for at least 3 hours before eventually starting to lose structural composition (**Figure 4**). However, the Dimora wound dressing was able to retain this absorbance for much longer. The experimental hydrogels had a statistically similar absorbency (single-factor ANOVA, $p > 0.05$) and anthocyanin did not have a significant effect on absorption or retention in the hydrogels (two-tailed t-test, $p > 0.05$).

Anthocyanin inhibits bacterial proliferation

The bactericidal of anthocyanin was tested through a Time-To-Kill assay, where increasing volume of anthocyanin (10, 20, 50, 50, 100, 200, and 500 milligrams) was incubated with *E. coli*. The optical density of cultures was read every 30 minutes and lower density indicated decreased viable

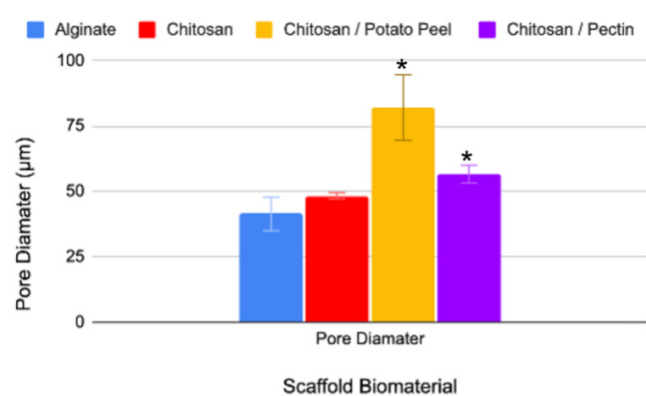


Figure 3. Potato and pectin hydrogels have physiologic pore diameters. Pore diameter was analyzed from scanning electron imaging (n=6). Potato peel and citrus pectin formulations were significantly different from the controls, alginate and chitosan in isolation (single factor ANOVA, $p < 0.05$). Data shown as mean \pm standard deviation. * $p < 0.05$

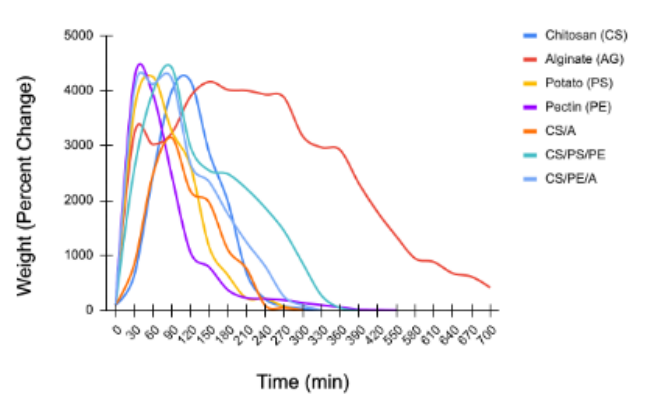


Figure 4. Potato peel and pectin hydrogels absorb 40 times dry weight. Hydrogels were soaked in cell culture medium and weight was recorded periodically (n=6). Experimental hydrogels absorbed similar amounts as the control hydrogels, as indicated by no statistical significance (single factor ANOVA, $p > 0.05$). The alginate hydrogel was able to retain water the longest period.

bacteria. Anthocyanin exhibited a concentration-dependent antibacterial effect on the bacterial colony: 50 milligrams demonstrated the ability to disrupt bacterial growth, 200 milligrams was able to stop bacterial growth completely, and 500 milligrams killed almost all bacteria present (Figure 5).

pH responsivity of anthocyanin-encapsulated hydrogels

The color change of anthocyanin encapsulated in hydrogels was compared to the color change of aqueous anthocyanin in various pH solutions. Skin that is healthy has a pH below 5.5, while infected skin has a pH between 7.2 and 8.5 (24). In all pH levels tested, the hydrogel readily changed colors, from a bright red to dark blue to orange in increasing pH solutions (Figure 6). This change in color is completely visible to the naked eye and can be used as a simple detector for infectious wounds.

Biological characterization of hydrogel's cellular viability

Fibroblasts (murine fibroblast NIH 3T3) were seeded into the hydrogels. To determine the cytotoxicity and ability to support cellular growth of each hydrogel, an MTT assay was performed which measures metabolically active cells by their conversion of the yellow water-soluble MTT compound to insoluble purple formazan crystals. The optical density was read at 560 nanometers and cell growth was quantified using a standard curve. The average viable cell counts were as follows: AG: 1257 ± 199 , CS: 533 ± 15 , CS+PS: 2252 ± 204 , CS+PE: 2404 ± 205 (Figure 7). The experimental plant waste hydrogels could support a statistically significant increase in cellular proliferation (one-factor ANOVA, $p < 0.05$). Anthocyanin was not cytotoxic as there was no statistically significant variance between samples with and without anthocyanin (two-tail t-test, $p > 0.05$).

DISCUSSION

Under physiological stresses in the rheology assay, the experimental hydrogels demonstrated a relevant stiffness and structure, as the G' , G'' , and complex moduli were all within the range of normal skin's extracellular matrix. Control samples

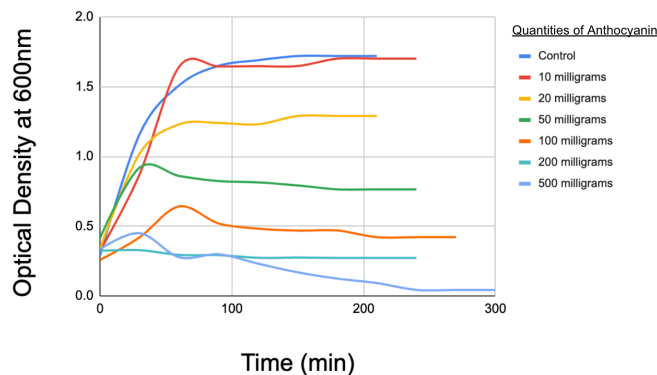


Figure 5. Anthocyanin inhibits bacterial growth in solution. *E. coli* K12 was cultured with varying concentrations of anthocyanin, and optical density was measured periodically to represent alive bacteria count through time-to-kill assay. Anthocyanin concentration was inversely related to bacterial survival, indicating its strong antibacterial activity.

were not able to endure as much stress as the experimental samples, resulting in lower complex moduli and loss of matrix structure under minimal stress. The skin mimicry seen in our hydrogels is essential to wound healing as it recreates natural matrix conduction conducive to fibroblast growth.

Scanning electron micrographs found physiologically relevant surface composition. Fibroblasts tend to settle in pores with a diameter ranging from 30 to 90 micrometers, and this range best facilitates cell survival as it mimics tissue architecture, enhances nutrient transport, and modulates cell behavior. All hydrogels tested were within this range and demonstrated statistically different pore sizes to the alginate and chitosan controls, which lacked skin-like pore morphology.

Our hypothesis that the hydrogels would be able to absorb and retain cell medium better than available hydrogels was not supported. All samples were capable of absorbing 40 times their weight, but the Dimora wound dressing was able to retain this absorbance for much longer. Anthocyanin did not have a significant effect on water absorption or retention in the hydrogels.



Figure 6. Hydrogels with encapsulated anthocyanin has visible color changes at various pH levels. Potato peel and citrus pectin hydrogels containing anthocyanin was placed in different pH solutions. pH levels of 7-9 best mimic a wound scenario, while 5-6 represent healthy skin. The visible color changes corresponded to varying pH levels, indicating anthocyanin's ability to detect significant jumps in pH.

Anthocyanin-encapsulated hydrogels did respond to various pH environments. This is completely visible to the naked eye and can be used as a simple detector for infectious wounds. Furthermore, anthocyanin was able to prevent bacterial growth in solution. Anthocyanin exhibited a reduction in bacterial growth starting at a concentration of 50 milligrams. In a real wound, anthocyanin would be released into the tissue and act as an antibacterial agent when the hydrogel naturally degrades (25). All materials in the experimental hydrogels exhibited no cytotoxic effects, and the test hydrogels supported cell growth.

Additionally, anthocyanin was a promising infection detector in these hydrogels. The distinct color changes of this dye-infused hydrogel were observed in relation to pH that simulates normal skin pH and infected skin pH levels. Patients could be given simple instructions to note the color of their wound dressing and seek medical assistance if the color indicates infection. Anthocyanin also has a dual purpose of killing bacteria associated with infections. We demonstrated that infusing scaffolds with anthocyanin could successfully prevent bacterial growth at a concentration of 200 milligrams. It would change colors with the accumulation of bacteria in the wound and be released in controllable amounts as the hydrogel degrades. It would then be able to disrupt bacterial membranes and disable biofilm growth, all while having no cytotoxic effect on murine cells.

Limiting factors to the validity and extent of this experiment were possible infections or cytotoxicity and model organism restrictions. Despite sterile techniques and procedures, infectious agents are always present and can alter cellular studies. Additionally, NIH 3T3 murine fibroblasts may have some limitations as immortalized cell lines may not always be representative. Different cell lines such as human primary epithelial cells or an *in vivo* model can be used to allow for direct observation of re-epithelialization within a wound as well the

interaction of the hydrogel with growth factors, extracellular matrix proteins, and cellular receptors. Furthermore, studying other common bacteria found in diabetic wounds, including *Staphylococcus aureus*, *Enterococcus spp.*, and *Pseudomonas aeruginosa*, can validate anthocyanin's antimicrobial properties in hydrogels.

Our investigation has implications in scientific biomaterial research as well as patient care. Four million people are diagnosed with diabetes, with millions more having prediabetes, and others remaining undiagnosed or unreported. Due to diabetic complications such as poor circulation and hyperglycemia-related nerve damage, natural wound healing is substantially impaired. More than 70,000 diabetic patients have had amputations due to wound care complications. Diabetic foot ulcers account for 80% of non-traumatic amputations (26). Diabetic foot ulcer treatment prices range from 15,000 to 65,000 dollars and include treatments such as Total Contact Casts and skin substitutes (27). They also require weeks to prepare and apply to wounds. Developing countries' medical infrastructure disproportionately struggles to support the overwhelming cases of diabetic ulcers (28). The natural hydrogels created in this project demonstrate an increase in viability and proliferation when fibroblasts are cultured within the matrices. This comes with a great cost reduction and has promising applications as a low-cost treatment (Figure 8). As hydrogels are already being explored as an alternative to allograft skin substitutes, with further experimentation and validation, these waste materials can be used for bulk production to be implemented as accessible treatments for clinical use.

The low-cost hydrogels developed in this investigation are promising alternatives to skin substitutes, capable of mimicking hospitable conditions and supporting wound regeneration while allowing patients to receive proper care for infections before amputations become necessary.

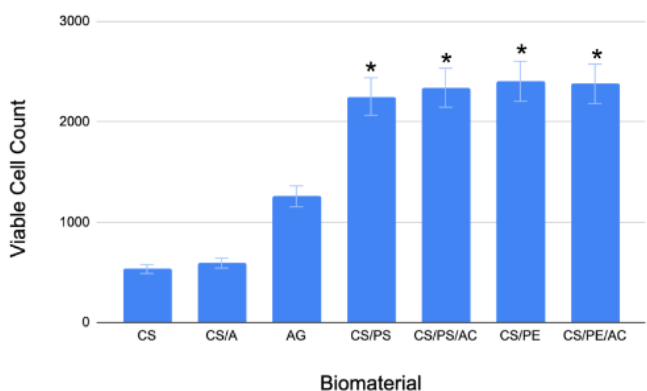


Figure 7. Potato peel and pectin hydrogels support 500 times cell proliferation. NIH 3T3 murine fibroblasts were cultured on each hydrogel (n=6). MTT dye was used to quantify cell proliferation through optical density readings. All formulations of potato peel starch and citrus pectin supported a significant increase of cell growth in comparison to the controls, alginate and chitosan in isolation (single factor ANOVA, $p < 0.05$). Data shown as mean \pm standard deviation. * $p < 0.05$

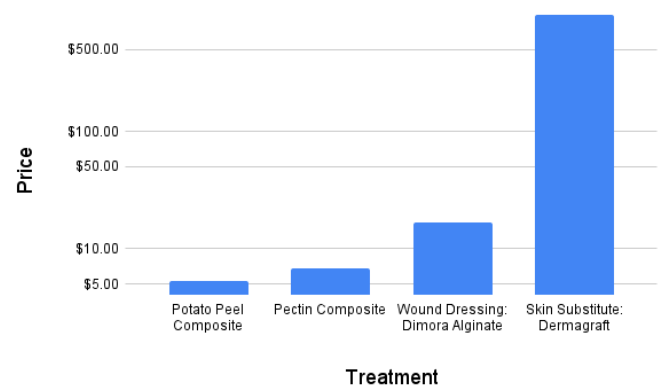


Figure 8. The plant waste hydrogels are 100 times cheaper than current standard treatments. The cost analysis of raw material prices per skin substitute is presented. The experimental waste-based hydrogels tested in this project are a cheaper and accessible alternative to commercially available treatments.

MATERIALS AND METHODS

Hydrogel preparation

To create the chitosan-based hydrogels, autoclaved chitosan was dissolved in 5% acetic acid to create a 3% (w/v) solution. The pH was adjusted to 4.5 with 0.1 M sodium hydroxide and then stirred at 450 rpm at room temperature for 24 hours. To create the pectin and potato peel composite hydrogels, autoclaved potato peel starch or pectin was dissolved in distilled water to create a 3% (w/v) solution. The solutions were immersed in a 10% sodium tripolyphosphate solution and crosslinked for 3 hours. The gel was then transferred into conical tubes and lyophilized for 3 days until the complete removal of solvent remnants. The hydrogels were sterilized with a 70% ethanol wash, followed by aspiration, 1 hour of UV radiation, and 3 phosphate-buffered saline (PBS) washes.

Characterization of hydrogels

A rheological frequency sweep assay was conducted on all samples. Hydrogel diameter was adjusted to 6 mm using a disc puncher and the height was adjusted to 1.5 mm using a sliding microtome. The sample was loaded, and the Peltier plate was set to 37°C. The rheometer was calibrated to a gap height of 1000 micrometers, a shear factor of 12.5, and a torque of 2 micro-N.m. The 25 mm geometry was rotated as it flattened the sample.

Cross sections of each hydrogel were taken to be analyzed under the scanning electron microscope. The diameter was adjusted to 32 mm using a disc puncher. The specimen was sputter coated with gold particles for 1 minute and 30 seconds. The specimen was loaded onto the stub, which was placed in the vented chamber before pumping the chamber to full vacuum. The parameters for magnification were 18X and 100X, and three images of randomized sections of each sample were analyzed through ImageJ software.

To conduct the swelling and degradation assay, the hydrogel was immersed in 200 mL of cell culture medium. The hydrogel was hydrated for 30 minutes (swelling), dried for 15 minutes, and weighed. This cycle was repeated until the hydrogel's mass approached zero and time for full degradation was recorded. The amount of media absorbed was determined for swelling capabilities and time of degradation indicated biodegradability.

pH responsivity of encapsulated anthocyanin in hydrogels

In phase 2, 15 mL solutions of specific pH levels were created: glacial acetic acid (pH 2), citric acid (pH 4), ammonium nitrate (pH 5.5), dimethyl sulfoxide (pH 6), distilled water (pH 7), magnesium sulfate (pH 8), sodium bicarbonate (pH 10), sodium hypochlorite (pH 12), and sodium hydroxide (pH 14). Hydrogels with anthocyanin encapsulated within them were soaked in each solution for 5 minutes and the color change was observed.

Time-to-kill anthocyanin antibacterial assay

The antibacterial properties of aqueous anthocyanin were tested through a time-to-kill assay. *E. coli* K12 was cultured in LB broth for 24 hours. Cultures were aliquoted into 10 mL vials, and the spectrophotometer was zeroed with a control vial of LB broth without bacteria or anthocyanin and a vial with bacteria but no anthocyanin. Increasing amounts of anthocyanin (10, 20, 50, 100, 200, and 500 milligrams) were introduced into each vial. The optical density at 600 nanometers was taken every 30 minutes until the reading became static.

3D cell culture and MTT assay

In the final phase, NIH 3T3 murine fibroblast cells from AddexBio were seeded at a concentration of 50,000 cells per well in 100 microliters of cell culture medium and incubated for 36 hours. We aspirated the medium and 30 microliters of trypsin was pipetted into each well and continuously resuspended to recover cells encapsulated deep within the matrix. The trypsin was incubated for 15 minutes before being deactivated with 100 microliters of fresh medium. The contents of each well plate were transferred into separate microcentrifuge tubes, and rapidly centrifuged at 7,000 rpm to obtain a cell pellet. This cell pellet was resuspended once again with 100 microliters of medium and 10 microliters of MTT labeling reagent before being transferred into 96 well plates and incubated for 4 hours. All but 25 microliters of medium were removed and the MTT reagent was solubilized with 50 microliters of DMSO. After 10 minutes of incubation and complete solubilization of the purple formazan crystals, the absorbance at 560 nanometers was read with a microplate reader.

Statistical analysis

Each assay was repeated three times to ensure statistically significant results. All results were analyzed using Microsoft Excel and the significance level was 0.05. When applicable, a two-tailed t-test was conducted between groups with and without anthocyanin. A one-factor ANOVA was conducted between all groups, comparing the results between experimental and control groups.

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