SEMARAK ILMU PUBLISHING

Journal of Advanced Research in Applied Mechanics

Journal homepage:
https://semarakilmu.com.my/journals/index.php/appl_mech/index
ISSN: 2289-7895

Physiochemical and In-Vitro Bioactivity Characterization of Polyvinyl Alcohol / Halloysite Nanotube / Collagen (PVA/HNT/Col) using Freeze-Thawing Method and Spin Coated Technique for Wound Healing Applications

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ARTICLE INFO

Article history:
Received 29 July 2024
Received in revised form 31 August 2024
Accepted 7 September 2024
Available online 30 September 2024

Keywords:
Hydrogel; Wound Healing; Polyvinyl alcohol; Collagen; Halloysite nanotube; Freeze thawing

ABSTRACT

Hydrogels are still relevant instruments that can support and improve the dynamic biological process of wound healing. This paper modified hydrogel film by combining polyvinyl alcohol (PVA), halloysite nanotube (HNT), and collagen using simple freeze-thawing and spin-coating techniques. The modification aims to improve the physiochemical and in-vitro bioactivity of the PVA/HNT hydrogel films via the inclusion of different concentrations of collagen. 0.092 mm PVA/HNT/Col3 hydrogel film was suggested to have excellent morphological properties with 94.11 % porosity. It also has good tensile properties with excellent hydrophilicity performance at 38.40° contact angle and 83.528% swelling capacity compared to other modified films. In-vitro bioactivity suggested that the PVA/HNT/Col3 are safe and non-toxic toward host tissue and can promote healing at 93.12% wound closure after 24 hours of treatment. Hence, this material displays huge potential as a wound healing matrix template.

1. Introduction

Wound healing is a dynamic process that the body performs with the aid of certain dressing modifications as the healing process advances. According to the depth, size, and extent of the damage to the epidermis and dermis layers of the skin, the healing of an acute wound takes 8 to 12 weeks [1]. The healing process of chronic wounds will recover slowly after 12 weeks due to the presence of tissue damage and frequently recur [1-3]. Every wound will proceed along the wound-healing pathway, which comprises the hemostasis, inflammatory, proliferative, and maturation phases [4,5]. There are two different types of wounds: acute wounds and chronic wounds. Surgery or an accident might cause an acute wound to occur suddenly. A chronic wound happens when the

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https://doi.org/10.37934/aram.125.1.6880

body cannot repair it in a timely and organized manner through the typical procedure or stages such as burns, decubitus ulcers, and leg ulcers [4].

The effectiveness of hydrogels in accelerating wound healing is well established and shown to be a potential material for wound dressings. Hydrogels, known as hydrophilic polymeric networks, were the first materials created for implantation inside the human body and entered the medical scene in the 1960s [6]. The initial wave of hydrogel technology merely focused on its capacity to swell up with water when touching one another. In contrast, the second generation anticipated making the hydrogel respond to stimuli like temperature, pH, or molecule concentration [7,8]. It is understood that hydrogel must made up of biocompatible substances such as polyvinyl alcohol (PVA) and polyethylene glycol (PEG), which has evolved into hundreds of new medical applications and has successfully changed the standards in many areas of medicine [6,7]. However, hydrogels have two significant flaws that prevent them from being used in regenerative medicine: limited adsorption capacity and poor mechanical qualities. The mechanical properties are weak due to inhomogeneity in the structure of hydrogel that dangles chains and loops as the hydrogel at an intermediate state between solid and liquid [9]. Therefore, different filler monomers, such as montmorillonite, layered double hydroxide, smectite, and halloysite, can be added to the composite hydrogel to improve the strength of the hydrogel [7,9-11]. Some of the hydrogel matrices are made up of a bioinert nature, which does not allow sufficient protein adsorption, thus not permitting a high capacity for tissue-substrate bonding to take place. Incorporation with bioactive monomers, such as pectin, collagen, peptide, and keratin, can help bind the cell and tissues and allow better cell proliferation [12,13].

Polyvinyl alcohol (PVA) formed by hydrolyzing polyvinyl acetate is a well-known polymeric material used in biomedical applications. PVA's biocompatibility makes it possible to absorb protein molecules, interact with cells with minimum adhesion, and have no hazardous side effects [14,15]. According to reports, PVA is hydrophilic, meaning it can absorb much water without dissolving. Due to its unique qualities, PVA polymer has been employed extensively in medical and pharmaceutical applications [14,16]. However, PVA exhibits low mechanical strength in several applications and is naturally considered a bioinert polymer [16-19]. Halloysite nanotubes (HNT) can improve the mechanical, thermal, and fire-retardant capabilities of polymers, as indicated by previous studies [10,20]. Unlike other nanoparticles, such as fumed silica and montmorillonite, HNT is easily dispersed in polymer matrix due to shearing due to rod-like geometry and inter-tubular contact area [21]. Collagen, a natural and biodegradable substance, has been employed extensively in the regeneration of skin tissue. According to some researchers, collagen has superior biological qualities for skin regeneration when used as a material for skin scaffolds [22,23]. Therefore, this study aims to investigate the effect of collagen inclusion on the PVA/HNT hydrogel film in terms of its physiochemical properties and in-vitro bioactivity activities.

2. Methodology

2.1 Preparation of PVA/HNT/Collagen Hydrogel Films

Polyvinyl alcohol (PVA) powder (*Mw* 89000 g/mol, Sigma Aldrich) was dissolved in distilled water at 90 ^{o}C and continuously agitated for 30 minutes to create a 13% (w/v) polymer solution. After that, type I collagen powder from fish skin (*Mw* 300000 g/mol, Sigma Aldrich) at 1, 3, and 5 wt% was gradually added to the PVA solution and mixed until completely dissolved. Then, 2wt% halloysite nanotubes (HNT) (*Mw* 258 g/mol, Sigma Aldrich) were added forming a PVA/HNT/Col solution. The mixing solution was agitated for 30 minutes until the composites were wholly dissolved [10]. After that, the mixture was dispensed on top of a glass dish and subjected to a 30-second spin-coated

process before being put through three cycles of freezing (at -20 °C for 24 hours) and thawing (at 23 °C for 24 hours) forming a hydrogel film as illustrated in Figure 1.

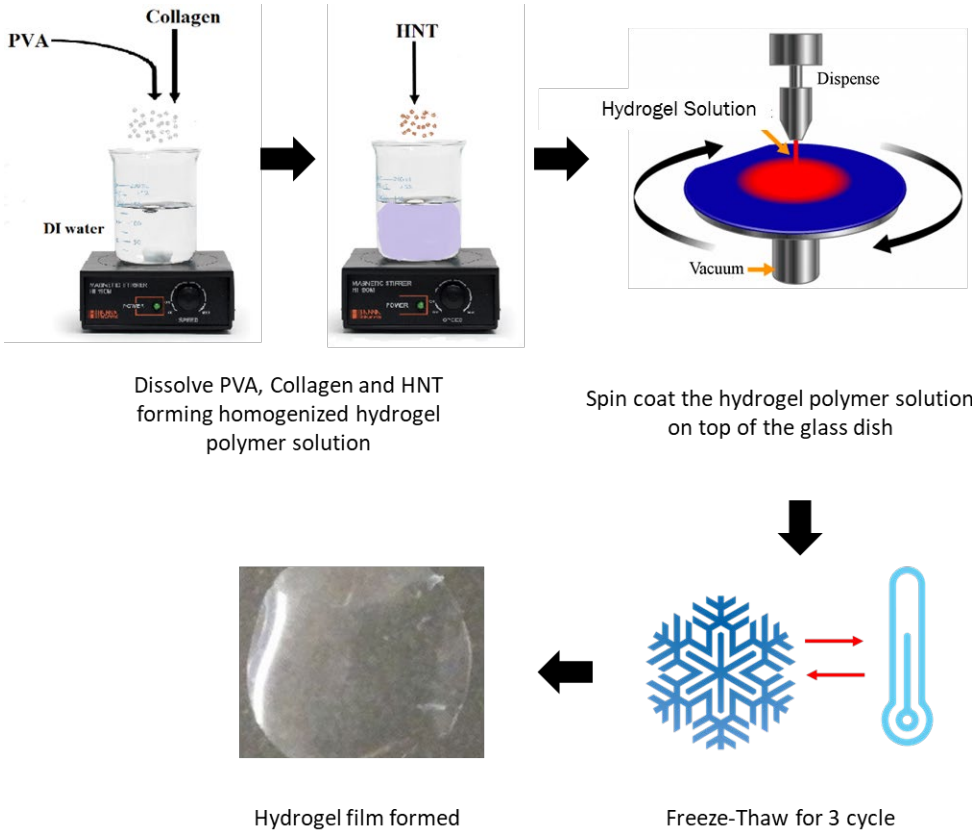


Fig. 1. The flow process for preparing PVA/HNT/Col hydrogel samples

2.2 Morphological Analysis via Scanning Electron Microscope

Composite PVA/Col/HNT hydrogel was morphologically observed using a scanning electron microscope (SEM) (FlexSEM 1000, Hitachi). The hydrogel was coated with gold, and the observation was conducted at 18 mA under magnification of 1000-10000 x.

2.3 Porosity Percentage

The porosity of the scaffolds was assessed using the liquid displacement method by submerging the samples in cyclohexane (p = 0.778 g/cm^3) [10,24]. The samples were immersed in a density bottle containing cyclohexane at 30 °C under vacuum conditions. Weight changes before and after submerging were recorded and used to calculate porosity percentage according to Eq. (1).

$$Porosity\ (\%) = \frac{v_p}{v_d + v_p}\ x\ 100 \quad (1)$$

v_p is the volume of cyclohexane in the pore (cm^3), whereas v_d is the volume of the hydrogel (cm^3).

2.4 Tensile Analysis

The hydrogel film was subjected to a tensile test according to ASTM D3039/D3039M, using a universal tester to create a stress-strain graph (Instron). The samples were prepared into dogbane

shapes of 80 mm x 10 mm (length x width) and subjected to a 5 N load range and 50 mm/min crosshead speed.

2.5 Hydrophilic Analysis – Water Contact Angle and Degree of Swelling Testing

The water contact angle was measured using a Video Contact Angle System (VCA Optima, Ast Products Inc.) according to ASTM D7334-08 standard. The system is used to capture the image of 5 µL of deionized water being dropped on the surface of the samples, and the angle was recorded after 3 seconds. The degree of swelling analysis was performed by placing the sample into phosphate-buffered saline (PBS) for 24 hours. The percentage of water adsorption was calculated using Eq. (2).

$$Degree\ of swelling\ (\%) = \frac{W_t - W_0}{W_t}\ x\ 100 \quad (2)$$

W_t is the weight of the sample at a predetermined time, and W_o is the initial weight of the sample.

2.6 Chemical Analysis via Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy, FTIR (Perkin Elmer Frontier) was used to determine the chemical interaction between PVA, HNT, and collagen of the hydrogel samples. The FTIR spectra were recorded at the range of 400-5000 cm^{-1} wavenumbers.

2.7 In-vitro Bioactivity Analysis using Human Fibroblast (HF) Cells

Human Fibroblast Cells were cultured in a completely supplemented DMEM medium containing 10 v/v% fetal bovine serum, 1 v/v% penicillin/streptomycin, and 1 v/v% pyruvate. The HF cells were cultured to $1x10^6$ cells/mL by incubating at 37 ^oC with 5 % carbon dioxide (CO^2) and 95% humidity. After reaching the desired cell density, the HF cells were indirectly cultured with PVA/Col/HNT hydrogel for 24 hours before being subjected to an MTT assay for cytotoxicity and scratch assay. Trixton X (positive) and complete medium (negative) were the controls used for the cytotoxicity assay. The samples were incubated with 50 ul MTT solution for 4 hours to allow formazan formation, and absorbance was read at OD = 590 nm. The cell viability was calculated using Eq. (3).

$$Cell\ Viability\ (\%) = \frac{OD_{sample} - OD_{background}}{OD_{control} - OD_{background}} x\ 100 \quad (3)$$

The scratch assay was performed by ensuring HSF cell at 80 % confluency in the 24-well plate under standard culture conditions. A 10 uL plastic tip was used to demonstrate the wound, as illustrated in Figure 2. Then, the eluted PVA/Col/HNT hydrogel with the fresh medium was added to the sample to investigate the potential of the sample to promote the proliferation of cells [25-27]. The reference lines (wound area) progression was observed, marked, and measured under an inverted microscope (Zeiss) at 10x magnification. The wound healing was quantified by calculating the percentage of wound closure using the following Eq. (4).

$$Wound\ Closure\ (\%) = \frac{[Wound\ Area\ (0h) - Wound\ Area\ (th)]}{Wound\ Area\ (0h)} x\ 10 \quad (4)$$

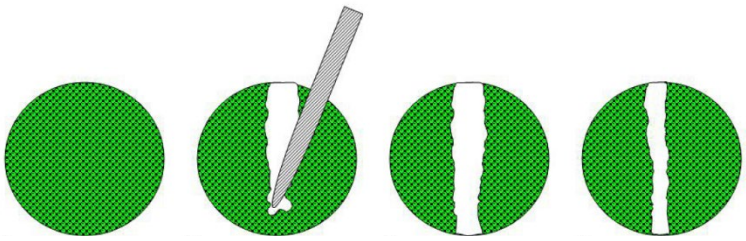


Fig. 2. Scratch Assay Process Illustration [25]

3. Results

3.1 Morphological Analysis: SEM Micrograph and Porosity Percentage

The physical characteristics of hydrogels, such as pore size, porosity, and interconnectivity, are all influenced by the freezing and thawing procedure used to create the hydrogel [28,29]. The SEM pictures in Figure 3 show how these amazing processes result in porous network topologies at the hydrogel surfaces. The smoother, less porous surfaces of the PVA/HNT hydrogel surface morphologies in Figure 3(a) are comparable to those of Azmi *et al.,* earlier work published in 2016 [10]. It shows no phase separation, representing that the HNT is thoroughly integrated into the hydrogel. The presence of HNT inclusion was confirmed via structural analysis in section 3.4. On the other hand, including collagen in the polymer solution introduces a significant change on the porous surface of the hydrogel that resembled. As illustrated in Figure 3, when the water molecules in the polymer matrix formed ice crystals, it led to mechanical stress on the surrounding on the crosslinking point of PVA/HNT/Col monomers. The thawing process leaves behind a dried polymer matrix, creating the opening cellular pore structure with a lamellar shape and random lumps due to collagen's presence, as shown in Figures 3(b-d). The SEM images showed the presence of fibrils with interconnected lamellae structures that resemble native nerve tissue matrix, especially at higher collagen inclusion (Figure 3(d)). Higher collagen concentration resulted in the hydrogel surface exhibiting homogenous pore structures with uniform distribution. These corroborated with the porosity percentage analysis where the PVA/HNT/Col3 hydrogel produces a higher porosity at 94.11% compared to 91.35% for PVA/HNT/Col2, 89.12%PVA/HNT/Col1 and 87.13% for PVA/HNT hydrogels as referred to Table 1. A higher porosity of over 90% allows greater controlled growth for tissue and drug delivery [30]. These porosity characteristics are highly interconnected and correlated with the hydrophilicity and mechanical properties of the hydrogel.

Table 1
The porosity percentage and thickness mean for hydrogel films

Sample Type	Porosity %	Thickness (mm)
PVA/HNT	87.13±1.2	0.082±0.011
PVA/HNT/Col1	89.12±2.4	0.091±0.041
PVA/HNT/Col2	91.35±1.8	0.093±0.025
PVA/HNT/Col3	94.11±1.1	0.092±0.021

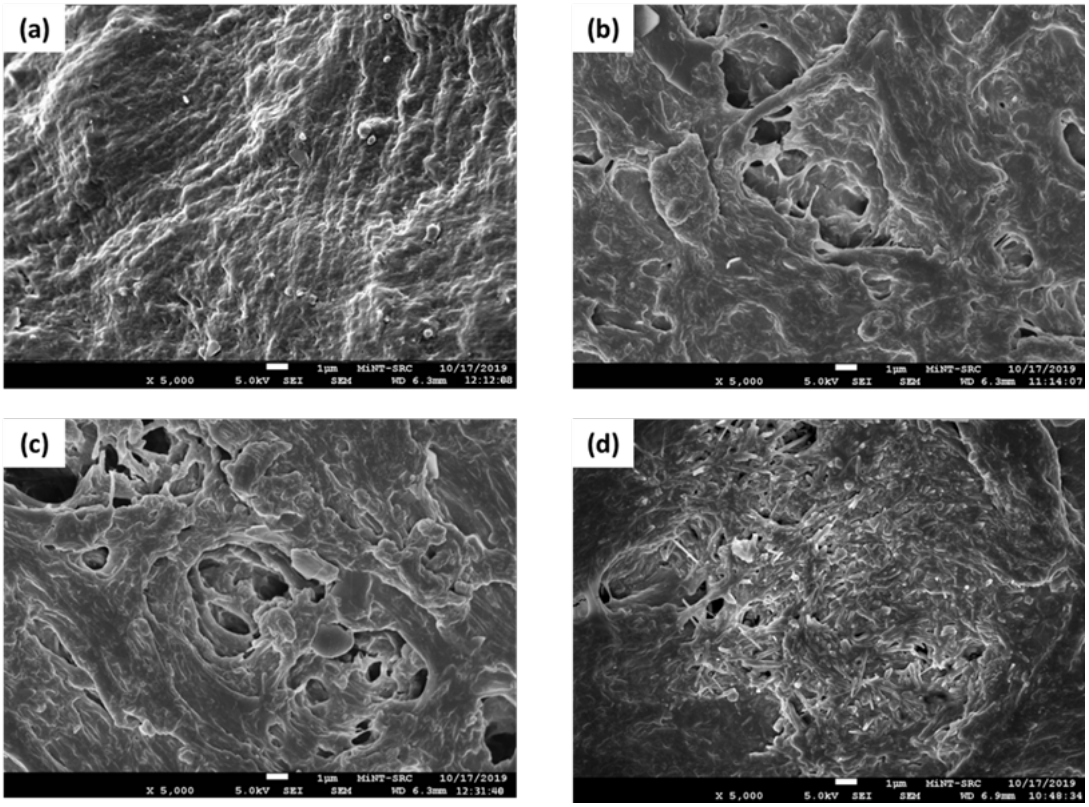


Fig. 3. SEM Micrograph of (a) PVA/HNT, (b) PVA/HNT/Col1, (c) PVA/HNT/Col2, and (d) PVA/HNT/Col3 hydrogel's surface

3.2 Mechanical Characterization – Tensile Testing

As can be seen in Table 2, the tensile properties show that the inclusion of collagen in the PVA/HNT hydrogel formulation revealed an increase of nearly 28% for the tensile strength (0.593 MPa to 0.761 MPa), 33% for the Young Modulus (0.546 to 0.725), and 29% for elongation at break (211.34% to 273.331%) comparing the PVA/HNT and PVA/HNT/Col hydrogel respectively. The plausible explanation for this behaviour is the existence of additional covalent bonds and hydrogen bonds from the triple helix collagen monomers (in the PVA/HNT/Col hydrogel) [31]. The same outcome was reported where the inclusion of collagen in matrix monomer promotes better mechanical properties in terms of gel strength and elasticity of 3D network composite [32-34]. Besides, this result corroborates with the SEM image that shows a well-connected pore structure, resulting in a more uniform stress distribution, mitigating the stress concentration at specific points, and contributing to reinforcement to the overall gel. Generally, the mechanical performance of hydrogel was recently reported at the range of tensile strength 0.5 – 10 MPa and elastic modulus 0.1 – 1.0 MPa [35]. The native skin has tensile strength values of approximately 5.0 to 30.0 MPa, Young's modulus in the range of 4.6-20.0 MPa, and elongation at break about 35.0-115.0%. However, the skin's mechanical properties may vary depending on the age, type, gender, and position of the skin [36]. Considering the performance of the hydrogels meets the properties range, it can be said that formulated hydrogel films can be potentially used as an alternative tissue scaffold.

3.3 Hydrophilicity Properties – Water Contact Angle and Degree of Swelling Analysis

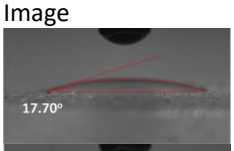
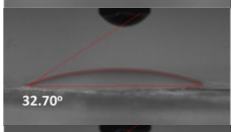
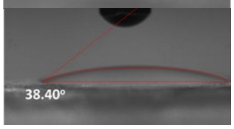
The hydrophilicity properties of the PVA/HNT and PVA/HNT/Col hydrogels were impacted by the water contact angle and degree of swelling as shown in Table 3. It is well-known that low water

contact angles <90° are referred to as hydrophilic towards water molecules, while high contact angles >90° correspond to hydrophobic towards water molecules [25,37]. The PVA/HNT hydrogel exhibited 17.70^{o} while the angle contacts slightly increased to 21.80^{o}, 32.70^{o}, and 38.40^{o} for 1, 2, and 3 wt% of collagen, respectively. The low contact angle of PVA/HNT may be attributed to the nature of HNT hydroxyl groups leading to an increase in water adsorption. Although collagen is slightly hydrophobic, its hydrophilic monomer (NH_2, COOH, OH, C=O) can help improve holding water capacity as indicated by the degree of swelling percentage [22]. The swelling capacity achieved the highest percentage after 48 hours at 83.538% for PVA/HNT/Col3 hydrogel film compared to PVA/HNT hydrogel at 48.380%. This result corroborated the porosity advantage of the PVA/HNT/Col3 hydrogel that gives extra space within the hydrogel structure for the liquid to infiltrate and cause the hydrogel to expand. As a crucial property, swelling hydrogels encapsulate and deliver cells to specific lesion sites. It also provides a hydrated environment, allowing cell expansion and differentiation, which is more effective in wound healing [15,25,27]. Based on the aforementioned analysis, the PVA/HNT/Col3 hydrogel showed good results in producing morphological porous structure with hydrophilic conditions and maintaining strong mechanical properties compared to other formulations. Therefore, it was suggested that further FTIR, cytotoxicity, and scratch assay analysis.

Table 2
Mechanical properties of the hydrogel

Sample Type	Tensile strength (MPa)	Young Modulus E	Elongation at Break (%)
PVA/HNT	0.593±0.018	0.546±0.311	211.340±0.098
PVA/HNT/Col1	0.690±0.290	0.610±0.045	232.201±0.188
PVA/HNT/Col2	0.728±0.013	0.691±0.201	254.146±0.057
PVA/HNT/Col3	0.761±0.023	0.725±0.013	273.331±0.032

Table 3
Water Contact Angle and Swelling Percentage of Hydrogel Composites

Sample	Water Contact Angle Image		Degree of swelling (%)	
		Value (°)	24 hours	48 hours
PVA/HNT		17.70±1.10	24.804±0.12	48.380±0.10
PVA/HNT/Col1		21.80±2.38	36.071±1.10	54.569±0.11
PVA/HNT/Col2		32.70±1.61	38.531±0.21	69.356±0.13
PVA/HNT/Col3		38.40±1.01	45.114±0.31	83.538±0.16

3.4 FTIR Analysis

Figure 4 shows that FTIR spectra for PVA, which are proved by characteristic bands of PVA, can be observed. O–H stretching vibrations at 3300 to 3500 cm^{-1}, C–H stretching vibrations near 2100 cm^{-1}, O–H deformation vibration due to absorbed water on the surface of the hydrogel at 1600 cm^{-1}, C–H bend and C–C stretching at 1400 and 1300 cm^{-1}, respectively, and the C–C–C bond stretching

vibration which corresponds to the crystalline domains of PVA at 1095 - 1150 cm^{-1} [10,18]. Adding HNT in PVA hydrogel shifted the range peak between 900 – 1300 cm^{-1} to one strong peak due to Si-O stretching. The new peak at 910 cm^{-1} represents the hydroxyl group of HNT, where the same peak is present at the PVA/HNT/Col3 hydrogel film [20]. The slight increase in O-H and C-O intensities could be correlated with the H bond between Si-O of HNT and the PVA band's OH, increasing the number of accessible OH, which will connected to collagen forming new interactions as shown in PVA/HNT/Col3 spectra. The hydrogel sample of PVA/HNT/Col hydrogel has a similar pattern, with a few peaks shifting its spectrum into broad peaks, such as at the 3315 cm^{-1} representing the amide A group in the collagen [38]. Several collagen peaks exhibited in the spectra at 1397, 1452, and 1670 cm^{-1} contribute to CH_2, CH_3, C-N, and N-H bonding. Collagen contains amino groups that may exist at a peak between 1600 to 1650 cm^{-1} that allow hydroxyl groups of PVA to form ester bonds, contributing to the strengthening of the materials. Cross-interaction between collagen and HNT may happen, as illustrated in Figure 5, because both monomers provide sites for H bonding interaction, contributing to better overall composite film stability.

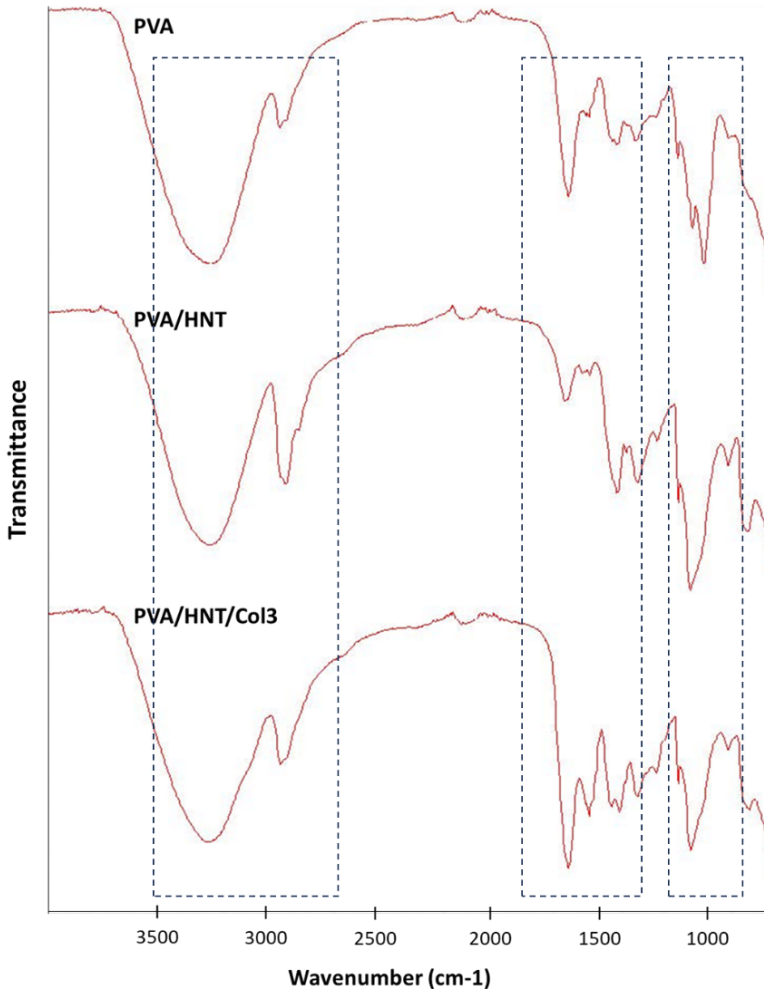


Fig. 4. FTIR spectra of PVA, PVA/HNT and PVA/HNT/Col3 hydrogel film

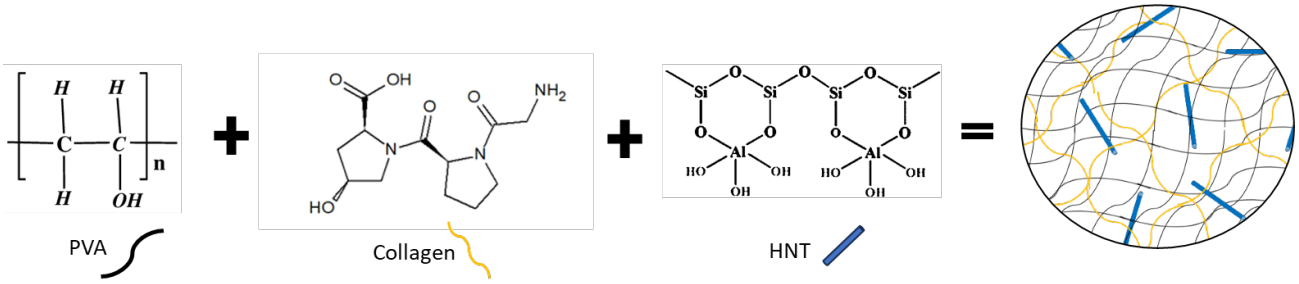


Fig. 5. Proposed interaction of the PVA/HNT/Col hydrogel

3.5 In-Vitro Bioactivity: Cytotoxicity and Scratch Assay Analysis

The cytotoxicity analysis proves that the PVA/HNT/Col3 will not cause adverse effects on host cells or tissues by using MTT tests and human fibroblast (HF) cells. In Figure 6, collagen addition improves the cell-substrate interaction after 24 hours by having 85.315% cell viability compared to PVA and PVA/HNT hydrogel at 75.911% and 79.134%, respectively. The hydrogel samples are not toxic to the host tissue and cells but help promote the growth of new cells, especially with the existing collagen. The results also contributed to the morphology of the hydrogel with a better-interconnected pore with a good porosity percentage and bioactive monomers that improve surface potential which allows migration and growth of HF cells at optimum conditions.

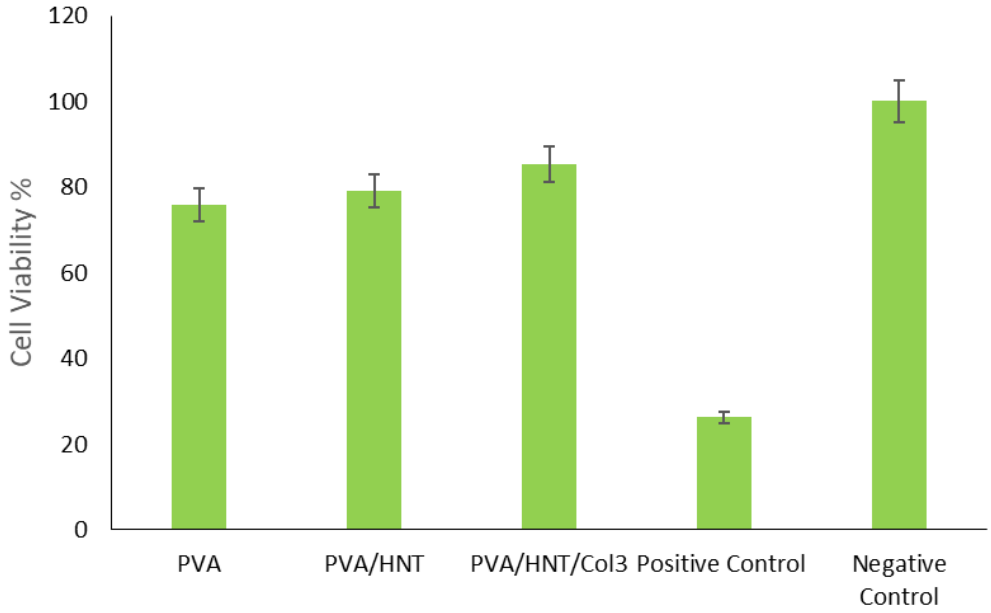


Fig. 6. Cell viability percentage of sample from MTT assay

Scratch assay was used to investigate the potential for the hydrogel film to allow cell migration during wound healing. The migration activities are required for tissue development, repair, and regeneration. Figure 7 shows the percentage of wound disclosure after 24 hours of treatment. The PVA/HNT/Col3 showed the wounded area covered up to 93.12% with HF cells compared to PVA/HNT up to 73.56%. The covered area was different in terms of its migration pattern, where collagen inclusion might provide signalling molecules that attract HF to the lesion site and promote differentiation of fibroblast to myofibroblast that help to close the wounded area.

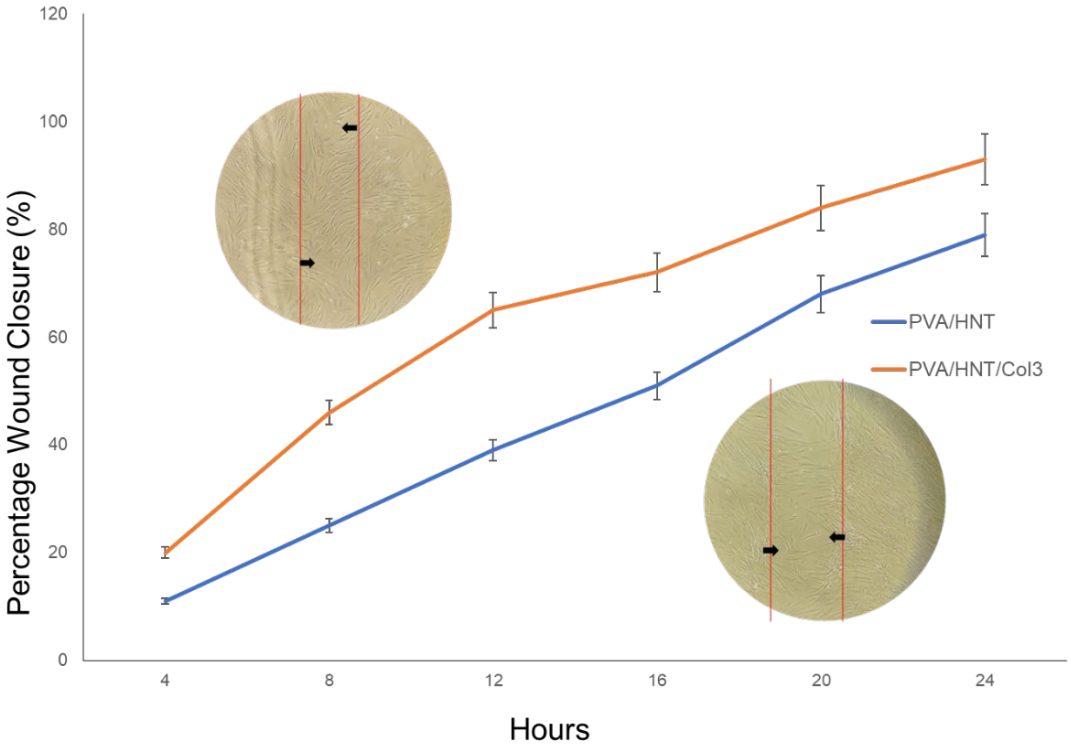


Fig. 7. Scratch assay and visualization for PVA/HNT and PVA/HNT/Col3 hydrogel films

4. Conclusions

The physiochemical and in-vitro bioactivity of the composite hydrogel films made of polyvinyl alcohol (PVA), halloysite nanotubes (HNT), and collagen (Col) were thoroughly examined in this study. Collagen improves the interconnectivity of pores with excellent porosity %, according to SEM micrographs. Additionally, collagen improves the mechanical integrity of hydrogel films. It offers benefits for chemical cues for wound healing: the hydrophilic surface and excellent swelling percentage of the hydrogel films allowed for enhanced matrix-cell interaction. After a thorough examination, it was concluded that the PVA/HNT/Col3 sample met all the criteria for a remarkable hydrogel film that may be used for wound healing. The amended sample improved cell migration, as demonstrated by in vitro bioactivity, which bodes well for wound film dressing. For future research, it is essential to extend these findings by investigating the hydrogel films' effectiveness in chronic wound environments, which involve more complex inflammatory processes. Additionally, in vivo studies are crucial to verify the practical applicability and safety of these hydrogel films in real-world scenarios. These further investigations will provide a more comprehensive understanding of the hydrogel films' therapeutic potential and pave the way for their clinical use in treating both normal and chronic wounds.

Acknowledgment

This research was funded by a Fundamental Research Grant Scheme (FRGS) FRGS/1/2022/TK09/UTHM/03/7 from the Ministry of Higher Education of Malaysia and Universiti Tun Hussein Onn Malaysia through Geran Penyelidikan Pascasiswazah (GPPS - H458).

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