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Original Article

Antimicrobial Assessment of Biopolymer Based Scaffold Prepared by Freeze-Thaw Process for Various Biomedical and Pharmaceutical Applications

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Abstract *- This present research work is based on soya protein isolates biopolymer cryogel, which was synthesized by the freeze-thaw method. Cryogel is a porous three-dimensional network material that is insoluble in water and can store much water. These materials have excellent mechanical properties, such as in medical fields, wastewater treatment, and other fields, and are widely used. With the development of modern science and technology, the research on hydrogel, a new kind of biomaterial, is increasingly extensive. Many researchers have researched bone tissue engineering based on the advantages of hydrogel, such as its adjustable mechanical properties, convenient production, and combination with bioactive factors. This method forms a spongy, flexible, and macroporous gel characterized by the Scanning Electron Microscopy technique (SEM). The morphology of the cryogel surface depicted irregular pores structure. After drug loading, the surface of the gel is relatively smoother with no voids and interconnected pores, showing the uniform distribution of and good adsorption of the antibiotic drug into the gel. Surface roughness parameters investigated by Atomic Force Microscopy (AFM). The particle size of gel and drug particles dispersed into gel were also studied using Transmission Electron Microscopy (TEM). Drug loaded gel examined by antimicrobial spectrum against gram positive and gram negative bacteria. The prepared drug loaded gel is used in wound dressing, target drug delivery, food packaging and tissue engineering.*

Keywords *- Macro pours cryogel, Surface roughness, Morphology, Biopolymers.*

1. Introduction

Today, bacterial infections constitute one of the greatest global public healthcare challenges. Specifically, wound infections, one of the most commonly acquired infections, are a leading cause of morbidity and mortality. Extensive efforts have been made to develop an effective wound care device with antibacterial properties that would dress the wound, maintain a moist wound microenvironment, and prevent bacterial infection, thereby enhancing and accelerating the wound healing process. Wound dressings made from biomaterials are often used to protect damaged skin or tissue from dehydration and microbial infections and are continually being developed to address emerging challenges. Commercial wound dressings include films, sponges, hydrocolloids, and hydrogels. Among them, hydrogels offer numerous advantages for antibacterial wound healing, including high water content and soft consistency, which provide a moisturized local environment for wound healing and prevent secondary infections caused by the entry of microorganisms into the wound [1]. Even though Antimicrobial Resistance (AMR) is a major and growing public health concern, the research relating to the growth of this phenomenon in environmental settings is remarkably limited. A key strategic objective of the European Antimicrobial Resistance Surveillance Network and the WHO is to strengthen AMR surveillance. However, they recognize that several countries in the region do not have systems for the surveillance of AMR, antibiotic use, and hospital acquired infections. Multi-drug resistance among bacterial pathogens is of particular concern because they are responsible for many severe infections in hospitals and the contamination of implants or devices introduced into the body as stents or catheters. Several reports have confirmed a rapid increase in rates of infections due to Methicillin-Resistant S. Aureus (MRSA) [2]. Drugs are chemicals that produce a biological effect when administered to living organisms. The term antibiotic originated from the word “antibiosis”, which literally means “against life”. Antibiotics, in the past, were considered to be organic compounds produced by one microorganism that are toxic to other microorganisms by selectively killing or inhibiting the growth of other microorganisms [3]. Azithromycin, a member of the macrolide class of antibiotics, has shown promising results when used to treat COVID-19.

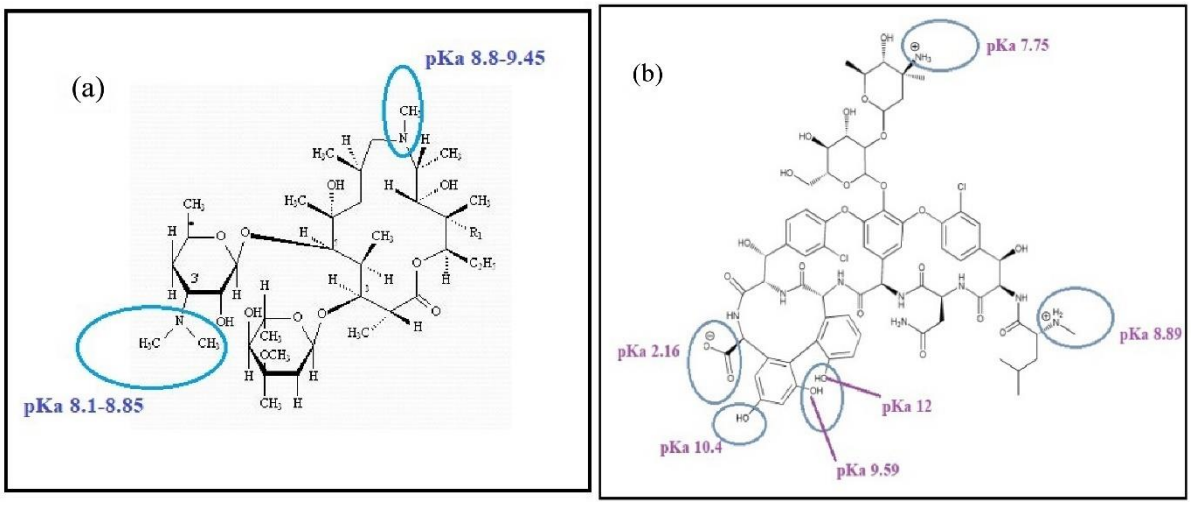


Fig. 1 Chemical structure of (a) Azithromycin and (b) Vancomycin antibiotic drug

Azithromycin has a broad spectrum of antibacterial properties that render it highly effective against both G^+ and G^- bacteria. Moreover, its anti-inflammatory, antiviral, immune modulatory, and antimalarial properties make it particularly well-suited to reign in this novel, highly contagious virus and mitigate the life-threatening symptoms (i.e., COVID-19) associated with its infection [4]. Azithromycin (AZM) is a semi synthetic 15-membered macrolide antibiotic with a lipophilic nature (log P = 4), (pK_a = 8.74), and a molecular weight of 749 g/mol. The drug, which is included in the model list of essential medicines on the World Health Organization website, is classified as the first azalide subclass among its family members, with superior antibacterial activity in the market for the last three decades. AZM is the drug of choice for treating various gastrointestinal, respiratory, and genitourinary infections.

Recently, it has gained increased popularity owing to its suggested important role in improving the ability of hydroxychloroquine to eradicate the global outbreak of COVID-19 virus at clinical levels [5]. Vancomycin is the most prominent glycopeptide antibiotic clinically and exhibits potent activity against Gram-positive bacteria. It functions by inhibiting cell-wall biosynthesis by targeting the peptidoglycan precursor lipid II and specifically by binding the D-Ala-D-Ala terminus of the lipid II pentapeptide via a network of five hydrogen bonds. In binding to this peptidoglycan precursor, vancomycin prevents cell-wall polymerization by bacterial transpeptidases and transglucosylases, leading to decreased bacterial cell-wall integrity and eventually resulting in the bacterial cell's lysis. Furthermore, the binding of vancomycin to lipid II is enhanced by cooperative dimerization, which increases the binding affinity of vancomycin to lipid II and enhances its antimicrobial activity. While lipid II is also present in Gram-negative bacteria, vancomycin cannot be used due to the additional Outer Membrane (OM) found in Gram-negative bacteria. The OM is characterized by an inner leaflet of phospholipids and an outer leaflet decorated by lipopolysaccharide (LPS) [6]. In recent years, the design of coatings suitable for localized treatment of surface-related infections of medical devices has been of great interest. Thus, several composite hydrogels were developed to prevent bacteria's adhesion to medical devices' surfaces by inhibiting quorum sensing and biofilm formation [7]. Smart hydrogels targeting bacterial infections and responsive to the bacterial microenvironment and their ability to adjust the release of antibiotics and/or antimicrobial compounds according to the bacterial contamination have been studied. These strategies limit the accumulation of drugs in healthy host tissues, minimizing the risks of toxicity and the selection of resistant bacteria [8]. A lower dose of antibacterial compounds could be administered with hydrogels than when administered systemically, thus overcoming the bacterial resistance [9]. Due to the multiple mechanisms of antibacterial ingredients loaded in hydrogels, it is difficult for bacteria to develop resistance.

At present, polymeric gels have applications in many different areas of biotechnology, including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immune diffusion, and as a gel basis for solid cultural media. Various problems associated with using polymer gels and the broad range of biological objects encountered lead to new, often contradictory, requirements for the gels. These requirements stimulate the developing and commercializing of new gel materials for biological applications. One of the new types of polymer gels with considerable potential in biotechnology is 'cryogels' (from the Greek krios (kryos), meaning frost or ice) [10]. Advantages of physically crosslinked hydrogels include non-toxicity, non-carcinogenicity, high elasticity, excellent biocompatibility, potential bioactivity and porosity of the resulting polymer. Nugent and co-workers prepared a novel poly (vinyl alcohol) composite hydrogel by freeze–thaw method for drug delivery applications [11]. This is somewhat

similar to freeze-drying, where a gel precursor solution or hydrogel is frozen to form a porous material and then lyophilized. No drying occurs after cryotropic gelation and a stable macroporous hydrogel forms after thawing. A cryogel forms due to physical or chemical cross-linking in a sample when stored under frozen conditions at −12ºC to −20ºC. The cryotropic gelation method is beneficial compared to other methods that rely on physico-chemical interruptions in the hydrogel solution achieved by using porogens such as salt, sugar, silica and others that should be removed after gelation. Carefully removing these pore-forming units is necessary to prevent them from adversely affecting cells, but this step is hard to achieve. It may cause chemical contamination of the material and an inadequate interconnection of the pores [12]. Cryogels continue to receive considerable scientific and industrial attention for their prominent features, such as superabsorbent properties, high loading capacities, the ability to modulate drug release profiles, and biocompatibility. They find application in tissue scaffolding, drug delivery, cell transplantation, and stem-cell-based therapies because of their excellent biocompatibility features. They exhibit significantly improved drug protection, excellent oxygen permeability, a high drug-loading ability, and better absorption capacity than parent hydrogels. Interestingly, depending on the application, their oxygen permeability may be modulated as a function of the dimension of the pores [13]. PVA hydrogels have been used in various biomedical applications. PVA cryogels have certain advantages which make them ideal candidates for biomaterials.

The advantages of PVA gels are that they are non-toxic, non-carcinogenic, and bio-adhesive. PVA also shows a high degree of swelling in water (or biological fluids) and a rubbery and elastic nature; therefore, it closely simulates natural tissue and can be readily accepted into the body. PVA gels have been used for contact lenses, the lining for artificial hearts, and drug-delivery applications [14]. SPI-based composites and nanocomposites are mostly used because of their eco-friendly nature. The important properties of SPI are solubility in water at the isoelectric point and the highest protein content compared to other protein products. It has an excellent tendency to blend with various biopolymers to prepare biodegradable products. Researchers often study SPI to improve its chemical and mechanical properties. Its excellent microbial properties make it suitable for packaging applications. It protects food during storage and transportation. It tends to secure its products from the harmful effects of microbes **[**15]. Soybean Protein Isolate (SPI) is an important raw material for producing food gels. However, SPI alone is insufficient to obtain good gelation properties and is often used with other ingredients to obtain the desired gel product [16]. This present research aims to synthesize drug-loading biomaterials used as a carrier for repairing skin wounds such as burns and surgical wounds, localized drug delivery for wound healing and tissue engineering. Its biodegradability, biocompatibility, nontoxicity and low cost reduce the economic burden of its implementation, and it does not sacrifice patient safety. One of the best ways to reduce antibiotic resistance is controlled drug delivery systems. This strategy improves drug absorption, allows targeted antibiotic delivery, improves tissue and biofilm penetration, and reduces side effects. In this research article, biopolymer-based cryogel is synthesized with different composition variations. After that antibiotic drug was unloaded, and the loaded cryogel was analyzed by Infra-Red Spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). The surface structure of the gel enhanced the drug loading, and the films showed that the antimicrobial properties of this edible film are applicable for artificial skin surgery, cell proliferation, tissue regeneration, wound dressing, and preventing from seviour microbial infections. Another useful application of this synthesized macro pour cryogel is water purification, a matrix used in separation techniques. The antibiotic drug loaded cryogels were also examined by antibacterial assy.

2. Experimental Section

2.1. Materials

Polyvinyl Alcohol (PVA) was obtained from Merck India (molecular weight: 14,000; hydrolysis rate: 90%; density: 1.3 g/cm^3), Mumbai and used without purification, soya Protein Isolate (SPI, isoelectronic point 4.5) was purchased from Sigma Aldrich. Sodium hydroxide (Mol. wt. 39.997), pure chems. Azithromycin Drug 250 mg tablets Brand- Azicip, Manufacturer name- CIPLA, Maharashtra, vancomycin tablet 250mg Cipla Verna indl. Estate, Goa 403 722 INDIA. All other chemicals and reagents used were of analytical grade, and double-distilled water was used throughout the experiments.

2.2. Method

2.2.1. Preparation of PVA-SPI Cryogel

Biopolymer based cryogel prepared by freeze-thaw method. In this method, the 2.0g of soya protein isolate was dissolved in 25ml of 0.05N NaOH solution with constant stirring for 15-20 minutes so that the protein was denatured and uniformly dispersed into the alkaline solution, followed by the addition of 1.0g of polyvinyl alcohol. The reaction mixture was continuously stirred for half an hour on a magnetic stirrer. The solution was poured into a petri dish and placed in a deep freezer (-15°C) for 20 to 25 hrs. The cryogel was removed from the freezer for thawing for at least 3 hrs at room temperature, and this process was repeated three to four times for complete polymerization and the formation of a macroporous cryogel. Each such cycle is termed a Freeze-Thaw Cycle (FTC). The whole reaction mixture was converted to a thick, yellow, and the synthesized gel was termed the SPI-PVA cryogel. The synthesized hydrophilic film was allowed to swell in a water bath for 72 hrs until equilibrium was reached for chemical and impurity separation. The purified cryogel film was desiccated at

ambient temperature for one week and subsequently preserved in hermetically sealed polyethylene pouches for further examination. The measured cryogel thickness was 0.24 cm.

2.2.2. Loading of Drug into Cryogel

Loading of the drug onto the cryogel was followed by the equilibrium swelling method. In these experiments, 100mg of dry cryogel was taken to swell in a drug solution in the prepared range of 0.1mg/mL to 0.4mg/mL concentration until the equilibrium was reached. The following equation is used for calculating the percentage of drug loading-

$$(\%)\ \text{Loading} = W_d - W_o / W_o \times 100 \quad (1)$$

Wd and Wo are the dry weights of drug loaded and unloaded cryogel. After being taken out, the gels were dried and used for antimicrobial examination.

2.2.3. Drug Loading Efficiency and Drug Loading Capacity

Drug loaded cryogels were prepared using the absorption method. Solutions of vancomycin drug (PBS) at different concentrations were prepared in the 0.01mg/ml range to 0.05mg/ml. Briefly, dry cryogels were immersed in 5.0 ml of each drug solution and were kept at 37°C in the dark.

Drug concentration before and after the soaking procedure was determined using a UV-Vis spectrophotometer at 261 nm for PBS (A = 0. 0264C + 0.0105, r = 0.9994. Subsequently, the drug loaded cryogels were frozen and freeze-dried for 24 hours. The tests were performed three times. Drug loaded cryogels were prepared using the absorption method. Solutions of azithromycin drug in PBS by adding 0.5M HCl to complete dissolution. Different concentrations of drug solution were prepared in the range of 0.01mg/ml to 0.05mg/ml.

Briefly, dry cryogels were immersed in 5.0 ml of each drug solution and were kept at 37°C in the dark. Drug concentration before and after the soaking procedure was determined using a UV-Vis spectrophotometer at 231 nm for PBS + 0.05M HCl (A = 0. 0273C + 0.0101, r = 0.9996. Subsequently, the drug loaded cryogels were frozen and freeze-dried for 24 hours. The tests were performed three times. The drug Loading Efficiency (LE %) and Drug Loading Capacity (DLC %) were calculated according to the following equations:

$$\text{DLE}\ (\%) = C_o - C_e / C_o \times 100 \quad (2)$$

C_o is the initial drug concentration (mg/ml), and C_e is the equilibrium drug concentration (mg/ml).

$$\text{Weight of drug in SPI-PVA cryogels / weight of SPI-PVA cryogel} \times 100 \quad (3)$$

2.2.4. In Vitro Drug Release Procedure

The in vitro release of vancomycin and azithromycin from the SPI-PVA cryogels was performed in Phosphate Buffer Solution (PBS-release medium) at pH 7.4 by immersing cryogel samples in 10.0 ml of PBS. The samples were incubated at 37°C and gently shaken at 100rpm. At regular intervals, 5.0 ml of supernatant was withdrawn and replaced with an identical volume of fresh PBS to keep the whole volume constant.

The drug release profile of drug loaded cryogels was determined spectrophotometrically (UV–VIS Shimadzu, Japan) by measuring the absorbance at a specific wavelength of 261 nm and 231nm (λmax), respectively. The findings are reported in terms of drug release as a function of time. At the end of this experiment, the cryogels were removed from the drug-release system and dried in the vacuum oven at 37°C. Drug release studies were performed in triplicate to ensure accuracy.

$$\%\ \text{cumulative drug release} = I_o / I_F \times 100 \quad (4)$$

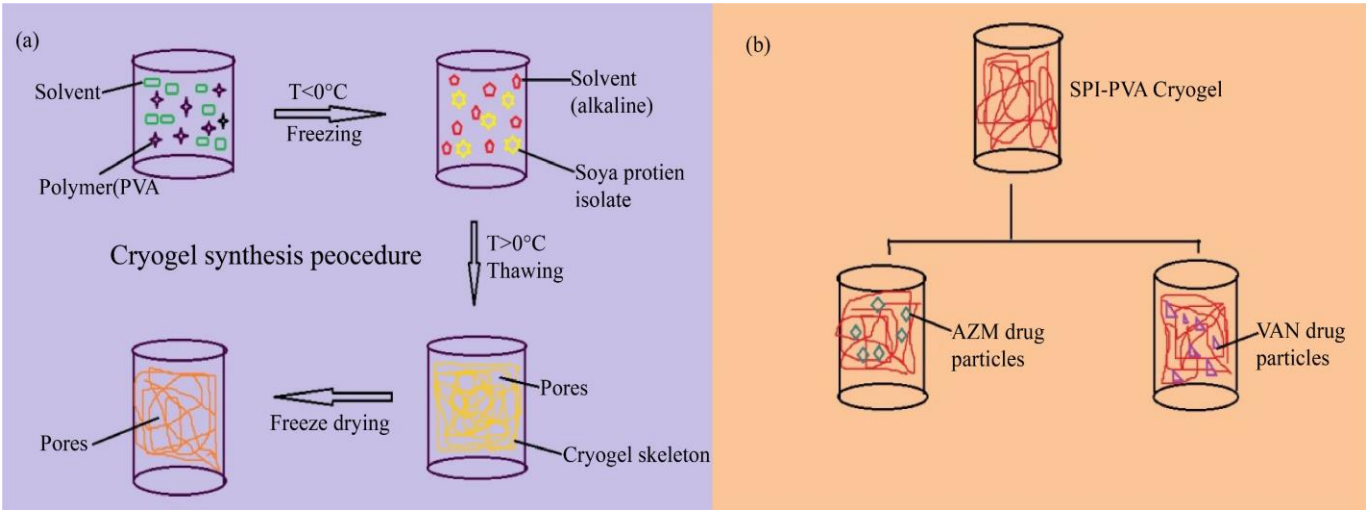


Fig. 2 (a) The procedure of SPI-PVA cryogel synthesis (b) Drug loading into the macroporous gel

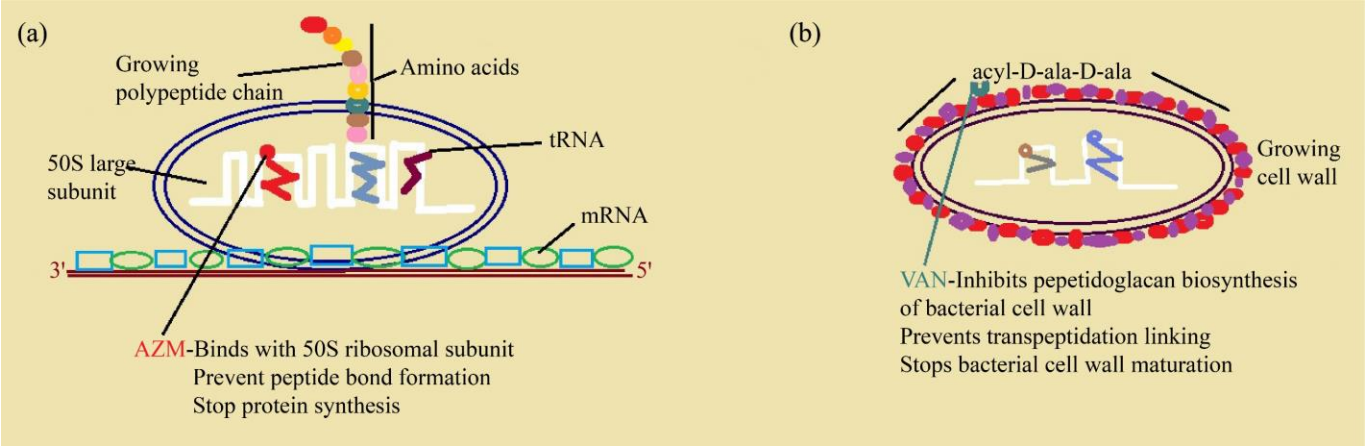


Fig. 3 Mechanism of action of antibiotic drug (a) Azithromycin and (b) Vancomycin

3. Mechanism of Antibiotic Drug Action

The glycopeptides bind to the D-alanyl D-alanine portion of the peptide side chain of the precursor peptidoglycan subunit. The drug molecule vancomycin prevents binding this D-alanyl subunit with the Penicillin Binding Proteins, inhibiting cell wall synthesis [17, 18]. Most of the antibiotics that belong to the glycopeptide class of antibiotics (e.g. vancomycin) inhibit the synthesis of peptidoglycan, leading to bacterial growth inhibition. This is done by binding themselves to peptidoglycan units and blocking the penicillin binding proteins (transglycosylase and transpeptidase) activities [19]. Macrolides: They target the conserved sequences of the peptidyl transferase center of the 23S rRNA of the 50S ribosomal subunit at translocation (early stage of protein synthesis). This action results in the premature detachment of the incomplete peptide chains. Lincosamides, streptogramins B and Macrolides show similar mechanism of action [20]. Examples of macrolides are azithromycin and erythromycin.

4. Characterization

4.1. Field-Emmision-Scanning Electron Microscopy (FE-SEM)

The surface morphology and chemical composition of soya protein isolate based cryogel were investigated by the FEG-Quanta FEG 200F, Field Emission Scanning Electron Microscopy (FE-SEM), which provides insights into the morphologies of the pure polyvinyl alcohol and soya protein isolate based cryogels.

4.2. Transmission Electronic Microscopy (TEM) -

The biopolymer based cryogel surface contains different nano size particles analyzed by JEM 2100F model Transmission Electronic Microscopy (FEG TEM 200kV) with high resolution power.

4.3. Atomic Force Microscopy (AFM)

Atomic force microscopy Model Park XE7 examined the surface roughness parameter, Make Park System, Korea.

4.4. Fourier Transport Infra-Red (FTIR)

The FTIR analysis provides the functional groups in the SPI-PVA cryogel and possible interaction between the biopolymer–hydroxyl group of polyvinyl alcohol. The FTIR spectral analysis was carried out by scanning a thin film of the cryogel in the range 4000-450cm^{-1} on an FTIR Spectrometer (Shimadzu, Japan).

4.5. Mechanical Properties

Mechanical behavior of cryogel analyzed by universal tensile machine (CIPET-BHOPAL). A Universal Testing Machine (UTM) (LS5, Lloyd Instruments Limited, West Sussex, UK) was used to measure the tensile strength and percent elongation at break. For this, the films were cut into dumbbell-shaped (3 cm × 0.5 cm) strips. The strips were subjected to extension by UTM at a speed of 0.5 cm·min−1 until breaking the films. The following equations were used to calculate the tensile strength.

Tensile Strength = Max. Load at break / Transverse section area

5. Antimicrobial Assay

The antibacterial activities of drug loaded cryogel were determined against E. coli and S. aureus microbes by agar disk diffusion method. The agar plate disk diffusion method performed the drug loaded gel's antimicrobial spectrum. Briefly, different concentrations of drug solutions ranging from 0.1mg/mL to 0.4mg/mL were prepared, and the 100mg pieces of cryogel were allowed to swell until equilibrium after loading that drug into the gel. These drug loaded gel pieces were carefully introduced into the agar petri plate and prepared under sterile conditions. The agar plate surface is inoculated by uniformly spreading the test gram negative *E.coli* and gram positive *S. aureous* microbes over the entire agar plate. Petri plates are incubated at a temperature of 37ºC. The antibiotic agent diffuses in an agar medium and inhibits the growth of microbes. After the incubation, the zone of inhibition was measured using a slide caliper scale of mm.

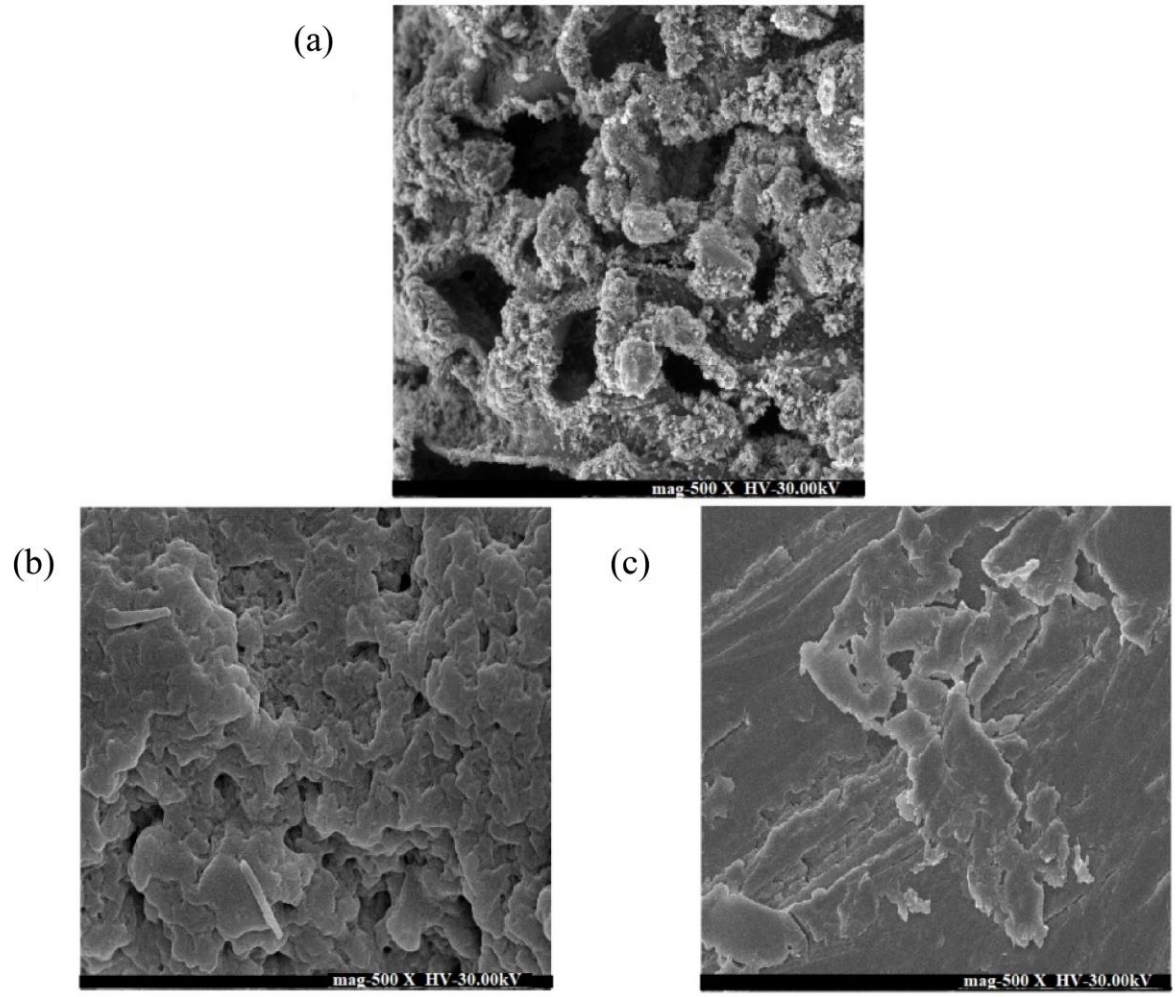


Fig. 4 FE-SEM images of (a) Drug unloaded gel, (b) Azithromycin drug loaded (79.4%) gel and (c) Vancomycin drug loaded (81.2%) gel at the magnification of 500X

6. Result and Discussion

6.1. Field Emission Scanning Electron Microscopy (FE-SEM)

FE-SEM images of drug unloaded and loaded cryogel are depicted in Figures 4 (a) and (b), (c), respectively, at 500x magnification. Cryogel synthesized by biopolymer shows a macroporous structure with irregular pore size. Physically crosslinked soya protein isolate and polyvinyl alcohol gel exhibit a fractured surface with significant cracks and many voids. These pores are the region for the interaction site of external stimuli with hydrophilic groups that support the adsorption of bioactive molecules like antibiotic drugs.

The pore size of SPI-PVA cryogel varies from 50μm to 300μm. When drug particles are embedded into these pores, the surface is relatively smooth, with no voids. The particles of the drug are uniformly distributed into the pore of the gel; hence, this network becomes a relatively smoother surface, as shown in Figure (b) AZM drug loaded and (c) VAN drug loaded gel. The smoother surface of the drug-loaded gel was examined using the SEM technique. The irregular pore structure of a network after drug loading shows an interconnected network with distinctive pores. In this structure, the distribution of drug particles enhanced the controlled drug release, the results of which were depicted via the gel antimicrobial assay.

6.2. Transmission Electron Microscopy (TEM)

As investigated by TEM images in Figures 5 (a), (b), and (c), the unloaded and drug-loaded cryogel was obtained to confirm the nanoscale particles and the distribution of the drug particles. These images depicted the pores nature of the gel, which held the maximum quantity of water molecules, improving their drug loading behaviour.

The size of the particles in the SPI-PVA matrix ranged from 50 nm to 400 nm, and the particles had a porous structure, which contributed to easy drug particle loading and good water holding capacity. Figures 5 (b) and (c) depicted the dark black spherical type structure representing the uniform distribution of antibiotic drug particles.

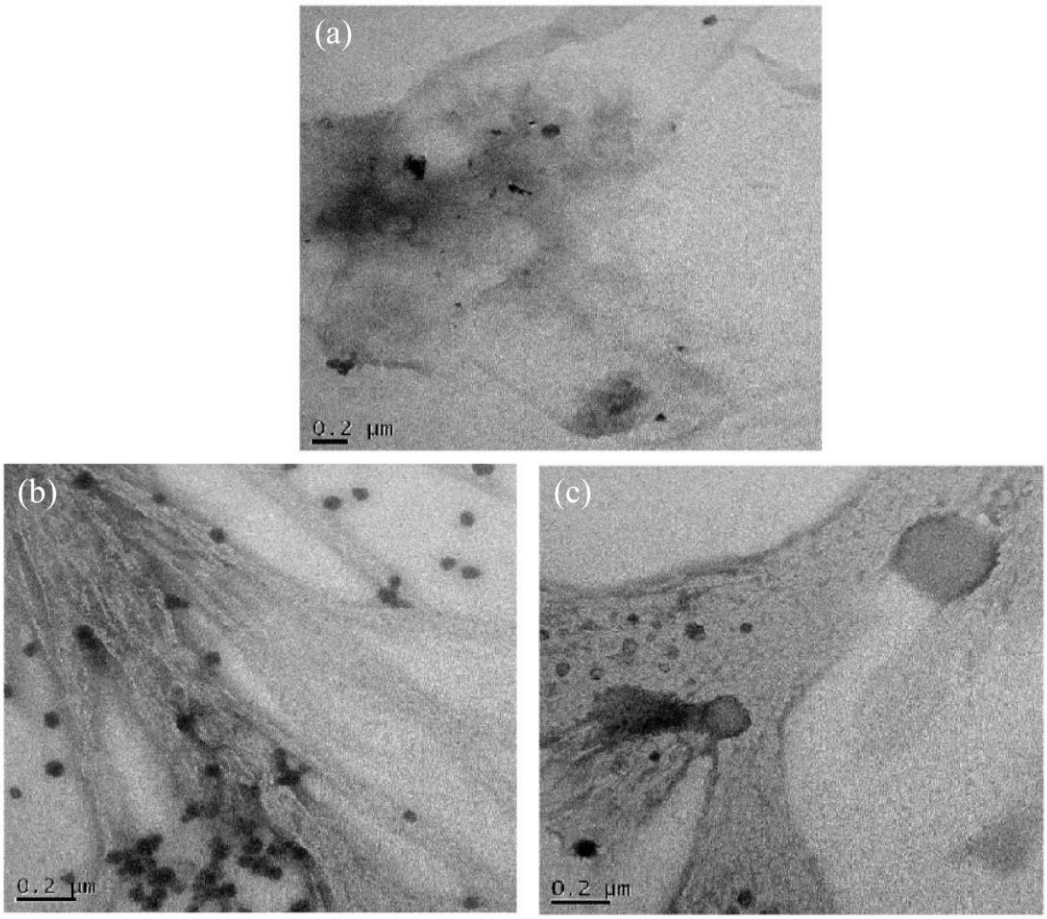


Fig. 5 (a) Drug unloaded cryogel and (b) Azithromycin,(c) Vancomycin drug loaded cryogel

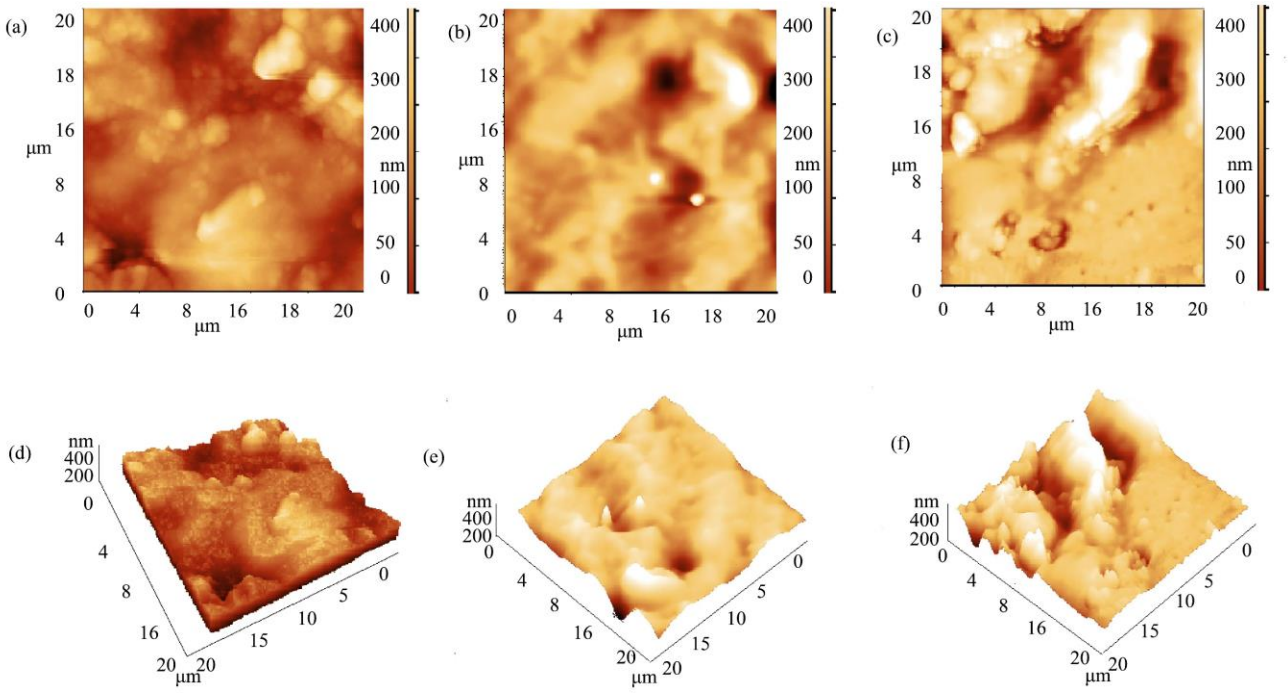


Fig. 6 (a), (b), (c) The 2D and (d), (e),(f) 3D AFM images of drug unloaded and Azithromycin, Vancomycin drug loaded cryogel

6.3. Atomic Force Microscopy (AFM)

There is an important balance between the relative scaling in the roughness of the particles and the relative size of the carrier and drug particles when considering the effects of surface roughness on particle–particle adhesion. The surface roughness is illustrated by the schematic in Figure 6 (a), (b), (c) 2D and (d), (e), (f) 3D AFM images of drug unloaded, AZM drug loaded and VAN drug loaded, respectively. Assuming no other changes in particle properties (i.e., surface energy, particle size, amorphous content, hygroscopicity, etc.), the rank order of drug-carrier adhesion would obey the following trend in the roughness of the larger carrier particle: macro-rough surface >smooth surface >micro-rough surface. This trend is strictly dependent on the contact area between the drug and carrier**.** Atomic force microscopy images reveal that the surface roughness of the biopolymer based 3D network was reduced after drug embedded into the gel pores, so the synthesized film was used as a good platform for appropriate drug adhesion.

6.3.1. Amplitude or Height Parameters

A sample's amplitude parameters (Ra, Rq and Rpv) describe the surface topography or roughness analysis, which provides information about the statistical average values, shape of the histogram heights and other extreme properties. The average roughness (R_a) is the mean height calculated over the measured length/area. R_a is typically used to describe the roughness of gels (soft) and metallic surfaces (hard). The root mean square (RMS) roughness (R_q) is the square root of the distribution of surface height, is considered to be more sensitive than the average roughness for large deviations from the mean line/plane and is also used in computing the skew and kurtosis parameters. The values of the principle parameters of surface roughness R_a, R_q and R_{pv} investigated by AFM analysis for the SPI-PVA cryogels and AZM, VAN-drug loaded matrix are shown in Table 1.

6.3.2. Skewness Parameter

The surface skewness (R_{sk}) measures the symmetry of the variations in a profile/surface about the mean line/plane and is more sensitive to occasional deep valleys or high peaks. R_{sk} illustrates nonconventional machining processes' load carrying capacity, porosity, and characteristics. A negative skew is a criterion for a good bearing surface. As mentioned earlier, the negative values of the skewness indicate that the valleys are dominant over the scanned area, i.e., porous surfaces. The positive R_{sk} value of drug loaded cryogels represents the drug particles' careering nature of the SPI-PVA matrix. The Gaussian distribution surface of this SPI-PVA cryogel investigated by the ratio of Rq./Ra is 1.20, i.e., all the surfaces are nearly symmetrical relative to the asperity height distribution; therefore, the statistical correlation pertaining to surface roughness; however, the drug-loaded matrix fails to demonstrate a Gaussian distribution in surface characteristics, as illustrated in the Table 1.

6.4. Infra-Red Spectral Analysis (FTIR)

The presence of functional groups and existing hydrophilic, hydrophobic, and covalent types of intermolecular interactions in cryogel was confirmed by interpreting Figure 7 (a) spectra of thin and spongy SPI-PVA biofilms. In order to acknowledge the study of proteins, the secondary and tertiary structure of proteins is analyzed using the FTIR technique. The secondary structures of the protein were commonly based on the primary amide band analysis (1700–1600 cm-1). Primary amide is the most intense absorption band of the polypeptides. ν (C= O) has a predominant role after that ν (C-N) signals. There are also some in-plane NH bending contributions to Amide I. The secondary structure of proteins corresponds to these bands as follows: 1610 ~ 1640 cm-1 for the β-sheet; 1640 ~ 1650 cm-1 for the random coil; 1650 ~ 1658cm-1 for the α-helix; 1660 ~ 1700 cm-1 for the β-turn**.** The main characteristic peaks of SPI were illustrated at 1633 cm−1, 1554 cm−1, and 1238 cm−1, presenting the Amide I (C=O stretching, C-N stretching), Amide II (N–H bending), and Amide III (C–H and N–H stretching), respectively. The FTIR spectra of azithromycin drug loaded cryogel depicted in Figure 7 (b), bands at 2782-2971 cm-1 and 1376 cm-1 related to the axial stretching and bending of C-H of the methyl groups. The axial stretching of the C=O was observed at 1719 cm-1. Figure 7 (c) vancomycin drug loaded cryogel revealed characteristics peaks at 3406cm-1 of hydroxyl stretching, 1658cm-1 of the C-O stretching, 1502cm-1 C-C and 1230cm-1 of the phenolic hydroxyl groups. The FTIR-spectra of drug loaded and unloaded gel showed all characteristic peaks of both antibiotic drugs and polymer composites (SPI-PVA) without any significant shift, indicating the particles of drug embedded on the surface of the matrix and drug-polymer interactions observed.

Table 1. The surface roughness parameters investigated by the AFM technique

Surface Parameter	SPI-PVA Cryogel	AZM drug loaded Cryogel	VAN drug loaded cryogel
Scanning area	20X20	20X20	20X20
Roughness average(R_a)	127.48nm	20.63nm	21.2
Root mean square roughness(R_q)	153nm	75nm	71nm
Skewness of surface(R_{sk})	-0.129	3.023	2.89
Kurtosis (R_{ku})	2.356	2.20	2.01
Ten point height R_z	756.18nm	69.72	67.4
R_{pv}	765.50nm	157.3	158.5
R_q/R_a	1.20	3.75	3.38

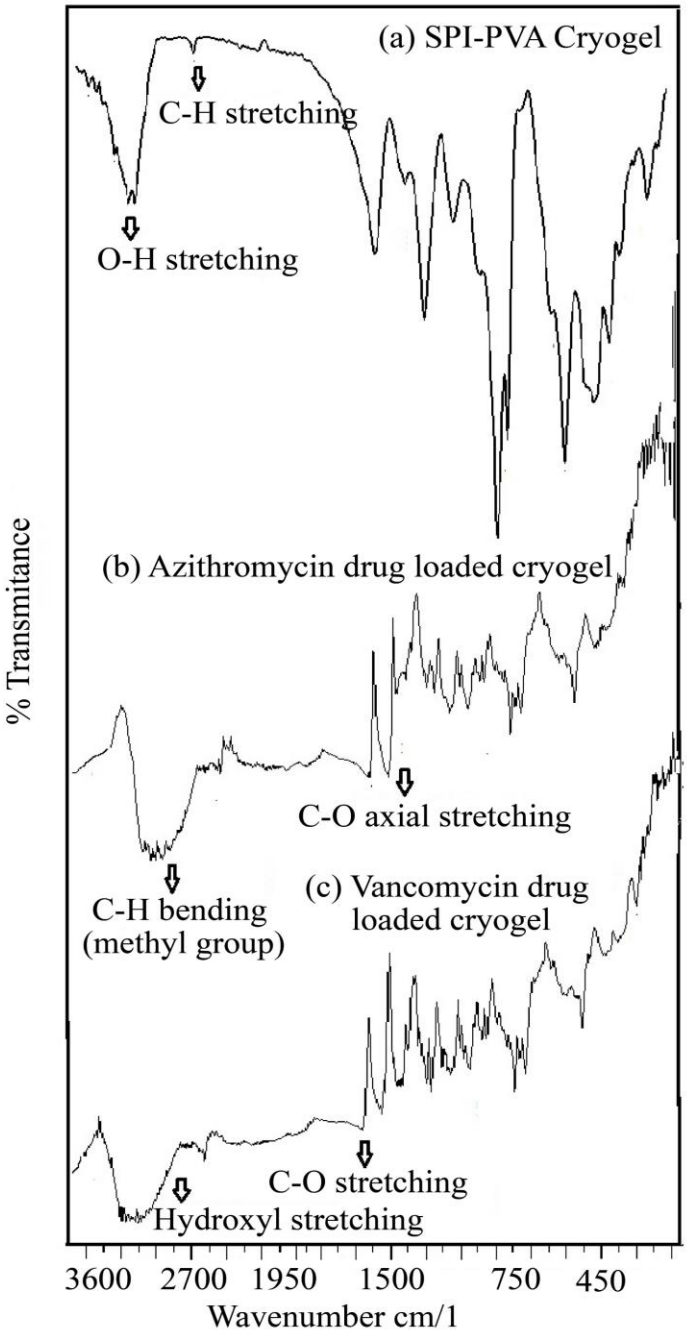


Fig. 7 FTIR spectra of (A) Unloaded drug cryogel (b) Azithromycin drug loaded cryogel (c) Vancomycin drug cryogel

6.5. Mechanical Strength

Due to the impact of porosity and the densification of polymers in the pore walls, determining the mechanical properties of cryogels is difficult without direct measurements. Several mechanical properties can be deemed as of significant interest. The characterization of bulk tensile or compressive strength could be important depending on the expected forces exerted on the tissue scaffold in any application. However, local elastic properties are also of significant interest. The biopolymer-based SPI-PVA cryogel had interconnected macro-porous structures, and their tensile strength was observed at 14.56MPa. These specimens exhibit similar stress–strain curves, where approximately 140% strain change occurs as the stress on the sample increases from 0 to about 24 MPa and slight strain change as the stress increases above 24 MPa. A comparison of stress–strain curves among PVA-SPI cryogels with different drug loaded cryogels indicates that gels loaded with azithromycin drug and vancomycin drug particles are stronger than the ones with polyvinyl alcohol- soya protein isolate cryogel.

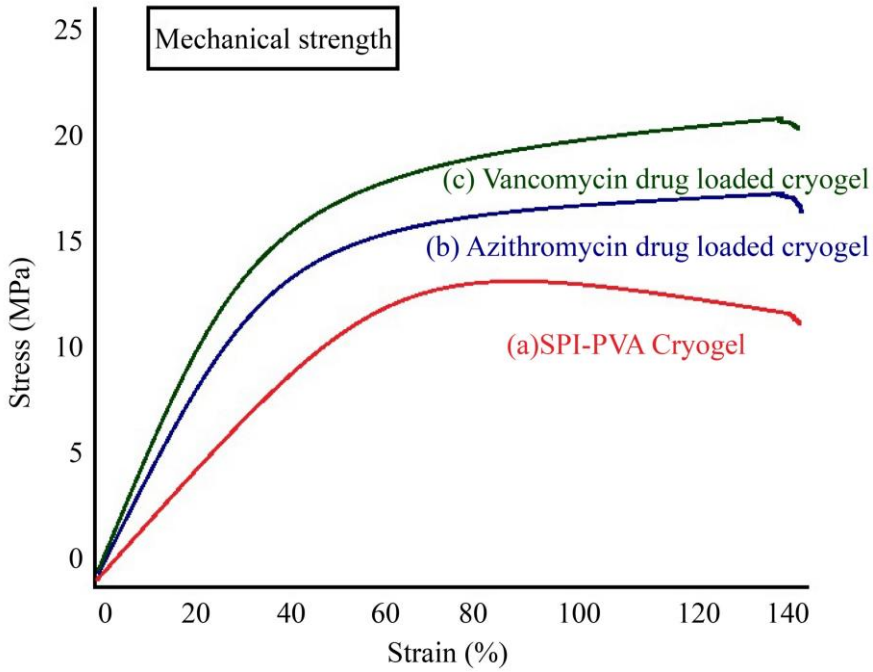
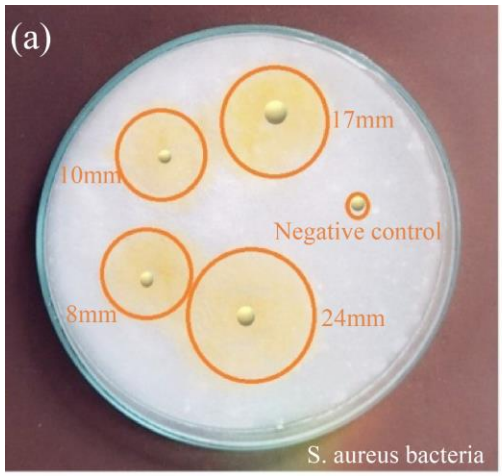
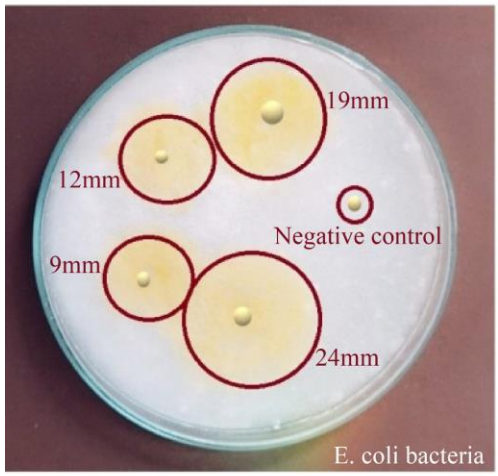
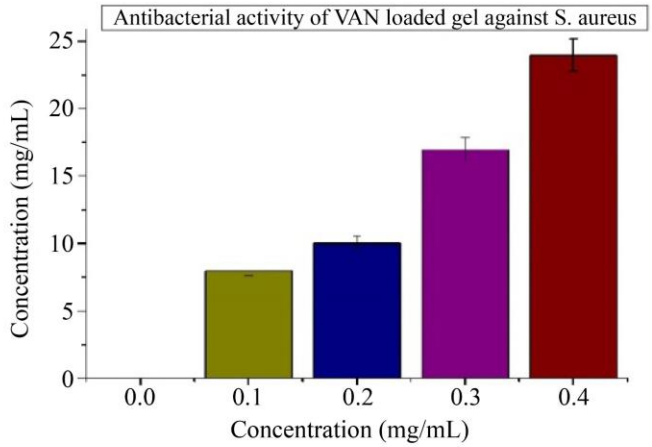
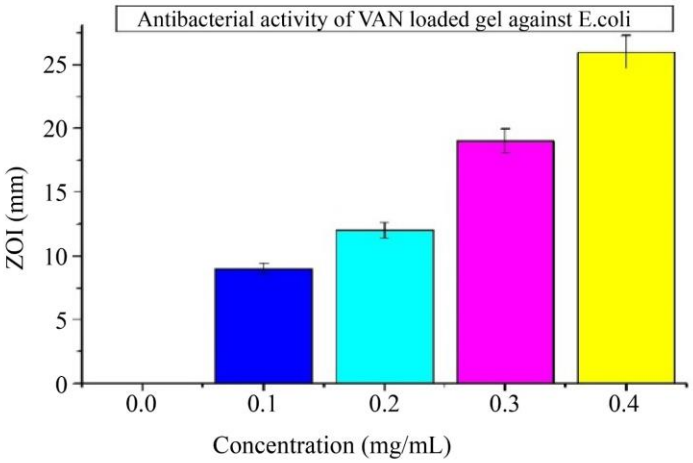


Fig. 8 Strain-stress curve of drug unloaded and drug loaded SPI-PVA cryogel









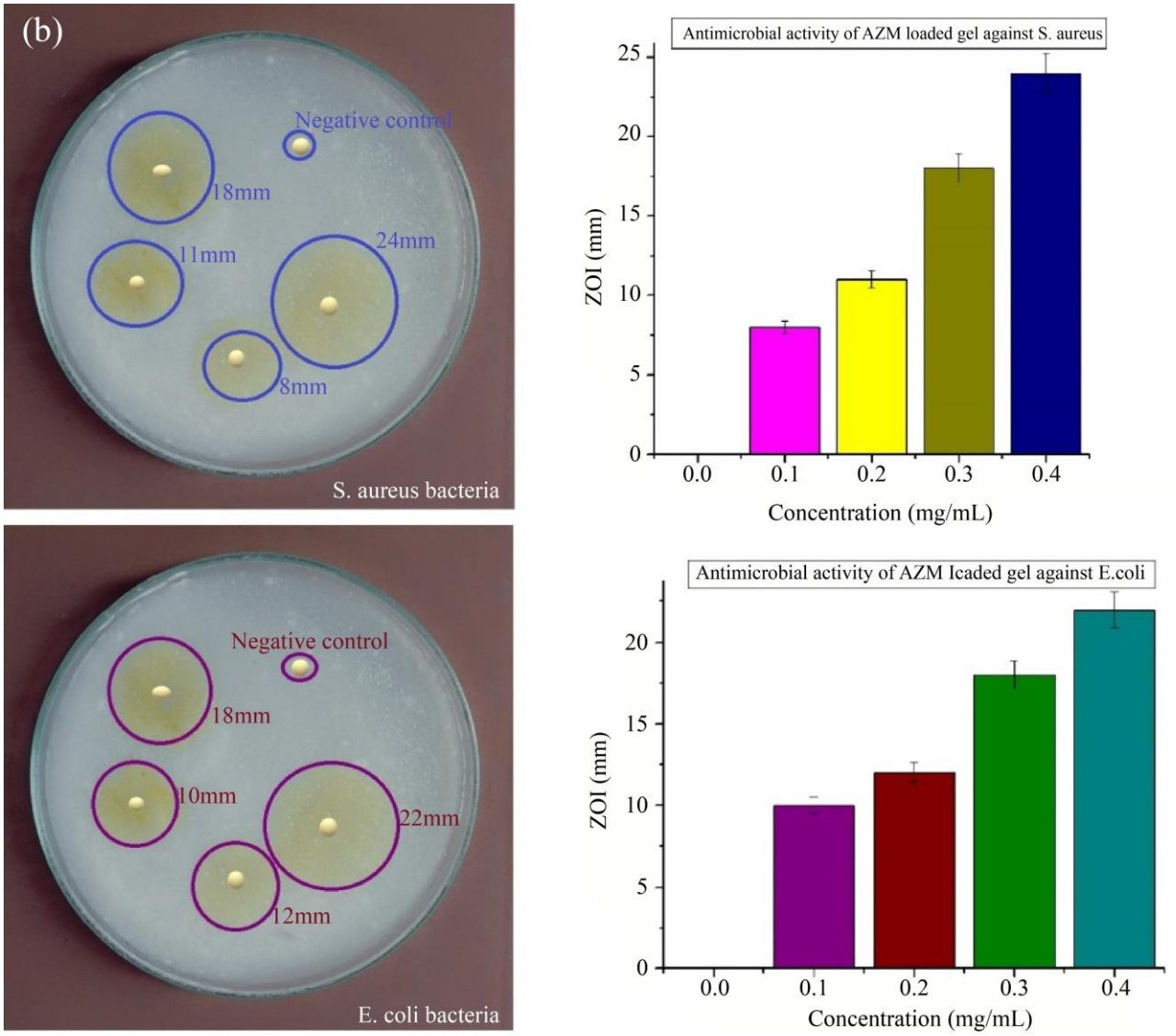


Fig. 9 Antimicrobial examination of (a) Vancomycin (b) Azithromycin antibiotic drug loaded gel against negative control (drug unloaded gel)

6.6. In Vitro Antibacterial Activity

The AZM-loaded SPI-PVA-based cryogel is further implemented for antibacterial activity. Bactericidal effects of AZM films were measured using inhibitory zone measurements against Gram-positive and Gram-negative organisms, including *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 35218), respectively. Gram-positive bacteria, such as the *Enterococcus* species, *S. aureus* and *Clostridium difficile*, can be treated with vancomycin and similar glycopeptides, which are last resort medications. Vancomycin's bactericidal effect was long hypothesized to be impervious to resistance as it binds to the bacterial cell envelope rather than a protein target, as is the case for other antibiotics.

Two complex resistance mechanisms threaten Vancomycin's therapeutic efficacy, each involving a multi-enzyme route, that have arisen and is becoming more common in pathogenic species. Precursor degradation and substitution with D-Ala-D-lac or D-Ala-D-Ser alternatives constitute resistance mechanisms for which vancomycin has a poor affinity. Vancomycin resistance has been studied extensively for >30 years, and significant progress has been made in understanding the molecular biology of the enzyme cascades involved**.** Notably, Shlaes and co-workers confirmed the ability of vancomycin to bind to Gram-negative lipid II from E. coli**.** The result of the antibacterial activity of antibiotic drug loaded gel examined against gram positive bacteria, gram negative bacteria and zone of inhibition is shown in Figures 9(a) and (b) with graph plot against vancomycin and azithromycin drug concentrations, respectively. When the concentration of the drug increases, the zone of inhibition also increases, proving the antimicrobial property of drug loaded cryogel.

7. Applications of Antibacterial Cryogels in Wound Dressing

Wound healing is a dynamic and interactive process involving four phases: coagulation and hemostasis, inflammation, proliferation, and wound remodeling with scar tissue formation. The four phases mediating the wound closure process involve the interaction between multiple cell populations, soluble mediators, cytokines, and so forth. The healing process is initiated immediately after the injury [21]. At the early phases of acute or chronic wound healing, degranulation of platelets induced the release of platelet basic protein. After proteolytic cleavage, it could release the neutrophil attractant NAP-2 family of proteins, which provides a bolus dose of neutrophil chemoattractant activity to recruit and activate neutrophil leukocytes to destroy planktonic bacteria [22].

Table 2. Some antibacterial matrices used in wound dressing

S.No.	Main components	Antibacterial agent	Bacteria	Ref.
1.	CS/ PVA	CS/gentamicin	E. coli, S. aureus	[23]
2.	PVP/HA	Antiseptic/ciprofloxacin	E. coli, S. aureus	[24]
3.	Carrageenan/ PEO	Streptomycin, diclofenac	E. coli, S. aureus, P. aeruginosa	[25]
4.	Astaxanthin/ collagen	Gentamicin	E. coli, S. aureus, P. aeruginosa	[26]
5.	SPI-PVA	Vancomycin, azithromycin	E.coli, S.aureus	Current work

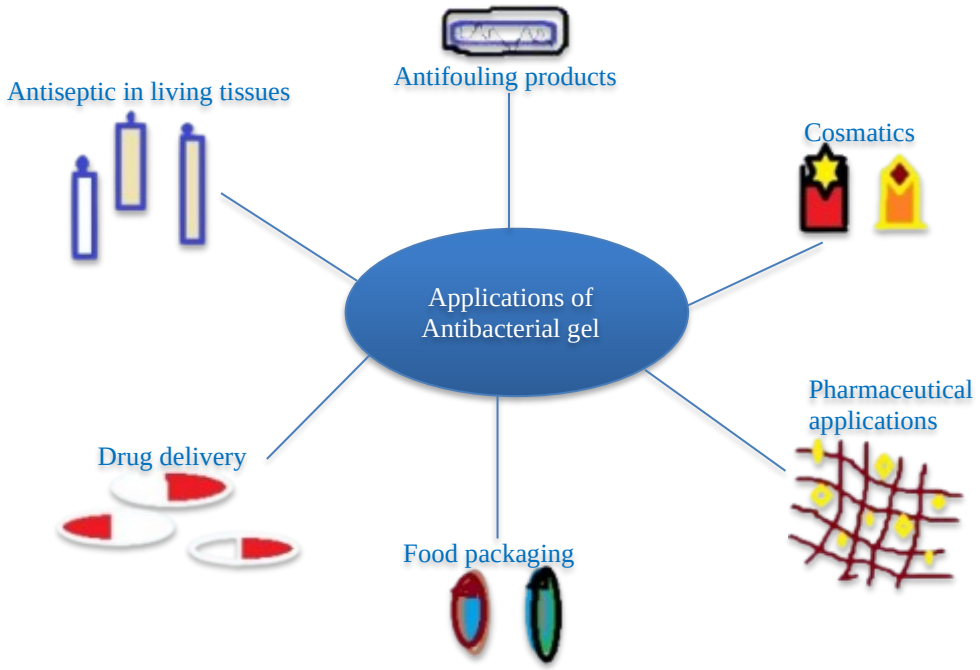


Fig. 10 Applications of antibacterial cryogels

8. Conclusion

Synthesized cryogel had good biocompatibility, non-toxicity, biodegradability, low cost and hydrophilic. Soya protein isolates and polyvinyl alcohol-based cryogel macro pores structure were investigated by Scanning Electron Microscopy (SEM), which shows that the gel surface had cracks with significant pores. This region supports binding hydrophilic groups like amino and carboxylic (-NH_2 and –COOH). After drug loading, the gel becomes smooth, which reveals the good adsorption of the drug into the film. The nanoscale particle size of the drug unloaded and loaded gel was investigated by transmission electron microscopy. Includes AFM images showing the good adhesion property of prepared film. This research article describes that antimicrobial cryogel is prepared by drug loading in known antibiotic concentrations until equilibrium. These drug loaded gel antimicrobial properties were examined by gram positive and gram negative bacteria using the disk diffusion method compared with negative control (drug unloaded gel). The Observed result shows the gel's great antimicrobial behaviour, so this gel applies to wound dressing, respiratory infections, gastrointestinal infections, tissue regeneration and targeted drug delivery.

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