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Synthesis and evaluation of a hydrogel that binds glucose and releases ciprofloxacin

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Abstract This study reports the formation of a hydrogel generated by polymerizing aminophenyl boronic acid in polyvinyl alcohol (PVA). The gel formed as a result of complexation between the –OH groups of PVA, and boronic acid moieties were stable and exhibited high degree of swelling proportional to the concentration of glucose. Extended swelling was attributed to the strong affinity of the gel to glucose and to the subsequent breaking of the bond formed between PVA and boronic acid groups. Interestingly, the gel was found to bind a high amount of glucose. We evaluated the hydrogel in terms of its ability to bind glucose and to release ciprofloxacin. Retention of antibacterial efficacy of the released drug was also demonstrated. Features such as swelling, drug release, and glucose binding reflect the possibility of tuning a new dressing for wounds particularly in diabetic patients.

Introduction

Wound healing is a complex process in which a variety of cellular and matrix components act in concert to establish the integrity of injured tissue. The response can be broadly divided into many phases in which specific processes occur such as inflammation, proliferation, and remodeling. Initially the hemostatic platelet plug re-establishes the infection and desiccation limiting barrier and exerts the first wave of cellular infiltrates [1–6]. The infiltrates consist mainly of leukocytes that provide both innate and acquired immunity. These cells produce an array of molecules to eliminate microbial contamination. Simultaneously, defense mechanism is detrimental to keratinocytes, fibroblast, and endothelial cells required to regenerate the lost tissue [7–11]. Thus, as healing proceeds the events and process of inflammatory phase need to regress. A particular challenge is offered in the case of skin wound repair which occurs at a contaminated surface. It is documented that if a wound become infected, the normal healing is disrupted as the inflammatory phase becomes chronic suppressing the regenerative phase [12]. Further the enzymes elaborated by both the microbes and leukocytes break down the wound tissue as well as the surrounding skin. Thus, it is critical to prevent infection for proper healing. Also many other medical conditions or treatments exist that impair or improve the process occurring during these phases. Any factor that affects these individual processes has the potential to affect the successful completion of healing. For example diabetes known to alter the function of leukocytes in which chemotaxis, phagocytosis, and intracellular bacterial killing are all diminished. These effects would be expected to lead to impaired healing due to a less effective inflammatory response and the altered function of neutrophils, macrophages, and lymphocytes causing diminished fibroblast proliferation and collagen deposition [13–15]. Control over serum glucose in diabetics can improve several healing defects including altered leukocyte function, fibroblast proliferation, and collagen synthesis. Thus, wound management in diabetics warrants the need for special materials capable of performing multitasks; apart from

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performing as wound coverage, the material should be capable of binding glucose from the wound site and should release an antibacterial drug preferably in a sustained fashion.

Polymer containing a phenyl boronic acid moiety can form reversible complexes with molecules that have diol units in which two hydroxyl groups are in a coplanar configuration and hence capable of binding sugars such as glucose and living cells without affecting its viability [16–18]. Among the variety of boronate containing materials water soluble synthetic phenyl boronic acid containing copolymer has the ability to bind with glycoprotein existing in the cell membrane which leads to the proliferation of lymphocytes and other such cells which leads to faster healing [19, 20]. Polyvinyl alcohol (PVA) has excellent film forming and adhesive properties together with high tensile strength and flexibility. It is biocompatible and is one of the widely employed materials for a variety of medical applications [21–24]. In the past Kitano et al. have explored extensively polymers consisting of PVA and phenyl boronic acid. These authors main focus was the design of novel glucose responsive drug delivery systems. Such systems could ultimately be tuned as insulin delivery vehicles for the management of diabetics [25–27].

Polyaniline is a well investigated conductive polymer [28–30]. Aniline derivatives can easily be polymerized to form a wide range of materials. An interesting aniline derivative is amino phenyl boronic acid. Bosi et al. have shown that this molecule can be polymerized using ammonium persulfate (APS) in ambient conditions to form polyaminophenyl boronic acid and coated as thin layer on the surface of polystyrene tissue culture plates [31]. Since boronic acid moieties can interact with –OH functionalities of PVA, it is reasonable to assume that when aminophenylboronic acid is oxidized in PVA solution, a polymer–polymer complex between PVA and polyaminophenyl boronic acid can be formed. A material of this kind may be useful in the generation of new class of matrices as scaffolds for cells as well as wound dressings. To the best of our knowledge, such a hydrogel has not been reported. It appears that hydrogel prepared by this approach complexing polyvinyl alcohol (PVA) and polyaminophenyl boronic acid and loaded with an antibacterial drug (e.g., Ciprofloxacin) provide an optimal healing milieu. We report herein the synthesis and evaluation of a hydrogel capable of binding glucose and having antibacterial efficacy.

Materials and method

Materials

3-Aminophenyl boronic acid hemisulfate salt ≥95% (ABA), APS, alpha-D-glucose anhydrous, 96%, and ciprofloxacin were purchased from Sigma Aldrich, Bangalore, India. Polyvinyl alcohol (PVA, MW 125,000) was purchased from SD Fine Chem. Ltd, Mumbai, India. The materials were used as received.

Synthesis of hydrogel

Hydrogel consisting of PVA and polyaminophenyl boronic acid (PBA) was prepared as cited below. Five grams of PVA and 150 mg ABA were dissolved in 100 mL distilled water by heating to 60 °C under stirring. Fifty milligrams of APS was added to this solution and stirred continuously for about half an hour to get a uniform solution. The solution was dialyzed against distilled water in order to remove unreacted components. It is then transferred into Petri dish and kept for evaporation at 40 °C. After drying, the film was peeled off, cut into 2 × 2 cm^2 strips and stored in an air-tight container at room temperature until use. Several compositions by changing the quantity of ABA were prepared. However, any substantial variation in properties could not be observed in the materials generated by varying the compositions. The results delineated here is based on the hydrogel obtained using the above-mentioned composition.

Incorporation of ciprofloxacin drug into the hygdrogel sheet

Ciprofloxacin was incorporated into the hydrogel by incubating it in 10 mL solution of ciprofloxacin (1 mg/mL) in 0.01 M sodium hydroxide at 37 °C. After overnight, incubation samples were removed from the solution and rinsed gently with distilled water and air dried. The extent of drug loading was determined from the absorption at 315 nm of the drug solution before and after placing the gel sheet.

Swelling index

The swelling index of the membrane was determined by immersing the material (1 cm^2) of a known weight in glucose solution having different concentrations (50, 100, 150, 200, 250, and 300 mg/dL) for a period of 4 h. The pH of the solutions was kept at 7.4. The percentage swelling of the membrane was determined using the equation

$$\text{Percentage swelling} = \{(W_w - W_D)/W_D\} * 100;$$

where W_D and W_W are the weights of the dry and wet films in grams, respectively.

Glucose binding studies

It is generally accepted that the reactive form of boronic acid as well as phenyl boronates is the anionic one. pK_a of

PBA is around 8.6 and the binding of glucose is known to be more at alkaline pH. Glucose solutions in water containing varied amount of glucose (50, 100, 150, 200, and 250 mg/dL) were prepared. The pH of the solutions was maintained at 7.4 by adding sodium hydroxide. The amount of bound glucose was estimated using glucose kit marketed by Enzyme Technologies Pvt. Ltd, Mumbai, India. The absorbance of standard and samples were read against reagent blank at 515 nm using UV–visible spectrophotometer (Carry model 100 Bio UV–Visible spectrophotometer).

In vitro drug release

In vitro drug release kinetics with respect to glucose solution was studied using UV–visible spectroscopy. Hydrogel strips having thickness of 0.5 mm and an area of 5 cm^2 (both sides) were used for the release studies. The films with an initial drug concentration of 15 ± 0.2 mg ciprofloxacin were immersed in 10 mL of glucose solution (1 mg/mL, pH 7.4) and were kept in an orbital shaker at 37 °C. Amounts of released ciprofloxacin at each 30 min intervals were measured from the absorbance at 315 nm. We chose 315 nm for quantifying the amount of drug released from the absorption spectrum of ciprofloxacin in water (pH was adjusted to 7.4). The spectrum depicted in Fig. 1 showed peaks at 315, 328, and 277 nm, respectively. The averages of three measurements were taken.

$$\text{Cumulative drug release } \% = \left(\text{Amount of drug released at time ``}t\text{''(mg)} / \text{Total amount of drug incorporated (mg)}\right) * 100.$$

Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectra of the materials were recorded on a Nicolet 5700 FTIR Spectrometer Madison, USA). All spectra were recorded in the range of 4000–400 cm^{-1}.

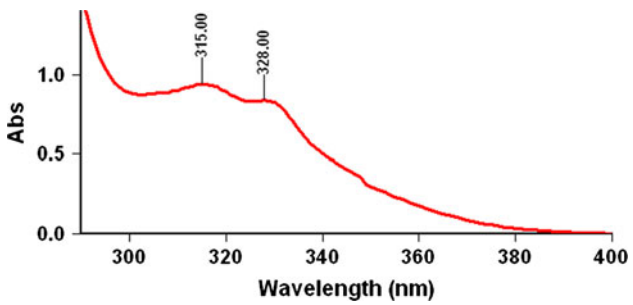


Fig. 1 Absorption spectrum of ciprofloxacin in water (pH 7.4)

Determination of tensile strength

Tensile strength and elongation at break of the hydrogels was measured with a universal testing machine (INSTRON 3365, UK). The membrane was cut into 5 mm × 40 mm strips, and thickness was measured. The test was carried out at room temperature (24 °C and 43% humidity). The gauge length was 20 mm. A maximum load of 500 N and a cross-head speed of 10 mm/min were used. The tensile strength and elongation at break percent were calculated by the following relationship.

$$\text{TS} = \text{breaking load}/A$$

$$\text{EB } \% = (L/L_0) * 100$$

where A = cross-sectional area of the samples (mm^2), TS = Tensile strength (MPa), L_0 = original length of the sample (mm), and L = increase in length at the break point (mm)

Differential scanning calorimetry

Heat flows associated with temperature were carried out for ciprofloxacin and ciprofloxacin loaded hydrogel using TA instrument model SDT Q6000 at heating rate of 10 °C/min in a dynamic atmosphere of nitrogen.

In vitro cytotoxicity

Cytotoxicity evaluation of materials was carried out by the direct contact test with monolayer of L929 mouse fibroblast cells according to ISO standards (ISO 10993-5, 1999). Briefly, L929 cells were subcultured from stock culture (National Centre for Cell Sciences, Pune, India) by trypsinization and seeded into multiwell tissue culture plates. Cells were fed with Dulbecco's minimum essential medium supplemented with bovine serum and incubated at 37 °C in 5% carbon dioxide atmosphere. After incubation of cells with hydrogel strips and controls at 37 ± 2 °C for 24 h, cell culture was examined microscopically for cellular response using a phase contrast inverted microscope (Leica, WLD MPS32, and Germany). The morphology of the cells was assessed in comparison with negative control (Ultra High density Polyethylene) and positive control (Polyvinyl chloride).

Antibacterial effect

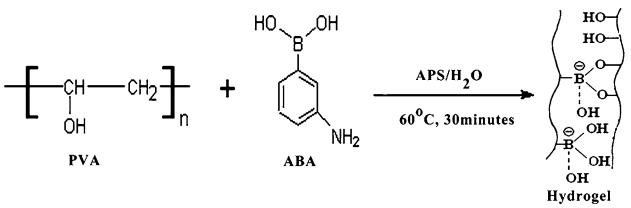
A strain of *Klebsiella pneumonia* and *Acinetobacter calcoaceticus anitratus* (isolated from patient's throat culture), *Klebsiella* sap (blood culture), *Micrococci* and *Staphylococcus aureus* (ATCC 25923) were used for these studies. Bactericidal efficacy of ciprofloxacin loaded

hydrogel was evaluated by disk diffusion method using Muller–Hinton agar (MHA) prepared as per the standard. Hydrogel membrane and ciprofloxacin loaded membrane were extracted separately in 1 mL of distilled water for a period of 1 h and 10 μL of the extract were incorporated onto 6 mm diameter sterile filter paper disk, which were then placed on the agar plates streaked with bacteria and incubated for 18 h at 37 °C.

Results and discussion

The hydrogel membrane was generated by polymerizing ABA to Poly (phenyl boronic acid) (PBA) using APS in 5% PVA solution. Color of the film turned to deep brown reflecting the formation of PBA [28]. Boronic acid moieties are well known to form reversible complexes with diol units in which two hydroxyl groups are in coplanar configuration. In fact this property has been explored in the detection of carbohydrates and in affinity chromatography [32, 33]. During the polymerization process in PVA, boronic acid moieties form bonding with –OH functionalities of PVA to form a hydrogel networks. The schematic representation for the formation of hydrogel is shown in Scheme 1. The formation of the hydrogel was confirmed by FTIR analysis. The spectrum showed characteristic peaks of both PVA and PBA. 1314 cm^{-1}(s, B–O), 1637 cm^{-1} (b, NH). B–O stretching peak is shifted to lower wavelength reflecting the interaction of this moiety with –OH groups in PVA. The strong broad peak centered on 3300 cm^{-1} (s, OH) was assigned to OH of PVA. A peak centered around 1719 cm^{-1} (s, CO) indicates the presence of residual acetate groups in PVA. The drug loaded hydrogel showed many characteristic peaks of ciprofloxacin namely aromatic C–H stretching and bending modes indicating the stabilization of the drug in the matrix (Spectra are given in supporting information).

The extent of swelling of PVA–PBA complex was evaluated by incubating gel in glucose solution at 37 °C. Figure 2 shows the kinetics of swelling of hydrogel on equilibration at different concentration of glucose solutions. As the concentration of glucose increased the percentage of hydrogel swelling also enhanced. This is accordance with many earlier reports of copolymers containing boronic acid



Scheme 1 Formation of hydrogel between PVA and PBA

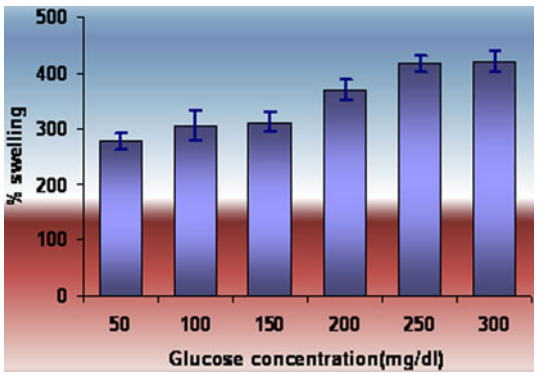


Fig. 2 Effect of glucose concentration on hydrogel swelling: Different concentrations of glucose solution, 50, 100, 150, 200, 250, and 300 mg/dL, were used for incubating the hydrogel membrane. Percentage swelling was calculated after equilibrating for a period of 4 h in the solution. Error bar represents standard deviation of the experiment performed in triplicate

moieties [34, 35]. At a concentration of 50 mg/100 mL of glucose, the percentage swelling was about 279 ± 14.63% and on increasing the concentration to about 300 mg/100 mL the percentage swelling reached up to 420 ± 18.93%. The degree of swelling is in agreement with the high affinity of boronic acid moieties toward glucose molecule which leads to the breakdown of reversible bond formed between boronic acid and hydroxyl groups of PVA. Glucose binding studies (Fig. 3) confirm the above results of increased swelling with glucose concentration. The amount of glucose bonded to the boronic acid moieties in the hydrogel increased proportionally with the concentration of glucose in the solutions.

It is generally accepted that the reactive form of phenyl boronates is the anionic form one. The pK_a of PBA is around 8.6, and the binding of glucose onto the polymer can be expected more in alkaline pH [36]. Several studies have shown that boronic acid derivatives show enhanced binding of sugars within a range of pH varying from 6. 5 to 8.5 [37]. Such results indicate that the pK_a of the boronic acid groups is the only factor deciding its binding to sugars.

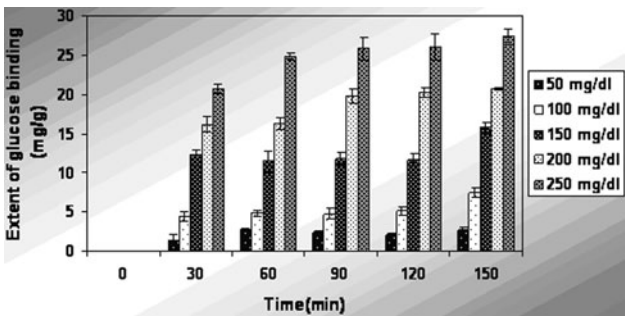


Fig. 3 Extent of glucose binding into the hydrogel membrane and its effect on glucose concentration: Different concentration of glucose solutions were studied for a period of 150 min. Error bar represents standard deviation of the experiment performed in triplicate

Complex formation is, largely, governed by steric arrangement of the interacting moieties and the composition of the media [38]. This study we performed at physiological pH and the data summarized in Fig. 2 apparently show that the polymer bind significant quantity of glucose reflecting the suitability of the material is using at physiological pH.

The mechanical properties provide an indication of the strength and elasticity of the film which can be reflected by tensile strength and elongation at break. It is suggested that films suitable for wound dressing should preferably be strong and flexible. The material showed a tensile strength of 31.74 ± 3.93 MPa and elongation at break of about 148 ± 0.45%. The parameters are indicative that the material has adequate strength to function as wound dressing.

One of the mandatory requirements of a material indented to be used in contact with living entity is the non-cytotoxicity. To confirm the nontoxic nature of the hydrogel a preliminary cytotoxicity evaluation using L929 mouse fibroblast cells was carried out. Neither the gel nor its extract induced any morphological changes to the cells confirming its nontoxic nature. The morphology of the cells growing on the surface (scored as zero) for a period of 24 h is depicted in Fig. 4.

Thermal behavior of the material after loading ciprofloxacin was measured to get an insight into the distribution of the drug in the material. The differential scanning calorimetric traces of the drug and polymer containing the drug are shown in Fig. 5a and b. Pure ciprofloxacin showed melting point at 271 °C. The melting transition is absent in the drug loaded polymer indicating that the drug is evenly distributed without the formation of any crystallites. Homogeneous distributions of ciprofloxacin in the film ensure uniform release over the total area of the wound site.

We examined the potential efficacy of the new material as a drug delivery vehicle. Ciprofloxacin was chosen as the model drug considering it broad activity against Gram-positive and Gram-negative bacteria. Here ciprofloxacin was loaded up to a concentration of 3 mg/cm^2 of the hydrogel membrane. The hydrogel system loaded with an initial amount of 3 mg/cm^2 of ciprofloxacin may result in

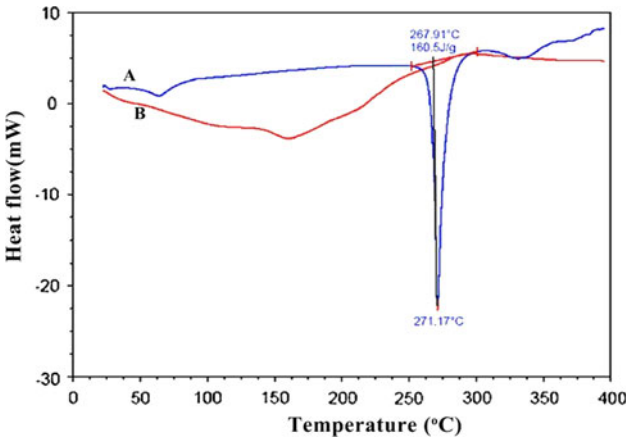


Fig. 5 Differential scanning calorimetric traces of (*A*) ciprofloxacin and (*B*) drug loaded hydrogel. Ciprofloxacin shows a sharp melting point at ~271 °C

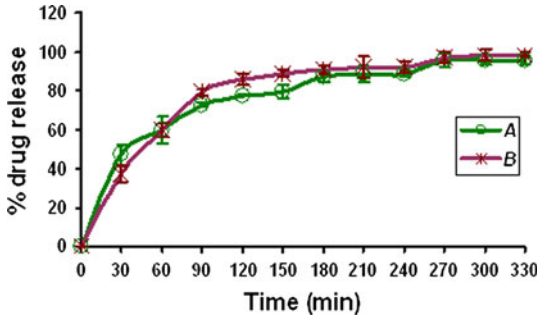


Fig. 6 In vitro release kinetics of ciprofloxacin in (*A*) water and (*B*) glucose solution (1 mg/mL). Cumulative percentage drug releases were calculated from the amount of drug released at time t and the total amount of drug encapsulated in the hydrogel matrix

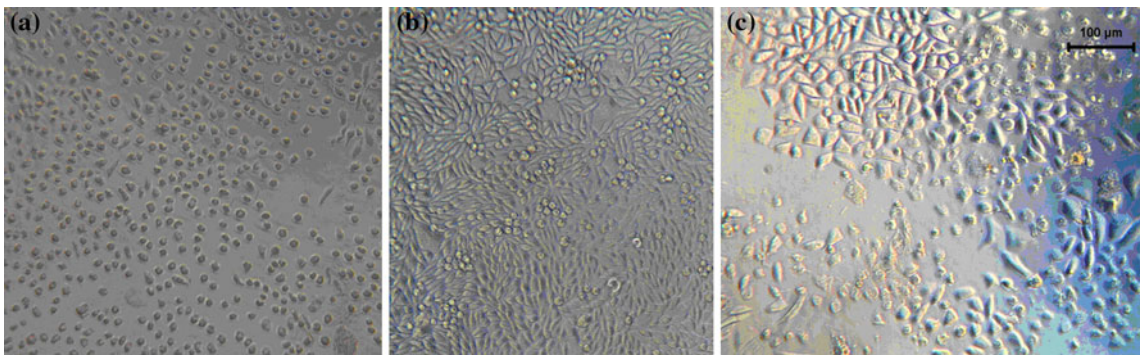


Fig. 4 Morphology of L929 mouse fibroblast cells after incubating with hydrogel membrane and control at 37 ± 2 °C for 24 h. Morphology of the cells captured using phase contrast inverted microscope for (**a**) Positive control, Polyvinyl chloride (**b**) Negative control, Ultra high density poly ethylene (**c**) hydrogel membrane generated by polymerizing PBA and PVA. There is no considerable change in the morphology of the cells in contact with the hydrogel membrane, confirming the nontoxic nature of the gel

an effective dosing and therapeutic response and thus produce high antibacterial efficacy at the wound site.

Figure 6 shows the release kinetics of ciprofloxacin from the hydrogel using 1 mg/mL glucose solution in distilled water. Glucose, due to its high affinity toward boronic acid groups, breaks the bonds existing between boronic acid and hydroxyl groups of PVA, thereby freeing the –OH functionalities of PVA. Within 30 min nearly 50% of the loaded drug was diffused out into the medium. With time, percentage release increased and more than 95% of the drug was diffused out of the system in 5 h. The faster release of the drug from the gel can be attributed to the increased swelling of the polymer gel in the presence of glucose. Increased swelling opens up additional pathways to accelerate the diffusion of the drug out of the matrix. The system of this kind deposits a higher quantity of drug at a desired site protecting the site from infection for a prolonged period. We observed nearly same release profile in the absence of glucose also. Considerable variation was not visible since the gel has a reasonably high degree of swelling in water (180%).

Table 1 Zone of inhibition of the drug loaded hydrogel on various bacterial strains

Bacterial strain	Zone of inhibition (mm)
Klebsiella pneumonia	18
Acinetobacter calcoaceticus anitratus	28
Klebsiella sap	18
Micrococci	10
Staphylococcus aureus (ATCC 25923)	34

The efficacy of the drug loaded hydrogel was studied in contact with various bacterial strains using MHA. Hydrogel membrane containing ciprofloxacin showed a clear zone of inhibition for most of the bacterial strains used in this study. The extent of clear zone of inhibition experienced by various bacterial strains is given in Table 1. Figure 7 shows zone of inhibition for 10 µl of the extract from 1 cm^2 of the hydrogel membrane with and without ciprofloxacin. It clearly demonstrates that ciprofloxacin containing hydrogel is highly active (marked as "T") while

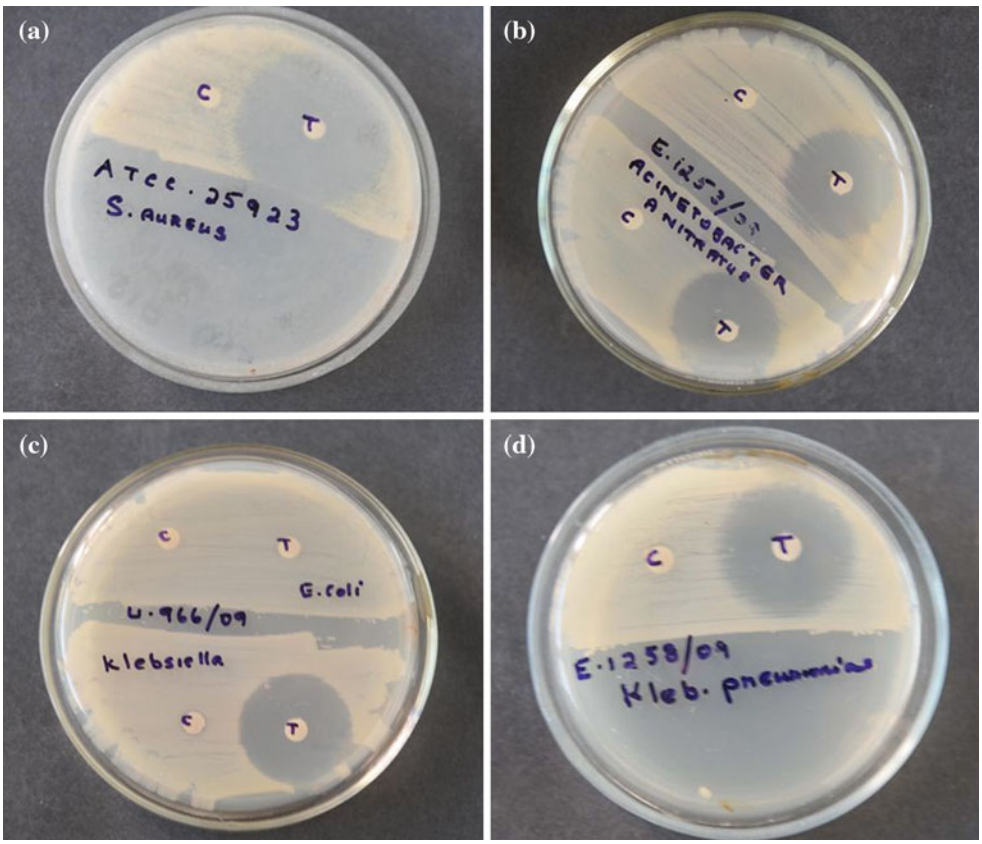


Fig. 7 Agar diffusion test. Clear zone of inhibition shown by ciprofloxacin incorporated hydrogel membrane using different strains were shown in the figure **a** *Staphylococcus aureus* (ATCC 25923), **b** *Acinetobacter calcoaceticus anitratus*, **c** *Klebsiella* sap in the bottom of the disk and *Micrococci* (*E. coli*) at the top, **d** *Klebsiella pneumonia*. Here hydrogel membrane with ciprofloxacin represented as "T" and hydrogel membrane without ciprofloxacin as "C" (control)

hydrogel with out drug is inactive (marked as "C"). No zone of inhibition was observed in the case of *E. coli* indicating ciprofloxacin is inactive against this strain.

As the diabetic condition alters the function of leukocytes, which leads to impaired healing due to less efficient inflammatory response, the uptake of glucose from wound site together with the drug release would possibly heal the wound relatively faster. Previous research shows that control over serum glucose level in diabetes improves the healing defects including altered leukocyte function, fibroblast proliferation, and collagen synthesis [14]. It is reasoned that the hydrogel reported here would effectively control the healing process, particularly in diabetics by manipulating the level of glucose at the wound site.

Conclusion

A novel hydrogel made of polyvinyl alcohol and poly aminophenyl boronic acid was synthesized and evaluated in terms of its ability to bind glucose and to release ciprofloxacin in a sustained fashion. The generation of the hydrogel was extremely simple. The data emerged suggest that new wound dressing materials for the wound management in diabetics could be optimized.

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