ISSN : 0974 - 7486 Volume 11 Issue 12

Materials Science

An Indian Journal

Full Paper

MSAIJ, 11(12), 2014 [391-398]

# Preparation of highly volatile gelatinous solution for wound healing applications

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## ABSTRACT

Attractive properties of gelatin, such as good biocompatibility, low immunogenicity, plasticity, adhesiveness, promotion of cell adhesion and growth, and low cost, make it ideally suitable as a biomaterial for tissue engineering. Highly volatile solution of gelatin (gel-PEG-DEE) and polyethylene glycol (PEG) in diethyl ether (DEE) was prepared for wound healing applications. The compatibility of gel-PEG-DEE with blood sample was performed that results demonstrated that the substance was fully compitable with human blood sample. In addition to this gel-PEG-DEE also showed antibacterial properties with no cytotoxicity as observed by brine shrimps analysis. The solidification time of the solution was found to be 4 sec which is extremely good as wound healing gel. The *in vivo* wound healing characteristics of the gel-PEG-DEE (highly volatile solution) was evaluated using rat (*Rattus norvegicus*) model test. Full-thickness wounds were created on the dorsal surface of the *Rattus norvegicus* and were treated with both gel-PEG-DEE and "eco-plaster" (control and commercially available wound healing materials) to evaluate the efficiency of the gel. The characteristic results revealed that the gel-PEG-DEE gelatinous solution was found to be more effective in the healing of rat wound than the commercial eco-plast.



## KEYWORDS

Highly volatile solutions;
Gelatin;
Polyethylene glycol;
Wound healing;
Bbiocompatibility..

## INTRODUCTION

The field of tissue engineering has developed to meet the tremendous need for organs and tissues[1,2]. In the most general sense, tissue engineering seeks to fabricate, living replacement parts for the body[3]. The necessity of tissue engineering is illustrated by the ever-widening supply and demand mismatch of organs and tissues for transplantation. A variety of syntheticand naturally derived materials may be used to form hydrogels for tissue engineering scaffolds. Synthetic materials include poly (ethyleneoxide) (PEO), poly (vinyl alcohol) (PVA), poly (acrylic acid) (PAA), poly (propylene furmarate-co-ethylene glycol) (P(PF-co-EG)), and polypeptides. Representative naturally derived polymers include agarose, alginate, chitosan, collagen,

fibrin, gelatin, and hyaluronic acid (HA)[4]. Naturally derived hydrogel forming polymers have frequently been used in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM. For example, collagens are the main protein of mammalian tissue ECM and comprise 25% of the total protein mass of most mammals[5,6].

Collagen is regarded as one of the most useful biomaterials. The excellent biocompatibility and safety due to its biological characteristics, such as biodegradability and weak antigenecity, made collagen the primary resource in medical applications[7,8]. It was also used for tissue engineering including skin replacement, bone substitutes, and artificial blood vessels and valves[9]. Collagen is an attractive material for biomedical applications as it is the most abundant protein in mammalian tissues[6], and is the main component of natural ECM[5]. Recently, it has been demonstrated to exhibit activation of microphage and high-haemostatic effect[10]. Consequently, it has been used in a wide variety of wound dressings and as a biomaterial in tissue engineering. The strength of the gelatin is due to the presence of triple helixes. The greater the triple helix content, the higher the strength and lower swelling property in water[11]. The selection of gelatin as a component for the preparation of the scaffold film rest up on two reasons; first, gelatin is a connective tissue protein, well known for its nontoxic, nonirritant and biodegradability properties, and good living body compatibility and therefore has been widely used in food, pharmacology, and cosmetic applications[12]. Second, the formation of specific intermolecular interaction through hydrogen bond of two or more polymers is responsible for the observed mixing behavior and properties of the films. The selection of gelatin is also justified with this point of view.

One of the drawbacks of collagen for tissue engineering applications is its solubility in aqueous media; therefore, collagen containing structures for long-term biomedical applications need to be cross linked[13]. In recent years polyethylene glycol (PEG) has attracted much attention as a polymeric excipient that can be used in cross-linking for different purposes. For instance, formulations with PEG have been extruded to make different products such as swell able and erodible implants, scaffolds for tissue engineering[14,15]. The incorporation of PEG with degraded collagen (gelatin) has an aim to develop a material which would have good mechanical properties, thermally stable in the human body, good swelling property and effective water absorption capacity. An ideal wound healing material would be biocompatible, nontoxic, odor-free, highly volatile, impermeable to microorganisms and yet permeable for necessary water vapors. In this experiment PEG was used as to modify the gelatin solution as well as to increase the swelling and adhesiveness during external dermal applications. Here in we wish to develop gelatin and PEG based flexible, bioactive, biocompatible and nontoxic bio-adhesive or highly volatile gelatinous solution in DEE that might show rapid healing properties and having soothing effect. This study was a successful endeavor to develop and investigate the suitability of such a bio-adhesive wound healing gel based on gelatin as an alternative to the conventional expensive wound dressing material

## EXPERIMENTAL

### Materials

Gelatin (Type B from cattle bones, Bloom strength-240 g, and Pharmaceutical grade) granules were supplied by the Global Capsules Limited (G.C.L.), Barishal, Bangladesh. PEG (400 USP/NF) was of obtained from BASF, and diethyl ether (DEE) from Merck, Germany. Rats (*Rattus norvegicus*) that used for *in vivo* study were purchased from Animal Resource Department, ICDDR, B, Mohakhali, Dhaka, Bangladesh.

## METHODS

### Preparation of gelatin and gelatin-PEG solution

15%, 20%, 30% and 40% gelatin solutions in water were prepared at 60°C by vigorous mixing with distilled water using magnetic stirrer and the viscosity of the solutions were measured. By considering the optimum viscosity among all gelatinous solutions, the 30% gelatin solution was taken as standard. Polyethylene glycol (PEG) was added to the prepared gelatin solution to make different ratios of gelatin-PEG (15:1, 15:2, 15:3, and 15:4 at w/v basis) solutions. The mixture was

allowed to stir for 10 minutes at 60°C for complete mixing. By using above procedure 200 mL of 30% gelatin solution was prepared and distributed in 4 different beakers such that each beaker contains 50 mL of 30% gelatin solution. Polyethylene glycol was added to the beakers in ratios of gel: PEG (15:1, 15:2, 15:3, and 15:4) in w/v basis. The mixtures were again stirred for another 10 minutes at 60°C.

**Preparation of highly volatile gelatinous solution**

Highly volatile gel was made by introducing diethyl ether (DEE) as highly volatile solvent. DEE as high volatile solvent was added to test tubes in different ratios of gel-PEG: DEE (5:1, 5:2, 5:3, 5:4, 1:1, 5:6, 5:7) respectively. The prepared mixtures were labeled properly and were shaken vigorously with Vortex mixer, VM-2000 and the temperature was maintained by 60°C to 65°C. After shaking and heating several times the miscibility of the solution was observed and it was found that a ratio 1:1 of gel-PEG: DEE was found complete miscible.

**Measurement of pH**

The pH of the pure gelatin solution and various gel-PEG and gel-PEG-DEE solutions were determined by using a digital pH meter (PHILIPS, PW-9409, UK) with an efficiency level of ±0.2. This test was performed to observe the acceptability of wound healing gel with the pH value of body fluid.

**Measurement of viscosity**

The viscosities of the solution were measured using capillary Brookfield digital viscometer and VT550 viscometer. These measurements were carried out at room temperature of 29.5°C and 37.6°C (body temperature).

**Membrane formation time measurement**

Gel-PEG-DEE solution of different compositions were sprayed on body skin and time require to solidify the gel were measured using a digital stop watch (Casio, Sf19).

**FT-IR measurements**

The FTIR spectroscopy of gelatin, gel-PEG, gel-PEG-DEE solutions were performed by IR Prestiged-21 (200VCE) Fourier Transform Infrared Spectrophotometer, SHIMADZU, FTIR Spectrophotometer (Paragon 500 Model, PerkinElmer, Beaconsfield, Buckinghamshire, UK) in the range of 400-4000 $cm^{-1}$ with resolution of 1$cm^{-1}$.

**Microbial sensitivity and in vitro cytotoxicity study**

Antimicrobial activity of the composites against *Bacillus subtilis*, and *Escherichia coli* were investigated by the disc diffusion method. This method was performed in Muller Hinton medium. The media used for antimicrobial activity was poured into sterile petridish and was allowed to be cooled. Then the test culture (Bacillus subtilis and Escherichia coli) was incubated properly onto the media. The samples (A-H numbered by 1 to 6) were autoclaved for 2 h and 10 m to remove any bacterial contamination. It is important to mention that PVA composites melted little bit at autoclave temperature though the zone was still possible to identify. The plates were incubated overnight at 37°C and the inhibition zone was measured in the evaluation of antimicrobial activity of the biocomposites.

In vitro, cytotoxicity test was performed using brine shrimp lethality bioassay method 1. Brine shrimps (Artemia Salina) were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile artificial seawater and pH was adjusted at 8.5 using 0.1 N NaOH under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. The composites were dissolved in artificial seawater at 0.20 $mgmL^{-1}$ concentration and were taken in petridishes where the active nauplii were inoculated. After overnight incubation, the nauplii were counted. 0.5 $mgmL^{-1}$ of vincristine sulfate (an anticancer drug) was considered as positive control.

**Biocompatibility test**

Heparinized human blood was used to assay biocompatibility of developed gelatin based volatile healing gel. Samples were diluted with different ratios of blood to analyze the *in vitro* biocompatibility. Blood sample of the same donor was also diluted at the same ratios with distilled water and normal saline for control. Samples were spread on glass slides after two hour incubation at room temperature and observed under light microscope.

**In-vivo wound healing**

The wound healing characteristics of gel-PEG-DEE solution were evaluated using a rat model. Female rats

of weight approximately 213 g of each were anesthetized with 5 mL diethyl ether using an inhalation anesthesia. The surgical area was shaved with an electric razor, the rat was strapped to a surgical board, and additional anesthesia was provided via a nose cone. After a deep surgical plane of general anesthesia had been reached, a wound, approximately 1-$cm^2$ in area, was created on the left (lateral) side of the rat using curved blade surgical scissors. Both the epidermal and dermal layers were removed creating a full-thickness wound with minimal bleeding. The wound site was marked and measured using digital calipers and averaged to determine the original wound diameter and area. The wound was then dressed with eco-plast (control: product of 'Sahnisons Manufacturing Co. New Delhi, India) and sprayed with gel-PEG-DEE solution. The surgery was repeated multiple times to give a sample size of 8 rats per treatment per time point. After 3 and 6 days of post-surgery, the rats were anaesthetized and the healing was monitored using real time photograph.

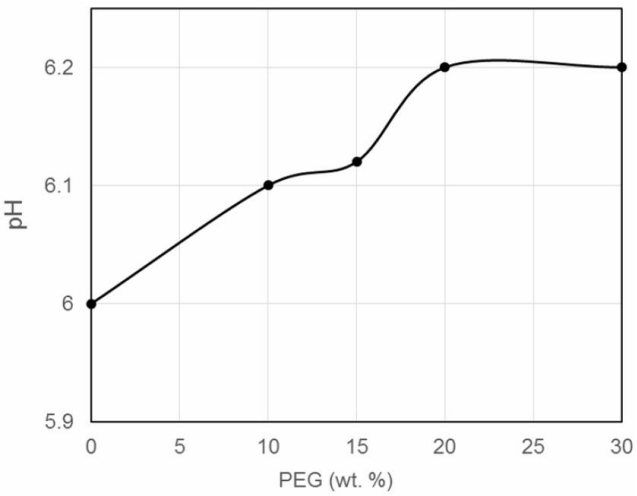


**Figure 1 : Variation of pH with increase in the amount of PEG**

## RESULTS AND DISCUSSION

### pH and viscosity

pH of the formulated gelatin solutions were measured with respect to PEG concentration and are shown in Figure 1. It was observed that the pH of gelatin solution increases slowly with the increasing PEG concentration. Because of slightly alkaline in nature (pH 6.4 in our measure), PEG lower the concentration of hydronium ion of the solution and thus the pH increase with the increase of concentration of PEG. It was also found that 20% of PEG containing solution has a pH of 6.2 which is a close proximate value with both the pH of skin (~5.5) and blood (7.4±0.04) and for the rest of the work of this study the mixture of 20 wt. % PEG and 80% gelatin (30% (w/v) will keep constant and will referred as standard gel-PEG. Addition of DEE slightly decreases the pH value and a ratio of 1:0.5 (gel-PEG: DEE) yielded pH of 6.0, however at a ratio of 1:0.75 and 1.1 respectively (gel-PEG: DEE) showed pH 5.9, and the solution became saturated with DEE. These results suggest that the ideal gelatin-PEG and DEE mixture for rest of experiments are (1:1) and were termed as gel-PEG-DEE.

The viscosity of gelatin, gel-PEG and gel-PEG-DEE were measured (the results of viscosity are given in Figure S2 and S3 and Table S1 the supporting informations). The viscosity was found slightly increases by increasing of PEG content in the gelatin solution. This indicates an increase in segment density within the molecules, which, in turn, results in a smaller hydrodynamic volume and a lower intrinsic viscosity. On the other hand, with the increase of DEE gradual increase of viscosity has been observed.

### FT-IR analysis

The width and intensity of the spectrum bands, as well as the position of the peaks, are all sensitive to environment changes and to the conformation of macromolecules on the molecular level. Intermolecular interaction occurs when different polymers are compatible, so that the FTIR spectrum of the film was different from those of pure polymers, which was advantageous to the study of the extent of polymer compatibility. The FTIR spectrum of pure gelatin solution, gelatin-PEG solution and gelatin-PEG-DEE gel is shown in Figure 2. The spectrum of gelatin showed an absorption 3400 $cm^{-1}$ due to N-H stretching of secondary amide, C=O stretching and at 1651 $cm^{-1}$, N-H bending between 1545 $cm^{-1}$ and 1500 $cm^{-1}$, N-H out of plane wagging at 654 $cm^{-1}$ and C-H stretching at 2960 $cm^{-1}$ and 3084 $cm^{-1}$. The FTIR spectrum of gelatin-PEG film showed a characteristic peak that range from 2163 $cm^{-1}$ to 2166 $cm^{-1}$ indicating the presence of a hydroxyl group with polymeric association and a secondary amide. The

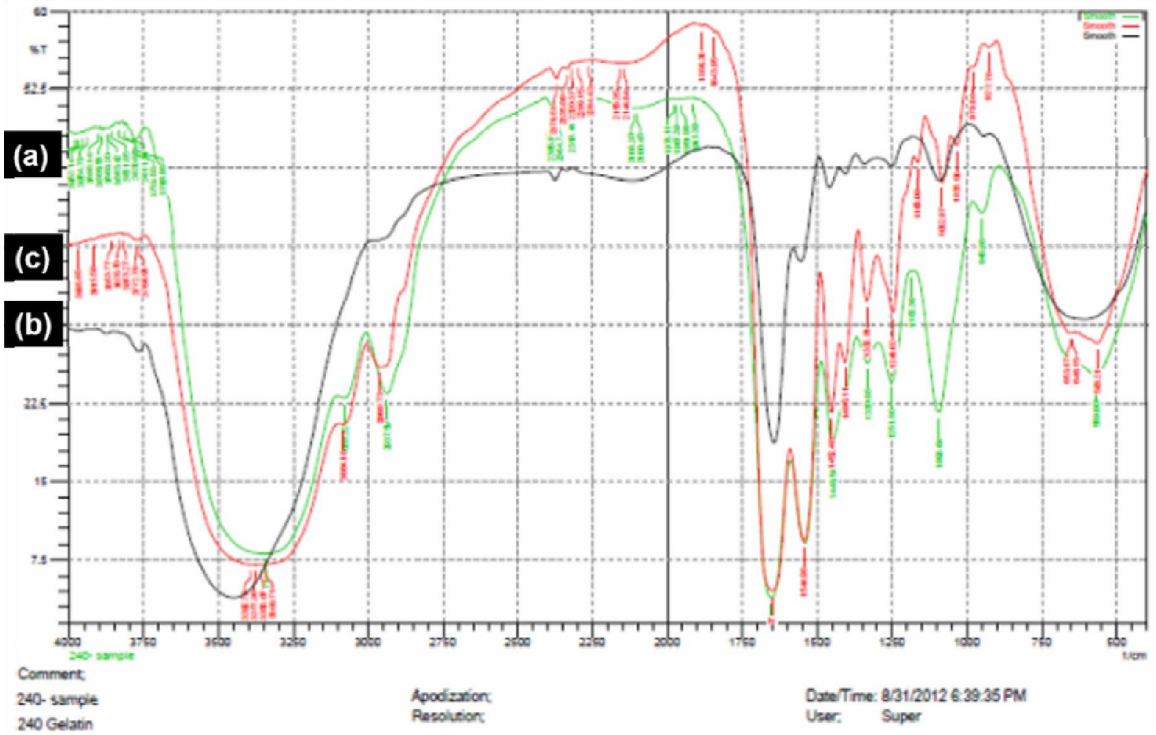


**Figure 2 : FT-IR spectra of (a) gelatin solution, (b) gel-PEG solution and (c) gel-PEG-DEE solution**

peaks at 1986.3 $cm^{-1}$and 2681.7 $cm^{-1}$ indicated the C-O stretching of secondary hydroxyl groups and ester. The spectra of the film also show a peak at 2735.7 $cm^{-1}$ indicating the presence of a secondary amide group.

## Membrane formation time measurement

Time required to form membrane of the highly volatile solution were plotted against DEE content in Figure S5 (in the supporting information). The results showed that, increasing DEE decreased the time requirement to a certain level and then it became steady. It was observed that gel-PEG: DEE=1:1 was solidified by 4 sec and at this ratio DEE becomes saturated with the gelatin-PEG solution. Additional amount of DEE did not affect the time requirement of membrane formation. Polyethylene glycol (PEG) also have similar effect on solidification time of gelatin solution.

## Antimicrobial properties

Standard methods agar was dissolved with distilled water at the amount of 17.5g agar/L liter of distilled water. After mixing completely by frequent stirring, the mixture was autoclaved for 15 minutes at 121°C. Small portion of pure 30% gelatin solution, gel-PEG solution and gel-PEG-DEE solution were added to three individual agar plates. Cultured pseudomonas bacteria was mixed with normal saline water then incubated in each of the three agar plates and the plates are given in Figure S4 in the supporting informations. The figure showed that the growth of bacteria in agar plates containing pure gelatin and gel-PEG were noteworthy which suggests that pure gelatin and gel-PEG solution have no antimicrobial effect. However no growth of bacteria in agar plate containing gel-PEG-DEE were found which reveals that gel-PEG-DEE showed antimicrobial sensitivity due to the presence of DEE.

## Biocompatibility test

Light microscopic analysis of prepared slides at 10×100 magnification revealed that the red blood cells (RBCs) of the blood sample were intact when incubated with 1:3 (w/v) sample/blood ratio. Normal saline also showed similar results but distilled water caused cell lysis at the same ratio (Figure 3) and 1:1 ratio. These results represent the biocompatibility of the prepared gelatin based wound healing gel which means compatible with Human blood.

## In-vivo wound healing

In rat model artificial wound was formed surgically and treated by developed wound healing materials.

Wound healing was assessed by monitoring wound contraction, re-epithelialization and wound morphology (real time photograph). Wounds supplemented with these new materials, had improved wound healing results compared to those wounds with the eco-plast alone. In addition, the underlying fibro-vascular tissue appeared more rapidly than for those wounds treated with eco-plast. Eco-plast, a thin adhesive membrane coated with a synthetic layer is permeable to both water vapor and oxygen but impermeable to microorganisms. It is generally used clinically in the treatment of minor burns, pressure areas, donor sites and a variety of minor injuries including abrasions and lacerations, and was selected for these experiments so that effects due to the bio-adhesive gelatin-PEG-DEE solution could be assessed in a controlled environment.

During wound healing no significant weight loss or fever was found. The edges of the wound pull inwards to reduce the overall wound area. Wound fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments and the establishment of cell–cell and cell–matrix linkages. The fibroblasts link to extracellular fibronectin and collagen and to each other through adherence junctions. Collagen bundles at the wound edge and the underlying dermis crosslink to form a collagen network. These cell–cell, cell–matrix, and matrix–matrix links provide a network through which the traction of the fibroblasts can be transmitted across the wound, leading to wound contraction. Re-epithelialization was the process by which new cutaneous tissue covers the wound defect. This process requires the uninjured keratinocytes along the wound edges to migrate laterally to cover the wound bed. Both wound contraction[16,17] and re-epithelialization[18] have been used as measures for monitoring wound closure and healing. Wound contraction was monitored by measuring the area within the wound's full-thickness margins, and re-epithelialization was determined by measuring the advancing epithelium. Combining gel-PEG films and gel-PEG-DEE membrane with Ciprofloxacin created a wound-healing environment that appeared to meet the criteria set forth above for ideal wound dressing. First, no signs of bacterial infection were apparent during gross examination of the wounds or histologically, suggesting that the materials effectively protected the wound from bacterial infection (Figure S6 and Figure S16). Second, the wounds exposed gel-PEG solution or gel-DEE-PEG gel was moist and hydrated, thus demonstrating that evaporative water loss and wound dehydration had been prevented. Third, abundant cellular proliferation suggested that oxygen and carbon dioxide permeability had been maintained. Fourth, the absorption of wound exudates was clearly visible in those wounds supplemented with a gel-PEG

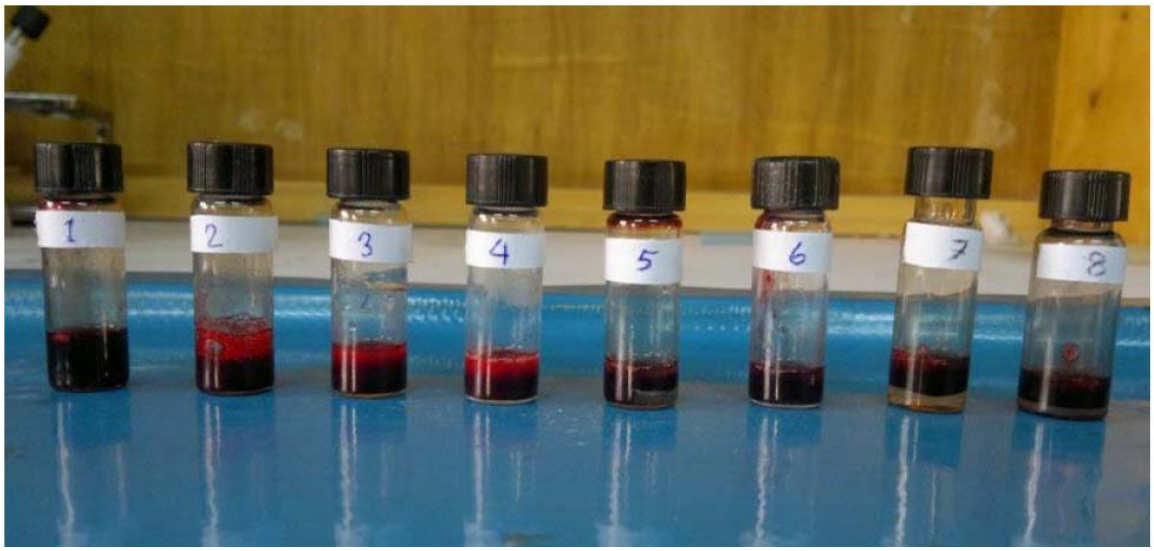


**Figure 3 : Blood sample with various concentration of pure gelatin, gel-PEG, gel-PEG-DEE, saline water and distilled water. Sample 1: blood and distilled water (1:1), Sample 2: blood and distilled water (1:3), Sample 3: blood and saline water (1:1), Sample 4: blood and saline water (1:3), Sample 5: blood and gel-PEG-DEE (1:1), Sample 6: Blood and gel-PEG-DEE (1:3), Sample 7: Blood and gel-PEG (1:1), Sample 8: Blood and pure gelatin (1:1)**

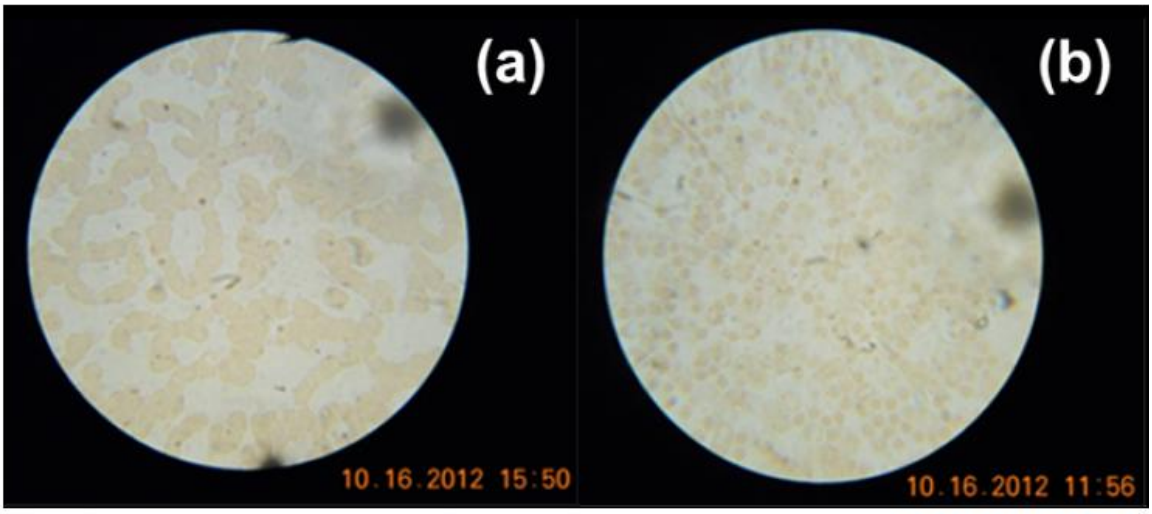


**Figure 4 : Microscopic view of lysed blood cell: (a) with distilled water after 2 h incubation (b) with gel-PEG-DEE**

film. Finally, the acceleration of re-epithelialization with the gelDEE-PEG membrane treatments indicated an enhancement of the overall healing process.

The mechanism by which the gelatin-DEE-PEG solution accelerates re-epithelialization is as yet unknown. However, reasonable explanations can be proposed. It might be that the gelatin solutions promote cell movement in early granulation tissue. Gelatin has been found to bind and act as a repository for a large family of cytokines[19]. It is likely that the gel-PEG gel and gelatin-DEE-PEG membrane are behaving as an artificial ECM, retaining cytokines and other growth factors made by the regenerating tissue. Wound exudate mixed with degrading film was clearly visible upon gross and histological examination. Therefore, it is believed that the gel-DEE-PEG solution provide a highly hydrated, peri-cellular environment that simulates the ECM. In this growth-conducive environment, assembly of other matrix components, presentation of growth and differentiation factor, and cell migration contribute to accelerated wound repair.

## CONCLUSION

In this highly volatile wound healing gel based on gelatin was prepared, characterized and evaluated for biomedical application. It was observed that 30% gelatin solution with 20% PEG at a ratio of (8:2) showed the maximum viscosity and was kept constant. Furthermore 1:1 ratio of gelatin-PEG and DEE formed saturated solution and was used for blood compitability and in vivo studies. It was observed that gel-PEG-DEE has extremely enhanced antimicrobial properties and didn't show any cytotoxicity in brine shrimp. The in vivo healing efficiency of the developed gel was compared with commercial eco-plast (wound healing materials based bandage) using rat model. Extremely quick healing was monitored using real time imaging technique and it was found that gel-PEG-DEE solution showed excellent rapid healing of the wound surface than eco-plast. There were no differences amongst experimental groups for wound contraction or inflammatory response on any day post-surgery. In conclusion the detailed experimental results revealed a successful development and application of a new bio-adhesive wound healing volatile material for wound healing application.

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