



## Research Article

# Comparative Analysis of Collagen and Chitosan-based Dressing for Haemostatic and Wound Healing Application

Divya Tripathi,<sup>1,2</sup> Kartikey Rastogi,<sup>3</sup> Priyanka Tyagi,<sup>4</sup> Harish Rawat,<sup>1</sup> Gaurav Mittal,<sup>1</sup> Abhinav Jamini,<sup>1</sup> Harpal Singh,<sup>2</sup> and Amit Tyagi<sup>1,5,6</sup>

Received 21 October 2020; accepted 24 January 2021; published online 16 February 2021

**Abstract.** Collagen and chitosan have haemostatic, tissue fix and wound healing properties but the poor mechanical property limits their application. Therefore, various concentrations of collagen (1–6%) and chitosan (1–2%) were used to develop biopolymer-coated gauzes, with and without glycerol as plasticiser. Glycerol-treated gauzes showed desired mechanical and adhesive property in comparison to polymer-coated gauzes alone. Developed gauzes were characterized using differential scanning calorimetry, thermal gravimetric analysis and Fourier transform infrared spectrophotometry to confirm the biopolymer coating and stability. Scanning electron microscopy showed multilayer coating of the biopolymer and faster clotting in chitosan gauzes in comparison to collagen. Surface plasmon resonance assay confirmed that chitosan exhibited more binding affinity of 65 RU in comparison to collagen, which showed 55 RU with erythrocytes. Decrease in the value of plateletcrit and mean platelet volume confirmed platelet adhesion and aggregation over the surface of polymer-coated dressings. Gamma scintigraphy studies showed  $85 \pm 2\%$  formulation retention up to 12 h at the wound site in comparison to  $40 \pm 3\%$  retention of the radiopharmaceutical alone. Collagen and chitosan-coated gauze showed  $226 \pm 15$  s and  $179 \pm 12$  s haemostasis time, respectively, which was significantly less from  $506 \pm 15$  s in standard gauze. Chitosan gauze showed faster wound healing in comparison to the collagen-coated gauze. Chitosan and collagen-coated gauzes showed  $55 \pm 4\%$  wound contraction on day seven in comparison to  $25 \pm 2\%$  in the control group, while chitosan gauzes showed complete wound contraction on day fourteenth, while the collagen-coated gauze showed  $90 \pm 3\%$  on the same day.

**KEY WORDS:** biopolymer; collagen; chitosan; gamma scintigraphy; haemostasis.

## INTRODUCTION

Massive bleeding is one of the driving reasons of deaths after battle and civil injury. Haemostasis is a key challenge to tackle such emergency scenario. Timely haemostasis after injury and during surgical interventions is a major challenge

in present day situation. Around 40–50% traumatic and up to 90% of battle casualty have been observed in the pre-hospital setting (1). Haemorrhage and wound healing in trauma patients are the leading cause of casualty if not operated timely and it may require reoperation or additional procedures of tissue recovery (2–4). Topical haemostatic and wound healing formulations have been used since the ancient times which include herbs, various types of beers, wax blends, seed oils, grains and animal shrouds blended with hot sand to quit bleeding. Recent development in biotechnology and biomaterials has achieved vast development of topical haemostatic and wound healing agents (5–7). There are several commercial formulations available in the market having haemostatic and wound healing applications, including biotherapeutics as well as biopolymers alone or in combination like fibrinogen, thrombin, co-factors, chitosan, cellulose, alginates *etc.* (8, 9). An ample availability of material and characteristics such as biocompatibility, stability and cost effectiveness has encouraged the researchers to search for the development of new or refabricated materials, which

<sup>1</sup> Division of Nuclear Medicines, Institute of Nuclear Medicine and Allied Sciences, DRDO, Delhi, 110054, India.

<sup>2</sup> Center for Biomedical Engineering, Indian Institute of Technology, New Delhi, 110016, India.

<sup>3</sup> Department of Pharmacology, KIET Group of Institutions, Ghaziabad, U.P. 201206, India.

<sup>4</sup> Department of Basic and Applied Sciences, School of Engineering and Sciences, G.D. Goenka University, Gurugram, Haryana 122103, India.

<sup>5</sup> Department of Combat Sciences, Institute of Nuclear Medicine and Allied Sciences, DRDO, Delhi, 110054, India.

<sup>6</sup> To whom correspondence should be addressed. (e-mail: amityagi@gmail.com)

could stop bleeding without any adverse effects. Biomaterials, which include synthetic as well as natural biopolymers such as cellulose, collagen, lactic acid and chitosan, are suitable material for desired applications. Chitosan and collagen are well-known biopolymers used for the haemostatic and wound healing applications are available abundantly. Collagen is a biopolymer that made up 25% to 35% of the content of human beings and is liable for holding the body all together as a single functioning system. Collagen plays a critical role in providing mechanical strength to the tissues in addition to restoring the tissue integrity during wound healing, through co-operations with other framework proteins (10–13). Collagen assimilates great amount of exudate, to cling to the wet injury, to keep up a damp recuperating condition and to support the recovery of granular and epithelial tissues at the wound site. Collagen has significant potential for tissue rebuilding, resistance against bacteria and anti-inflammatory action (14–16). Collagen as a burn dressing agent, allows fast healthy granulation of the tissue over the burn surface and helps in a rapid healing, in addition to maintaining the sterility of the wound site. The large surface area of collagen fibres attracts the fibrogenic cells, which help in healing and interaction with blood platelets to form tough haemostatic plug. After cellulose, chitin is the second most bounteous biopolymer and is generally found in spineless creatures such as shellfish shells or insect cuticles, certain mushroom envelopes, green growth cell dividers and yeasts (17). Molecular weight of chitosan commonly varies from 300 to 1000 KDa, contingent upon the source and arrangement of units. Chitin and chitosan are biocompatible polymers yet there are a few confirmations that chitosan is more cytocompatible *in vitro* than chitin. At the point when the quantity of positive charges expands, the connection amongst cells and chitosan increments also, which improves biocompatibility (18, 19).

Chitosan has been widely used in biomedical and pharmaceutical applications on account of its low toxicity and biocompatibility (9). It can enhance the reepithelialisation and typical skin recovery, and provide extensive antibacterial environment. Chitosan provides an excellent haemostatic property due to the positive charges over its surface and is therefore used extensively in civil and battle field applications (20, 21). But still there is no evidence to prove the effect of concentrations of collagen and chitosan on haemostatic and wound healing properties. Chitosan possesses diverse diversity in atomic weight, deacetylation value, and can be synthetically adjusted. Similarly, collagen is also found to have variable but definite concentrations and it is conceivable to prepare distinct physical structures like gels, froths, films, wipes, fibres *etc.* (22). Collagen, chitosan and their derivatives are generally utilized for biomedical applications because of their antioxidative, mitigating, antimicrobial and tissue fixing properties (23–25). Chitosan derivatives are utilized as haemostatic agents (26–29). Poor mechanical properties of both the biopolymers limit their applications in severe blood haemorrhage and pre-hospital applications. The effect of collagen and chitosan concentration on natural reaction of platelets and other wound healing applications is still unclear. The present study is emphasized towards comparative analysis of collagen and chitosan-based dressings for haemostatic and wound healing efficacy. The efficacy of the developed biopolymer-coated gauze was evaluated using gamma scintigraphy on *Sprague–Dawley* rats and *in vitro* analysis was performed using

Surface Plasmon Resonance (SPR) for determining the blood interaction affinity.

## MATERIALS AND METHODS

Chitosan (source shrimp shells) of low viscosity was obtained from Sigma-Aldrich, USA. Marine collagen peptide was obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Glacial acetic acid was obtained from Merck India Ltd., Mumbai. All experiments were carried out using deionized water of resistivity at  $18.2 \text{ M}\Omega \text{ cm}^{-1}$ . Technetium 99 m pertechnetate ( $^{99\text{m}}\text{TcO}_4^-$ ) was procured from BRIT (Board of Radioisotope and Technology), INMAS-DRDO, New Delhi, India. N-hydroxysuccinimide NHS, N-ethyl-N-dimethylaminopropyl carbodiimide (EDC) was obtained from GE Healthcare, Uppsala, Sweden. All chemicals used in the experiments were of analytical grade and utilized as received, without any further purification.

### Preparation of Haemostatic Dressings Using Collagen and Chitosan

For impregnation of dressings, 1 and 2% (w/w) chitosan solution was dissolved in 0.75% glacial acetic acid by constant stirring at  $25 \pm 2^\circ\text{C}$  overnight at 200 rpm using a magnetic stirrer and stored for further studies. Similarly, marine collagen peptide at various concentrations (1, 2, 4 and 6% w/w) was prepared using deionized water at  $25 \pm 2^\circ\text{C}$  overnight at 200 rpm using a magnetic stirrer. Spray pyrolysis machine (Holmarc Mechatronics Model No: HO-TH-04C3) was used for coating biopolymers at a stream pace of  $1 \text{ mL min}^{-1}$  and pneumatic force of  $40 \text{ lb./in}^2$  for 3 min. Chitosan and collagen peptide-based biopolymeric hydrogels thus obtained were used separately for single and multiple coating on the cellulose-based gauze (with and without glycerol) as given in Table I.

### Gauze–Chitosan (G-Ch)

Cellulose-based standard gauze, with predefined porosity and vein size, was single, double, triple and quadruple coated using 1% chitosan-based hydrogel by spray pyrolysis method. Chitosan-coated gauze thus obtained, was dried using thermostat at  $36^\circ\text{C}$  for 24 h. Six sets of the chitosan-coated gauze were further coated with 1% (w/w) of glycerol and dried again at  $36^\circ\text{C}$  for 24 h as given in Table I.

### Gauze Collagen Peptide (G-Cp)

Similarly, cellulose-based gauze was double-coated using various concentrations (1, 2, 4 and 6% w/w) of marine collagen peptide-based biopolymeric hydrogel and dried at  $36^\circ\text{C}$  using thermostat for 24 h. Six sets of collagen-coated gauze, thus obtained, were further coated with 1% (w/w) glycerol and dried.

**Table I.** Chitosan and Collagen Peptide-coated Gauzes

| Sr. no.                                      | Types of gauzes            |                                 |                                |
|--|----------------------------|---------------------------------|--------------------------------|
| 1. Chitosan-treated gauze                    |                            |                                 |                                |
| Gauze Name                                   | No. of 1% glycerol coating | No. of chitosan coating         | Chitosan concentration         |
| G-Ch 1% SC                                   | None                       | Single                          | 1%                             |
| G-Ch 1% DC                                   | None                       | Double                          | 1%                             |
| G-Ch 1% TC                                   | None                       | Triple                          | 1%                             |
| G-Ch 1% QC                                   | None                       | Quadruple                       | 1%                             |
| 2. Chitosan + glycerol-treated gauze         |                            |                                 |                                |
| Gauze name                                   | No. of 1% glycerol coating | No. of chitosan coating         | Chitosan concentration         |
| G-Ch 1% SC                                   | Single                     | Single                          | 1%                             |
| G-Ch 1% DC                                   | Single                     | Double                          | 1%                             |
| G-Ch 1% TC                                   | Single                     | Triple                          | 1%                             |
| G-Ch 1% QC                                   | Single                     | Quadruple                       | 1%                             |
| 3. Collagen peptide-treated gauze            |                            |                                 |                                |
| Gauze name                                   | No. of 1% glycerol coating | No. of collagen peptide coating | Collagen peptide concentration |
| G-Cp 1%                                      | None                       | Double                          | 1%                             |
| G-Cp 2%                                      | None                       | Double                          | 2%                             |
| G-Cp 4%                                      | None                       | Double                          | 4%                             |
| G-Cp 6%                                      | None                       | Double                          | 6%                             |
| 4. Collagen peptide + glycerol-treated gauze |                            |                                 |                                |
| Gauze name                                   | No. of 1% glycerol coating | No. of collagen peptide coating | Collagen peptide concentration |
| G-Cp-Gly 1%                                  | Single                     | Double                          | 1%                             |
| G-Cp-Gly 2%                                  | Single                     | Double                          | 2%                             |
| G-Cp-Gly 4%                                  | Single                     | Double                          | 4%                             |
| G-Cp-Gly 6%                                  | Single                     | Double                          | 6%                             |

## CHARACTERIZATION

### Differential Scanning Calorimetry (DSC)

DSC of collagen and chitosan biopolymer was carried out using a differential scanning calorimeter DSC 6000, Perkin Elmer, Massachusetts, USA. Both samples were precisely weighed ( $5 \pm 0.25$  mg) and protected in aluminium compartments. The samples were heated from  $-50$  to  $300^\circ\text{C}$  at the rate of  $20^\circ\text{C min}^{-1}$  under constant dynamic nitrogen atmosphere and the thermogram of each sample was recorded.

### Thermo Gravimetric Analysis (TGA)

TGA of collagen and chitosan polymer-coated gauze were carried out using a thermogravimetric analyzer TGA 4000, PerkinElmer, Massachusetts, USA. All samples were kept at  $50^\circ\text{C}$  to  $800^\circ\text{C}$  in an inert nitrogen atmosphere with a flow rate of  $60 \text{ mL min}^{-1}$  at heating rate of  $20^\circ\text{C min}^{-1}$ . Thermogram of each coated gauze was recorded to observe the amount and recurrence of weight variation of the samples.

### Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflectance- FTIR of chitosan powder, collagen peptide powder, collagen and chitosan polymer-coated gauze and standard gauze were recorded on a Spectrum Two spectrometer, Perkin-Elmer, Massachusetts, USA. All samples were analysed in the range of  $450$  to  $4000 \text{ cm}^{-1}$  in the transmittance mode.

### Scanning Electron Microscopy (SEM)

The surface morphology of chitosan and collagen polymer-coated gauze (with and without blood haemostasis) was studied using MIRA3 TESCAN, Brno, and Cze hia. Sample was kept on a double-sided carbon adhesive tape and sputter-coated with gold particles under reduced pressure conditions and observed under SEM at  $4.0 \text{ KV}$  accelerating voltage.

### IN VITRO STUDIES

*In vitro* studies were carried out to evaluate the efficacy of biopolymer-coated gauze using various test parameters.

### Surface Plasmon Resonance Assay

The surface plasmon resonance assay was carried out using Biacore T-200 from GE Healthcare, Uppsala, Sweden to investigate the kinetics and association/dissociation interactions between human erythrocytes with collagen peptide and chitosan. The CM-5 chip was used for the analysis, which is pre-dextran-coated with gold base. Briefly, baseline stabilization was carried out by interactive association for  $500 \text{ s}$ , dissociation for  $400 \text{ s}$  and surface regeneration for  $12 \text{ s}$ . The carboxyl groups present on the surface of dextran-coated CM5 sensor chip were used for amine coupling using  $10 \text{ mL}$  of a blend of equivalent volumes of N-hydroxysuccinimide (NHS,  $115 \text{ mg mL}^{-1}$ ) and N-ethyl-N-dimethylaminopropyl carbodiimide (EDC,  $750 \text{ mg mL}^{-1}$ ), trailed by the infusion of chitosan at a stream pace of  $1 \text{ mL min}^{-1}$ . A total of  $15 \text{ mL}$  aliquot was immobilized on the surface of the flow cell

number four. The unreacted dextran surface was treated with 10 mL of ethanol amine at a flow rate of 1 mL/min to block the surface of the CM-5 chip. Phosphate-buffered saline (PBS pH 7.4) was used as the running buffer. Collagen was immobilized on the surface of other CM5 sensor chip at flow cell number 2 and unreacted dextran was treated in the similar manner as chitosan. Human erythrocytes were suspended in PBS (pH 7.4) for 1 min and various haematocrits of (0.05%, 0.10%, 0.15%, 0.20%) were prepared and injected at a flow rate of 3  $\mu\text{L min}^{-1}$  for 4 min in flow cell immobilized with chitosan/collagen, separately. The contact time was set at 120 s while dissociation was observed for 180 s. The surface was recovered by infusing 10 mM Glycine-HCl (pH 2.5) with a stream pace of 30  $\mu\text{L min}^{-1}$  for 30 s (30).

### Haemostatic Dressing pH Determination

It is well-known that pH is a critical parameter that plays a vital role in the study of bimolecular interactions and can also influence the efficacy. All dressings were suspended into de-ionized water (1:100 *w/v*) and incubated at room temperature for 24 h. pH was determined at 3 h and 24 h using a pH meter, Hanna Instruments, India (31).

### BLOOD INTERACTION ANALYSIS

The standard operating procedure was endorsed by the Institutional Human Ethical Committee of INMAS-DRDO, Delhi, India. An appropriate informed consent was obtained from each healthy human volunteer. A total of 35 mL of blood was drawn from two healthy volunteers (age ~24 years) by the registered medical practitioner. Briefly, 2.5 mL of blood was poured in Ethylenediaminetetraacetic acid Becton Dickinson Vacutainers® for complete blood count (CBC) trial of the control blood. The gauze strips of G-Cp, G-Ch, G-Cp-Gly, G-Ch-Gly weighing  $100 \pm 5.0$  mg each, were placed in Becton Dickinson Vacutainers® separately and incubated in thermostat at 36°C for 10 min. Each gauze sample was removed and treated blood was used separately for haematology analysis.

### Human Haematological Profile

Complete blood count was done using the haematology analyser NIHON KOHDEN MEK-6450 K. Various parameters like haemoglobin level (HGB, g/dL), red blood cells (RBC, M/mL), platelets (PTL, K/mL), white blood cells (WBC K/mL), haematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dL), red cell distribution width (RDW, %), plateletcrit (PCT, %), mean platelet volume (MPV, fL) and platelet distribution width (PDW, fL) were observed.

### Erythrocyte Agglutination

A total of 20 mL blood was centrifuged at 1000–2000  $\times g$  using the Heraeus Biofugestratos centrifuge (Thermo Scientific) for 10 min at 4°C. A total of 5 mL of erythrocytes was diluted in 95 mL of saline to acquire 5% erythrocyte mass.

Collagen solution of 125  $\mu\text{L}$  having concentrations of 1%, 2%, 4% and 6% was moved to 'U' bottom of a 96-well micro plate separately. Similarly, 125  $\mu\text{L}$  of chitosan solution with concentrations of 1% and 2% was moved to a 'U' bottom 96-well microplate separately and 25  $\mu\text{L}$  of 5% erythrocyte mass was added to each well, respectively. The 96-well plate was incubated at 37°C for 40 min. Hemagglutination analysis was done as per the standard Stavitsky scale (32).

- +++++ Compact granular agglutinate;
- +++smooth mat on base of well with collapsed edges;
- ++Smooth mat on base of well. Edges fairly worn out;
- +narrow ring of red around edge of smooth tangle;
- – Discrete red button in focus of base well.

### Blood Sorption

All samples removed from Becton Dickinson Vacutainers® with human blood were weighed to assess the sorption of blood. It was determined utilizing the following Eq. (1):

$$S = W_1/W_2 * 100 \quad (1)$$

where,  $S$  is the sorption of blood (% age),  $W_1$  is the test weight of blood sorbed (mg);  $W_2$  is the test weight of sample (mg).

### Ethics Statement

All animal methods and care conventions were followed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals regulation and the exploratory protocol was affirmed by the Institutional Animal Ethics Committee of INMAS.

### IN VIVO STUDIES

#### Animals and Experiment Design

Thirty male *Sprague-Dawley* rats weighing 250–300 g were obtained from the Experimental Animal Facility of Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, Delhi. All animals were housed in the standard environmental conditions of temperature 21–23°C, moisture 45–50% with 12-h light and 12-h dark cycle in polypropylene confines. The animals were provided with standard feed and water *ad libitum*.

#### Formulation Retention Studies Using Gamma Scintigraphy

$^{99m}\text{Tc}$  SPECT Gamma scintigraphy is an approved nuclear medicine diagnostic tool since the 1950s to monitor the real-time distribution of radiolabelled formulation. Various gauzes were radiolabelled with  $^{99m}\text{Tc}$  using in-house developed method. Briefly, 50  $\mu\text{g}$  stannous chloride was

added to the vial and mixed well. pH was adjusted to 6.5 using 0.1 N HCl and the contents were passed through a 0.2- $\mu$ m filter (Millipore Corporation, Bedford, MA, USA). Freshly eluted sodium pertechnetate (2 mCi) was added to the above solutions, followed by incubation at room temperature for 20–30 min, to achieve maximum radiolabelling efficiency. The stability of the labelled product was evaluated up to 24 h by ascending chromatography method using 100% acetone as the solvent and examined by a gamma counter (CH CAPRAC-t, USA). *In vivo* animal imaging studies were also carried out using Siemens Symbia-T, USA. Six animals were caged in each group for the determination of formulation retention capacity of gauzes. SGD, G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly were dip-coated in the pre-defined amount of radiolabelled solution of chitosan and collagen separately. All the labelled batch of products were dried at 36°C for 30 min and placed on freshly prepared wound in the animal model. The images were captured at different time intervals of 0 min, 30 min, 1 h and 12 h to assess the retention of biopolymer-coated bandages over the wounded area.

### Screening of Haemostasis

Thirty *Sprague–Dawley* rats weighing 250–300 g, were separated into five groups of six animals each, to assess the haemostatic potential of G-Cp, G-Cp-Gly, G-Ch and G-Ch-Gly dressings and compared with SGD as control. All animals were administered with ketamine hydrochloride dose of 20 mg/kg intra-peritoneally. The dorsal surface of the thigh region was shaved and superficial femoral artery was exposed to half using the surgical blade for evaluation of haemostatic property of the developed formulations. The second, third, fourth and fifth group animals were treated with chitosan and collagen polymer-based formulations. In all groups, Standard Manual Pressure (SMP) was maintained for 60 s and if the bleeding continued, SMP was applied for the next 60 s and repeated up to 10 min. After the haemostasis was attained, SGD, G-Cp, G-Cp-Gly, G-Ch and G-Ch-Gly were weighed and the amount of blood absorbed was resolved calculated using the following Eq. (2):

$$S = W_1 - W_2 \quad (2)$$

where,  $S$  is the sorption of blood (mg),  $W_1$  is the test weight after trial (mg),  $W_2$  is the test weight before trial (mg) (33).

### Wound Healing Studies

Thirty-six animals were randomly chosen and divided into six groups with six animals in each group. All animals were anaesthetised by intramuscular infusion of ketamine and xylazine 20 mg/kg. The dorsal surface was shaved and the underlying skin was cleaned with 70% ethanol. Full thickness from the outlined zone was excised off by utilizing 8 mm punch biopsy to create wound (34). The developed dressings were applied on wound and changed alternately up to 14 days. Wound contraction was estimated consistently utilizing a digital Vernier calliper and change in wound diameter was determined. Animals of Group I were taken as control and

left untreated. Group II were taken as the sham-operated group and treated with SGD. Group III animals were treated with G-Cp, while in Group IV, the animals were treated with G-Cp-Gly. In Group V, animals were subjected to G-Ch, while in Group VI, animals were dressed utilizing G-Ch-Gly dressings, to decide the rate of wound contraction until complete. Area of the wound at day 0 was considered 99%, to figure the percentage reduction in the wound area. The percentage of wound contraction was determined utilizing the following equation.

$$\% \text{Wound contraction} = \frac{\text{healed area}}{\text{total area}} \times 100 \quad (3)$$

## TOXICITY

### Animal Histopathological and Haematological Analysis

The granulation tissue was excised from the wound site on day 7 and day 14 by punch biopsy technique and immersed in 10% buffered formalin solution for histopathology investigation. Skin tissue of each group was procured by the standard histopathological method to observe the minuscule changes in the morphology. The histopathological assessment of skin tissues of animals, applied with G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly, was observed under fluorescence magnifying lens Olympus BX 60.

Haematological evaluation of all animals was done on day 14. The blood samples were collected from retro-orbital plexus and blood was collected in EDTA (ethylenediaminetetraacetic acid) Becton Dickinson Vacutainers® (35). The collected blood samples were analysed by haematological assays like haemoglobin, red blood cell, white blood cell, packed cell volume and differential counts.

### Skin Irritation Test

The wound site was analysed after 24 h for symptoms of erythema and oedema as per the OECD test guideline 404. The dressing materials were evaluated for potential skin irritation, if any. Animals with healthy, flawless skin were kept up on standard animal feed and free access to fresh water under standard environmental conditions. Body hairs were depilated from the dorsal body surface of animals and cleaned with 70% ethanol. Introduction of the skin to G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly dressings was accomplished by means of patch test using micropore non-irritating surgical tape on the back of each of the six *Sprague–Dawley* rats, covering a zone of 3 cm<sup>2</sup> (36).

### Statistical Analysis

Statistical information was communicated as mean  $\pm$  standard deviation. An unpaired t-test was designed to evaluate the measurable importance of the distinction between the outcomes obtained. Statistical significance was expected at certainty level of 95% ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

### Haemostatic Dressing Fabrication

Chitosan and collagen peptide biopolymers were used for gauze coating considering their characteristic properties such as haemostasis, antimicrobial activity, high absorbency, moisture retention, inertness and non-toxic chemical nature (37). Briefly, 1% w/w chitosan gel was prepared, which was light pale in colour, while the 2% gel was yellowish. Chitosan hydrogel with 1% w/w concentration was found better as the coating agent over the gauze. Chitosan hydrogel of 2% was highly viscous and was found to cause blockage in the syringe coating and therefore, could not be used for coating. In view of this, only 1% w/w chitosan gel was utilized for further studies. Similarly, spray coating of collagen peptide gel with concentration of 1%, 2%, 4% and 6% w/w was also performed to evaluate the efficacy of the gauze. It was observed that, gauze coated with chitosan and collagen had brittle texture. The 1% w/w glycerol was coated on the gauze as a plasticiser to overcome the brittle texture of the gauzes. Physical properties of the polymer coated gauzes were also evaluated as given in Table II. It was observed that polymer-coated gauzes were rough, while the glycerol-treated gauzes were soft with even texture.

### Differential Scanning Calorimetric Analysis

In case of collagen, the endothermic peak was observed at 51.13°C, due to the loss of water associated with the hydrophilic groups of collagen peptide as given in Fig. 1. The occurrence of another peak at around 210.47°C was due to conversion of triple helix of collagen into random fibres. In chitosan, the endothermic peak was observed at 54.88°C. In the solid state, chitosan and collagen have disordered structure and strong affinity for water and as a result they can be easily hydrated. The peak suggested that chitosan and collagen were not completely dried and still there was some bound water, which was not removed during drying. The second primary thermal event for chitosan was depicted as a wide exothermic peak in the range of 250–350°C when heated between 50 and 400°C. The peak may be attributed to the thermal degradation of the biopolymers. The study was carried out to attain the thermal and molecular stability of the biopolymer undergoing physiochemical changes.

### Thermogravimetric Studies

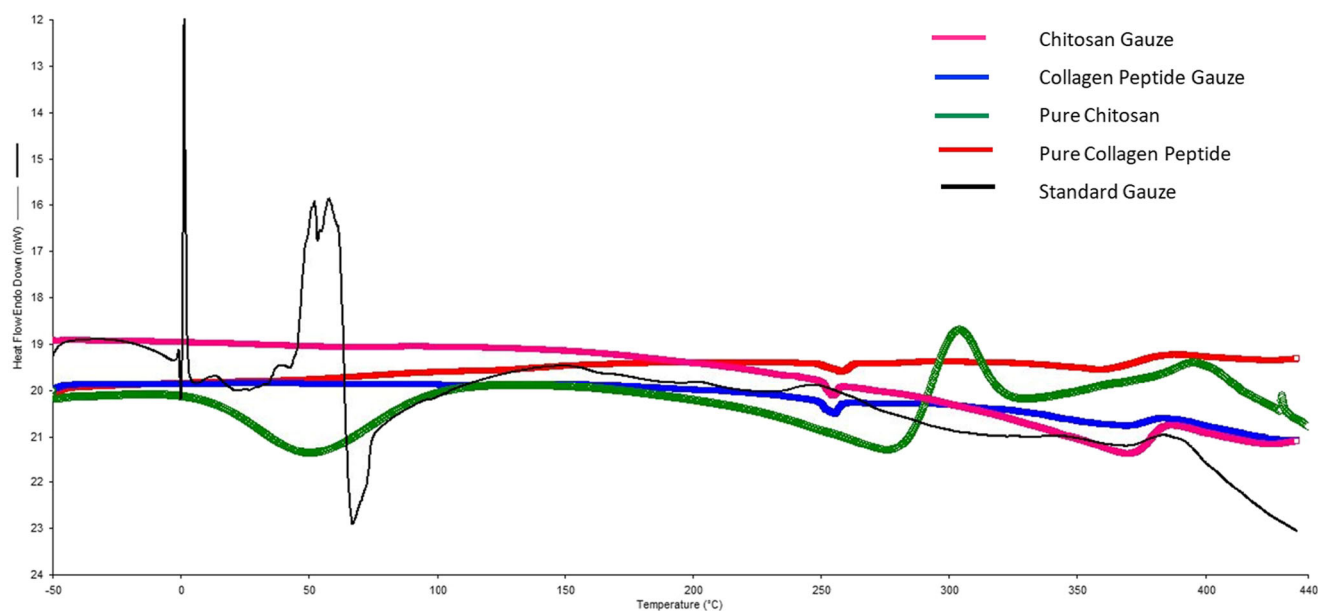
TGA of collagen and chitosan-treated gauzes was carried out to observe changes in the weight of samples over a particular range of 50–800°C as shown in Fig. 2. The thermal degradation and oxidative stability of coated materials was affirmed by thermogram under continuous stream of dry nitrogen gas. TGA of collagen peptide and chitosan showed loss of bound water and conformational changes occurring within the fibrils due to breaking of internal cross links in the temperature extension range from 80 to 150°C. The weight reduction of 6.73% and 9.72% was observed from the collagen and chitosan, respectively. Second decomposition was found at 250–450°C for collagen and 280–400°C for chitosan with corresponding weight reduction of 30.37% and 32.11%, respectively. This thermal disintegration step suggests the deacetylation of chitosan, vaporization and elimination of volatile products. In addition, chitosan and collagen-based materials were found to decompose at temperature range of 250–480°C. Weight loss at this stage may be due to thermal disintegration of the biopolymer with the amino groups to form unsaturated structures. During the pyrolysis of polysaccharides, a random split of the glycosidic bonds occurred, which are followed by decomposition to form acetic acid, butyric acid and a series of lower fatty acids. When the temperature reached 500°C, the residual weight of SGD, G-Cp, G-Cp-Gly, G-Ch and G-Ch-Gly were 7.09, 6.14, 4.70, 6.37 and 0, respectively. As reported previously, these are the weight estimations of carbon and debris after complete deterioration. TGA analysis confirmed the oxidative stability, shelf life and kinetics of the biopolymer after the coating cycles.

### Fourier Transform Infrared Spectroscopy

All the chitosan and collagen-coated gauzes were analysed using ATR-FTIR to observe the chemical stability of the material as shown in Fig. 3. Integration of chitosan and collagen peptide with the coated gauzes was confirmed in the respective samples. G-Ch gauze spectra showed the band at  $\sim 3260\text{ cm}^{-1}$  for O–H stretching and further increase in intensity was observed in case of G-Ch-Gly-coated gauze because of the presence of additional hydroxyl groups of the glycerol, while lowest intensity band was observed in case of standard gauze, as expected. C=O peak was observed at  $\sim 1700\text{ cm}^{-1}$  in G-Ch and uncoated gauze samples, while the peak shifted to  $\sim 1550\text{ cm}^{-1}$  in the form of band, probably due

**Table II.** Physical Characteristics of Chitosan and Collagen Peptide Gauze

| Sr. no. | Gauze type      | Physical properties |         |           |           |
|---------|-----------------|---------------------|---------|-----------|-----------|
|         |                 | Colour              | Texture | Rigidity  | Odour     |
| 1       | Chitosan gauzes |                     |         |           |           |
|         | G-Ch            | Pale yellow         | Rough   | Very hard | Pungent   |
| 2       | G-Ch-Gly        | Pale white          | Fine    | Soft      | Off smell |
|         | Collagen gauzes |                     |         |           |           |
|         | G-Cp            | Off white           | Uneven  | Hard      | Fishy     |
|         | G-Cp-Gly        | White               | Even    | Soft      | Off smell |



**Fig. 1.** Differential scanning calorimetric (DSC) thermograms of **a** standard gauze, **b** collagen powder, **c** collagen gauze, **d** chitosan powder and **e** chitosan gauze

to merging with strong C–H stretching at  $\sim 1400\text{ cm}^{-1}$ . Similarly, collagen-coated gauze spectra of G-Cp, G-Cp-Gly, Cp and standard gauze showed the band at  $\sim 3300\text{ cm}^{-1}$  for O–H stretching. C=O peak was observed at  $\sim 1700\text{ cm}^{-1}$  in G-Cp and uncoated gauze samples while the peak shifted to  $\sim 1550\text{ cm}^{-1}$  in the form of band probably due to merging with strong C–H stretching at  $\sim 1400\text{ cm}^{-1}$ . FTIR spectra confirmed the coating of biopolymers over the gauze and their stability after coating.

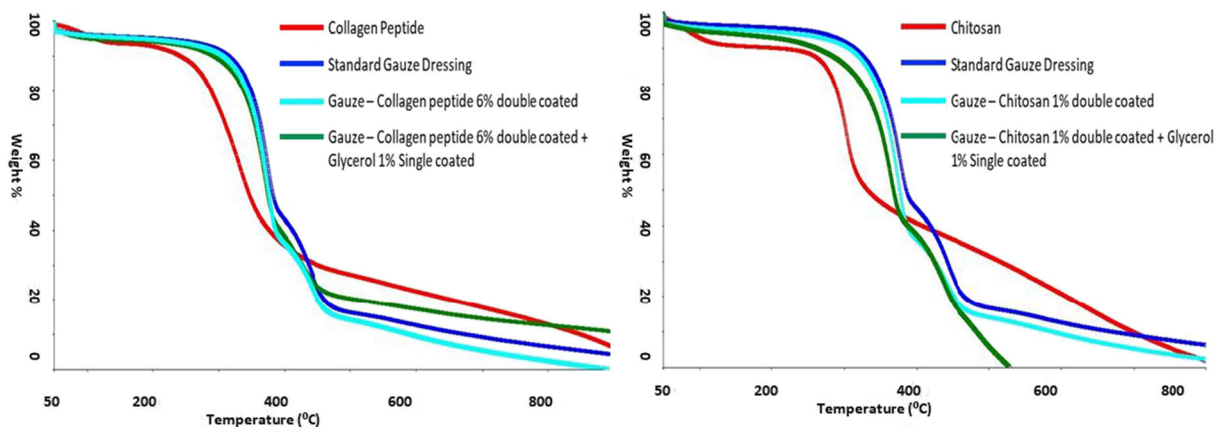
### Scanning Electron Microscopy

Surface morphology of the biopolymer treated and untreated gauzes was observed using a field emission scanning electron microscope as shown in Fig. 4. Standard gauze dressing (SGD), collagen and chitosan-coated gauzes were evaluated at 4.0 KV using appropriate magnification. Standard gauze dressing showed the linen thickness of  $\sim 10\text{ }\mu\text{m}$  and did not show any coating over the surface. While, in case of collagen-coated gauze, thick coating in the

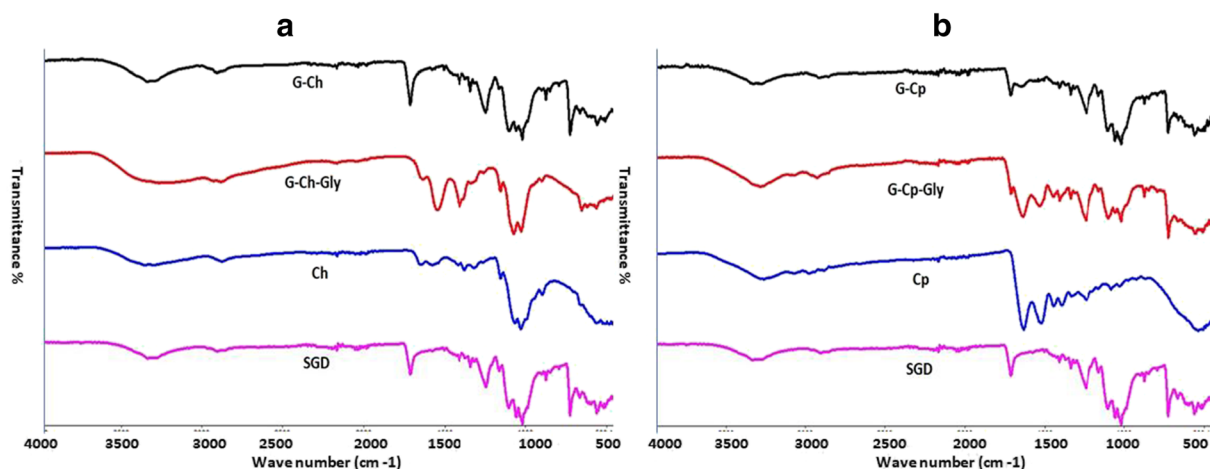
form of multiple layers was observed. In case of chitosan-coated gauze, thick coating with heterogeneous surface was observed, while in case of collagen-coated gauze, thick coating with homogeneous surface was observed. Surface morphology of chitosan and collagen-coated gauze after blood haemostasis was tested. Collagen-treated gauzes showed mesh-like porous structure with an average pore size of  $\sim 2\text{ }\mu\text{m}$  appearing on the surface, while in case of chitosan-treated gauze, smooth surface with lumps was observed. The difference in the haemostatic properties of biopolymer-treated gauze also confirmed the faster blood coagulation by chitosan in comparison to collagen. Delayed haemostatic action of the collagen resulted in mesh-like structure over the surface to impact the haemostatic properties to the treated gauzes.

### Surface Plasmon Resonance Assay

Surface plasmon resonance assay was used to investigate the kinetics and association/dissociation interaction between



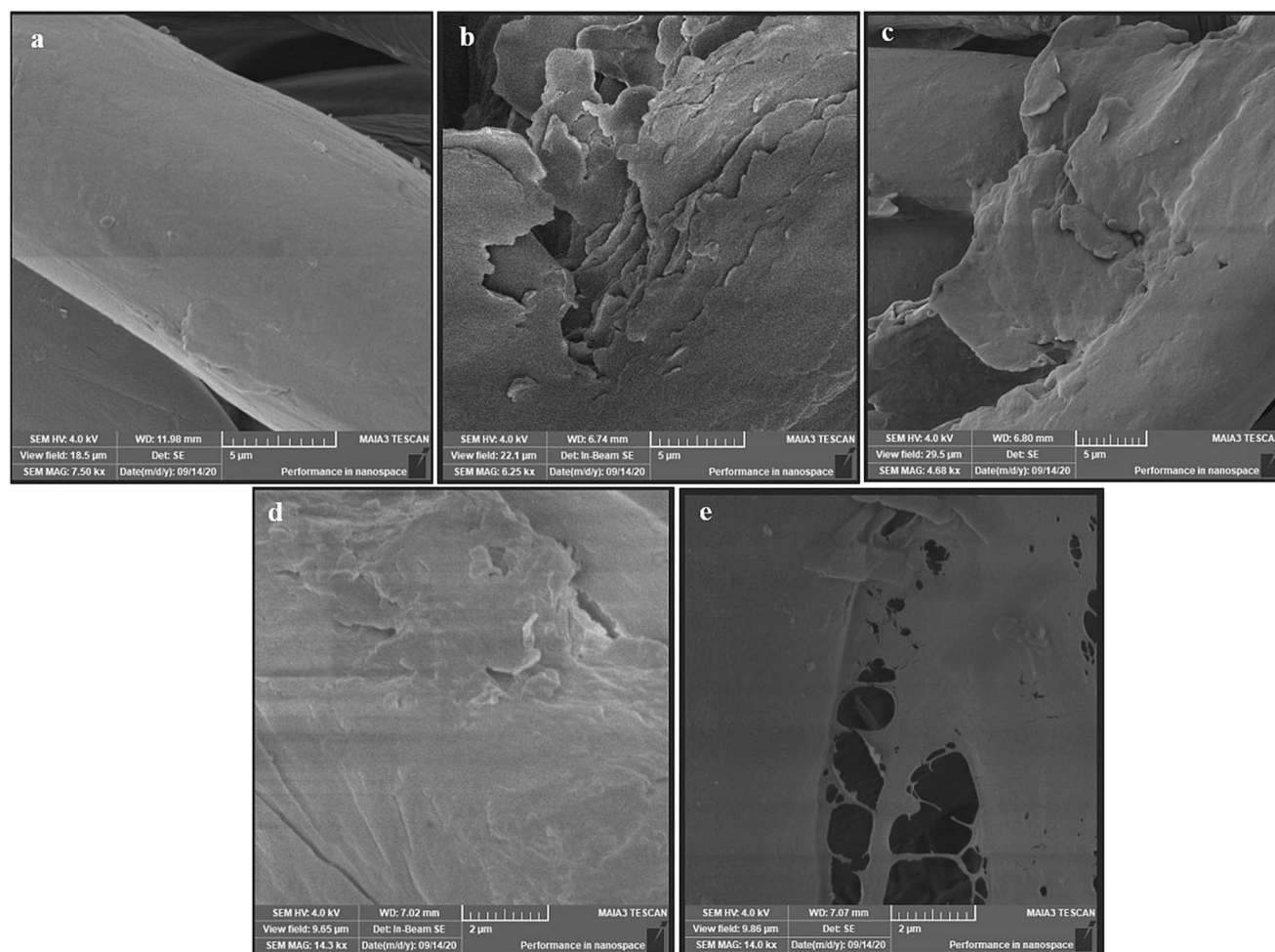
**Fig. 2.** Thermal gravimetric analysis (TGA) curves of collagen-coated materials and chitosan-coated materials



**Fig. 3.** Fourier transform infrared spectrum of **a** Chitosan-based materials **b** Collagen based-materials

human erythrocytes and collagen peptide and chitosan, respectively, as given in Fig. 5. In case of collagen-erythrocyte interaction, response unit (RU) increased from 42 to 63 RU, with the increase in the concentration of the erythrocytes from 0.5 to 0.20%. While in case of chitosan,

increase in the response units from 6 to 65 RU was observed. However, no change was observed in the RU of chitosan after further increase in concentration. It may be due to the saturation of collagen surface coated in CM5 chip by erythrocyte binding. It may be concluded that, chitosan has



**Fig. 4.** Scanning electron microscopy indicating **a** microstructure of standard gauze dressing **b** gauze-chitosan **c** gauze-collagen peptide. **d** gauze-chitosan with blood. **e** Gauze-collagen peptide with blood



more affinity towards erythrocyte even at lower concentration, in comparison to collagen as it showed higher affinity at lower concentration.

### Dressing pH Determination

Various dressing gauzes showed variation in pH with time, which shifted towards neutral when kept in deionized water as shown in Table III. It is probably due to ionization of the gauze surface and release of collagen and chitosan moieties with time. It was observed that pH of the solution was about 7 at 24 h, which is well versed with limit for the dermal application. In addition, it also indicates the release of biopolymer with time, which may further improve the local environment for faster wound healing. There was imperative distinction in the pH estimations of the dressing materials following 24 h. In all the coated dressings, the pH value increased when tested for a soaking time of 24 h. Following 3 h, pH change ranged from 6.4 to 6.9, however following 24 h, the pH changed from 7.1 to 7.9. The change in the pH may be attributed to the decrease in the acidification of the dressings, as the application time of the dressings increased. Shifting of the pH value may likewise antagonistically influence the injury recuperating process. As the injury advances towards healing, the pH value moves to neutral and afterward becomes acidic. In past investigations, the pH condition of interminable injuries has been recorded to be within a range of 7.15–8.9 (38).

### Blood Interaction Studies

The modifications in blood cells, due to the interaction of collagen and chitosan, were determined using complete blood count examination as shown in Table IV. All parameters were found to be within the biological reference range and there

was no significant variation between control blood (untreated) and blood that interfaced with collagen and chitosan-based materials with RBC, haemoglobin level, platelets, HCT, MCV, MCH, MCHC, RDW and PDW fixation. On the other hand, G-Ch and G-Cp dressings showed decreased plateletcrit (PCT) and mean platelet volume (MPV) compared to the standard gauze dressing and control blood. In chitosan-coated dressings, PCT increased from  $0.13 \pm 0.2$  to  $0.21 \pm 0.3\%$ , MPV from  $5.5 \pm 1.0$  to  $6.8 \pm 1.3$  fL while PCT from  $0.15 \pm 0.1\%$  to  $0.18 \pm 0.2\%$  and MPV  $5.1 \pm 0.8$  to  $5.9 \pm 0.5$  fL in collagen-coated materials. Decrease in the value of PCT and MPV may be due to the platelet adhesion and aggregation over the surface of the coated dressings as reported earlier (39). It also suggests that the coated gauze have better haemostatic property due to the platelet adhesion and aggregation capability.

### Erythrocyte Agglutination

Hemagglutination index of chitosan and collagen solution was determined using the Stavitsky scale and it was observed that total hemagglutination index (++++) was present in the solution of 6% collagen and 1% chitosan as shown in Fig. 6a. On the other hand, almost no agglutination was observed in 1 and 2% collagen hydrogel, probably due to weaker haemostatic property of the collagen peptide in comparison to the chitosan. In case of 2% chitosan hydrogel, there was quick haemostasis due to high viscosity and the presence of active moieties over the surface of hydrogel for erythrocyte.

### Sorption of Blood

In collagen-coated samples, G-Cp 1%, 2%, 4% double-coated and G-Cp 1%, 2%, 4% double-coated with glycerol (Gly), single-coated biopolymer assimilated significantly less

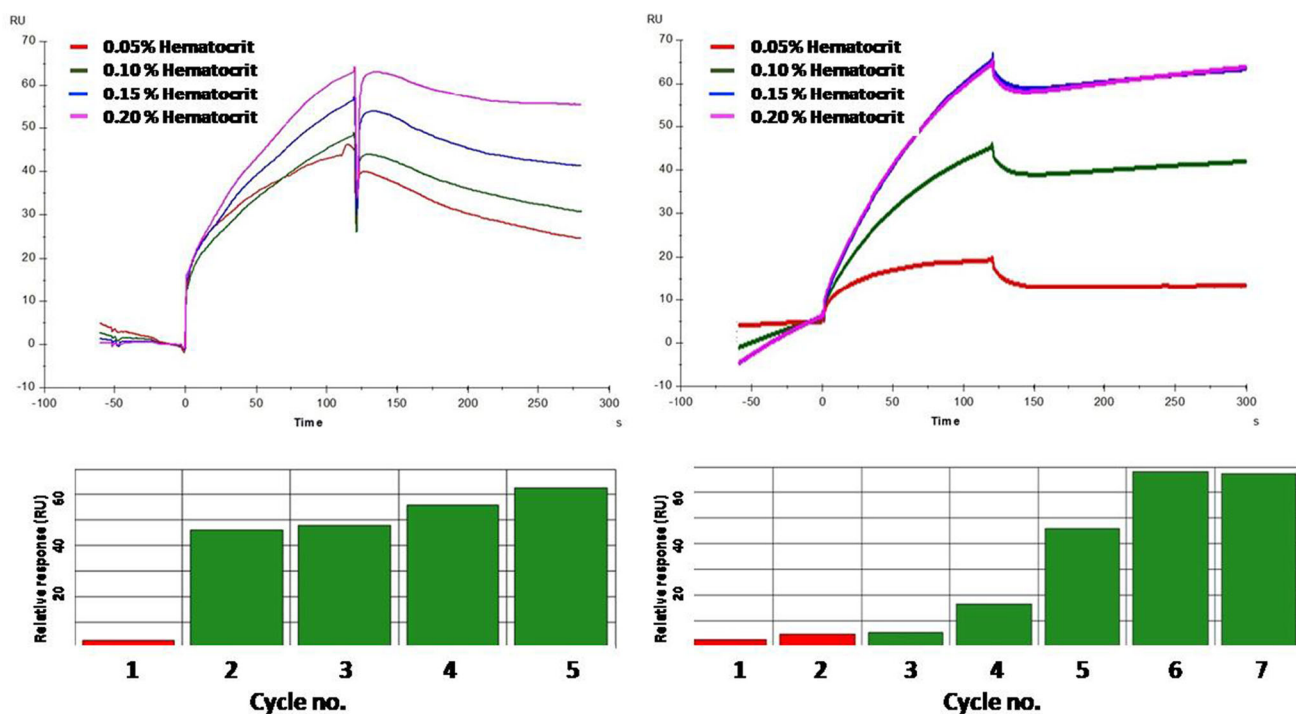


Fig. 5. Surface plasmon resonance analysis of human erythrocytes binding to a Collagen and b Chitosan

**Table III.** Comparison of pH in Dressing Materials at 3 h and 24 h

| Sr. no. | Gauze type           | pH Value |      |
|---------|----------------------|----------|------|
|         |                      | 3 h      | 24 h |
| 1       | SGD                  | 7.1      | 7.2  |
| 2       | G-Cp 1% DC           | 6.6      | 7.1  |
| 3       | G-Cp 1% DC+Gly 1% SC | 6.9      | 7.2  |
| 4       | G-Cp 2% DC           | 6.7      | 7.5  |
| 5       | G-Cp 2% DC+Gly 1% SC | 6.8      | 7.8  |
| 6       | G-Cp 4% DC           | 6.5      | 7.4  |
| 7       | G-Cp 4% DC+Gly 1% SC | 6.7      | 7.7  |
| 8       | G-Cp 6% DC           | 6.8      | 7.5  |
| 9       | G-Cp 6% DC+Gly 1% SC | 7.0      | 7.8  |
| 10      | G-Ch 1% SC           | 6.7      | 7.2  |
| 11      | G-Ch 1% SC+Gly 1% SC | 6.9      | 7.9  |
| 12      | G-Ch 1% DC           | 6.7      | 7.3  |
| 13      | G-Ch 1% DC+Gly 1% SC | 6.8      | 7.5  |
| 14      | G-Ch 1% TC           | 6.3      | 7.4  |
| 15      | G-Ch 1% TC+Gly 1% SC | 6.7      | 7.6  |
| 16      | G-Ch 1% QC           | 6.6      | 7.0  |
| 17      | G-Ch 1% QC+Gly 1% SC | 6.9      | 7.4  |

blood compared to G-Cp 6% double-coated (DC), G-Cp 6% double-coated Gly 1% single-coated (SC) and SGD. Gauze made with 6% collagen and SGD retained blood from 72% to 83% as shown in Fig. 6b. However, in chitosan-coated samples, G-Ch 1% double-coated and G-Ch 1% double-coated + Gly 1% single-coated retained more blood than SGD and other chitosan-coated samples of single, triple and quadruple coats. The measure of blood absorbed by G-Ch 1% double-coated and G-Ch 1% double-coated + Gly 1% single-coated ranged from 81 to 87%, which was found to be relatively more than SGD and G-Cp-covered materials. SGD retained more blood in comparison to the collagen or chitosan-coated gauzes as a result of large surface availability due to the presence of the loose open weave structure, which was blocked after coating with the collagen and chitosan-based polymeric hydrogel. The blockage of the polymers due to the loose open weave structure of the standard gauze was also confirmed by SEM images. Blood haemostasis is also the reason for the surface blockage of the polymer-coated gauze and results in lesser blood assimilation in comparison to the uncoated gauze.

### Haemostasis

The time of haemostasis and amount of blood loss in animals after application of various collagen and chitosan-coated dressings was determined as shown in Table I. In collagen-based materials, average time of haemostasis after the use of G-Cp 6% DC was  $226 \pm 15$  s and  $237 \pm 15$  s in G-Cp 6% DC + Gly 1% single-coated dressings, which was 50% less in comparison to SGD ( $506 \pm 15$  s). In case of chitosan-based materials, the time of haemostasis, after utilization of G-Ch 1% double-coated, was  $179 \pm 12$  s and  $191 \pm 14$  s in G-Ch 1% double-coated + Gly 1% single-coated gauzes. On the other hand, animals treated with G-Cp 6% double-coated depicted blood loss  $149 \pm 16.21$  mg and  $167 \pm 18.43$  mg in G-Cp 6% double-coated + Gly 1% single-coated dressing while in G-Ch

1% double-coated, measure of blood loss was  $141 \pm 14.32$  mg and  $131 \pm 19.43$  mg in G-Ch 1% double-coated + Gly 1% single-coated dressing. However, the use of SGD led to massive blood loss of  $1168 \pm 137.48$  mg during the haemostatic procedure (Table V). This finding suggests positive charge carried by chitosan and quickly activate the agglutination of erythrocytes and blood platelets, and plasma proteins were assimilated on the outside of the injury dressing upon contact with blood. Similarly, haemostatic plugs in animals treated with collagen have additionally indicated the presence of fibrin fibres at the outside of the plug in the prompt region (40). Glycerol-coated gauzes consumed more time in haemostasis because of an extra layer of glycerol coated as plasticiser, which resulted as barrier for the blood to come in contact with the polymeric coatings.

### Excision Animal Wound Model

The rate of wound contraction in the excision animal wound model was found to be higher in G-Cp, G-Ch, G-Cp-Gly and G-Ch-Gly, as compared to the control, and sham group (SGD) animals. On day second, 29% healing was observed in the treatment groups in contrast to 8% and 15% in the control and sham groups, respectively. On the seventh day of treatment, 26% and 30% healing was observed in the control and sham groups, respectively, while it was approximately 57% in all treatment group animals. On the 14th day, 86% to 100% healing was observed in the case of treatment groups compared to 52% and 61% in the control and sham groups, respectively. The application of G-Ch-Gly and G-Cp-Gly resulted in complete wound contraction within 14 days; 6 days earlier than the normal control group and 5 days earlier than the sham group. It was also observed that chitosan dressings were more efficient in wound healing in comparison to other gauzes, probably due to faster degradation of biopolymer over the wound site that enhances the wound healing as shown in Fig. 7. Similarly, chitosan dressing coated with glycerol showed further better results due to less sticky nature of the dressings at the wound site which helped in easy and quick removal of the dressings during alternate day replacements.

### Skin Irritation Test

At the end of the exposure period, G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly dressings were evaluated without adjusting the response or the integrity of the epidermis. There were no indications of oedema and erythema. The conduct and physical appearance of the animal was normal and there was no significant change in feeding and water intake behaviour. Therefore, it may be inferred that collagen and chitosan dressings have no unfavourable susceptible impact on the tested lab animals.

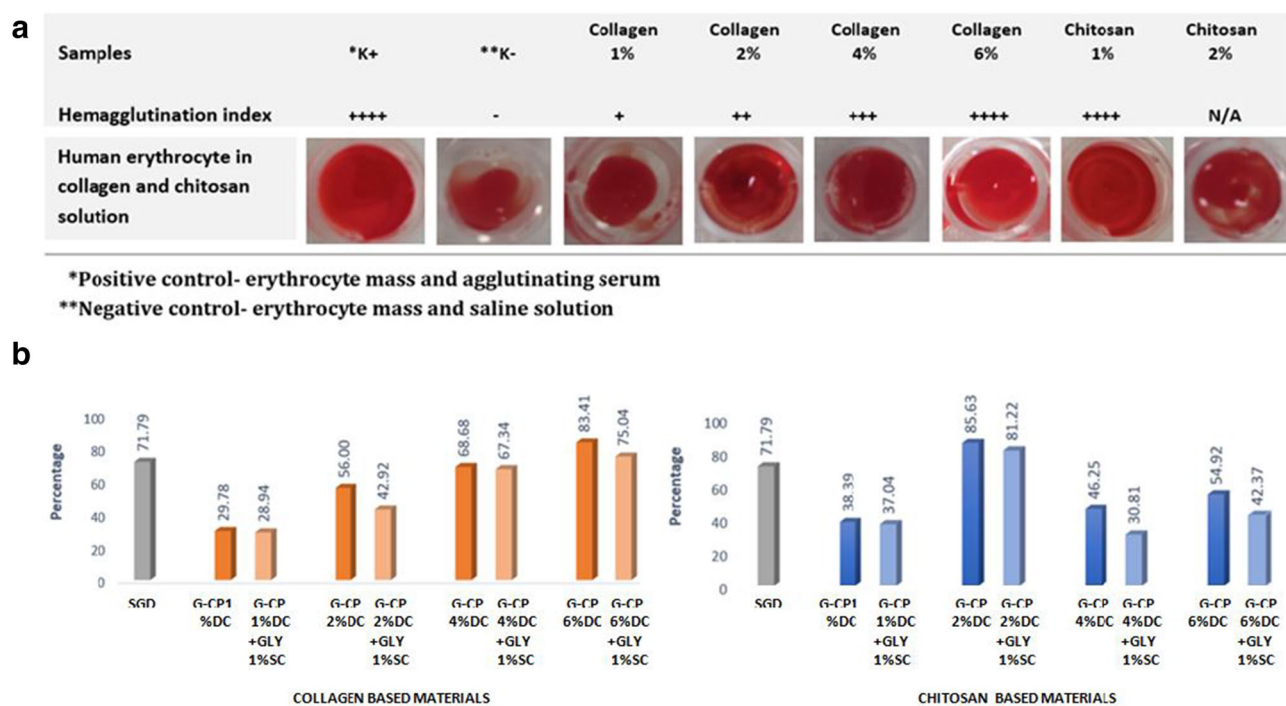
### Gamma Scintigraphic Studies

The *in vitro* stability of radiolabelled formulation was determined and labelling efficiency was found to be  $\geq 98\%$  up to 24 h. The scintigraphic images of the animal wound model showed no release of radiolabelled formulation-coated gauzes in the systemic circulation. Gamma scintigraphy studies

**Table IV.** Human CBC profile After Interaction of Collagen and Chitosan-based Materials

| Complete blood count reference range units | WBC 4.8–10.8 K/mL | RBC 4.7–6.1 M/mL | HGB 14–18 g/dL | HCT 42–52% | MCV 80–100 fl | MCH 27–32 pg | MCHC 32–36 g/dL | PLT 150–450 K/mL | RDW 11.5–14.5% | PCT 0.22–0.24% | MPV 7.5–11.0 fl | PDW4–19 fl |
|--|-------------------|------------------|----------------|------------|---------------|--------------|-----------------|------------------|----------------|----------------|-----------------|------------|
| Control blood SGD                          | 9.61±0.13         | 4.75±0.7         | 14.8±0.9       | 46.8±0.2   | 98.5±0.5      | 31.2±0.3     | 31.6±0.7        | 279±4.1          | 13.6±0.2       | 0.22±0.1       | 8.9±0.5         | 17.7±0.8   |
| G-Cp 1% DC                                 | 9.9±0.12          | 4.8±0.4          | 15±0.2         | 47.3±0.4   | 98.5±0.7      | 31.3±0.6     | 31.7±0.5        | 274±3.2          | 13.1±0.6       | 0.20±0.2       | 7.2±0.4         | 17.5±0.3   |
| G-Cp 1% DC+Gly 1% SC                       | 12.71±0.9         | 5.01±0.3         | 16±0.7         | 49.4±0.5   | 98.6±0.6      | 31.9±0.8     | 32.4±0.3        | 267±2.4          | 14±0.5         | 0.14±0.3       | 5.4±0.3         | 17.8±0.2   |
| G-Cp 2% DC                                 | 11.31±1.0         | 5.02±0.5         | 16±0.5         | 49.6±0.6   | 98.8±0.8      | 31.9±0.5     | 32.3±0.2        | 289±2.6          | 14±0.4         | 0.16±0.6       | 5.6±0.7         | 17.8±0.5   |
| G-Cp 2% DC+Gly 1% SC                       | 10.71±0.13        | 4.9±0.8          | 15.8±0.2       | 48.4±0.4   | 98.8±0.8      | 32.2±0.9     | 32.6±0.8        | 276±3.7          | 14±0.3         | 0.16±0.2       | 5.7±0.9         | 17.4±0.7   |
| G-Cp 4% DC                                 | 11±0.6            | 4.97±0.11        | 15.9±0.3       | 49.1±0.3   | 98.8±0.2      | 32.2±0.1     | 32.4±0.7        | 274±4.6          | 13.4±0.6       | 0.16±0.5       | 5.5±0.4         | 17.4±0.6   |
| G-Cp 4% DC+Gly 1% SC                       | 11.31±0.6         | 5.02±0.7         | 16.1±0.6       | 49.7±0.5   | 99±0.5        | 32.1±0.3     | 32.4±0.5        | 280±4.1          | 13.4±0.9       | 0.15±0.1       | 5.4±0.2         | 17.8±0.8   |
| G-Cp 6% DC                                 | 11.23±0.8         | 5.03±0.5         | 16.1±0.8       | 49.6±0.7   | 98.6±0.3      | 32±0.6       | 32.5±0.3        | 274±2.4          | 13.6±0.4       | 0.16±0.3       | 5.9±0.5         | 17.4±0.9   |
| G-Cp 6% DC+Gly 1% SC                       | 11.71±0.7         | 5.17±0.9         | 16.4±0.9       | 51±0.6     | 98.6±0.4      | 31.7±0.5     | 32.2±0.1        | 263±1.9          | 13.9±0.3       | 0.16±0.3       | 5.9±0.7         | 17.4±0.5   |
| G-Ch 1% SC                                 | 11.3±0.4          | 5.07±1.2         | 16.1±0.3       | 50.4±0.2   | 99.4±0.5      | 31.8±0.2     | 31.9±0.5        | 325±4.0          | 13.8±0.5       | 0.18±0.2       | 5.1±0.8         | 17.9±0.3   |
| G-Ch 1% SC+Gly 1% SC                       | 11.51±0.5         | 5.07±0.4         | 16.5±0.2       | 50.2±0.9   | 99±1.0        | 32.5±0.6     | 32.9±0.2        | 266±3.8          | 13.6±0.8       | 0.15±0.4       | 5.5±1.0         | 17.8±1.0   |
| G-Ch 1% DC                                 | 12±1.0            | 4.9±0.3          | 15.9±0.5       | 48.4±0.8   | 98.8±0.8      | 32.4±0.7     | 32.9±0.7        | 362±2.9          | 13.6±0.4       | 0.20±0.3       | 6.3±1.6         | 18.2±0.6   |
| G-Ch 1% DC+Gly 1% SC                       | 10.71±0.14        | 5.31±0.12        | 17.1±0.4       | 52.5±0.3   | 98.9±0.4      | 32.2±0.4     | 32.6±0.8        | 261±4.3          | 13.6±0.8       | 0.15±0.2       | 5.8±1.2         | 18.6±0.7   |
| G-Ch 1% TC                                 | 11.2±0.21         | 5.01±0.16        | 16.1±0.7       | 49.6±0.4   | 99±0.9        | 32.1±0.8     | 32.5±0.6        | 236±2.5          | 13.5±0.2       | 0.14±0.1       | 5.9±0.9         | 18.6±0.3   |
| G-Ch 1% TC+Gly 1% SC                       | 9.21±0.12         | 4.69±0.52        | 15.3±0.7       | 46.5±0.6   | 99.1±0.3      | 32.6±0.7     | 32.9±0.5        | 324±3.6          | 13.4±0.3       | 0.18±0.5       | 5.5±1.6         | 17.9±0.4   |
| G-Ch 1% QC                                 | 8.81±0.6          | 4.65±0.41        | 15.2±0.6       | 46.2±0.1   | 99.4±0.7      | 32.7±0.5     | 32.9±0.8        | 365±4.8          | 13.7±0.5       | 0.21±0.3       | 6.8±1.3         | 19.6±0.2   |
| G-Ch 1% QC+Gly 1% SC                       | 8.61±0.34         | 4.66±0.60        | 14.8±0.3       | 46.5±0.4   | 99.8±0.2      | 31.8±0.8     | 31.8±0.4        | 238±3.7          | 13.1±0.9       | 0.13±0.2       | 5.4±1.9         | 17.8±0.6   |
| G-Ch 1% QC+Gly 1% SC                       | 8.31±0.22         | 4.73±0.44        | 15.1±0.5       | 46.9±0.9   | 99±0.6        | 31.9±0.3     | 32.2±0.3        | 267±4.2          | 13.9±0.7       | 0.16±0.5       | 6.1±1.5         | 18.6±0.4   |

**CBC** complete blood count



**Fig. 6. a** Results of hemagglutination of human erythrocyte in collagen and chitosan solutions. **b** Comparative analysis of blood sorption between collagen-based materials and chitosan-based materials with reference to SGD in 10 min after blood incubation

showed  $85 \pm 2\%$  formulation retention up to 12 h at the wound site in comparison to  $40 \pm 3\%$  retention of the radiopharmaceutical alone. The scintigraphic images did not show significant release of formulation up to 12 h in G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly, while in SGD, aggregation in blood was observed with significant reduction in counts as shown in Fig. 8b. The study confirmed the formulation retention up to 12 h at the wound site without any significant release in the systematic circulation.

### Histopathological and Haematological Analysis

The haematological parameters were found within the typical range among the sham-operated and treatment groups (G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly), depicting safety of collagen and chitosan gauzes as described in Fig. 8a. Furthermore, the images of skin tissue segments stained with haematoxylin and eosin showed histological changes during wound-healing process in the sham-treated and G-Ch, G-Ch-Gly, G-Cp and G-Cp-Gly-treated rats at day 0, day 7 and day

14. Histopathological examination indicated a general early recuperation and recovery. Contrasted with the sham operated group, the treatment groups exhibited a significant recovery of skin extremities with systematic collagen, more fibroblasts, conspicuous epidermal thickening, nearness of granulation tissue and fundamentally better re-epithelization on the 14th day (Fig. 8a). Animals of the sham group were not completely epithelialized and indicated lopsidedness of the epidermis with more prominent inflammation and diminished collagen.

### CONCLUSION

The *in vitro* and *in vivo* experimental study demonstrated that collagen and chitosan-coated gauze promoted advanced wound healing compared to the commercial dressings, proving their potential as dressings for full thickness skin wound healing. The results were validated by *in vitro* human blood studies on surface plasmon resonance followed by *in vivo* wound healing studies in *Sprague-Dawley*

**Table V.** Effectiveness of Haemostasis During *In Vivo* Experiment

| Group  | Time taken in complete haemostasis | % of effectiveness in terms of blood loss |
|--|------------------------------------|---|
| SGD  | $506 \pm 25$                       | 25  |
| G-Cp 6% double coated                        | $226 \pm 15$                       | 87.5                                      |
| G-Cp 6% double coated + Gly 1% single coated | $237 \pm 15$                       | 75  |
| G-Ch 1% double coated                        | $179 \pm 12$                       | 100                                       |
| G-Ch 1% double coated + Gly 1% single coated | $191 \pm 14$                       | 87.5                                      |

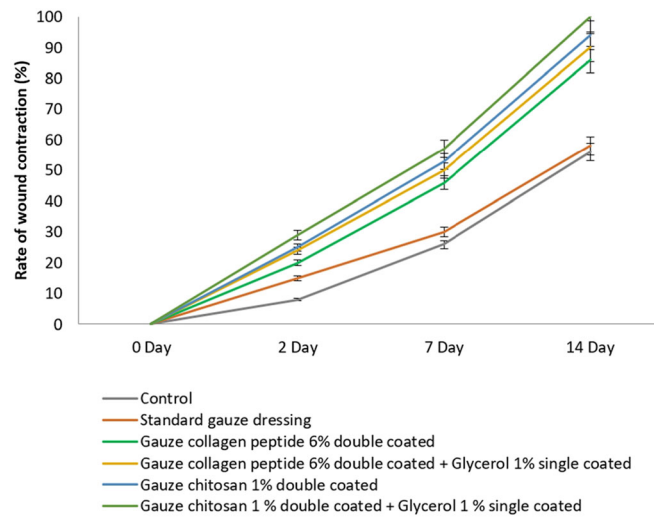


Fig. 7. Rate of wound contraction in excision animal wound model

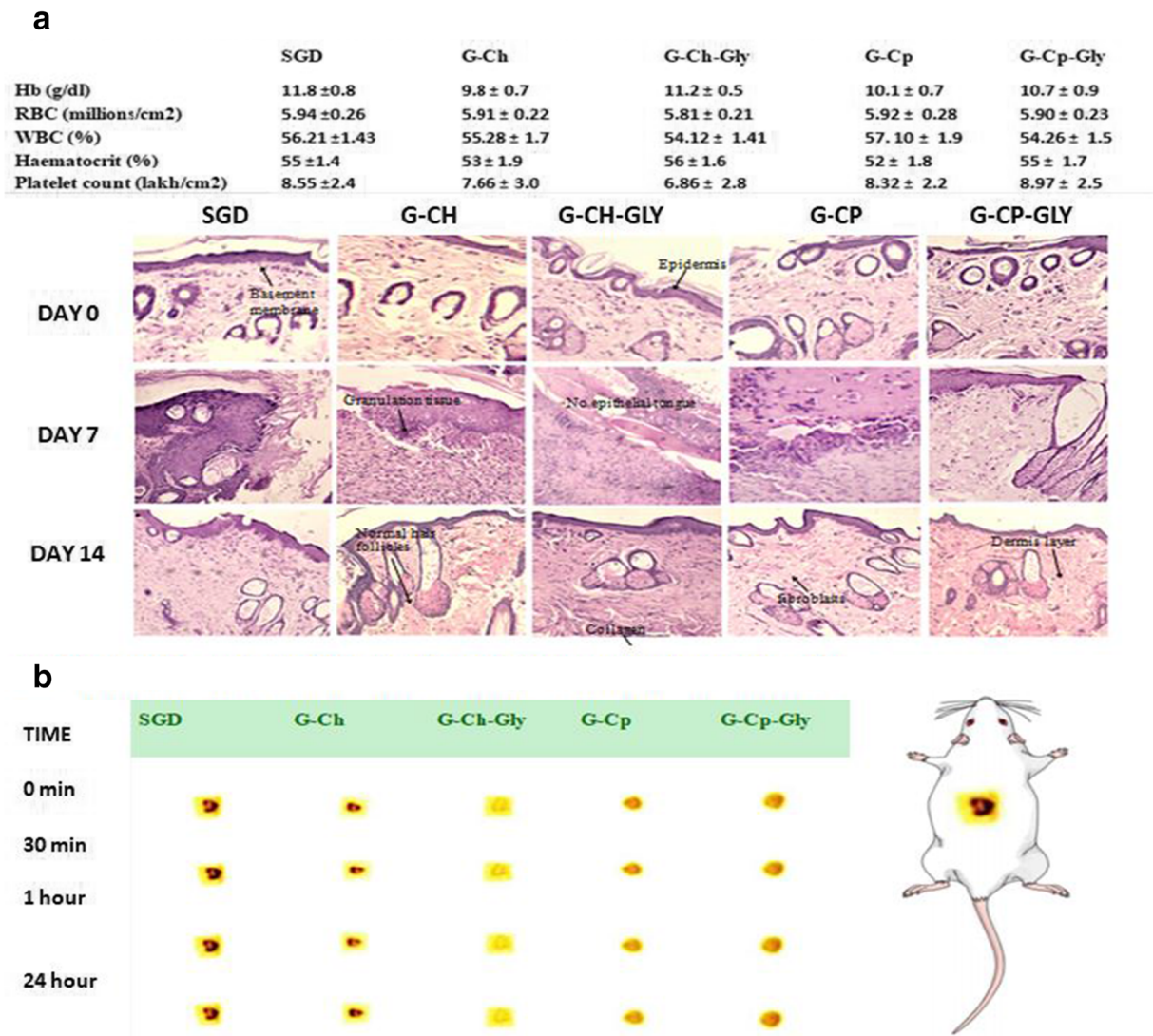


Fig. 8. Haematological profile, histopathological analysis and scintigraphic animal imaging at different time intervals

rats. Comparative viability research may be utilized as a tool to evaluate topical therapy for wound care. The permeable, breathable, non-adhesive chitosan/collagen-based wound dressings have been found to impart better *in vivo* haemostatic and wound healing action.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Institute of Nuclear Medicine and Allied Sciences-Defence Research and Development Organization (INMAS-DRDO), New Delhi. Grant No. INM/321.

## DECLARATIONS

**Conflict of Interest** The writers proclaimed no conflicts of interest as for the research, authorship and publication of this article.

## REFERENCES

- Achneck HE, Sileshi B, Jamiolkowski RM, Albala DM, Shapiro ML, Lawson JH. A comprehensive review of topical haemostatic agents: efficacy and recommendations for use. *Ann Surg*. 2010;251:217–28.
- Sauaia AMD, Moore FAMD, Moore EEMD, Moser KSPRA, Brennan RRNMS, Read RAMD, et al. Epidemiology of trauma deaths. *Epidemiology of trauma deaths: a reassessment*. *J Trauma Inj Infect Crit Care*. 1995;38(2):185–93.
- Hirshberg A, Wall MJ, Ramchandani MK, Mattox KL. Re-operation for bleeding in trauma. *Arch Surg*. 1993;128:1163–7.
- Enoch S, Leaper DJ. *Basic science of wound healing*. *Surgery (Oxford)*. 2005;26:31–7.
- Ong S-Y, Wu J, Moochhala SM, Tan M-H, Lu J. Development of a chitosan based wound dressing with improved Haemostatic and antimicrobial properties. *Biomaterials*. 2008;29:4323–32.
- Pusateri AE, Kheirabadi BS, Delgado AV, Doyle JW, Kanellos J, Uscilowicz JM. Structural design of the dry fibrin sealant dressing and its impact on the haemostatic efficacy of the product. *J Biomed Mater Res B Appl Biomater*. 2004;70:114–21.
- Pusateri AE, Modrow HE, Harris RA, Holcomb JB, Hess JR, Mosebar RH. Advanced haemostatic dressing development program: animal model selection criteria and results of a study of nine Haemostatic dressings in a model of severe large venous hemorrhage and hepatic injury in swine. *J Trauma*. 2003;55:518–26.
- Liu X, Ma L, Mao Z, Gao C. Chitosan-based biomaterials for tissue repair and regeneration. *Chitosan for biomaterials II*. In: Jayakumar R, Prabakaran M, Muzzarelli RAA, editors. *Advances in polymer science*. Heidelberg: Springer Berlin; 2011. p. 81–127.
- Rao SB, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and haemostatic potential. *J Biomed Mater Res*. 1997;34:21–8.
- Ghica MV, Albu MG, Popa L, Moisescu S. Response surface methodology and Taguchi approach to assess the combined effect of formulation factors on minocycline delivery from collagen sponges. *Pharmazie*. 2013;68:340–8.
- Ghica MV, Albu MG, Leca M, Popa L, Moisescu S. Design and optimization of some collagen-minocycline based hydrogels potentially applicable for the treatment of cutaneous wounds infections. *Pharmazie*. 2011;66:853–61.
- Antoniac IV, Albu MG, Antoniac A, Rusu LC, Ghica MV. Collagen-bioceramic smart composites. In: Antoniac IV, editor. *Handbook of bioceramics and biocomposites*. Basel: Springer; 2016.
- Albu MG. Collagen gels and matrices for biomedical applications. Saarbrücken: Lambert Academic Publishing; 2011. ISBN 978-01-9850-970-7
- Lee CH, Lee Y. Collagen-based formulations for wound healing applications. In: Agren M, editor. *Wound healing biomaterials*, vol. 2. Cambridge: Woodhead Publishing, Elsevier; 2016. p. 135–49. ISBN 978-1-78242-456-7.
- De Almeida EB, Cardoso JC, Lima a AKD, Oliveira a NLD, Pontes-Filho c NTD, Lima a SO, Souza a ICL, Cavalcanti de RL, Júnior a AJ. The incorporation of Brazilian propolis into collagen-based dressing films improves dermal burn healing. *J Ethnopharmacology*. 2013;147:419–25.
- Akturk O, Tezcaner A, Bilgili H, Deveci MS, Gecit MR, Keskin D. Evaluation of sericin/collagen membranes as prospective wound dressing biomaterial. *J Biosci Bioeng*. 2011;112:279–88.
- Aranaz I, Harris R, Heras A. Chitosan amphiphilic derivatives. *Chemistry and applications*. *Curr Org Chem*. 2010;14:308–30.
- Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials*. 2000;22:261–8.
- Pusateri AE, Holcomb JB, Harris RA, MacPhee MJ, Charles NC, Beall LD. Effect of fibrin bandage fibrinogen concentration on blood loss after grade V liver injury in swine. *Mil Med*. 2001;166:217–22.
- Brian TG. Effects of Celox and trauma DEX on hemorrhage control in a porcine model. *AANA J*. 2010;78(2):115–20.
- Mercy HP, Halim AS, Hussein AR. Chitosan-derivatives as haemostatic agents: their role in tissue regeneration. *Regen Res*. 2012;1:38–46.
- Arafat MT, Tronci G, Yin J, Wood DJ, Russell SJ. Biomimetic wet-stable fibres via wet spinning and diacid-based crosslinking of collagen triple helices. *Polymer*. 2015;77:102–12.
- Tronci G, Grant CA, Thomson NH, Russell SJ, Wood DJ. Multi-scale mechanical characterization of highly swollen photo-activated collagen hydrogels. *J R Soc Interface*. 2015;12:20141079.
- Tronci G, Doyle A, Russell SJ, Wood DJ. Structure-property-function relationships in triple-helical collagen hydrogels. *Mater Res Soc Symp Proc*. 2013;1498:145–50.
- Wedmore I, McManus JG, Pusateri AE, Holcomb JB. A special report on the chitosan based haemostatic dressing: experience in current combat operations. *J Trauma*. 2006;60:6558.
- Rhee P, Brown C, Martin M, Salim A, Plurad D, Green D. QuikClot use in trauma for hemorrhage control: case series of 103 documented uses. *J Trauma*. 2008;64:1093–9.
- Bettini R, Romani AA, Morganti MM, Borghetti AF. Physico-chemical and cell adhesion properties of chitosan films prepared from sugar and phosphate containing solutions. *Eur J Pharm Biopharm*. 2008;68:74–81.
- Park BK, Kim MM. Applications of chitin and its derivatives in biological medicine. *Int J Mol Sci*. 2010;11:5152–64.
- Hunt TK, Beckert S. Therapeutical and practical aspects of oxygen in wound healing. In: Lee B, editor. *The wound management manual*. New York: McGraw-Hill Medical. 2000;44–54.
- Rydzak J, Kaczmarek R, Czerwinski M, Lukaszewicz J, Tyborowska J. The Baculovirus-expressed binding region of Plasmodium falciparum EBA-140 ligand and its Glycophorin C binding specificity. *PLoS One*. 2015;10(1):e0115437.
- Tsukada K, Tokunaga K, Iwama T, Mishima Y. The pH changes of pressure ulcers related to the healing process of wounds. *Wounds*. 1992;4(1):16–20.
- de Lima JM, Sarmiento RR, de Souza JR, Brayner FA, Feitosa APS, Padilha R, et al. Evaluation of hemagglutination activity of chitosan nanoparticles using human erythrocytes. *Biomed Res Int*. 2015; I.D.247965, 1–6.
- Pogorielov M, Kalinkevich O, Deineka V, Garbuzova V, Solodovnik A, Kalinkevich A, et al. Haemostatic chitosan coated gauze: in vitro interaction with human blood and in-vivo effectiveness. *Biomater Res*. 2015;19(22):1–6.

34. Chhabra P, Tyagi P, Bhatnagar A, Mittal G, Kumar A. Optimization, characterization, and efficacy evaluation of 2% chitosan scaffold for tissue engineering and wound healing. *J Pharm Bioallied Sci.* 2016;8:300–8.
35. Sharma A, Fish BL, Moulder JE, Medhora M, Baker JE, Mader M, et al. Safety and blood sample volume and quality of a refined retro-orbital bleeding technique in rats using a lateral approach. *Lab Anim.* 2014;43(2):63–6.
36. [https://www.oecd-ilibrary.org/environment/test-no-404-acute-dermalirritation\\_corrosion\\_9789264070622-en](https://www.oecd-ilibrary.org/environment/test-no-404-acute-dermalirritation_corrosion_9789264070622-en).
37. Pan H, Fan D, Cao W, Zhu C, Duan Z, Fu R, et al. Preparation and characterization of breathable hemostatic hydrogel dressings and determination of their effects on full-thickness defects. *Polymers.* 2017;9:727.
38. Schneider LA, Korber A, Grabbe S, Dissemond J. Influence of pH on wound-healing: a new perspective for wound-therapy? *Arch Dermatol Res.* 2007;298:413–20.
39. Stavitsky AB. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J Immunol.* 1954;72(5):360–7.
40. Sixma JJ, Wester J. The Haemostatic plug. *Semin Hematol.* 1977;14:265–99.

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.