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# *In vitro* and *In vivo* Biocompatibility Evaluation of Freeze Dried Gelatin Haemostat

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**Abstract:** Gelatin sponges developed by crosslinking gelatin with formaldehyde followed by controlled freeze-drying results in soft, spongy porous structures, which are useful in arresting bleeding during surgical procedures. The aim of this article is to conduct the *in vivo* evaluation of the gelatin sponge as per ISO 10993: Biological evaluation of medical devices; and prove its biocompatibility. The sponges were also evaluated by morphology analysis, tensile strength and blood-absorption studies. Sponges were found to be microporous with considerable mechanical strength to withstand any rupture during its application. Blood absorption studies showed good absorption behaviour. As per ISO 10993; cytotoxicity, skin irritation, sensitisation and blood compatibly studies has to be conducted to check the biocompatibility of a biomaterial. Gelatin sponge was found to be non-cytotoxic in Balb/c 3T3 cells. The experiments conducted on albino rabbits and guinea pig concluded that the material does not cause any irritation and sensitisation *in vivo* and is non-haemolytic when in contact with blood. Hence the material meets the requirements of a biocompatible haemostatic agent for the management of blood loss during surgical procedures.

**Keywords:** Gelatin, Cytotoxicity, Irritation studies, Sensitisation studies, Haemocompatibility

## Introduction

Uncontrolled bleeding is one of the major reasons for death among trauma patients during surgical procedures [1]. If not managed properly, surgical bleeding can extend the length of surgical procedure and necessitate blood transfusion. It can also damage wound healing and increase the risk of infection [2]. Thus, one of the greatest challenges for a surgeon is the minimization of blood loss during surgery and for this purpose wide variety of haemostatic agents has been used.

Different haemostatic agents have been developed for the past several decades, with variable efficacies but none being perfect. Biomaterials with plant and animal origin such as chitosan [3], gelatin [4], collagen [5], starch [6] and oxidised regenerated cellulose [7] offer excellent biocompatibility, accelerated tissue regeneration and reconstruction capacity [8]. Though chitosan is a good haemostat, this marine polysaccharide can contribute to presence of impurities, pyrogens, endotoxins, cytotoxic agents and increased bioburden in the end product [9]. Poor mechanical properties along with high sensitivity towards environmental conditions are the main drawbacks of starch [10]. The source variability, limited range of physical and chemical properties of collagen [11] and rare adverse side effects of cellulose [10,12] makes them a lesser choice as haemostat.

Gelatin is an ideal choice for making a haemostat owing to its safe use in terms of biodegradability and biocompatibility, in addition to other contributing factors like ease of raw material availability, cost of economy, wide use, good foaming properties [13] and low immunogenicity [14]. It is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) [15] and compliant with the US, European and Japanese pharmacopoeia. The major limitation of gelatin is its weak mechanical strength and poor hydrolysis resistance and this can be circumvented by cross-linking of gelatin through physical, chemical or enzymatic cross-linking [16-21]. The use of Glutaraldehyde (GTA) vapour as a cross linking agent is time-consuming and may result in poor crosslinking degree, while use of GTA solution may pose a difficulty in complete removal of GTA residues post cross-linking and may lead to cytotoxicity [22]. High cost, limited sources and difficulty in extraction are the major disadvantages associated with genipin as a cross linker [23]. The factors like ease of availability, low cost and very fast cross-linking action of formaldehyde [24,25] was utilized for developing gelatin sponges in our present study. Gelatin in the sponge form shows increased haemostatic performance because of its increased adhesion to the bleeding site and porous structure which activates thrombocytes when blood comes in contact with it. These activated thrombocytes initiate the release of a variety of substances that enable their aggregation, and leads to fibrin formation [26].

The development of gelatin based absorbable sponge in the present work was attempted by cross-linking gelatin with formaldehyde followed by controlled freeze-drying to obtain a soft, spongy material having high blood absorption characteristics. Freeze dried gelatin sponges prepared using different crosslinking agents, comparison of their properties and cell culture evaluations are already reported [27-31]. But as

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of now there are no *in vivo* biocompatibility studies reported on gelatin sponge. As per ISO 109993: Biological evaluation of medical devices; cytotoxicity, irritation, sensitization and blood compatibility studies has to be conducted on a haemostatic device prior to commercialization of the product. Hence in this article we are reporting the biocompatibility studies such as cytotoxicity, skin irritation, sensitisation and haemolytic studies along with other physico-chemical evaluations.

## Experimental

### Materials

Type B Gelatin granules conforming to USP/BP standards were obtained from Nitta Gelatin India Limited, Cochin, India. Formaldehyde (37 % w/v) ACS, Ph.Eur. from Merck, Pepsin from porcine gastric mucosa (≥ 250 units/mg solid) and Complete Freund's Adjuvant from Sigma-Aldrich was purchased. All other chemicals and solvents used for various tests were obtained from reputed chemical suppliers.

### Development of Gelatin Sponge

Prepared 5 % w/v gelatin solution in distilled water at room temperature. The prepared gelatin suspension upon swelling for 10-15 minutes was allowed to dissolve by heating the suspension at a temperature of 45-50 $^{o}$C for 20 minutes at 300 rotations per minute (rpm) using a magnetic stirrer (WiseStir MSH 30D, Korea South). The gelatin solution upon cooling was cross-linked with formaldehyde (0.025 ccm) and stirred using an over-head stirrer (IKA RW20 digital, India) at a speed of 1400-1500 rpm to produce a stable foam. The development of the gelatin sponge was done by optimizing various parameters such as concentration of gelatin, amount of cross-linking agent used, time of stirring and the cycle of freeze drying. The gelatin foam produced upon stirring was then poured into suitable teflon coated moulds and freeze-dried in a programmable controlled freeze dryer (LYODRYER, India). The freezing rate of a material directly influences subsequent drying behaviour and other quality attributes of the final product like its pore size, uniform pore distribution, water absorption capacity etc. Controlled freeze drying enables precise control of both time and temperature at which nucleation occurs, which offers better product morphology [32,33]. For the process of freeze drying, stable gelatin foam was poured into pre-cooled Teflon coated moulds, which were then freezed to -40 $^{o}$C for 5 hours, subsequently increasing the temperature to -30 $^{o}$C, -10 $^{o}$C, 10 $^{o}$C and 45 $^{o}$C over a time period of 14 hours.

### Characterization Studies on the Optimized Formulation of Gelatin Sponge

#### *Morphological Studies*

The morphology of the gelatin sponges were examined using a scanning electron microscope (SEM) (Vega 3,Tescan, Czech Republic) after coating the samples with a thin layer of gold under vacuum at 15 kV. The SEM images obtained were then evaluated for their pore size distribution using Image J software (ImageJ 1.36b, Wayne Rasband, NIH, Washington, DC). Atleast 100 pores were assessed. Porosity of the sponge was evaluated by liquid displacement method [34]. Pre-weighed gelatin sponges were immersed into a known volume of the displacement fluid-ethanol ($V_1$) and degassed for 5 minutes with a vacuum pump. $V_2$ was recorded as the total volume of ethanol-impregnated sponge and residual volume of ethanol was recorded as $V_3$. Porosity ($\epsilon_1$) of the sponge was calculated as follows:

$$\epsilon_1\ (\%)=(V_1 - V_3)/(V_2 - V_3) \times 100$$

The density of the freeze-dried gelatin sponge was calculated from the ratio of weight of the gelatin sponge to its volume. The density measurements was performed using samples with a volume of 7×5×1 cm$^3$ and the final densities were obtained from the mean of values obtained for three specimens. Fourier transfer infra-red spectroscopy (FTIR) of gelatin and gelatin sponge was recorded with a Perkin Elmer-Frontier FTIR (UK) in order to detect possible chemical cross-linking of the gelatin sponge using attenuated total reflectance (ATR) accessory [35] in the range of 400-4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$. The cross-linking degree of gelatin sponges were further determined by trinitro benzene sulfonic acid (TNBS) assay [36]. (Details of liquid displacement method and degree of cress linking are given as supplementary information).

#### *Mechanical Analysis*

The mechanical properties of gelatin sponges were investigated using a universal testing machine, UTM (Shimadzu-AGX-10 kN, Kyoto, Japan) at 23+/-2 $^{o}$C according to U.S patent protocol US6693180B2 [37]. The rectangular sponge samples (approximately 20 mm×50 mm×10 mm) were tested in the machine at a cross-head speed of 50 mm/ minute and tensile strength was noted. Five samples of the test item were used for analysis and calculated the average tensile strength of the samples. The wet tensile strength of the samples was also studied using similar procedure.

#### *Blood Absorption Studies*

Blood absorption study was done as per British Pharmacopoeia, 1995 [38] protocol. A 5×5 cm piece of gelatin sponge was accurately weighed ($w_1$) and placed in a petridish. Whole blood corresponding to a volume of 40 times the weight of the gelatin sponge was warmed at 37 $^{o}$C and added to the petridish, which was then incubated at 37 $^{o}$C for 30 minutes. Using forceps, the gelatin sponge being tested was suspended in air for 30 seconds by one corner and reweighed ($w_2$). The testing was repeated on 5 samples and the weight of blood retained using the formula $[(w_2-w_1)/w_1]\times100$ was calculated. The same test was conducted with water and calculated the water absorption.

***In vitro Cytotoxic Study***

Cytotoxicity test were carried out based on ISO 10993-5: 2009 (E); Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity [39]. The cytotoxicity of the gelatin sponge was studied *in vitro* using human foreskin fibroblast cell lines procured from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala using the direct as well as indirect contact method. Fibroblast cell lines were seeded onto 24 well plates with $0.8\times10^6$ cells per well, which were then incubated at 37 °C for 24 hours. The sponges weighing 0.1 g was added onto the wells for direct contact testing. 1 m*l* of the media in which the sponge was soaked overnight was used for indirect contact testing. The plates after both treatments were incubated at 37 °C with 5 % $CO_2$ (Leec Culture Safe $CO_2$, Touch 190S, United Kingdom) for a period of 24 hours, followed by addition of MTT reagent (Merck Millipore, United States) and reading was done at 570 nm. The percentage cell cytotoxicity of the haemostatic sponge was calculated and their quantitative evaluation was done using an inverted microscope (Magnus Invi Trinocular, India).

$$\% \text{ Cell Cytotoxicity} = [100 - (\text{Optical density of sample}/\text{Optical density of control})] \times 100$$

***In-vitro Haemolytic Test***

Haemolytic tests are usually done on any device which comes in contact with blood [40]. The test was done in order to evaluate the toxic reactions that may impact the red blood corpuscles (RBC) and may cause bursting of RBC. Disk shaped specimens of gelatin sponges in triplicates were placed in polystyrene culture plates and agitated with phosphate buffered saline for 5 minutes before they were exposed to blood taken from human volunteer. To each plate, 5.0 m*l* blood was added and 1.0 m*l* was taken immediately for analysis and remaining 4.0 m*l* blood was exposed to the materials for 30 minutes under agitation at 70±5 rpm using a shaker thermostated at 35±2 °C.Three empty polystyrene petridishes were exposed with blood as reference. The blood count was analysed initially and after 30 minutes using automatic haematology analyser (Sysmex-K 4500, USA). The free haemoglobin liberated in to the plasma after exposure was measured in each sample using Diode Array Spectrophotometer (Hewlett Packard 8453, United States) and the percentage haemolysis was calculated using the formula

$$\text{Haemolysis (\%)} = (\text{Hb in test sample}/\text{Total Hb}) \times 100$$

where Hb refers to Haemoglobin content.

***Skin Irritation Studies***

Skin irritation studies [41] were carried out based on ISO 10993-10: 2010 (E); Biological evaluation of medical devices standard to evaluate the local responses of the gelatin sponge after applying topically on albino rabbits. Rabbits weighing not more than 3.5 kg was procured from Sree Chitra Institute of Medical Sciences and Technology, Thiruvananthapuram, India. The study was done at Small Animal Experimentation Facility, HLL CRDC, HLL Lifecare Limited, Akkulam, Thiruvananthapuram, India (IAEC study No. HLL/CRDC/SAEF/IAEC/01/02/2015). The gelatin sponge and control was cut into the size of approximately 6.25 $cm^2$ (2.5 cm×2.5 cm) under sterile conditions and placed as such on to the fur clipped area of rabbit skin in the dorsal region on the left cranial end and right caudal end. Right cranial and left caudal end was treated with absorbent cotton wool and secured with a path followed by a suitable semi occlusive dressing. The patches were removed after 4 hours and the test sites were marked with a non-irritant permanent marker pen. The grading of erythema and oedema of the test and control sites of all animals at 24, 48 and 72 hours were recorded as per ISO 10993-10: 2010 (E).

***Skin Sensitisation Test***

Skin sensitization study [41] was designed according to ISO 10993-10: 2010 (E); Biological evaluation of medical devices-Part 10: Tests for irritation and skin sensitisation to determine the skin sensitisation potential of the gelatin sponge in physiological saline extracts of the sponge and is compared with absorbent cotton as a reference material. The tests were performed on Dunkin-Hartley Guinea pig of either sex (290-450 g) procured from Sree Chitra Institute of Medical Sciences & Technology, Thiruvananthapuram, India and the tests were held at Small Animal Experimentation Facility, HLL CRDC, HLL Lifecare Limited, Akkulam, Thiruvananthapuram, India (IAEC Study No. HLL/CRDC/SAEF/IAEC/01/03/2015). Extracts of the gelatin sponge was prepared by extracting 100 g of sponge in 30 m*l* of physiological saline (Sodium Chloride 0.9 %, Varni Corporation, Ahmedabad). Induction of sensitisation consists of a two stage procedure with intradermal injections initially administrated, followed by a closed topical patch exposure and on day 7. Intradermal injections of the test extract vehicles and complete freunds adjuvant (CFA) in various mixtures were administrated to the vehicle control and test groups. On day 6, following the intradermal injections, test area was treated with 0.5 m*l* of 10 % sodium lauryl sulphate. On the following day, topical patch of size 8 $cm^2$ loaded with 0.5 m*l* of test extract and vehicle respectively was applied topically to respective groups of guinea pigs, on the same site as that of intradermal injections. This occlusive dressing was held in place for 48 hours. Two weeks after following the topical patch induction, challenge exposure was administered as a topical patch of size 8 $cm^2$. Patch loaded with 0.5 m*l* of the test item extract was applied on left side and the patch with 0.5 m*l* of vehicle was applied on right side of each animal in respective groups for 24 hour at sites other than those used for intradermal injections/topical application and the application sites were marked with non-irritant marker pen. Grading of skin reactions was performed visually at 24 hour and 48 hour after removing the challenging phase. The

challenge application sites were assessed for erythema and edema using Magnusson and Kligman scale. After the observation period the animals were humanely sacrificed.

## Results and Discussion

### Development of Gelatin Sponge

The final product of freeze-dried gelatin sponge was observed visually as well as microscopically and were found to be white, flexible and porous in structure. Initially, the sponges were optimized based on the quality and stability of the foam produced upon stirring at different time intervals for different concentration of gelatin. 5 % w/v gelatin crosslinked with 0.025 ccm formaldehyde was found to produce the best quality foam upon stirring. Prepared gelatin sponges were gamma sterilised at 25 kGy before performing all the studies.

### Characterization of Optimized Gelatin Formulation

#### *Morphological Examination*

The images obtained from SEM were evaluated for pore size and distribution of pores along the matrix. The sponges were found to be microporous in structure with pore distribution as shown in Figure 1(A) & 1(B). The pores are in the size range of 65 µm to 740 µm and are found to be interconnected [42]. The pore size of the gelatin sponge was studied using Image J software and the pore size distribution curve under 50 X magnification and 100 X magnification have been plotted as shown in Figure 1(C) and Figure 1(D). It shows maximum pore size in the range of 100 µm to 350 µm. Haemostatic ability of a gelatin sponge formulation is related to its sponge porosity, such that they can absorb many more times blood than their own weight. According to Babitha *et al*. [43] a huge surface area promotes platelet aggregation, forming a solid clot embolism, which promotes platelet aggregation and consequently helps in wound healing. In order to further probe the porosity, porosity % was determined using liquid displacement method and was found to be 55.99±2 % (supplementary data). Similar studies were conducted on porous cross-linked gelatin carriers by Lai *et al*., which yielded a porosity value in the range of 30-60 % [44]. The density of the sponge was calculated as per the equation given in materials and methods section and was found to be 0.017 g/cm$^3$.

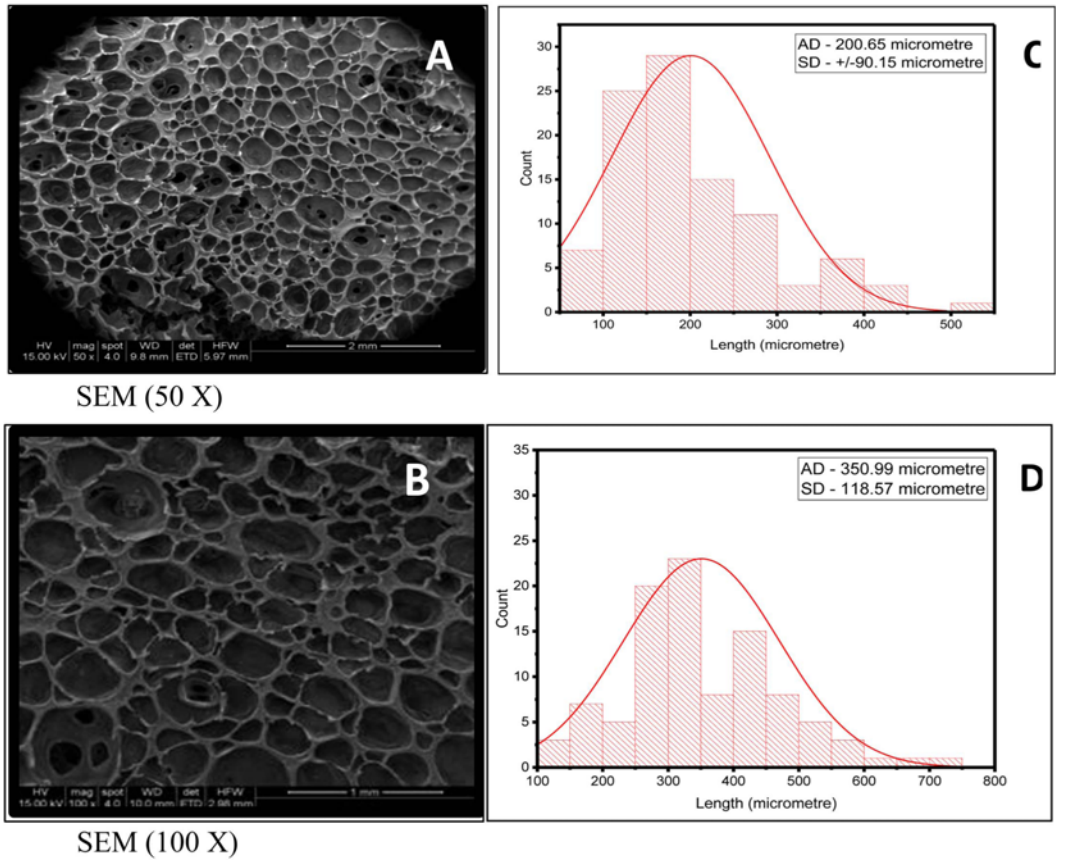


**Figure 1.** (A) SEM images of gelatin haemostatic sponge under 50 X magnification, (B) SEM images of gelatin haemostat under 100 X magnification, (C) pore size distribution of gelatin sponge under 50 X magnification, and (D) pore size distribution of gelatin sponge under 100 X magnification.

The FTIR spectra of gelatin and cross linked gelatin were compared and an increase in intensity in the case of the amide band I, II and III of gelatin sponge is seen (supplementary data). The cross-linking degree of gelatin haemostatic sponge was further quantified as 43.65±4 % using TNBS assay. Khadidja *et al*. [36] performed similar studies on various formulations of alginate/gelatin cross-linked systems and the cross-linking degree was found to be comparable.

***Mechanical Analysis***

Determination of break stress gives an idea regarding the strength of the sponge to withstand any rupture during its application. Break stress for gelatin sponge was found to be 0.066 N/mm$^2$; which is comparable to the already marketed products of gelatin haemostat - Abgel (Sri Gopal Krishna Labs Pvt. Ltd., Mumbai) and Spongostan (Johnson & Johnson Medical N.V., Belgium) with a break stress of 0.064 N/mm$^2$ and 0.051 N/mm$^2$ (supplementary data). Our own studies on chitosan haemostat also showed a break stress of 0.058 N/mm$^2$ [42]. Thus the haemostat has considerable strength to withstand any rupture during application and is comparable to marketed formulations. Since gelatin haemostat is used during surgery; mechanical property was found out in the wet state with a value of 0.0148 N/mm$^2$. Eventhough the tensile strength is lowered upon wetting the gelatin sponge, it shows considerable strength and ease of handling, and as such they can be applied directly to wounded site without any difficulty.

***Blood Absorption Studies***

According to British Pharmacopoeia; haemostats that absorb less than 12 g of liquid per 100 cm$^2$ is regarded as low absorbing capacity and more than 12 g per 100 cm$^2$ as of high absorbing capacity [45]. One gram of blood is regarded equivalent to 1 m*l* of blood. After 30 minutes of blood exposure, gelatin haemostat showed absorption of approximately 40 times its own weight of blood. Similar absorbing capacity was found for the marketed formulation ABGEL (Sri Gopal Krishna Labs Pvt. Ltd., Mumbai). After 24 hours, the gelatin sponge fell in weight by about 40-50 %, which can be attributed to the dissolution of the material in the blood. Both gelatin sponge and ABGEL were observed to cause clotting of blood despite the presence of an anticoagulant post 24 hours. A comparison of water and blood absorption capacity of gelatin sponge is given in Figure 2.

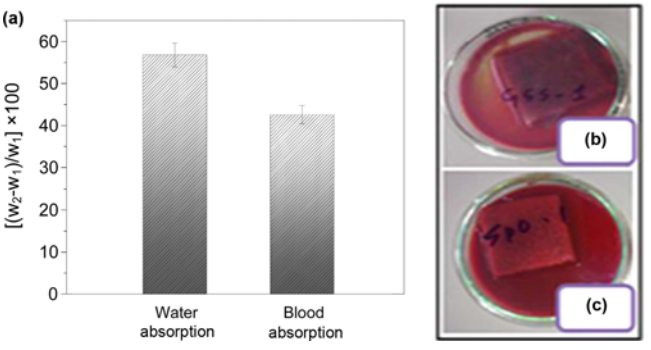


**Figure 2.** (a) Water and blood absorption capacities of gelatin sponge, (b) photograph showing blood absorption capacity of gelatin haemostat, and (c) photographs showing blood absorption of marketed formulation.

***In-Vitro Cytotoxicity Studies***

The *in-vivo* cytoxicity of a potential test substance is a complex process showing direct damage to cells as well as variations in physiological and biochemical functions along with inflammatory and other systemic effects happening at the site of application and also at other sites [46]. Generally *in vitro* cytotoxicity studies are conducted to quantify cell and tissue response, when a test substance is in contact with it and to calculate its toxic potential or probability for cell lysis [47]. Fibroblast cells upon direct contact with gelatin sponge induced less than 15 % cytotoxicity and showed slight or no significant effect in the inhibition of growth of cells upon indirect contact. The cytotoxic effect of the haemostatic sponge extract on the human foreskin fibroblast cell lines is presented as percent cell cytotoxicity with reference to a control sample. The average percentage cytotoxicity of gelatin sponge upon direct contact and indirect contact was calculated using the equation given in the materials section and was found to be 12.86 % and 3.02 % respectively. Thus the gelatin sponges were found to be non-cytotoxic (percentage cytotoxicity < 30 %), as the sample did not evoke any cytotoxic response upon direct and indirect contact with cells. The qualitative results of cytotoxicity by microscopic examination of the haemostatic sponge samples upon direct and indirect contact are as shown in Figure 3. A cytotoxic grade (Scale range: 0-4) is

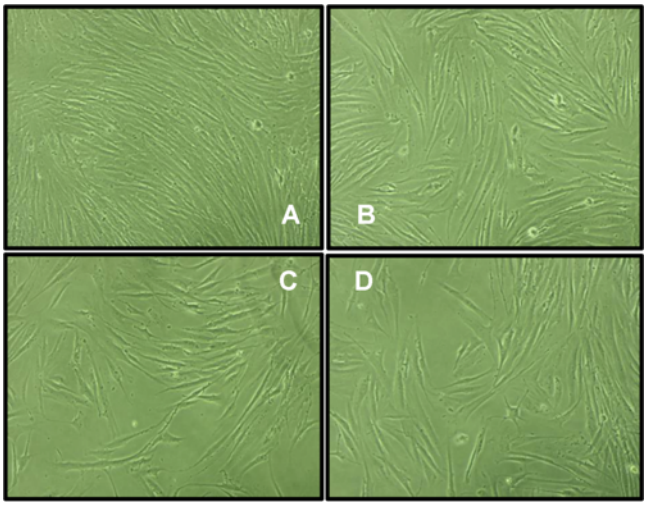


**Figure 3.** Microscopic evaluation of cell viability upon cytotoxicity studies; (A) control cells for direct contact study, (B) cell viability after direct contact, (C) control cells for indirect contact, and (D) cell viability after indirect contact study.

assigned to samples based on an estimated percent lysis and morphology of cells. The samples are found to be non-cytotoxic with a cytotoxic score ≤ 2 (≤ 50 % lysis).

### *In-vitro* Haemolytic Study of Gelatin Sponge

Haemolysis is regarded as a significant screening test to evaluate the measurement of RBC membrane's fragility when in contact with materials and devices [48]. Thus the development of new blood contacting biomaterials with an improved haemocompatibility not only increases the tolerability of the medical device but also minimizes any unwanted side-effects like thrombus formation [49]. Upon haemolysis, the released haemoglobin will cause serum/ plasma to appear pale red or cherry red in colour [50]. The average percentage haemolysis in plasma samples after 30 minutes exposure to gelatin sponge was found to be 0.03±0.02 % (n=3), while normal haemolysis with normal saline and isotonic sodium acetate solutions showed haemolysis < 0.1 %. [51]. Hence the data suggests that the gelatin sponge samples offer an acceptable level of haemolysis and is a non-haemolytic [52]. Timmons performed haemolytic tests on various alginate dressings and the test criteria for non-haemolytic samples was set at <5 % [53].

### *Skin Irritation Study on Albino Rabbits*

Cytotoxicity, skin irritation and skin sensitization tests are mandated for all medical devices by the International biocompatibility standards ISO 10993-1 "Guidance on the Selection of tests", and its FDA counterpart blue book memorandum #G95-1. Upon contact with chemicals released from medical devices, the body may produce a local tissue response characterized by usual signs of infection like redness and swelling (54). The skin irritation tests can serve as a fast and easy way to substantiate the safety of blood-contacting medical devices prior to their use. This study was conducted in accordance with ISO 10993:10: Biological evaluation of medical devices - Tests for irritation and delayed-type hypersensitivity, IAEC approval No: HLL/ CRDC/SAEF/IAEC/01/02/2015. The extent of skin reaction in humans can be directly correlated to the amount of irritability response in an animal [55]. In this study animals were observed for 3 consecutive days for morbidity, mortality, abnormal clinical signs and for the classic acute skin reactions following topical administration of gelatin haemostat. The observed skin reactions were then graded and primary irritation index was calculated. The dermal irritation scores were evaluated in conjunction with the nature and severity of lesions, and their reversibility or lack of reversibility (Table 1). The results indicated that animals treated with gelatin haemostat neither showed any skin reactions like erythema or eschar formation, oedema etc. nor any abnormal clinical symptoms as shown in Figure 4. The primary irritation index of test and control was obtained by adding scores for an animal and dividing the sum of all scores by 6 (taking into account two test/observation sites and three time points). The experimental animals used in the study showed no signs of morbidity or mortality and no signs of illness or overt toxicity. Hence it was concluded that the haemostatic device was a non-skin irritant under standard conditions of the study.

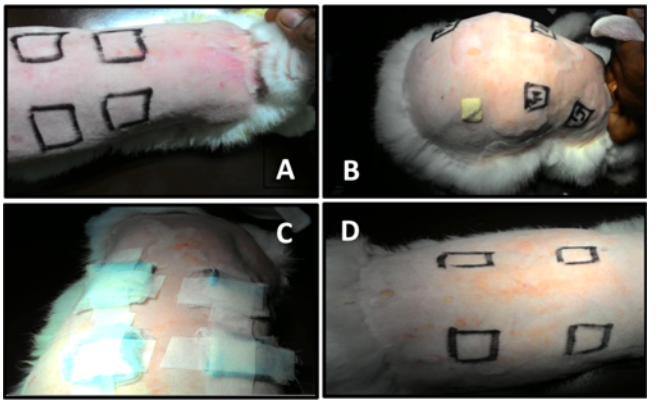


**Figure 4.** Skin irritation study on albino rabbits; (A) topical surface before application of sponge, (B) after application of sponge, (C) sponges left to be in contact with skin, and (D) topical surface of rabbit after 72 hours.

**Table 1.** Individual grades of skin reactions based on skin irritation study in albino rabbits

| Skin reaction | Observation time (h) | Individual score | | | | | |
|---|---|---|---|---|---|---|---|
| | | Animal No. 1 | | Animal No. 2 | | Animal No. 3 | |
| | | C | T | C | T | C | T |
| Erythema and Eschar formation | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 48 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 72 | 0 | 0 | 0 | 0 | 0 | 0 |
| Oedema formation | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 48 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 72 | 0 | 0 | 0 | 0 | 0 | 0 |

C: Control site and $T_1$: Test item site.

***Skin Sensitization Test***

Skin sensitization is a hypersensitivity reaction which usually occurs as a result of prolonged or repeated contact of a chemical substance with body's immune system [56]. The haemostatic devices is tested for the presence of sensitizing chemicals using guinea pigs, a species known to be nearly as responsive to dermal sensitizers as human beings are [57]. This study was conducted in accordance with ISO 10993:10: Biological evaluation of medical devices - Tests for irritation and delayed-type hypersensitivity, IAEC approval No: HLL/CRDC/SAEF/IAEC/01/03/2015. This method can be used for the identification of contact sensitizers in order to establish whether a chemical is a skin sensitizer or not. The results of skin sensitization indicated that animals treated with gelatin sponge or reference control did not show any significant sensitization reactions as shown in Figure 5. Post sensitization reactions, dorsal skin surface of guinea pig was evaluated for histopathological changes. There was no significant difference in the structural pathology of the test as well as control animal as shown in Figure 6. Epidermis of the test as well as the control skin sample was observed as intact stratified squamous epithelium with the most of the superficial keratin layer present and the stratum basale close to the dermis. The dermis contained occasional mononuclear cells infiltration in the papillary layer, while rest of dermis contained collagen, intact hair follicles and glands. Based on such findings the gelatin haemostatic sponge is classified as a non-skin sensitizer as per the Magnusson and Kligman classification, since 0 % sensitization rate was observed in the evaluation of challenge application.

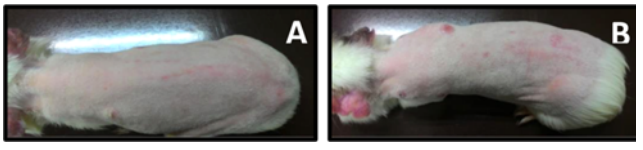


**Figure 5.** Skin sensitization test on Guinea pig; (A) dorsal surface of guinea pig before sensitization test and (B) dorsal surface of guinea pig post sensitization test.

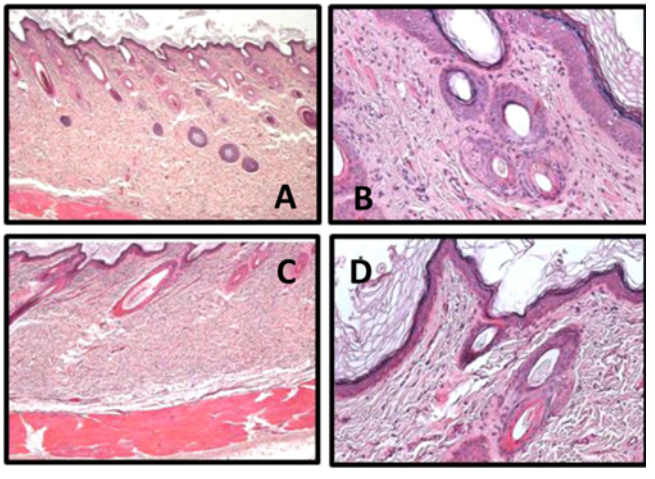


**Figure 6.** Histopathological evaluation of dorsal skin surface of guinea pig post skin sensitization reactions: A&B represents dorsal skin surface post application of control, while C&D represents test formulation.

## Conclusion

The present study was conducted to evaluate the gelatin sponge developed by the controlled freeze drying process. Physical characterisation, along with *in vitro* and *in vivo* evaluations were conducted to prove its biocompatibility. Controlled freeze drying results in a soft pliable gelatin sponge which can be applied easily on the wounds. Gelatin sponge is a good haemostatic agent with a porous structure enabling high blood absorption capacity. The sponge has comparable tensile properties to other gelatin based products available in the market and is found to be non-cytotoxic as well as non-haemolytic. Since gelatin sponge will be applied on the skin for arresting bleeding; it was very important to conduct the sensitization and irritation studies. The study results prove that the haemostatic device is a non-skin irritant and a non-skin sensitizer. Hence all the studies conducted as per ISO 10993: cytotoxicity, skin irritation, skin sensitisation and blood compatibly studies prove gelatin sponge to be biocompatible.

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**Electronic Supplementary Material (ESM)** The online version of this article (doi: 10.1007/s12221-021-0268-7) contains supplementary material, which is available to authorized users.

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