RESEARCH PAPER

Physicochemical Evaluation and in Vitro Hemocompatibility of Goat Gelatin: Bioactive Dressing to Promote Wound Healing

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Abstract: *Gelatin (GEL) is extensively used in various fields, particularly in therapeutics and pharmaceuticals. In this study GEL was extracted from goat skin using hot temperature extraction process and compared with that of commercial GEL. The physico-chemical characterization and functional properties were investigated by using temperature denaturation (T_d), water-holding and fat-binding capacities (WHC and FBC), colour measurement and UV-light spectrum. In vitro biocompatibility was studied for the first time and was evaluated by blood coagulation index (BCI) and haemolytictests for wound dressing applications. The results revealed thermal stability of goat GEL at T_d 37°C. WHC and FBC capacities were determined as 2.5 and 1.2 g/ml, respectively. The hunter colour spaces a*, b* and L* were -0.27, -1.97 and 25.23respectively. UV-Vis absorption spectrum of the goat GEL showed a maximum absorption peak at 280 nm. The in vitro anticoagulant activities of extracting GEL were higher than 70% after incubation for one hour. After being in contact with red blood cells for 1 h, the haemolysis ratio increased from to 0.46 to 1.4 when the concentration of goat GEL increased from 1 to 50 mg/ml suggesting the safety of the tested samples. These results suggest that thromboresistivity and hemocompatibility of this biopolymer retained the biological activity of our samples for biomaterial applications. According to this, goat GEL successfully competes with, and significantly could be useful for substitution of bovine in wound healingapplications.*

Keywords: *Goat Gelatin, Biocompatibility, Natural Polymer, Wound Dressing.*

1. INTRODUCTION

Biomaterials are substitutes for biological tissues in the human body that have the ability to interact with the body [1]. They can either be natural or synthetic [2]. Gelatin (GEL) is one of the most commonly used biopolymers in medical applications and pharmaceuticals [3]. Being natural biomaterials, GEL contains a number of functional groups, such as amino acids. This property makes GEL the main choices for tissue engineering applications [4]. GEL has shown promising results for skin wound healing. The largely used, studied and commercialized is the bovine GEL [5]. However, bovine products are deeply evaded in Europe and the United States owe to concerns with mad cow illness and bovine spongiform encephalopathy (BSE), which is a mortal neurodegenerative infection in cattle that induces spongy degeneration of the brain and spinal cord [6]. Search for new gelling agents to replace bovine GEL was appeared. The goat GEL is still at thepremature levels and lack large-scale clinical trials. For that, in the present work, goat GEL is studied and the analysed physicochemical properties correlated with those of biological properties to have anideal scaffold for wound healing. The conventional wound dressings, such as paraffin gauze, traditionally cause trauma to wounds at dressing change [7]. Several modern dressing types still give rise to pain. Subsequent experimental data have identified the characteristics required for an ideal dressing. Advanced wound care has been developed to provide modern dressings that effectively interact with the wound environment to aid healing. Some researchers showed better gelation capacity than commercial bovine GEL [8]. At pH7, the minimum protein concentration needed to obtain GELs was lower in goat GEL than in bovine GEL and at pH 4, goat GEL were firmer than bovine GEL gels [8]. According to these gelation properties, goat GEL could be very useful in the pharmaceutical industry. The objectives of this

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work were to evaluate the main characteristics (functional properties, colour, and biocompatibilityfor the first time of heat-induced gels from goat and so to determine if goat GEL could be useful for substitution of bovine in wound healing.

2. MATERIAL AND METHODS

2.1. Gelatin extraction

Gelatin was extracted from the goat skin by extrusion-pre-treatment and hot water extraction as described previously with slight modifications [9]. About 50grams of goat skin were soaked in NaOH (0.5 M) at 4°C (1:5, w: v) for 48 hours. The soaked sample was then washed until the pH neutralisation. GEL was extracted by stirring the skin in distilled water (1:5, w: v) at 60°C for 6 h. The supernatant was filtered using filter paper in order to remove insoluble materials. The prepared samples were frozen at -20°C, and dried. GEL powder was subjected to analyses.

2.2. Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described previously with some modifications [10]. Ten milligrams of Goat GEL was dispersed in 1 ml of 5% (W/V) SDS solution. The mixtures were incubated at 85°C for 1 h and centrifuged at 5000 x g for 10 min to remove un-dissolved debris. The samples were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8) containing 5% SDS (w/v) and 20% glycerol (v/v) at the ratio of 4:1 (v/v). Sample was then loaded onto polyacrylamide gels (8% resolving gel, 3% stacking gel) and electrophoresed using a Bio-Rad electrophoresis under a constant voltage of 100 V. The gel was then stained with 0.1% (w/v) Coomassie blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for one hour followed by 3 hours of de-staining in 30% (v/v) methanol and 10% (v/v) acetic acid.

2.3. Colour measurement

A colorimeter was used to determine the colour of the goat GEL as described previously [11] using aportable Konica Minolta colorimeters CR 300. The values of L^*, a^*, b^*, and ΔE were recorded and three replicate samples were tested. The white board was used as a reference.

2.4. Denaturation temperature (T_d)

Denaturation temperature (T_d) was measured following the methods previously described [12]. The viscosity of the goat GEL solution (0.6%: W/V) at different temperatures (from 4°C to 44°C with a heating rate of 4°C) was measured using rotational viscometer (Brookfield; 20 rpm, mobile S1). Sample suspension was incubated for 30 min before to viscosity determination at the designated temperature and T_d was defined as the temperature at which the relative viscosity was 50% in comparison with that obtained at 4°C.

2.5. Determination of water-holding and fat-binding capacities

To be used as biomaterial for tissue engineering, the hydrophobicity of goat skin gelatin, is one of the most important factors responsible for the absorption and transport of metabolites and cell nutrients. Water-holding (WHC) capacity and fat-binding capacity (FBC) were determined as described by [13]. In brief, 0.5 g of dried gelatin was immersed in 50 ml of distilled water or 10 ml of soybean oil and kept at room temperature for 1 h. After the centrifuged at 450 × *g* for 20 min, the upper phases were removed. The difference between the initial volume of distilled water or oil added to the gelatinsolution and the volume of the supernatant after incubation was determined. WHC and FBC were reported as ml of water or fat absorbed per gram of goat GEL. The experiments were conducted in triplicate.

2.6. Spectre UV-light

The UV absorption spectra of goat GEL was determined using the method of [14]. The sample was prepared by dissolving the dried GEL in deionized water with a sample/ solution ratio of 1:1 000 (w/v).The supernatants were collected after centrifugation at 18,000 rpm at 4°C for 5 min. Then, UV absorption was measured from 200 to 400 nm

2.7. Biocompatibility

The blood biocompatibility of goat GEL was performed by haemolysisand BCI assays.

2.7.1. Haemolysis

The haemostatic performance of goat gelatin was tested *in vitro* as described previously [15]. 800 µl of goat gelatin at different concentrations (1 mg/ml, 3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml and 50 mg/ml) were incubated with 200 µl of fresh rabbit whole blood (whole blood: normal saline= 8:10) at 37°C for an hour. After that, the

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mixture was centrifuged at 4000 rpm for 5 min and the absorbance of supernatant was measuredat 545 nm. Distilled water and normal saline added with whole blood were used as positive and negative controls, respectively. The experiments were run in triplicate. Haemolysis rate (HR) was calculated as follows:

$$HR= [(AS - AN) / (AP - AN)] X 100\%$$

Where AS, AP and AN presents the absorbance of the goat GEL at different concentrations, the positive control and the negative control, respectively.

2.7.2. Blood anticoagulantIndex

Blood anticoagulant index was investigated according to the method reported by [16]. Briefly, 100 µl of goat GEL at different concentrations (1 mg/ml, 3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml and 50 mg/ml) were incubated with 100 ml whole blood anticoagulatedby 3.8 wt% citrate sodium for 1 min. 25 µl of $CaCl_2$ solution (0.1 M) was then added to each sample. Samples were taken out at indicated time points (5, 10, 30, 40 and 60 min) after the addition of $CaCl_2$. After the incubation, each sample was immersed into 30 ml deionized water and the absorbance (AS) of the suspension was measured at 545 nm.

The blood anticoagulant index (BCI) was determined as follows:

$$BCI= AS / Aw \times 100\%$$

Where Aw represents the absorbance of the solution containing 100 µl whole blood and 30 ml deionized water. Then, the BCI values were plotted against the corresponding time points.

3. RESULTS AND DISCUSSION

3.1. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The protein pattern of GEL extracted from goat skin using acid pre-treatment is shown in Figure 1. Pre-treated skin sample clearly showed thepresence of α and β chains. In fact, the stabilizing hydrogen bonds of collagen are destroyed by heating at a higher temperature (60°C) resulting in the conversion of native helix to coil structure, which leads to the transformation of collagen to gelatin. Similar protein patterns were found by Mad-Ali et al [17].

3.2. Colour

Colour of Goat gelatin and commercial Bovine gelatin expressed as L*, a* and b*are shown in Table 1. Lower L* and b*-values of GoatGEL was observed, compared with Bovine GEL. The variation in colour characteristics was affected by different factors such as raw material and extraction process. However, as reported previously, changes in colour did not affect the biological properties of gelatine [18].

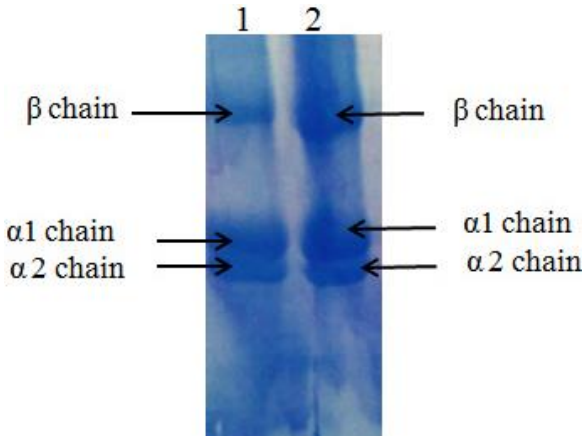

Fig. 1. SDS-PAGE pattern of Goat gelatin (Goat GEL) (line 1) and commercial Bovine GEL (line 2).

Table 1. Colour characteristics of gelatin extracted from goat skin (Goat GEL) in comparison with commercial Bovine gelatin (Bovine GEL)

Colour	Goat GEL	Bovine GEL
L*	25.23	35.1
a*	-0.27	-0.13
b*	-1.97	13.75

3.3. UV–Vis spectroscopy analysis

The UV-Vis absorption spectrum of the goat GEL was presented in Figure 2. The maximum absorption peakwas determined at 280 nm. It is well known that the maximum absorption wavelength of protein in the near ultraviolet region is 280 nm because of the absorbance of aromatic amino acids such as Phe, Trp and Tyr [19]. Previous reports indicated that if the protein is a collagen and not a gelatin it should have a maximum absorption near 210– 240 nm Which was related to the groups C=O, –COOH and $CONH_2$ in polypeptide chains of collagens [14].

3.4. Denaturation temperature

The thermal stability of goat GEL (T_d) is revealed in Figure 3. Bovine gelatine (Bovine GEL) was used as a control. As shown in Figure 3, the T_d values of goat GEL and Bovine GEL were 37°C and 38°C, respectively. These results were in concordance with the previous studies on

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mammalian GELs [20]. However, goat GEL showed higher thermal stability as compared to gelatin extracted from fish species such as *skipjack tuna* (29.7°C), *Japanese seabass* (26.5°C) and *Paper nautilus* (27°C). It is reported that the difference in the thermal stability between GEL of different species was associated with the content of amino acids especially hydroxyproline and proline [21]. Thermal stability plays a key role to preserve the biological, mechanical and physicochemical properties of GEL during themanufacturing process for tissue engineering applications. GEL having highly T_d is mostly preferred. Therefore, T_d value is an important property in stabilising the triple helix structure of GEL. Our result confirmed that the helix structure of goat GEL, was more stable than those extracted from fish species and are of particular interest for biomedical applications such as wound healing dressing.

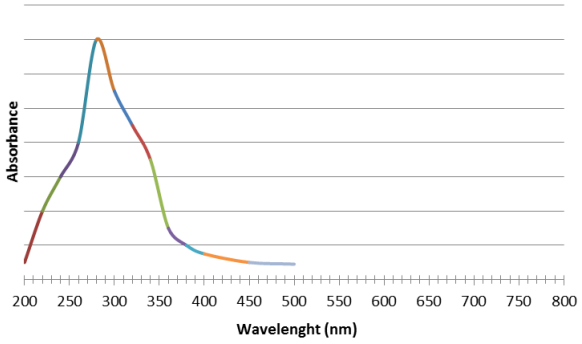

Fig. 2. UV spectra of goat gelatine from 200 to 400 nm

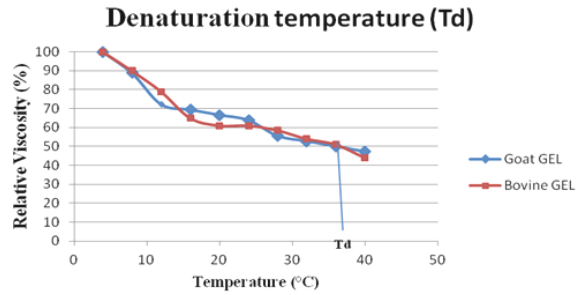

Fig. 3. Relative viscosity changes of goat gelatine (%) in comparison with bovine gelatine at different temperatures (from 4°C to 44°C with a heating rate of 4°C).Denaturation temperature (T_d) was determined as 50% of relative viscosity.

3.5. Water-Holding and Fat-Binding Capacities

WHC and FBC of goat GEL are shown in Figure 4. GEL presents an important water holding capacity. A value of 5– 10 times of water absorption of its own weight was observed. This is a key factor which is highly desirable for wound-healing applications. The WHC of a biopolymer may reflect its behaviour in an aqueous environment. The WHC ratio of goat GEL was 2.5. Similar result was obtained with gelatin extracted from Golden grey mullet [22]. This high value could be due to hydrophilic amino acids, particle size and the great number of pores in the GEL structure [23]. The excellent WHC suggested that the goat GEL could retain its behaviour onto the surface resulted in stabilisation of material for a longer period in aqueous environment. The ability to absorb oil is another essential characteristic for wound dressingfor absorbing exudates, metabolites, and body fluids. The FBC (1.2) of goat GEL was lower than Golden grey mullet and thornback ray skin gelatins [24]. The difference in WHC and FBC may be attributed to the presence of hydrophilic groupsand hydrogen bonding forces that are responsible for lipid-protein interactions [25-26].

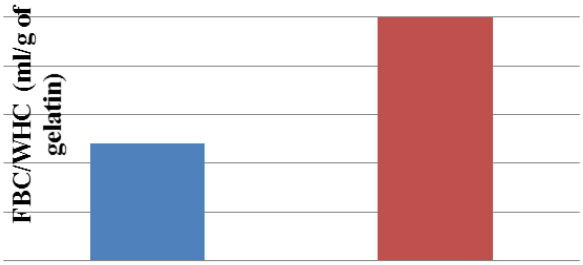

Fig. 4. Water-holding (WHC) and fat-binding capacities (FBC) of goat gelatine after incubation for 1 hour at room temperature.

3.6. Biocompatibility

3.6.1. Determination of Haemolysis Assay

The effect of goat GEL on blood coagulation time was investigated *in vitro*, as shown in Figure 5. The investigation results revealed that goat GEL had a higher procoagulant effect when compared with the negative control. In general, after being in contact with red blood cells for 1 h, the haemolysisrates of all goat GEL concentrations were below 1.8% and no visible hemoglobin release was observed. The results of the haemolysis assay in the present study were in accordance with to ISO/TR 7406 [the critical safe biomaterials haemolytic ratio (5%) suggesting the safety of the tested samples [27]. Furthermore,

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our results are consistent with other reported studies regarding gelatin based-membranes [28].

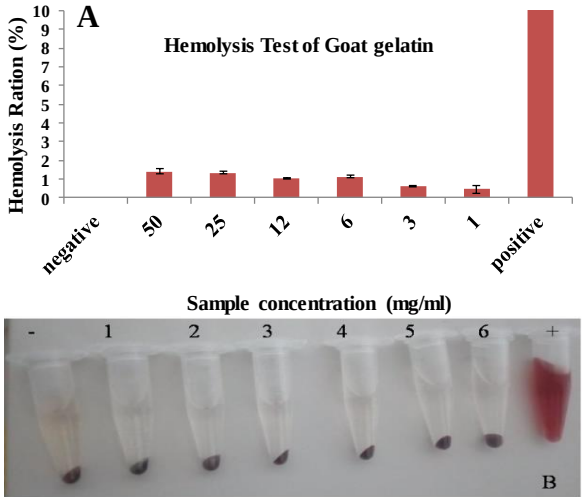

Fig. 5. (A) Percent haemolysis of RBCs incubated with different concentrations (from 1 to 50 mg/ml) of goat gelatin. (B) Photographs of the centrifuged erythrocytes after incubation with goat gelatin for one hour (from the left to right: negative control, 1 mg/ml, 3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml, 50 mg/mland positive control).left; where 1 represents the positive control, and 2 represent the negative control).

3.6.2. Determination of blood coagulation index (BCI)

BCI is an important indicator of biosafety assessment of materials. Haemolysis reaction was initiated when erythrocytes in uncoagulated blood exposed to deionised water. As a higher BCI value indicates better thromboresistance of the material contacted with blood [29]. Thrombus formation and dependence of BCIon time is shown in Figure 6. Goat GEL at different concentrations showed higher BCIvalues, and decreased slightly with the increase of contact time. Goat GEL did not influence red blood cells at high concentration (50 mg/ml). These results suggest that thromboresistivity and hemocompatibility of this biopolymer retained the biological activity for biomaterial applications.

4. CONCLUSIONS

In this study, goat GEL was successfully extracted using thermal extraction. The physico-chemical properties and its hemocompatibility were determined. Protein patterns of goat GEL showed the presence of α and β chains as major compounds. Desired physicochemical properties were found indicating satisfactory T_d (37°C), water-holding and fat-binding capacities and colour measurement, witha maximum absorbation peak at 280 nm. The goat GEL showed less than 5% haemolysis, suggesting that it is highly hemocompatible. The blood anticoagulant index demonstrates that the blood incubated with the goat GEL did not coagulate. This study summarizes that the extracted goat GEL is a highly hemocompatible biomaterial and could potentially be utilized as a potential substitution of commercial analogues in biomedical applications such as wound healing dressings.

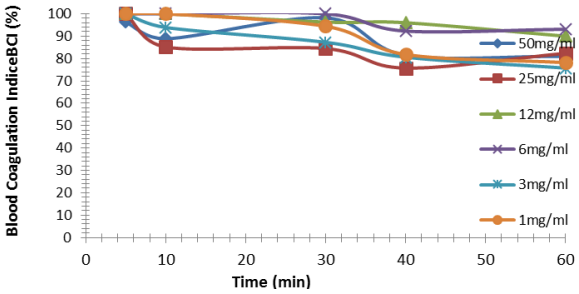

Fig. 6. Blood coagulation process for goat gelatin at different concentrations (1, 3, 6, 12, 25 and 50 mg/ml)and times (5, 10, 30, 40 and 60 min).

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