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Rejoining of cut wounds by engineered gelatin–keratin glue

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ABSTRACT

Background: Rejoining of cut tissue ends of a critical site challenges clinicians. The toxicity, antigenicity, low adhesive strength, flexibility, swelling and cost of the currently employed glue demands an alternative. Engineered gelatin–keratin glue (EGK-glue) described in the present study was found to be suitable for wet tissue approximation.

Methods: EGK-glue was prepared by engineering gelatin with caffeic acid using EDC and conjugating with keratin by periodate oxidation. UV–visible, ^{1}H NMR and circular dichroism analyses followed by experiments on gelation time, rheology, gel adhesive strength (*in vitro*), wet tissue approximation (*in vivo*), H&E staining of tissue sections at scheduled time intervals and tensile strength of the healed skin were carried out to assess the effectiveness of the EGK-glue in comparison with fibrin glue and cyanoacrylate.

Results: Results of UV–visible, NMR and CD analyses confirmed the functionalization and secondary structural changes. Increasing concentration of keratin reduces the gelation time (<15 s). Lap-shear test demonstrates the maximum adhesive strength of 16.6 ± 1.2 kPa. Results of hemocompatibility and cytocompatibility studies suggested the suitability of the glue for clinical applications. Tissue approximation property assessed using the incision wound model (Wistar strain) in comparison with cyanoacrylate and fibrin glue suggested, that EGK-glue explicitly accelerates the rejoining of tissue with a 1.86 fold increase in skin tensile strength after healing.

Conclusions: Imparting quinone moiety to gelatin–keratin conjugates through caffeic acid and a weaker oxidizing agent provides an adhesive glue with appreciable strength, and hemocompatible, cytocompatible and biodegradable properties, which, rejoin the cut tissue ends effectively.

General significance: EGK-glue obtained in the present study finds wide biomedical/clinical applications.

1. Introduction

Adhesives in modern surgical practices are unavoidable and they are majorly used as an adjunct in several surgical procedures (tissue approximation, wound closure/sealant, grafting and hemostatic procedures) [1,2]. Tissue approximation using glues/sealants of both natural and synthetic origin are currently in use. Cyanoacrylate has long been recognized as a performing synthetic adhesive for external use. Despite the enormous adhesive strength, release of toxic components, insufficient flexibility and lipid membrane damage prevents cyanoacrylate from internal applications [3,4]. Although adhesives of biological origin (fibrin glue, gelatin resorcinol formaldehyde, glutaraldehyde stabilized bovine serum albumin) were found helpful, the biocompatibility, low adhesive strength, immune response and potential infection restricts the clinical applications and thus demands high strength, non-toxic and safe agents for tissue approximation. Since, the internal wound milieu is completely different from the external; agents with additional characteristic features (high adhesive strength and compatible with wet tissue surface) are highly required. Hence, approaches were made to mimic the natural adhesives (adhesives of sessile organisms, mussels, and sandcastle) [5–8] which should also ensure that the demands on efficacy, safety, usefulness, ease of preparation, cost and regulatory approvability in addition to different surgical applications could be met. It has been found that these organisms exhibit adhesive properties because of the presence of diversified functional groups (–NH_2, –SH, –COOH, –OH, Aromatic-OH) of proteins present at the interface of glue and substrate. Thus, proteins with a wide variety of functional groups are well suited for the fabrication of adhesives. However, the utility of many proteins is limited due to inconsistencies in composition, performance, limited supply and high cost compared to synthetic adhesives. Despite this, engineering the proteins for tissue approximation for both external and internal applications, especially in wet tissue surface is still going on.

Gelatin, a denatured product of the matrix protein collagen was widely explored in the field of food and biomedical technology [9]. The resorbable, low immunogenicity nature of this protein is used to prepare scaffolds, wound dressing material and hydrogels. Gelatin/collagen has long been used to prepare surgical adhesives however, without a crosslinker gelatin alone cannot be used as tissue glue [10]. But, in the presence of enzymes (transglutaminase) and chemical crosslinkers (EDC/NHS, glutaraldehyde, resorcinol/formaldehyde, genepin) gelatin can be transformed into an adhesive product, however, the major

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disadvantages are the presence of aldehydes and byproducts, low adhesive strength, flexibility and swelling.

Caffeic acid, is a bi-functional plant phenolic, widely present in vegetables, fruits, coffee, tea, olive oil, red wine etc. The presence of dihydroxyl groups and α,β unsaturated carboxylic group of caffeic acid imparts several functions like wound healing, antimicrobial, antioxidant, anti-inflammatory, antitumor, antianxiety, antimetastatic and MMP 2,9 inhibitor activities [11–13]. Most importantly, caffeic acid has a similar structural and functional resemblance to DOPA, a potential candidate for imparting adhesive strength and curing rate to mussel adhesives [14].

Keratin is a fibrous structural protein providing outer coverings such as hair, wool, feathers, nail, and the horns of mammals, reptiles and birds. The disulfide bonds in keratin contribute strength to the hair. It has been employed as a biomaterial in the form of 2D and 3D scaffolds and hydrogels [15–17]. Keratin and gelatin blended films are also used to prepare 2D film and skin graft mesh to treat wounds [18,19]. However, for the preparation of surgical glues no reports are available on the use of keratin alone or in combination with natural proteins/polysaccharides.

In the present study, an attempt was made to prepare a protein based adhesive with high strength and compatible for wet tissue approximation. In brief, both gelatin and keratin proteins were interacted *via* (i) functionalization using bi-functional phenolics, followed by (ii) oxidation (either chemical/enzymatic), and the resultant glue like material, hereby named as EGK-glue (engineered gelatin–keratin-glue) was subjected to wet tissue approximation using both *in vitro* and *in vivo* models. Mechanical property (rheology and tensile strength), *in vitro* biodegradability and biocompatibility (cyto and hemo-compatibility) were also assessed in addition to ^{1}H NMR and circular dichroism analyses to demonstrate functionalization and interaction.

2. Materials and methods

2.1. Materials

Gelatin, type A (300 bloom strength), EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), NHS (*N*-hydroxysuccinimide), caffeic acid and collagenase, type I (*Clostridium histolyticum*), were purchased from Sigma Aldrich, USA. MES (2-(*N*-morpholino)ethane sulfonic acid), and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid) were purchased from HiMedia, India. All other chemical reagents were of analytical grade and commercially available. Periodate was obtained from Sd Fine Chem, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma, USA. Ketamine hydrochloride was obtained from Neon Laboratories, Mumbai, India. Fibrin glue was obtained from Reliance Life Sciences, Mumbai, India, in the form of Reliseal and cyanoacrylate was obtained from Ethicon Inc. in the form of Dermabond.

2.2. Preparation of EGK-glue

2.2.1. Functionalization of gelatin using caffeic acid

Caffeic acid functionalized gelatin was prepared using EDC–NHS [20]. In brief, caffeic acid (0.1 M) dissolved in 0.1 M MES buffer (pH 5.5) was treated with EDC (0.2 M) and NHS (0.2 M) and stirred for 45 min at 25 °C and then mixed with gelatin dissolved in HEPES buffer (pH 7.0). The resulting solution was extensively dialyzed against 5 mM HCl and then against the same medium containing 1% NaCl. Final dialysis was made against 0.1 M phosphate buffer (pH 6.5) and the dialyzed samples were freeze dried, stored at 4 °C and hereby named as functionalized gelatin.

Proton NMR spectra of gelatin, functionalized gelatin and caffeic acid was recorded at an operating frequency of 500 MHz in a JEOL ECA-500 FT NMR spectrometer. Ten milligrams of gelatin, functionalized gelatin and 1 mg of caffeic acid was dissolved in 750 μl D_2O individually and ^{1}H-spectra was recorded by keeping Tetramethylsilane (0.01%) as an internal reference and at 45 °C (for better solubility) without suppressing the water signal. The acquisition parameters for (^{1}H) NMR were as follows: 32 scans for signal to noise averaging, relaxation time of 5 s, sweep width of 20 ppm, number of data points was 16,384 and 45 pulse width is 6.5 μs.

UV–visible spectrum for gelatin, functionalized gelatin and caffeic acid was recorded using UV-2450 (Shimadzu, Japan). In brief, caffeic acid (1 μg), gelatin and functionalized gelatin (1 mg) were dissolved separately in 1 ml of phosphate buffer (pH 6.5). The spectra were recorded in the wavelength region of 200 to 600 nm by keeping the buffer as a blank.

2.2.2. Keratiene (reduced form of keratin) preparation

Human hair keratin was extracted according to the Shindai method [21]. In brief hair was obtained from a local hair salon, intensively washed with water containing 0.5% SDS, rinsed with fresh water and air-dried and then treated with chloroform and hexane to remove the external lipids and dried at 50 °C. About 10 g of hair was cut into small pieces and mixed with the 250 ml of extraction medium containing 10 M urea, 5% SDS, 5% 2-mercaptoethanol. The mixture was kept at 50 °C for 24 h and centrifuged at 5000 rpm for 10 min. The supernatant was dialyzed against water, until the keratin solution was free from mercaptoethanol. The dialyzed solution was concentrated using an ultraconcentrator using 10 kDa cut off membrane. The resulting thiol functionalized keratin (keratiene) solution was used for conjugation with functionalized gelatin.

2.2.3. Conjugation of caffeic acid functionalized gelatin and thiol-functionalized keratin

Different percentage weight ratios, 10:0, 10:2, 10:3, 10:4, 10:5 and 10:6 respectively to functionalized gelatin and keratin in 0.1 M phosphate buffer (pH 7.5) were taken and then treated individually with 1.0% sodium meta periodate. The time taken by the proteins to transform from solution to gel state (by inverting the reaction tube) was measured as curing (gelation) time. The resulting product was named as EGK-glue.

2.3. Surface morphology of EGK-glue

The surface morphology of the EGK-glue was studied using a scanning electron microscopy (SEM). The EGK-glue sample was freeze-dried and the cross sections of the freeze-dried samples were placed on the carbon ribbon and gold coated. The cross sectional view was observed and captured at different magnifications using a HITACHI-S3400N SEM operated at 5 kV.

2.4. Circular dichroism

All circular dichroism (CD) measurements were performed using a Jasco J715 spectropolarimeter at room temperature, using a circular quartz cell with a path-length of 0.1 cm. The instrument was calibrated with ammonium D-camphor-10-sulfonate as described by the instrument manufacturer. All CD spectra were measured between 190 and 300 nm with a scanning speed of 100 nm/min. The bandwidth, response time and data pitch were set to 1 nm, 1 s and 0.5 nm, respectively. All CD spectra represent the average of three individual scans and all the spectra were solvent subtracted.

2.5. Rheological study

Rheological analysis was carried out using an oscillatory rheometer (Anton Paar Rheometer MCR-301) with a cone and plate geometry of 1°, 25 mm diameter with 0.1 mm gap. The experiments were carried out at 37 °C using 1% strain and the frequency was varied from 0.1 to 100 rad/s. The change in viscoelasticity was recorded as