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A Novel Topical Biocompatible Tissue Adhesive Based on Chitosan-modified Urethane Pre-polymer

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ABSTRACT

A novel tissue adhesive composed of urethane pre-polymer and chitosan gel was synthesized in a two-step procedure. The first step of the procedure was carried out under nitrogen atmosphere at 60°C and 24 h and the final formulation was prepared using a urethane pre-polymer and 2% chitosan gel. The presence and quantity of NCO groups in the adhesive were determined using FTIR spectroscopy and titration methods. The presence of the urethane band and NCO free groups in the adhesive were confirmed at 1514 cm^{-1} and 2261 cm^{-1}, respectively and the quantity of NCO free groups was calculated as 21%. The molecular weight of urethane pre-polymer in the adhesive was measured 2444.7 g/mol using GPC. The relative peel resistance of the adhesive bonds between flexible adherents was 15.1 N for urethane pre-polymer and 14.6 N for the final formulation of tissue adhesive. Chitosan gel was used in this tissue adhesive to increase its biocompatibility. The structure of the synthesized adhesive which exhibited a porous structure due to the presence of chitosan gel was illustrated using SEM technique. The surface energy of the adhesive which was lower than its values for gelatin, skin and blood was determined by contract angle measurment as 33.65 mN/m. Cytotoxicity of the tissue adhesive was determined on growth and viability of various cells, using $CaCO_2$, T47D, HT29 and NIH3T3 cell lines which showed adhesive's non-cytotoxic nature. The skin irritation test, carried out according to ISO 10993-10, suggested that the compound is non-irritant.

Key Words:

tissue adhesive;
urethane pre-polymer;
chitosan;
biocompatible;
biodegradable.

INTRODUCTION

Tissue adhesives serve as suitable alternatives to suturing or stapling [1-3]. They represent fast and easy closure methods which are less painful and do not require local anesthesia, thus, eliminating the problems of suture application and removal [4].

Tissue adhesive is defined as any substance with characteristics which allow adherence of two substrates together [5] and have been developed as an advanced method for the approximation of superficial wounds, optimally those that are almost linear or demonstrated minimal or no tension [6-9].

In the past few decades, many synthetic or natural tissue adhesives, e.g., cyanoacrylate-based glue [10-13], biomacromolecules and aldehyde-based glue [14], fibrin-based [15,16] and other glues

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[17,18] have been developed and applied in the biomedical field. Among these tissue adhesives, fibrin and cyanoacrylate-based adhesives are the main and most popular type to be used.

Because of their biocompatibility, fibrin adhesives have been applied in pterygium surgery [19], ophthalmology [20], cardiovascular surgery [21], nerve cell reconnection [22], etc. Furthermore, Cyanoacrylates have been used widely as tissue adhesives [23]. Their advantages include speedy wound closure, decreased pain scores, and equivalent cosmetic outcomes [24,25]. However, there are limitations that restrict the use of these adhesives, i.e., cyanoacrylate is not biodegradable and releases formaldehyde upon degradation [26,27] which causes chronic inflammation and delays wound healing due to residual reactions [28]. The drawbacks concerning fibrin adhesive are low bonding strength for load-carrying and flexible body parts [29], as well as, its immunogenicity and risk of blood transmission diseases such as HIV and BSE [30,31], and its high price cannot be afforded by many patients [32].

Therefore an ideal tissue adhesive with promising properties such as high tensile strength, acceptable biocompatibility, biodegradability and good adhesion has been investigated for many years [33-35]. Chitosan and its derivatives, due to their biological properties [36,37] biodegradability, bioactivity and biocompatibility have been examined in a wide variety of biomedical applications [38-40]. In addition to accelerating wound healing, chitosan is also capable of activating host defensive immune system to prevent infection, thereby offering anti-bacterial properties [41].

In this study, for the first time, urethane pre-polymer was combined and synthesized with a chitosan gel (ChitoHeal®) to be used as tissue adhesive. The aim of such combination was twofold: first exploiting the properties of polyurethane in order to achieve good adhesion and to manipulate the biocompatibility and healing properties of chitosan.

EXPERIMENTAL

Materials

The following materials were purchased and used without further purification: isophorone diisocyanate ($C_{12}H_{18}N_2O_2$, Merck 818586, Germany), castor oil (CO, Aldrich 18722, USA) for synthesizing urethane pre-polymer, 2-propanol (C_3H_8O, Merck 818766, Germany), hydrochloric acid (HCl, Merck 113386, Germany), butyl amine ($C_4H_{11}N$, Merck 801539, Germany), bromophenole blue ($C_{19}H_{10}Br_4O_5S$, 3',3",5',5"-tetrabromophenolsulphonphthalein, Sigma Aldrich B0126, Germany), tetrahydrofuran (C_4H_8O,THF, Merck 108107, Germany) for titration of urethane pre-polymer and chitosan gel (ChitoHeal®, ChitoTech Inc., Iran) for modifying urethane pre-polymer.

The following materials were purchased for cytotoxicity tests: Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAAs), HEPES, and Hank's balanced salt solution (HBSS), Antibiotics and *N*-glutamine were obtained from Gibco Invitrigen (Life Technologies, Paisley, UK). Filter inserts (Nunc, Denmark). Cell proliferation reagent MTT solution was purchased from Roche Diagnostics (Roche, Germany). Standard compound methotrexates were purchased from Sigma-Aldrich (USA). Methotrexate stock solutions were prepared in dimethyl sulphoxide (DMSO). Cell lines Caco-2, T47D, HT29 and NIH-3T3 were prepared by Pasteur Institute, Tehran, Iran.

Synthesis

To synthesize urethane pre-polymer, 6.3 mL of isophorone diisocyanate (IPD) was mixed with 4.85 mL of castor oil (CO). The two components were mixed in the reaction vessel by stirring at 60°C under nitrogen atmosphere for 24 h. The final formulation was prepared using this mixture with 2% chitosan gel mixed for 20 min in a stoppered vessel in ambient temperature.

FTIR Spectroscopy

To confirm the presence of urethane band and NCO free groups following the reaction, FTIR spectroscopy was carried out. The FTIR spectra of samples were measured between 4000 and 400 cm^{-1} using a FTIR spectrometer (Bruker - Equinox 55, Germany) and recorded on an average of 16 scans at a resolution of 4 cm^{-1}.

Gel Permeation Chromatography

The molecular weight of urethane pre-polymer was determined according to ASTM D6579-06 using an Agilent 1100 system with 20000 microliter of the sample injected to the column as follows:

Eluent: THF, concentration: 1.000 g/L, detector 1: RID A, refractive index signal, flow rate: 1.000 mL/min, inject volume: 20.000 µL, delay volume: 0.000 mL, and acquisition interval: 0.430 s.

Titration Method

The NCO free group was determined quantitatively by titration against HCl. At first, 1 g of the sample was taken into a stoppered conical flask along with few silicon carbide chips. A volume of 7 mL of butyl amine was added into 10 mL of THF and allowed to stand for 15 min. Then, 10 mL of isopropanol and two drops of bromophenole blue were added to the above mixture and the final solution was titrated against 1 N HCl until the color changed from blue to yellow. A blank titration was conducted as above, except having no pre-polymer. The NCO% was calculated using the following equation.

$$NCO\ (\%) = \frac{[B - S] \times 42 \times 100}{W \times 1000} \quad (1)$$

where, B, S, and W represent blank reading, sample reading and weight of the sample in g, respectively.

Adhesion Test

The relative peel resistance of the urethane pre-polymer and tissue adhesive was measured based on ASTM F2256-05. This test was performed by a computer-controlled testing apparatus of mechanical properties (Zwick/Roell-HCr 25/400) at a crosshead speed of 20 mm×min^{-1}. Two layers of gelatin sheets of dimension 5 × 20 cm were used. The first layer was fixed on a holder; followed by spreading the urethane pre-polymer and applying two drops of water. For proper placement of the two sheets on the apparatus, 2 cm of the upper part of the sheets was kept free of the compound.

Scanning Electron Microscopy

Morphology of the urethane pre-polymer and that of the final tissue adhesive was demonstrated using SEM. The two compounds were coated with gold in ambient temperature. The analysis was performed using scanning microscope XL30, Philips, Holand.

Surface Energy Determination by Contact Angle Measurement

Surface energy of the adhesive, using contact angle measuring system (Kruess - G10, USA), with LBW02 method based on Owens, Wendt, Rabel and Kaelble calculations [31] was evaluated with four liquids: water, formamide, ethylene glycol and diiodomethane. In separate test, one drop of each liquid was put on the surface of the cured tissue adhesive film at 23 ± 5°C.

Cytotoxicity Test

Cytotoxic effect of the tissue adhesive was determined on the growth and viability of cells according to ISO 10993-5. Cytotoxicity tests after 72 h were performed within concentration range of 3.98 to 1020 µg/mL tissue adhesive, using the four cell lines Caco-2 colon carcinoma, T47D ER+ breast carcinoma, HT29 colon carcinoma and NIH-3T3 normal cell line fibroblast. Control culture was maintained in RPMI 1640 similar to the treated cultures.

A quantity of 3×10^3 cells/mL was plated in microwell plates. MTT solution was prepared at 5 mg/mL in PBS; filter sterilized and stored in the dark at 4°C for a maximum of 1 month. MTT reagent (20 µL) was added to each 100 µL of culture. After incubation for 3 h at 37°C a water insoluble formazan dye was formed which was solubilized by addition of 100 µL acidified isopropanol inside the culture wells. The plates were further incubated for 20 min at room temperature and optical densities (OD) of the wells were determined using an Anthos 2020 (Salzburg) ELISA micro-plate reader at a test wavelength of 570 nm and a reference wavelength of 690 nm. Each plate contained blank background control wells containing an appropriate volume of media but no cells.

Statistical analysis was performed with the student's paired t-test. ANOVA test with Post-hoc tests were used between groups. P values < 0.05 were considered to be significant. The vales presented are means ± SD.

Skin Irritation Test

Four rabbits (white, New Zealand, male, 2223-2262 g) were used to perform the test. The back's right caudal and left cranial areas of each tested animal were treated with the examined substance, while the non-treated left tail and the right cranial area of the back of each rabbit was used as control. Independent negative and positive controls were tested. Approximately, 24 h before the test, the fur was removed from a surface of 240 cm^2 area by clipping and shaving the dorsal and flank zones of the animals. The test substance was directly applied to the skin in an area of 25×25 mm on cranial site of each rabbit. The application sites were covered with non-occlusive dressing and wrapped with a semi-occlusive bandage. Normal saline and histamine were negative and positive controls, respectively.

RESULTS AND DISCUSSION

The FTIR spectra of samples confirmed the presence of intense bands of O=C-NH and the NCO free groups of urethane pre-polymer at 1514 and 2261 cm^{-1}, respectively as shown in Figure 1. The N-H stretching vibration band appeared at 3385 cm^{-1}. The C-H symmetric and asymmetric stretching vibrations of CH_2 groups were detected at 2929 and 2854 cm^{-1}, respectively. The intense band at 1743 cm^{-1} is due to C=O groups and the absorption bands at 1462 and 1364 cm^{-1} are attributed to bending vibrations of CH_2 and C-H, respectively (Figure 1). As described in the method section, urethane pre-polymer had been modified with 2% of chitosan. In this reaction, the dominant mechanism was between free isocyanate groups in IPD and amino groups in chitosan.

Table 1. Number average molecular weight ($\overline{M}_n$), weight average molecular weight ($\overline{M}_w$) and the size average molecular weight ($\overline{M}_z$) of urethane pre-polymer.

Molecular weight	Experimental data (g/mol)
$\overline{M}_n$	1899.3
$\overline{M}_w$	2444.7
$\overline{M}_z$	3478.5

The molecular weight ($\overline{M}_w$) of urethane pre-polymer was measured by GPC as 2444.7 g/mol (Table 1). The polydispersity index was obtained by the ratio of $\overline{M}_w$ to $\overline{M}_n$ is 1.2872 (Figure 2). By titration, the NCO percentage was calculated to be 21% of the total compound used as in eqn (1).

The relative peel resistance of the adhesive bonds between flexible adherents was measured where, forces of 15.1 N and 14.6 N were applied to fracture the gelatin sheet glued with urethane pre-polymer and tissue adhesive, respectively.

The morphologies of urethane pre-polymer and tissue adhesive were observed by SEM technique. Figure 3a shows the plain structure of urethane pre-polymer which is completely different from porous structure of tissue adhesive (Figure 3b). The surface energy of tissue adhesive was measured 33.65 mN/m, which is less than values of gelatin, skin

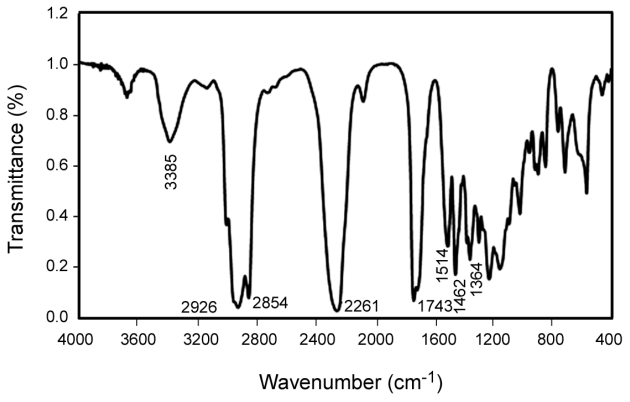


Figure 1. FTIR spectrum of urethane pre-polymer.

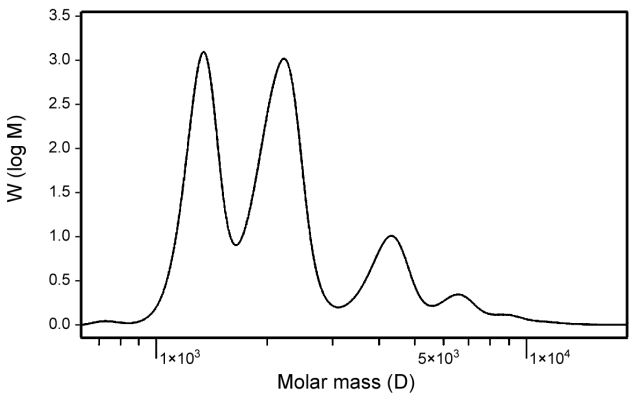
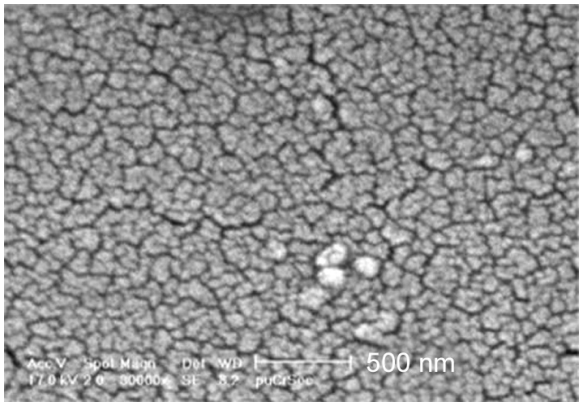
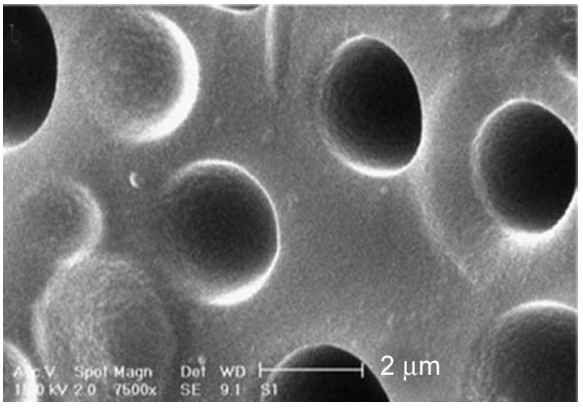


Figure 2. GPC Chromatogram of urethane pre-polymer.



(a)



(b)

Figure 3. SEM Micrographs of: (a) urethane pre-polymer and (b) tissue adhesive.

Table 2. Surface energy values and the corresponding dispersive and polar components.

Substrate	Surface energies and surface tensions (mN/m)		
	σ_s	σ_s^D	σ_s^P
Gelatine	44.24	5.00	39.24
Skin	38-56	-	-
Blood	47.50	11.20	36.30
PU + ChitiHeal (2%)	33.65	32.21	1.45

and blood surface energies which are 44.24, 38-56, and 47.5, respectively [31] (Table 2).

Figures 4-7 show the effects of tissue adhesive on the viability of Caco-2, T47D, HT-29 and NIH-3T3 cell lines, respectively. As it is shown in these figures, the product did not show significant cytotoxicity compared to control and other groups. Table 3 shows the results of skin irritation test and reveals that the tissue

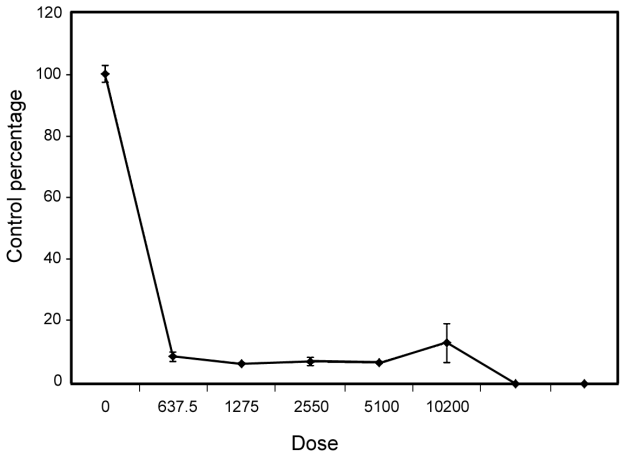


Figure 4. Cytotoxicity test of tissue adhesive on Caco-2 cell line.

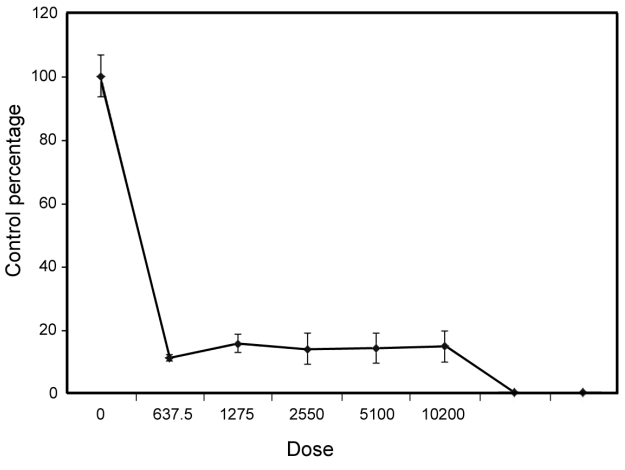


Figure 5. Cytotoxicity test of tissue adhesive on T47D cell line.

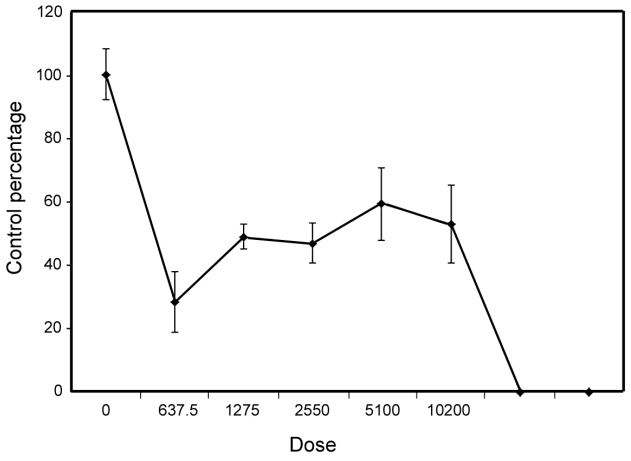


Figure 6. Cytotoxicity test of tissue adhesive on HT-29 cell line.

Figure 7. Cytotoxicity test of tissue adhesive on NIH-3T3 cell line.

adhesive produced no skin irritation.

The urethane pre-polymer was prepared in a reaction with isophorone diisocyanate, an asymmetric aliphatic diisocyanate with two different NCO groups, and castor oil, a triol with ester linkages, at a ratio of 3/0.5. The overall mechanism of the adhesion process [31] is shown in Scheme I. The NCO free groups react with water and form aminated molecules and CO_2. The resulting NH_2 groups react with NCO groups to form urea units and the NH groups of the resulting urea attach to other NCO groups in the next step and form a cross-linked material,.

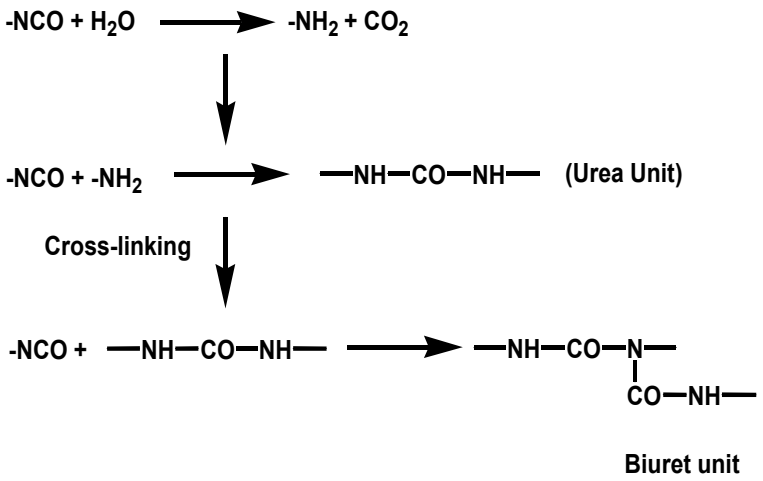
There are two types of NCO groups with different chemical reactivity in the urethane pre-polymer. The NCO free group, bonded to a secondary carbon is more reactive than those bonded to a tertiary carbon. This is primarily due to the more empty space around the secondary carbon. By adding a small amount of chitosan gel, NH_2 groups of chitosan react preferably with more reactive NCO groups of the pre-polymer. Hence, the less reactive NCO free groups would be available to react with amino acids of biological molecules where the adhesive is applied. The addition of the chitosan gel reduces the bonding capacity of the tissue adhesive by 3.31% as measured by adhesion peeling test, compared to urethane pre-polymer. However, chitosan, as a natural biopolymer, is known for its biocompatibility and biodegradability [41] and its presence increases the biocompatibility of the adhesive.

The morphologies of urethane pre-polymer and tissue adhesive are demonstrated using SEM

Table 3. Skin irritation test of tissue adhesive on four Albino rabbits using normal saline and histamine as negative and positive controls, respectively.

Positive control	Negative control	Observation +24 h	Animal No.
Er++++	Er-	Er-	1
Ed++++	Ed-	Ed-	
Er++++	Er-	Er-	2
Ed++++	Ed-	Ed-	
Er++++	Er-	Er-	3
Ed++++	Ed-	Ed-	
Er++++	Er-	Er-	4
Ed++++	Ed-	Ed-	

Er: erythema; Ed: edema



Scheme I. Mechanism of the reaction between tissue adhesive and biological molecules.

technique. As it is evident in Figure 3a, prior to the addition of the chitosan gel, there is no pores present in the surface of the urethane pre-polymer, however, following modification with chitosan gel, probably due to the release of CO_2 gas, a porous structure is formed (Figure 3b). Hence, the use of chitosan gel enhances the formation of a porous structure which can serve as an anchor for cells to grow and consequently accelerating tissue regeneration, as well as increasing the biocompatibility of the compound. These aspects could be regarded as one of the novelties of the synthesized adhesive.

The surface energies of gelatin, skin and blood have been reported previously [31]. The surface energy measurement of the synthesized tissue adhesive was 33.65 mN/m (Table 4) which is lower than those of the above natural tissues. For an adhesive to adhere to a surface, thermodynamically speaking, measured surface energy of the adhesive must be equal or lower than that of the surface [31]. Therefore, it appears that because the surface energy of the tissue adhesive synthesized in this study is lower than the tissues it is designed to adhere to, i.e., skin and blood clot [31], therefore it spreads effectively and adheres acceptably.

To determine the biocompatibility and safety of the adhesive prepared in this study, cytotoxicity experiment was performed. Studies on chitosan have been intensified since 1990 due to its low cytotoxicity, anti-microbial activity and excellent biodegradable properties in the human body [42-45]. The experiments for determination of cytotoxicity of the present tissue adhesive indicated its non-cytotoxic response. Selection of the cell lines employed was based on their features such as high proliferation, similarity to columnar epithelium, hormone dependence. By having primary cell lines, most aspects of possible cytotoxic effects of the synthesized adhesive were assured.

The molecular weight of the urethane pre-polymer determined by GPC suggests that the reaction was completed and the extra peak appearing in the chromatogram, possibly attributed to the residual monomers, is comparatively very small with respect to the peaks of main pre-polymer obtained. This minute amount has shown to be non-toxic, non-irritant with no sensitization side effects [46].

CONCLUSION

The use of tissue adhesive to replace sutures is an exciting new approach in wound closure. The present study claims to have achieved a novel biocompatible tissue adhesive, with acceptable adhesion, and accelerated tissue regeneration properties with topical applications. Considering the enhanced biocompatibility of the adhesive using chitosan gel it appears that, unlike cyanoacrylate adhesives, its degradation product does not promote any toxicity (unlike fibrin-based glues) and it adheres to tissues even in the presence of water with acceptable adhesion strength and

with no skin irritation effect. Researches on further optimization of this compound for using externally as well as on its application for various tissues will be advantageous.

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SYMBOLS AND ABBREVIATIONS

NCO : Isocyanate group
CO : Castor oil
FTIR : Fourier transform infrared spectroscopy
GPC : Gel permeation chromatography
HCl : Hydrochloric acid
THF : Tetrahydrofuran
ASTM : American Society for Testing and Materials
SEM : Scanning electron microscopy
MTT : 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyl-tetrazolium bromide
Er : Erythema
Ed : Edema

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