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Effect of hemostatic agents on properties of gelatin–alginate soft tissue adhesives

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Lacerations and traumatic wounds are considered to be among the most prevalent scenarios encountered in hospitals and emergency rooms. Reattachment of the lacerated soft tissue edges is traditionally performed using sutures. Use of tissue adhesives, i.e. substances that have the ability to firmly attach lacerated tissues back together, has raised interest as an alternative, due to several advantages. Novel tissue adhesives based on the natural polymers gelatin and alginate, and cross-linked by carbodiimide (EDC), were recently developed by our research group. In the current research, two types of hemostatic agents, tranexamic acid and kaolin, were loaded into our gelatin–alginate bioadhesive, in order to improve the adhesion abilities in the hemorrhagic environment of the wound. Their effects on the ex vivo adherence properties, physical properties, and biocompatibility were investigated. Incorporation of kaolin significantly improved the ex vivo bonding strength of the gelatin–alginate–EDC bioadhesives through a combination of three physical mechanisms and decreased the swelling ratio without affecting weight loss. In contradiction, incorporation of tranexamic acid into the bioadhesive formulation resulted in a lower ex vivo bonding strength and a higher swelling ratio and weight loss, probably due to reduced efficiency of the cross-linking reaction between the molecules of the natural polymers and the cross-linking agent EDC. The hemostatic agent-loaded bioadhesives showed good biocompatibility when tested in vitro on fibroblast cells. This research clearly shows that the incorporation of kaolin in our gelatin–alginate tissue adhesives may be a very promising novel approach for improving the bonding strength and physical properties of the tissue adhesives for use in hemorrhagic environments.

**Keywords:** gelatin; alginate; hemostatic agents; soft tissue adhesives; bonding strength

1. Introduction

1.1. Soft tissue adhesives

Lacerations and traumatic wounds are considered to be among the most prevalent scenarios encountered in hospitals and emergency rooms.[1] Reattachment of the lacerated soft tissue edges is traditionally performed using sutures or staples. Use of tissue adhesives, i.e. substances that have the ability to firmly attach lacerated tissues back together, as an alternative to these conventional applications, has raised interest in the last few decades due to several major advantages. Tissue adhesives exhibit hemostasis sealing of air leakage, can be applied more quickly, may require less
equipment, relatively less time-consuming procedure and removal is not required. Use of tissue adhesives prevents the painful procedure that is involved when using sharp instruments and was proven to be less expensive, without compromising the cosmetic outcome.[2–4]

Although extensive efforts were made in the past, an ideal tissue adhesive has not been developed to date, probably due to the various rigid requirements that a substance must fulfill in order to serve as a medical tissue adhesive for clinical use.[5–7] Nonetheless, a few soft tissue adhesive products were approved for medical use – cyanoacrylates, fibrin and gelatin-based adhesives. These products were approved for restricted use only, due to the low biocompatibility of cyanoacrylates which were cross-linked with formaldehyde, or glutaraldehyde, or due to the low mechanical strength of the fibrin adhesives.[8,9]

Novel tissue adhesive formulations based on a combination of gelatin with alginate as a polymeric additive, cross-linked by carbodiimide (EDC), have recently been developed and studied in our laboratory in order to combine high bonding strength with biocompatibility and other desired properties (suitable viscosity, curing time and flexibility).[10]

1.2. Our bioadhesives’ components

Gelatin is a water-soluble natural polymer derived from collagen. It has become one of the most investigated materials for tissue adhesives due to its suitable natural properties. Gelatin is considered to be biocompatible, biodegradable and non-immunogenic.[11] It can form physically cross-linked hydrogel structures,[12] has a natural tacky behavior in solution and is highly accessible in nature.[13] In spite of its promising qualities, the mechanical strength of physically cross-linked gelatin adhesives is not sufficient as an adhering substance on its own.[13] A chemical cross-linking agent and a polymeric additive (with suitable available functional groups for the cross-linking reaction) can be added to the gelatin solution in order to create gelatin-based hydrogel formulations with suitable mechanical properties for soft tissue adhesion.[6,13–16]

Using this concept, our study focuses on novel tissue adhesives based on a combination of gelatin with an alginate polymeric additive and cross-linked by carbodiimide. Carbodiimide, which is mainly used for modification and conjunction of proteins and other biological macrostructures, was chosen as the cross-linking agent because carbodiimides and their cross-linking by-products have been reported to be less cytotoxic than other conventional cross-linking agents such as formaldehyde and glutaraldehyde.[17] For example, gelatin-based adhesive, gelatin–resorcinol–formaldehyde/glutaraldehyde was developed in the 1960s, has been clinically utilized in Europe and Japan for the past few decades. Despite this, there is currently no FDA-approved gelatin–resorcinol–formaldehyde/glutaraldehyde glues in the USA.

Alginate is a natural polysaccharide which is extracted from marine algae and was chosen to be the polymeric additive for the gelatin adhesive in the current research, due to its natural source and high concentration of carboxylic groups which are essential for the cross-linking reaction of carbodiimides. The carbodiimide couples to a carboxylic group (originally from the gelatin or the alginate) to form an O-iso-acylurea intermediate, which is highly reactive and has an extremely short life. This intermediate goes through a nucleophilic attack by a primary amino group (originally from the gelatin) to form an amide bond. As a result of the nucleophilic attack, an urea molecule (derivative of the carbodiimide type) is released as a by-product.[18]
O-iso-acylurea intermediate can be stabilized by reaction with N-hydroxysuccinimide (NHS), forming an NHS-carboxyl group. This active complex is less susceptible to hydrolysis and prevents rearrangements for other by-products, thus increasing the efficacy of the carbodiimide cross-linking reaction. The molar ratio between NHS and EDC must be optimized for the specific cross-linking reaction in order to prevent undesired reactions.[19]

Since lacerated tissues contain exposed amino and carboxylic groups which can take part in the cross-linking reaction, our new gelatin–alginate–carbodiimide bioadhesives have the potential to be especially attractive for tissue adherence, and indeed demonstrated very good ex vivo bonding strength and high biocompatibility.

1.3. **The hemostatic agents used in the current study**

Two types of hemostatic agents, tranexamic acid and kaolin, were loaded into the gelatin–alginate (Ge–Al) hydrogel in the current research, in order to improve the adhesion abilities in the hemorrhagic environment of the wound. These hemostatic agents were chosen for our research due to evidence from other studies that indicate their hemostatic properties in topical applications.[20–22] Tranexamic acid, a synthetic derivative of the amino acid lysine, is an antifibrinolytic agent that acts by binding to plasminogen and blocking plasminogen’s interaction with fibrin, thereby preventing dissolution of the fibrin clot. Large randomized controlled trials demonstrated that intravenous and oral administration of tranexamic acid generally significantly reduced perioperative blood loss compared to placebo in a variety of surgical procedures, including cardiac surgery, total hip and knee replacement and prostatectomy. It also reduced blood loss in gynecological bleeding disorders, trauma patients with significant bleeding, traumatic hyphema, gastrointestinal bleeding, and hereditary angioneurotic edema.[22–25]

Kaolin is a clay mineral which has a 1:1 layered silicate structure and the formula Al₂(Si₂O₅)(OH)₄ in which the silica tetrahedral layer (Si₂O₅)²⁻ is rendered electrically neutral by an adjacent Al₂(OH)₄²⁺ layer. The midplane consists of O²⁻ anions from the (Si₂O₅)²⁻ layer, as well as OH⁻ ions that are a part of the layer. A kaolinite crystal is made of a series of these double layers or sheets stacked parallel to each other which form small flat plates that are typically less than 1 μm in diameter and nearly hexagonal.[26–31] Surface charge properties of kaolin are important for the interaction with the polymers solution and affect the hemostatic capability. Kaolin has negative charge in the faces (basal planes) due to imperfections in the kaolinite crystals.

Kaolin is a strong contact pathway activator agent that initiates rapid clot formation in wounds. It is used as an activator in different laboratory evaluations of hemostasis. Quikclot® (Z-Medica) is one of the many applications of kaolin that received FDA approval as a topical hemostatic gauze with kaolin impregnated as a hemostatic agent in non-woven standard medical gauze. This application is widely used by military forces and hospitals around the world.[20,21]

In the current research, we studied the physical properties of our unique gelatin–alginate–carbodiimide bioadhesive system when loaded with the hemostatic agents kaolin and tranexamic acid. We investigated the bonding strength, viscosity, swelling behavior, and weight loss of the loaded adhesives. These properties are the most relevant for the soft tissue adhesive application. Biocompatibility was studied as well.
2. Materials and methods

2.1. Materials

Gelatin ‘type A’ from porcine skin (90–100 bloom), alginic acid sodium salt (viscosity ~250 cps, 2% (25 °C)), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), kaolin (K1512), and tranexamic acid (857653) were all purchased from Sigma–Aldrich, Rehovot, Israel.

2.2. Preparation of hemostatic agent-loaded tissue adhesives

The bioadhesive preparation was based on dissolving various amounts of gelatin and alginate (Ge–Al) and hemostatic agent (kaolin and tranexamic acid) powders in distilled water, under heating up to 60 °C. Various amounts of the cross-linking agent (EDC) were added to the Ge–Al solution containing the hemostatic agents just prior to the adhesive’s use. All studied formulations are presented in Table 1. The effect of kaolin and tranexamic acid was studied in concentrations of 1, 3, 5, and 7% w/v and 0.5, 1.5, 3% w/v, respectively. The formulations are presented in the form of Ge–Al–EDC–NHS, where Ge is the concentration of gelatin (mg/mL), Al is the concentration of alginate (mg/mL), EDC is the concentration of the carbodiimide cross-linking agent (mg/mL), and NHS is the concentration of N-hydroxysuccinimide (mg/mL).

2.3. In vitro bonding strength measurements

The mechanical testing procedure was inspired by the standard test method for strength properties of tissue adhesives in tension (ASTM F-2258-03) [32] for evaluating the effect of each hemostatic agent on the bonding strength of the adhesives. Porcine skin (Kibbutz Lahav, Israel) was cut into 2 × 2 cm² square-shaped pieces and their epidermis side was attached firmly to metal testing holders with a matching surface area. The porcine skin specimens were kept wet using double distilled water and then the water was blotted by wipe immediately before application of the adhesive. One hundred and forty microliters of the adhesives (with various concentrations of the hemostatic agents) were then spread uniformly on the dermis side of two porcine skin pieces (that were attached to the testing holders) which were immediately attached by applying a 1.25 N load on the pieces and placed in an environment of 37 °C and 100% humidity. After 30 min, the bonding strength was measured in tension mode at room temperature using a 5500 Instron universal testing machine (Instron Engineering Corp.) with a 10 N or 100 N load cell. The two parts of the joint were strained at a constant velocity of 2 mm/min until separation was achieved. The bonding strength was defined as the maximum strength in the stress–strain curve, measured by the Instron Merlin software. A minimum of 10 repetitions were carried out for each formulation.

2.4. Microstructure characterization

The microstructure of the kaolin-loaded adhesives was investigated in order to characterize the dispersion of kaolin in the adhesive matrix. For this purpose, 420 μL of cubic drug-loaded adhesive specimens were air-dried in a chemical hood, freeze fractured, and their cross-section was observed using an environmental scanning electron microscope (Quanta 200 FEG ESEM) in a high vacuum mode, with an accelerating voltage of 10 kV.
2.5. **Viscosity characterization**

The initial viscosity of the Ge–Al-based bioadhesive at the moment when it is applied on the tissue is affected mainly by the viscosity of the Ge–Al solution. Viscosity measurements of Ge–Al solutions were performed using a controlled stress rheometer (model AR2000, TA Instruments Ltd), fitted with a cone-and-plate geometry (4° cone angle, 40 mm diameter, 400 μm gap), at a constant temperature of 37 °C, and a constant shear rate of 10 Hz in order to investigate the effect of the hemostatic agents on the adhesive’s initial viscosity.

2.6. **Swelling ratios and weight loss**

The tissue bioadhesives were poured into 6.2 mm × 6.2 mm × 3.5 mm silicon molds and after gelation they were carefully removed and dried for 24 h. The bioadhesives were then weighed \( (W_i) \) and immersed in 2 mL PBS (pH 7.0), placed in a static incubator at 37 °C and 100% relative humidity for 2, 6, and 24 h. The adhesives were then weighed \( (W_s) \) by removing the PBS and blotting using Kimwipes and then dried for 24 h and weighed again \( (W_f) \). The swelling ratio and the weight loss were calculated according to the following equations:

\[
\text{Swelling ratio: } \left( \frac{W_s}{W_f} \right) \times 100\% \quad (1)
\]

\[
\text{Weight loss: } \left( \frac{W_i}{W_f} \right) \times 100\% \quad (2)
\]

Three repetitions were carried out for each formulation at each point of time.

2.7. **Cytotoxicity evaluation**

Human neonatal foreskin fibroblast cell cultures were exposed to adhesive extracts for certain periods of time as described in the ISO 10,993 standard (parts 5 & 12) for biological evaluation of medical devices,[4,33] in order to evaluate the cytotoxic effect of the various bioadhesive formulations.

2.7.1. **Preparation of adhesive extract**

The tissue bioadhesives were poured into 6.2 mm × 6.2 mm × 3.5 mm silicon molds. After gelation, the adhesives were carefully removed and dried overnight. These samples were sterilized by ethylene oxide. Adhesive extracts were obtained by immersing the sterilized samples in culture medium at a concentration of 0.2 g/mL and incubation for 24 h at 37 °C.

2.7.2. **Cell cultures**

Primary human fibroblast cultures were obtained from neonatal foreskins (HFFn). The cells were thawed and cultured in 75 mm³ flasks with modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 0.1% penicillin–streptomycin–nystatin. The cells were kept in a humidified 37 °C and 5% CO² environment. After reaching a confluence of 70%, the cells were separated from the bottom of the flasks using a ‘trypsin A’ solution and were seeded into 96-well plates at concentrations of 5000 cells per well with 0.2 mL of fresh culture medium and incubated for 24 h. This cell concentration was chosen based on preliminary
experiments using cell densities between $0.5 \times 10^3$ and $50 \times 10^3$ per well, which demonstrated that optimal growth was obtained using this initial cell concentration. After 24 h, the medium was removed and replaced with 0.2 mL per well of adhesive extract. Cells cultured without adhesive extract served as a negative control. The cells were cultured for an additional 24, 48, and 72 h. Three repetitions were carried out for each formulation at each point of time.

2.7.3. Alamar Blue assay for cell viability

An Alamar Blue (AB) assay was used to evaluate cell growth and viability in the presence of adhesive extracts. The AB assay was performed at 24, 48, and 72 h after the addition of the adhesive extracts to the wells. The procedure included replacing the original medium with 0.25 mL of fresh medium containing 10% (v/v) AB and incubating the cells for 4 h. Subsequently, 100 μL duplicates from each well were transferred into a 96-well plate for spectrophotometer analysis (Spectra max 340 PC384, Molecular Devices). The percent reduction of the AB was calculated according to the manufacturer’s protocol. The %AB reduction after exposure to the adhesive extracts for different periods was compared to the %AB reduction in the control cells’ environment (cells that were not exposed to the extracts), in order to evaluate the cytotoxicity of the drugs.

2.8. Statistical analysis

All data were processed using the Excel software. Statistical comparison between more than two groups was performed using the ANOVA (with Tukey Kramer) method via the SPSS (V. 15) software. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

In the current study, we investigated the effect of the hemostatic agents kaolin and tranexamic acid on the function and physical properties of soft tissue adhesives based on gelatin and alginate cross-linked by carbodiimide (EDC). Four formulations (see Table 1) with different concentrations of gelatin, alginate, and cross-linking agents were chosen for most studies. NHS was added to two of them in order to increase the efficacy of the carbodiimide cross-linking reaction. We have previously found that these formulations are suitable for application as soft tissue adhesives with high bonding strength and relatively low cytotoxicity.[10] The statistical significant differences mark with (*) in all figures.

<table>
<thead>
<tr>
<th>Ge–Al–EDC–NHS formulation</th>
<th>Gelatin [mg/mL]</th>
<th>Alginate [mg/mL]</th>
<th>EDC [mg/mL]</th>
<th>NHS [mg/mL]</th>
</tr>
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<tr>
<td>300-30-20</td>
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<td>200-40-10-1</td>
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3.1. Ex vivo bonding strength

The effect of the hemostatic agents, tranexamic acid and kaolin, on three of the basic selected formulations is presented in Figures 1 and 2. Tranexamic acid is highly watersoluble and kaolin is dispersed in water very easily even at high concentration, forming stable suspension. Therefore, the hemostatic agents mixing with the aqueous Ge–Al solution resulted in the preservation of homogenous solutions.

The bonding strength of the three basic formulations without hemostatic agent is similar, exhibiting an average value of 16.7 ± 0.7 kPa. The results indicate that the presence of the 1 mg/mL NHS in the carbodiimide reaction compensates for the reduction in the EDC concentration from 20 mg/mL to 10 mg/mL. For comparison, the bonding strength of the fibrin sealant Evicel™ is six times lower (approximately 2.5 kPa) as measured in our system [34].

The incorporation of tranexamic acid was found to decrease the bonding strength of all examined formulations (Figure 1). The decrease in bonding strength observed from the minimal concentration of 0.5% was more significant for the formulation based on 200 mg/mL gelatin than for formulations based on 300 mg/mL gelatin. For example, formulations with 3% tranexamic acid exhibited a decrease of 68, 53, and 44% in the bonding strength of the 300-30-20, 300-30-10-1, and 200-40-10-1 formulations, respectively. This effect was probably due to the chemical structure of tranexamic acid which consists of both an amine group and a carboxylic group, which are the functional groups that react in the carbodiimide cross-linking reaction which leads to the formation of an amide bond. Increasing the concentration of tranexamic acid leads to its conjugation with the two natural polymers and decreases the cross-linking density of the adhesive network. As a result, both the cohesion forces inside the adhesive and the adhesion forces between the adhesive and the tissue decrease. The formulation based on 00 mg/mL gelatin was greatly affected by tranexamic acid, probably because it consists of only two-thirds of the amino groups of the formulation based on 300 mg/mL gelatin.

A totally different strength behavior was obtained for formulations with kaolin (Figure 2). The incorporation of kaolin did not reduce the bonding strength and was
found to even improve it, when a kaolin concentration of up to 5% was used. The bonding strength at higher concentrations was similar to the non-loaded formulations. Observation of the surface fraction indicates that kaolin improved the cohesion forces of the adhesive. This may be attributed to the stiff layers of the kaolin-loaded samples compared to the non-loaded or tranexamic acid-loaded adhesives. This improvement in the bonding cohesion strength due to kaolin incorporation is probably achieved through the three following mechanisms:

- Kaolin has a high water absorbance capacity and forms a hydrate layer on its surface. This results in a decrease in water availability in the adhesive and the cross-linking efficiency therefore increases. It is known that free water disturbs the carbodiimide reaction.[35]

- A kaolin-polymer interaction due to surface charges of kaolin edges and basal planes. Reactive hydroxyl groups on the kaolin basal surface exhibit negative charges and can form hydrogen bonds with the positive charges of the gelatin amines. In addition, the edge surface yields positive charges which can interact with the alginate carboxylic groups.[36–39]

- Kaolin–kaolin interactions due to the abovementioned surface charges of kaolin could reinforce the structure by adding new bonds formed in three different modes of particle interactions: edge to face, edge to edge, and face to edge.[27,28,30]

Reduction of the bonding strength in the relatively high kaolin concentration of 7% can be attributed to a loss of the adhesive’s homogeneity, which is prepared by manual mixing of the Ge–Al and the cross-linker solutions.

In conclusion, incorporation of tranexamic acid reduced the adhesive bonding strength due to competition over the cross-linker, whereas incorporation of kaolin between 1 and 5% w/v (depending on the composition of the adhesives basic formulation) increased the cohesion strength of the adhesive, and therefore increased its bonding strength.
3.2. Microstructure

The bulk cross-sections of the Ge–Al 200-40 formulations, non-loaded and loaded with 1 and 7% w/v kaolin, were observed using ESEM. The non-loaded reference sample did not demonstrate any phase separation. Some cracking was exhibited, which probably resulted from the fracturing process (Figure 3(a)). Although the microstructure observation was performed under dehydrated conditions, it can still provide some information on the ability of kaolin to improve the bonding ability of a semi-liquid adhesive. The fractographs of the adhesive loaded with kaolin demonstrated that kaolin is uniformly dispersed in the bioadhesive at both the low and the high concentrations (Figure 3(b)–(f)). Furthermore, the kaolin layers remain integral and kaolin delamination was not observed. In fact, the incorporation of kaolin in the adhesive formed a micro-composite structure of kaolin layers integrated in the polymeric matrix. This

Figure 3. ESEM fractographs of the Ge–Al 200-40 formulation (a) unloaded, (b)–(c) loaded with 1% w/v kaolin and (d)–(f) loaded with 7% w/v kaolin.
microstructure indicates a partial phase separation between the kaolin and the bioadhesive matrix which demonstrated kaolin’s characteristic as a non-expanding layered silicate. If kaolin could be further dispersed in the bioadhesive matrix, we would probably obtain nanostructures of the kaolin within the bioadhesive. Such nanostructuring can result in markedly improved mechanical strength compared to the pure polymer or micro-composite structure, as obtained in the present formulation. The microstructure characterization of bioadhesive loaded with tranexamic acid was done and no difference was observed, when compared to unloaded bioadhesive (not shown).

3.3. Viscosity
Rheological tests were performed in order to elucidate the effect of kaolin on the adhesive’s viscosity, which is a highly important characteristic of the adhesive and determines the ease of use. The measurements were carried out at 37 °C, when the Ge–Al solution is in the liquid state without cross-linking. The viscosity of the non-loaded Ge–Al solution is 12.5 Pa s, and the kaolin-loaded Ge–Al 200-40 samples exhibited viscosity values of 11.2, 11.8, and 11.4 Pa s, for samples loaded with 1, 5, and 7% kaolin, respectively. Thus, our results show that the incorporation of kaolin (1–7% w/v) in the 200-40 Ge–Al formulation has practically no effect on the viscosity of the Ge–Al

![Figure 4](image-url)  
Figure 4. The effect of tranexamic acid content (– 0%, – 0.5%, – 1.5%, and – 3% w/v) on the weight loss of the four Ge–Al-EDC-NHS formulations (a) 300-30-10-1, (b) 300-30-20, (c) 200-40-10-1, and (d) 200-40-20. Significant differences are marked with (*).
solution. This means that the resistance of Ge–Al to sheer or tensile stress was not affected only by the addition of kaolin to the solution, and indicates that no significant bonds were formed between these natural polymers and the kaolin in the liquid solution.

3.4. Swelling ratio and weight loss

Both the swelling ratio and the weight loss of bioadhesives are very important physical parameters, indicating the density of the network between the polymer chains formed by the cross-linking reaction. A high swelling ratio indicates a relatively weak and less dense network structure of the polymer. A denser hydrogel structure reduces the accessibility of water molecules to enter the hydrophilic parts of the polymer molecules and as a result less water can penetrate into the hydrogel structure. Similarly, high weight loss indicates that polymer chains are detached from the network more easily or that the portion of polymer involved in the connected network is smaller.[19,40] In our study, all four bioadhesive formulations loaded with tranexamic acid and kaolin were tested concerning swelling ratio and weight loss behavior. The weight loss and swelling ratio of formulations loaded with tranexamic acid are presented in Figures 4 and 5, respectively. The weight loss and swelling ratio of formulations loaded with kaolin are presented in Figures 6 and 7, respectively.

![Figure 5](image)

Figure 5. The effect of tranexamic acid content (– 0%, – 0.5%, – 1.5%, and – 3% w/v) on the swelling ratio of the four Ge–Al-EDC-NHS formulations (a) 300-30-10-1, (b) 300-30-20, (c) 200-40-10-1, and (d) 200-40-20. Significant differences are marked with (*).
It is important to note that most of the weight loss and the swelling ratio in all four formulations of non-loaded bioadhesives are obtained during the first 2 h of immersion, followed by only a moderate increase over the test periods. Thus, the initial weight loss and swelling ratio well reflect the system’s behavior. The initial weight loss is obtained due to detachment of uncross-linked chains. The initial swelling occurred in the amorphous and uncross-linked regions of the matrix.

Although the swelling ratio of our bioadhesive is relatively high in water, practically it showed minimal effect on the bonding of the injured tissue because it is applied in a very thin layer between the lacerated tissues. Also, it should be noted that the swelling ratio and weight loss tests were performed on dry adhesive samples that were cast into cubic molds (3D instead of thin layers) and not on thin films between two skin layers, as practically will be applied, due to technical reasons.

### 3.4.1. The effect of the EDC concentration

The cross-linking agent’s (EDC) concentration was found to have strong effect on the swelling ratio and weight loss of both types of formulations, based on 300 mg/mL gelatin and 200 mg/mL gelatin. The weight loss values of the EDC-10 mg/mL and

![Figure 6. The effect of kaolin content (0%, 1%, 3%, 5%, and 7% w/v) on the weight loss of the four Ge-Al-EDC-NHS formulations (a) 300-30-10-1, (b) 300-30-20, (c) 200-40-10-1, and (d) 200-40-20. Significant differences are marked with (*).](image-url)
NHS-1 mg/mL formulations are approximately 2.5 times higher than those of the EDC-20 mg/mL formulations (Figures 4 and 6). The swelling ratios of the former are two times higher than those of the latter (Figures 5 and 7). It is therefore suggested that cross-linking with 20 mg/mL EDC is more effective than with 10 mg/mL EDC and 1 mg/mL NHS, in terms of physical properties, i.e. stability. On the other hand, a positive effect of NHS on the bonding strength was demonstrated (see Section 3.1).

3.4.2. The effect of tranexamic acid incorporation

Tranexamic acid-loaded bioadhesives show an increase in the swelling ratio and the weight loss values with the increase in the tranexamic acid concentration (Figures 4 and 5). For example, the initial weight loss (after 2 h) of 3% loaded tranexamic acid was 1.5–3 times higher than the weight loss obtained for non-loaded bioadhesives (Figure 4). As explained earlier (see bonding strength results, Section 3.1), incorporation of tranexamic acid into the bioadhesive formulation reduced the cross-linking reaction’s efficiency between the Ge–Al polymers and the EDC due to a reaction between the amine and carboxyl groups in the tranexamic acid structure and the EDC. This phenomenon not only decreases the bonding strength of the bioadhesive, but also enables a higher degree of swelling and weight loss. Despite these changes,
inclusion of tranexamic acid in our bioadhesives could lead to improvement when applied in vivo as a result of its antifibrinolytic quality that strengthens the clot within the tissue adhesive application area.

3.4.3. The effect of kaolin incorporation

Kaolin-loaded bioadhesives did not show significant differences in weight loss over the range of loading concentrations. Kaolin is hydrophilic and has high ability to disperse in water to form suspension. Therefore, it can be assumed that when the kaolin concentration is increased, the weight loss is influenced mainly by the detachment of the kaolin from the adhesive rather than by detachment of the cross-linked gelatin and alginate chains. However, although kaolin is a highly hydrophilic molecule with high water capacity, the swelling ratio of the bioadhesives significantly decreased with the increase in the kaolin concentration. This phenomenon indicates that kaolin and Ge–Al probably create a dense cross-linked structure and the water-reactive groups of kaolin are

Figure 8. Cytotoxicity of adhesives composed of (a) 200-40 and (b) 300-30 Ge-Al unloaded (□ – 20, □ – 10-1 EDC-NHS) and loaded with tranexamic acid (■ – 20+0.5%, ○ – 20+3%, ▢ – 10-1+0.5%, □ – 10-1+3% EDC-NHS+tranexamic acid). Measured after 24, 48hr and 72hr. Significant differences are marked with (*).
occupied. The decrease in the swelling ratio without affecting the weight loss is exceptional.

3.4.4. The hemostatic agent $\rightarrow$ swelling $\rightarrow$ bonding strength effects

As thoroughly described above, each hemostatic agent affects the bonding strength, weight loss, and swelling ration. In addition, the bonding strength is probably also affected by the weight loss and swelling ration. When tranexamic acid is added to the bioadhesive, the weight loss and swelling ration are increased (Figures 4 and 5) and as a result the bonding ability of the bioadhesive is also decreased (Figure 1). In contradiction, when kaolin is added to the bioadhesive in concentrations lower than 5% w/v, the swelling ratio is decreased (Figure 7) and it helps achieving some increase in bonding strength (Figure 2). Hence, in addition to the effect of the hemostatic agent on both, welling ration and bonding strength, through the cross-linking reaction, additional hemostatic agent $\rightarrow$
swelling → bonding strength effects help to explain the significant influence of the hemostatic agent on the bonding strength even at relatively low contents.

3.5. In vitro cytotoxicity evaluation

The Alamar Blue assay was performed on human fibroblasts that participate in the wound healing process, in order to test cell viability. The effect of the tissue adhesives based on the Ge–Al 300-30 and Ge–Al 200-40 formulations on the cells is demonstrated in Figures 8 and 9, respectively. Each Ge–Al combination was tested when cross-linked with 20 mg/mL EDC or 10 mg/mL EDC and 1 mg/mL NHS. The four non-loaded formulations exhibited high viability when exposed to the bioadhesive extracts for 24, 48, and 72 h. The differences in cell viability due to the Ge–Al or EDC concentrations are insignificant for practical purposes.

All formulations loaded with kaolin or tranexamic acid also showed relatively low cytotoxicity, i.e. kaolin-loaded formulations exhibited 87–100% viability after 24 h, 78–92% viability after 48 h, and 65–83% viability after 72 h (Figures 8(a) and 9(a)). Tranexamic acid-loaded formulations exhibited 83–98% viability after 24 h, 67–82% viability after 48 h, and 55–78% viability after 72 h (Figures 8(b) and 9(b)). Thus, tranexamic acid-loaded bioadhesives are generally more cytotoxic than kaolin-loaded ones. The maximal change was observed after 72 h for the 200-40-10-1 formulation with 0.5% tranexamic acid (55% viability, Figure 9(b)) and for the 300-30-20 formulation with 3% tranexamic acid (59% viability, Figure 8(b)). Since it has already been shown that the presence of tranexamic acid in the extraction medium may cause detachment of the cells from the culture plates, it is possible that the cytotoxicity of tranexamic acid-loaded bioadhesives is similar to that of kaolin-loaded bioadhesives. Tranexamic acid is used in medical applications and we can therefore assume that it is a biocompatible material.

Our novel bioadhesive faced the main obstacle in the tissue adhesive discipline, i.e. the trade-off between cytotoxicity and strength. In comparison, cyanoacrylates have a good bonding strength but high cytotoxicity; hence, their application is limited to topical use only and are not suitable for internal use in surgery. Tissue adhesives based on natural polymers, cross-linked via biochemical reactions, offer a more compatible alternative to most of the synthetic adhesives. This is obtained due to their composition, since they can mimic the same cross-linking process that naturally occurs during the blood coagulation. In contrast, fibrin sealant exhibit lower cytotoxicity and better biocompatibility then our bioadhesive; however, their bonding strength (cohesive and adhesive) is significantly lower.[3,4]

4. Conclusions

In the current study, we developed and studied a gelatin–alginate bioadhesive which is loaded with the hemostatic agents kaolin and tranexamic acid. Our thought was that in addition to providing an attractive alternative for sutures and other traditional wound closing applications, our bioadhesive will also induce hemostatic effects and thus improve adhesion and overall function in a hemorrhagic environment. Such unique bioadhesives may be used for both external and internal adhesion applications. Two hemostatic agents were chosen for this purpose, kaolin and tranexamic acid, and their effects on the ex vivo adherence properties, physical properties, and biocompatibility were investigated.
Incorporation of kaolin resulted in formation of a micro-composite structure of kaolin layers integrated in the bioadhesive matrix. It was found to significantly improve the ex vivo bonding strength of the Ge–Al–EDC bioadhesives through a combination of three physical mechanisms, and to decrease the swelling ratio without affecting weight loss. The kaolin-loaded bioadhesives showed high biocompatibility when tested on fibroblast cells.

Incorporation of tranexamic acid into the bioadhesive formulation reduced the cross-linking reaction’s efficiency between the natural polymers Ge–Al and the cross-linking agent EDC, and therefore resulted in a lower ex vivo bonding strength and higher swelling ratio and weight loss compared to non-loaded samples.

In conclusion, the incorporation of kaolin in our Ge–Al tissue adhesives seems to be a very promising novel approach for improving the tissue adhesive, whereas incorporation of tranexamic acid does not seem to be advantageous in terms of bonding strength and physical properties, but could lead to improvement when applied in vivo due to its antifibrinolytic quality that strengthens the clot within the tissue adhesive application area.

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References


