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Novel gelatin/alginate soft tissue adhesives loaded with drugs for pain management: structure and properties

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Interest in tissue adhesives as alternatives for conventional wound closing applications, such as sutures and staples, has increased in the last few decades due to numerous possible advantages, including less discomfort and lower cost. Novel tissue adhesives based on gelatin, with alginate as a polymeric additive and crosslinked by carbodiimide were developed and loaded with two types of drugs for pain relief, bupivacaine and ibuprofen, in order to improve the therapeutic effect. The release of the drugs from the adhesive matrix was found to be controlled mainly by the adhesive’s characteristics, i.e. swelling and hydrophilic group concentration. The drug characteristics, i.e. hydrophilicity and electrical interactions between the drug and the polymeric components, were also found to have some effect. Incorporation of bupivacaine was found to improve the bonding strength of the adhesive due to its inert nature and the reinforcing effect of its fibrous crystals, whereas incorporation of ibuprofen was found to have an adverse effect on the bonding strength, probably due to its reaction with the other adhesive components which increased the crosslinking density. Overall, the novel drug-eluting gelatin-based bioadhesives investigated in this research, especially those loaded with bupivacaine, demonstrated a promising potential for use in wound closing applications.

Keywords: gelatin; alginate; carbodiimide; bupivacaine; ibuprofen; adhesive; controlled drug delivery; bonding strength; cytotoxicity

1. Introduction

Lacerations and traumatic wounds are considered to be among the most prevalent scenarios treated in hospitals and emergency rooms.[1] Re-attachment of the edges of lacerated tissues is traditionally carried out using sutures or staples. The use of tissue adhesives, i.e. substances that have the ability to firmly attach lacerated tissues back together, as an alternative for these conventional applications has raised interest in the last few decades due to several major benefits [2,3]: Tissue adhesives can be applied more quickly, may require less adhesive equipment and are considered to be a relatively less time-consuming procedure. Use of tissue adhesives also prevents the painful procedure involved in using sharp instruments and was proven to be less expensive, without compromising the cosmetic outcome.[4] Tissue adhesives can also be used to control bleeding, seal air leakage from the lungs, repair aortic dissections as well as for external fixation of certain devices.[5,6] Tissue adhesives have a further potential for use as drug delivery systems.

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An ideal soft tissue adhesive has not been developed to date in spite of extensive efforts that have been made in the past, probably due to the various strict requirements that a substance must meet in order to serve as a tissue adhesive for clinical use. The major demands include efficient bonding strength to the tissue in a moist environment, sufficient biocompatibility to the tissue and its surrounding, ease and convenience of application, sufficient flexibility for ensuring that it will remain adhered to the tissue, penetrable to cell migration, biodegradability, stability during storage and economical feasibility.[7–9] Nonetheless, a few products have been approved for restricted medical use—cyanoacrylates, fibrin, and gelatin-based adhesives.[5,6]

Gelatin, a water-soluble natural polymer derived from collagen, has become one of the most extensively investigated materials for tissue adhesives due to its suitable natural qualities. Gelatin is considered to be biocompatible, biodegradable, and nonimmunogenic.[10] It also has an ability to form physically crosslinked hydrogel structures,[11] has a natural tacky behavior in solution and is very abundant in nature.[12] These characteristics have not only turned gelatin into a promising candidate for tissue adhesives, but also for a wide range of other medical applications such as sealants,[13] hydrogels,[14] and microspheres.[15] In spite of its promising qualities, the mechanical strength of physically crosslinked gelatin adhesives is not sufficient as an adhering substance on its own.[12] A chemical crosslinking agent and a polymeric additive (with appropriate available functional groups for the crosslinking reaction) were, therefore, usually added to the solution in a wide range of published attempts, in order to create gelatin-based hydrogel formulations with suitable mechanical properties for soft tissue adhesion.[8,12,16–18]

A novel tissue adhesive based on a combination of gelatin with alginate as a polymeric additive, crosslinked by carbodiimide, has recently been developed and studied in our laboratory.[19] Carbodiimide, which is mainly used for modification and conjunction of proteins and other biological macrostructures, was chosen as the crosslinking agent since carbodiimides and their crosslinking by-products have been reported to be less cytotoxic than other conventional crosslinking agents such as formaldehyde and glutaraldehyde.[20] Alginate is a natural polysaccharide that is extracted from marine algae and is widely used in the food and beverage industry as a gelling agent, stabilizer, and emulsifier.[21] It is also applied in the medical and the pharmaceutical industries as a drug delivery vehicle,[22] a dental impression material,[23] part of a synthetic extracellular matrix for cell immobilization[24] and for wound dressing.[25] Alginate was chosen as the polymeric additive for the gelatin adhesive in the present study, since it is a natural source for high concentrations of carboxylic groups that are essential for the crosslinking reaction of carbodiimides. Carbodiimide binds to a carboxylic group (originally from the gelatin or the alginate) to form an o-iso-acylurea derivative which is highly reactive and has an extremely short life. This activated structure 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, a urea molecule (derivative of the carbodiimide type) is released as a by-product.[26] Since lacerated tissues consist exposed amino and carboxylic groups, which can take part in the crosslinking reaction, this adhesive has the potential to be attractive especially for tissue adhering.

In the current study, two types of widely used drugs with pain relief effects—bupivacaine and ibuprofen, were loaded inside the gelatin hydrogel in order to investigate the possibility of expanding the functionality of the adhesive from being used solely for closing lacerations to also serving as a device for local painkiller
release, for alleviating the pain caused by the laceration. Tissue adhesives’ quality of adhering to live organs makes them especially suitable for local controlled release at specific target sites. The benefits of local release, as opposed to systemic release, are a reduction in the amount of drug needed for achieving the therapeutic effect and prevention of significant undesirable side effects. Furthermore, local drug release abolishes the need for the patient to take an active part in delivering the drug to his body.[5,27]

Bupivacaine – an anesthetic drug, and ibuprofen – an analgesic drug, have different pain-killing mechanisms of action. Analgesics reduce pain without blocking any sensory system,[28] whereas the pain inhibition which results from anesthetics is usually an outcome of total sensation elimination.[29] The controlled release of a wide range of anesthetic and analgesic drugs from various types of matrices (such as hydrogels, [30] microspheres,[31] and wound dressing scaffolds [32]) has been reported. However, it is important to note that hydrogels designed for controlled release of pain killers for bioadhesion of lacerated tissues have not been developed and studied to date.

The effect of the adhesive’s components on the release profiles of the drugs from the adhesive matrix, the effect of each drug on the bonding strength of the adhesive to the tissue, and the effect of each drug on the biocompatibility of the adhesive were studied in vitro in order to achieve the goal of this research.

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), bupivacaine hydrochloride, and ibuprofen sodium salt were all purchased from Sigma-Aldrich, Rehovot, Israel.

2.2. Preparation of the drug-loaded adhesive

Adhesive preparation was based on dissolving various amounts of gelatin, alginate, and drug (GAD) powders in distilled water, under heating up to 60 °C.

Various amounts of the crosslinking agent (EDC) were added to the GAD solution immediately prior to the adhesive’s use. All studied formulations are presented in Tables 1 and 2.

2.3. In vitro drug release study

In order to evaluate the effects of the adhesive’s components on the release profile of each drug, cubic drug-loaded adhesive specimens (140 μl) were air-dried in a chemical hood. The air-dried specimens were immersed in 1 ml of phosphate-buffered saline (PBS), pH 7.0, with 0.02% w/v sodium azide (S.A) and kept at 37 °C for 14 days. The entire medium was removed and replaced with fresh medium at specific time points during the experiment – 6 h, 1, 2, 3, 7, and 14 days.

2.3.1. Bupivacaine and ibuprofen assays

The medium’s bupivacaine and ibuprofen content was determined using a Jasco High Performance Liquid Chromatography (HPLC) with a UV 2075 plus detector.
set at 210 nm, and a reverse-phase column (ACE 5 C18, inner diameter $d =$ 4.6 mm, length = 250 mm) with a guard cartridge (ACE 5 C18, inner diameter $d =$ 3.0 mm, length = 10 mm), under a constant temperature of 40 °C. The mobile phase for monitoring bupivacaine consisted of a mixture of PBS (pH 3.3) and acetonitrile (72/28, v/v) at a flow rate of 1.5 ml/min, with a quaternary gradient pump (PU 2089 plus) without gradient. The mobile phase for monitoring ibuprofen consisted of a mixture of PBS (pH 3.3) and acetonitrile (40/60, v/v) at a flow rate of 2 ml/min. Samples were injected with an auto sampler (AS 2057 Plus). The area of each eluted peak was integrated using the EZstart software, version 3.1.7, using a calibration curve.

### 2.3.2. Extraction of drug remainders from the adhesive

At the end of the release experiment, the adhesive samples were immersed in a ‘trypsin A’ solution at 37 °C for 4 h, in order to dissolve the samples and extract the drug that was not released during the first 14 days. The dissolved solutions were filtered using a disposable filter unit (Whatman, 0.2 μm) and the drug concentration was estimated using the aforementioned assays.

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<th>Gelatin concentration [mg/ml]</th>
<th>Alginate concentration [mg/ml]</th>
<th>EDC concentration [mg/ml]</th>
<th>Bupivacaine concentration [% w/v]</th>
<th>Burst effect (six hours) [%]</th>
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<th>EDC concentration [mg/ml]</th>
<th>Ibuprofen concentration [% w/v]</th>
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2.4. **In vitro bonding strength measurements**

Porcine skin (Kibbutz Lahav, Israel) was used as soft tissue model for evaluating the effect of each drug on the bonding strength of the adhesive. The porcine skin was cut into $2 \times 2$ cm$^2$ square-shaped pieces and their epidermis side was attached firmly to metal testing holders with a matching surface area (all dimensions of the holders are specified in Figure 1). About 140 μl of the adhesive (with various concentrations of the drugs) were then spread uniformly on the dermis side of two porcine skin pieces (that were attached to the testing holders) that were immediately attached by applying a 1.25 N 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.) and a 10 N load cell. The two parts of the joint were strained at a constant velocity of 2 mm/min until separation was achieved. The mechanical testing procedure was inspired by the standard test method ASTM F-2258-03. The bonding strength was defined as the maximum strength in the stress/strain curve, measured by the Instron Merlin software. Five repetitions were carried out for each formulation.

2.5. **Microstructure characterization**

The microstructure of the drug-loaded adhesives was investigated in order to characterize the dispersion of both types of drugs in the adhesive matrix and to examine the

![Figure 1. Illustration of the adhesive bonding system.](image-url)
effect of the microstructure on the relevant properties, i.e. drug release profiles and bonding strength. 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. The mean diameter of the drug crystals and aggregates was analyzed using the Sigma Scan Pro software.

2.6. Cytotoxicity evaluation

2.6.1. Cell cultures
Primary human fibroblast cultures were obtained from neonatal foreskins. The cells were thawed and cultured in 75 mm² 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 6-well plates at concentrations of 10⁵ cells per well. About 1.5 ml of fresh culture medium was then added to each well, and the plates were returned to incubation. After a confluence of 70% was reached in the wells, 100 µl of aqueous solutions of the drugs at various concentrations were added in triplicates into the cultured wells with fresh medium for 24 h of incubation.

2.6.2. Alamar Blue (AB) assay for cell viability
An AB assay was used to evaluate cell growth and viability in the presence of aqueous drug solutions. AB is a dark blue nontoxic fluorogenic redox indicator that turns red as a result of reduction in living cells. The oxidized blue form has little intrinsic fluorescence, whereas its red reduced form is highly fluorescent. The extent of AB reduction, which indicates cell viability, can be quantified spectrophotometrically at wavelengths of 570 and 600 nm.[33]

The AB assay was performed before and 24 h after the addition of the aqueous drug solutions to the wells. The procedure included replacing the original medium with 1.5 ml of fresh medium containing 10% (v/v) AB and incubating the wells for 4 h. After the 4 h of incubation, triplicates of 100 µl 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:

\[
\% \text{Alamar Blue reduction} = \frac{(E_{600}^{o})(A_{570}^{c}) - (E_{570}^{o})(A_{600}^{c})}{(E_{570}^{c})(A_{600}^{o}) - (E_{600}^{c})(A_{570}^{o})} \times 100
\]

where \(E^{o}\) and \(E^{r}\) represent the molar extinction coefficient of oxidized and reduced AB, respectively, at 570 and 600 nm. \(A^{t}\) and \(A^{c}\) represent the absorbance of the test and the control well (media with AB with no cells), respectively, at 570 and 600 nm. The % AB reduction after 24 h in the presence of the aqueous drug solutions was compared to the % AB reduction in the control cells’ environment (cells that were not exposed to the drug), in order to evaluate the cytotoxicity of the drugs. Percentage AB reduction
was also calculated before introducing the drug solutions, in order to ensure comparable initial cell growth in all wells.

2.7. Statistical analysis

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

3. Results and discussion

3.1. In vitro drug release

Investigating the effect of an adhesive’s components on the drug release profiles enables not only designing a controlled release adhesive that fits the specific therapeutic demands, but also provides better insight into the basic fundamentals of the release kinetics from the adhesive. The effect of the adhesive’s components on the drug release profiles were therefore examined for both types of drugs. A formulation consisting of 200 mg/ml gelatin, 40 mg/ml alginate, 20 mg/ml EDC, and 3% w/v drug (bupivacaine or ibuprofen) as a reference were used, because when developing the neat bioadhesive, these concentrations enabled a relatively high in vitro bonding strength and easy handling.[19] 3% w/v drug was chosen as the reference due to the limited solubility of bupivacaine in aqueous solutions with mild heating. The effect of each adhesive component on the release profile was examined by changing its concentration, while maintaining the same concentration of the other components as in the reference formulation. Representative extraction tests that were performed at the end of the release experiments showed that neither bupivacaine nor ibuprofen remainders were left inside the specimens.

3.1.1. Bupivacaine release

The effects of the bioadhesive’s components (i.e. gelatin, alginate, and EDC contents) on the bupivacaine release profiles were examined, and the results are presented in Figure 2. Bupivacaine exhibited a burst release in the range of 44–74% during the first six hours of release in all examined adhesive formulations, followed by a release rate which decreased with time. Approximately, 99% of the drug was released during the first 3 days of the experiment. Such release profile is beneficial for treating wounds, because the pain in usually decreased with the healing process.

The burst effect of bupivacaine from the examined adhesive formulations is presented in Table 1. It should be noted that the EDC concentration has a dominant effect on the burst release of bupivacaine, while the effects of the gelatin and alginate concentration are less notable.

3.1.1.1. Effect of EDC concentration. The dominant effect of the EDC concentration on bupivacaine release indicates that the release of drugs from the adhesive is in fact basically controlled by the swelling rate and water penetration into the adhesive matrix. Due to the chemical crosslinking of the hydrophilic adhesive, it can absorb and retain a relative large volume of the surrounding solution, without dissolving. As a result, the mesh size of the swollen polymeric adhesive increases and the drug manages to diffuse
EDC, as the crosslinking agent, is responsible for the chemical crosslinking between the adhesive’s polymeric chains. Increasing the EDC concentration increases the density of the adhesive’s crosslinked network. This is expressed in a smaller mesh size and lower structure porosity. As a result, there is less available space in the adhesive for water penetration, the swelling rate decreases as does the drug release rate. A higher EDC concentration contributes to a decrease in the burst effect of the drug also by reducing the relative part of chemically noncrosslinked gelatin in the adhesive. When exposed to 37 °C, the noncrosslinked gelatin dissolves due to the breakdown of hydrogen bonds between adjacent gelatin chains. This result in a huge burst release of 100% during the first six hours of release (Table 1).

3.1.1.2. Effect of the gelatin and alginate concentration. Gelatin and alginate, the two polymeric components in the adhesive, exhibit a different effect on the release profile of bupivacaine. Addition of gelatin increases the burst effect of the drug, whereas addition of alginate decreases the burst effect. We expected an increase in the burst effect of the drug with the increase in the concentration of both polymers, due to the large number of hydrophilic groups in these polymers (carboxylic groups in alginate and carboxylic and amino groups in gelatin) which increase the ability of the adhesive to absorb water. In addition, at a given EDC concentration, increasing the gelatin and alginate concentrations decreases the relative EDC concentration, which results in a less dense adhesive network and a more porous structure that leads to easier and faster water penetration. The effect of the gelatin concentration on the release profile fits this trend, while the alginate concentration unexpectedly resulted in a slight decrease in the
burst release of bupivacaine. We suggest that this behavior is probably related to the drug/polymer electrical interactions effect on the drug release profile. The isoelectric points of bupivacaine and alginate are 8.1 and 3.4–4.4, respectively.[34,35] This means that the bupivacaine molecules are positively charged (due to their amine groups) in the release medium (pH 7.0), which resembles the body’s environment, while the alginate molecules are negatively charged (due to their carboxylic groups). Thus, an electrostatic attraction occurs between the bupivacaine molecules and the alginate chains, which probably causes a delay in the diffusion of bupivacaine from the adhesive. Therefore, although increasing the alginate concentration increases the swelling rate of the adhesive, it also increases the bupivacaine/alginate attraction interactions and the latter is more effective.

In contradistinction, the isoelectric point of gelatin type A is 7.0–9.0, due to the presence of both amino and carboxylic groups in its side chains. This means that if it is not completely neutral (which prevents any type of electrostatic attraction with bupivacaine), the gelatin chains are positively charged in the release medium and therefore repulse the bupivacaine molecules that are also positively charged. This repulsion actually accelerates the release of bupivacaine from the adhesive, and by doing so enhances the effect that gelatin already has on bupivacaine release due to its contribution to the swelling of the adhesive.

3.1.2. Ibuprofen release

Our experience with bupivacaine release shows that it is greatly affected by the EDC concentration. We therefore chose to examine the effect of EDC also on ibuprofen release. Our results show that all examined formulations exhibited a burst release, followed by a release rate which decreased with time for three days (Figure 3(a)). The cumulative release of ibuprofen was in the range of 36–68%, and the burst release values during the first six hours were in the range of 27–48%. Table 2 summarizes the burst effect of ibuprofen from all studied adhesive formulations, as well as its cumulative release after two weeks.

Increasing the EDC concentration was found to significantly decrease the burst effect of ibuprofen and the total amount of released drug. The second series of experiments shows that increasing the loaded ibuprofen concentration in the adhesive resulted

Figure 3. Effect of EDC (a) and ibuprofen (b) concentrations on the release profile of ibuprofen from the adhesive (a formulation based on 200 mg/ml gelatin, 40 mg/ml alginate, 20 mg/ml EDC and 3% w/v bupivacaine as a reference).
in an increase in the release rate of ibuprofen during the first 3 days but had practically no effect on the burst release (Figure 3(b)).

Our results indicate that only part of the loaded ibuprofen was released in its pure form during the first 2 weeks. This was inferred not only from the release profiles but also from the appearance of unidentified peaks in the HPLC diagrams, which could not be attributed to any other background element. It was assumed that the ibuprofen probably reacted with the other adhesive components. This is related to its carboxylic group, to which the crosslinking agent probably binds during the initial stage of the crosslinking reaction. In contradistinction to ibuprofen, bupivacaine is chemically inert to the other adhesive components because it does not contain any functional groups that are sensitive to the crosslinking reaction. Our suggestions for possible reactions of ibuprofen with the other adhesive components are presented in Figure 4. Our explanation of ibuprofen’s reaction with EDC is supported by the drug release results. When the EDC concentration is increased under a constant concentration of the drug, the relative fraction of ibuprofen that reacts with the EDC increases. Similarly, increasing the ibuprofen concentration in the adhesive under a constant concentration of EDC results in a higher fraction of drug that is released in its pure form.

Due to its reactive behavior, ibuprofen has a potential for exhibiting two levels of release under the human body’s multifunctional enzymatic activity – initial release by swelling of ibuprofen did not react with the adhesive components, followed by release of ibuprofen that was originally covalently bonded inside the adhesive network as a result of degradation of the natural polymers.

Our results show that the drug release profiles from our new studied bioadhesive platform are affected mainly by the swelling rate of the adhesive. The swelling rate is determined by the crosslinking density of the adhesive network and by the concentration of the hydrophilic groups that promote water penetration. A minor effect on the

![Diagram](image-url)

**Figure 4.** Possible side reactions of ibuprofen with the other adhesive components.
drug release profile is attributed to the drug characteristics, i.e. hydrophilicity. Ibuprofen is more water-soluble than bupivacaine (100 mg/ml at room temperature and 50 mg/ml under mild heating, respectively, according to the manufacturer’s protocols) and the release rate of ibuprofen is therefore slightly higher than that of bupivacaine. Electrical repulsion or attraction interactions between the drug and the polymeric components in the adhesive matrix may also affect its mobility and as a result its release profiles, although this effect does not seem to be very dominant. Figure 5 summarizes our suggested physical model for drug controlled release from the adhesive.

3.2. In vitro bonding strength

The bonding strength of both types of drug-loaded adhesives was measured with various drug concentrations – 1, 2 and 3% w/v. The concentrations of gelatin (200 mg/ml), alginate, (40 mg/ml) and EDC (20 mg/ml) were kept constant. The bonding strength results were compared which the bonding strength of the unloaded adhesive. The results are presented in Figure 6. Significant differences are marked with ‘(*)’.

Bupivacaine and ibuprofen exhibited an opposite effect on the bonding strength of the adhesive. Increasing the bupivacaine content of the adhesive improved its bonding strength (from 9.8 ± 1.9 kPa for unloaded adhesive to 15.0 ± 1.9 kPa for 3% w/v bupivacaine-loaded adhesive), whereas increasing the ibuprofen content significantly decreased the bonding strength (4.5 ± 0.8 kPa for 3% w/v ibuprofen-loaded adhesive). Although a clear visual determination of the failure mode could not be achieved, our impression is that bupivacaine-loaded adhesives exhibited a cohesive failure (in the adhesive itself), while the ibuprofen-loaded adhesives exhibited a combined failure, where part of the failure occurred between the adhesive and the tissue and the other part in the adhesive itself.

The improvement in the bonding strength of bupivacaine-loaded formulations is probably achieved due to the stiffness of the inert bupivacaine particles, which restrain some of the movement of the adhesive’s strings in their vicinity. As a result, the mechanical properties of the adhesive are improved and the bonding strength is therefore also improved. The opposite effect of these two drugs on the bonding strength of

Figure 5. Schematic representation of a qualitative model describing the dependence of the drug release profile on both the adhesive and the drug characteristics.
the adhesive is due to the difference in their reactivity with the other adhesive components, i.e. in the current system bupivacaine is inert whereas ibuprofen is reactive. Since ibuprofen is chemically attached by the crosslinking agent through its carboxylic group and has only one such group per molecule, the carbodiimide is less effective as a crosslinking agent in our system. Increasing the ibuprofen load in the adhesive decreases the crosslinking density of the adhesive network and as a result, both the cohesion forces inside the adhesive and the adhesion forces between the adhesive and the tissue decrease. The bonding strength of the adhesive to the tissue therefore also decreases and a mixed failure occurs. In contradistinction, bupivacaine has a positive effect on the bonding strength of the bioadhesive and thus actually acts as a reinforcing filler. The fact that the bupivacaine-loaded adhesives failed in a cohesive mode indicates that the bonding strength of bupivacaine-loaded adhesives is determined by the strength of the cohesive forces inside the adhesives. Since the reinforcing effect of bupivacaine improves the mechanical properties of the adhesives, the bonding strength also increases.

3.3. Microstructure analysis
The bulk cross-sections of air-dried adhesive specimens loaded with 1% w/v drug (bupivacaine or ibuprofen) were observed using ESEM. The concentrations of gelatin (200 mg/ml), alginate (40 mg/ml) and EDC (20 mg/ml) were kept constant. The unloaded reference sample did not demonstrate any phase separation. Some cracking was exhibited, which probably results from the fracturing process (Figure 7(a)).

3.3.1. The microstructure of bupivacaine-loaded adhesives
The fractographs of bupivacaine-loaded adhesives provide a clear perspective of the dispersion and crystallization of the drug within the adhesive matrix. As can be seen in Figure 7(b–d), bupivacaine is uniformly dispersed within the matrix and crystallizes into two levels of structure – a primary structure of needles that form fiber-shaped secondary structures. This type of crystallization apparently turns the bupivacaine adhesive into a kind of fiber-reinforced composite material. Although the microstructure observation was made under dehydrated conditions, it can still provide some information on the ability of bupivacaine to improve the bonding state of a semi-liquid
adhesive. Since dissolving the bupivacaine in the adhesive required mild heating due to its low solubility in water, it is reasonable to assume that the crystallization process of the drug inside the adhesive starts already in the cooling stage, when the adhesive is removed from heating conditions in order to be applied on the tissue.

Evaluation of the fibers’ diameter was performed by measuring the diameters of 50 fibers from the same area for 3 different specimens (total of 150 fibers). Evaluation of the needles’ diameter was performed by measuring the diameters of 40 needles per fiber, for 3 different fibers (total of 120 needles). The bupivacaine-loaded adhesive exhibited mean fiber and needle diameters of 11.69 ± 2.49 and 0.49 ± 0.09 μm, respectively. Regardless of the size of the bupivacaine crystals, their homogenous dispersion in the matrix indicates that the bupivacaine-loaded adhesive is actually a type of monolithic system. Indeed, the bupivacaine release profiles are typical for monolithic devices in which the release rates decrease with time, as was observed in the drug release experiments.

3.3.2. The microstructure of ibuprofen-loaded adhesives

The structure of the ibuprofen-loaded samples was more difficult to characterize. The presence of ibuprofen in the adhesive could be detected only in some of the examined

Figure 7. ESEM fractographs of adhesives (200 mg/ml gelatin, 40 mg/ml alginate and 20 mg/ml EDC) unloaded (a) and loaded with 1% w/v bupivacaine (b–d).
specimens, and even in those specimens only in certain domains. An example for such a domain is shown in Figure 8. The ibuprofen crystals were randomly distributed in the adhesive in needle-shaped structures without any secondary structure. Analysis of the mean diameter of the ibuprofen needles was performed by measuring the diameters of 60 needles per area, for three different areas (total of 180 needles). The mean needle diameter was $0.21 \pm 0.04 \, \mu m$, which is smaller than that of bupivacaine. These differences can probably be attributed to the difference in the solubility of bupivacaine and ibuprofen in aqueous solutions. As was mentioned earlier, according to the manufacturers’ protocols, bupivacaine exhibits a solubility of 50 mg/ml under heating, while a solubility of 100 mg/ml can be achieved for ibuprofen at room temperature. It should also be remembered that a significant portion of ibuprofen reacts with the adhesive components and therefore less pure drug is left in the adhesive solutions for crystallization.

Although it was difficult to observe, we can assume that ibuprofen, similarly to bupivacaine, is also uniformly dispersed in the matrix since it was mixed with the adhesive components in the same way, and because its release profiles exhibited the same behavior of a decrease with time which is typical for monolithic systems.

### 3.4. In vitro cytotoxicity evaluation

Fibroblast viability was examined after exposure to aqueous solutions of 1, 2, and 3% w/v bupivacaine and ibuprofen for 24 h, by measuring their AB reduction relative to the AB reduction of control cells. Significant differences are marked with ‘(*)’. As can be seen in Figure 9, some cytotoxic effects were obtained for both bupivacaine and ibuprofen when testing concentrations higher than 1% w/v. The cell viability values in the presence of bupivacaine (67.6% and 58.8% for 2% w/v and 3% w/v drug, respectively) were lower than those obtained in the presence of ibuprofen (93.5 and 79.7% for 2% w/v and 3% w/v drug, respectively). It is important to note that at least 50% cell viability was achieved in all cases, compared with the control. It should also be mentioned that this test is ‘pessimistic’, because the bioadhesive gradually releases its loaded drug over three days, which is different from the conditions in this experiment.

![Figure 8](image.png)

Figure 8. ESEM fractographs of adhesives (200 mg/ml gelatin, 40 mg/ml alginate and 20 mg/ml EDC) loaded with 1% w/v ibuprofen.
4. Summary and conclusions

In the current study, we developed and studied a gelatin/alginate bioadhesive which is loaded with drugs for pain relief. Our thought was that in addition to providing an attractive alternative for sutures and other traditional wound closing applications, our bioadhesive will also alleviate the patient’s pain sensation by locally releasing an analgesic or anesthetic drug at the lacerated area in a desired controlled manner. Two well-known drugs were chosen for this goal – bupivacaine (anesthetic) and ibuprofen (analgesic), which were incorporated into the gelatin/alginate adhesive already during the preparation stage.

The release of drugs from the adhesive matrix was found to be controlled mainly by the bioadhesive’s characteristics, i.e. swelling and hydrophilic group concentration. The drug characteristics, i.e. hydrophilicity and the electrical interactions between the drug and the polymeric components, were also found to have some effect. In addition to restraining the swelling rate, proper analysis of the drug’s physical and chemical reactive behavior in the presence of the other adhesive components may therefore be essential for designing a device with a desirable controlled release.

Bupivacaine and ibuprofen exhibited a different effect on the bonding strength of the adhesive. Loading bupivacaine improved the bonding strength due the reinforcing effect of its crystals, while loading ibuprofen caused a decrease in the bonding strength probably due to its reaction with the EDC crosslinking agent. Thus, although bupivacaine is slightly more cytotoxic than ibuprofen, it can be considered as the preferred drug for our novel tissue adhesives.

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