In vitro microbial inhibition, bonding strength, and cellular response to novel gelatin–alginate antibiotic-releasing soft tissue adhesives†

Maytal Foox, Ayelet Raz-Pasteur, Israel Berdichevsky, Norberto Krivoy and Meital Zilberman

INTRODUCTION

Soft tissue adhesives

Traumatic wounds, including skin lacerations, are among the most common problems encountered in emergency departments.[1] Some lacerations are more serious than others, depending on a variety of characteristics such as the angle, force, depth or the object that caused the wound, leading to severe bleeding, wound infections, unsightly and dysfunctional scars, and even mortality. Natural wound healing is a process that involves several stages in which cellular and matrix components act together to rebuild and replace the damaged tissue. It is important to ensure proper healing and prevent infection or unattractive scar formation. The common procedure is therefore to clean the wound and re-attach its edges using sutures or staples.[2] Tissue bioadhesives have raised interest in the last few decades as an alternative to sutures or staples in wound closing applications because of various advantages. They are less time consuming, less painful, and can be less expensive, without compromising the cosmetic outcome. Even though extensive efforts have been made, an ideal tissue adhesive has not been developed to date, mostly because of toxicity or weak bonding strength issues. Novel bioadhesives comprised gelatin and alginate with carbodiimide (N-ethyl-N-(3-dimethylaminopropyl) carbodiimide [EDC]) as the cross-linking agent were recently developed by our research group. In the current research, N-hydroxysuccinimide (NHS) was added to the cross-linking reaction to enable a decrease in the EDC content and therefore also the cytotoxicity, without decreasing the bonding strength. The antibiotic drug clindamycin was added to the bioadhesive formulation. It was selected because of being inert toward the cross-linking reaction. The effects of EDC, NHS, and clindamycin concentrations on the ex vivo bonding strength, drug release profile, and fibroblast viability, as well as the microbial inhibition, were studied. Incorporation of clindamycin was found to improve the bonding strength of the adhesive. Its release profile was highly effective against the two relevant bacterial strains, Staphylococcus albus and Staphylococcus aureus, which were eradicated within less than 48 h. The good cytotoxicity results indicate that our new antibiotic-eluting bioadhesives represent an effective and selective treatment option for bacterial infections. Delivering an antibiotic drug locally using our bioadhesive could decrease the risk of infections and increase the therapeutic effect of the bioadhesive itself. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: gelatin; alginate; carbodiimide; clindamycin; bioadhesive; controlled drug delivery

Tissue bioadhesives have raised interest in the last few decades as an alternative to sutures or staples in wound closing applications because of various advantages. They are less time consuming, less painful, and can be less expensive, without compromising the cosmetic outcome. Even though extensive efforts have been made, an ideal tissue adhesive has not been developed to date, mostly because of toxicity or weak bonding strength issues. Novel bioadhesives comprised gelatin and alginate with carbodiimide (N-ethyl-N-(3-dimethylaminopropyl) carbodiimide [EDC]) as the cross-linking agent were recently developed by our research group. In the current research, N-hydroxysuccinimide (NHS) was added to the cross-linking reaction to enable a decrease in the EDC content and therefore also the cytotoxicity, without decreasing the bonding strength. The antibiotic drug clindamycin was added to the bioadhesive formulation. It was selected because of being inert toward the cross-linking reaction. The effects of EDC, NHS, and clindamycin concentrations on the ex vivo bonding strength, drug release profile, and fibroblast viability, as well as the microbial inhibition, were studied. Incorporation of clindamycin was found to improve the bonding strength of the adhesive. Its release profile was highly effective against the two relevant bacterial strains, Staphylococcus albus and Staphylococcus aureus, which were eradicated within less than 48 h. The good cytotoxicity results indicate that our new antibiotic-eluting bioadhesives represent an effective and selective treatment option for bacterial infections. Delivering an antibiotic drug locally using our bioadhesive could decrease the risk of infections and increase the therapeutic effect of the bioadhesive itself. Copyright © 2014 John Wiley & Sons, Ltd.
prepared for clinical use. Extensive efforts have been made to develop appropriate tissue adhesives. However, most of the bioadhesives that have been approved for use are either too toxic or have weak bonding strength to tissue, and none seem to have noticeable advantages. For these reasons, we recently developed novel tissue bioadhesives composed of gelatin and alginate with \( N \)-ethyl-\( N \)-(3-dimethylaminopropyl) carbodiimide (EDC) as the cross-linking agent.

The polymeric component in our study

Gelatin and alginate were selected for this study because of their suitable range of properties. Gelatin is a water-soluble polypeptide that is obtained from the hydrolysis of collagen. The formation of a thermo-reversible gel along with its other qualities, such as being biocompatible, biodegradable, and non-immunogenic, has made gelatin popular in medical applications such as tissue adhesives, sealants, hydrogels, microspheres, drug delivery systems, and wound dressings. Gelatin has polar groups, such as amine and carboxyl groups, which enable it to bind to other compounds. Alginate is a naturally occurring polysaccharide extracted mainly from three species of brown algae. It has controllable gel porosity (allowing high diffusion of macromolecules), and its biodegradability under normal physiological conditions turns it into a good candidate for protein and cell delivery. Alginites are also known to induce cytokine production (such as TNF-\( \alpha \) by human monocytes) and can thus influence the healing process. It also possesses a bioadhesive nature and is classified, with its carboxyl end groups, as an anionic mucoadhesive polymer.

The cross-linking reaction in our study

In order to achieve bonding ability, the gelatin–alginate solutions should be cross-linked with an appropriate cross-linking agent. When a nonzero-length cross-linking agent is incorporated into the bioadhesive, it may leach into the body during the biomaterial’s biodegradation and may cause damage due to toxicity. In contradistinction, a zero-length cross-linking agent activates the carboxylic acid groups to react with free amine groups, resulting in the formation of an amide bond without incorporation of foreign structures into the network. Carbodiimide cross-linking is one of the representatives of this class of cross-linkers, and involves the use of less harmful reagents. EDC, which is water-soluble, is the most popular cross-linker. It activates carboxylic acid residues of aspartic and glutamic acid to form O-acylisourea groups. Then, there is a nucleophilic attack on the activated carboxylic acid residues by the free amine groups of lysine and hydroxylysine residues, resulting in the formation of peptide amide bonds and the release of urea. Possible side reactions are the hydrolysis of the O-acylisourea group and the formation of a carboxylic acid group and an \( N \)-acylurea group (Fig. 1).

Figure 1. Cross-linking reaction of gelatin and alginate with \( N \)-ethyl-\( N \)-(3-dimethylaminopropyl) carbodiimide (EDC) and \( N \)-hydroxysuccinimide (NHS) (Gel—gelatin, Al—Alginate). This figure is available in colour online at wileyonlinelibrary.com/journal/pat.

We therefore added \( N \)-hydroxysuccinimide (NHS) to our basic formulations in order to improve the cross-linking reaction, and reduce the extent of side reactions and the amount of EDC needed for the cross-linking. The addition of NHS to the cross-linking reaction created an NHS-activated carboxylic acid group, which is less susceptible to hydrolysis and prevents rearrangements. However, at high concentrations, NHS can react with EDC. This may lead to the formation of reactive isocyanate and NHS-ester residues, which can reduce the effective amount of EDC for cross-linking. The conditions for the cross-linking of gelatin and alginate with EDC and NHS therefore need to be optimized for cross-linking density and the prevention of undesired side reactions.

In the current study, we developed a method for the preparation and application of a bioadhesive, which enables us to reduce the concentration of the cross-linking agent and thus...
increase biocompatibility without reducing the bioadhesive’s bonding strength. The formulation-strength effects and cytotoxicity of these novel bioadhesive formulations are described in the current article.

**Antibiotic drugs used in the current study**

Controlled release of antibiotic drugs to the wound area can prevent infections and help the natural tissue healing process. We examined the incorporation of three antibiotic drugs into our bioadhesive: clindamycin, vancomycin, and ofloxacin. Clindamycin is a lincosamide antibiotic, which has been shown to have activity against Gram-positive organisms (staphylococci, streptococci), Gram-negative anaerobes, mycoplasmas, and some protozoa.\(^{[24]}\) It is used for treatment of a wide spectrum of diseases such as infections of the respiratory tract, skin and soft tissue infections, osteomyelitis, and gynecological infections.\(^{[25,26]}\) Vancomycin is a glycopeptide antibiotic and has been shown to have activity against methicillin- and gentamicin-resistant strains of *Staphylococcus aureus*.\(^{[27]}\) Ofloxacin is a synthetic fluoroquinolone, which has been shown to be effective against skin infections caused by *S. aureus* and *Streptococcus pyogenes*.\(^{[28]}\)

In the current research, we studied formulation-strength effects and cytotoxicity as well as the strength of antibiotic-loaded formulations, their drug release profile, and the resulting antimicrobial effects.

## EXPERIMENTAL

### 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), NHS, vancomycin hydrochloride, ofloxacin, and clindamycin hydrochloride were all purchased from Sigma-Aldrich, Rehovot, Israel. The chemical structure of the antibiotic drugs is shown in Table 1.

### Preparation of the adhesive

The preparation of the bioadhesive was based on dissolving 200 mg/ml gelatin and 40 mg/ml alginate in double-distilled water, under heating up to 60 °C. EDC and NHS were dissolved in double-distilled water, and various amounts of these solutions were added to the gelatin–alginate solution immediately prior to the adhesive’s use. The formulations are presented in the form of EDC–NHS–Drug, where EDC is the concentration of the carbodiimide cross-linking agent (mg/ml), NHS is the concentration of the NHS (mg/ml), and Drug is the drug content (% w/v) relative to the total volume of the bioadhesive. The pH values of the studied solutions are approximately 6. All studied formulations are presented in Table 2.

### Table 1. The chemical structure of the antibiotic drugs used in this research

<table>
<thead>
<tr>
<th>Antibiotic drug</th>
<th>Chemical structure</th>
<th>Functional groups that can react with the cross-linking agent EDC (circled)</th>
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<tr>
<td>Clindamycin</td>
<td><img src="https://example.com/clindamycin.png" alt="Clindamycin structure" /></td>
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<td>Ofloxacin</td>
<td><img src="https://example.com/ofloxacin.png" alt="Ofloxacin structure" /></td>
<td>Carboxyl groups</td>
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<td>Vancomycin</td>
<td><img src="https://example.com/vancomycin.png" alt="Vancomycin structure" /></td>
<td>Carboxyl and amine groups</td>
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**Ex vivo bonding strength measurements**

Porcine skin (Kibbutz Lahav, Israel) was used as a soft tissue model. The porcine skin was cut into $2 \times 2$ cm$^2$ square-shaped pieces, and their epidermis side was firmly attached to metal testing holders with a matching surface area (all dimensions of the holders are specified elsewhere ($^{[13]}$)). One hundred and forty microliters of the adhesive was then spread uniformly on the dermis side of two porcine skin pieces (that were attached to the testing holders). These two porcine skin pieces were immediately attached by applying a load of 1.25 N on them and were incubated at 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. Norwood, Massachusetts, USA) 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. Fifteen repetitions were carried out for each formulation. The bonding strength of the bioadhesive loaded with 1% w/v ofloxacin, 3% w/v vancomycin, 1%, 3%, 5%, and 7% w/v clindamycin was also tested.

**In vitro drug release studies**

The tissue adhesive was prepared in a $6.2 \times 6.2 \times 3.5$ mm$^3$ silicon mold with various amounts of the antibiotics. After gelation, the adhesive was carefully removed and dried in air. The samples ($n=5$) were immersed in 2 ml phosphate buffered solution (PBS) with 0.02% w/v sodium azide and placed in a static incubator at 37 °C and 100% relative humidity in order to determine the drug release kinetics. The PBS was removed completely, and fresh medium was added at 2, 4, 6, and 24 h, and 2 and 3 days.

The residual drug was extracted by dissolving the tissue adhesive in trypsin A for 24 h in a static incubator at 37 °C and 100% relative humidity. The residual drug was estimated using high-performance liquid chromatography (HPLC) (as described later).

**Clindamycin assay**

The medium’s clindamycin content was determined using a Jasco HPLC (Jasco corporation, Tokyo, Japan) with a UV 2075 plus detector and a reverse phase column (ACE®, Aberdeen, Scotland 5 μm, inner diameter $d=4.6$ mm, length = 250 mm), kept at 35 °C. The mobile phase consisted of a mixture of monobasic potassium phosphate (pH 7.5) and acetonitrile (55/45, v/v) at a flow rate of 1 ml/min with a quaternary gradient pump (PU 2089 plus) without gradient. Ten-microliter samples were injected with an auto sampler (AS 2057 Plus). The column effluent was eluted for 15 min and detected at 210 nm. The area of each eluted peak was integrated using the EZstart software version 3.1.7. (Scientific Software Inc. Pleasonton CA, USA). A calibration curve was prepared for concentrations ranging from 25.0 to 1500.0 μg/ml (correlation coefficient >0.99).

**Microbiological evaluation**

Two strains were chosen for the microbiological assay: S. aureus and Staphylococcus albus. Both strains were clinically isolated at the Microbiological Laboratory of Rambam Medical Center (Haifa, Israel), and their minimal inhibitory concentration values were evaluated (Table 3). The strains were grown overnight on Müller-Hinton agar plates (HyLabs, Rehovot, Israel) at 37 °C prior to use. The bacterial cells were collected and re-suspended in saline, and adjusted to $1 \times 10^{6}$ CFU/ml (colony forming units). Five groups of bioadhesives were tested for drug release-induced bacterial inhibition. All studied bioadhesive groups contained gelatin (200 mg/ml) and alginate (40 mg/ml). The EDC, NHS, clindamycin,

<table>
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<tr>
<th>Gelatin concentration (mg/ml)</th>
<th>Alginate concentration (mg/ml)</th>
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<th>NHS concentration (mg/ml)</th>
<th>% of NHS from EDC (%)</th>
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and cross-linker concentrations are as follows: 20 mg/ml EDC (20–0) and also loaded with 7% (w/v) clindamycin (20–0–7%) or loaded with 3% (w/v) clindamycin (20–0–3%) or cross-linked with 10 mg/ml EDC and 1 mg/ml NHS (10–1), loaded with 7% (w/v) clindamycin (10–1–7%). The bioadhesives were poured into a 6.2×6.2×3.5 mm³ silicon mold to create cube-shaped samples. Drug release-induced bacterial inhibition from these bioadhesives was evaluated using the following two methods.

**Corrected zone of inhibition**

The corrected zone of inhibition test (CZOI) was used to determine the antimicrobial activity of the bioadhesives, which is time-dependent. In this modified version of the Kirby-Bauer disk diffusion test, which is typically used to determine bacterial susceptibility to antibiotics, cubic-shaped samples of the bioadhesive loaded with clindamycin were placed on a bacterial lawn (100 μl of inoculums, ~10⁶ CFU/ml, seeded on Müller-Hinton agar plates), and incubated overnight at 37 °C and then photographed. The effective diameter of the inhibition zone was measured from the images, and the area of inhibition was calculated. The CZOI was then calculated by subtracting the adhesive area (36 mm). This procedure was repeated on bioadhesives, which were incubated in PBS for 24, 48, and 72 h prior to testing. The test was performed in triplicates for each of the microorganisms.

**Viable counts**

A release study from the cube-shaped bioadhesive in the presence of bacteria was performed in order to study the effect of drug release on the kinetics of residual bacteria. One milliliter of PBS containing the bacterial strains at a concentration of 1×10⁶ CFU/ml was added at the beginning of the release study, and the effect of the antibiotics released from the bioadhesives on residual bacteria was tested. Microorganisms in the presence of PBS only served as the control. Ten-microliter samples were collected at time 0 and after 24, 48, and 72 h, and spread on agar plates containing Müller-Hinton. CFU/ml was counted after 24 h incubation at 37 °C. The test was performed in duplicate for each of the microorganisms.

**Cytotoxicity evaluation**

The Alamar Blue (AbD Serotec Ltd, Kidlington, Oxford, United Kingdom) assay was used to evaluate cell growth and viability in the presence of the bioadhesives. Human fibroblasts were obtained from neonatal foreskins. Fibroblasts (14th passage) were thawed and cultured in 75 mm³ flasks with modified Eagle’s medium supplemented with heat-inactivated fetal bovine serum (10% v/v), l-glutamine (1% v/v), and penicillin-streptomycin-nystatin (1% v/v). The cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air. The tissue culture medium was changed every 2 days until confluence was reached. After the fibroblast confluence reached 70% (14th–20th passages), they were separated from the bottom of the flasks by a “Trypsin A” solution and were seeded into 96-well plates at a concentration of 5000 cells per well. Former experiments (data not shown) determined this optimal seeding density and culture time. Added to each well was 0.2 ml of fresh culture medium, and the plates were returned to incubation for 24 h until a confluence of 70% was achieved.

In order to create the testing medium, 100 μl of gelatin–alginate solutions were mixed with 40 μl EDC and NHS solutions, with different concentrations, to create cuboids. All five cuboids were weighed, and placed in scintillation vials, and fresh culture medium was added at a ratio of 1:5 (w/v). The scintillation vials were placed in a 37 °C and 5% CO₂ humidified incubator environment for 24 h. The Alamar Blue assay included replacing the original medium with 0.2 ml of fresh medium containing 10% (v/v) Alamar Blue and incubation of the wells for 4 h. After the incubation, 100 μl duplicates from each well were transferred into a 96-well plate for spectrophotometer analysis (Spectra max 340 PC384, Molecular Devices, Sunnyvale, CA, USA) at 570 and 600 nm. The percentage of Alamar Blue reduction was calculated according to the manufacturer’s protocol:

\[
\text{%Alamar Blue reduction} = \left( \frac{E_{570} - E_{600}}{E_{570} - E_{600}} \right) \times 100
\]

where \( E_0 \) and \( E_r \) represent the molar extinction coefficient of the oxidized and reduced Alamar Blue, respectively, at 570 and 600 nm; \( A_c \) and \( A_r \) represent the absorbance of the test and control well (medium with Alamar Blue with no cells), respectively, at 570 and 600 nm. All samples were tested in triplicate.

**RESULTS AND DISCUSSION**

In the current study, we investigated the effect of the addition of NHS to bioadhesives based on gelatin and alginate, cross-linked with EDC, on the adhesives’ bonding strength and cytotoxicity. As explained in the introduction section, NHS was chosen in order to improve the cross-linking reaction. The incorporation of antibiotic drugs was also studied in order to increase the bioadhesives’ therapeutic effect. The main results and their discussion are presented later.

**Bonding strength**

High bonding strength to the tissue is crucial in the application of tissue bioadhesives. Gelatin and alginate mixtures exhibit a range of mechanical, bioactivity, biocompatibility, and bioadhesive properties. However, these mixtures should also be cross-linked with an appropriate cross-linking agent in order to maintain the mechanical properties for long-term use. The cross-linking agent carbodiimide is considered to be less toxic than the other cross-linking reagents and was therefore chosen for the current study. A formulation consisting of 200 mg/ml gelatin and 40 mg/ml alginate was chosen, based on our previous study.[13] The combined effect of the EDC and NHS concentrations is presented in Fig. 2. Addition of NHS in relatively low concentrations (0–2 mg/ml) increased the bonding strength at all EDC concentrations, whereas addition of higher NHS concentrations had no further effect on the bonding strength for most EDC concentrations. In the cross-linking reaction, EDC activates carboxylic acid in the gelatin and alginate molecules to form O-acylisourea groups. Then there is a nucleophilic attack on the activated carboxylic acid residues by the free

| Table 3. Minimal inhibitory concentration (MIC) of clindamycin (µg/ml) |
|-----------------------------|-----------------------------|
| Microorganism              | MIC (µg/ml)                 |
| Staphylococcus aureus      | 0.064                      |
| Staphylococcus albus       | 0.047                      |
amine residues (found in the gelatin molecules), resulting in the formation of peptide amide bonds and the release of urea. Possible side reactions are the hydrolysis of the O-acylisourea group and the formation of carboxylic acid and N-acylurea groups.[21] The addition of NHS to the cross-linking reaction yields NHS-activated carboxylic acid groups, which are less susceptible to hydrolysis, prevents rearrangements, and shifts the reaction toward the creation of peptide bonds while reducing the side reaction.[22] This enables us to increase the bonding strength of our formulations. However, at relatively high NHS concentrations, it probably reacts with EDC, which may lead to the formation of reactive isocyanate and NHS-ester residues, leading to a decrease in the effective amount of EDC for cross-linking and thus lowering the bonding strength. As can be seen in Fig. 2, addition of NHS enabled us to increase the bonding strength from 20 to 10 mg/ml without decreasing the bonding strength. The maximal bonding strength, 14.3 ± 2.5 kPa, was measured for formulations cross-linked with 10 mg/ml EDC and 1 mg/ml NHS. The bonding strength of bioadhesives loaded with the selected antibiotic drugs (vancomycin, ofloxacin, and clindamycin) was also measured. As can be seen in Fig. 3a, addition of only 1% w/v vancomycin or ofloxacin molecules into the formulation resulted in a significant decrease in the bonding strength, from 10.1 ± 1.6 kPa to 7.2 ± 1.6 and 6.9 ± 1.9 kPa, respectively. In contrast, addition of different concentrations of clindamycin did not decrease the bonding strength and even slightly increased it (Fig. 3b). These results can be explained by the chemical structure of the drugs (Table 1). The drugs’ primary amine and carboxyl groups can be cross-linked with gelatin or alginate by the EDC reaction, reducing the amount of EDC molecules available for the cross-linking of gelatin and alginate, and as a result decreasing the cross-linking degree of the bioadhesive and its bonding strength. Vancomycin contains an amine and carboxyl groups, and ofloxacin contains carboxyl groups. However, clindamycin does not contain either of these groups, which is why it was probably inert and did not interfere with the chemical cross-linking reaction. Clindamycin was thus found to be the most suitable drug for our system, and was therefore used for the following stages of the research.

Cytotoxicity evaluation

One of the requirements from an ideal bioadhesive is to be safe, with minimal cytotoxic byproducts. The Alamar Blue assay was performed on human fibroblasts that participate in the wound healing process in order to test the cell viability, and the entire experiment was performed according to a standard test method.[29] The high reactivity of EDC and its urea derivative to amino and carboxyl groups in living tissues and their positive charge, which is known to be irritative to cultured cells, can make them cytotoxic at high concentrations.[12] Figure 4 presents the fibroblasts’ viability as a function of different EDC (Fig. 4a) and NHS (Fig. 4b) concentrations. At relatively low EDC concentrations...
formulations was very similar. All profiles exhibited a relatively high burst effect of 76–81% during the first 2 h of release, and approximately 99% of the encapsulated clindamycin was released after 4 h (Fig. 5). Our results indicate that the drug release mechanism in our system is probably diffusion, which is strongly enhanced by the swelling of the gelatin–alginate matrix in an aqueous medium. Water penetrates the bioadhesive because of its hydrophilic nature, thus facilitating clindamycin’s diffusion to the surrounding.

Microbiological evaluation
Drug eluting bioadhesives offer many advantages compared with conventional dosage forms or bioadhesives without antibiotic drugs. The incorporation of antibiotic drugs can increase the therapeutic effect and help in the healing process. It is important to examine the effect of the drug release profile on the inhibition of bacterial growth. The time-dependent antimicrobial efficacy of our selected bioadhesives loaded with different concentrations of clindamycin was tested in vitro by two complementary methods, CZOI and viable counts.

Corrected zone of inhibition test
A modification of the disk diffusion test, which is a good representation of the clinical situation, was performed with cube-shaped samples of our bioadhesive (instead of disks). This method represents the situation when the bioadhesive is applied on the wound surface and the drug can diffuse to the wound area. Bacterial inhibition depends on the diffusion rate of the drug from the bioadhesive, the environment, and the sensitivity and growth rate of the bacterial species. Representative photographs of zones of inhibition created around the bioadhesive cubes that were loaded with different concentrations of clindamycin are shown in Fig. 6. Both S. aureus and S. albus demonstrated susceptibility to clindamycin at the initial time point. A slight inhibition was also seen in the control groups that did not contain clindamycin, probably because of the acidic nature of NHS. The presence of bacterial inhibition in an area that exceeds the dressing material (CZOI > 0) can be considered beneficial in keeping the surrounding sterile. The largest zone of inhibition for both types of microorganisms, at the initial time point, was found for the EDC-NHS-clindamycin 10–1–7% formulation. EDC-NHS-clindamycin 20–0–7% showed similar results,

In vitro clindamycin release
Skin infections limit the healing process, may attract harmful organisms, and may lead to bacteremia, sepsis, or multi-system failure. It is therefore crucial to respond immediately to the presence of large numbers of bacteria. In order to respond to this risk, a local antibiotic release profile should exhibit a high initial release rate followed by a sustained released phase at an effective inhibitory level.28 Based on ex vivo bonding strength measurements and the cytotoxicity study, our preferred formulation for the drug release profile and the microbiological study was the EDC-NHS 10-1 formulation. This formulation was loaded with different concentrations of clindamycin (1%, 3%, 5%, and 7% w/v). The release profile of clindamycin from all studied formulations was very similar. All profiles exhibited a relatively high burst effect of 76–81% during the first 2 h of release, and approximately 99% of the encapsulated clindamycin was released after 4 h (Fig. 5). Our results indicate that the drug release mechanism in our system is probably diffusion, which is strongly enhanced by the swelling of the gelatin–alginate matrix in an aqueous medium. Water penetrates the bioadhesive because of its hydrophilic nature, thus facilitating clindamycin’s diffusion to the surrounding.

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Corrected zone of inhibition test
A modification of the disk diffusion test, which is a good representation of the clinical situation, was performed with cube-shaped samples of our bioadhesive (instead of disks). This method represents the situation when the bioadhesive is applied on the wound surface and the drug can diffuse to the wound area. Bacterial inhibition depends on the diffusion rate of the drug from the bioadhesive, the environment, and the sensitivity and growth rate of the bacterial species. Representative photographs of zones of inhibition created around the bioadhesive cubes that were loaded with different concentrations of clindamycin are shown in Fig. 6. Both S. aureus and S. albus demonstrated susceptibility to clindamycin at the initial time point. A slight inhibition was also seen in the control groups that did not contain clindamycin, probably because of the acidic nature of NHS. The presence of bacterial inhibition in an area that exceeds the dressing material (CZOI > 0) can be considered beneficial in keeping the surrounding sterile. The largest zone of inhibition for both types of microorganisms, at the initial time point, was found for the EDC-NHS-clindamycin 10–1–7% formulation. EDC-NHS-clindamycin 20–0–7% showed similar results,

In vitro clindamycin release
Skin infections limit the healing process, may attract harmful organisms, and may lead to bacteremia, sepsis, or multi-system failure. It is therefore crucial to respond immediately to the presence of large numbers of bacteria. In order to respond to this risk, a local antibiotic release profile should exhibit a high initial release rate followed by a sustained released phase at an effective inhibitory level.28 Based on ex vivo bonding strength measurements and the cytotoxicity study, our preferred formulation for the drug release profile and the microbiological study was the EDC-NHS 10-1 formulation. This formulation was loaded with different concentrations of clindamycin (1%, 3%, 5%, and 7% w/v). The release profile of clindamycin from all studied formulations was very similar. All profiles exhibited a relatively high burst effect of 76–81% during the first 2 h of release, and approximately 99% of the encapsulated clindamycin was released after 4 h (Fig. 5). Our results indicate that the drug release mechanism in our system is probably diffusion, which is strongly enhanced by the swelling of the gelatin–alginate matrix in an aqueous medium. Water penetrates the bioadhesive because of its hydrophilic nature, thus facilitating clindamycin’s diffusion to the surrounding.

Microbiological evaluation
Drug eluting bioadhesives offer many advantages compared with conventional dosage forms or bioadhesives without antibiotic drugs. The incorporation of antibiotic drugs can increase the therapeutic effect and help in the healing process. It is important to examine the effect of the drug release profile on the inhibition of bacterial growth. The time-dependent antimicrobial efficacy of our selected bioadhesives loaded with different concentrations of clindamycin was tested in vitro by two complementary methods, CZOI and viable counts.

Corrected zone of inhibition test
A modification of the disk diffusion test, which is a good representation of the clinical situation, was performed with cube-shaped samples of our bioadhesive (instead of disks). This method represents the situation when the bioadhesive is applied on the wound surface and the drug can diffuse to the wound area. Bacterial inhibition depends on the diffusion rate of the drug from the bioadhesive, the environment, and the sensitivity and growth rate of the bacterial species. Representative photographs of zones of inhibition created around the bioadhesive cubes that were loaded with different concentrations of clindamycin are shown in Fig. 6. Both S. aureus and S. albus demonstrated susceptibility to clindamycin at the initial time point. A slight inhibition was also seen in the control groups that did not contain clindamycin, probably because of the acidic nature of NHS. The presence of bacterial inhibition in an area that exceeds the dressing material (CZOI > 0) can be considered beneficial in keeping the surrounding sterile. The largest zone of inhibition for both types of microorganisms, at the initial time point, was found for the EDC-NHS-clindamycin 10–1–7% formulation. EDC-NHS-clindamycin 20–0–7% showed similar results,
while the smallest zone of inhibition was found with EDC-NHS-clindamycin 20–0–3% (Fig. 7). Similar bioadhesives, which were incubated in PBS for 24, 48, and 72 h prior to exposure to the bacteria, demonstrated lower levels of bacterial inhibition, because of drug release from the bioadhesives (Fig. 7). The in vitro clindamycin release studies showed that approximately 99% of the clindamycin was released after 4 h. The later inhibition that was found in this assay can therefore be explained by the ability of the bioadhesive cubes to absorb the surrounding liquid, which contains drug that was released while they were incubated in PBS, prior to exposure to the bacteria.

Viable counts

The purpose of these experiments was to monitor the effectiveness of cumulative antibiotic release from the bioadhesive in terms of the residual bacteria compared with initial bacterial inoculations of $1 \times 10^7$ CFU/ml, which correspond to severe infection. This investigation focused on samples containing 3% and 7% (w/v) clindamycin. Bacteria present in PBS only served as the control. The results are presented in Fig. 8. Relatively high numbers of bacteria, above $10^6$ CFU/ml, survived over 72 h in $S. aureus$ and $S. Albus$ controls (without the antibiotic), whereas total eradication of $S. aureus$ and $S. albus$ occurred within 24 h because of clindamycin release from the EDC-NHS-clindamycin 10–1–7% formulation. Similar results were obtained for the EDC-NHS-clindamycin 20–0–7% bioadhesive. However, when the EDC-NHS-clindamycin 20–0–3% bioadhesive was used, total eradication of both types of microorganisms occurred only after 72 h (Fig. 8a and b).

Despite the favorable effective antimicrobial activity of clindamycin against Gram-positive organisms (staphylococci and streptococci), Gram-negative anaerobes, mycoplasmas, and some protozoa, concerns regarding its systemic administration have always been emphasized, because clindamycin is associated with a serious antibiotic-related complication. As mentioned previously, the drug is inert to our cross-linking reaction and therefore does not decrease the adhesion ability of the bioadhesive. Delivering this drug locally using our bioadhesive could thus decrease the risk of systemic complications and increase the therapeutic effect of the bioadhesive itself.

**SUMMARY AND CONCLUSIONS**

In the current study, we developed and studied a gelatin–alginate bioadhesive, cross-linked with EDC and NHS and loaded with antibiotic drugs. Our thought was that the addition of NHS to these bioadhesives may improve the efficiency of the cross-linking reaction. In addition to providing an alternative for sutures and other traditional wound closing applications, our bioadhesive can have a therapeutic effect when it is loaded with antibiotic drugs. Three antibiotic drugs were examined for this purpose—clindamycin, vancomycin, and ofloxacin, and their effects on the ex vivo adherence properties were investigated. Clindamycin was selected as the preferred drug, as it is inert
Bacteria in the presence of phosphate buffered saline only served as control. This indicated total eradication of S. albus and S. aureus. The zone of inhibition results showed that clindamycin-eluting bioadhesives preserved their antibacterial activity, as well as microbial inhibition, when compared to the bonding strength. This release of clindamycin from the bioadhesive could decrease the risk of infections and increase the therapeutic effect of the bioadhesive itself.

Despite severe toxicity to bacteria, the studied bioadhesive formulations were not found to have a toxic effect on cultured fibroblasts, indicating that in addition to their regular role as soft tissue adhesives, our new antibiotic-eluting bioadhesives represent an effective and selective treatment option for bacterial infections. Delivering an antibiotic drug locally using our formulations could decrease the risk of infections and increase the therapeutic effect of the bioadhesive itself.

Acknowledgement

The authors are grateful to the Office of the Chief Scientist [OCS] in the Israel Ministry of Industry, Trade and Labor, for supporting this research.

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