Cellulose fibres enhance the function of hemostatic composite medical sealants

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Abstract

Tissue adhesives and sealants offer promising alternatives to traditional wound closure methods, but the existing trade-off between biocompatibility and strength is still a challenge. The current study explores the potential of a gelatin-alginate-based hydrogel, cross-linked with a carbodiimide, and loaded with two functional fillers, the hemostatic agent kaolin and cellulose fibres, to improve the hydrogel's mechanical strength and hemostatic properties for use as a sealant. The effect of the formulation parameters on the mechanical and physical properties was studied, as well as the biocompatibility and microstructure.

The incorporation of the two functional fillers resulted in a dual micro-composite structure, with uniform dispersion of both fillers within the hydrogel, and excellent adhesion between the fillers and the hydrogel matrix. This enabled to strongly increase the sealing ability and the tensile strength and modulus of the hydrogel. The fibres' contribution to the enhanced mechanical properties is more dominant than that of kaolin. A combined synergistic effect of both fillers resulted in enhanced sealing ability (247%), tensile strength (400%), and Young's modulus (437%), compared to the unloaded hydrogel formulation. While the incorporation of kaolin almost did not affect the physical properties of the hydrogel, the incorporation of the fibres strongly increased the viscosity and decreased the gelation time and swelling degree. The cytotoxicity tests indicated that all studied formulations exhibited high cell viability.

Hence, the studied new dual micro-composite hydrogels may be suitable for medical sealing applications, especially when it is needed to get a high sealing effect within a short time. The desired hemostatic effect is obtained due to kaolin incorporation without affecting the physical properties of the sealant. Understanding the effects of the formulation parameters on the hydrogel's properties enables the fitting of optimal formulations for various medical sealing applications.

Keywords: Hydrogels, cellulose fibres, composite bioadhesives, Kaolin, medical sealants**.**

1. Introduction

The management of wound leakage poses a significant challenge in general surgery, where complications can manifest as bleeding or the release of body fluids and gases from tissues.[1,2]. Surgical sealants are usually absorbable materials, which, as opposed to tissue adhesives, are not used to attach organs or tissues, but rather to control internal bleeding and to seal the tissue in order to prevent leaks of fluid or gas from a surgical incision [3]. Currently, surgical sealants are classified into two major groups: natural sealants, i.e., based on proteins or polysaccharides, and synthetic sealants, i.e., based on synthetic polymers.

Natural Sealants include, among others, fibrin, collagen gelatin dextran, and albumin-based materials[4]. While their main advantages are biocompatibility and biodegradability, they are limited in their adhesion ability, especially in wet conditions. Additionally, they carry the risk of disease/infection transmission or allergic reaction and high costs. Due to their low bonding strength, some are recommended only as an adjunct treatment to sutures[5].

Synthetic sealants are usually based on cyanoacrylates, polyurethane, or polyethylene glycol. Cyanoacrylates provide high bonding strength and rapid hemostasis; however, they are stiff and have the potential toxicity of degradation products or complications through prolonged persistence in the body, which is why they are mainly approved for topical applications[3]. Polyethylene glycol hydrogels induce rapid cross-linking to inherent extracellular matrix proteins, such as collagen, thus readily sealing the wound without the need for additional hemostatic agents. One prominent disadvantage of polyethylene glycol is its high swelling ratio, which limits the application to organs that are not sensitive to mechanical pressure induced by the swelling[6]. Polyurethanes possess good wettability and

strong bonding to tissues. However, they exhibit long setting time, and non-biocompatible degradation products can be created[3].

Hence, the main challenge facing tissue adhesives and sealants today is the existing trade-off between biocompatibility and strength. Therefore, the design of a hydrogel sealant system which combines high bonding ability and low cytotoxicity is desirable.

Our research group has recently developed bioadhesives and medical sealants based on the well-known natural polymers, gelatin and alginate[7–9]. The crosslinking reaction in this system is based on N-(3-dimethy laminopropyl)-N-ethylcarbodiimide hydrochloride (EDC). This zero-length crosslinker is known to be significantly less toxic than other crosslinkers used for crosslinking of gelatin, such as formaldehyde and glutaraldehyde, which are non-zero-length crosslinkers.

To improve the hydrogel's mechanical strength and hemostatic properties for use as a sealant, kaolin and cellulose fibres were added to the hydrogel as fillers. Kaolin, also known as China clay, is a versatile mineral used in various industries. Its main component, kaolinite, has been recognised for its hemostatic properties[10,11]. As a hemostatic agent, kaolin promotes blood clotting and may be useful in situations where rapid hemostasis is important, such as in surgical procedures or wound management. Cellulose, a widely abundant organic polymer, finds applications in industries like paper and different biomedical applications and would healing [12–15]. The cellulose fibres can reinforce the hydrogel through physical entanglement with the polymeric network, enhancing mechanical strength. Additionally, fibres incorporation can provide pathways for load transfer within the hydrogel, improving overall cohesion.

This study aims to explore the combination of cellulose fibres and kaolin clay in surgical sealants for enhanced mechanical strength and hemostatic properties. This concept of dual composite hydrogel is new in the field of natural polymeric biomaterials, especially in the field of bioadhesives/sealants. This study aims to investigate the effects of the formulation parameters on the hydrogel's mechanical and physical properties, microstructure, and cell viability. It addresses the need for biocompatible surgical sealants with enhanced mechanical and hemostatic properties.

2. Materials and Methods

2.1. Preparation of the composite hydrogel

The preparation of the sealant system involved two aqueous solutions. The first, a polymeric solution, was prepared by dissolving gelatin and alginate (Coldwater fish skin ‘‘type A” gelatin, G7041 and alginic acid sodium salt A1112, Sigma-Aldrich, Rehovot, Israel.) into a suspension of cellulose fibres (TECHNOCEL® 300, fibre length ~500 µm, CFF GmbH and Co., Germany.) and kaolin (K1512, Sigma-Aldrich, Rehovot, Israel) in double-distilled water at 60°C.

The polymeric solution was kept at room conditions, allowing it to reach room temperature before application. The second solution was prepared by dissolving the cross-linking agent, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, E7750, Sigma-Aldrich, Rehovot, Israel) in double distilled water.

The crosslinking agent solution was mixed with the polymeric solution just before use. In all experiments, the dual-component bioadhesive was applied using a double syringe with a static mixer at a 4:1 volume ratio (Mixpac L-System, Sulzer, Switzerland), which provided consistent and homogeneous mixing of the polymeric and crosslinker solutions.

A sealant system consisting of 400 mg/mL gelatin, 10 mg/mL alginate, and 20 mg/mL EDC (400–10–20 mg/mL Gelatin–Alginate–EDC) was chosen as the basic formulation for this study since it exhibited good mechanical and physical properties as well as low cytotoxicity in our preliminary studies [7–9].

The effect of loading the basic gelatin-alginate adhesive with both, kaolin and CF, was studied on a total of 25 formulations, loading kaolin in concentrations of 0, 10, 20, 30, and 50 mg/ml and CF in concentrations of 0, 10, 20, 30 and 50 mg/mL. All studied kaolin/CF formulations are presented in Table 1.

Table 1. The composite hydrogel formulations (400:10:20 gelatin-alginate-EDC) loaded with different Kaolin/ Cellulose fiber concentrations

		Cellulose fibres [mg/mL]				
		0	**10**	**20**	**30**	**50**
Kaolin [mg/mL]	**0**	0,0	0,10	0,20	0,30	0,50
	10	10,0	10,10	10,20	10,30	10,50
	20	20,0	20,10	20,20	20,30	20,50
	30	30,0	30,10	30,20	30,30	30,50
	50	50,0	50,10	50,20	50,30	50,50

2.2. Evaluation of the composite hydrogel's mechanical properties

Burst strength measurements

The burst strength, which measures the sealing ability of the hydrogel, was evaluated using a custom-built mechanical burst device according to the standard method for Burst Strength of Surgical Sealants ASTM F2392-04. The principle of this method is to measure the maximal pressure that can be held by the hydrogel at the tissue leakage point. A collagen casing (S1 Fibran, Spain) with a uniform 3.0 mm diameter hole was used as the tissue substrate. Approximately 0.5 mL of hydrogel was applied to the collagen casing substrate, sealing the defect with a measured thickness of approximately 1 mm. The sample was placed in the test unit, and pressure was applied. The pressure at which hydrogel failure occurred was recorded as the maximal burst pressure. A minimum of 10 repetitions was carried out for each formulation**.**

Bulk properties of the hydrogel

Cylindrical samples (8 mm diameter, 40 mm height) were prepared in a silicon mold and analysed 24 hours after casting. They were subjected to tensile displacement at a rate of 5 mm/min until failure, using a 5500 Instron Universal Testing Machine (Instron Engineering Corp.) in order to measure their Young's modulus and tensile strength. 5-7 specimens were tested for each formulation.

2.3 Evaluation of the composite's physical properties

Gelation time

Crosslinking time, i.e., gelation time, was determined by the time required for the magnetic bar to cease its motion after mixing the polymers and crosslinker solutions together. Approximately 1 mL of hydrogel and 0.25 mL of crosslinker solution at various concentrations were poured into a 1.6 cm diameter plate under mixing at 300 rpm with a 1.4 cm magnetic bar at room temperature.

Viscosity

The initial viscosity of the polymeric hydrogel on the tissue is affected mainly by the viscosity of the aqueous gelatin-alginate solution. Viscosity measurements of the polymer solutions were performed using a rheometer (model DHR2, TA Instruments Ltd.) with a plate-plate geometry (40 mm), at a constant temperature of 25°C and a constant shear rate of 10 Hz. The viscosity was measured with and without the addition of kaolin and cellulose fibres.

Swelling degree

The hydrogels were injected into 7×7×3.5 mm^3 silicon molds, and following gelation (after about 5 minutes), were carefully removed and weighed (Wi). These adhesive plugs were then placed in a 24-well plate and immersed in 1.5 ml of water each. The plate was placed in an incubator at 37°C and 100% relative humidity for 2 or 24 hours. Following these time frames plugs were taken out of the water, blotted with a Kim wipe and weighed a second time (Ws).

The swelling degree was calculated according to the following equation:

$$\textbf{Swelling degree} = (Ws - Wi)/Wi \times 100\%$$

2.4 Microstructure characterisation

The composite hydrogel's microstructure was investigated to characterize the dispersion of both fillers in the hydrogel matrix. For this purpose, cubic specimens of approximately 0.5 mL 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 Cytotoxicity evaluation

Human dermal fibroblast cell cultures were exposed to hydrogel extracts for specific periods of time, as described in the ISO 10993 Standard (parts 5 & 12) for biological evaluation of medical devices[16], in order to evaluate the cytotoxicity of the various formulations.

Preparation of hydrogel extract

The hydrogel was poured into 7.0×7.0×3.5 mm^3 silicon molds. After gelation, the specimens were carefully removed and dried overnight. These samples were sterilised by gamma irradiation. Hydrogel extracts were obtained by immersing the sterilised samples in a culture medium at a 60 mg/mL concentration and incubating for 24 h at 37°C.

Cell cultures

Human dermal fibroblast cultures were obtained from neonatal foreskins (HFFn). The cells were thawed and cultured in 75 mm^3 flasks with modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin-nystatin.

The cells were kept in a humidified 37°C and 5% CO_2 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 5,000 cells per well and incubated for 24 h. After 24 h, the medium was removed and replaced with 0.1 mL per well of hydrogel extract. Cells cultured without the extract served as a negative control. The cells were cultured for an additional 72 hours. Eight repetitions were carried out for each formulation.

Alamar Blue assay for cell viability

An Alamar Blue assay was used to evaluate cell growth and viability in the presence of hydrogel extracts. The Alamar Blue assay was performed 24 and 48 hours after adding the hydrogel extracts to the wells. The procedure included replacing the original medium with 0.25 mL of fresh medium

containing 10% (v/v) Alamar Blue and incubating the cells for four hours. 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 Alamar Blue reduction was calculated according to the manufacturer's protocol. The percent reduction after exposure of the hydrogel extracts for different periods was compared to the reduction in the control cells' environment (cells not exposed to the extracts) in order to evaluate the cytotoxicity of the hydrogel.

2.7 Statistical analysis

All data were processed using the GraphPad Prism 10.1.0 software. Statistical comparison between more than two groups was performed using the ANOVA (with Tukey Kramer post hoc). A value of $p<0.05$ was considered statistically significant.

Error bars indicate the standard deviation. In all figures, statistically significant differences are indicated with asterisks where:

Symbol	Meaning
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

.

Spearman’s ranks correlation test was also conducted in order to test for statistical significance in terms of monotonic relations between fillers’ concentration and the mechanical/physical property. Calculations were performed using R’s “test.cor” method, with the “spearman” method. p value<0.05 was considered significant.

3. Results

The current study explored the potential of a gelatin-alginate-based hydrogel loaded with two functional fillers, the hemostatic agent kaolin and cellulose fibres, for use as a medical sealant. The rationale for adding cellulose fibres was to reinforce the hydrogel and provide pathways for load transfer within the structure, improving overall cohesion and mechanical strength. The hemostatic abilities of the Kaolin may assist in the wound healing process, especially for applications where rapid hemostasis is crucial, such as in surgical procedures or wound management.

3.1. Mechanical Properties

Burst Strength

The sealing ability of the medical sealants was evaluated by measuring the maximal pressure at burst. Surgical sealants must meet the threshold set by the systolic blood pressure, i.e., 200 mmHg. As shown below (Figure 1), the incorporation of kaolin resulted in a significant monotonic increase in the bioadhesive's bulk strength. The formulations loaded only with kaolin (with no cellulose fibres) reached burst strength between 259-425 mmHg. A constant increase in burst strength was also apparent when cellulose fibres were added. The unloaded formulation (400:10:20 mg/ml gelatin-alginate-EDC, no Kaolin) reached 383 mmHg when the maximal concentration of fibres was incorporated (50 mg/ml), and the formulation loaded with the highest filler concentrations (50 mg/ml Kaolin and 50 mg/ml Cellulose fibres) reached a burst strength of 639 mmHg. Examining the effect of increasing levels of CF while maintaining a constant kaolin concentration reveals a positive monotonic effect in most kaolin concentrations ($p<0.05$).

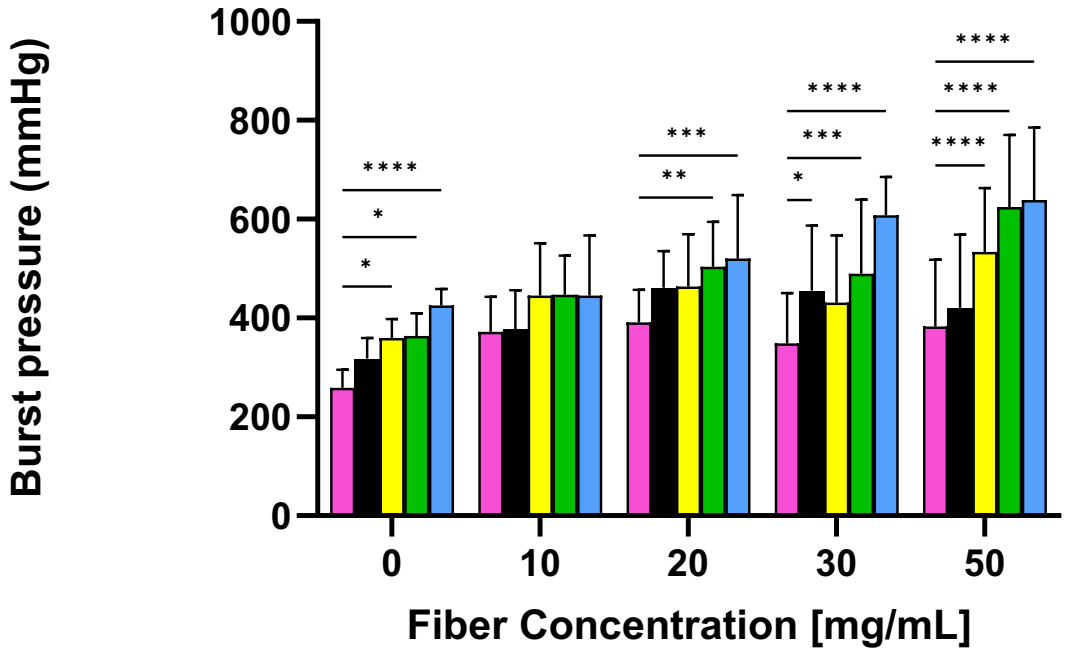

Fig. 1. The Effect of cellulose fibres and Kaolin concentrations (- 0, - 10, - 20, - 30 and - 50 (mg/mL) on the burst strength of the hydrogel. Significant differences are marked with asterisks.

Young's Modulus and Tensile Strength

The bulk properties of the hydrogels were studied in tensile mode using cylindrical specimens. Two bulk properties were measured: the specimen's Young's modulus, a characteristic of the sealant's elasticity, and the tensile strength - a measure of the cohesion strength of the bulk material.

Young's modulus was dramatically increased with the increase in cellulose fiber concentration (Figure 2). For example, the Modulus of the formulation without kaolin (40 KPa) was more than three times higher when loaded with 50 mg/mL CF (125 KPa). The modulus of the formulation containing 50

mg/ml kaolin was increased from 50 KPa to 178 KPa when the CF concentration was increased from 0 to 50 mg/ml. The same trend was observed for the sealants' tensile strength (Figure 3).

A less prominent effect was observed when kaolin was incorporated. The addition of kaolin seemed to increase the Modulus of formulations containing 10, 30, and 50 mg/ml CF (Fig. 2), and the strength of formulations containing 30 and 50 mg/ml CF (Fig. 3). The incorporation of kaolin and cellulose fibres, both at highest concentrations (50 mg/ml) resulted in a tremendous increase in the Modulus and tensile strength. Both are up to 4.5 folds compared to the basic unloaded formulation.

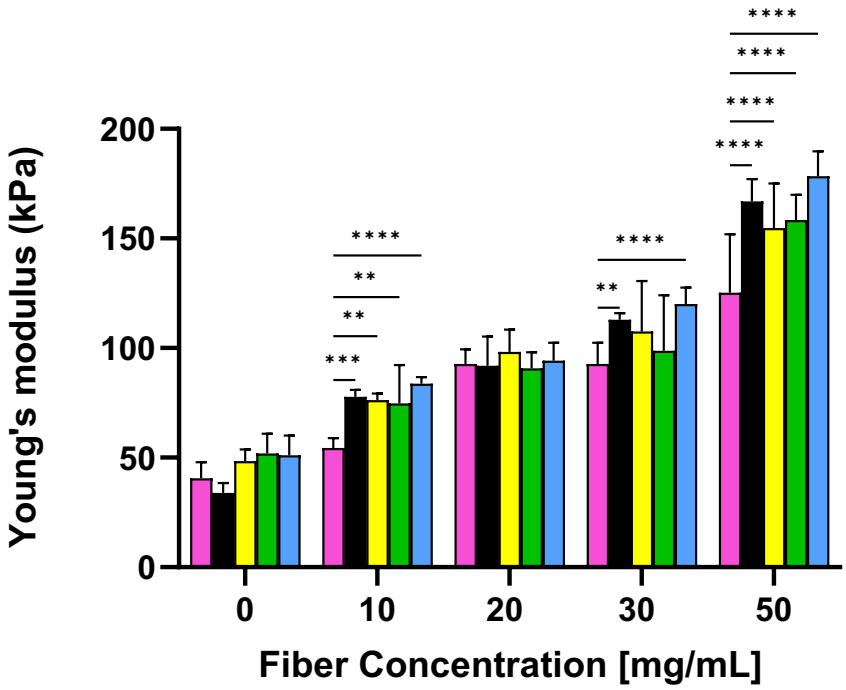

Fig. 2: Effect of the cellulose fibres and Kaolin concentrations concentration (- 0, - 10, - 20, - 30, and - 50 (mg/mL) on Young's modulus of the hydrogel. Significant differences are marked with asterisks.

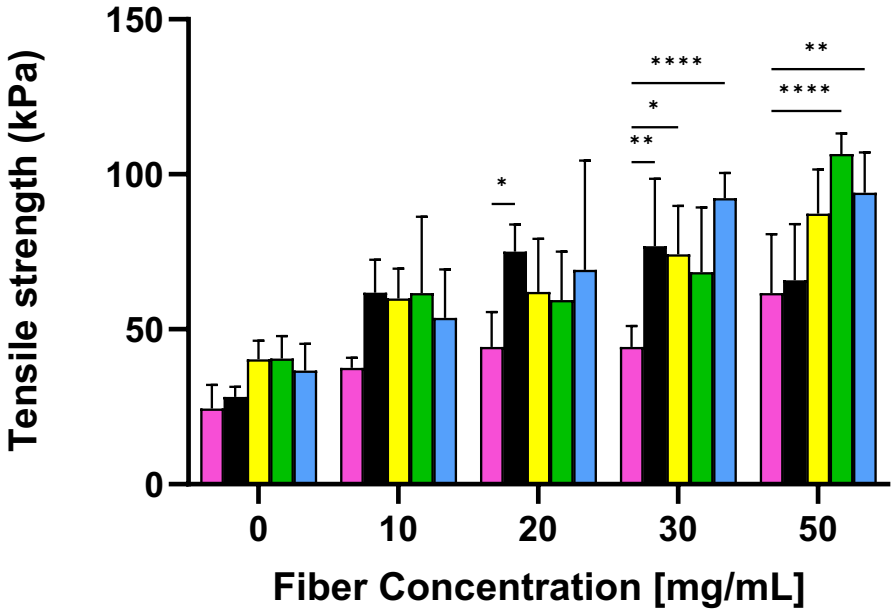

Fig. 3: Effect of the cellulose fibres and Kaolin concentrations (- 0, - 10, - 20, - 30, and - 50 (mg/mL) on the tensile strength of the hydrogel. Significant differences are marked with asterisks.

3.2 Physical properties

Gelation time

The gelation time test provides information regarding the duration the liquid formulation cures upon crosslinking, i.e., converted to a hydrogel and reaches its desired strength. The requirements regarding gelation time change dramatically according to the sealant's application. For example, a short gelation time is desired for specific applications such as sealing blood vessels, where the sealing effect must be achieved immediately after injecting to the relevant site. In contrast, a longer gelation time is desired for gastroenterological applications, where locating and shaping the sealant takes more time.

The basic gelatin-alginate-EDC formulation (400-10-20 mg/mL) exhibited a gelation time of 13.3 seconds. The incorporation of a CF dramatically decreased the gelation time (Fig. 4). For example, the formulation containing 50 mg/ml CF without kaolin exhibited a gelation time of 5 sec. In contrast, incorporating kaolin almost did not affect the gelation time.

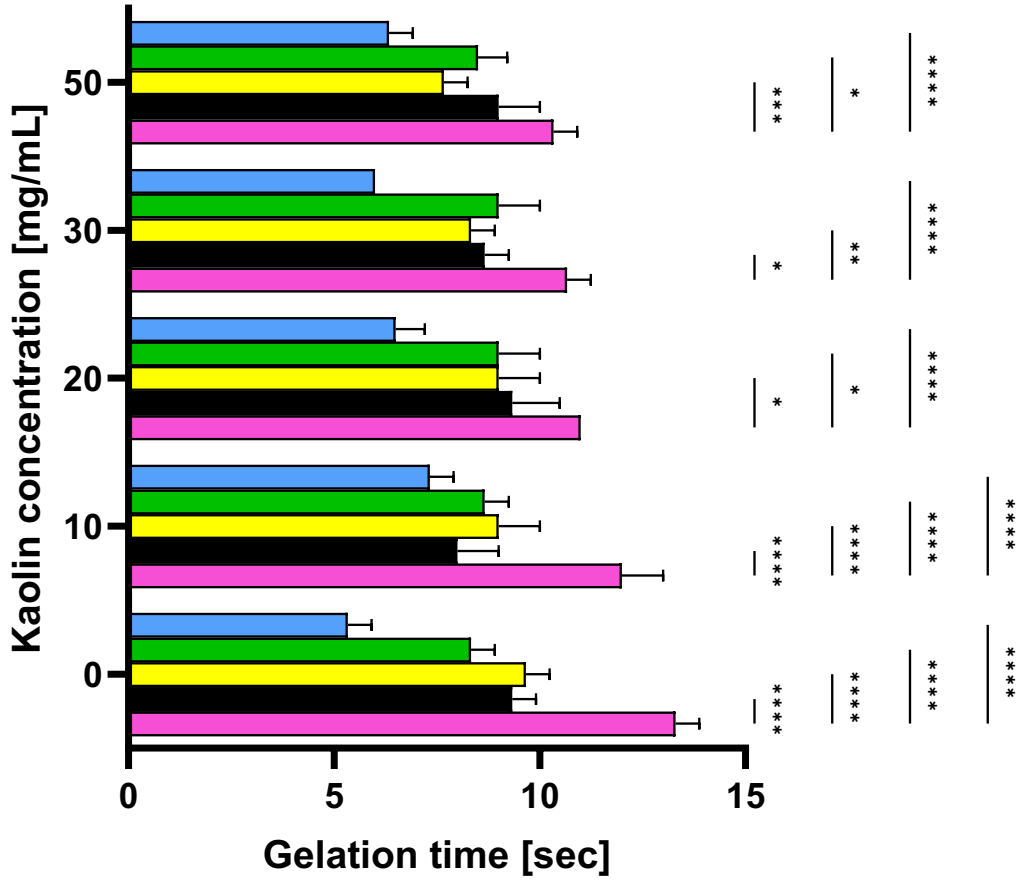

Fig. 4. The effect of kaolin concentration and CF concentration (- 0, - 10, - 20, - 30 and - 50 (mg/mL) on the gelation time.

Swelling degree

The degrees of swelling of a hydrogel are of tremendous importance when used as a surgical sealant and indicate the density of its structure. The swelling degree of the sealants was measured following two incubation times- 2 hours and 24 hours. The results are presented in Fig 5. As can be seen, the swelling degree increased during incubation. For example, the unloaded reference formulation exhibited a swelling degree of 54% after incubation of 2 hours in water and 120% after incubation of 24 hours. The addition of cellulose fibres had a prominent effect on the degree of swelling. It resulted in 36% after 2 hours and 65% after 24 hours, as a result of the incorporation of 50 mg/ml CF without the incorporation of kaolin. In contrast, kaolin incorporation almost did not change the swelling degree (Figure 5).

Viscosity

Viscosity measurements were performed on the gelatin-alginate-based polymer solution (without the incorporation of an EDC crosslinking agent) because its viscosity is the closest approximation to the viscosity at the moment of the adhesive's application. While the viscosity was dramatically increased by the incorporation of cellulose fibres, from 1.5 Pa·s to 11 Pa·s (Figure 6), the addition of kaolin had almost no effect on the viscosity of the solution.

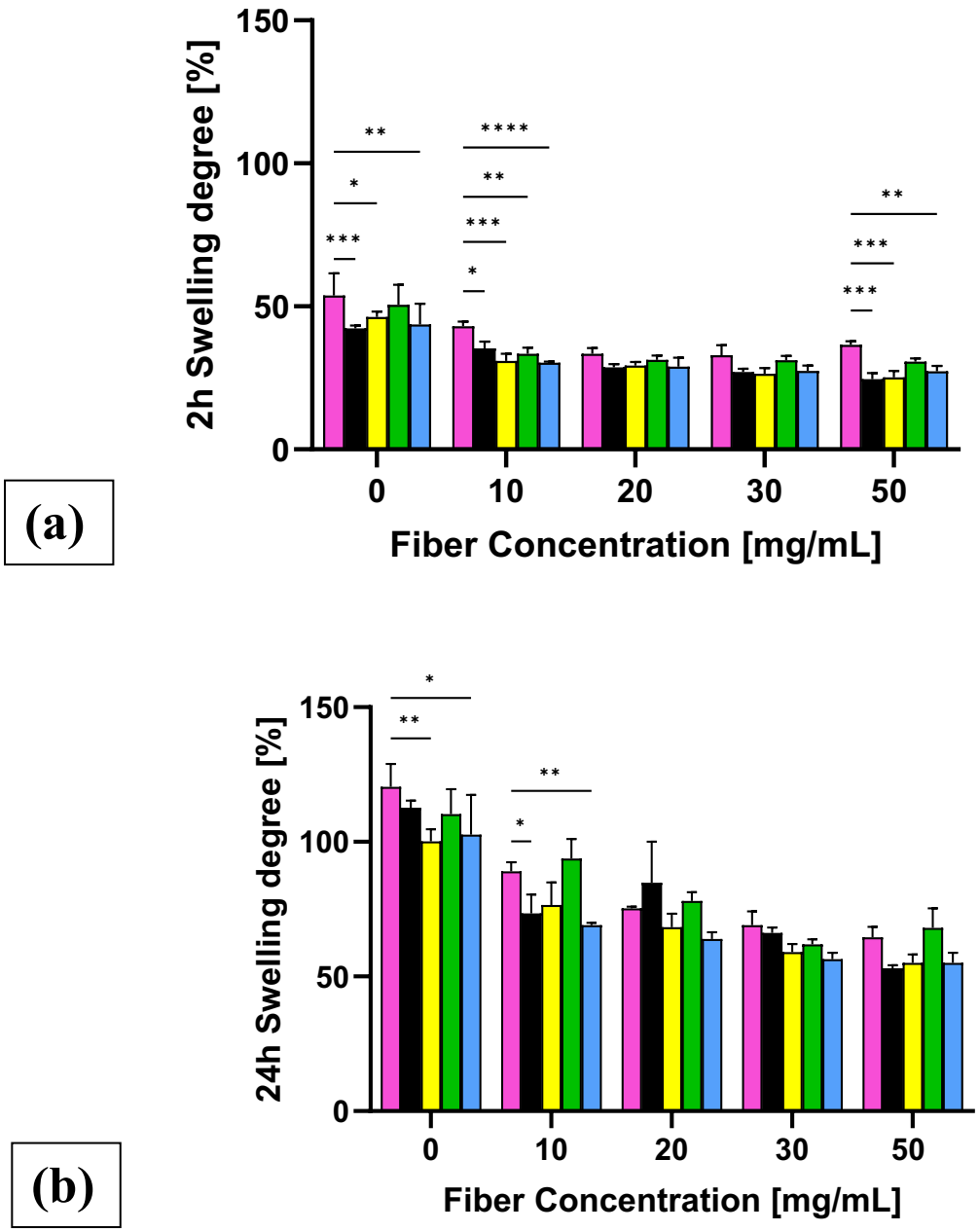

Figure 5: Effect of CF concentration and Kaolin concentration (- 0, - 10, - 20, - 30, and - 50 (mg/mL) on the swelling degree after (a) 2 h and (b) 24 h. Significant differences compared to the non-loaded formulation are marked with asterisks.

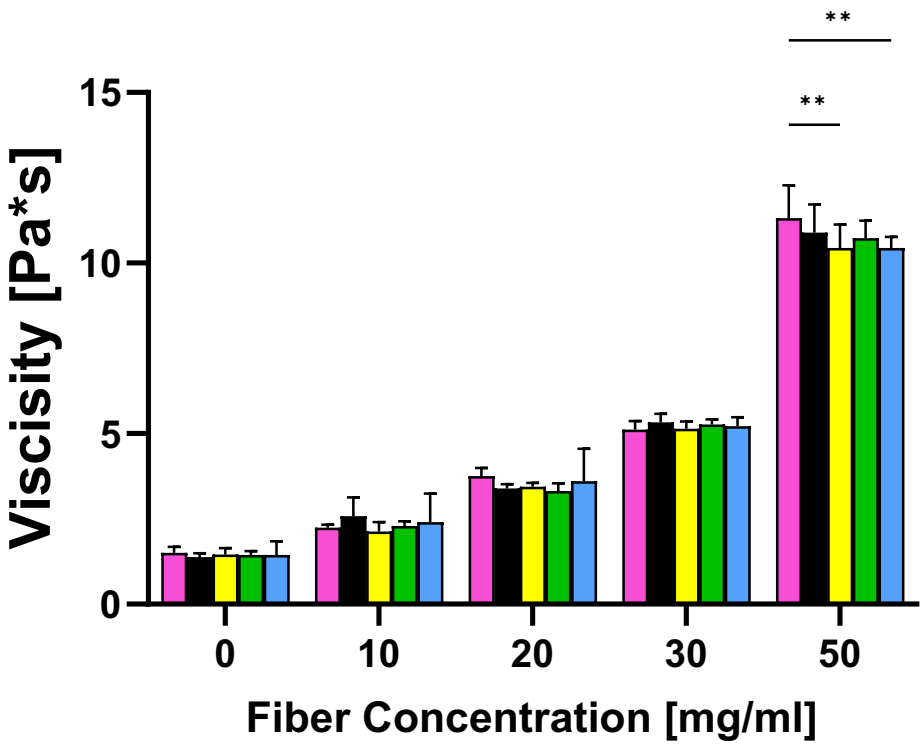

Fig. 6: The viscosity of the gelatin-alginate solution as affected by the cellulose fibres and Kaolin concentrations (- 0, - 10, - 20, - 30, and - 50 (mg/mL) . Significant differences compared to the unloaded formulation are marked with asterisks.

3,3 Cytotoxicity evaluation

Assessment of cytotoxicity and cell viability is a necessary step in the evaluation of biocompatibility and is critical for application of medical sealants. The crosslinking agent and fillers combined in the sealant have different cytotoxicity mechanisms and might be toxic to tissue cells. They must therefore be evaluated *in vitro* before use. The Alamar Blue assay was performed on human fibroblasts participating in the wound healing process to evaluate the cell viability in the presence of the selected

formulations. Formulations that decrease by more than 30% in cell viability are considered cytotoxic. The results show that the cells in the presence of all formulations exhibited high viability, i.e., above 80% after 24 hours (Fig. 7a) and above 70% after 48 hours (Fig. 7b) of incubation. The incorporation of kaolin practically did not affect the cell viability. Some increase in the cell viability was obtained for all CF-loaded formulations after 48 hours of incubation.

3.4 Microstructural Characterization

Cross sections of dry formulations were observed using an Environmental Scanning Electron microscope. The dispersion of both kaolin and CF in the hydrogel matrix and their adhesion to the hydrogel were observed. The cellulose fibres (20 micron diameter) were dispersed homogeneously, at both low and high concentrations (Figure 8 b, d, e and f), and no gaps were observed between the matrix and the fibres (Figure 8 c), meaning that excellent adhesion between the hydrogel matrix and the CF was obtained. Kaolin was dispersed into very small particles (less than 10μm) within the hydrogel matrix, and only part of these particles can be observed within the typical texture of the kaolin-loaded hydrogel (Fig. 8a). Perfect adhesion between the kaolin particles and the hydrogel matrix was observed.

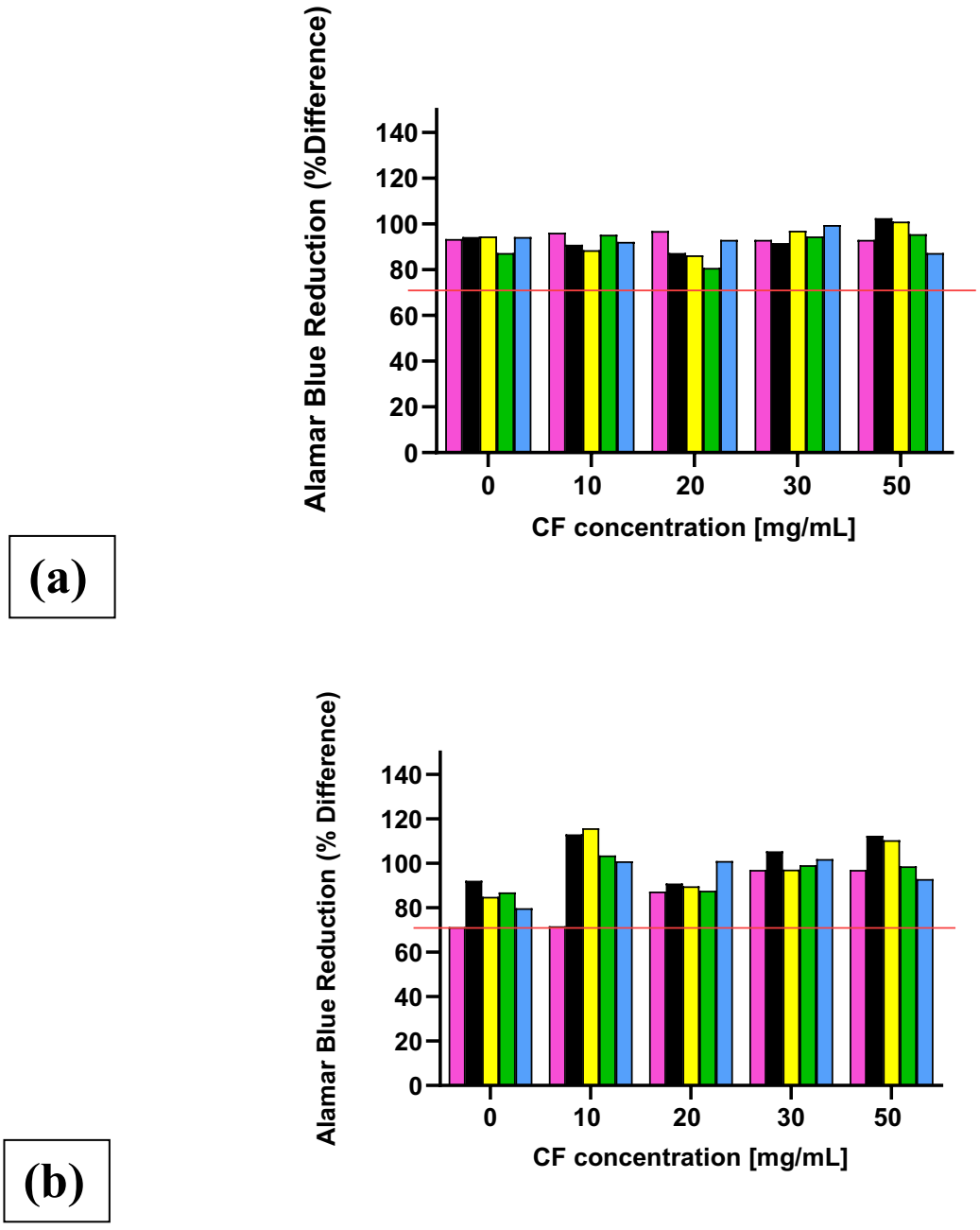

Fig. 7: The effect of kaolin (- 0, - 10, - 20, - 30, and - 50 mg/mL) and cellulose fibres on cells viability of fibroblast cells after (a) 24 and (b) 48 hours incubation.

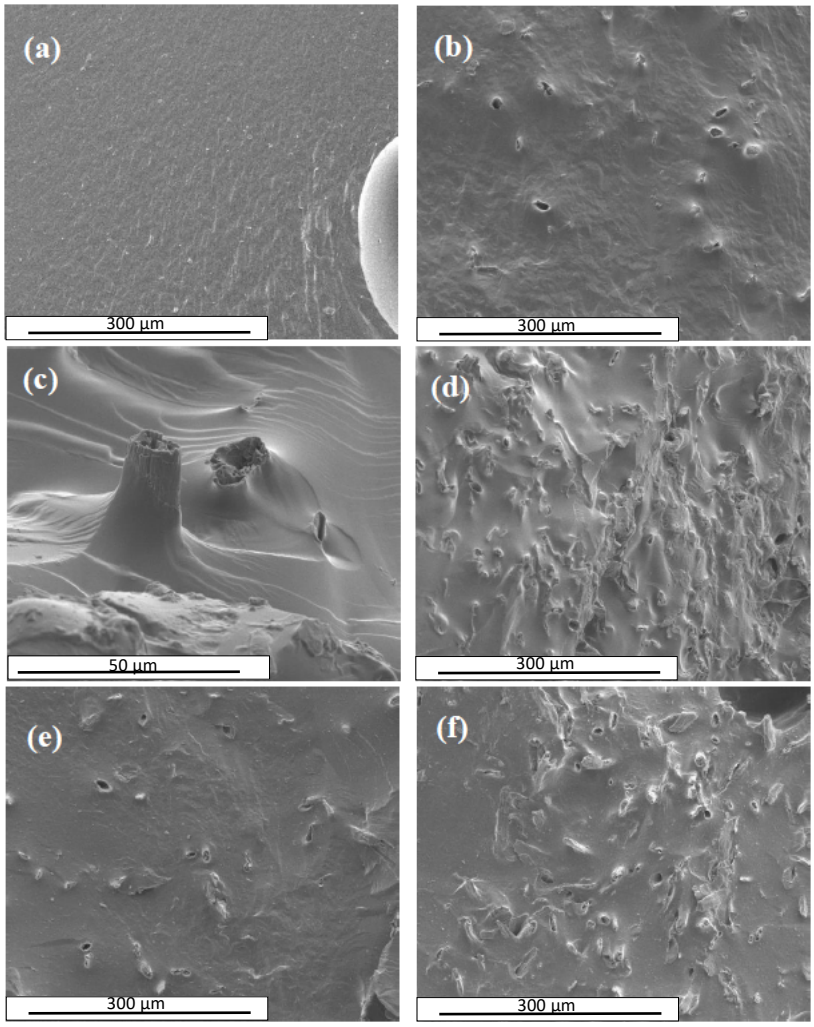

Fig. 8: ESEM fractographs of the hydrogel loaded with: (a)10mg/mL kaolin, (b)10mg/mL CF, (c) 10mg/mL CF, tilted view and high magnification, (d) 50mg/mL CF, (e) 10mg/mL CF and 20mg/mL kaolin, (f) 50mg/mL CF and 50mg/mL kaolin.

4. Discussion

This study aimed to develop hydrogel with improved mechanical strength and hemostatic properties to be used as a medical sealant. Cellulose fibres were incorporated to enhance the bulk properties of the gelatin-alginate-EDC hydrogel sealant, and kaolin was incorporated to induce a hemostatic effect. The combination of the two fillers created a dual composite hydrogel, resulting in a diverse range of physical and mechanical properties for the medical sealant system.

4.1. Mechanical Properties

The results clearly show that both fillers enhance the mechanical properties compared to the basic hydrogel formulation. The sealing ability of the medical sealants was evaluated by measuring the maximal pressure at burst. Surgical sealants must meet the threshold set by the systolic blood pressure, i.e., 200 mmHg. The unloaded formulation could withstand the pressure of 250 mmHg, and the reinforcement using fibres and kaolin succeeded in enhancing it (Fig. 1).

The short structural fibres probably reduce crack initiation and propagation, which strengthens the bulk material, increases the cohesion strength, and, as a result, elevates the burst strength. The load transfer mechanism from the soft hydrogel matrix to the strong cellulose fibres also contributes to the hydrogel strength. This mechanism is obtained due to the excellent adhesion between the fibres and the hydrogel matrix (Fig. 8). We noticed that formulations containing high CF concentrations exhibited more adhesive failures, compared to those of low CF concentrations, which occurred before they could reach their potentially higher value of cohesive burst strength. This may be attributed to the higher viscosity of formulations containing high CF concentrations, which may disturb the mechanical interlocking between the hydrogel and the surface.

The addition of the kaolin silicate to the hydrogel also resulted in increased burst strength. Our experience with composite hydrogels shows that clays are known for their ability to reinforce a hydrogel while creating a nanocomposite structure[17]. Kaolin is a non-expanding clay due to the binding of hydroxyl groups on the surface of its layers, thus forming a micro-composite that contributes to the reinforcement of the matrix to a lower extent than that of a nano-composite. It should be noted that incorporating both functional fillers, kaolin and CF, demonstrated a synergistic effect on the burst strength.

As presented in Figs 2 and 3, both tensile strength and Young's modulus are affected mainly by the addition of CF, especially at high concentrations, where the effect of CF becomes more prominent. In contrast, the influence of kaolin is almost negligible. A possible explanation for this is the role of these functional fillers. While particles and fibres play a similar role in blocking cracks propagation in burst test, in the tensile test there is higher significance to the presence of longitudinal high-modulus filler in the stretched matrix since a load transfer from the hydrogel matrix to the strong cellulose fibres is more effective.

It is important to note that when our basic hydrogel was loaded with a relatively high concentration of both functional fillers (50 mg/ml), it presented high mechanical cohesive strength and, therefore, excellent sealing ability (639 mmHg) compared to other surgical sealants such as Bioglue (Cryolife Inc., Kennesaw, GA) and PreveLeak (Mallinckrodt Pharmaceuticals, St. Louis, MO) with a burst pressure of 596 and 235 mmHg, respectively[18]. It should also be noted that the biocompatibility of our hydrogel is higher than that of Bioglue [19]. This is attributed to the mild crosslinker used (EDC), compared to glutaraldehyde used in Bioglue.

In order to assess the combined effect of kaolin and CF (relative to the reference formulation) on each mechanical property, the equation 1 was used, as follows:

Equation 1: The relative result of x:y formulation = 100 * (Vxy – Vb) / Vb

Where x=0,10,20,30 or 50 mg/mL of kaolin, y=0,10,20,30 or 50 mg/mL of CF, Vxy=test result of a hydrogel with x:y relative quantities of kaolin: CF (mg/mL), and Vb=test result of the basic formulation without fillers. A scatter chart was plotted accordingly, considering the relative results of all three mechanical properties (Figure 9a); each result normalised and represented by a coloured dot according to its score.

The three values of each formulation were then averaged, and an averages scatter chart was plotted (Figure 9b). The averages chart highlights the effect of incorporation of both kaolin and CF on the hydrogel's mechanical properties. The diagonal (0:0 to 50:50) shows a clear monotonic increase in mechanical properties as the concentration of both fillers are increased. Moreover, it is clearly demonstrated that the presence of kaolin contributes to mechanical properties mostly for high CF concentrations, where a synergistic effect is evident. In formulations containing low concentrations of CF, however, mechanical performance is less enhanced by the addition of kaolin.

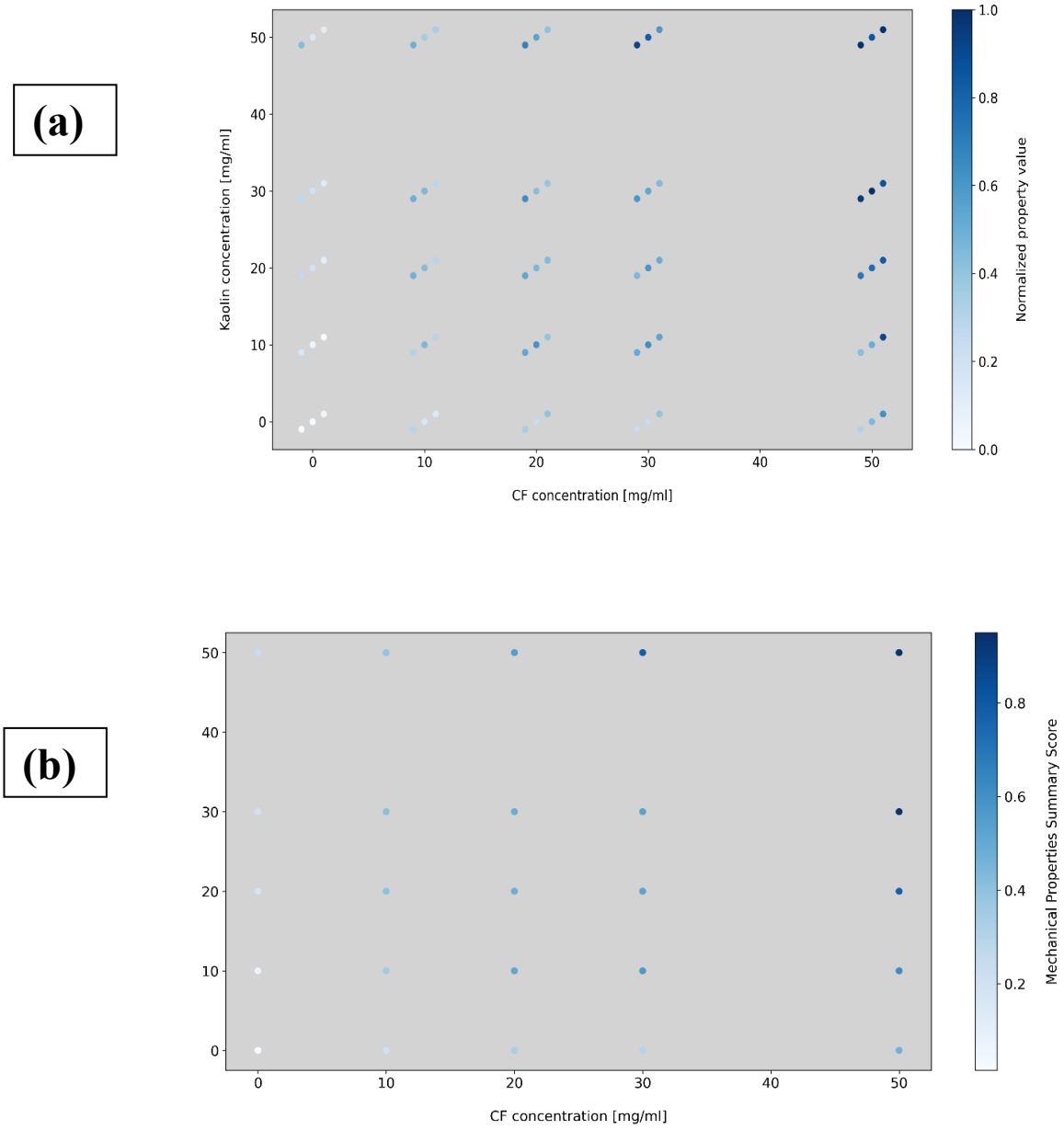

Fig 9: (a) Scatter plot of all three mechanical properties. White dot symbolises the lowest normalised score, and darkest blue dot represents highest normalised score. The three dots for each formulation represent the normalised values of the burst strength- tensile strength - Young modulus, respectively (left to right). (b) Scatter plot of the average normalised value of mechanical properties. White dot

symbols the lowest normalised score, and darkest blue dot represents the highest normalised score. The dot of each formulation is the average of all properties' normalised results of this formulation.

4.2. Physical Properties

Gelation time measures the curing duration of the hydrogel, which is the time to reach its gel point and lose its ability to flow. This characteristic is essential when handling a sealant since it shouldn't cure too fast, allowing the surgeon to manipulate the tissue and perform the procedure. On the other hand, it should cure fast enough to stop the bleeding and complete the procedure.

Gelation time range of 5 – 13.3 sec (Fig. 4) was achieved for all studied formulations, which enables perfect fit to various procedures that require a relatively short gelation time. While kaolin incorporation almost does not affect the gelation time, CF incorporation dramatically decreases the gelation time. Incorporation of 50 mg/ml CF resulted in a gelation time value of 38% of that of the reference formulation, and 57-60% of that of formulations containing kaolin (Fig. 4). The reason for these changes is that the CF fibres probably enhance chain entanglements, and this change in structure probably contributes to faster gelation. It is important to note that the gelation time range of our studied formulations is similar to this of commercial products, such as Bioglue (Cryolife Inc 16.8 sec), Progel (C. R. Bard, Inc., 14 sec) and Coseal® (IFU, 1.2 sec)[20].

Swelling degree is important so as not to lose the mechanical properties of the hydrogel, and especially when subcutaneous applications of the sealant are considered, for which minimal water adsorption is desired. The swelling behaviour of the hydrogel is a measure of the density of its polymeric chains since a dense network will be less absorptive, and fewer water molecules will be able to infiltrate.

The swelling degree following 2 hours and 24 hours of immersion exhibited similar trends. The presence of cellulose fibres in the hydrogel, even at low concentrations, significantly decreased swelling degree. This effect is more significant after 24 hours, compared to 2 hours. For example, incorporation of 50% CF (without kaolin) resulted in a decrease in the swelling degree from 54% to 38% after 2 hours of incubation, and from 120% to 55% after 24 hours of incubation. This effect of CF incorporation is probably obtained due to the formation of a thicket of chains structue, with more entanglement. the higher density which is created, making it difficult for water to infiltrate.

It is interesting to notice that even though kaolin is considered a relatively absorbent material (hydrous phyllosilicate), it does not increase the swelling ratio but rather decreases it, as do the fibres. A possible explanation can be found in the work of Pourjavadi et al.[21]which suggested that the hydroxyl groups on the surface of kaolin may act as a crosslinking agent. In our hydrogels, these hydroxyl groups can react with carboxylate groups from either gelatin or alginate, resulting in ester formation. If kaolin indeed contributes to the cross-linking effect, its incorporation can lead to some higher density of crosslinking sites, resulting in a lower degree of swelling.

The viscosity of polymeric solution (before curing) at the moment of application affects not only the ease of use during injection, but also the bonding strength and sealing ability through the mechanical interlocking mechanism. The results show that the CF concentration had a clear exponential effect on the hydrogel's viscosity. Adding 50 mg/mL CF to the basic formulation multiplied the viscosity by 7. This can be explained by the entanglement of polymeric chains obtained due to the CF incorporation, which makes it harder for the solution to flow. In contrast, Kaolin did not affect the viscosity of the polymeric solution. This is advantageous when it is desired to increase the hemostatic effect of the sealant without affecting its physical properties.

The combined effect of kaolin and CF (relative to the reference formulation) was studied on each physical property separately. Then, an average "physical result" for each formulation was calculated according to Equation 1 (see above). A scatter chart was plotted accordingly, considering the relative results of all three physical properties (Figure 10a); each result normalised and represented by a coloured dot according to its score.

An improved sealant, in terms of physical properties, usually means lower swelling degree and gelation time, and higher viscosity (in a range that prevents excessive spreading yet does not complicate the application). Such improvements can be highly effective in various applications. Hence, in order to examine the average improvement trend, we look here at an inversion of the gelation time and swelling degree results (e.g., the highest gelation time and swelling degree receive the lowest score and vice-versa). The three values of each formulation were then averaged and an averages scatter chart was plotted (Figure 10b). The averages chart highlights the effect of incorporation of both kaolin and CF on the hydrogel's physical properties.

The plot clearly shows a significant improvement in the physical properties of the hydrogel when relatively high fiber concentrations are loaded, and a negligible effect to kaolin incorporation in all CF concentrations. This could be a real advantage when it is desired to enhance the hemostatic effects of a sealant without altering its physical properties.

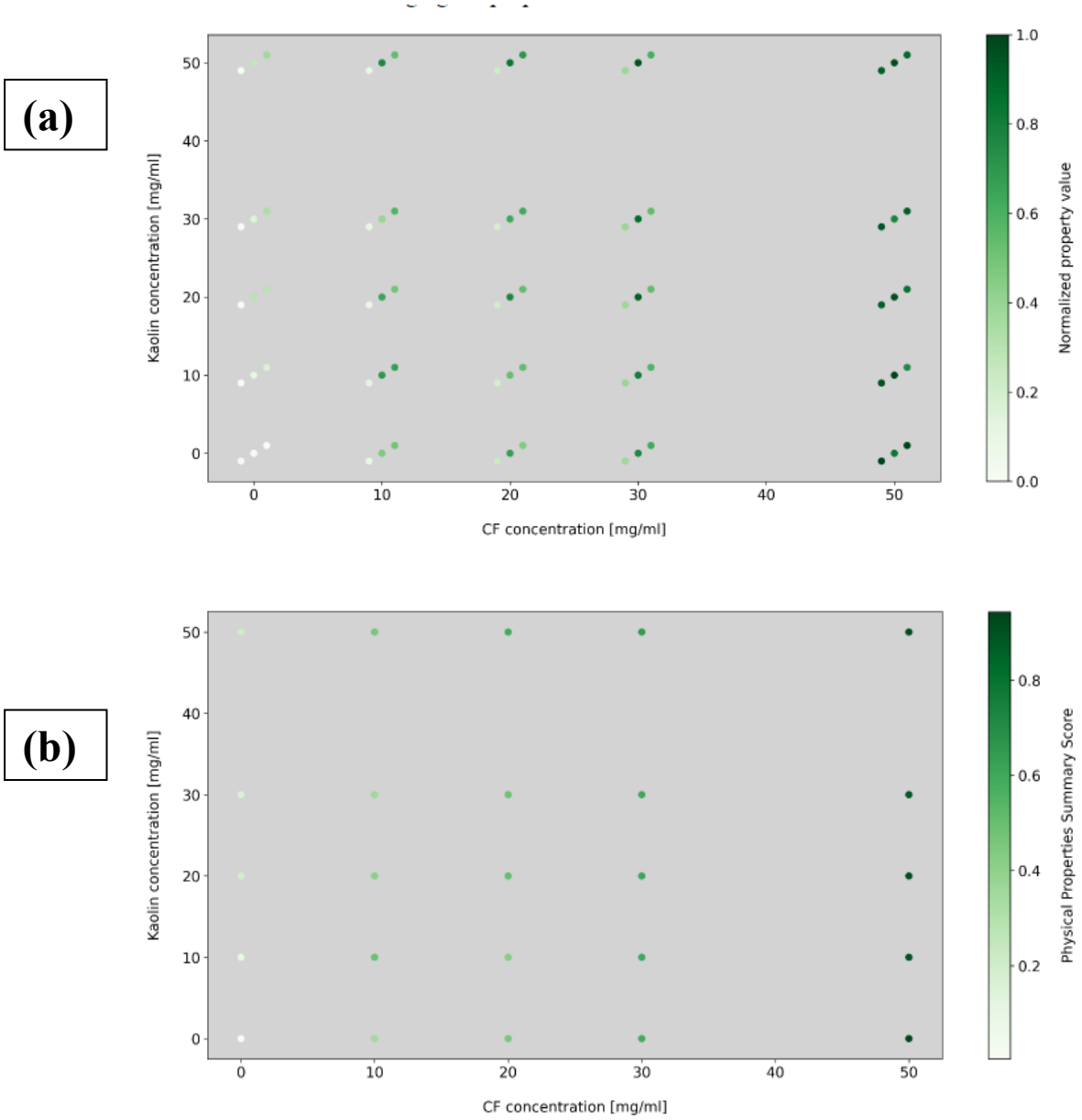

Fig 10: (a) Scatter plot of all three physical properties. White dot symbolises the lowest normalised score, and darkest green dot represents highest normalised score. The three dots for each formulation represent the normalised values of the viscosity- swelling- gelation time, respectively (left to right). (b) Scatter plot of the average normalised value of physical properties. The white dot symbolises the

lowest normalised score, and darkest green dot represents the highest normalised score. The dot of each formulation is the average of all properties' normalised results of this formulation.

4.3. Cytotoxicity Evaluation

The carbodiimide cross-linking agent was chosen because it is considered less cytotoxic than other crosslinkers, such as formaldehyde and glutaraldehyde. EDC and gelatin/alginate form together a reactive O-acylisourea intermediate, which, in turn, goes through a nucleophilic attack, forming an amide bond, with a soluble urea derivative as the only by-product. Our experience with the unloaded gelatin-alginate-EDC formulations indicates no cytotoxic effect for EDC concentrations lower than 30 mg/ml [22]. After 48 hours of incubation, the cells exposed the fillers-containing extract have shown slightly higher viability compared to the cells exposed to the basic formulation's extract. A possible explanation could be that the cellulose fibres have some positive effect on the cells due to their high modulus, which improves cell adherence and induces proliferation.

It is important to mention that the biocompatibility of the formulations in the current study was significantly higher than that of commercially available wound closure and sealing products such as Histoacryl®, Dermabond® and Bioglue®. Our previous study [17] indicated cell viability results of only 7.5%, 5%, and 60%, respectively for these products.

4.4. Microstructural Characterization

The microstructure of a composite hydrogel can strongly affect its mechanical properties. A smooth transition between the phases/domains, without gaps, enables an effective reinforcement by the fillers, which enables load transmission between the phases. The ESEM fractographs of the hydrogel

demonstrated homogeneously dispersed CF and kaolin in all specimens' cross sections, without aggregates, and with a high-quality interface (no gaps between fibres and matrix). The kaolin particles appear as well-dispersed elongated domains, with good adhesion to the matrix (Figure 8). These observations definitely explain the enhancement of the hydrogel's mechanical properties by both functional filers, CF and kaolin.

4.5 Limitations

Although this study has thoroughly examined the mechanical, physical, structural, and cytotoxicity properties of the hydrogel, there are some limitations. The in vitro cytotoxicity assessment using human fibroblasts may not fully reflect in vivo responses. Therefore, further research using an in-vivo model is required. Additionally, long-term stability, degradation kinetics, adhesive strength, and inflammatory response were not investigated. Further research is needed to assess the hydrogel's feasibility for clinical applications.

5. Conclusions

The current study aimed to develop and investigate novel gelatin-alginate-EDC hydrogels loaded with two functional fillers: the hemostatic agent kaolin and cellulose fibres. The mechanical and physical characteristics relevant to medical sealant applications were investigated, together with their microstructure and cytotoxicity.

Our study shows that these dual micro-composite hydrogels can successfully function as medical sealants with enhanced physical and mechanical performance, mainly due to the incorporation of fibres. This unique system also enables better control and tuning the hydrogel's properties,

customising its final application.

The uniform dispersion of both fillers within the hydrogel and excellent adhesion between the fillers and the hydrogel matrix enabled enhanced cohesion strength of the material and resulted in higher burst strength (sealing ability). Higher Young's modulus and tensile strength were obtained mainly due to CF incorporation.

The incorporation of CF strongly affected the physical properties of the hydrogel, i.e., decreased the gelation time and swelling degree, mainly due to chain entanglements, which act as physical crosslinking. In addition, the viscosity significantly increased due to the formation of thicket-like domains, which increased the resistance to flow.

The hemostatic effect induced by kaolin incorporation can be enhanced by increasing the kaolin content without the risk of harming the ease and effectiveness of the sealant application, since kaolin has little to no effect on the gelation time, viscosity, and swelling degree of the hydrogel.

The cytotoxicity tests indicated that all studied formulations exhibited high cell viability (>70%) and therefore are safe to use in medical applications. The CF incorporation also induced some positive effects on cell viability. Hence, it can be concluded that cellulose fibres enhance the function of hemostatic composite medical sealants, in all studied aspects.

Understanding the effects of each functional filler on the mechanical and physical properties enables the fitting of desired formulations to specific medical applications.

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Data Availability Statement

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

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