Hemostatic Ability of Thermosensitive Biologically Active Gelatin-Alginate Hydrogels Modified with Humic Acids and Impregnated with Aminocaproic Acid

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Abstract: The article studies the hemostatic ability of thermosensitive biopolymer hydrogels containing 14% by weight of gelatin and 6.4% by weight of sodium alginate, impregnated with a hemostatic agent aminocaproic acid ~0.2 g/ml, with the addition of humic acids with antioxidant, antibacterial, fungicidal and anti-inflammatory properties. Modification of hydrogels with humic acids slightly increases viscosity, but maintains the gel-sol transition temperature close to the physiological temperature of about 37 °C, which allows them to melt on human skin or inside a wound, ensuring the delivery of aminocaproic acid. SEM images showed that the developed hydrogels have a layered internal morphology, which is improved due to better swelling of the hydrogels contained humic acids, which promotes the dissolution of aminocaproic acid inside the hydrogels and its subsequent rapid delivery to the bleeding site when applying a hydrogel dressing. It has been experimentally established that the concentration of humic acids in hydrogels of no more than 5 wt.% promotes blood clotting due to the entry of aminocaproic acid into it from the hydrogels. The aminocaproic acid delivered at physiological temperature from these hydrogels can shorten the blood clotting time to the lower limit of the normal clotting time range. The clotting time of the hydrogel with 5 wt.% humic acid is only 95 s, which confirms its particularly effective hemostatic ability.

Keywords: Hemostasis, biopolymer hydrogel, gelatin, alginate, humic acid, aminocaproic acid, thermosensivity, blood coagulation.

1. INTRODUCTION

Effective stopping of bleeding, despite the development of modern medicine, still remains a significant problem both at the pre-hospital stage and in inpatient surgical care. Among the numerous methods of stopping bleeding, one of the priority ones is the use of local hemostatic agents in the form of thermoresponsive hydrogels contained a hemostatic drug, which melt at a physiological temperature of approximately 37 °C, releasing hemostatic agent, thus delivering it directly to the site of bleeding. Such hydrogels may be affordable, effective and easy-to-use materials that can be used either alone or in combination with other methods. The growing desire to find innovative ways to produce environmentally friendly hydrogel materials explains the interest in natural biopolymers, which possess many remarkable characteristics including light weight, excellent mechanical properties, biocompatibility, non-toxicity and low cost [1-5]. Biopolymers are materials obtained from biological sources such as vegetables, plants, microorganisms, trees, algae, animals, fish, crustaceans, etc. Compared with synthetic materials, they have many advantages such as natural distribution, strong structure, hydrophilicity, multiple active sites, mechanical flexibility, non-toxicity and biocompatibility, biodegradability and renewability. The use of biopolymers makes it possible to create “smart” biologically active hydrogels that can swell/shrink depending on the amount of water present in them,

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which allows them to react and be sensitive to various external influences, such as heat, pH, molecular interactions, ionic strength, etc. [6,7]. Biopolymer hydrogels, as a family of three-dimensional (3D) polymeric materials in which the main part of the matrix consists of water (usually 75-90% by weight), have excellent diffusion properties and are therefore widely used in regenerative medicine, tissue engineering, artificial organs and related fields [8,9]. In particular, these hydrogels have long been used as carriers for targeted drug delivery systems due to the large number of functional groups for drug immobilization, excellent biocompatibility with the *in vivo* environment, and controlled degradation period for self-destruction [8,10-12]. It has been found [13,14] that, compared with weak conventional single-network hydrogels, double-network and multiphase hydrogels exhibit superior properties, including increased mechanical strength, better flexibility and thermal sensitivity. A promising double-network and multiphase hydrogel based on biopolymers is a hydrogel containing gelatin (GN) and sodium alginate (ALG), the structure of which is formed by homopolymer and heteropolymer blocks [13,15,16]. In [6], the addition of humic acids (HA) as antioxidant, antibacterial, fungicidal and anti-inflammatory agents was used as a method to regulate the gelation of gelatin hydrogels, as well as to improve their wound healing ability. According to [15,16], GN-ALG hydrogels modified with humic acids should become promising materials for hemostasis. Recently, the authors [1] developed a biocompatible and biodegradable biopolymer hydrogel from a chitosan derivative grafted with aminocaproic acid (AA) that has antibacterial and hemostatic properties that prevent secondary bleeding [17], since exhibits antiplasmin activity [18], slowing down the destruction of thrombi and reducing secondary wound bleeding [19,20]. It was shown in [15], that the hemostatic gelatin-alginate hydrogels modified with humic acids and impregnated with aminocaproic acid have a high swelling rate and swelling ability, allowing the initiation of a blood clotting cascade within 30 seconds of application to a bleeding wound. Their strong and rapid swelling contributed to the dissolution of aminocaproic acid in hydrogels and its subsequent delivery to the wound in the amount of 300-400 mg AA from 5 ml of hydrogel [15].

In this work, we investigated the rheological properties of thermosensitive biologically active gelatin-alginate hydrogels impregnated with aminocaproic acid, containing 14% by weight of NG, 6.4% by weight of ALG and ~0.2 g/ml of AA, including those modified with humic acids (2.5-7.5% by weight), and also studied *in vitro* their ability to clot blood at physiological temperature in order to optimize the composition to ensure high hemostatic efficiency.

2. MATERIALS AND METHODS

2.1. Materials

For the preparation of gelatin-alginate-humic hydrogels, we used food grade gelatin P-11 (molecular weight ≈ 4.9 10^4 g/mol) produced by TM Mriya, PJSC Ukroptbakaliya, Ukraine, sodium alginate (molecular weight ≈ 216 g/mol) produced by Lianyungang Fengyun Seaweed Manufacturer Co., Ltd., China, and aminocaproic acid powder produced by UmanHimTrade Co., Ukraine. Humic acids with nanodispersity in the range of 52 - 380 nm were obtained by extraction from lignite, as described in [11,12,16]. Blood was obtained directly from the hearts of rabbits under anesthesia, similar to that described in [21].

2.2. Methods

2.2.1. Sample Preparation

To prepare gelatin-alginate-humic hydrogels containing 14% by weight of NG, 6.4% by weight of ALG and from 2.5 to 7.5% by weight of HA, first, a pre-calculated amount of gelatin was placed in distilled water pre-heated to 90±2°C and stirred in a water bath using a VEVOR 85-2 magnetic stirrer with a heating plate until a pure gelatin sol was obtained. Then sodium alginate was added to the gelatin sol and stirred with a VEVOR 85-2 magnetic stirrer with a heating plate until a homogeneous gelatin-alginate sol was obtained. Before adding to the gelatin-alginate sol, humic acids were partially dissolved in aqueous 1 wt. % alkaline solutions of NaOH. To impregnate the hydrogels with aminocaproic acid, 1.018 g of AA powder was added to 5 ml of a sol sample corresponding to each of the gelatin-alginate hydrogels, including those modified with humic acids, heated to a temperature above 50°C and thoroughly mixed until a suspension was obtained, which after cooling was transformed into hydrogels GN-ALG-AA, GN-ALG-HA2.5-AA, GN-ALG-HA5-AA or GN-ALG-HA7.5-AA, respectively.

2.2.2. Characterization

In this work, rheological measurements were performed to study the gel-sol transition temperature of the obtained biopolymer hydrogels. For this purpose, a

standard method for determining the kinematic viscosity (mm^2/s) was used using a VPZh-2 3.35 glass viscometer with a capillary diameter of 3.35 mm and a viscometer constant of 10. As the temperature increased, the gel-to-sol transition temperature was recorded as the temperature at which the sol began to flow freely through a 3.35 mm diameter capillary. Temperature has been controlled during the measurement of the kinematic viscosity by precisely regulating the temperature in the water bath in which the viscometer was immersed.

Scanning electron microscopy (SEM) was used to characterize the morphology of the dried hydrogels using a Zeiss ULTRA Plus SEM with a secondary electron objective. Optical micrographs of hemostatic hydrogels with blood dripped onto their surface were obtained using a HD color CMOS Sensor digital microscope (China).

The hemostatic ability of the gelatin-alginate hydrogels modified with humic acids and impregnated with aminocaproic acid was determined *in vitro*. A preliminary test consisted of visual observation of the darkening of blood dropped onto the surface of a hemostatic hydrogel heated to a physiological temperature of 37°C. We then used a modified clotting time test, the Morawitz and Althausen drop method, to determine the clotting time, which is a general term for the time it takes for a blood sample to form a clot, i.e., coagulate [22]. In clinical settings, healthcare providers measure clotting time as the time it takes for fibrin to form in the blood, which is one of the first signs of clotting. The normal range of clotting time is 2-15 minutes [22]. In the test, we measured the time it took for blood to clot on a glass plate coated with a hemostatic hydrogel at a temperature of 37°C. To do this, a drop of fresh rabbit blood was transferred to the surface of the hemostatic hydrogel so that a blood spot with a diameter of 4-6 mm was formed on the surface of the hydrogel with an area of 1 cm^2, and a glass rod with a ball on the end was applied to the blood spot every 30 seconds. The moment of appearance of the first fibrin threads along the rod is considered the beginning of blood clotting. The clotting time was determined by the interval between application of a drop of blood to the surface of the hydrogel and the formation of fibrin threads.

Experiments on kinematic viscosity and clotting time of hemostatic gelatin-alginate hydrogels modified with humic acids and impregnated with aminocaproic acid were performed in three replicates to ensure reproducibility. The obtained data were presented as average values and standard deviations. For statistical analysis, data normality was assessed by Tukey's multiple comparison post hoc test using ANOVA. The parameters used in the ANOVA analysis had a significance value of $p < 0.05$ at the 95% confidence level.

3. RESULTS AND DISCUSSION

The results of the study of the rheological properties of thermosensitive gelatin-alginate hydrogels impregnated with aminocaproic acid depending on the presence of humic acids in them are shown in Figure **1**. As can be seen in Figure **1**, humic acids slightly increase the viscosity of hydrogels, but do not noticeably change the gel-sol transition temperature. The gel-sol transition temperature in GN-ALG-AA, GN-ALG-HA2.5-AA and GN-ALG-HA5-AA hydrogels is equal to the physiological temperature of about 37°C, which allows the hydrogel to soften and melt on human skin or inside a wound, ensuring the delivery of the hemostatic agent AA in accordance with that described in [15]. However, additional experiments showed that with an increase in the humic acid content to 7.5% by weight in the thermosensitive gelatin-alginate hydrogel with aminocaproic acid GN-ALG-HA7.5-AA, the gel-sol transition temperature shifts to the region of 45-50 ºC.

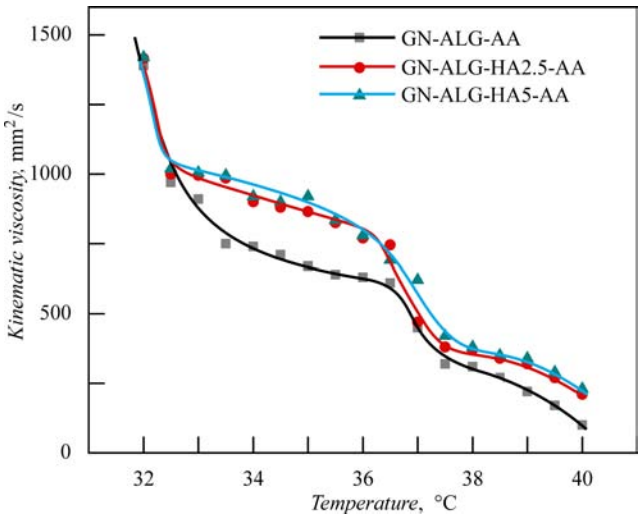

Figure 1: Rheological studies of hemostatic gelatin-alginate hydrogels containing 14 wt.% GN and 6.4 wt.% ALG and impregnated with ~0.2 g/ml aminocaproic acid (GN-ALG-AA), as well as modified with humic acids: 2.5 wt.% HA (GN-ALG-HA2.5-AA) and 5 wt.% HA (GN-ALG-HA5-AA).

As can be seen from the SEM images in Figure **2**, the hydrogels have a layered internal morphology, which is improved at HA content of 5 wt.% and 7.5 wt.% due to better swelling, described for gelatin-

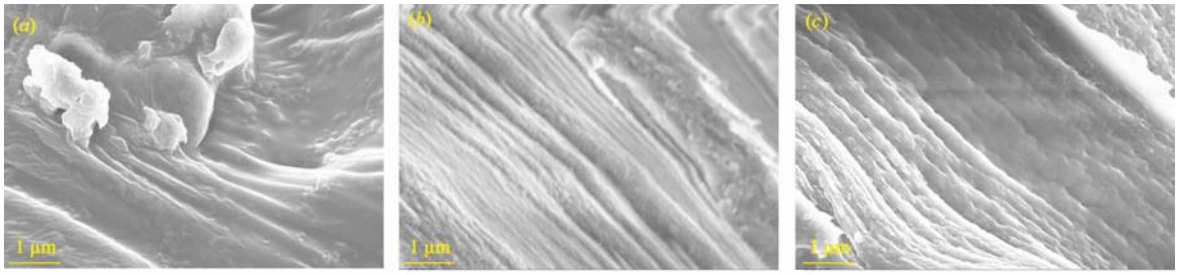

Figure 2: SEM images of the developed gelatin-alginate hydrogels modified with humic acids and impregnated with aminocaproic acid: (**a**) – GN-ALG-HA2.5-AA; (**b**) – GN-ALG-HA5-AA; (**c**) – GN-ALG-HA7.5-AA.

alginate-humic hydrogels in [16], which promotes the dissolution of aminocaproic acid inside the GN-ALG-HA5-AA and GN-ALG-HA7.5-AA hydrogels. The AA clumps are clearly visible in the SEM image of GN-ALG-HA2.5-AA in Figure **2a**, but they are not present in the SEM images of GN-ALG-HA5-AA and GN-ALG-HA7.5-AA hydrogels in Figure **2b** and **c**, respectively. This indicates that HA promotes the dissolution of AA and its subsequent rapid delivery to the bleeding site upon application of the hydrogel dressing.

Figure **3** shows photographs of hemostatic hydrogel samples on glass plates dripped with fresh blood, which were used in preliminary experiments for visual observation of the darkening of blood dripped onto the surface of hemostatic hydrogels heated to a physiological temperature of 37°C, as well as for clotting time tests.

Optical micrographs of these samples, presented in Figure **4**, demonstrate the results of preliminary tests

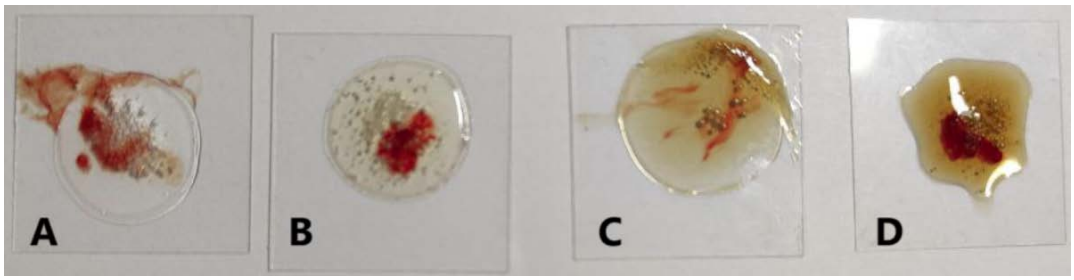

Figure 3: Photographs of hemostatic hydrogel samples on glass plates dripped with fresh blood: (**A**) – GN-ALG-AA; (**B**) – GN-ALG-HA2.5-AA; (**C**) – GN-ALG-HA5-AA; (**D**) – GN-ALG-HA7.5-AA.

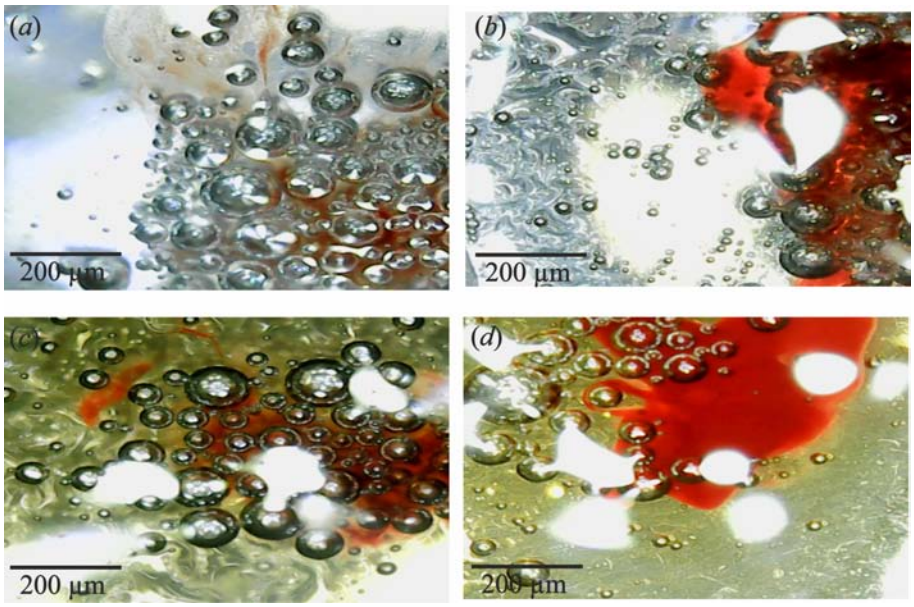

Figure 4: Optical micrographs of hemostatic hydrogel samples on glass plates dripped with fresh blood: (**a**) – GN-ALG-AA; (**b**) – GN-ALG-HA2.5-AA; (**c**) – GN-ALG-HA5-AA; (**d**) – GN-ALG-HA7.5-AA.

on the darkening of blood dropped onto the surface of various hemostatic hydrogels. It is evident that concentrations of humic acids of 2.5 wt.% and 5 wt.% in the developed hemostatic hydrogels do not prevent darkening of the blood, which indirectly indicates its coagulation due to the entry of aminocaproic acid into the blood from the hydrogels GN-ALG-GA2.5-AA and GN-ALG-GA5-AA, as well as from the hydrogel GN-ALG-AA, which have a melting point of about 37 °C. At the same time, on the surface of the GN-ALG-GA7.5-AA hydrogel, the blood remains scarlet, which is explained by its high gel-sol transition temperature, significantly exceeding the physiological temperature, as a result of which AA at 37 °C is not released from the solid gel and does not interact with the blood.

The results of the clotting time tests shown in Figure **5** demonstrate that aminocaproic acid delivered at physiological temperature from the developed GN-ALG-AA and GN-ALG-HA2.5-AA hydrogels can reduce the blood clotting time to the lower limit of the normal clotting time range of 120-900 s. At the same time, the even shorter clotting time of the GN-ALG-HA5-AA hydrogel is 95 s, which confirms its particularly effective hemostatic ability.

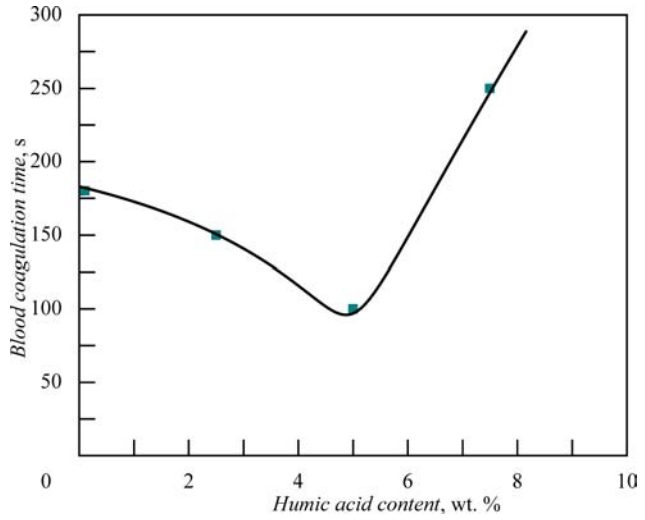

Figure 5: Effect of humic acid content on the clotting time of developed hemostatic gelatin-alginate hydrogels containing 14 wt.% GN and 6.4 wt.% ALG and impregnated with ~0.2 g/ml aminocaproic acid.

CONCLUSIONS

The results of the study of the rheological properties of thermosensitive gelatin-alginate hydrogels impregnated with aminocaproic acid show that their modification with humic acids slightly increases the viscosity of the hydrogels, but does not noticeably change the gel-sol transition temperature. The gel-sol transition temperature of the GN-ALG-AA, GN-ALG-HA2.5-AA and GN-ALG-HA5-AA hydrogels is equal to the physiological temperature of about 37 °C, which allows them to soften and melt on human skin or inside a wound, providing delivery of the hemostatic agent aminocaproic acid. In addition, SEM images showed that the developed hydrogels have a layered internal morphology, which is improved due to better swelling of the hydrogels modified with humic acids, which promotes the dissolution of aminocaproic acid inside the hydrogels and its subsequent rapid delivery to the bleeding site when applying a hydrogel dressing.

An indirect indicator of blood coagulability is its darkening on the surface of the developed hemostatic hydrogels. It was experimentally revealed that concentrations of humic acids in them of no more than 5 wt.% do not prevent blood coagulation caused by the entry of aminocaproic acid into the blood from hydrogels GN-ALG-GA2.5-AA and GN-ALG-GA5-AA with a melting point of about 37 °C, as well as from the hydrogel GN-ALG-AA. The results of the blood clotting time tests show that aminocaproic acid delivered at physiological temperature from the developed GN-ALG-AA and GN-ALG-HA2.5-AA hydrogels can shorten the blood clotting time to 180 s and 150 s, respectively, which is close to the lower limit of the normal clotting time range. Moreover, the clotting time of GN-ALG-HA5-AA hydrogel is even shorter, being 95 s, which confirms its particularly effective hemostatic ability. Thus, these thermosensitive bioactive hydrogels can be used in hemostatic dressing materials for wound covering. The main limiting factor for the use of the developed thermosensitive bioactive hydrogels is the temperature of the organism during bleeding, which is not lower than 37°C. Perspective of further research of the developed thermosensitive bioactive hydrogels is their practical study within the framework of studying the process of stopping bleeding in real animals.

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