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Research Article

In Vitro Evaluation of Real-Time Viscoelastic and Coagulation Properties of Various Classes of Topical Hemostatic Agents Using a Novel Contactless Nondestructive Technology

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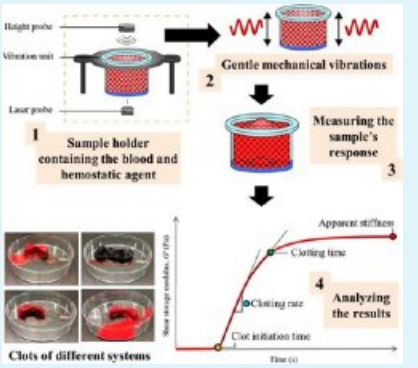
ABSTRACT: Hemorrhaging is the main cause of death among combat and civilian injuries and has significant clinical and economic consequences. Despite their vital roles in bleeding management, an optimal topical hemostatic agent (HA) has yet to be developed for a particular scenario. This is partly due to a lack of an overarching quantitative testing technology to characterize the various classes of HAs in vitro. Herein, the feasibility of a novel, contactless, and nondestructive technique to quantitatively measure the shear storage modulus (G') and clotting properties of whole blood in contact with different dosages of eight topical HAs, including particulates and gauze-like and sponge-like systems, was assessed. The real-time G'−time profiles of these blood/HA systems revealed their distinct biomechanical behavior to induce and impact coagulation. These were analyzed to characterize the clot initiation time, clotting rate, clotting time, and apparent stiffness of the formed clots (both immediately and temporally), which were correlated with their reported hemostatic mechanisms of action. Moreover, the HAs that worked independently from the natural blood clotting cascade were identified and quantified through this technology. In sum, this study indicated that the nondestructive nature of the technology may offer a promising tool for accurate, quantitative in vitro measurements of the clotting properties of various classes of HAs, which may be used to better predict their in vivo outcomes.



KEYWORDS: *blood coagulation, hemostatic agents, in vitro, shear storage modulus G′, ElastoSens Bio²*

1. INTRODUCTION

Hemorrhaging and uncontrolled bleeding have significant clinical and economic consequences.[1−3] For example, ∼40% of traumatic and ∼90% of combat mortalities occur prior to hospitalization, half of which being due to uncontrolled blood loss.[2] Topical hemostatic agents (HAs) are used in different situations to help control the bleeding and complement the natural blood clotting action.[1−8] Although traditional topical HAs, such as various herb mixes, have been used since ancient times, the introduction of topical HAs (as topical fibrin) into modern surgery occurred around 1909.[2,6] Since then, topical HAs have been widely used in various medical fields, surgeries, and clinical scenarios. Given that any surgery can potentially be associated with bleeding and the risk of its subsequent complications, HAs have been applied in neurosurgery, urology, cardiovascular surgeries, gastrointestinal bleeding, dermatologic procedures, and dental surgeries.[3,5,6,8−10]

Topical HAs can be classified into either active (biological), nonactive (physical or mechanical), or flowable agents. They can be also classified in other ways according to their mechanisms of action, forms, and textures.[2,4−9,11,12] Unlike active agents, nonactive HAs (including mechanical HAs and synthetic sealants) do not contain natural clotting agents (thrombin), whereas flowable HAs are known to have properties common to both active and nonactive HAs.[5,6,9] The thrombin-based active (biological) agents can be derived from different sources of bovine, pooled human plasma, or more recently via the recombinant technology.[3,4]

The complex natural blood coagulation process involves liquid blood converting into a gel as the result of the transformation (polymerization) of its fibrinogen content into fibrin and activation of platelets through a coagulation cascade reaction followed by clot formation and stabilization.[3,13−15] The role and involvement of the blood cells and plasma proteins in such hematology-related processes are both mechanical and biological.[16] Under uncontrollable bleeding, hemostasis can be promoted and achieved using topical HAs through different mechanisms, including concentrating coagulation factors and/or providing a scaffold (mostly as a result of high fluid absorption), adhesion to the bleeding site and

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Table 1. HAs Used in This Study

HA form	HA type	composition	example	dosages (g) incorporated with 5 mL media[a]		
gauze (membrane or dressing)	non-active	kaolin-based	QuikClot (Z Medica)	0.2	0.4	0.6
		oxidized regenerated cellulose	Surgicel Original (Johnson & Johnson/Ethicon Inc.)			
		non-oxidized, non-regenerated cellulose structure	WoundClot (Core Scientific Creations Ltd.)			
sponge (foam)	non-active	collagen-based	Helistat (Integra Lifesciences)	0.01	0.02	0.04
		porcine gelatin-based	Surgifoam (Johnson & Johnson/Ethicon Inc.)	0.05	0.1	0.2
powder (granular)	non-active	chitosan-based	Celox (Medtrade Products Ltd.)	0.4	0.6	0.8
		clay-based	Hemospray (Cook Medical)			
gel (paste)	active or flowable	bovine-derived gelatin matrix component + human-derived thrombin component	FloSeal (Baxter Healthcare Corporation)[b]			

[a]Media: recalcified blood (RB), citrated blood (CB), and PBS. [b]Note that this agent can be considered and has been referred to as a "liquid" or "foam"-like agent as well.[22]

tissues, and delivering procoagulant factors and substances.[2,4,8,11] For example, the mechanism of action of nonactive mechanical HAs, such as oxidized cellulose-, gelatin-, and polysaccharide-based beads, normally relies on their high surface area and absorption capacity leading to the formation of a matrix as a physical barrier over the bleeding site. This helps activate an extrinsic clotting pathway and thrombus deposition and provides a scaffold for platelet aggregation.[2,8,9,14] On the other hand, active HAs promote coagulation through a multi-action mechanism, wherein the native fibrinogen is rapidly converted into a fibrin clot; factors V, VIII, and XI are activated; and simultaneously platelet aggregation and adherence are promoted.[2,4,8,9] Recent advancements have resulted in the development of an increasing number of biocompatible HAs without any animal- or human-derived components involved (nonactive) that are less dependent on the natural blood clotting cascade.[3,12] Considering this fact and the development and introduction of recombinant thrombin, a shift from more traditional active HAs can be expected.[1,3,9] In general, an ideal topical HA must be safe and effective in different situations and cases, self-activating, easily removable and/or biodegradable, cost-effective, and may also be bacteriostatic and/or bactericidal and promote the healing process.[1,4,10]

Along with animal models and clinical evaluations, visual inspections of blood/HA systems have been commonly used to evaluate their clotting properties and in vitro performances.[1,3,7,17] On the other hand, the ability to measure and monitor blood biomechanics (e.g., viscoelastic properties) in vitro can provide information on the disorders, diseases, and conditions affecting the blood coagulation such as von Willebrand disease and hemophilia.[13,16] It can also provide valuable information on blood coagulation under different conditions, for example, in the presence of various HAs. Since 1948 when Hellmut Hartert first attempted to measure the viscoelastic and clotting properties of blood,[18] various technologies have been developed and used for this purpose. These include thromboelastography (TEG), rotational thromboelastometry (ROTEM), rheology, as well as more recently developed techniques such as (magnetomotive) optical coherence elastography and laser speckle rheology.[13,15,19,20] However, there is still a need for technology that is able to accurately and quantitatively characterize, in real-time, the clotting properties of the various classes of HAs, including gauze-like and sponge-like agents when in contact with blood (i.e., all blood/HA systems including those of heterogeneous nature which cannot be conventionally characterized). Such an in vitro characterization technology can contribute to optimizing the choice of HAs leading to improvement in bleeding-related complications and mortality, as well as cost by enhancing bleeding management and hemostasis. Also, having an effective in vitro technology may reduce the cost and delay to clinically assess and qualify a HA.[5,6,11,18] Currently, topical HAs are mainly selected for use without fully considering factors such as composition, type, clotting mechanism, or efficacy.[3−6,9]

In this study, a contactless, nondestructive technique, is used for the first time to quantitatively measure the shear storage modulus (G') and coagulation properties of whole blood (recalcified and citrated) in the presence of different dosages of eight topical HAs, including particulates and gauze-like and sponge-like systems. Due to their highly heterogeneous nature, performing such in vitro analysis on these blood/HA systems has not been feasible using the above-mentioned characterization methods. Therefore, the main goal of this study was to assess the feasibility of the viscoelastic testing of bilayered materials (VetBiM) technique (used in ElastoSens Bio^2; Rheolution Inc.) in characterizing the clotting process of various blood/HA systems in vitro. This characterization technique uses gentle, low-amplitude mechanical vibrations and lasers to measure the temporal progression of G' of the blood/HA samples.[13,21] It is important to note that the accuracy and sensitivity of the technique in measuring the mechanics of whole blood clotting as well as hydrogels used in biofabrication processes have been recently validated.[13,21] The G'−time outputs of the technique were then mathematically analyzed to obtain the clot (or gel) initiation time, clotting (or gelation) rate, clotting (or gelation) time, and the apparent stiffness of the clots (or gels) in contact with various media. Furthermore, the overall change in height (or volume) of the clotting systems as well as their post-clotting properties and behavior over 5 days were monitored using this technology. These results were then discussed and correlated with the reported mechanisms of action of the various HAs.

2. MATERIALS AND METHODS

2.1. Materials. The topical HAs, which can be classified into granular (powder); gauze-like; sponge-like (foam); and gel-like

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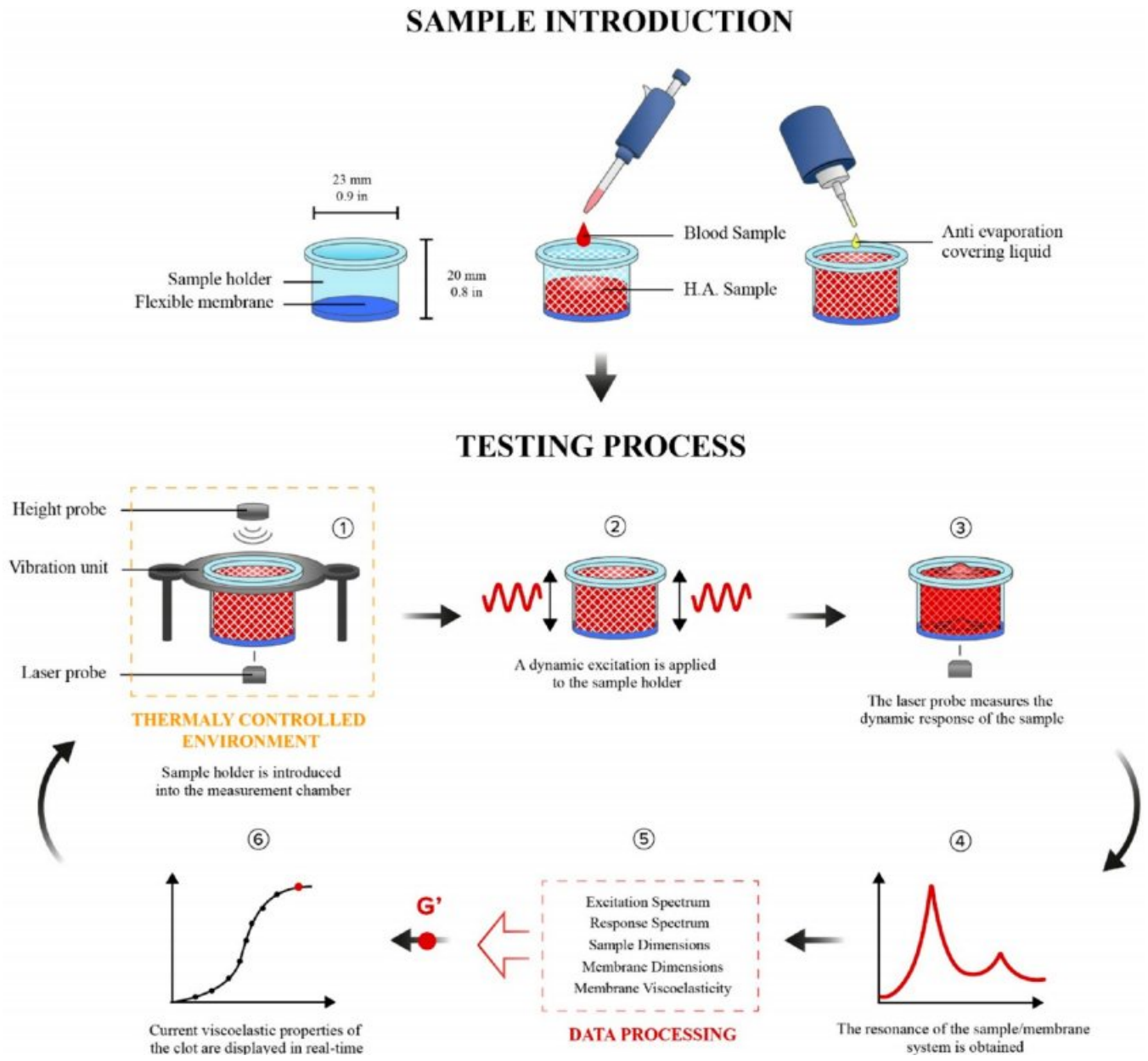

Figure 1. Schematic of the contactless, nondestructive characterization technology used in this study. Sample introduction and the testing process via the contactless, nondestructive technology used in ElastoSens Bio[2]. The same procedure was applied for all three media used in this study: recalcified blood, citrated blood, and PBS. The two arrows on the bottom panel of the figure indicate that the samples can be further characterized even after completing the analysis due to the nondestructive nature of this technology.

(liquid paste) in terms of their texture (Table 1) were each used according to their Instructions for Use (IFU) provided by their manufacturer. All the agents were kept tightly sealed and away from air and humidity before analysis. Bovine whole blood (sterile, Citrated, CL1700-500C; purchased from Cedarlane, Canada), calcium chloride ($CaCl_2$; Sigma-Aldrich, anhydrous, ACS reagent, ≥96%), and phosphate buffered saline (PBS; Fisher Scientific) were all used as received.

2.2. Sample Preparation and Shear Storage Modulus (*G′*) Measurements. Three media were used in this study: as-received citrated blood, recalcified blood, and PBS. Recalcified and citrated blood were used to simulate healthy and impaired clotting cascades, respectively, whereas PBS was used to represent a non-blood physiological fluid medium. Through these three media, the mechanism of action of HAs, for example, those that either rely on the natural blood clotting cascade or work independently, was identified.

Recalcified blood (5 mL) was prepared by adding 0.5 mL of 35 mM $CaCl_2$ solution (in deionized water) to 4.5 mL of citrated blood at 37 °C.[13] Citrated blood was kept in an incubator for at least 20−30 min to reach 37 °C prior to recalcification or G' measurements. Four 500 mL batches of citrated whole blood were used in this study, which upon receipt were sealed and stored at 4 °C and used mostly within 7 days. The results of the G' measurements on the recalcified blood of these batches were similar, and thus their average was used as the recalcified blood control.

Granular agents were used as received, whereas gauze-like and sponge-like agents were cut into smaller pieces followed by weighing prior to their incorporation within the media. The gel-like FloSeal agent was prepared according to its IFU and kept sealed in a syringe for up to a few hours while being used in analyses at different times. Because these agents have very different densities and textures, various amounts of each were incorporated with the liquid media in the sample holders for analysis (i.e., the various dosages in Table 1).

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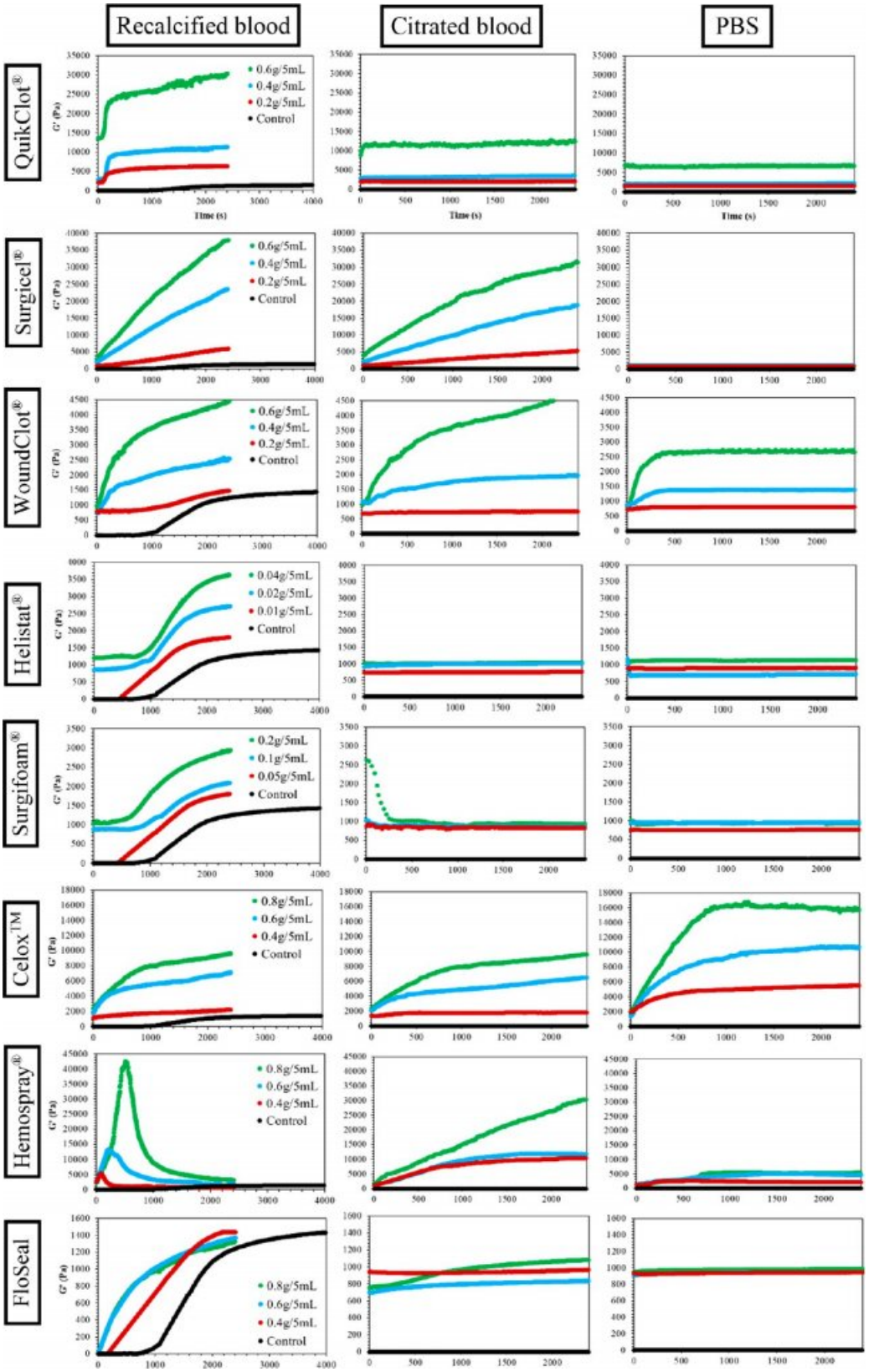

Figure 2. Clotting or gelation profiles. Clotting or gelation profiles (representative G'−time graphs) of various systems of recalcified blood (left column), citrated blood (middle column), and PBS (right column), pure (control) and mixed with different dosages of HAs.

The highest dosage for each agent was first identified (via preliminary trial and error tests) based on the maximum amount of the HA that could be incorporated into 5 mL of blood (or PBS) without overflowing from the sample holders during the test. The objective was to characterize the maximum dosage of each HA in vitro, as no specific dosages are recommended in the IFUs of these agents (i.e., it is normally recommended to use as much HA as needed until the

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Figure 3. Images of the systems at the end of their test. Images of various systems of pure media (recalcified blood, citrated blood, and PBS as controls) and mixed with different dosages of HAs, captured at the end of their 40 min tests at 37 °C (except for the pure media which were tested at 37 °C for a longer time as explained in the Materials and Methods section). The resulting systems were cut open to better show their consistency and liquid residue. The label "N" indicates no clot or gel was formed. The images of the clots formed in recalcified blood for all systems are presented in Figure S3 in a larger size to better show clot consistency, texture, and blood residue (along with the images of the systems of citrated blood and PBS for further comparison).

bleeding stops). Based on the determined highest dosages, two lower dosages for each agent were added to compare their effects.

Figure 1 provides a schematic overview of the VetBiM technology (ElastoSens Bio^2; Rheolution Inc., QC, Canada) used to measure the G' properties of the various systems. Briefly, the ElastoSens Bio^2 applies at each measurement point a pulse vibration to the bilayered structure (i.e., the flexible membrane of the sample holder and the sample being tested) in order to induce its free resonance. The spectrum of resonance of the bilayered structure is measured using an optical probe and processed to extract the resonance frequency of the system. Knowing the geometrical properties of the bilayered structure and the mechanical properties of the flexible membrane, the resonance properties of the bilayered structure only depend on the unknown viscoelastic properties of the sample. An inverse problem is automatically applied by the instrument to obtain the viscoelastic properties of the sample.

The device was calibrated at 37 °C with the empty sample holders in place to record baseline readings prior to the addition of the HA specimens. The cylindrical sample holders were made of polycarbonate with a special silicone rubber base (Figure 1). Next, the weighed amounts of HAs were loaded in the sample holders and followed by the addition of 5 mL of the various liquid media, also at 37 °C. The pieces of the sponge-like and gauze-like HAs were folded and placed in the sample holders such that they occupied their volume that were then filled by the media (with no mixing) to maximize the homogeneity of the final liquid media/HA system. In the case of Celox, media addition was followed by quick and brief mixing to create a more homogeneous mixture as the powder tended to remain on the bottom of the sample holders. In contrast, mixing

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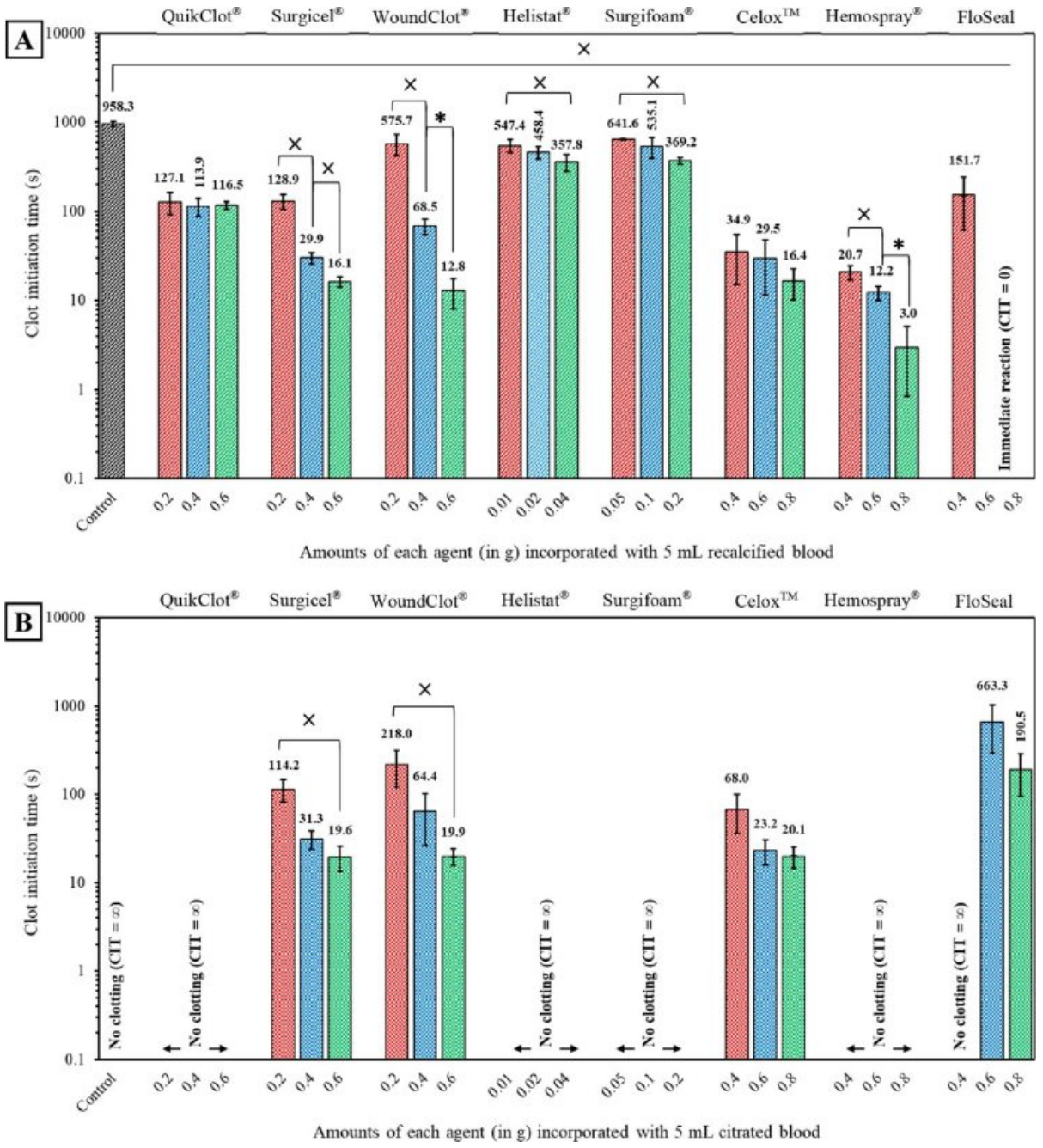

Figure 4. CIT of various systems. CIT of various systems of pure blood (controls) and mixed with different dosages of HAs: (A) recalcified blood, and (B) citrated blood ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 4$ or 5). Each bar is labeled with its average value (the error bars are standard deviation). Note that the clot initiation was considered as "∞" within the testing time(s) defined here for those systems of citrated blood that did not coagulate within the testing time frame (up to 120 min as explained in Section 2.2).

the Hemospray powder with the liquid media was not possible as it created a sticky paste upon contact. Similarly, the weighed gel-like FloSeal agent was placed on the bottom of the sample holder followed by the incorporation of the different media without mixing (the reaction was immediate upon incorporation). During analysis, an anti-evaporation covering liquid was added on top of the all test specimens to prevent evaporation (and artificial hardening of the sample) as well as to remove surface bubbles.

The real-time changes in shear storage modulus (G') of the systems were temporally measured as an indication of blood (citrated and recalcified) coagulation ($n = 4$ or 5) or PBS gelation ($n = 3$ or 4). Analyses were performed for 40 min at 37 °C for HA-containing systems, and up to 120 min for pure media (controls) with a 5 s resolution between each measurement point. Note that the accuracy and reproducibility of the results produced by ElastoSens Bio^2 for whole blood have previously been studied and verified.[13]

The G'−time plots were then analyzed to obtain the coagulation or gelation characteristics of each system, including clot (or gel) initiation time, clotting (or gelation) rate, clotting (or gelation) time, and the apparent stiffness of the clots (or gels), as schematically shown in Figure S1. The clot (or gel) initiation time was considered as the time at which G' indicates a greater than 5% increase over G_0' (the initial G' at the beginning of the analysis) and progressively continues to increase. The clotting (or gelation) rate was considered as the slope of the line fitted ($R^2 \geq 0.99$) to the part of the G'−time graphs where G' rapidly increases. Clotting (or gelation) time was considered as the time when the rapid increase in G' ends (the clot or gel is formed), which was approximated from the detachment point of

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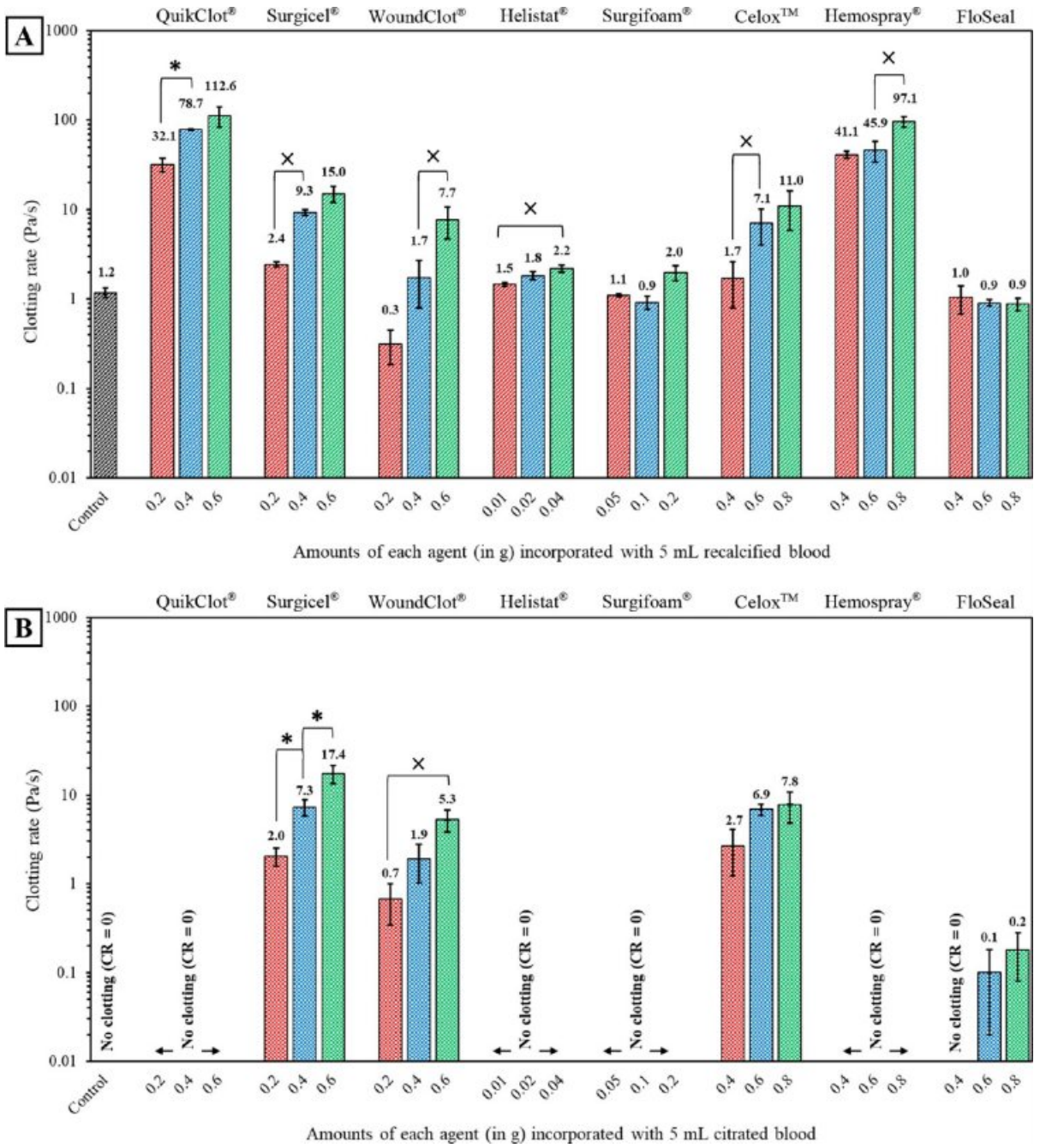

Figure 5. CR of various systems. CR of various systems of pure blood (controls) and mixed with different dosages of HAs: (A) recalcified blood and (B) citrated blood ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 4$ or 5). Each bar is labeled with its average value (the error bars are standard deviation). Note that the CR was considered as "zero" within the testing time(s) defined here for those systems of citrated blood that did not coagulate within the testing time frame (up to 120 min as explained in Section 2.2).

the second fitted line (the dashed lines in Figure S1) to the graphs. This method allowed for a systematic calculation of clotting time (CT) from all G'−time curves with different shapes with or without reaching a plateau. The apparent stiffness of the clots (or gels) was considered as the G' value of the systems at the end of analysis, that is, at 40 min for all systems of HAs mixed with media and ∼60−65 min for pure recalcified blood (where the G'−time plateaued). At the end of analysis, all systems were removed from the sample holders and photographed using a high-definition digital camera.

2.3. Height Measurements (Volume Change in Samples). Using an ultrasound probe (Figure 1) within its dimensional measurement resolution (200 μm), the change in height of the systems of HAs mixed with recalcified blood was also monitored using an ElastoSens Bio^2. The (%) change in height of the systems ($n = 3$) was then calculated as [(final height − initial height)/initial height] × 100 and used as an indication of volumetric change in real-time.

2.4. Clot Aging Studies. The as-formed clots of recalcified blood mixed with each HA at their highest respective dosages (Table 1) were further tested through a 5-day aging experiment. The as-formed clots (in their sample holder with the surfactant oil on top) were sealed by Parafilm and aluminum foil and stored in an incubator at 37 °C in between measurements. Note that despite all these measures, minor evaporation to some extent was unavoidable over the 5-day experiments. The G' of each system was tested ($n = 3$) at the 120 min, 1 d, 2 d, 3 d, 4 d, and 5 d time points by placing the sample holders back into the ElastoSens Bio^2. At the end of analysis, all systems were

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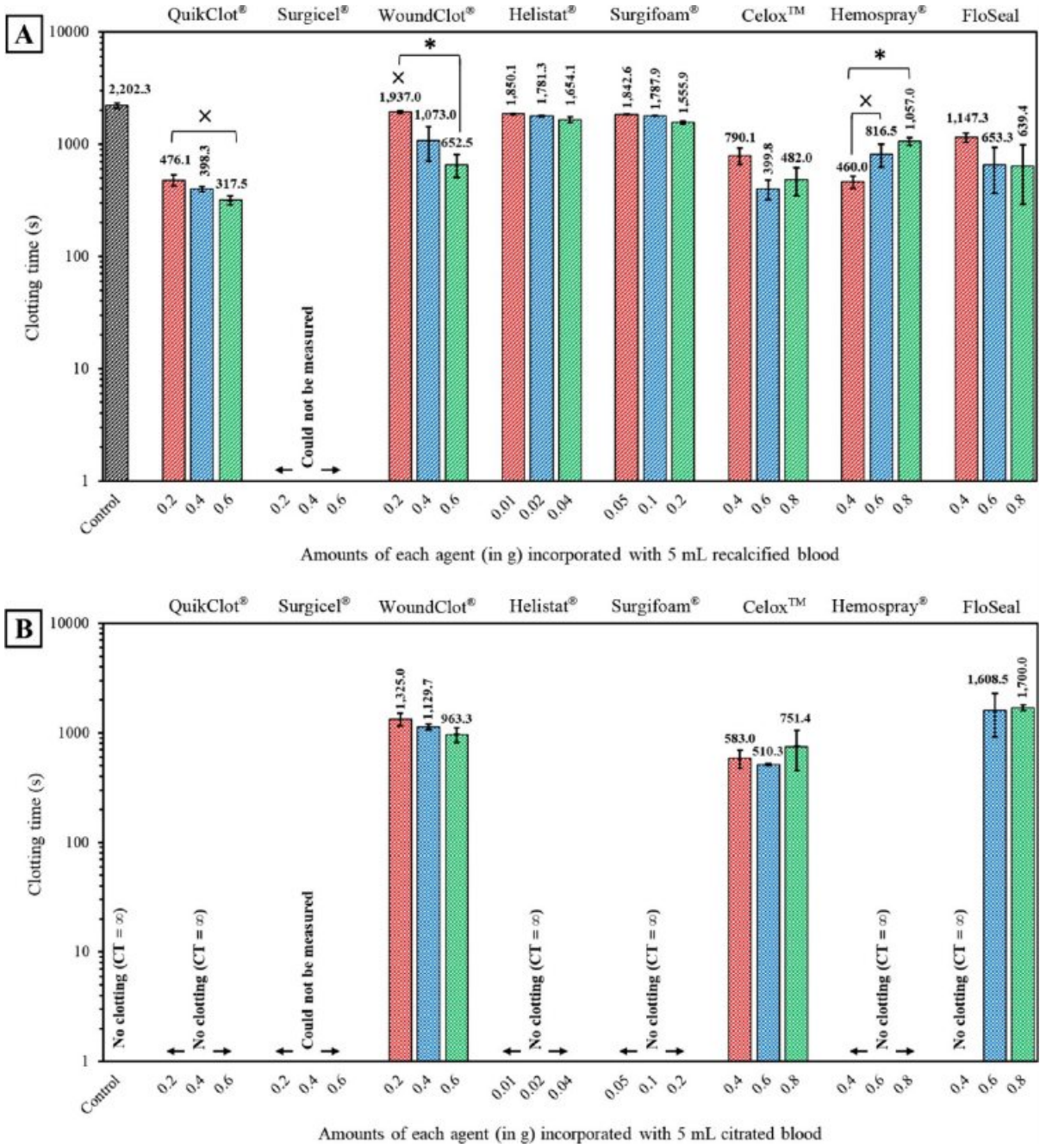

Figure 6. CT of various systems. CT of various systems of pure blood (controls) and mixed with different dosages of HAs: (A) recalcified blood and (B) citrated blood ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 4$ or 5). Each bar is labeled with its average value (the error bars are standard deviation). Although the systems of recalcified and citrated blood mixed with a Surgicel agent underwent clotting, their CT could not be measured due to the linear shape of their corresponding G'−time curves (Figure 2). Note that the CT was considered as "∞" within the testing time(s) defined here for those systems of citrated blood that did not coagulate within the testing time frame (up to 120 min as explained in Section 2.2).

removed from the sample holders and photographed as described above.

2.5. Statistical Analysis. Statistical significance of all values in each set of results was determined using one-way ANOVA multiple comparison test in Excel at two significance levels of $^{\times}p < 0.05$ and $^{*}p < 0.01$.

3. RESULTS

The various topical HAs used in this study are presented in Table 1. The change in G' (stiffness) of the systems of pure media (controls) and when mixed with different HA dosages as a function of time is presented in Figure 2. Unlike in pure recalcified blood, both citrated blood and PBS did not show any signs of clotting or gelation as there was no change in the G' versus time profiles. On the other hand, the different G'−time profiles and values for the HA/media systems, in particular those mixed with recalcified blood, revealed their distinct behavior to induce and impact coagulation.

The recalcified blood clotting profiles for Surgicel and Hemospray displayed distinct trends compared to the other HA systems. For Surgicel, G' linearly increased with time during analysis indicating that the stiffness of the clots was continuously increasing. For Hemospray, the powder could not

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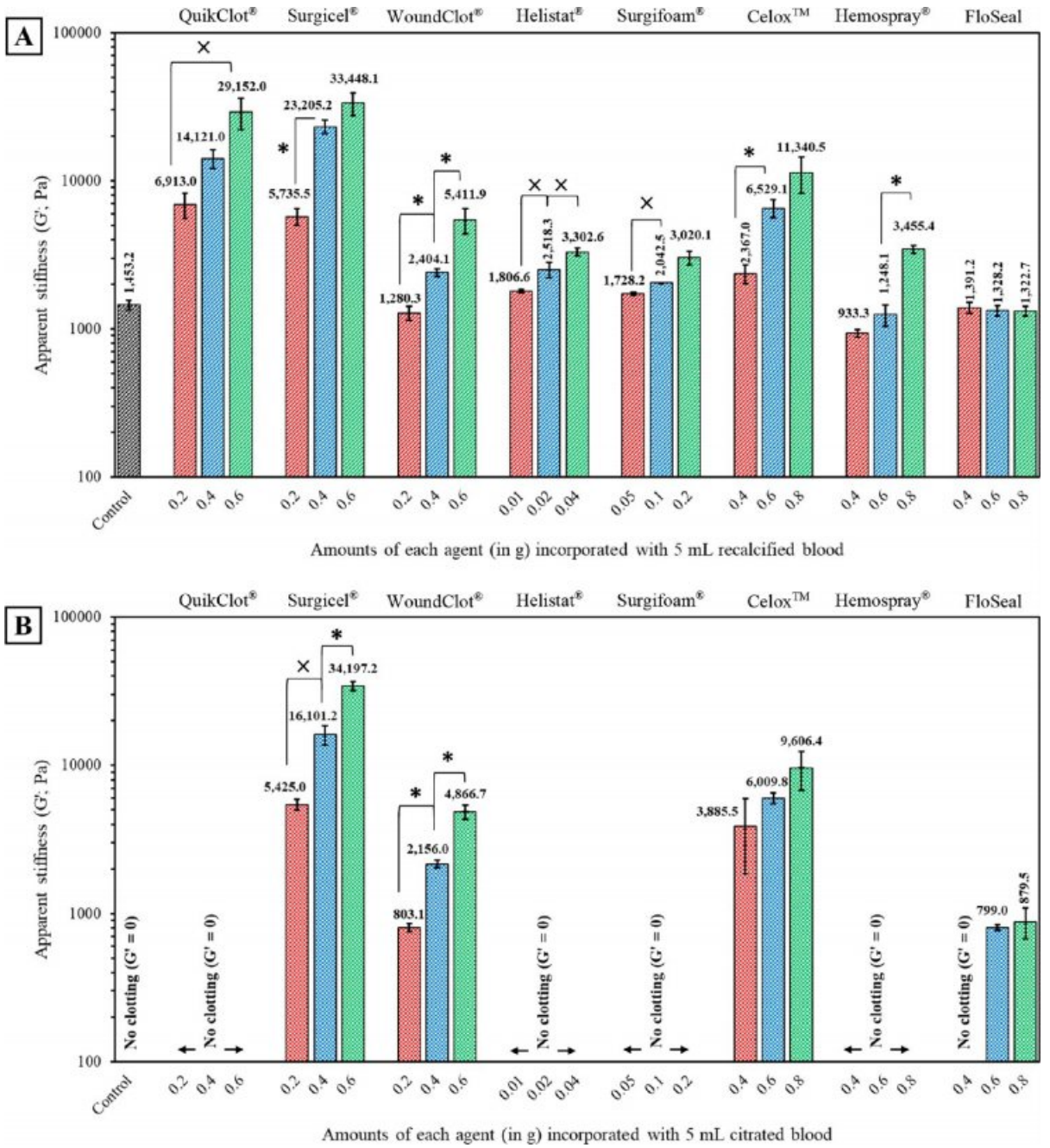

Figure 7. Apparent stiffness (G') vs dosages of various HA systems. Apparent stiffness (G') of various systems of pure blood (controls) and mixed with different dosages of HAs at the end of their 40 min tests at 37 °C (except for the pure blood which was tested at 37 °C for a longer time): (A) recalcified blood and (B) citrated blood ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 4$ or 5). Each bar is labeled with its average value (the error bars are standard deviation). Note that the apparent stiffness was considered as "zero" within the testing time(s) defined here for those systems of citrated blood that did not coagulate within the testing time frame (up to 120 min as explained in Section 2.2).

be mixed with blood and was initially placed in the sample holders and followed by the addition of the various liquid media. Because the reaction with recalcified blood initiated immediately, it created a stiffer layer on the base of the sample holder as reflected by the rapid initial increase in G' at all dosages. However, after reaching a maximum peak, there was a decrease in G' as the whole system underwent clotting.

The analysis indicated no significant increase in G' over time for QuikClot (the lower dosages), Helistat, Surgifoam, and FloSeal (for the lowest dosage) in citrated blood and for QuikClot, Surgicel, Helistat, Surgifoam, and FloSeal in PBS. Fluid absorption may be responsible for the slight increase in G' of QuikClot in citrated blood (the highest dosage), and Hemospray in both citrated blood and PBS because no clot or gel was formed at the end of analysis in these systems.

Complementary to the quantitative results of Figure 2, the images of all systems at the end of their analyses are shown in Figure 3. As expected, all recalcified blood samples indicated clotting, while only Surgicel, WoundClot, Celox, and FloSeal (at higher dosages) formed clots in citrated blood, among which only WoundClot and Celox were also gelled in PBS. This indicated that Surgicel and FloSeal still need to be in

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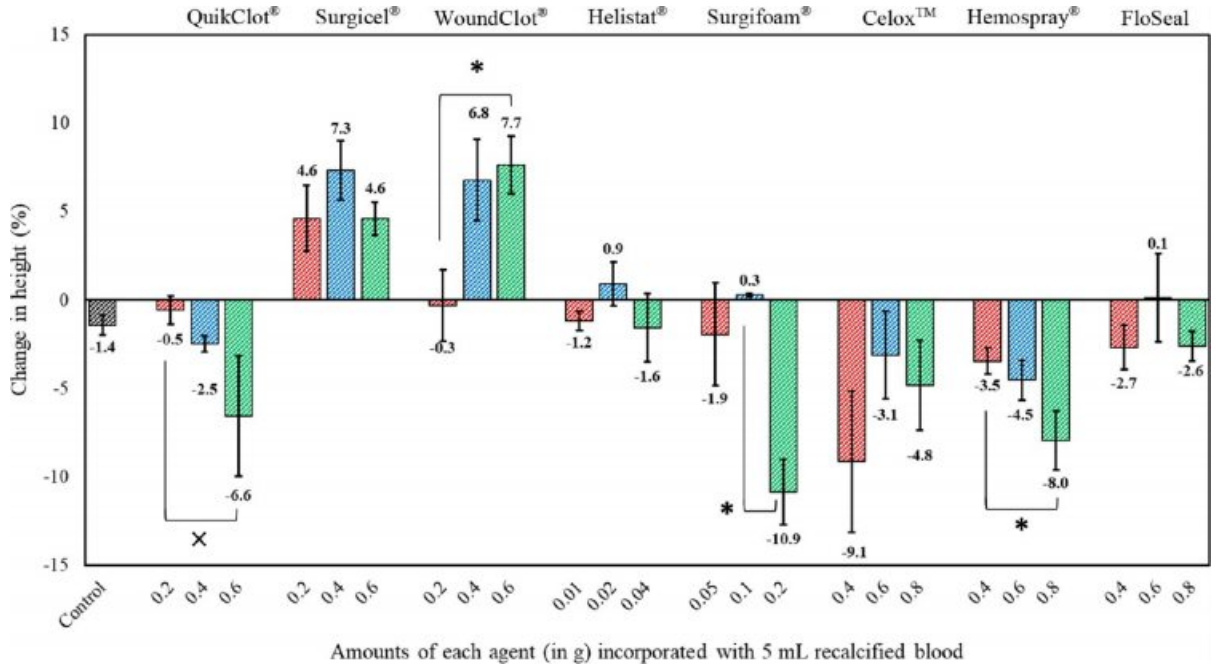

Figure 8. Overall changes in the height of the systems during clotting. Overall change in the height (%) of the systems of pure recalcified blood (control) and mixed with different dosages of HAs ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 3$). Note that the final height is measured at the end of its 40 min test at 37 °C (except for the pure recalcified blood which was tested at 37 °C for a longer time).

contact with blood (even in citrated blood with an impaired clotting cascade) to be able to induce clotting or gelation. On the other hand, the clotting mechanisms of WoundClot and Celox appeared to be independent of the blood. Also, Figure 3 shows that in these systems, the incorporation of higher dosages of the agents resulted in less fluid residue in the formed clot or gel.

Figures 4−7 present the characteristic clotting values of clot initiation time (CIT), clotting rate (CR), CT, and apparent stiffness for the various systems of pure blood (recalcified or citrated) and those mixed with different HA dosages generated from their corresponding G'−time graphs (as described in Figure S1). The gelation characteristic values for systems of PBS mixed with different dosages of HAs are presented in Figure S2.

CIT can be used to indicate how rapidly clot formation can initiate and hinder bleeding, which can be critical in severe cases of bleeding.[7,11] Figure 4A shows that the CIT of the recalcified blood was significantly ($p < 0.05$) decreased when mixed with each HA at all respective dosages. Also, there was a decrease in CIT with an increase in HA dosages for all these systems (Figure 4A). A similar trend was observed for the systems forming a clot in citrated blood (Surgicel, WoundClot, Celox, and FloSeal) where higher dosages decreased the CIT (Figure 4B).

Figure 5A shows that the incorporation of HAs can, in most cases, accelerate the CR of recalcified blood with more noticeable impacts with higher dosages of each agent (except for FloSeal). In some systems, the CRs appeared to be lower compared to those in recalcified blood, including the lower dosages of WoundClot and Surgifoam as well as all dosages of FloSeal. For the systems forming a clot in citrated blood, the CR also increased with increasing dosages (Figure 5B).

CT is an important parameter as it approximates the time expected for the clots to form that then continue to stiffen and stabilize.[13,16−18] In other words, CT indicates the point at which the incipient clots are relatively stable after the rapid increase in G', which can then be followed by either a plateau or gradual stiffening and further stabilization (e.g., QuikClot or WoundClot, respectively, in Figure 2). Figure 6A reveals that there is a decrease in CT of recalcified blood when mixed with HAs at all dosages. A similar trend was observed for those systems forming a clot in citrated blood (Figure 6B).

In the case of Hemospray, the CT is calculated using the approach described in Figure S1, but on the "after the peak portion" of the bell-shaped curves. As a result, this CT may be prolonged in Figure 6A due to the difference in its in vitro (mixing with blood) and in vivo (by spraying a layer of the powder as instructed[6]) applications. This is also the case for the other characteristics, including the final apparent stiffness of Hemospray clots (Figure 7A).

The final apparent stiffness of the clots formed from pure recalcified blood and its mix with HAs at different dosages is presented in Figure 7A. It appears that, in most cases, incorporating HAs increased the stiffness of the recalcified blood clot. This is partly due to the presence of the HAs in the clots, in particular those of the gauze-like agents with a higher strength texture. Interestingly, the stiffness of the systems that formed a clot in citrated blood appeared to be mostly lower than those in recalcified blood, indicating that the interaction of the natural blood clotting cascade with these HAs may also contribute to the final stiffness.

Figure 8 reveals that recalcified blood and its mix with HAs at different dosages experience a change in height (which can be translated to a change in volume) during the analysis. In fact, the change in the height of these systems during clotting appeared to be very different. Recalcified blood exhibited an overall small shrinkage, whereas systems of gauze-like HAs appeared to swell during clotting, except for QuikClot, which exhibited a dose-dependent shrinkage. Systems of sponge-like HAs as well as the gel-like FloSeal showed an interesting trend with a greater shrinking tendency for systems of their lowest

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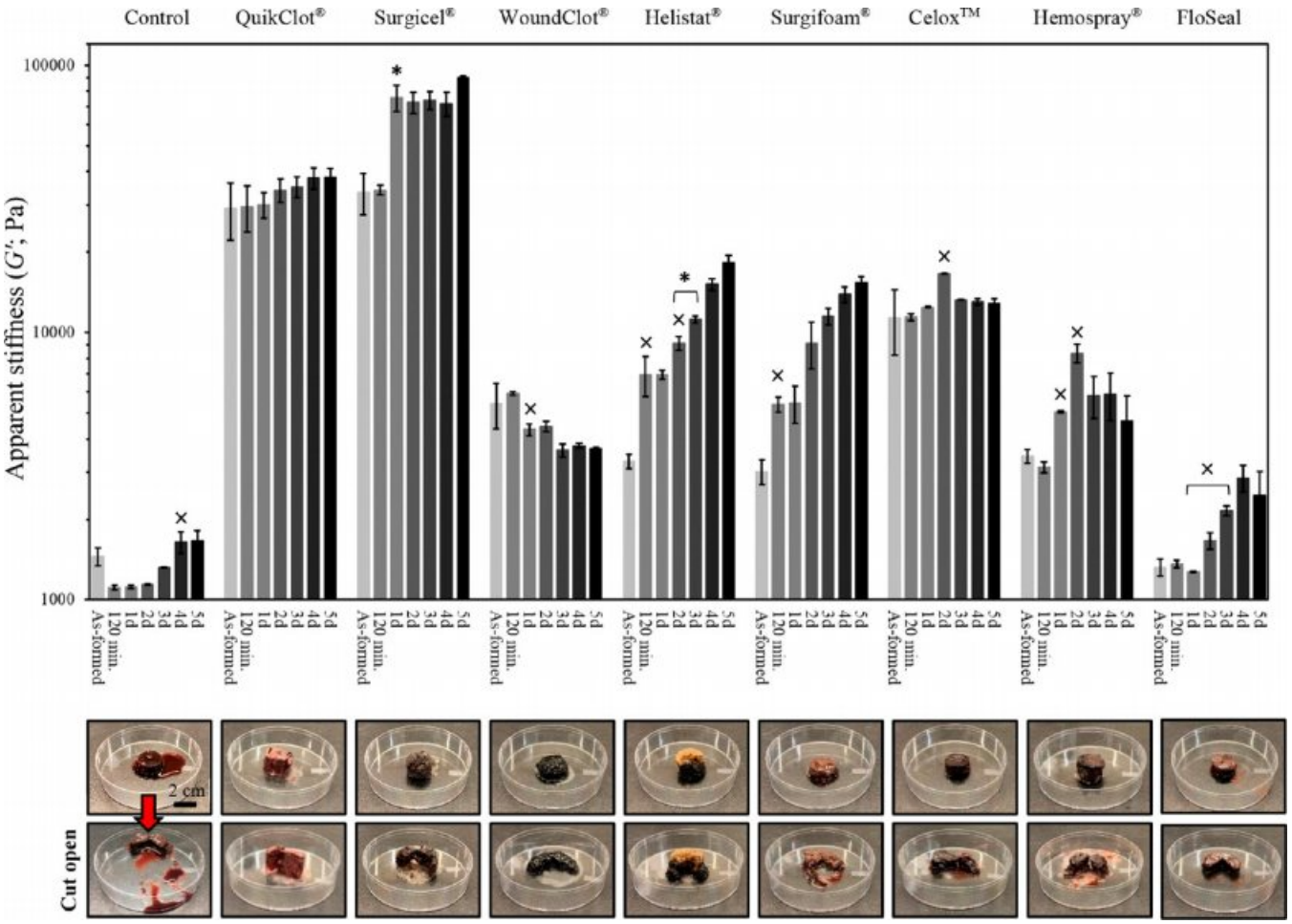

Figure 9. Changes in the apparent stiffness of the as-formed clots of various systems in recalcified blood. Clot aging process as indicated by the apparent stiffness (G') of the clots of pure recalcified blood (control) and mixed with the respective maximum dosages of each HA (Table 1) at different timepoints ($^{\times}p < 0.05$ and $^{*}p < 0.01$; $n = 3$). The images of the clots at the end of the 5-day aging process are also presented. These images can be compared with the corresponding images of the as-formed clots before aging in Figures 3 and S2.

and highest dosages. Both systems of powder-like agents exhibited shrinkage, although with different trends at varying dosages.

The change in clot stiffness as a function of time can be used to indicate clot stability, which may impact the healing process as well as limitations on the removal of the HA, if required.[7] For example, it has also been suggested that the final clots must be mechanically stable but not too stiff which otherwise may prevent proper dissolution after wound healing.[16] To this end, the nondestructive nature of the technology applied in this study was used to monitor the stiffness of the as-formed clots in recalcified blood at 37 °C over a 5-day aging period (Figure 9). This provided quantitative data to assess the longer term performance of the HAs, in vitro. The stiffness of the as-formed clot of pure recalcified blood (control) initially decreased and then increased up to day 3, whereas other systems exhibited different behaviors. Among the gauze-like agents, QuikClot and Surgicel showed an overall increase in stiffness (with a noticeable spike at day 1 for Surgicel), whereas the WoundClot systems seemed to degrade over time. The slow and poor biodegradability of QuikClot has been previously reported.[1,11] On the other hand, the clot stiffness of the sponge-like agents (Helistat and Surgifoam) gradually increased over time. The powder-like agents displayed a similar trend with their clot stiffness reaching a maximum at day 2 and then decreasing and stabilizing up to day 5. In contrast, in the case of the gel-like agent, FloSeal, the as-formed clot appeared to continue to stiffen over this timeframe.

4. DISCUSSION

The results of this study demonstrate that the contactless technology can both measure clotting properties of blood/HA systems and differentiate between different HAs at varying dosages. While it is important to note that these results are not intended to compare the performance of the various HAs (e.g., as the dosages used here and their recommended methods of in vivo application are different), they may be correlated with the reported hemostatic mechanism of action of each HA.[1−8]

Kaolin, which is used in QuikClot, is an inert inorganic aluminum silicate compound that is known to be an activator of the intrinsic blood clotting pathway.[5,7,8] Upon contact with blood, kaolin transforms factor XII into its activated form (XIIa), which then activates factor XI and pre-kallikrein, all of which contribute to the formation of clots.[4,8] Additionally, this agent absorbs and holds water molecules via hydrogen bonding, and results in the concentration of platelets and clotting factors, further contributing to the coagulation process.[11] The hemostatic properties of the kaolin-based QuikClot agent mainly depend on the blood clotting cascade that is, there was no clotting or gelation in citrated blood and

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PBS, respectively (Figures 2 and 3). Thus, this agent may be less effective in coagulopathic cases.[7] It has also been reported that kaolin may not provide immediate hemostasis upon application onto the wound,[7] which was reflected in this study, as indicated by the lag phase observed in the G'−time curves of QuikClot (Figures 2 and 4A).

The hemostatic mechanism of cellulose is not yet fully understood.[10] Surgicel is a bioabsorbable gauze that generates a tamponading effect while providing a matrix to promote clot initiation and platelet activation, adherence, and aggregation.[3,4,10] The acidity of the oxidized regenerated cellulose component of this agent caused the dark brown color of the product when in contact with blood (Figure 3), which is a result of local acidosis inducing red blood cell lysis.[4,5,22] On the other hand, upon contact with the non-oxidized, non-regenerated cellulose structure in WoundClot, water molecules are cross-linked via polymeric chains to create a network that can also significantly absorb blood and concentrate procoagulants, thereby promoting clot formation without manual pressure.[4] It has been reported that, once in contact with the bleeding site, the WoundClot gauze turns into a "thick, tenacious, expanding 3D gel"[23] and facilitates hemostasis through multiple mechanisms of absorption, adhesion, and aggregation, as well as activation of Plasma Thromboplastin Antecedent PTA(XI) and Hageman factor (XII).[4,23] The ability to cross-link water molecules most likely explains its gelation in PBS (Figures 2, 3, and S3).

Helistat is a nonactive topical HA based on microfibrillar collagen (MFC), a water-insoluble acid salt of bovine collagen that provides a large surface area to facilitate platelet adherence, aggregation, degranulation, and the intrinsic pathway induced thrombus formation.[2−4,14] Collagen also facilitates cell adhesion and growth during wound healing.[4] Because the hemostatic mechanism of MFCs depends on platelet activation, no clotting or gelation was observed for Helistat in citrated blood and PBS, respectively (Figures 2, 3, and S3). Thus, it has been reported that MFC agents may be less effective in the presence of severe thrombocytopenia.[2,8]

Surgifoam, which is an absorbable gelatin sponge, initiates a two-step hemostatic mechanism involving contact activation and platelet aggregation.[4,5,22] The highly hygroscopic gelatin content of this agent creates a structure that absorbs blood and promotes clot propagation and tissue granulation.[4,5,22] It has been reported that gelatin sponges can absorb fluid, including blood, up to 40 times their mass.[5,8,12,22] Consistent with this reported mechanism of action, no clotting or gelation was observed for Surgifoam in citrated blood and PBS, respectively (Figures 2 and 3).

Attributable to their highly hydrophilic nature, the chitosan particles in Celox swell to form a gel through absorption and dehydration of blood. This phenomenon triggers the observed Celox tamponading effect when put in contact with blood, while simultaneously concentrating serum proteins, platelets, and other factors involved in clot formation.[2−4] The electrostatic interaction between the positively charged chitosan granules and negatively charged red blood cell membranes is mainly responsible for its hemostatic properties.[2,3,5,7] Chitosan may also promote the release of platelet-derived growth factor-AB and contribute to transforming growth factor-β1, thus aiding in wound healing.[3] Chitosan-based HAs possess no intrinsic hemostatic properties and work independently from the natural blood clotting cascade. Thus, they can still work in the presence of anticoagulants.[3,5,7,11] This is consistent with the results of Figures 2 and 3 showing clot formation in citrated blood and gelation in PBS, that is, in the absence of a healthy blood clotting cascade.

The mechanism of action of the inert bentonite clay-based powder, Hemospray, relies on its high capacity for water absorption.[24] This consequently leads to a greater concentration of clotting factors and cellular components, with additional mechanical tamponade provided as a result of particle swelling, and possible activation of the clotting cascade.[12] The rapid water absorption capability of the finely ground clay (high specific surface area), which is known to also promptly activate the intrinsic blood clotting pathway,[7,14] can explain the immediate clotting of recalcified blood when in contact with this agent (Figure 4A). While fluid absorption in Hemospray played an essential role in increasing G' in all three media (Figure 2), coagulation only occurred in recalcified blood (Figures 2 and 3), which indicates that this agent interacts with the natural blood clotting cascade. Rapid hemostasis is expected from Hemospray in vivo,[6] and this is in line with the very short CIT demonstrated in this study, in vitro (Figure 4A). Additionally, when in contact with moisture in vivo (e.g., in the gastrointestinal tract), the Hemospray powder becomes cohesive and adhesive, thus creating a mechanical barrier at the bleeding site.[6] Moreover, the rapid initial increase in G' in recalcified blood followed by it reaching a maximum (Figure 2) suggests that the effects of Hemospray cannot only be attributed to its absorption capability. Rather, when mixed with citrated blood, it exhibited a different (linear) increase in G' as a result of fluid absorption, thus creating a paste with no clot formation. This trend was similar to that observed in PBS but with a less noticeable change in G' (Figure 2). Therefore, in addition to absorption, Hemospray seems to interact with the clotting cascade leading to rapid clot initiation in recalcified blood (Figure 4A).[25] Note that, Hemospray powder is used for gastrointestinal bleeding control and is only applied through a dispenser, that is, by spraying the powder onto the bleeding site.[6,24]

Upon contact with blood, FloSeal initiates several mechanisms including expansion of crosslinked gelatin particles (tamponade effect), while its thrombin component simultaneously activates FV, FVIII, FXIII, thereby converting fibrinogen to fibrin to accelerate clot formation.[2−4,12,14,22] Additionally, it has been reported that this agent can also firmly attach to the wound.[10] Since the physiologic clotting agent (thrombin) in FloSeal maintains its effect even in the case of blood with an impaired clotting cascade (e.g., platelet dysfunction), clots were also formed in citrated blood (Figures 2 and 3).[4,5,10] Moreover, the lack of gelling in PBS (Figures 2, 3, and S3), indicates that FloSeal requires contact with blood (even an impaired clotting cascade) to enable hemostasis. As an active agent, FloSeal activates and promotes the blood clotting cascade, which was confirmed by its shorter initiation (Figure 4A) and clotting (Figure 6A) times. However, this agent did not enhance the stiffness of the final clot (Figure 7A).

In contrast to the other HAs studied here, the agents Surgicel and FloSeal (except for its lowest dosage) exhibited unique behaviors, as they formed a clot in citrated blood, but did not gel in PBS (Figures 2, 3 and S3). This indicates that these two agents can be activated as long as they are in contact with blood, even when citrated, where the natural clotting cascade is impaired. Note that, the clotting characteristics of these two agents are different in recalcified and citrated blood

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(Figures 4−7), highlighting the interaction of the agents with the natural blood clotting cascade.

While it may be expected that the presence of higher dosages of HAs increases the apparent stiffness of the final clots (except for FloSeal), their hemostatic mechanism of action also plays a role in this phenomenon. Indeed, both gauze- and powder-like agents exhibited significant ($p < 0.05$) increases in stiffness with higher dosages in recalcified blood. Interestingly, for the gauze-like agents, Surgicel and WoundClot, higher dosages significantly increased ($p < 0.01$) the clot stiffness in both recalcified and citrated blood, whereas for Celox, the impact was less significant ($p < 0.05$) in citrated than in recalcified blood. This may indicate that the contribution of powder-like agents to the final clot stiffness relies more on their interaction with the blood clotting cascade.

Although there is some information on the long-term behavior (e.g., biodegradation) of the formed blood clots when in contact with various HAs, in vivo, there is no corresponding quantitative data, in vitro.[3−5,10,12,22] Therefore, the availability of a technique that characterizes the longer-term mechanical properties (e.g., stiffness) of blood clots, in vitro, can provide important information on their stability and degradation behavior. Furthermore, the ability to anticipate a change in clot stiffness over time (with or without HAs), in particular for cases of blood-related diseases, can be vital.[16] The non-destructive nature of the technology used in this study permitted such characterization (Figure 9). In addition to aging studies, it is anticipated that this technology can also be potentially used to investigate the effects of different physiological fluid or enzyme addition on the degradation of blood clots, in vitro.[4,14] In fact, due to the highly heterogeneous nature of the systems (in particular, the gauze-like HAs mixed with blood), such quantitative in vitro analyses cannot be performed using other techniques such as TEG, ROTEM, or rheometer. This is mainly due to their sample loading process and set up which limit the types of HA systems that can be tested. Moreover, the lack of ability to monitor the real-time changes in viscoelastic properties, insufficient sensitivity, and the destructive nature of these techniques prevent further complementary studies.[13,15,19,20] Similarly, the ability of to predict the change in height (i.e., volume) of the systems may be clinically relevant since an unexpected change in clot volume, in particular in confined spaces in vivo, can potentially damage surrounding blood vessels and nerves.[2,4,5,12] For example, the Surgicel IFU cautions that, after hemostasis is achieved, in certain anatomical locations, the agent must be carefully removed as it may swell and apply unwanted pressure to surrounding tissues.[26] Similarly, the WoundClot IFU describes an "expanding gel" capable of absorbing a significant amount of fluid.[23] These reports are consistent with the quantitative aging results of Figure 8. Although this study only analyzed the initial and final height of the samples, the contactless technology allows for real-time monitoring of sample height, which may be useful since some systems can exhibit changes in their height (volume) at different stages (e.g., an initial expansion followed by a shrinkage).

While TEG and ROTEM have been commonly used to characterize pure blood and its clotting properties,[16,18] there are limited studies reporting the use of these techniques in characterizing blood in contact with HAs, even in granular form. Therefore, a direct comparison between the findings of this study with those in literature is challenging, for example, various types of blood have been recalcified to different levels and used in combination with different dosages of hemostatic materials, thus making a comparison difficult. Nevertheless, Zhang et al.[27] used TEG to characterize the clotting properties of rabbit blood when mixed with Celox (~20 mg in 1 mL blood) and reported a reaction time of 450 ± 54 s. However, the Celox dosage in that study was approximately 4 times less than the lowest dosage used in this study. Similarly, Fischer et al.[28] used TEG to report a 33.7 min lag time for thrombin generation when 3 mg of chitosan was mixed into 1 mL of platelet-rich plasma. Li et al.[29] also used TEG to measure the CT of blood in contact with layered clays (montmorillonite and bentonite), which were reported to be ~6 min for dosages in the range of 3−7 mg/mL. Once again, the dosages (concentrations) used in that study were lower than the lowest dosage of 0.4 g of either Celox or Hemospray in 5 mL blood, which may be due to the limitation of TEG in measuring high concentration systems. On the other hand, Xu et al.[19] used optical coherence elastography to report that the reaction time for recalcified porcine whole blood in contact with a kaolin activator was 121 ± 41 s. This value is close to the corresponding CIT (127 ± 35.79 s) of the kaolin-based QuikClot, when tested at its lowest dosage (Figure 4A). Moreover, Holster et al.[25] used ROTEM in their study to report that the average recalcification time of whole human blood (time needed for clot formation) decreased from 187.5 s to 60 and 45 s after addition of 1 and 10 mg/mL Hemospray, respectively. They also reported a similar impact on the plasma prothrombin time upon incorporation of Hemospray.

It is anticipated that the ability to quantify the clotting characteristics of HAs when in contact with blood, in vitro, can in the future be used to predict in vivo and clinical outcomes and may contribute to the selection of an appropriate HA in various clinical scenarios.[3−5,9,16] Since healthy or impaired blood clotting cascades and their distinct interactions with various classes of HAs affect the clotting kinetics in vivo, this in vitro technology may potentially be used as a device to evaluate patient specific blood when in contact with different types of topical HAs in advance of a procedure. Moreover, the ability to predict swelling when different HAs come into contact with blood can help with adjusting HA dosages when used in vivo and prevent potential damage to the surrounding tissues. However, the differences between in vivo and in vitro conditions, that is, the presence or absence of other tissues as well as dynamic versus static conditions, respectively, constitute some limitations for making direct conclusions. For example, most of the nonactive mechanical HAs perform as intended under mechanical pressure to stop the bleeding.[4,5,9,11] However, implementing this critical step in vitro was not feasible. Another limitation of this technology is the volume of blood required for each test (e.g., ~5 mL for each sample) that may be large and may not be feasible in some cases.

In summary, it can be predicted that this technology can potentially play an important role in developing new and improved HAs for not only the current needs but also future generations of HAs.[8,10,11,22] In time, this technology can also assist in better understanding, diagnosing, and potentially treating blood-related complications; for example, conditions affecting natural blood clotting, such as hemophilia and von Willebrand disease,[5,10,13] or illnesses promoting hypercoagulability such as that seen in severe cases of COVID-19 patients.[30−32]

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5. CONCLUSIONS

In this study, the feasibility of a novel contactless, nondestructive technology for in vitro characterization of different dosages of eight commercially available HAs, which were selected from various classes (active, nonactive, and flowable) was evaluated. The real-time changes in G' as a function of time for each system were analyzed to generate information on the clotting (or gelation) characteristics and behavior of the HAs, which were then correlated with their reported homeostasis mechanisms. By comparing the results generated from citrated blood and PBS with those of recalcified blood, the HAs that can affect hemostasis independently of blood, potentially performing well, even in cases where the natural blood coagulation cascade is inadequate (i.e., coagulation deficiencies), were identified and quantified. The contactless, nondestructive nature of the technology also allowed for further quantitative investigations on the as-formed clots including longer term aging analysis, which are not feasible with other characterization techniques. Therefore, the technology used in this study offers a promising tool for accurate, quantitative measurements of the clotting properties of various blood/HA systems, which may be used to better predict their in vivo performance. Such in vitro characterization is essential, considering the new HAs being introduced, and the increasing number of studies in this field attempting to address the knowledge gaps.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c01741.

Clotting (or gelation) characteristics on a schematic of a typical G'−time graph; gelation characteristics of PBS/HA systems obtained from their corresponding G'−time graphs; and images of various systems of pure media and mixed with different dosages of HAs (PDF)

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Notes

The authors declare the following competing financial interest(s): Dr. Schmitt and Dr. Hadj Henni are the co-founders and shareholders of Rheolution Inc. They participated in the design and the scientific guidance of the study.

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