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ORIGINAL ARTICLE

A Cryopellet Containing Silica Can Activate Intrinsic Blood Coagulation in Rotational Thromboelastometry

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SUMMARY

Background: **The use of colloidal silica contained in different cryopellet formulations is examined as a contact activator of blood in an intrinsic coagulation test. In particular the necessary amount of silica per cryopellet was determined, as well as the interfering effects of cryopellet components on coagulation.**
Methods: **Cryopellets were prepared using an established technique and were tested by rotational thromboelastometry using recalcified whole blood. The clotting time (CT) and maximum clot firmness (MCF) were determined from the dynamic coagulation profiles.**
Results: **Levels of silica of 25 - 75 µg per cryopellet weight of 1.25 mg reduced CT to below 200 seconds and gave good MCF. Trehalose had no effects of coagulation, whereas mannitol reduced CT by a third. The incorporation of silica in the liquid feed used for cryopelletization did not inhibit uniformity of droplet and hence cryopellet weight. The activating activity of the silica [CT and MCF] was the same as that of a classic intrinsic assay using ellagic acid.**
Conclusions: **A silica-containing cryopellet can be prepared and works as well as ellagic acid as an activator in intrinsic rotational thromboelastometry. A cryopellet avoids dilution of the whole blood sample and hence also of coagulation-relevant blood components that can alter the coagulation result.**
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INTRODUCTION

A rapid, quantitative assessment of blood coagulation is a vital tool for use in laboratory medicine, for example for FVIII dose adjustment in hemophilia A [1] as well as in peri-operative care [2]. The classical methods of activated partial thromboplastin time (APTT) and prothrombin time (PT) each determine an endpoint for coagulation expressed as a convenient, workable single value [3,4]. Although PT is standardized using an internationally-recognized sensitivity index [5], APTT has a DIN procedure [6] but has yet to be harmonized internationally [7]. An alternative technique to these single-point determinations is the method of rotational throm-

boelastometry. This records the dynamic coagulation profile over time and, therefore, provides information about initiation and maintenance of coagulation as well as subsequent fibrinolysis in real time [8]. The elasticity of a sample of whole blood contained in a fixed-cuvette/ oscillating-plunger set-up is measured. As the blood clots and anastomosed fibrin fibres are formed across the sample between the cuvette wall and pin, the oscillation amplitude of the pin becomes more and more restricted. This is measured mechanically or optically and expressed as a plot of pin amplitude versus time [9]. With rotational thromboelastometry the coagulation process is started by adding some type of reagent to the blood sample. The ROTEM system can use blood-dispersible cryopelletized reagents [10] in place of the previously used ready liquids which had to be pipetted into the blood sample. Further advantages to the cryopellets are the exact dosing of reagent, avoidance of dilution of blood sample, good storage stability at room temperature, and ease of handling. The system can be used for a number of applications which include: recalcification only without activator; 'extrinsic' activation with tissue factor [11]; 'intrinsic' activation with a surface contact activator acting as a surrogate for kallikrein [12]; and activation with snake venom protein from echis carinatus [13]. Intrinsic activation, like classic APTT, requires a surface activator, either kaolin, ellagic acid, or silica [14]. Ellagic acid is used together with calcium chloride for recalcification. Ellagic acid activates FXII to FXIIa, an active serine protease in the intrinsic coagulation cascade [15]. This occurs as soon as plasma contacts the anionic charge on the surface of ellagic acid [16].

This is the starting point of the work that we report in this paper. We wished to determine if silica could be used instead of ellagic acid as a contact activator in a cryopellet formulation suitable for rotational thromboelastometry. It is known that aqueous colloidal dispersions of silica work for APTT, although silica was reported to be less sensitive than ellagic acid at low concentrations of the phospholipids [a platelet substitute] that are also required for APTT [17]. The incorporation of finely dispersed silica into a cryopellet formulation is, however, a more complex matter. It is known that colloidal silica dispersions can be lyophilized and reconstituted without aggregation [18], but nothing is known about cryopelletization. The issues that we address are: 1) what amount of silica per cryopellet is required to give adequate activation of intrinsic coagulation of a recalcified whole blood sample of 300 µL? 2) do the bulking agents in the amounts used for cryopellet formation [9] interfere with coagulation? 3) does the gelling effect of aqueous dispersed silica [19] impair the uniform droplet formation necessary for cryopelletization to a uniform particle size? and 4) does the colloidal silica still function as an activator after release from a carbohydrate cryopellet? Indeed, is it possible to prepare a silica-based cryopellet that works with 'intrinsic' rotational thromboelastometry? In this paper we address these issues and demonstrate that cryopelletized silica is a feasible substitute for ellagic acid.

MATERIALS AND METHODS

D-Mannitol and D-(+)-trehalose dihydrate were both used as received from Sigma-Aldrich (Steinheim, Germany). Silica dioxide powder, Aerosil 200, was obtained from Evonik/Degussa (Frankfurt, Germany). Water was double-distilled from an all-glass apparatus and passed through a 0.1 µm pore-diameter membrane filter. The cryopelletization process has been fully described before [20] and is only given here in brief to aid understanding. A filtered, aqueous liquid-feed solution was pumped through a Sterican hypodermic needle of internal diameter 0.514 mm, 21 gauge (Braun, Melsungen, Germany). The droplets formed fell into a bath of liquid nitrogen (LN2) contained in a stainless steel bowl. The individual droplet weight was measured on droplet samples taken at various times over the dropping procedure by being collected into a special collecting tube before any freezing. At the end of this procedure, the bowl containing the dispersion of the frozen droplets in LN2 was transferred to the pre-cooled shelf (-40°C) of a Virtis lyophilizer (SP Scientific, Warminster, PA, USA) of total shelf area 0.57 m^2. The freeze-drying cycle used and shown in Table 1 was developed from a standard cycle for trehalose-based formulations [21]. After drying, the finished cryopellets were transferred to a glass vial and sealed under a dry nitrogen gas atmosphere and stored at room temperature until examined.

For scanning electron microscopy (SEM) the cryopellets were fixed to an Al stub (Model G301; Plano, Wetzlar, Germany) using a self-adhesive film. The sample was then Au sputtered for 1.5 minutes at 20 mA/ 5 kV (Hummer JR Technics) and examined on an Amray 1810T Scanning Electron Microscope at 20 kV. Rotational thromboelastometry was performed using a ROTEM model Delta machine (tem International, Munich, Germany). Each measurement was done on a 300 µL sample of citrated whole blood. The reference measurement was, in most cases, the NATEM assay which used the addition of 20 µL of 0.2 M $CaCl_2$ solution to the whole blood sample but no activator. In the single case of the final silica-cryopellets, the reference was the INTEM assay that used the addition of 0.2 M $CaCl_2$ and ellagic acid as a surface activator. The result produced by the thromboelastometry is the dynamic coagulation profile of pin oscillatory amplitude (mm) versus time (s) during coagulation. From this profile the empirical parameters of clotting time (CT, s) and maximum clot firmness (MCF, mm), were calculated [9], as described under Results and Discussion. Three series of experiments were done:

1) The effect of the amount of silica on intrinsic coagulation. 50 µL volumes of variously concentrated dispersions of silica in 0.9% w/v sodium chloride were added

Table 1. Lyophilization cycle used to prepare cryopellets. The total cycle process time was 56.6 hours.

Time (minute)	0	60	180	2680	3040	3400
Shelf temperature (°C)	-40	-40	-40	-40	+20	+20
Chamber pressure (mtorr)	760	760	30	30	30	30
Phase	equilibration		ramp	1° dry	ramp	2° dry

to the recalcified whole blood sample.
2) The effect of the bulking agents trehalose or mannitol on intrinsic coagulation. Various amounts of either bulking agent were added to the recalcified whole blood sample.
3) The efficacy of the trehalose-based or mannitol-based silica-cryopellets when used to measure intrinsic coagulation of recalcified whole blood.
Citrated whole blood samples were obtained from healthy volunteer blood donors who gave samples for this study prior to a plasma or plateletpheresis donation. This study was approved by the Institutional Ethics Committee of the University of Erlangen, Blood Donation and Transfusion Service of the University of Erlangen, Nuremberg. All donors met the relevant guidelines and tested negative for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus.

RESULTS AND DISCUSSION

Effects of silica on intrinsic coagulation

Figures 1A and B show the dynamic coagulation profiles we obtained at two different silica concentrations, c_{Aer}, i.e., 0 mg/mL and 0.5 mg/mL in isotonic saline. These are equivalent to weights of silica added to a recalcified whole blood sample, w_{Aer}, of 0 µg and 25 µg, respectively. The recalcified whole blood with no silica coagulates slowly (Figure 1A), but this is greatly accelerated in the presence of the silica (Figure 1B). We characterise a kinetic coagulation profile by a number of empirical parameters. Two have proven themselves to be the most useful, i.e., the initial clotting time, CT (minute), i.e., the time to reach a pin amplitude of 2 mm, and the maximum clot firmness, MCF (mm), i.e., the largest amplitude reached before fibrinolysis sets in [9]. Figure 2 shows that the addition of increasing amounts of the silica rapidly decreases the CT which reaches its minimal value at 25 µg - 150 µg added silica. Amounts equal to or above 375 µg silica cause CT to lengthen again as clotting is evidently hindered, but variation is now large. The MCF shows a clear downward tendency as the amount of added silica increases. The presence of the aggregates of silica nanoparticles [22] evidently disturbs the density of the anastomosing network of fibrin fibres formed between the cuvette wall and the pin and reduces final clot density. *In vitro* studies have indeed shown that fibrinogen self-assembles at the surface of silica nanoparticles [23]. This must disturb the formation of a fibrin network across the blood sample contained in the cuvette. This does not affect activation of coagulation or CT, but results in the lowered MCF, i.e., a lowered clot density. Silica nanoparticles have also been shown to be taken up by erythrocytes and cause their hemolysis [24]. How this might affect clot density is, however, unclear. These results show us that the activation effect of silica on coagulation can be measured and quantified by rotational thromboelastometry. Based on the data in Figure 2, we selected 25 and 75 µg silica for use in a cryopellet formulation.

Effects of trehalose and mannitol on coagulation

We tested placebo cryopellets made of either pure trehalose or pure mannitol, each weighing 1.25 mg, for their possible detrimental effects on the dynamic coagulation profile of recalcified whole blood. The addition of up to 10 cryopellets of trehalose (= 12.5 mg solid material) to a sample of 300 µL whole blood plus 20 µL of 0.2 M $CaCl_2$ produced no change in either CT or MCF (Figure 3A). This agrees with the results of a previous study which showed that disaccharides do not interfere with whole blood coagulation [9]. The mannitol cryopellets, however, behave differently: we find that the addition of 5 cryopellets already accelerates coagulation and CT drops from 742 s to 523 s in a quite surprising result (Figure 3B). The MCF is not changed up to this level of mannitol, but decreases when 10 cryopellets are added. The reduction in CT with mannitol has not been observed before. We attribute the change in MCF with 10 cryopellets to the resulting high concentration of mannitol in the blood sample. The reduction in CT with 6.26 mg added mannitol is still much less than that detected with 2.5 µg of added silica in Figure 1. Mannitol is therefore a much weaker activator than silica.

Effects of silica on uniform droplet formation

The droplet volume produced during the cryopelletization process determines the pellet weight and, hence, is the vital factor that governs the amount of silica added in one cryopellet to the whole blood sample for rotational thromboelastometry. It is necessary to show that any variation in droplet volume on dropping caused by the presence of the dispersed nanoparticles of silica must be insufficient to alter the measured coagulation

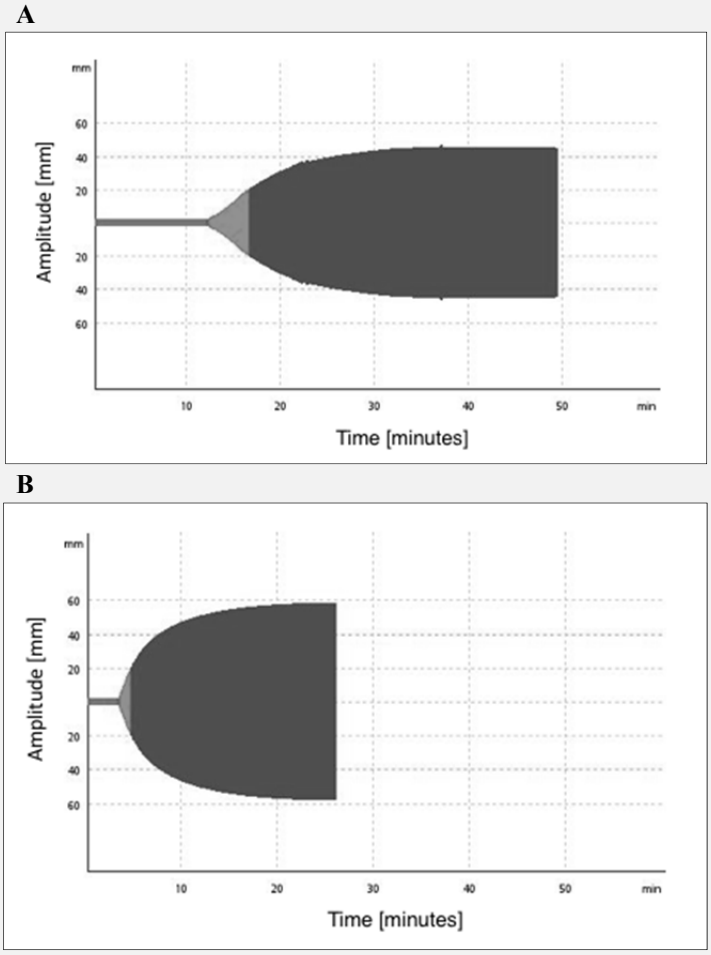

Figure 1. Kinetic coagulation profiles obtained from rotational thromboelastometry for different amounts of colloidal silica dispersion added to recalcified whole blood.

Each shows the pin oscillatory amplitude (mm) versus time (second) during coagulation. A) c_{Aer} = 0 mg/mL silica dispersion (w_{Aer} = 0 mg), B) c_{Aer} = 0.5 mg/mL silica dispersion (w_{Aer} = 25 µmg).

behavior. We found no dependence of uniformity of droplet size on the silica content over a process time of 120 minutes. The mean average values ± standard deviation were as follows at the two levels of added silica, i.e., 25 µg and 75 µg: mannitol plus 75 µg silica = 10.40 ± 0.062 mg; mannitol plus 25 µg silica = 10.66 ± 0.071 mg; trehalose plus 75 µg silica = 10.49 ± 0.043 mg; trehalose plus 25 µg silica: = 10.59 ± 0.057 mg. In all 4 cases the coefficient of variation of the mean value is < 0.7%. In particular, the gelling action of aqueous silica [19] does not cause a viscosity that is high enough to hinder or make highly-variable droplet formation. These results mean that a uniform addition of an exact amount of silica to the whole blood samples in a precise weight of cryopellet can be achieved.

Silica cryopellets to induce intrinsic coagulation

The clotting times with silica in either trehalose or mannitol cryopellets are shown in Figure 4A. At both the 25 µg and 75 µg levels of silica the CT values are not significantly different from that for INTEM that uses ellagic acid ($p < 0.05$). The trend to slightly lower values for the mannitol cryopellets agrees with the lower CT values found for pure mannitol in Figure 3B. The values for MCF do not differ between either of the cryopellet formulations and the silica dispersion (Figure 4B) and are also not significantly different from that for INTEM ($p < 0.05$). The cryopellets containing silica, therefore, work just as well as ellagic acid in an intrinsic rotational thromboelastometry test.

The silica cryopellets of trehalose or mannitol all showed very rapid dispersion on adding to 0.3 µL water.

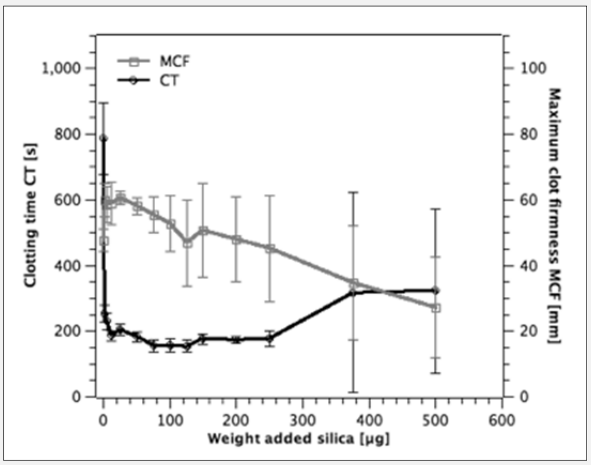

Figure 2. Effects of increasing amounts of added colloidal silica on clotting time, CT, and maximum clot firmness, MCF, from rotational thromboelastometry of recalcified whole blood.

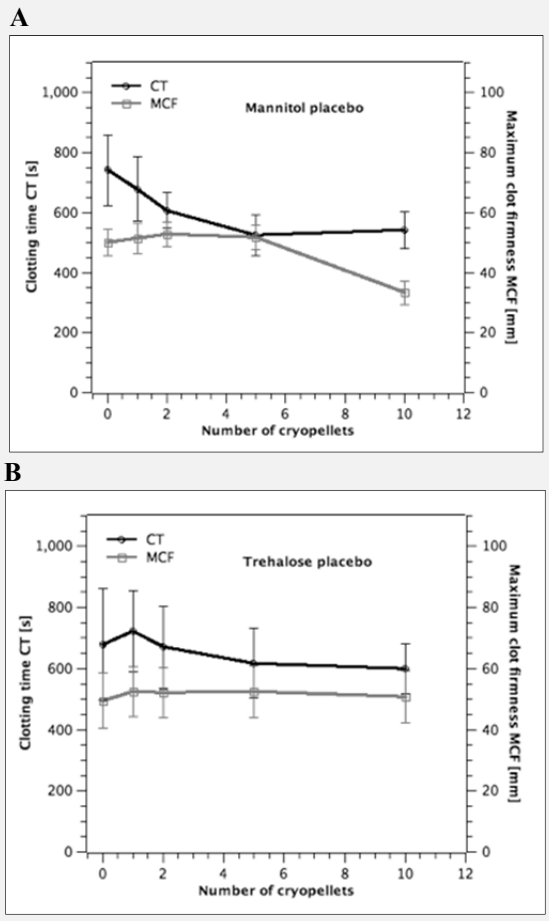

Figure 3. Effects of added bulking agent on clotting time, CT, and maximum clot firmness, MCF, from rotational thromboelastometry of recalcified whole blood. A) trehalose, B) mannitol.

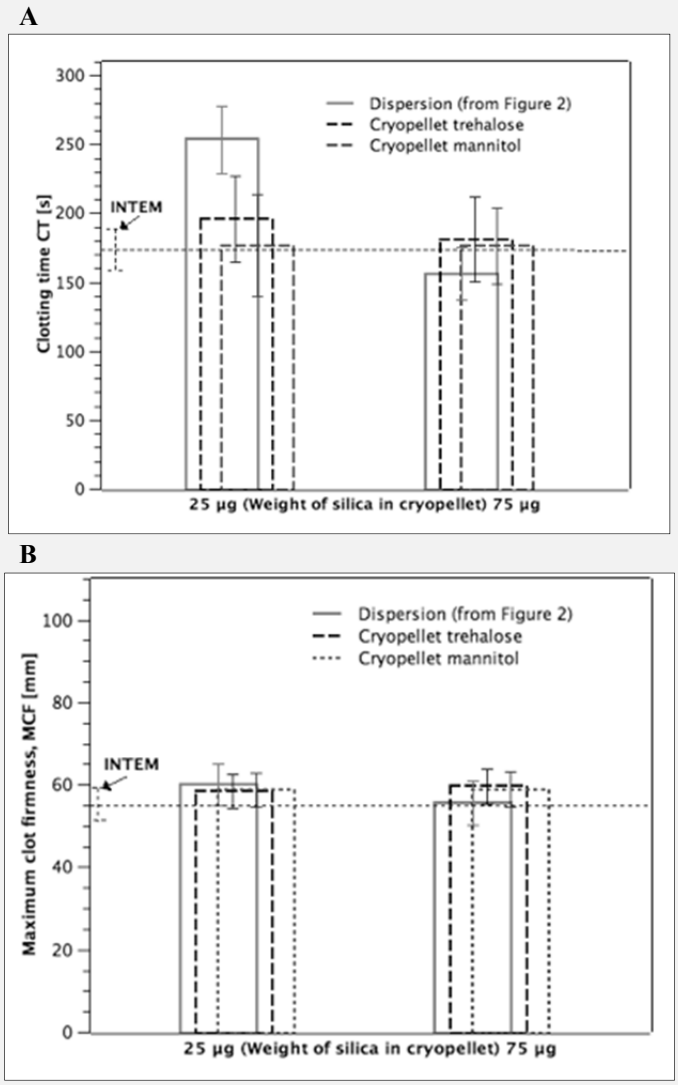

Figure 4. Results of rotational thromboelastometry using cryopellets on recalcified whole blood.

A) Clotting time - CT. B) Maximum clot firmness - MCF. In each figure the comparison is made with the colloidal silica dispersion from Figure 1 and also with the INTEM results using ellagic acid.

Within 5 seconds we could detect no visible solid residue. We use this model as an approximation of the dissolution behavior in blood which cannot be determined visibly. This behavior can be explained by the highly porous nature of the cryopellets. Figures 5 shows a scanning electron micrograph of a section through a trehalose cryopellet loaded with 25 µg silica (mannitol was very similar). The presence of the silica is not evident in any differences in structure compared with cryopellets without silica [25]. The highly porous carbohydrate cryopellet offers a high specific surface area and hence rapid dissolution.

CONCLUSION

We draw the following conclusions from this work:
1) 25 - 75 µg of silica produces the activation of intrinsic coagulation of a recalcified whole blood sample of 300 µL volume and a CT of < 200 seconds;
2) of the two bulking agents examined, trehalose and mannitol, only the mannitol influences coagulation directly and reduces the clotting time;
3) the presence of the colloid silica dispersed in the liquid feed does not impair the formation of a uniform droplet size necessary for uniform cryopelletization;
4) cryopellets of trehalose or mannitol containing 25 or

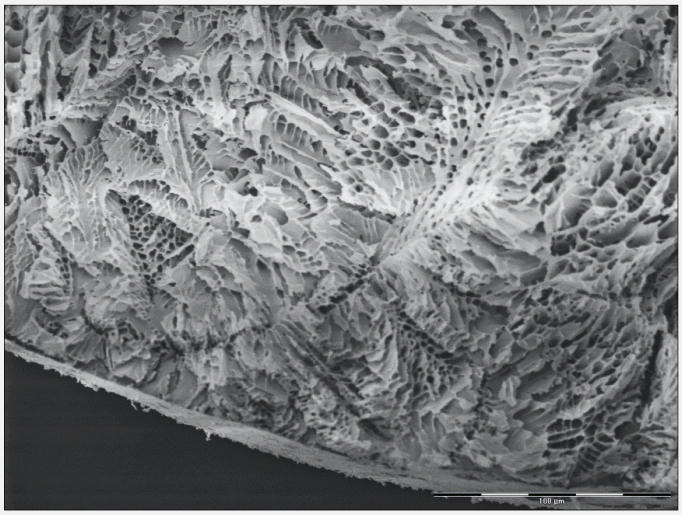

Figure 5. Scanning electron micrograph of sectioned cryopellet of trehalose containing 25 µg silica.

75 µg silica produce the same clotting behavior as ellagic acid in recalcified whole blood.

In this paper we have, therefore, shown that a silica-containing cryopellet can be prepared that works as an activator in intrinsic rotational thromboelastometry. The advantage to the use of silica is that in contrast to a liquid activator the silica cryopellet does not lead to dilution of the whole blood sample. This avoids diluting the concentrations of fibrinogen, coagulation factor, and platelets in the blood sample as well as changing hematocrit, all of which can alter the coagulation result [26, 27]. Furthermore, we note that there is a substantial economic benefit to be gained compared with the expense of ellagic acid.

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Author Contributions:
JH performed all of the laboratory work; RZ provided the whole blood samples; GL conceived the project and wrote the paper.

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Declaration of Interest:
None.

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