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Development of zinc chelating resin polymer beads for the removal of cell-free hemoglobin.

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Abstract

Objective: Red blood cell (RBC) hemolysis is one of the most common storage lesions in packed RBCs (pRBC). Older units of pRBCs, especially those >21 days old, have increasing levels of hemolysis leading to increased oxidative stress and premature platelet activation. This effect can mostly be attributed to the increase of cell-free hemoglobin (Hb). Therefore, removal of cell-free Hb from pRBCs prior to transfusion could mitigate these deleterious effects. We propose a new method for the removal of Hb from pRBCs using zinc beads.

Approach and Results: Prepared Hb solutions and pRBCs were treated with zinc beads using two different protocols. UV-Vis spectrophotometry was used to determine Hb concentrations, before and after treatment. Experiments were run in triplicate and paired t-tests were used to determine significant differences between groups. Zinc beads removed on average 94% of cell-free Hb within 15 minutes and 78% Hb from pRBCs ($p < 0.0001$), demonstrating a maximum binding capacity $\sim 66.2 \pm 0.7$ mg Hb/mL beads. No differences in RBC morphology or deformability were observed after treatment.

Conclusion: This study demonstrates the feasibility of using zinc beads for the rapid and targeted removal of Hb from pRBC units. Further investigation is needed to scale this method for large volume removal.

Keywords

hemolysis; RBC; blood products; transfusion; zinc resin; Hb

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Introduction

Approximately 14.2 million units of whole blood or packed red blood cells (RBCs) are transfused each year in the United States (US).^{9, 25} These blood products are often obtained from healthy donors and stored by blood banks. In recent years, various technologies such as improved additive solutions, cell separators, and pathogen reduction techniques have improved the quality of stored products. However, it is well established that RBCs experience qualitative and functional decline throughout their 42 day storage period.^{12, 20, 21} Storage is generally limited by the level of bacterial contamination and/or other storage lesions that impact cell function. However, there remains no concrete consensus on the length of storage, especially with RBCs. For example, several countries including China, Germany, the Netherlands and the United Kingdom, have shortened their RBC storage length to 35 or even 21 days, while the US continues to use a storage period of 42 days.¹² While most first-world countries agree that storage of RBC products up to 42 days is acceptable, there is still debate regarding the efficacy of older stored RBCs versus newer.¹³ The transfusion of older RBC units has been implicated in adverse patient outcomes in several studies, but the exact effect of older RBC transfusions is not well defined.¹²

Part of the difficulty in defining an ideal storage period for RBCs is the lack in definitive criteria for what constitutes “good” versus “bad” RBCs. Federal recommendations in the US and Europe generally rely on two factors: the amount of RBC hemolysis (to be kept below 0.8% at the end of the storage period), and the number of surviving cells 24 hours after transfusion (>75% is required).¹⁰ On average, the natural lysis of aging RBCs which releases cell-free hemoglobin (Hb), commonly referred to as hemolysis, reaches 0.2 to 0.4% within 42 days of storage; however, this level can be significantly affected by handling, storage temperature, donor genotype, and administration protocols.^{10, 22} Transfusion of RBC units with increased levels of hemolysis is worrisome because it can cause adverse reactions, interfering with several important mechanisms and pathways within the body.²⁵ Under normal circumstances, the body has built-in mechanisms to protect itself from cell-free Hb, by sequestering the molecule and breaking it down into less toxic metabolites. However, this process can become oversaturated during situations which result in higher-than-normal levels of cell-free Hb, such as sickle cell disease (SCD), sepsis, hemorrhagic shock, trauma, and the administration of multiple blood transfusions.²⁵

Large amounts of cell-free Hb can cause a wide array of deleterious effects throughout the body. For example, cell-free Hb has been identified as a scavenger of nitric oxide (NO) which leads to increased oxidative stress. Hb-induced reduction of NO levels explains the acute hypertensive response seen in patients with extreme amounts of hemolysis, as well as the subsequent effects of the increased levels of heme within the blood.²⁵ Overall, Hb-induced NO scavenging can lead to a hypercoagulable state and poorer patient outcomes. In addition to NO scavenging and an increased inflammatory response, cell-free Hb has been identified as a powerful platelet activator. ADP bound to cell-free Hb activates platelets both *in vitro* and *in vivo* by binding to the P2Y1 and P2Y12 G protein-coupled receptors on the platelet surface.¹⁵ In response the platelet releases thromboxane A2, adding to the activation cascade.^{19, 27}

These detrimental effects of hemolysis and release of bioactive cell-free Hb are especially relevant in patients experiencing massive hemorrhage. These individuals are often receiving multiple units of RBCs,⁶ and thus would most likely benefit from receiving RBC transfusion units with lower levels of hemolysis, to promote primary hemostasis. Currently, there is no well adopted method for the removal of cell-free Hb. RBC units can be washed with saline solution to remove storage lesions; however, most hospital facilities do not have RBC washers and the washing process can take approximately 1.5–2 hours, meaning it is not a feasible solution in emergency or trauma situations where large volumes of blood products are needed immediately.^{18, 26}

To address this problem, we developed a rapid method to remove cell-free Hb from RBC units using zinc (Zn^{2+}) chelating resin beads. Zinc chelating resin beads use immobilized metal ion affinity chromatography to remove histidine tagged proteins. The zinc resin used in this study consists of iminodiacetate coupled to 6% cross-linked agarose beads charged with Zn^{2+} at a capacity of 20–40 $\mu\text{moles Zn}^{2+}/\text{mL}$ resin (Figure 1A).⁴ The microscopic pores in the beads (~50nm wide) lined with Zn^{2+} ions create a larger surface area for capture and allow for smaller molecules (such as Hb) to become bound in the bead while larger cells flow past unaffected.³

Hb contains four heme groups and numerous histidine residues that allow it to bind with the zinc resin beads. It has been proposed in previous literature that the primary binding site of zinc ions to Hb molecules is located at the $\beta 143\text{His}$, as illustrated in Figure 1B, in addition to $\beta 93\text{Cys}$.²⁴ We hypothesized that cell-free Hb would be captured at these sites to the zinc resin via binding of histidine residues within Hb molecules, allowing normal (unlysed) RBCs to flow through the chromatography column unaffected. Thus, the objective was to develop a rapid and efficient method for RBC hemolysis removal using these zinc resin beads.

Materials and Methods

Materials

Packed RBCs were obtained from the regional Interstate Blood Bank (Asheville, NC) as well as from the Internal Blood Bank at Wake Forest Baptist Medical Center. All RBC units were stored at 4°C following standard procedures. Zinc chelating resin beads were purchased from G-Biosciences (Cat. #786–287) and chromatography columns were obtained from Bio-Rad (Cat. #7321010). Phosphate buffered saline (PBS) (Cat. 2944–100, Fisher-Scientific, Fair Lawn, NJ) was used as a blank control. Binding buffer (50mM Na_2HPO_4 , 0.3M NaCl, pH 8.0) was prepared for the zinc resin as per vendor instructions, as well as an elution buffer (50mM Na_2HPO_4 , 0.3M NaCl, 0.25M imidazole, pH 8.0).

Cell-free Hb preparation

Cell-free Hb stock solutions were prepared by the Kim-Shapiro lab at Wake Forest University, as previously described.¹⁵ Leuko-reduced RBCs were washed thrice with PBS, hypotonically lysed (5:1 by volume), and centrifuged for one hour at 17,211×g to remove cell membranes. The resulting supernatant was used to prepare stock solutions containing up

to 4.1 mM Hb. Cell-free Hb solutions were prepared from this stock solution, which ranged from 0–4mM. All stock solutions were kept at –80°C and thawed to 37°C prior to experiments.

Hb quantification via absorption spectrophotometry

The Hb concentration of each sample was quantified prior to and after testing using absorbance spectra obtained on a UV-Vis spectrophotometer (NanoDrop 2000c, ThermoFisher Scientific, Wilmington, DE), as previously described.^{17, 27} Briefly, the Hb content of each sample was determined from the full absorption spectra at 450–700 nm. PBS served as a blank control. To determine the cell-free Hb content in packed RBC hemolysate, aliquots (~2mL) were spun at 500g for 3 minutes to obtain a cell-free supernatant. This could then be diluted with PBS to a final volume of 300µL and analyzed. The absorbance of the sample was compared to known basis spectra of Hb, using nonlinear least squares curve fitting. This analysis software was developed by Ivan A. Azarov at Wake Forest University, using methods comparable to Jensen.¹⁷ Each sample was measured in triplicate and the average total-Hb concentration was calculated.

Zinc Chelating Resin Beads

The zinc chelating resin beads were washed following commercial vendor protocols. They were placed in a centrifuge tube and pelleted by centrifugation at 500×g for 2 minutes. The supernatant was carefully decanted. Distilled water (5× bead volume) was added and mixed with the beads end-over-end several times before being pelleted again by centrifugation, and the supernatant decanted. This process was repeated twice more. After washing, the beads were resuspended in an equal volume of binding buffer and set aside until needed. Prior to use, the beads were pelleted and the supernatant decanted. After use, the beads were washed with the elution buffer to remove any bound Hb and were regenerated for future experimentation, following vendor protocol.

Experimental Design

Incubation Protocol—The ability for zinc resin beads to capture Hb was first determined using the cell-free Hb solution prepared from RBCs. Hb solutions (ranging from 0.06–0.4 mM Hb) were incubated with zinc beads for up to 15 minutes with gentle agitation at room temperature, as per vendor protocol. The 0.2 mM concentration of Hb was used as our initial starting point since previous studies have shown that it is near the maximum concentration found in the stored blood.^{11, 27} The mixture was then transferred to a chromatography column for gravity filtration, allowing the zinc beads to remain in the column while the cleaned solution dripped through (Figure 2A). Hb concentration was quantified both pre- and post-column to determine percent removal of Hb. The binding capacity of zinc-to-Hb was also calculated. This process was then repeated using packed RBCs with a range of hemolysate (i.e. released Hb from RBC lysis) levels (0.1–0.38 mM).

Drip Protocol—Once adequate zinc-to-Hb binding was confirmed, the method was adjusted to meet the time constraints typically seen in emergency and trauma situations. In these cases, there would be no time to incubate the beads in the RBC unit prior to transfusion. Rather, if the zinc beads were a component of the intravenous (IV) line, Hb

could be cleared simultaneously from the RBC unit while being delivered to the patient. The zinc chamber could be inserted prior to any traditional IV components, allowing the RBCs to be dripped through the zinc resin column prior to flowing through the IV line.

To determine if the drip rate would affect the binding capacity of the zinc beads, the prepared cell-free Hb solution was dripped onto packed zinc resin beads in a chromatography column (Figure 2B) at a rate of 2 mL/min (commonly used flow rate in transfusion medicine).¹ Hb concentrations were quantified pre- and post-dripping protocol. This process was repeated using packed RBCs.

RBC Morphology & Deformability

RBC morphology and shape were qualitatively evaluated pre- and post-zinc bead treatment by taking microscopic images of a standard blood smear. RBCs were smeared onto a glass slide and imaged using a brightfield microscope (Nikon Eclipse FN1, Nikon Instruments, Melville, NY). Images were obtained with a 40× objective.

RBC deformability was analyzed using osmotic gradient ektacytometry on packed RBCs before and after being dripped through the zinc resin column (Technicon Ektacytometer, Technicon Instrument Corp., Tarrytown, NY). Osmotic gradient ektacytometry quantifies RBC deformability through a laser diffraction technique using the equation for the deformability index (DI) proportional to the cell elongation:

$$DI = \frac{L - W}{L + W}$$

where L and W are length and width of the diffraction pattern, respectively that corresponds to the dimensions of the deformed cell.⁷ The deformability index was measured under constant shear over a range of osmolarities. Several key parameters can be observed from the DI curve, such as DI_{Max} (maximum deformability), O_{Min} (osmotic fragility), and O' (intracellular viscosity) (Figure 3).¹⁶

Deformability of the RBCs were analyzed using a standard protocol, as previously described.⁷ Briefly, RBCs (150 μ L) were diluted to 4 mL and pumped into the ektacytometer where they were exposed to a range of osmotic pressures (40–290 mOsm). The diffraction patterns were then analyzed to determine RBC deformability.⁷

Statistical Analysis

Data is reported graphically as mean $\pm$ SEM (standard error of the mean). Differences in Hb concentrations pre- and post-zinc beads were analyzed using a student's paired t-test. All analyses were conducted using GraphPad Prism (version 7, La Jolla, CA) software and SAS (version 9.4, Cary, NC). Statistical significance was set at the 0.05 level.

Results

Incubation Protocol: Cell-free Hb Solution

Cell-free Hb solutions were added to the zinc resin beads (0.75 mL) and incubated for 15 minutes at room temperature with gentle agitation and passed through a chromatography column. This was repeated with increasing volumes of the Hb solution (0.2 mL increments, starting at 0.4 mL) until the resin beads were fully saturated. The maximum binding capacity of the zinc beads was calculated to be 66.2 ± 0.7 mg Hb/mL of beads (Table 1), resulting in the removal of 94% cell-free Hb, on average (Figure 4) ($p < 0.0001$).

The effect of incubation time was also analyzed. Cell-free Hb solution was incubated with zinc beads for various lengths of time (0.5, 5, 15, and 30 minutes). As seen in Figure 5, the binding of Hb to the zinc beads is fairly rapid and is saturated within 10–15 minutes. Increasing the incubation time beyond 15 minutes did not significantly improve Hb removal.

Incubation Protocol: Packed RBCs

Packed RBCs from a transfusion unit were incubated with the zinc resin beads to determine how the presence of RBCs affected the binding capacity. It was found that the binding buffer suggested by the manufacturer led to additional RBC lysis (due to the alkaline pH relative to the pH of 7.35–7.45 in human blood). Therefore, PBS was substituted in the place of the binding buffer and resulted in adequate Hb removal.

RBCs were incubated with zinc beads under the same conditions (15 minutes with gentle rocking) at various ratios by volume to determine the Hb binding capacity of the beads in the presence of RBCs. Incubating packed RBCs (0.5–2 mL) with zinc beads (0.75 mL) resulted in a lower binding capacity of 35.1 ± 3.7 mg Hb / mL zinc beads (Table 1). Despite this lower binding capacity, the zinc beads were still able to efficiently remove 78% of hemolysate, on average, from packed RBCs (Figure 6, $p < 0.0001$). Compared to the binding capacity of zinc incubated with cell-free Hb solution, the presence of RBCs reduced the binding capacity of the zinc beads by 17%, perhaps due to the increased cell-cell interactions.

Drip Protocol: Cell-free Hb Solution

Following confirmation of zinc-Hb binding, we attempted to develop a drip protocol that would provide more rapid and translational implementation. Cell-free Hb solution (0.06–0.23 mM) was dripped onto packed zinc resin beads in a chromatography column at a rate of 2 mL/min. On average, the zinc beads removed 78% cell-free Hb ($p < 0.0001$), with a binding capacity of 57.8 ± 2.8 mg Hb / mL of zinc beads through this drip method (Table 1). Compared to the incubation protocol, dripping the Hb solution through the zinc beads resulted in a 13% reduction in binding capacity, potentially due to the reduced interaction time between zinc beads and Hb molecules.

Drip Protocol: Packed RBCs

Similarly, packed RBCs (2 mL, 0.17–0.23 mM) were dripped through zinc resin beads (0.75 mL) at a rate of 2 mL/min. On average, the zinc beads removed 70% cell-free Hb ($p < 0.0001$), with a binding capacity of 24.1 ± 1.8 mg Hb / mL of zinc beads (Table 1).

Compared to the incubation protocol, dripping RBCs through the zinc beads resulted in a 31% reduction in binding capacity, again potentially due to the reduction in time interacting with the zinc beads. A summary of the measured binding capacities under the two methods has been provided in Table 1.

RBC Morphology & Deformability are unchanged with Zn-bead treatment

Images were taken of the RBCs prior to and after the zinc protocol, to ensure the process was not affecting RBC morphology and that no zinc beads were present (Supplemental Figure 1). RBC morphology did not appear to be changed after the zinc protocol and there were no zinc beads present. Packed RBCs (before and after the zinc protocol) were analyzed using osmotic gradient ektacytometry to determine if the zinc beads affected RBC deformability (Figure 7). There were no significant changes in the deformability parameters (DI_{Max} , O_{Min} , O') post-zinc protocol (Table 2).

Discussion

This study demonstrated that zinc chelating resin beads are successful in removing significant amounts of Hb from both controlled Hb solutions and packed RBCs. Both the incubation and drip protocols of Hb-removal have potential to rapidly remove hemolysate from RBC units. This method has substantial potential to be translated into a self-containing device that could improve the quality of RBC transfusions. By rapidly removing hemolysate, this method can prevent several of the deleterious effects of free-Hb and potentially benefit patient populations where urgent blood transfusions are needed to achieve hemostasis (e.g. major hemorrhage, trauma patients) and in patients that receive RBC transfusions relatively often (SCD, glucose-6-phosphate dehydrogenase deficiency, etc.).

It has been previously shown that cell-free Hb from hemolysis can lead to a variety of adverse patient reactions, including decreased oxygen delivery, inflammation, and premature platelet activation.^{15, 25, 28} In an effort to address this problem and other storage lesions that accumulate during RBC storage, some hospital facilities have begun to use cell washers on packed RBCs prior to transfusion. Briefly, packed RBCs are washed repeatedly with saline and then resuspended in the additive solution containing saline, adenine, glucose, and mannitol, commonly referred to as SAGM.¹⁴ While more recently developed technology such as the Continuous Autotransfusion System (CATS) can wash blood products relatively quickly, traditional washers can take over an hour to complete the wash cycle.³¹ In comparison, the zinc resin bead methodology developed in this study provides even more rapid removal of cell-free Hb. We observed up to 94% removal of Hb within 15 minutes of incubation with zinc resin beads and 78% removal of Hb through the drip protocol which involved the instantaneous removal of Hb in less than one minute. While the drip protocol exhibited lower binding capacities and less percent removal of Hb, we believe this is largely due to the limitation of the current drip protocol. The total contact time between RBCs and the zinc beads in the drip protocol is significantly less than that of the incubation protocol. Therefore, future development of a column that strategically optimizes the surface contact area of the zinc beads to RBCs could significantly improve the binding capacity and total percent removal of hemolysate.

Apart from the time required to complete the protocol, cell washers are costly and generally not very portable. They are also not high-throughput machines, since they can only be used to clean blood for one patient at a time. Furthermore, a study by Bennett-Guerrero *et al.* revealed that washing of packed RBCs might not remove significant amounts of cell-free Hb and that some washing procedures can actually increase the amount of hemolysate within the unit due to osmotic and/or osmolality differences.⁸ In comparison, our zinc resin beads (in either incubation or drip protocols) did not increase and actually decreased the amount of RBC hemolysate present within the processed samples as there were no changes in osmolality. Finally, our newly developed zinc bead method for hemolysis removal offers significant cost savings as it is much cheaper than existing cell washers; 10 mL of zinc beads cost ~\$100.⁴ Further, the zinc beads can be washed and prepared prior to use, and if unused within a reasonable period, the beads can be placed in ethanol solution and stored long-term. A comparison of our newly developed method to traditional cell washers is provided in Table 3.

The use of microbeads and microparticles for the removal of hemolysis is not new to our study. In fact, other polymer bead products have been proposed and are currently being developed. For example, HemoDefend™ and CytoSorb® use porous polymer beads to remove a broad range of cytokines and toxins from blood.² However, these products are not specific in the removal of cell-free Hb; rather they remove all substances between 1kDa and 150kDa. Patients who are at-risk for transfusion complications related to cell-free Hb (trauma or SCD) might benefit from having a more targeted removal of Hb, rather than a broad removal of cytokines and other substances.

This study was a first demonstration that zinc chelating resin beads can be used for hemolysis removal; however, there are some limitations. A potential concern for the zinc bead method of hemolysis removal might be the use of heavy metal ions within the resin that could potentially contaminate the processed blood. Given that Zn^{2+} ions are strongly bonded to the iminodiacetate crosslinker in the resin, we do not expect leakage or release of these ions into the blood.⁴ Even if a small portion of Zn^{2+} ions escape from the resin and enter the blood sample, zinc is a low toxicity metal with very little risk for humans at these low concentrations. The most common side effects of zinc in humans is the suppression of copper and iron absorption.^{5, 23, 29, 30} In fact, Zn^{2+} ions have been shown to increase Hb oxygen affinity at high concentrations.²⁴ Regardless, there are no known serious or long-term effects of excessive zinc levels and excessive hematological levels of zinc can be safely excreted through urine.²⁹

Another limitation is that this study only processed small sample volumes to test the feasibility of the zinc-Hb binding capacities. To translate this work to the hospital, we must be able to scale up this method to easily process up to 300 mL of packed RBCs, a typical volume for a single RBC unit. We believe our methods can be easily scaled up for large-scale volume removal of Hb. Given that federal recommendations limit RBC hemolysis to <0.8% and that the average RBC unit contains 300mL of RBCs, a standard packed RBC unit should contain between 0–210 mg of Hb.¹⁰ Given the measured binding capacities we estimate as little as 10 mL of zinc beads could theoretically remove 100% of Hb from packed RBCs. Considering most units of packed RBCs will contain less than 0.8%

hemolysis, both the incubation and drip protocols have the potential to significantly reduce the presence of Hb in RBC units.

In conclusion, zinc resin chelating beads are a promising method to remove cell-free Hb from packed RBC units that may be translated to various clinical environments where the removal of cell-free Hb could improve patient outcomes. Future work is needed to further validate this method for processing larger volumes and designing a self-contained device for translation into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Illustration of zinc bead and three-dimensional representation of the hemoglobin molecule.

A) The zinc bead is a porous microsphere approximately $90\mu m$ in diameter on average but can range from $45\text{--}165\mu m$. The agarose bead is lined with the chelating ligand iminodiacetate, which is bound to the Zn^{2+} ion. Histidine containing proteins (such as Hb) bind to the bead. Larger cells and/or molecules are not trapped by beads, and the beads can later be washed to remove any bound protein.^{3,4} **B)** The four polypeptide subunits (two α and two β chains) are labeled. Histidine residues are shown in red ball-and-stick β His-143 and β 93Cys, sites of potential Zn^{2+} binding, are highlighted in yellow.

Figure 2. Illustration of incubation and drip protocols.

A) Incubation Protocol: packed RBCs (pRBC) and cell-free Hb solution were incubated with zinc beads for 15 minutes, before being poured through a chromatography column. The zinc beads bound to Hb remained in the column, while the rest of the solution flowed through unaffected. B) Drip Protocol: pRBCs and cell-free Hb solution were dripped through a packed zinc resin chromatography column at a rate of 2 mL/min and pRBCs were recovered at the end of the drip column.

Figure 3. Representative ektacytometry curve with key parameters illustrated.

RBCs are exposed to a range of osmotic pressures (40–290 mOsm) and the changes in cell length and width are recorded using laser diffraction patterns. Key parameters from this analysis include DI_{MAX} (maximum cell deformability), O_{Min} (osmotic fragility) and O' (intracellular viscosity or the hydration state).¹⁶

Figure 4. Representative Hb absorption curve before and after incubation of cell-free Hb solution with zinc beads.

Zinc beads (0.75 mL) were incubated with cell-free Hb solution (4 mL). **A)** Absorption spectra of cell-free Hb solutions before and after incubation with zinc resin beads. Shaded region around line represents SEM. **B)** Mean Hb concentration $\pm$ SEM before and after incubation with zinc beads. Average Hb concentration was 0.209 ± 0.004 mM prior to incubation with zinc beads, and 0.012 ± 0.001 mM after the removal ($p < 0.0001$).

Figure 5. Effect of incubation time on percent removal of Hb.

Cell-free Hb solutions and packed RBCs (pRBC) were incubated with zinc beads for up to 30 minutes, where maximal removal of Hb was observed. Dotted lines indicate 95% confidence interval. Efficiency of Hb removal was higher in cell-free Hb solutions compared to pRBC units.

Figure 6. Representative Hb absorption curve before and after incubation of pRBCs with zinc beads.

Packed RBCs (2 mL) were added to 0.75 mL of zinc bead slurry. **A)** Absorption spectra of pRBC hemolysate before and after incubation with zinc resin beads. Shaded regions represent SEM. **B)** Mean Hb concentration $\pm$ SEM before and after incubation with zinc beads. Hemolysate concentration was 0.163 ± 0.01 mM prior to incubation with zinc beads, and 0.018 ± 0.002 mM after the removal ($p < 0.0001$).

Figure 7. Ektacytometry curve of packed RBCs before and after zinc drip protocol. Exposure to zinc beads did not significantly change the deformability curve or any ektacytometry parameters.

Table 1.

Summary of maximum Zn-Hb binding capacities by protocol.

	Cell-free Hb		Packed RBCs	
	Incubation Protocol	Drip Protocol	Incubation Protocol	Drip Protocol
Zn-Hb Binding Capacity (mg of Hb/mL of beads)	66.2 ± 0.7 [*]	57.8 ± 2.8 [†]	35.1 ± 3.7 [§]	24.1 ± 1.8 ^{§†}

Mean ± SEM reported.

^{*} Maximum binding capacity recorded.[†] Presence of RBCs significantly reduced the binding capacity of the zinc beads (p<0.0001)[§] Incubation with zinc beads resulted in significantly greater binding capacity compared to drip method (p<0.0001)

Table 2.

Deformability parameters for packed RBCs before and after zinc drip protocol.

Parameter	Pre- Zn treatment	Post- Zn treatment	p-value
DI _{Max}	0.360 ± 0.007	0.362 ± 0.002	0.2663
O _{Min}	119 ± 2	117 ± 1	0.4597
O'	300 ± 19	312 ± 4	0.5761

Mean ± SEM reported.

Table 3.

Comparison of zinc bead treatment for hemolysis removal to traditional cell washing.

		Zn-bead method	
		Incubation Protocol	Drip Protocol
Easily portable	No	Yes	Yes
Additional reagents required at bedside	Yes ⁸	No	No
Processing time	Up to 2 hr	15 min.	<1 min
Cost	\$\$\$\$	\$\$	\$\$