

# Influence of Methylation and Polymerization on Flocculant Properties of Bovine Blood

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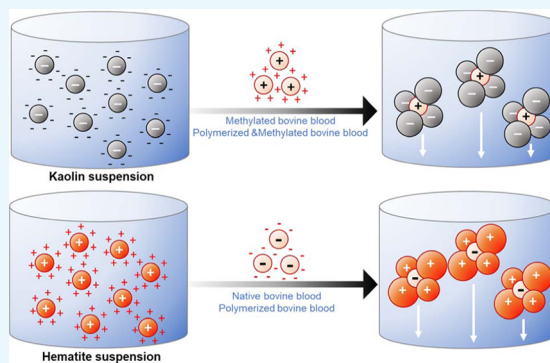
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**ABSTRACT:** Flocculants are used in the primary step of wastewater treatment to precipitate solids. Bovine blood is a slaughterhouse byproduct, and there is limited evidence in the literature demonstrating that it can be used as a flocculant. In this study, native bovine blood (NBB) and three types of chemically modified blood (methylated bovine blood (MeBB), polymerized bovine blood (PolyBB), and polymerized & methylated bovine blood (PMBB)) were tested against suspensions of negatively charged kaolin or positively charged hematite. The methylation reaction had the expected effect of increasing the apparent isoelectric point of MeBB and PMBB relative to that of the NBB starting material, and the polymerization reaction had the intended effect of increasing the average molar mass. NBB and PolyBB performed well with kaolin suspensions at pH  $\leq 5.5$ , and MeBB showed high and consistent performance, across the pH range of 4.5–8.5. Relative to NBB, MeBB had improved potency and pH independence but also the disadvantage of increased sensitivity to overdosing. The performance of PolyBB was very similar to that of NBB. PMBB had performance enhancements similar to those of MeBB, with a modest improvement in its overdose sensitivity. The performances of MeBB and PMBB with hematite suspensions were poor at all tested doses (2–100 mg/g hematite), whereas a 30 mg/g dose of PolyBB showed 81% precipitation in an hour. The results show that simple chemical treatments can improve the utility of blood as a flocculant for negatively charged solids.



## 1. INTRODUCTION

Synthetic polymer flocculants, especially varieties of polyacrylamide, are broadly used industrial substances most often employed to facilitate solid–liquid separation. While these substances can be highly effective at low doses, they have disadvantages including being made from nonrenewable resources, environmental persistence, and potential human health impacts.<sup>1</sup>

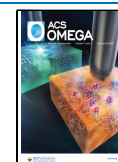
There is significant scientific and commercial interest in developing flocculants made from biopolymers, ideally sourced from agricultural byproducts. Such bio-based flocculants could offer the advantages of renewability, biodegradability, and reduced potential for unintended hazards to health and the environment.

Blood is a protein-rich byproduct of the meat industry.<sup>2</sup> If not captured and otherwise utilized, it can be a potent contributor of biochemical oxygen demand and nitrogen pollutants to facility wastewater.<sup>3</sup> Researchers have shown that slaughterhouse blood can function as a surprisingly good flocculant<sup>4</sup> and is capable of clarifying suspensions of kaolin clay or lignin.<sup>5</sup> Blood's most abundant protein, hemoglobin (Hb), was shown to be primarily responsible for the flocculant properties; blood's second most abundant protein, serum albumin, had no contribution.<sup>6</sup>

Purified Hb functions as a flocculant, and treatments to enhance the function have been investigated. Methylation of carboxylic acid groups to reduce the number of negative charges has the benefits of reducing the required dose, improving peak clarification, and reducing pH sensitivity, but also the disadvantage of increasing the sensitivity to overdosing.<sup>7,8</sup> Polymerization (intermolecular cross-linking) produces very high molar mass structures that function at lower doses and show improved peak clarification.<sup>9</sup> Unexpectedly, both treatments impart purified serum albumin with flocculant properties.

While serum albumin is not a flocculant, and whole plasma is a poor flocculant, the inclusion of these components in a blood-flocculant product does not have the expected effect of diluting the flocculant performance achieved with pure Hb. Rather, a flocculant containing all blood components is as potent as pure Hb at low doses and is very insensitive to

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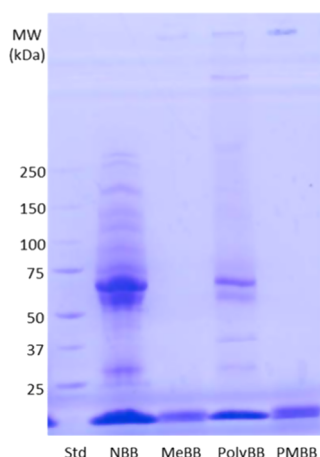


overdosing. Inclusion of the other blood components does not increase the chemical oxygen demand or nitrogen in treated suspensions when the flocculant is used at an appropriate dose.<sup>10</sup> Consequently, the expense required to purify Hb from whole blood may not be justified.

The present study takes the logical step of investigating whether the modifications applied to pure protein can be applied to whole blood, either individual or sequentially, to produce a high-performance flocculant. It also expands the range of applications studied by applying protein flocculants to a suspension of positively charged particles for the first time and addresses concerns about the red color in suspensions treated with a blood-based flocculant.

## 2. RESULTS AND DISCUSSION

**2.1. Molecular Weight and Compositions of Flocculants.** Blood is an extraordinarily complex substance containing, along with other substances, dozens of proteins.<sup>11</sup> The red cell cytoplasm is largely a solution of the protein hemoglobin. In the surrounding plasma, serum albumin is the most abundant protein along with significant amounts of various serum globulins and fibrinogen and small amounts of many other proteins. SDS-PAGE analysis of NBB (Figure 1,



**Figure 1.** SDS-PAGE of native bovine blood (NBB), methylated bovine blood (MeBB), polymerized bovine blood (PolyBB), and polymerized & methylated bovine blood (PMBB).

lane 2) reflects this known composition of blood and shows a prominent band consistent with the known molar mass of bovine serum albumin (66.5 kDa), the subunits of hemoglobin (~15.5 kDa), which are expected to dissociate under the conditions of SDS-PAGE, and  $\gamma$ -globulin (~30 kDa), the most abundant globulin in cattle blood, along with several fainter bands.<sup>12–14</sup> PolyBB (lane 4) displayed many of the same bands, although more faintly, along with a band that would be consistent with the transglutaminase added (~38 kDa),<sup>15</sup> a band at a position that would indicate a molar mass much greater than 250 kDa, and a stained material in the loading well, possibly indicating a cross-linked protein too large to enter the gel. Overall, these results suggest successful but incomplete cross-linking of blood proteins, consistent with Essandoh, Garcia, and Nieman.<sup>9</sup> The lanes for both types of methylated samples (MeBB, lane 3 and PMBB, lane 5) are mostly free of protein bands except for the band believed to be the hemoglobin subunit and the stained material in the loading wells. These results are consistent with the findings that when

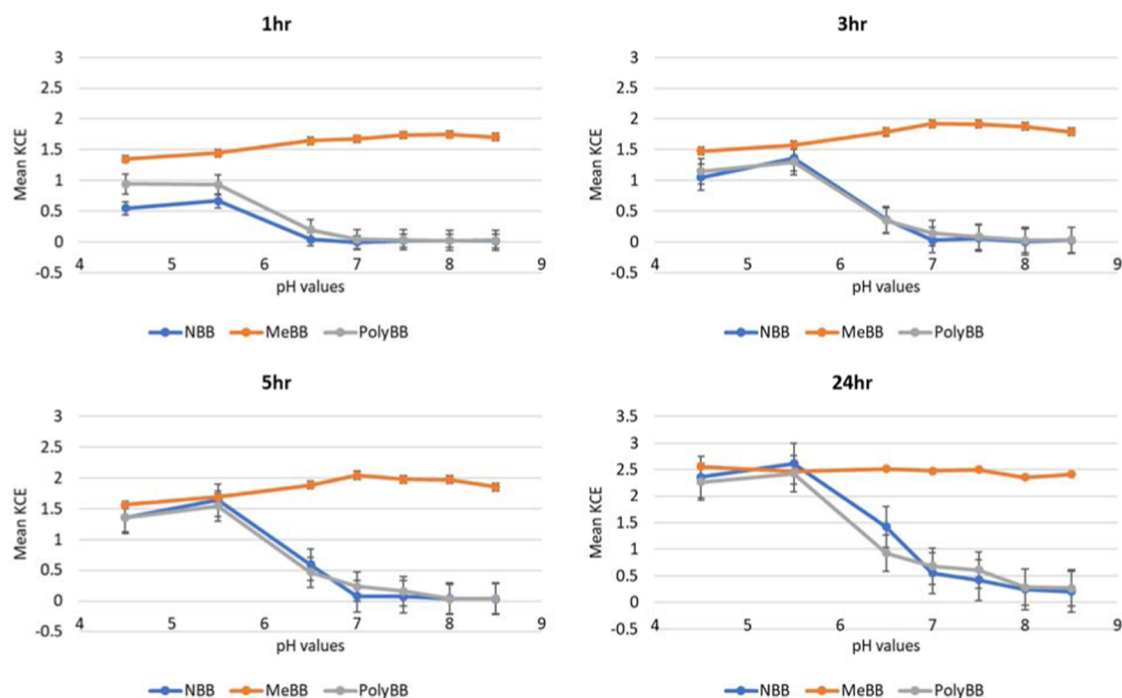
used on pure hemoglobin or serum albumin, the methylation treatment had the side effect of creating very large protein aggregates, both soluble and insoluble.<sup>8</sup>

### 2.2. Flocculation Performance in Kaolin Suspension.

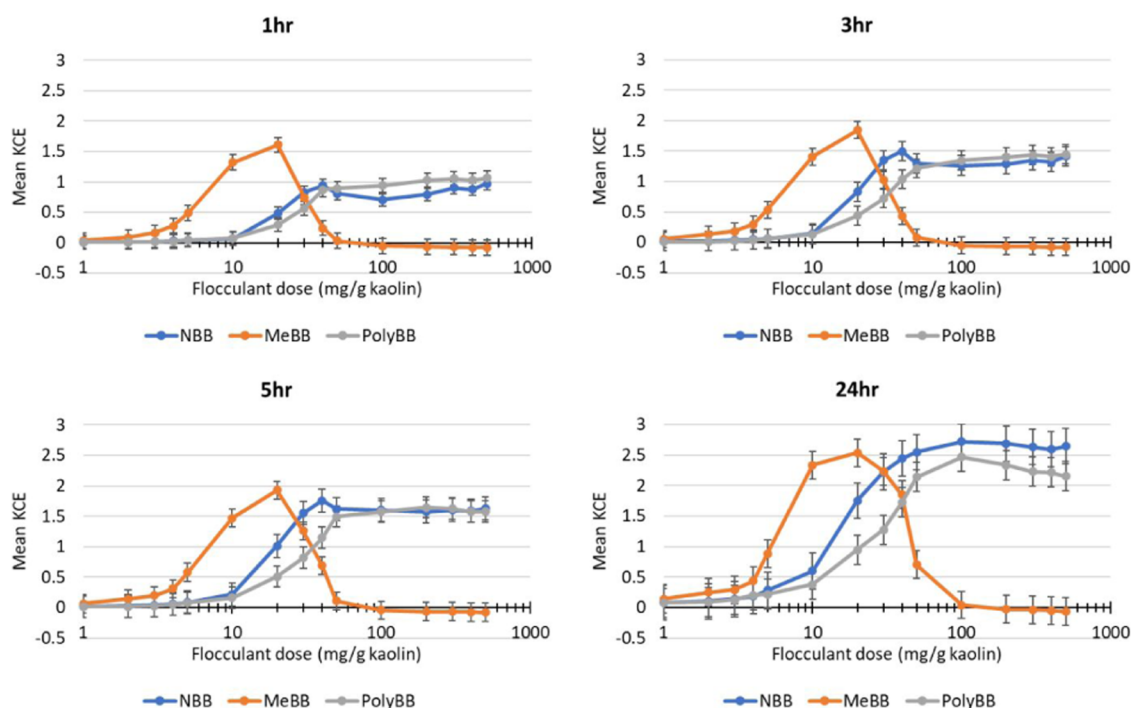
The performance of each type of blood in clearing a kaolin suspension was determined across a range of pH values (Figure 2). NBB and PolyBB showed a high clarification efficiency at pH 5.5 and below, but decreased with increasing pH values. MeBB, however, showed consistently high precipitation in all measured pH values. This is consistent with the results of Garcia, Qi, Essandoh, and Bumanlag,<sup>8</sup> which found that methylation of pure Hb or BSA yielded a pH-insensitive flocculant. Our results are the first report using this flocculant-improving reaction on an impure protein substrate, showing its potential to be used with less refined agricultural proteins. Since all types of blood showed high performance at pH 5.5, this condition was used for flocculant dosage tests. Trials were conducted over a very wide range of flocculant doses (1–500 mg/g kaolin), and KCE values were measured at a range of settling times (Figure 3). Flocculation activity of native bovine blood (NBB) was observed at 20 mg/g kaolin, and peak clarification (KCE ~1.0) was achieved with 40 mg/g kaolin at 1 h. The peak clarification was maintained even with excess NBB (up to 500 mg/g kaolin tested) added. The clarification performance of polymerized bovine blood (PolyBB) followed a similar trend. The same peak clarification was reached with a dose of 40 mg/g kaolin at 24 h and was not diminished by overdosing. For most dosages and settling times, the KCE of PolyBB was less than or equal to that of NBB, suggesting that this treatment on its own was not beneficial. Methylated bovine blood (MeBB) showed a higher peak clarification (KCE ~1.5) with a much lower dose at 20 mg/g kaolin at 1 h. However, the effective dose window of MeBB was narrow. Excess MeBB (over 40 mg/g kaolin) resulted in lower KCE values, and the flocculation activity was not detectable with doses over 100 mg/g kaolin. The charge reaction in flocculation was studied using the  $\zeta$ -potential values of flocculants (Table 1). The surfaces of NBB and PolyBB were negatively charged, whereas the surfaces of MeBB and PMBB were positively charged. The charge reaction of flocculants in the kaolin suspension resulted in clean water. Clean water after flocculation had a  $\zeta$ -potential of  $0 \pm 10$  mV, which was the same as the value of pure water ( $0 \pm 5$  mV).

The combination of polymerization and methylation treatments could produce high-molar-mass molecules with relatively high isoelectric points (Figure 8). This combination was applied to bovine blood with the intent of producing a flocculant with a high KCE of MeBB and the insensitivity to overdosing of PolyBB. Polymerized & methylated bovine blood (PMBB) had the same peak clarification as MeBB at a dose 20 mg/g kaolin, and the dose window was broader, showing improved activity of 50 mg/g compared to MeBB (Figure 4). Such overdose insensitivity is a substantial benefit in applications where the solid load of the wastewater is inconsistent.

Bovine blood has a dark-red/brown color due to the abundant hemoglobin. Most end-users would not accept a flocculant that adds color to the wastewater being treated. When applied at the appropriate dosage, the red hemoglobin presumably becomes a part of the flocs and precipitates without coloring the supernatant. The supernatant of kaolin suspensions treated with NBB, MeBB, and PolyBB remained clear at a dose of 50 mg/g kaolin and below (Figure 5).



**Figure 2.** Kaolin clarification efficiency (KCE) values of native bovine blood (NBB), methylated bovine blood (MeBB), and polymerized bovine blood (PolyBB) versus pH.



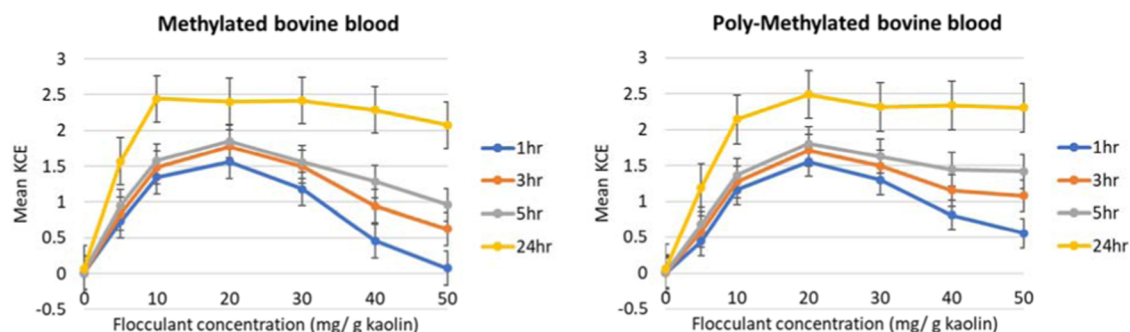
**Figure 3.** Kaolin clarification efficiency (KCE) values of native bovine blood (NBB), methylated bovine blood (MeBB), and polymerized bovine blood (PolyBB) at different dosages.

**Table 1.**  $\zeta$ -Potential Values of Native Bovine Blood (NBB) and Its Derivatives, Methylated Bovine Blood (MeBB), Polymerized Bovine Blood (PolyBB), and Polymerized & Methylated Bovine Blood (PMBB)

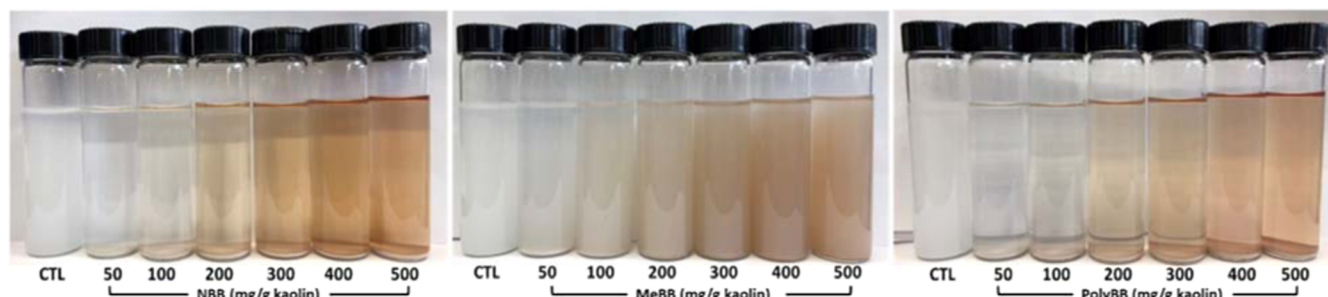
	NBB	MeBB	PolyBB	PMBB
$\zeta$ -potential (mV)	$-24.3 \pm 0.51$	$38.7 \pm 1.58$	$-22.0 \pm 0.38$	$37.7 \pm 2.50$

**2.3. Flocculation Performance in Hematite Suspension.** The flocculation performance of the bovine blood

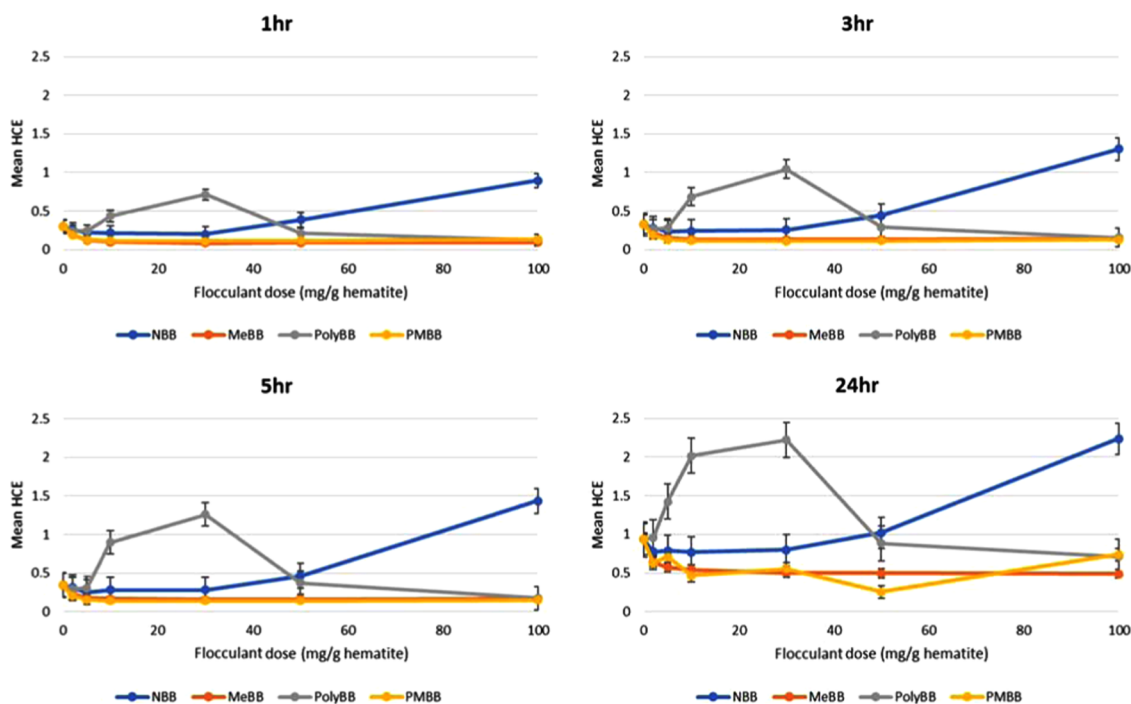
flocculants was tested against a hematite suspension at pH 7.0 (Figure 6). PolyBB had peak clarification (KCE  $\sim 1.5$ ) when



**Figure 4.** Kaolin clarification efficiency (KCE) values of methylated bovine blood (MeBB) and polymerized & methylated bovine blood (PMBB) at different dosages.



**Figure 5.** Visual comparison of the kaolin clarification efficiencies (KCEs) of native bovine blood (NBB), methylated bovine blood (MeBB), and polymerized bovine blood (PolyBB) after 24 h settling time.

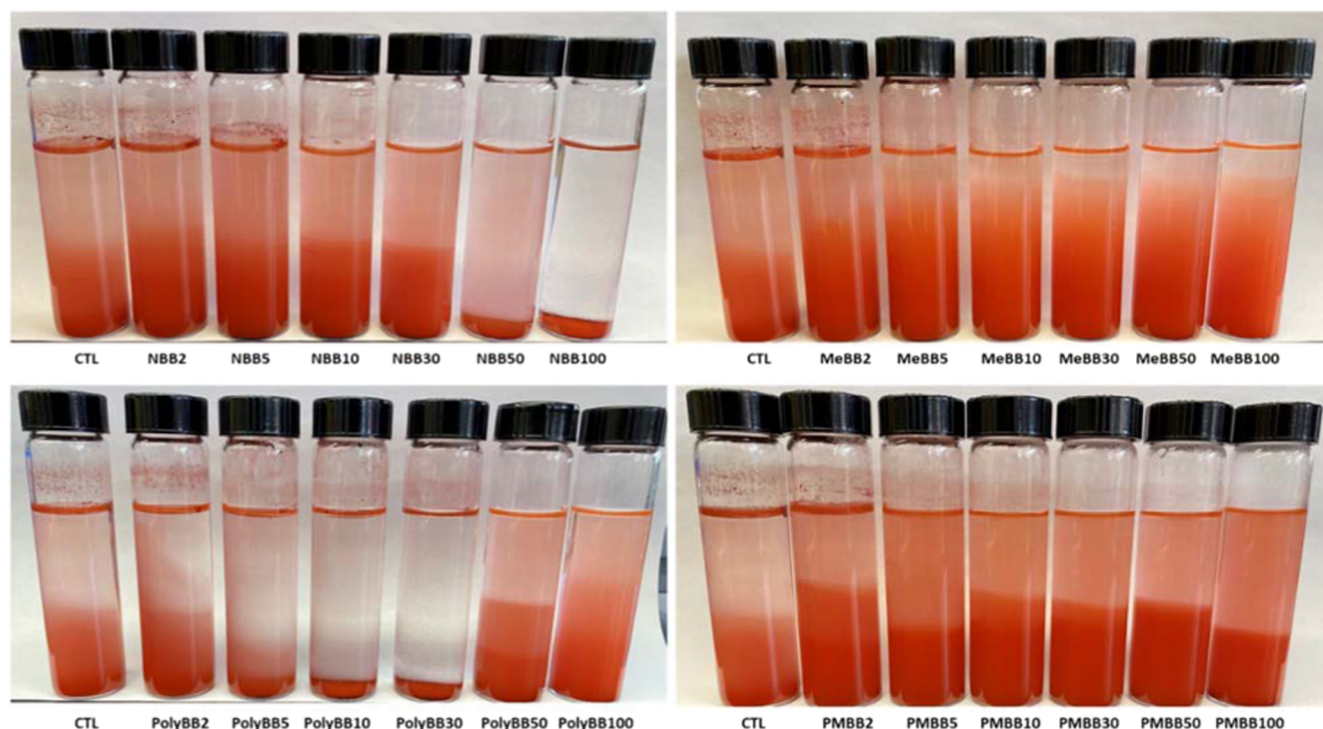


**Figure 6.** Hematite clarification efficiencies (HCEs) of native bovine blood (NBB), methylated bovine blood (MeBB), polymerized bovine blood (PolyBB), and polymerized & methylated bovine blood (PMBB).

dosed at 30 mg/g hematite. NBB reached the same clarification at 100 mg/g. However, no flocculation was detected when hematite suspensions were dosed with 2–100 mg/g MeBB or PMBB. Li et al.<sup>16</sup> reported that starch-based flocculants have good flocculation efficiencies on hematite suspensions due to charge neutralization. Ferretti et al.<sup>17</sup> found that flocculation occurred between particles and polymers with

a high molecular weight compared to the size of particles in a hematite system. Hematite particles acted as ligands to associate with polymer chains and form flocs by a bridging mechanism. Similar to the treated kaolin suspensions, the treated hematite suspensions were not substantially discolored by NBB or PolyBB at their effective doses (Figure 7).





**Figure 7.** Visual comparison of hematite clarification efficiencies (HCEs) of native bovine blood (NBB), methylated bovine blood (MeBB), polymerized bovine blood (PolyBB), and polymerized & methylated bovine blood (PMBB) after 24 h settling time.

**2.4. Interactions of Bovine Blood Flocculants in Two Suspensions.** Electrostatic interaction between suspended solids and flocculants can be elucidated through  $\zeta$ -potential measurements. Suspended kaolin has a negative surface charge in the pH range from 2.0 to 11.0.<sup>16</sup> Apparent isoelectric (pI) values of NBB and PolyBB are 5.86 and 5.59, respectively (Table 2), indicating net positive charges at pH below their pI.

**Table 2.** Apparent Isoelectric Point of Native Bovine Blood (NBB) and Its Derivatives, Methylated Bovine Blood (MeBB), Polymerized Bovine Blood (PolyBB), and Polymerized & Methylated Bovine Blood (PMBB)

	NBB	MeBB	PolyBB	PMBB
isoelectric point (pI)	5.86	9.80	5.59	9.80

High flocculation efficiency was associated with flocculants carrying opposite charge to the suspended solids.<sup>18</sup> The flocculation activity was initiated by electrostatic interactions, as evidenced by the loss of the clarification ability at pH 6.5 and above (Figure 2). The higher apparent pI of MeBB and PMBB (Table 1) may contribute to their higher efficiency at pH 5.5 due to the net positive charges. The positively charged nature of MeBB and PMBB below pH 9.8 enabled the pH adaptability from 4.5 to 8.5.

As opposed to kaolin suspension, the surface charge of suspended hematite is positive in conditions below pH 9.5.<sup>16</sup> The like-charge repulsion among MeBB, PMBB, and hematite impeded the flocculant activity. The clarification effectiveness of NBB and PolyBB in the hematite suspension may also be attributed to electrostatic interactions, as they are negatively charged at pH 7. Ferretti, Stoll, Zhang, and Buffle<sup>17</sup> found that increasing the chain size of flocculants results in decreasing the optimal dosage of flocculants. The observed higher molar mass of PolyBB by SDS-PAGE (Figure 1) contributed to bridging

interactions; therefore, a lower required dose was associated with PolyBB compared to NBB.

### 3. CONCLUSIONS

Chemical modifications can improve the flocculant properties of bovine blood. Turbidity tests with kaolin and hematite suspensions were performed at different pH and flocculant dosages, and selective flocculation was found. Kaolin represents a negatively charged contaminant, and hematite indicates a positively charged contaminant. The mechanism to flocculate via bovine blood was explained with charge neutralization and molecular weight through isoelectric point and electrophoresis. As the positive charge is dominant on the surface of MeBB and PMBB, aggregation with kaolin particles was excellent. Conversely, NBB and PolyBB have mostly negative charges on their surface, resulting in flocculation with the positive charges of hematite particles. According to the different chemical modifications, bovine blood can be used as effective flocculants in the various conditions of wastewater.

### 4. EXPERIMENTAL SECTION

**4.1. Materials.** Fresh bovine blood was obtained from a slaughterhouse, and disodium ethylenediaminetetraacetic acid ( $\text{Na}_2\text{EDTA}$ ) was added to a final concentration of 10.8 mM to prevent coagulation.<sup>5</sup> Commercial transglutaminase (Moo Gloo TI, Modernist Pantry, ME) composed of the enzyme plus maltodextrin was used for cross-linking. Kaolin clay powder was obtained from KaMin (Polygloss 90, Macon, GA), and hematite powder (iron(III) oxide) was bought from Sigma-Aldrich (St. Louis, MO).

**4.2. Preparation of Bovine Blood Flocculants.** Anti-coagulated blood was stored at  $-20\text{ }^\circ\text{C}$  until usage. Blood was lyophilized to produce a dry powder, termed native bovine blood (NBB). NBB was used to prepare methylated bovine

blood (MeBB) according to Essandoh et al.<sup>19</sup> with a slight modification (Figure 8). Lyophilized NBB (3% w/v) was

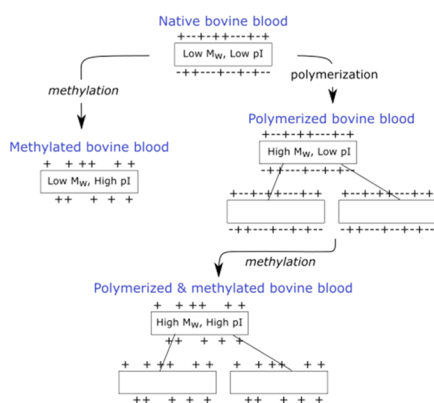


Figure 8. Schematic of blood protein modifications studied.

dispersed in methanol with 6.6% (v/v) HCl added. The suspension was shaken at 140 rpm for 24 h and then centrifuged at 10 000g for 15 min. The pellet was resuspended in deionized water and lyophilized. Polymerized bovine blood (PolyBB) was prepared from NBB following a modified method of Essandoh, Garcia, and Strahan.<sup>7</sup> NBB (4% w/v) was dissolved in 100 mM sodium phosphate buffer (pH 7.2), and transglutaminase (2% w/v) was added. After incubating the suspension at 4 °C for 24 h, it was lyophilized and stored in a freezer until further usage.

**4.3. Kaolin Clarification Efficiency (KCE) Test.** The kaolin clarification efficiency (KCE) test was performed according to Garcia et al.<sup>20</sup> The kaolin suspension (1 g/L) was prepared in 25 mM Malic-MES-Tris (MMT) buffer at the required pH. Test vials were filled with a 24 mL suspension, and their initial turbidities were measured using an infrared turbidimeter (2100AN IS, Hach, Loveland, TX). After addition of the bovine blood flocculants, vials were shaken using an orbital shaker at 400 rpm for 1 min and then at 200 rpm for 15 min. Vials were then placed in a 20 °C incubator, and turbidities were recorded at a range of settling times (1, 3, 5, and 24 h). KCE was calculated as follows

$$\text{KCE} = \log_{10} \left( \frac{T_i}{T_f} \right)$$

where  $T_i$  is the initial turbidity and  $T_f$  is the final turbidity. KCE is a logarithmic value, and a KCE value of 1 indicates a 90% reduction in turbidity and that of 2 represents 99% reduction.

**4.4. Hematite Clarification Efficiency (HCE) Test.** The hematite (iron oxide) suspension (0.25 g/L) was prepared in nanopure water and kept at room temperature for 24 h to stabilize. To completely disperse the hematite powder, the suspension was sonicated for 15 min using an ultrasonicator set at 50% amplitude, running at a 5 s pulse–5 s rest cycle. After fully mixing the suspension, the HCE test was performed in the same way as the KCE test.

**4.5.  $\zeta$ -Potential Measurements.** The  $\zeta$ -potential of the suspended bovine blood flocculants was measured as a function of pH using an autotitrator (MPT-2, Zetasizer Nano Z, Malvern Instrument Inc., Westborough, MA). Each sample was suspended in a 25 mM MMT buffer at 1 mg/mL and vortexed for 30 s. All values were measured in triplicate.

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### Notes

The authors declare no competing financial interest.

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