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Encapsulation of atomized erythrocytes in liposomes as source of heme iron for oral supplementation strategies

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Abstract

This work aimed to develop and characterize liposomes of atomized bovine erythrocytes (ABE-liposomes) for potential use as a heme iron supplement or to fortify food with heme iron. Soy lecithin (SL) and Tween 80 (T80) were chosen to prepare empty liposomes (8 types), and ABE-liposomes (16 types) by a rotary-evaporated film method. Variables of interest for our study were: ABE concentration, proportion of SL:T80 and ultrasonic bath time. ABE-liposomes were characterized according to appearance, color, morphology, Fourier transform infrared spectroscopy (FTIR), size, zeta potential, total iron content, encapsulation efficiency (EE) and in vitro iron release under gastrointestinal conditions. ABE-liposomes presented a brown color and spherical shape. Hydrogen bridges were the main interactions in ABE-liposomes. Their size ranged from 379 to 964 nm, the zeta potential from −56 to −74 mV, the iron concentration range was 0.8–9.0 mg/100 mL, and EE ranged from 29% to 48%. Ultrasonic bath time was the most important variable on the reduction of ABE-liposomes' size and EE. Under in vitro gastrointestinal conditions, iron release from ABE-liposomes occurred mainly at the intestinal level. In conclusion, ABE-liposomes were effectively developed, which could become a complement for supplementation or food fortification strategies with nonheme for the prevention of iron deficiency anemia.

Practical Applications

In this study atomized bovine erythrocytes (ABE)-liposomes were developed, due to the: (1) lack of supplements or products to fortify foods based on heme iron, (2) higher bioavailability of heme iron than nonheme iron, especially when working with ABE, and (3) ABE is a low value-added product that is generated in slaughter plants and in turn is an excellent source of underutilized heme iron. In this study it was possible to develop ABE-liposomes, where ABE was encapsulated inside liposomes, obtaining a product that could be used as a complement in iron supplementation strategies or food fortification to prevent or treat iron deficiency or iron deficiency anemia, especially in developing countries that have low consumption of heme iron.

1 | INTRODUCTION

Iron deficiency anemia is the most important nutritional deficit in the world. Although various strategies such as iron oral supplementation and food fortification have been studied and implemented, they have not had the expected results (Radhika et al., 2019; Trivedi & Barve, 2021). Anemia affects nearly 1.5 billion people worldwide and generates significant public health disruption and economic costs (Fischer et al., 2022). This deficiency is more prevalent in developing countries. It is estimated that in Latin American countries, anemia affects between 4% and 61.3% of the child population under 6 years old and 5.1%–45.5% of women of childbearing age (Mujica-Coopman et al., 2015). India has the highest prevalence of iron deficiency anemia worldwide, affecting 70% of children, 55% of women of reproductive age, and 85% of pregnant women (Mehta et al., 2017). In some regions of Africa, anemia affects 60% of preschool children and 40% of women of reproductive age (Petry et al., 2016; Turawa et al., 2021).

Iron deficiency anemia mostly affects developing countries due to low socioeconomic status, leading to a diet based on cereals/legumes with low consumption of animal-based ingredients that provide heme iron (Ngui et al., 2012), which is the most bioavailable source of iron (Buzała et al., 2016; Chang et al., 2019). These foods are among the most expensive in the diet (Mantadakis et al., 2020). In addition, a cereal-based diet that is common in these countries may contain abundant phytates, polyphenols or other inhibitors of intestinal non-heme iron absorption, favoring the presentation of anemia (Schümann & Solomons, 2017). Also, the low educational level in these countries explains the high prevalence of anemia, as it results in misinformation about the importance of having a balanced diet that covers the essential nutrients for good health (Ali et al., 2020; Kassebaum, 2016).

Different forms of iron are found in foods; heme iron has higher bioavailability (15%–37%) than nonheme iron (2%–20%) (Björn-Rasmussen et al., 1974; Carpenter & Mahoney, 1992). The increased bioavailability of heme iron is based on several aspects. (1) This form of iron is absorbed by a different transporter, heme carrier protein 1 (HCP1), where heme iron does not compete with other divalent metals as is the case of divalent metal transporter 1 (DMT-1), receptor of nonheme iron (Yanatori et al., 2010). (2) Heme iron moves through the gastrointestinal tract as a complete molecule (iron bound to the porphyrin ring) and is absorbed in this form, which reduces interactions with other compounds of the diet that can reduce its absorption and/or chelate it, as occurs with nonheme iron (Candia et al., 2018; Rouault, 2005). (3) Heme iron absorption may be decreased by calcium, which is still controversial (Gaitán et al., 2011; Hallberg et al., 1991; Roughead et al., 2002), compared to the absorption of nonheme iron that is affected by multiple dietary factors (Hallberg et al., 1993; Ma et al., 2011; Olivares et al., 2016).

Due to the higher bioavailability of heme iron, some supplementation and fortification strategies have used it to reduce the prevalence of iron deficiency anemia. Generally, these studies have used purified heme iron extracted from erythrocytes or hemoglobin (Alemán et al., 2014; González-Rosendo et al., 2010; Vaghefi et al., 2005). Studies from the 1970s, described that these forms of heme iron had higher absorption and bioavailability (Björn-Rasmussen et al., 1974; Hallberg et al., 1979; Hertrampf et al., 1978). However, it has recently been described that the most bioavailable form of oral heme iron is the administration of whole erythrocytes (Pizarro et al., 2016). This is advantageous because it is not necessary to apply the complex and costly protocols of extracting heme iron from blood. In addition, erythrocytes are generated in large quantities in animal slaughter plants and converted into blood meal. Blood meal processed by heat treatment by spray drying is safe for human consumption and is a low-cost source of heme iron (Ofori & Hsieh, 2014). In general, the use of animal blood meal and its derivatives for human consumption are permitted and strictly regulated in several countries. For example, in the United States, the use of blood products for human consumption is permitted (Food Safety and Inspection Service, 2021). For the European Union, in Regulation (EC) No. 1069/2009 (European Commission, 2009), the consumption of blood is allowed under the denomination "edible products of animal origin", where meat and offal are also included (Food Standards Agency, 2011). In Chile, according to the Food Sanitary Regulations, blood is considered an edible animal by-product and can be used if it comes from authorized establishments, has been heat-treated and does not present organoleptic alterations or hormone or pesticide residues (Ministerio de Salud, 2022).

The use of erythrocytes in fortification strategies would alter the organoleptic properties of foods, due to their intense reddish brown color, unpleasant "blood" odor, and metallic taste (Álvarez et al., 2018; Wang et al., 2017). To improve some of these unpleasant sensory properties of heme iron sources, encapsulation strategies have been used (Churio et al., 2018; Churio & Valenzuela, 2018; Durán et al., 2020; Valenzuela et al., 2014). Heme iron has been encapsulated to increase its bioavailability, but these types of studies are scarce (Churio et al., 2019; Yuan et al., 2013). Several nonheme sources of iron have been encapsulated by entrapment in liposomes, which is the main technique used for iron fortification in the food industry (Gupta et al., 2015; Khanniri et al., 2016; Zimmermann., 2004). To our knowledge, the use of erythrocytes as a source of heme iron and its encapsulation by entrapment in liposomes as a nutritional supplement has not been reported. Therefore, this work aimed to develop and characterize liposomes of atomized bovine erythrocytes for potential use as a heme iron supplement or to fortify food with heme iron.

2 | MATERIALS AND METHODS

2.1 | Materials

Spray-dried atomized bovine erythrocytes (ABE) were purchased from LICAN Alimentos S.A., Chile. Total iron content of ABE was determined by atomic absorption spectrophotometer (GBC, 905AA, Australia), after acid digestion (method 999.11, AOAC

International, 2000). Nonheme iron was obtained by acid extraction (Rebouche et al., 2004) and quantified by atomic absorption spectrophotometry. These measurements were validated against a standard curve assessed at $\lambda = 248.3$ nm using a commercial iron standard (J.T. Baker, USA) at a concentration of 1000 mg/mL. Heme iron content was calculated from the difference of total iron and nonheme iron.

The color of ABE was measured with a colorimeter (Konica-Minolta, CR-300, Japan) using the HunterLab color scale.

To prepared liposomes, food-grade liquid soy lecithin (SL) acquired from Prinal S.A. (Santiago, Chile), composed of phosphatidylcholine (min. 90%), other phospholipids (max. 5%), and fatty acid (approximately 2%) and Tween 80 (T80) purchased at Droguería Michelson (Santiago, Chile) was used. All reagents were of analytical grade and purchased from Merck S.A. or Sigma-Aldrich Company.

2.2 | Preparation of liposomes and ABE-liposomes

Different liposome formulations were prepared to determine the effects of different variables in their formation: (1) ABE content: 0%, 1%, and 5% (w/v). (2) ratio between soy lecithin (SL):Tween 80 (T80): 10:4 and 10:2 (v/v). (3) Ultrasonic bath exposure time: 0, 15, 30 and 45 min. Liposomes were prepared following a rotary-evaporated film method (Yuan et al., 2013). Blends of 10 mL of SL and T80 (in the proportions of 10:4 and 10:2, v/v) were dissolved in 20 mL of a chloroform: methanol solution (2:1, v/v). Removal of solvents was done by rotary evaporation (Heidolph, VV-2000, Germany), to yield a thin lipid membrane (films) over the inner surface of a round-bottom flask. These films were hydrated with 100 mL PBS 0.1 M at pH 6.5 and magnetically stirred in the flask to obtain a lipid suspension (liposomes). For the preparation of ABE-liposomes, we proceeded in the same way as the liposomes, with the modification that different concentrations of ABE were added at the time of hydration of the films, dissolved in PBS, and incorporated by magnetic stirring for 30 min. To reduce the size of liposomes and ABE-liposomes, these suspensions were subject to an ultrasonic bath (Elma, Elmasonic E30H, Germany) with an ultrasonic frequency of 37 kHz and an effective ultrasonic power of 40 W. The viscosity of liposome and ABE-liposome-forming solutions was measured with a viscometer (Brookfield, RVT, England) at 25°C. The liposome and ABE-liposome formulations and their respective codes are presented in Table 1.

TABLE 1 Formulations of liposomes and ABE-liposomes.

Code	Type	Ratio (SL:T80)	Time (min)	ABE (% p/v)
A	Liposome	10:2	0	0
B	Liposome	10:2	15	0
C	Liposome	10:2	30	0
D	Liposome	10:2	45	0
E	Liposome	10:4	0	0
F	Liposome	10:4	15	0
G	Liposome	10:4	30	0
H	Liposome	10:4	45	0
A1	ABE-liposomes	10:2	0	1
B1	ABE-liposomes	10:2	15	1
C1	ABE-liposomes	10:2	30	1
D1	ABE-liposomes	10:2	45	1
E1	ABE-liposomes	10:4	0	1
F1	ABE-liposomes	10:4	15	1
G1	ABE-liposomes	10:4	30	1
H1	ABE-liposomes	10:4	45	1
A5	ABE-liposomes	10:2	0	5
B5	ABE-liposomes	10:2	15	5
C5	ABE-liposomes	10:2	30	5
D5	ABE-liposomes	10:2	45	5
E5	ABE-liposomes	10:4	0	5
F5	ABE-liposomes	10:4	15	5
G5	ABE-liposomes	10:4	30	5
H5	ABE-liposomes	10:4	45	5

Abbreviations: ABE, atomized bovine erythrocytes; SL, soy lecithin; T80, Tween 80.

2.3 | Characterization of liposomes and ABE-liposomes

2.3.1 | Appearance, color, and morphology.

The appearance of ABE, liposomes and ABE-liposomes was recorded using a digital camera (Sony Corporation, DSC-HX1, Japan) and the color of liposomes and ABE-liposomes was measured with a colorimeter (Konica-Minolta, CR-300, Japan) using the HunterLab color scale.

Liposomes and ABE-liposomes were observed by transmission electron microscopy (TEM) to characterize the morphology. Briefly, 3 μL drop samples were placed on a carbon-coated copper grid, followed by air-drying for 1 min. Then, a 10 μL drop of uranyl acetate solution was placed on the grids for 10 min*. The samples were observed with a transmission electron microscope (Philips Tecnai 12, BioTwin, The Netherlands), operated at 80 kV.

2.4 | Fourier-transform infrared spectroscopy

All materials used for the formation of the liposomes (SL, T80 and ABE), liposomes and ABE-liposomes were analyzed using an ATR/FTIR spectrometer (Interspec 200-X, Estonia). The spectroscopic measurements were performed directly with the PIKE Miracle™ accessory in a Ge single reflection crystal plate. Spectra were obtained by averaging 20 scans over the spectral range of 600–4000 cm^{-1}.

2.5 | Size and zeta potential

Size and zeta potential was assessed in samples of liposomes and ABE-liposomes (0.5 mL) mixed with 5 mL of KCl at 1 mM solution. Size was measured by a dynamic light scattering particle size analyzer (Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). Zeta potential was measured using the Zetasizer Nano ZS90, using the laser Doppler method.

2.6 | Iron content and encapsulation efficiency

For total iron determination, samples were processed by acid digestion (method 999.11, AOAC International, 2000). Then, total iron content was quantified by atomic absorption spectrophotometry (GBC, 905AA, Australia).

Encapsulation efficiency (EE%) was calculated using the following equation:

$$EE\% = \frac{(I_t - I_s)}{I_t} \times 100 \tag{1}$$

where EE% is the encapsulation efficiency percentage, which is defined by I_t (total iron content) and I_s (surface iron defined as the iron adhered to the outer surface of the liposomes, that was not encapsulated).

For surface iron determination, 200 mL samples of ABE-liposomes were loaded in a centrifuge (Hermle Z230A, Germany) at 2900×g for 15 min. Then, total iron content in supernatant (iron surface) was determined by atomic absorption spectrophotometry.

2.7 | In vitro iron release studies

Gastric and intestinal conditions were simulated to perform an in vitro iron release study according to Churio et al. (2018). A solution containing 2 g/L of NaCl and 10 g/L of pepsin, adjusted to pH 2.0 by adding HCl 1 N solution was prepared to simulate gastric conditions.

For the release study, 1 g of sample (ABE-liposome and liposome empty as control) was suspended in 100 mL of this blend and incubated for 1 h at 37°C and constantly agitated by magnetic stirring. Then, this mixture was converted to intestinal conditions, dissolving 50 g/L of pancreatin, 31.2 g/L of bile extract, 8.76 g/L NaCl and PBS 0.1 M at pH 7.4 to reach pH 6. Incubation was continued for 3 h at 37°C.

For the quantification of iron released, five replicates of 5 mL aliquots were taken in triplicate at the end of each incubation step. Total iron content was measured with atomic absorption spectrophotometry techniques as described above. Incubation samples that did not include liposomes were used as blanks.

2.8 | Statistical analysis

Data from color and iron content analyses from iron release were subjected to the Shapiro–Wilk test for normality and Levene's test for homoscedasticity. As the data were normal and homoscedastic, analysis of variance (ANOVA) and Tukey ($p < 0.05$) tests were performed for color. As the iron content data did not have a normal distribution, a Wilcoxon test ($p < 0.05$) was applied. All these tests were performed with Statistix 8 software (Analytical Software 2003, Tallahassee, FL).

The data for size, zeta potential, iron content and encapsulation efficiency were normally distributed and homoscedastic and were therefore subjected to an ANOVA test using the GLM procedure of the SAS® statistical software package. Means were presented by LS, adjusted by Tukey's test, with a value of $p < 0.05$. In this analysis the effect of different variables such as: SL:T80 ratio (either 10:2 or 10:4), ultrasonic bath time and ABE concentration and their interactions on different properties of the ABE-liposomes (size, zeta potential, iron content, and encapsulation efficiency) were determined. The following mathematical model was used:

$$Y = \mu + \alpha i + \beta j + \gamma k + \alpha i^* \beta j^* \gamma k + \varepsilon ijk$$

where Y is the ABE-liposomes properties; μ is the general mean of all observations; αi is the SL:T80 proportion.

βj is the ultrasonic bath time; γk is ABE concentration; $\alpha i * \beta j * \gamma k$ represent interactions between factors; εijk is the random error.

3 | RESULTS AND DISCUSSION

ABE is obtained from animal slaughter plants and is used in human and animal feed (Valenzuela et al., 2014). This by-product has great potential as animal feed due to its high protein content; ranging from 78% to 90% (Duarte et al., 1999; Toldrà et al., 2004) and high amino acid digestibility (Gómez-Juárez et al., 1999). In addition, in recent years, the functional properties of ABE have been studied, such as high antioxidant and antimicrobial activity (Bah et al., 2016; Przybylski et al., 2020). The risk of transmission of spongiform encephalopathy, blood allergens or pathogens associated with ABE consumption is low, when the product is properly processed, similar to other animal products (Ofori & Hsieh, 2014). A scarcity of animal-derived ingredients, which will become more acute in the coming years with the trend toward circular economy, will cause by-products to gain importance and added value (Reynolds et al., 2015).

The development of ABE-liposomes was conceived as a potential strategy for supplementation or fortification with highly bioavailable iron to prevent or treat iron deficiency anemia and/or its previous stages as deficiency. Thus, the present study could contribute to the fulfillment of two of the 17 Sustainable Development Goals of the United Nations, such as "zero hunger" by improving people's iron nutrition and "good health and well-being", since anemia affects 40% of the world's population, and has a negative effect on growth, cognitive development and academic performance, causes fatigue and low productivity, affecting the health and well-being of the population (Tandoh et al., 2021).

The liposome encapsulation technique was selected because it is the most widely used technique for the development of oral supplements and food fortification with nonheme iron, due to its multiple advantages, such as: low cost, simple procedure, wide application, and use as supplements or to fortify solid and liquid foods (Ajeeshkumar et al., 2021; Subramani & Ganapathyswamy, 2020). The challenge of this study was to determine whether it was possible to encapsulate whole erythrocytes in this system.

Figure 1 shows the appearance of liposomes without ABE (Figure 1ai), with the lowest ABE concentration of 1% (Figure 1aii) and with 5% ABE (Figure 1aiii). The yellow color of the liposomes without ABE is similar to other formulations described in the literature and corresponds to the color of soy lecithin blended with Tween 80.
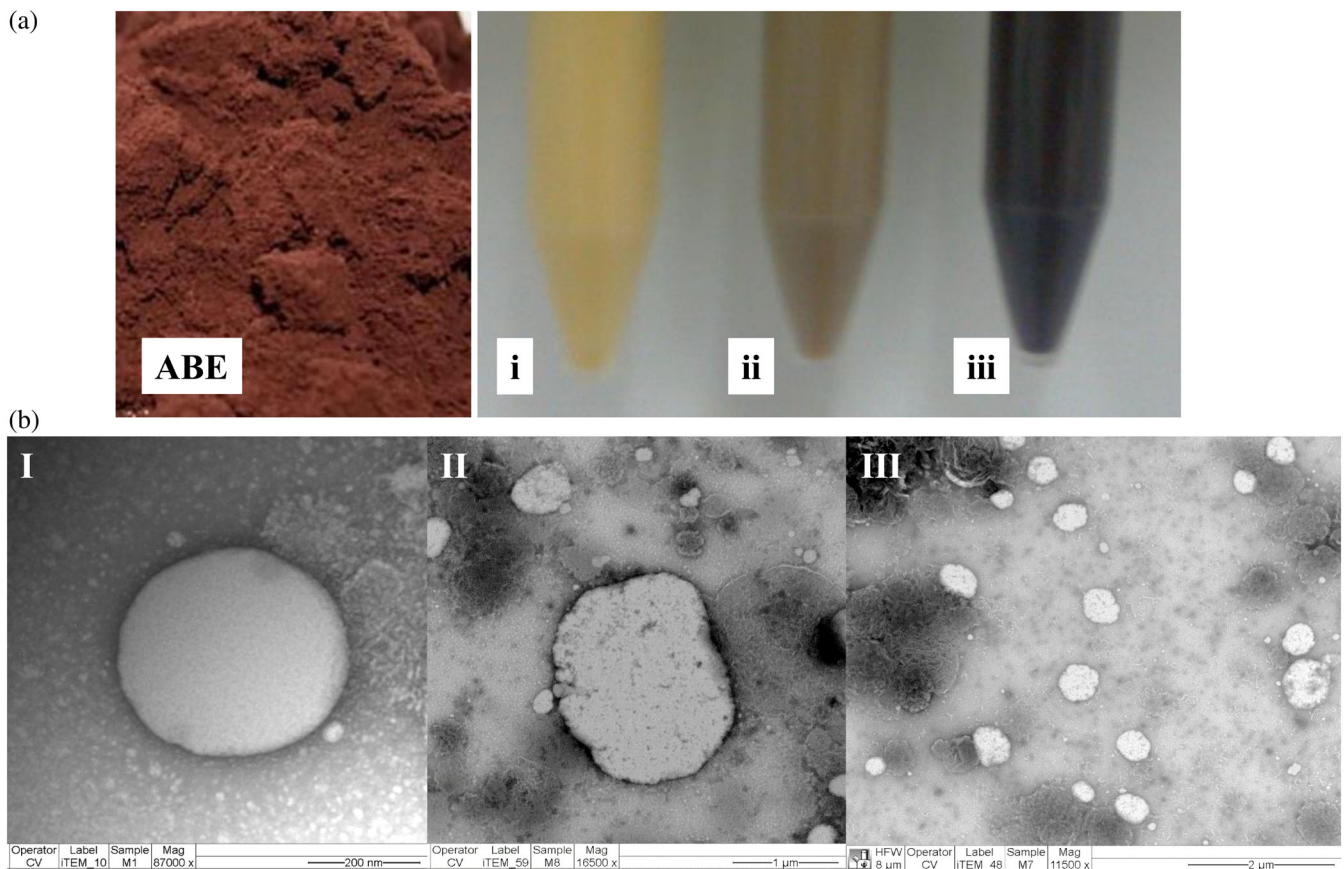

FIGURE 1 (a) Appearance of atomized bovine erythrocytes (ABE), liposome without ABE (i), ABE-liposome with 1% ABE (ii), and ABE-liposome with 5% ABE (iii). (b) Transmission electronic microscopy image of liposome without ABE (I), ABE-liposome with 1% ABE (II), and ABE-liposome with 5% ABE (III).

Upon addition of ABE, the color changed to light brown for 1% ABE-liposomes and dark brown for 5% ABE-liposomes, due to the potent reddish-brown color of ABE (Figure 1a).

The process of encapsulation of ABE in liposomes generates a color change, which was corroborated by the changes in the color parameters. Liposomes without ABE presented similar ranges of color parameter L^* (54–60), a^* (0.3–0.4), and b^* (20–23); that significantly shifted toward darker shades for 1% ABE-liposomes, which was evidenced by the reduction of parameter L^* (35–40) and toward more reddish ($a^* = 1.3$–1.5) and less yellow ($b^* = 2.2$–2.4). This trend was more pronounced for 5% ABE-liposomes ($L^* = 10$–14; $a^* = 10$–15; $b^* = 0.9$–1.1). Although the encapsulation of ABE in liposomes changes the reddish color of ABE, brown colorations are observed that might not be well perceived by consumers, therefore, the recommendation would be to use ABE-liposomes as supplements, which could be delivered in gelatin capsules or in brown colored solid foods (e.g., chocolate cookies, whole wheat pastas, others). This is to avoid browning of the product, described in foods fortified with nonencapsulated heme iron that cause consumer rejection (Martínez Graciá et al., 2000; Quintero-Gutiérrez et al., 2012).

Figure 1b also shows TEM images of liposomes and ABE-liposomes with 1% and 5% ABE. Liposomes were spherical and had an uninterrupted membrane with a smooth and homogeneous surface (Figure 1bI). However, after adding ABE, ABE-liposomes became irregularly spherical and their surface turned heterogeneous and rough (Figure 1bII,bIII). Several investigators have reported the finding of liposomes with spherical shapes that are similar to our observations (de Almeida et al., 2019; Yuan et al., 2013; Yuwanda et al., 2022). In all cases, liposomes with conserved structure were observed (Battistelli et al., 2018). The irregular shape adopted by the ABE-liposomes possibly occurred due to an increase in the viscosity of the formulations that generated the ABE-liposomes (Fujiwara & Yanagisawa, 2017). Since the viscosity of the liposome-forming solutions increased significantly when ABE was added to the formulations, ranging from 3.99–4.22 Pa s for liposomes without ABE, 7.42–8.08 Pa s for liposomes with 1% ABE, and 9.88–10.12 Pa s for liposomes with 5% ABE. This higher deformation is produced by a physical effect, when a low viscosity fluid comes in contact with a high viscosity fluid, the interface does not diffuse uniformly and generates shape fluctuations (Fujiwara & Yanagisawa, 2017). A loss of water to the outside is generated which favors this structural change (Fujiwara & Yanagisawa, 2017). This phenomenon could have occurred during the hydration of lipid films with PBS. In a previous work, we reported that alginate beads with ABE resulted in a higher viscosity of the bead-forming droplet, which generated irregularities in the bead shape (Valenzuela et al., 2014).

Figure 1bII shows an ABE-liposome surrounded by a dark coloration, which is explained by the staining effect, where the surface of the vesicle is stained (Battistelli et al., 2018).

Figure 2 presents FTIR spectra of SL, T80 and ABE (Figure 2a) and ABE-liposomes (Figure 2b). In the case of SL, the most important peaks were found at 2858 and 2929 cm^{-1}, which represent hydrophobic interactions typical of phospholipids ($—CH_2$ vibrations).

Another important peak was found at 1744 cm^{-1}, representing vibrations originating from stretching of C=O bonds and bending H—O—H bonds (Whittinghill et al., 2000). Meanwhile, T80 presented bands that are characteristic of $—CH_2$ stretching (2917 cm^{-1}), C=O stretching (1740 cm^{-1}) and C—O—H stretching (1112 cm^{-1}) (Hillgren et al., 2002). The ABE spectrum shows typical bands of O—H bonds (3305 cm^{-1}) and C—H bonds of CH_2 and CH_3 aliphatic chains (2970 cm^{-1}). It also presented two strong peaks at 1651 and 1538 cm^{-1} that are associated with stretching vibrations of C=O and N—H bonds, respectively (Churio et al., 2018).

Figure 2b shows the interactions in the ABE-liposomes. A different FTIR-spectrum is observed, where the main interactions were O—H bonds located in the FTIR spectrum at 3383 cm^{-1}. This could indicate that new interactions were established after the formation of the ABE-liposomes. O—H interactions could have been established when ABE were dispersed in PBS, where the hydroxyl groups of the amino acids of ABE are exposed in solution and interact with the polar groups of phosphatidylcholine molecules of soy lecithin on the water/membrane interface (Pawlikowska-Pawlęga et al., 2013; Urano et al., 1990; Xi & Guo, 2007). ABE are composed of 93% crude protein (Valenzuela et al., 2014), whose amino acids contain hydroxyl groups on the serine, threonine and tyrosine side chains of hemoglobin (Tristram & Smith, 1963).

Hydrophobic interactions (2978 cm^{-1} band) were rather reduced in ABE-liposomes, unlike those in the original encapsulation materials. Other authors have pointed out that hydrophobic interactions decrease after the formation stage, whereas hydrophilic interactions (O—H bonds) increase where water is present within liposomes (Arsov & Quaroni, 2007; Mady et al., 2009).

The spectrum of ABE-liposomes kept the characteristic peaks of the spectrum of ABE, characterized by vibrations resulting from stretching C=O and N—H bonds (located at 1634 and 1535 cm^{-1}, respectively). Such peaks signal that these liposomes had effectively incorporated ABE, which is consistent with other studies where authors encapsulated ABE within alginate beads (Churio et al., 2018; Valenzuela et al., 2014).

Figure 3 shows the effect of the variables SL:T80 material ratio, ABE content and ultrasonic bath time on the size, zeta potential and iron content of liposomes and ABE-liposomes. Size is an important parameter because it can influence several properties, such as aggregation, pharmacokinetic behavior, absorption and release properties (Hupfeld et al., 2006). Our results showed a size distribution ranging from 347 to 903 nm for liposomes, from 533 to 912 nm for 1% ABE-liposomes and from 379 to 964 for 5% ABE-liposomes (Figure 3a). In the case of liposomes, significant differences were observed with increasing ultrasonic bath time. The longer the time, the smaller the liposome size. This effect was also observed for liposomes with 5% ABE, but was less pronounced for liposomes with 1% ABE. Other authors have also described a significant reduction in liposome size using ultrasonic bath (Papahadjopoulos & Watkins, 1967; Schroeder et al., 2009), because the high-energy collisions between liposomes caused by the ultrasonic waves produced by the equipment cause the rupture of fragments of the phospholipid bilayer, which expose their
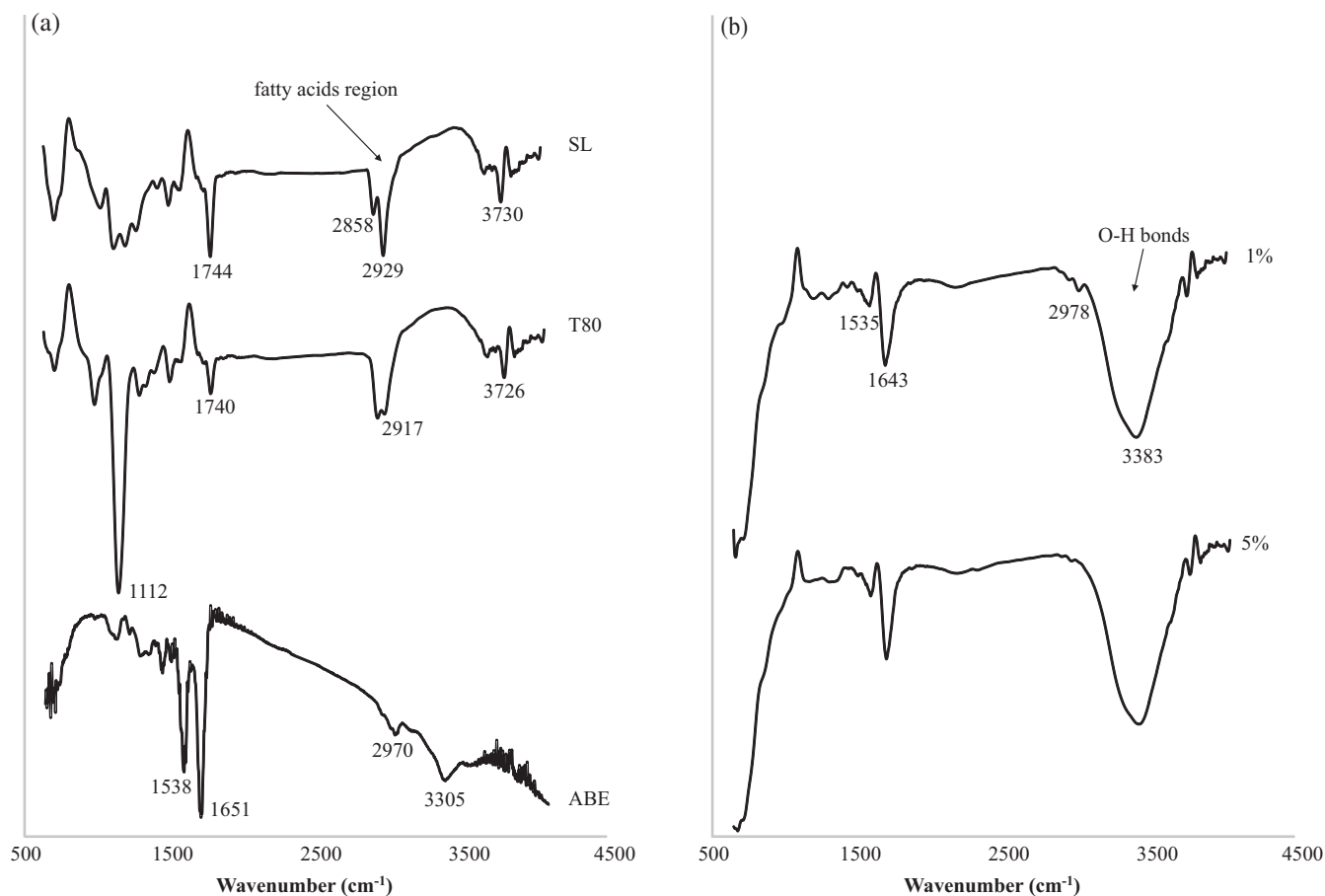
FIGURE 2 FTIR spectra of soy lecithin (SL), Tween 80 (T80) and atomized bovine erythrocytes (ABE) (a) and ABE-liposomes with 1% ABE and ABE-liposomes with 5% ABE (b).

hydrophobic regions to the aqueous phase and reorganize into smaller vesicles (Finer et al., 1972; Lasic, 1988). Ultrasound technology is especially effective in inhibiting agglomeration, reducing particle size and polydispersity through the effect of cavitation (Floris et al., 2013; Koshani & Jafari, 2019; Yamaguchi et al., 2009; Yu et al., 2022). Cavitation can be defined as ultrasonic waves, which release energy over a small area, generating an increase in local temperature and pressure, which upon collision with the particles reduce their volume (Jadhav et al., 2016). The effect of frequency on liposome size reduction may be related to that of cavitation force caused by the difference in bubble dynamics (Yamaguchi et al., 2009).

According to the size classification described by Pandur et al. (2020), the liposomes and ABE-liposomes in this study could be classified as large vesicles (100–1000 nm). The submicron size of the vesicles may favor gastrointestinal absorption compared to larger particles, as they have a greater penetration into the intestinal mucosa (Desai et al., 1996; Florence, 1997). This size is considered more suitable for nutrient vehicleization, since smaller sizes (<100 nm) have lower loading capacity, lower encapsulation efficiency and lower stability due to increased surface energy, and are more prone to rupture (Nakhaei et al., 2021; Pandur et al., 2020).

Figure 3b presents the zeta potential values of liposomes and ABE-liposomes, which were negative, and their absolute value was

high. Significant differences were observed between liposomes and ABE-liposomes. However, there were no significant differences between 1% ABE-liposomes and 5% ABE-liposomes. The importance of the surface charge of the particles lies in their stability in suspension and their absorption at the intestinal level (Guimarães et al., 2021; Niu et al., 2016). In general, liposomes can form a stable suspension when the absolute value of the zeta potential is >30 mV, as the electrical repulsion between particles inhibits coalescence/aggregation and improves storage stability (Chen et al., 2012; Kalra & Bally, 2013). The absolute values of the zeta potential of ABE-liposomes were above 30 mV, so they are considered physically stable particles, which is an important factor for oral delivery of liposomes (Rasti et al., 2012; Wang et al., 2019).

On the other hand, in this study the charge of liposomes and ABE-liposomes was negative, because soybean lecithin is negatively charged (Fricker et al., 2010). The zeta potential was significantly more negative in ABE-liposomes as the concentration of ABE increased, because the surface charge of ABE is also negative (Valenzuela et al., 2014). The negative charge of ABE is related to its composition, mainly by globulin and albumin fractions and the behavior of these proteins at neutral blood pH. For example, the isoelectric point of albumin is 4.9 (Pandey et al., 2019), and its functional groups tend to deprotonate at neutral pH and acquire negative charge,
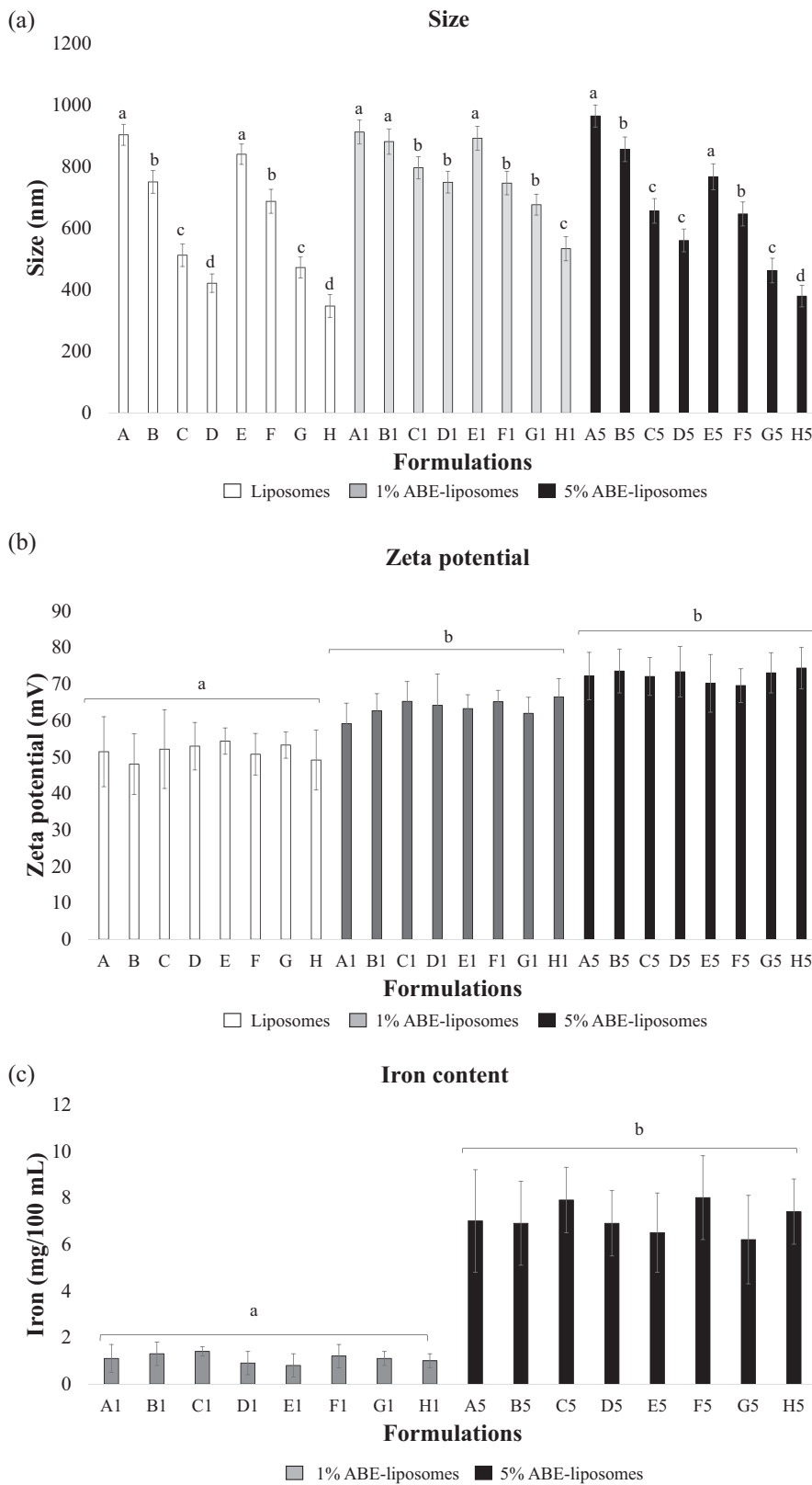
(a)

Size

(b)

Zeta potential

(c)

Iron content

FIGURE 3 Properties of liposomes and ABE-liposomes. Different letters indicate significant differences ($p < 0.05$).

explaining the observed negative zeta potential. It is important to mention that major organic compounds, such as blood, have a negative charge that is not physiologically toxic (Fernandes et al., 2011). Similar results were reported by Yuan et al. (2013) who described that the addition of heme into liposome suspensions resulted in more negative zeta potential values than those of empty liposomes (−56.5 mV vs. −44.1 mV, respectively). The surface charge of particles is related to their behavior as they pass through the gastrointestinal tract. In

general, negative or neutral particles have adequate permeability in the intestinal mucosa (Niu et al., 2016). Huang et al. (2011) developed negatively charged catechin liposomes that showed promising results, as these remained stable under gastrointestinal conditions. Another study by Liu et al. (2015), developed liposomes loaded with bovine serum albumin, which presented a zeta potential of −39 mV, which remained stable after in vitro digestion. Machado et al. (2019) obtained similar results encapsulating phenolic extracts of spirulina in negatively charged liposomes, where they observed that the liposomes were stable under gastric conditions and that the release of the compound occurred at the intestinal level.

Figure 3c presents the iron content values of ABE-liposomes. Liposomes showed very low iron values (between 0.002 and 0.009 mg/100 mL), as these do not contain ABE. The presence of trace amounts of iron in liposomes is explained by its natural abundance in food ingredients such as SL (van Nieuwenhuyzen & Tomás, 2008). In the case of ABE-liposomes, the iron content values increased significantly with the addition of ABE, as expected. The total iron content of ABE-liposomes (0.8–9.0 mg/100 mL) was low compared to iron supplements based on inorganic iron salts, such as liposomes prepared from nonheme iron salts (77–143 mg/g) (Gupta et al., 2015; Kosaraju et al., 2006). These differences are explained by the iron sources used, as inorganic salts, such as ferrous sulfate, contain 178 mg iron/g (Churio et al., 2018). In contrast, ABE contains 2.512 ± 0.226 mg iron/g, of which 99% is heme iron, as ABE is an organic source of iron, consisting mainly of proteins (80%–90%) (Valenzuela et al., 2014) such as hemoglobin, which contains iron (Kosmachevskaya & Topunov, 2018). Importantly, because ABE is composed of erythrocytes, it is considered a more bioavailable source of iron (15%–37%) than inorganic salts (2%–20%) (Björn-Rasmussen et al., 1974; Carpenter & Mahoney, 1992; Conrad & Umbreit, 2000; Pizarro et al., 2016). Heme iron is the least consumed form of iron by the anemic and iron-deficient population in developing countries (Carpenter & Mahoney, 1992), and the development of ABE-liposomes could contribute to increased heme iron consumption in those populations. Pizarro et al. (2016) reported that whole erythrocytes had an iron bioavailability of 25%, far exceeding any of the heme iron sources they tested (hemoglobin, hemin, heme group, meat), which had a bioavailability range between 4.9% and 13.7%. Therefore, the use of ABE-liposomes as supplements or to fortify foods could reduce the need to consume high levels of daily iron to meet the requirement. Gassmann (1991) indicated that the daily intake of iron depends on the bioavailability of the iron sources used in the diet. For example, in diets formulated with sources with a bioavailability of 15%, the recommended daily iron intake is 7 mg/day for children, while diets formulated with sources with 5% bioavailability require 21 mg/day.

There are few studies that have prepared heme iron-based liposomes for nutritional purposes. Yuan et al. (2013) prepared heme liposomes to enrich food to prevent inflammatory anemia, but they did not report the values of the iron content obtained, which was surely low, like our study. These liposomes, when inoculated intraperitoneally in rats, significantly increased hemoglobin compared to unencapsulated heme iron. Another study by Xu et al. (2014) obtained similar results by administering heme iron liposomes intragastrically, in rats with exercise-induced anemia. This work also does not indicate the total iron content of the liposomes. The administration of heme iron liposomes generated a significant increase in hemoglobin, red blood cells, hematocrit, serum iron and liver iron levels compared to nonencapsulated heme iron, and is considered a potential supplement to treat exercise-associated iron deficiency anemia (Xu et al., 2014). These studies suggest that encapsulation of heme iron in liposomes may increase its bioavailability, which is an important and beneficial factor to consider.

Encapsulation efficiencies ranged from 29% to 48% for ABE-liposomes, which is consistent with the 36% previously reported by Yuan et al. (2013) for heme liposomes. Heme iron encapsulation in liposomes is less efficient than nonheme iron sources, such as ferrous sulfate, where encapsulation efficiencies of 58% (Kosaraju et al., 2006), 63% (Jash et al., 2020), 67% (Xia & Xu, 2005) or even 85% (Abbasi & Azari, 2011) are reported. One possible explanation is that the negative charge of ABE generates repulsion against the negative charge of soybean lecithin, which decreases encapsulation efficiency. However, it is important to consider that although heme iron has a lower encapsulation efficiency, it has a higher bioavailability in the body compared to nonheme iron sources (Björn-Rasmussen et al., 1974; Carpenter & Mahoney, 1992).

We observed a significant effect for the variable ultrasonic bath time, as the encapsulation efficiency decreased with longer ultrasonic bath time (Table 1). This could be explained by the fact that the longer the time, the smaller the liposomes and consequently the smaller the volume of charge (Schroeder et al., 2009). Also, the rupture effect produced by ultrasound waves can lead to iron leakage (Abbasi & Azari, 2011; Perkins et al., 1993; Xia & Xu, 2005).

Figure 4 shows the release of iron from ABE liposomes when subjected to gastric and intestinal incubation media. No significant
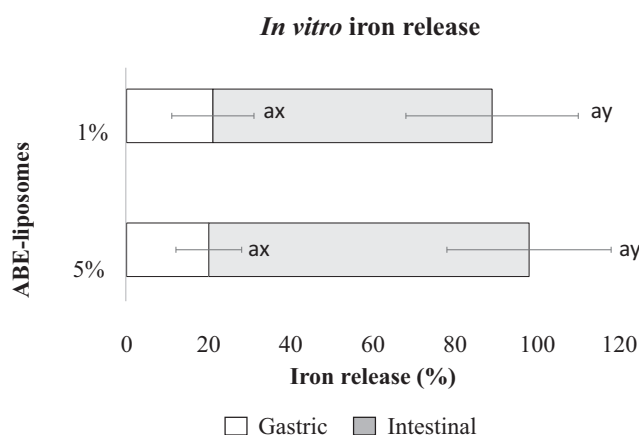

FIGURE 4 Total iron release after incubating ABE-liposomes with 1% and 5% ABE in simulated gastric and intestinal conditions. Different letters indicate statistically significant differences between hemoliposomes formulations ($p < 0.05$). Different letters (x,y) indicate differences between gastric and intestinal incubation ($p < 0.05$).

differences were observed between 1% ABE-liposomes and 5% ABE-liposomes, for gastric and intestinal conditions. However, significantly more iron was released in intestinal condition for both formulations. Thus, iron release was low (<20%) in simulated gastric incubation. This suggests that the liposomes remained stable during gastric incubation, which is consistent with previous studies (Liu et al., 2013; Tian et al., 2019). The iron concentration of ABE-liposomes did not influence their release under gastric mock conditions. The low gastric release is explained by the fact that lipid digestion does not occur primarily in this tract (Acevedo-Fani & Singh, 2022). Therefore, bile fluids and enzymes that could destabilize liposomes, such as lipases, are not present.

Under intestinal conditions, a high iron release occurs for both formulations, where the entire iron contained in the liposomes is eventually released. This phenomenon has been seen in other studies, where the release of the encapsulated compound occurred mainly in the simulated intestinal fluid (Aslan et al., 2023; Tan et al., 2014). This could be explained because bile salts and lipase present in pancreatin destabilize the liposomes, breaking the lipid membrane and thus degrading the encapsulation system (Beltrán et al., 2019; Chen et al., 2021; Liu et al., 2015). Once the encapsulated compound, in this case ABE, is released, it could be absorbed in the small intestine by heme carrier protein (HCP1) (Ma et al., 2010). This release behavior could be considered a benefit of using ABE-liposomes as an oral supplement or to fortify foods, as it allows ABE to be released at the site of absorption, the small intestine. However, further in vivo studies are required to establish whether this encapsulation system could, for example, increase the bioavailability of ABE when released in a controlled and localized manner.

4 | CONCLUSIONS

In this work ABE-liposomes were obtained, characterized as large vesicles (379–964 nm) with negative surface charge (-56 to -74 mV), brown color and an irregular spherical shape. The interactions that formed were mainly hydrophilic, such as hydrogen bonds. The presence of ABE in the liposomes was evident, since the characteristic bands of this material were maintained in the FTIR spectrum. The ABE-liposomes with 5% ABE showed higher iron content (9 mg/100 mL). The encapsulation efficiency ranged from 29% to 48%. In the in vitro digestion assay, iron release from the liposomes occurred mainly at the intestinal level and was very low at the gastric level. Thus, ABE-liposomes could be used as an adjunct to nonheme iron supplements and/or vehicles for fortification, currently used for the prevention and treatment of iron deficiency anemia.

AUTHOR CONTRIBUTIONS

Fabrizzio Valdés: Writing – original draft; writing – review and editing. **Raúl Carrillo:** Investigation; methodology; writing – original draft. **Francisca Campos:** Writing – original draft; writing – review and editing. **Leonardo Sáenz:** Formal analysis; investigation; writing – original draft. **Carolina Valenzuela:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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