EFFECT OF STORAGE ON RHEOLOGY AND HEMOLYSIS OF LIPOSOME-ENCAPSULATED HEMOGLOBIN IN LAMINAR SHEAR FLOW

تأثير التخزين على الريولوجي ونفاذية خضاب الدم في الحجيرات القابضة للخضاب تحت اجهاد التدفق الرقيق

TAHA M. ALKHAMIS
CHEMICAL ENGINEERING DEPARTMENT
MU'TAH UNIVERSITY
P.O.BOX: 7
MU'TAH - KARAK - JORDAN

AND
JOHN L. GOSSAGE AND RICHARD L. BRISSINGER
DEPARTMENT OF CHEMICAL ENGINEERING
ILLINOIS INSTITUTE OF TECHNOLOGY
CHICAGO - ILLINOIS

ملخص

تم في هذه الدراسة انتاج حجيرات قابضة لخضاب الدم (الهيموغلوبين) بطريقة تميه الطبقة الرقيقة. وتم في هذه الدراسة فحص ثباتية هذه الحجيرات من ناحية الخصائص الريولوجية ونفاذيتها لخضاب الدم تحت تأثير مدة التخزين على درجة حرارة 4° م وتأثير اجهاد الانسياب الرقيق.

تشير النتائج ان الخصائص الريولوجية لهذه الحجيرات ثابتة تحت تأثير مدة التخزين وكذلك فان نفاذيتها ايضا ثابتة تحت تأثير مدة التخزين ومجالات الاجهاد . كما ان جودة محتوى خضاب الدم تبقى ثابتة تقريبا بعد اضافة انزيم الكاتاليز عندما يقارن بالخضاب قبل احتوائه وخزنه. والخلاصة فان الطريقة تعتبر واعدة بالنسبة للخصائص المختبرة وان الحجيرات الناتجة بهذه الطريقة يمكن اعتبارها واعدة ايضا.

ABSTRACT

Liposome encapsulated hemoglobin (LEH) are produced in this study by lipid film hemoglobin hydration method LEH produced were tested for rheological and hemolytic stability as a function of storage at 4 °C (as in blood bank practice) and under the influence of laminar shear flow Results indicate that LEH produced by the method described in this study are rheologically stable as a function of storage age LEH samples demonstrated stability with respect to hemolysis as a function of storage age in laminar shear fields Quality of encapsulated Hb as a function of storage age are suggested to be improved by addition of catalase when compared to Hb before it is encapsulated and stored. LEH produced by the method described seem to be promising with respect to the properties tested.

Key Words liposome, hemoglobin, shear field,laminar flow, hemolysis, rheology, viscosity

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INTRODUCTION

Characterization of liposome encapsulated hemoglobin (LEH) and their susceptibility to shear induced damage, represented by monitoring hemolysis, is an important step towards evaluation of LEH efficacy as a red blood cell substitute. Characterization must not be limited to freshly produced samples, but rather to stored ones, since any blood cell substitute has to sustain reasonable shelf life to be considered as a suitable blood replacement. Other properties that any blood replacement must possess in order to be truly desirable involve: adequate oxygen carrying capacity, universal transfusibility, appropriate half life in circulation, no toxic side effects, and sterilizability [1-4].

Previous studies dealt, in one way or another, with most of the above properties [5-8]. However, only limited studies dealt with the property of prolonged shelf life (i e.) stability of LEH with storage in accordance to variation in rheological properties and resistance of such synthetic corpuscles to low shear forces as a function of time.

Simultaneous rheological characterization of blood along with the evaluation of its susceptibility to damage can be considered as a novel approach [9]. The evaluation of such properties as related to LEH indicate the suitability of this product to flow within the circulation. Viscosity of a fluid is considered the basic rheological property. Variation in viscosity helps in determination of a fluid behavior Therefore, evaluation of viscosity as a function of storage time will indicate how effective will the produced LEH to be utilized in the blood exchange process when they are used after a certain shelf life Moreover, integrity of produced synthetic LEH corpuscles under the influence of shear forces and as they aged when they are stored in conditions similar to that used for normal blood storage, are of quite similar importance. Resistance of LEH membranes to shear fields as a function of storage time, will indicate free hemoglobin (Hb) we will expect in the circulation if similar product is used after being stored

The limitation that is experienced in total blood exchange in large animal models involves preparing adequate quantities which has been tried to overcome by searching for suitable scale-up processes [5,8] This practice leads to a requirement for producing certain LEH products that are stable with storage. Moreover, dealing with LEH as a blood substitute on commercial basis require evaluation of their properties as a function of storage.

Therefore, the objective of this experimental study was to establish a clear vision about variation of LEH, produced by film hydrating method, rheological properties and susceptibility to damage under shear. This simultaneous characterization is considered to be a novel approach since it deals with both the rheology of the suspension and the integrity of the synthetic phospholipid membrane The suspension is characterized first as being fresh and the fresh properties are compared to long range properties

MATERIALS AND METHODS

Hb solution: Hb solution is prepared using a modification of the procedure of Farmer and Gaber [10]. To preserve Hb functionality, all steps are performed at 4 °C. Outdated packed red blood cells (RBCs) are washed three times in 1% NaCl solution and centrifuged at 4000Xg in a Beckman J2-21 refrigerated centrifuge (Beckman Instruments, Palo Alto, CA). The washed RBCs are then lysed in deionized water, and then resulting stroma is removed via centrifugation at 30000Xg. The Hb solution is next concentrated to the desired value of approximately 20 g% (20 g/dl) with a Minitan^{R} cross flow ultrafiltration system (Millipore, Bedford, MA) Finally, the concentrated Hb solution is dialyzed overnight in pH 7 4, 30 mM phosphate buffer, after which the following antibiotics are added (all values are per liter) Penicillin; 50000 units, Gentamicin; 40 mg, Polymyxin; 25000 units, and Streptomycin; 50 mg.

Lipid solution Hydrogenated soy phosphatidylcholine (HSPC, American Lecithin Co., Atlanta, GA), cholesterol (Chol, Sigma Chemical Co , St Louis, Mo), dicetyl phosphate (DCP, Sigma), and alpha tocopherol (AT, Sigma) are dissolved in chloroform in the molar ratio of HSPC:Chol:DCP:AT, 5 4 1 0 2

Phosphate buffered saline. Phosphate buffered saline (PBS) is composed of 115 mM NaCl, 3 mM KCl, and 30 mM phosphate at pH 7 4 When used with the liposomes, antibiotics reported above in Hb solution section are added at the same concentration.

Tyrode's albumin solution. Tyrode's albumin solution (TAS) consists of 5.4 mM KCl, 134 mM NaCl, 0.98 mM magnesium sulfate, 9 5 mM sodium phosphate, and 0.35% human albumin (Sigma) at pH 7 4 [11]

Acid citrate dextrose: Acid citrate dextrose, formula A (ACD) is anticoagulant/preservative which contains 22 0 g trisodium citrate, 8.0 g citric acid, 24.5 g dextrose, and enough deionized water to make 1000 ml of solution [12]

Blood: The blood used in this study is obtained from two sources. The outdated packed RBCs used tn make Hb solution is provided by Michael Reese Hospital and Medical Center (Chicago, IL) and comes originally from volunteer blood donors. The blood used as whole blood (WHB) as well as that which is resuspended is obtained from healthy volunteers who had fasted overnight and had not taken medications for 14 days. This blood is collected in plastic tubes containing ACD in the ratio of 15 ml ACD per 100 ml blood and is received by our laboratory 10 minutes of collection.

RBCs suspensions: RBCs are obtained by centrifuging the WHB at 3000Xg for 10 minutes and then removing the plasma. The packed RBCs are next washed three times with either PBS or TAS and then resuspended in the same aqueous phase at a hematocrit (volume percent solids) of 30%. The suspension is then stored at 4 °C until tested. Shear tests on fresh suspensions are carried out within 2

hours of preparation. Part of WHB was stored and tested for viscosity measurements and shear induced hemolysis.

LEH production: Figure 1 shows a schematic of LEH production steps. The lipid solution is dried to a thin, homogeneous film on the walls of a round-bottom flask by means of a rotary evaporator (Buchi, Fisher Scientific, Itasca, IL). Next, a sufficient quantity of concentrated Hb solution is added to the flask to bring the final lipid/Hb concentration to the desired values (i.e.) in this study, either 100 mM/ml (batch 1) or 200 mM/ml (batch 2). At this point, the lipid molecules spontaneously orient themselves into lipid bilayers which then form multilamellar vesicles (MLV) - large (up to 100 mm), multichambered objects which contain Hb solution. The MLV suspension is then processed at 5-7 °C through the Microfluidizer^{R} M-110 (MFZ, Microfluidics Corp., Newton, MA). The MFZ forces the dispersion at 2500 psi through the interaction chamber (figure 2). There are two high velocity jets in the thin microchannels strike each other with such force that MLV are broken down into small unilamellar vesicles (SUV). The dispersion is passed eight times through the MFZ so that the average particle size is reduced by two orders of magnitude (i.e.) from some tens of microns to a few tenths of a micron. Next, the SUV dispersion is washed three times in PBS at 30000Xg to remove the unencapsulated Hb. The SUV are finally resuspended in PBS. At this point, the dispersion is referred to as liposome-encapsulated Hb.

Shear tests: The shearing tests are performed using a modified Weissenberg rheogoniometer model R16/R18 rotational viscometer with a 10 cm aluminum cone (0.3°) in a cone-and-plate geometry. This geometry is extremely useful, since for small rotational velocities used in this study a good approximation to viscometric flow is achieved [13].

Before each run, the surface is cleaned with phosphate detergent solution, rinsed with deionized water, and dried with a cool-air blower. The sample is placed on the lower, conical, platen and the upper, flat, platen is then lowered until the required gap is achieved. Next, the lower platen is rotated for 10 minutes, during which time the viscosity data are obtained. Then the sample is collected for further testing after the shearing period.

For the WHB and RBCs suspension solutions, the sample is centrifuged at 3000Xg for 10 minutes and the supernatant is collected. On the other hand, for LEH solutions the sample is transferred to heparinized microhematocrit tubes and calcium chloride is added to each tube to ensure that the liposomes will spin down. The tubes are next centrifuged to obtain supernatant for aqueous phase Hb concentration determination.

Hb concentration determination: The concentration of Hb the filtered supernatant samples of this study is always quite small, in fact, almost all samples had concentrations below 100 mg%. Therefore, the more traditional spectrophotometric methods of Hb concentration determination such as

the cyanomethemoglobin method of Drabkin [14] cannot be used. Crosby and furth [15], which is reportedly accurate to concentrations as low as 1 mg%, and is described thoroughly elsewhere [2].

In order to measure the concentration of the Hb encapsulated, it is first necessary to dissolve the lipid membrane As found by Stratton and Farmer [2], the detergent n-octyl-beta-D-glucopyranoside will, when heated to 30-35 °C, lyse the vesicles and thus permit the Hb concentration to be determined by Drabkin's method [14]

RESULTS AND DISCUSSION

FRESH SAMPLES RESULTS:

LEH size distribution: The size distributiou for LEH product was obtained on Nicomp particle size analyzer and is shown in figure 3. The average size of the particles proved to be just under 0.3 micron for batch 1 and just over 0.35 micron for batch 2. LEH with such size can easily pass through capillaries of the circulation which exhibit an average diameter of 3 microns Therefore, efficacy with respect to particle size seemed to he proved by this procedure.

Viscosity. Figure 4 shows the viscosity versus shear rate data for WHB, RBCs in PBS, RBCs in TAS, and representative runs from batch 1 and batch 2. In the shear rate range of 500-4500 s^{-1}, all of the suspensions exhibit Newtonian behavior Moreover, the ratio of the viscosity of the RBCs in TAS to the viscosity of the RBCs in PBS solution is 1 17, which is about the same as the ratio of the viscosity of TAS to that of PBS alone. This may indicate that effects of RBCs at this hematocrit (30%) in both suspensions cancel each other. Moreover, interaction between albumin (high molecular weight protein) and RBCs membrane seem not to alter the rheological behavior of TAS except in the obvious increase in the level of viscosity itself.

Figure 5 shows the effect of LEH lipocrit on viscosity as a function of shear rate For purposes of comparison the figure also contains data on WHB viscosity for the same shear rate range employed. Non-Newtonian behavior is observed for all curves below 100 s^{-1}. However, asymptotic value is reached and a Newtonian behavior is observed for all curves above 100 s^{-1} (i.e.)for physiological conditions This may indicate a olose rheological behavior is seen for WHB and LEH produced by the process described in this study. Lipocrit is also seen to affect viscosity of LEH suspensions. Moreover, it is obvious that viscosity of 50% lipocrit LEH suspension is close to 43% hematocrit WHB, which may suggest that exchange of WHB with LEH of this concentration seems to be feasible with respect to their rheological behavior

Effect of the suspending medium on viscosity is shown in figure 6. All curves show Newtonian behavior within the shear rate range studied (500 - 4500 s^{-1}). Suspensions of both LEH and RBCs in PBS show the lowest viscosity values, never the less TAS suspensions do not exhibit significantly high

viscosity This may indicate that addition of material that is required for balancing colloid osmotic pressure, such as albumin or may be dextran, does not really alter the rheological properties of LEH suspensions significantly. Therefore, LEH produced can still be modified to contain colloidal material in the outer aqueous phase. Suspension of 7.5% albumin in PBS is presented in the figure, and when compared to the curve of 30% RBCs in TAS, it can be seen that the viscosity increased due to existence of cells but to a value that is lower than that of 43% WHB.

STORED SAMPLES RESULTS:

Viscosity Effect of storage on LEH viscosity as compared to WHB is shown in figure 7. Three distinct viscosity regions are obvious in the figure The lowest viscosity values are for 30% LEH of 100 mM lipid loading, followed by those for 34% LEH of 200 mM lipid loading, and finally the highest values are for WHB samples. For all curves it is obvious that storage age does not have any effect on viscosity. Therefore, rheological stability as a function of storage age can be assumed for the produced LEH Differences in viscosities of batch 1 LEH and Batch 2 are due to lipocrit (28% to 34%) and to the lipid loading (1 to 2).

Hb quality: Figure 8 shows results on the quality of LEH encapsulate Hb as a function of storage age compared to free Hb which is stored at the same conditions. Functional Hb represents oxy and reduced forms of Hb, Where as non functional Hb represent met-Hb form.Storage seems to affect Hb quality of encapsulated type, while stored free Hb was not significantly affected. However, a plateau (an asymptotic value) seems to be reached for LEH-Hb curve after 7 days of storage. Conversion rate of oxy to met form of Hb appears to be slow after the first week of storage. After 32 days of storage the change in percent conversion was about 12% while it was about 1.4% for free stored Hb Existence of synthetic phospholipids seems to catalyze Hb oxidation. Therefore addition of certain enzymes, that are known to preserve Hb, such as catalase [7] may help in stabilization of encapsulated Hb quality.

Shear induced hemolysis: Hemolysis percent as a function of shear for fresh samples of RBCs suspensions in either PBS or TAS, WHB, and LEH batches are presented in figure 9. Higher percent hemolysis is observed for RBCs suspensions than other samples. Initial percent hemolysis for these samples as well as aged samples are shown in table 1. Changes in percent hemolysis for all samples as a function of shear rate were not significant. All curves show a highest hemolysis level of 1% which is not physiologically significant. In fact, LEH samples are seen to sustain shear rate more than WHB or RBCs suspensions. Therefore, LEH membranes can be considered stable under the influence of shear fields

Figure 10 shows percent hemolysis of different LEH samples as a function of storage age and shear rate and are compared to WHB. Shear rate and storage age seem not to influence the hemolysis

level of all samples tested. It is, therefore, reasonable to suggest that LEH membranes are stable against application of shear as a function of storage age. It is also obvious that percent hemolysis levels are all below 1% which can be considered physiologically not significant. Therefore, we experience a reasonably good LEH product with respect to hemolysis as a function of storage age in shear fields.

In conclusion LEH produced by the method described in this study are rheologically stable as a function of storage age. LEH samples demonstrated stability with respect to hemolysis as a function of storage age in laminar shear fields. Quality of encapsulated Hb as a function of storage age can be improved by addition of catalase to Hb before it is encapsulated and stored. LEH produced by the method described seem to be promising with respect to the properties tested.

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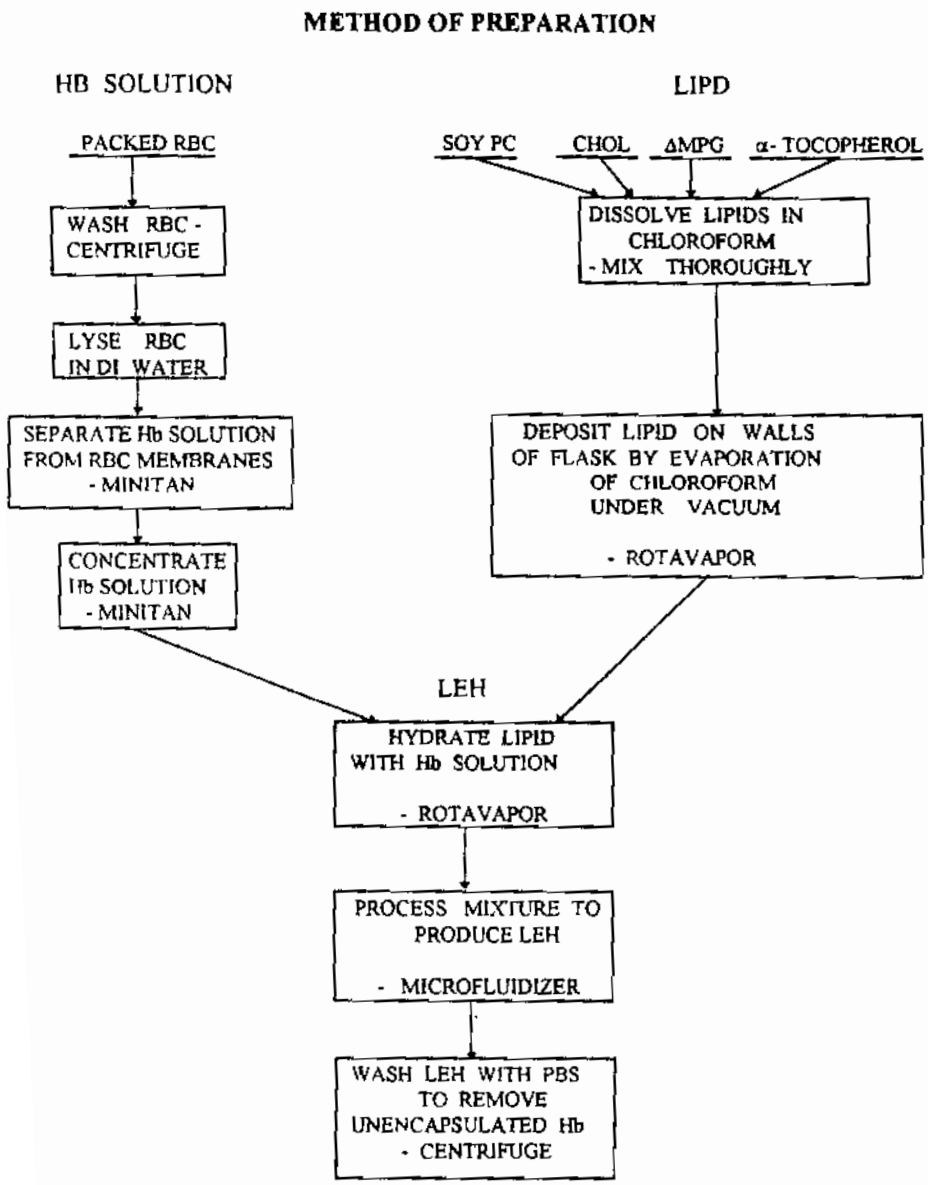


Figure 1 : Schematic of LEH production process

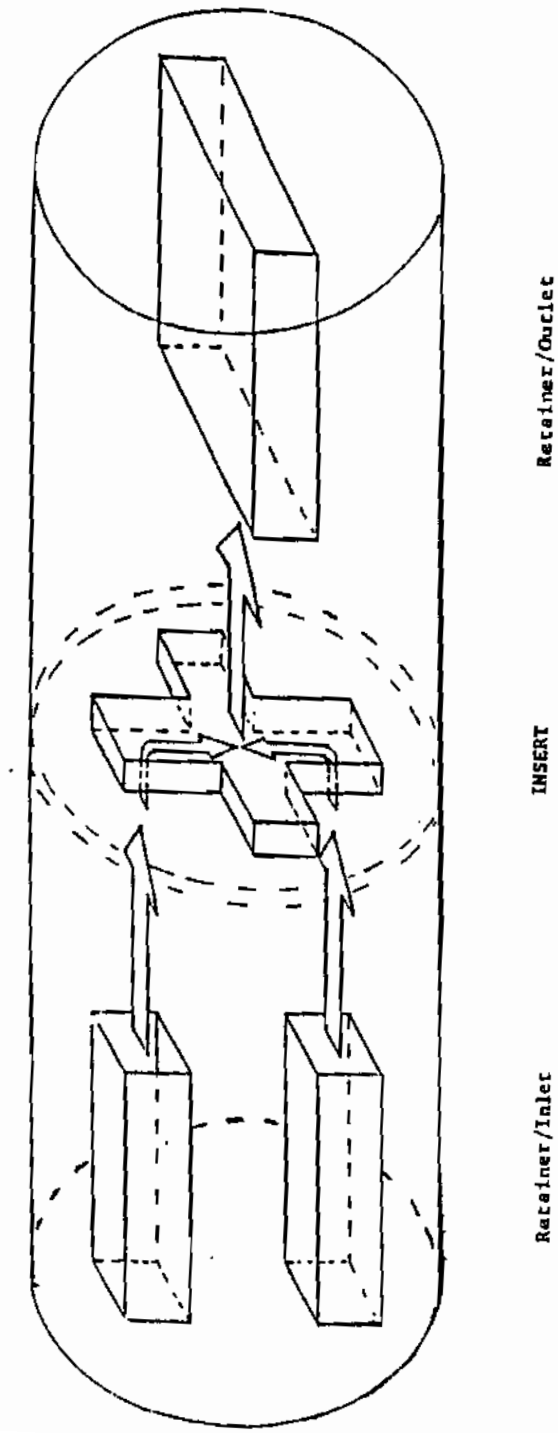


Figure 2: Schematic of Microfluidizer interaction chamber with flow patterns

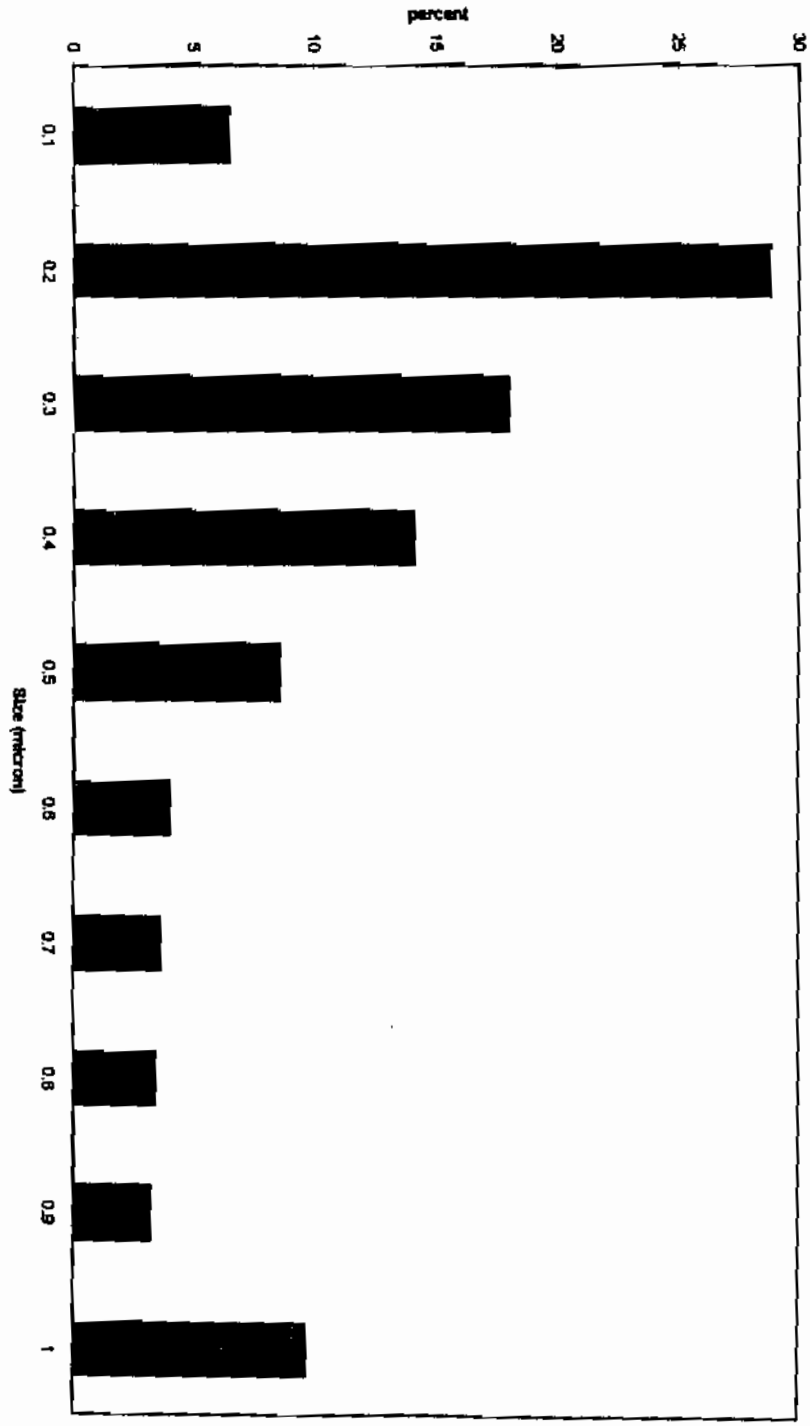


Figure 3: Size distribution of LEH

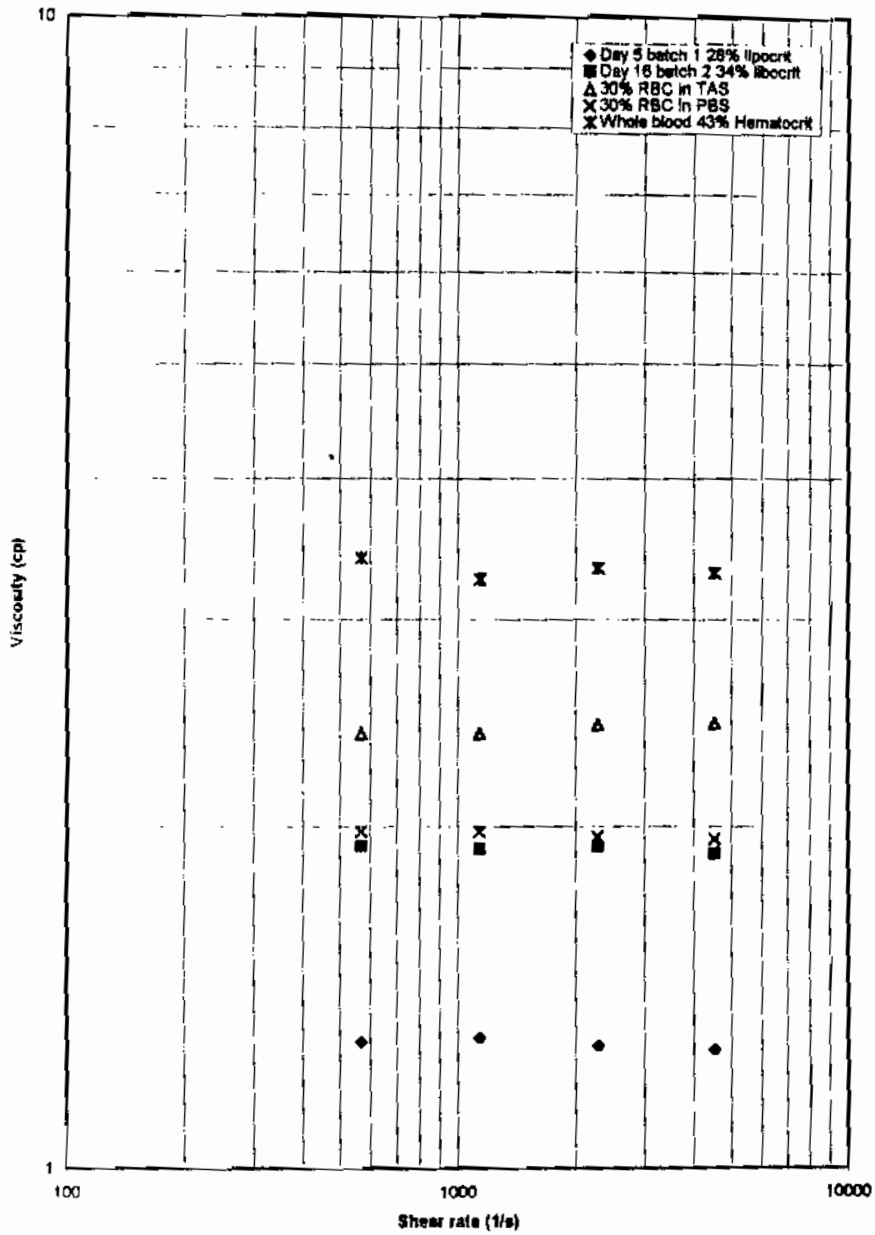


Figure 4: Viscosity versus Shear rate for representative tested samples

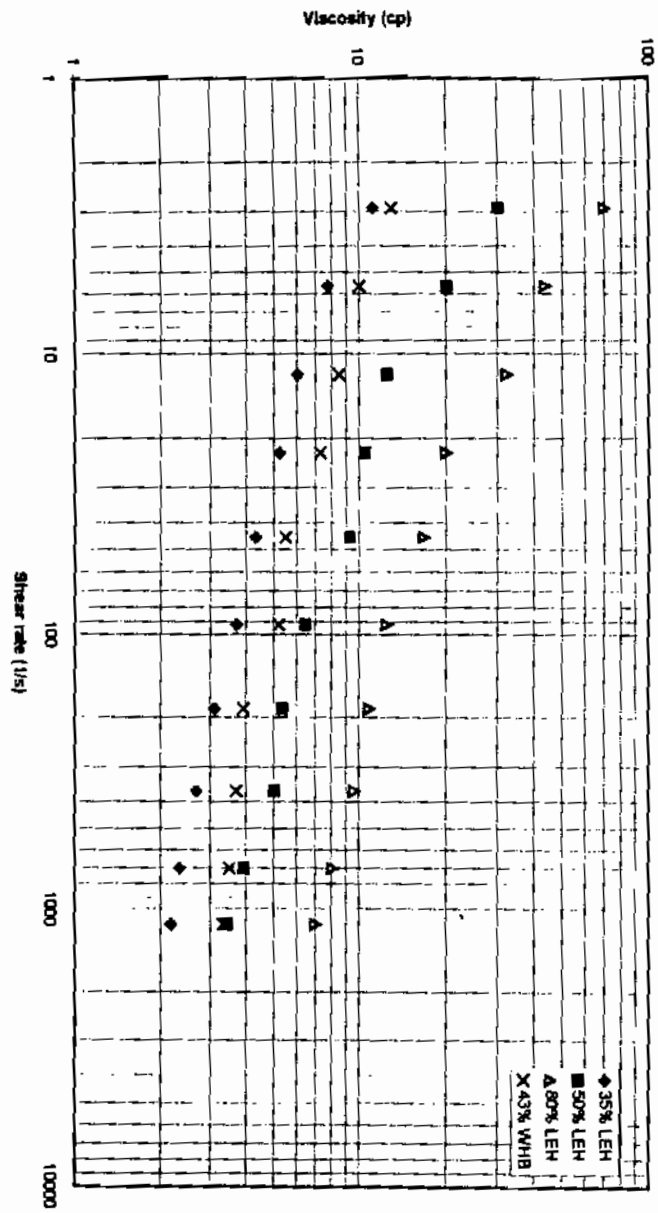
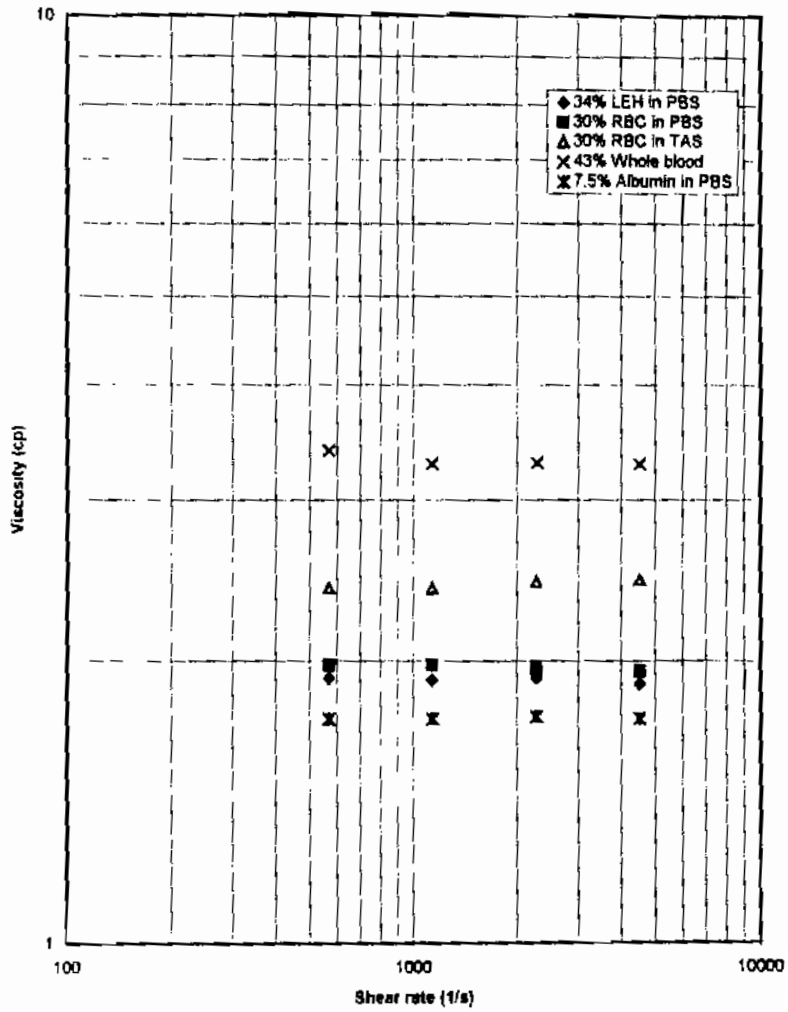


Figure 5:Viscosity of different libocrit LEH samples compared to whole blood as a function of shear rate



Figere 6: Effect of suspending medium on RBCs viscosities as compared to LEH viacosity as a function of shear rate

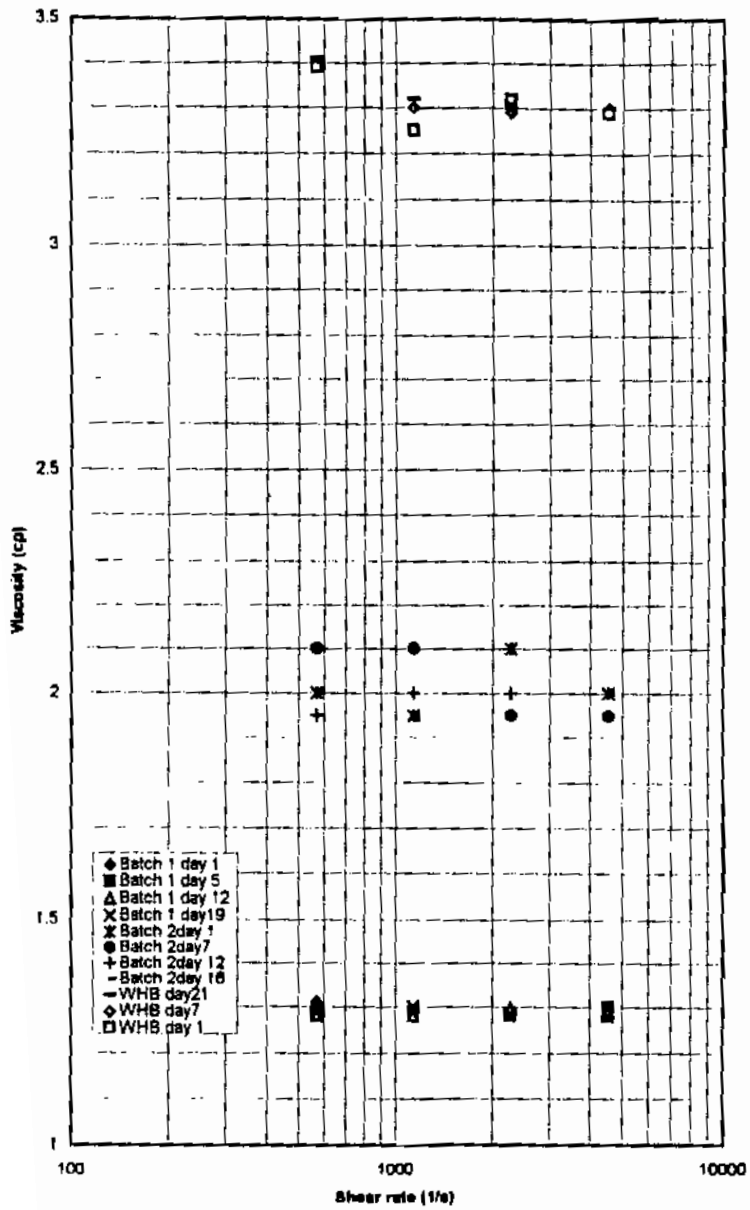


Figure 7: Effect of storage on LEH viscosity as a function of shear rate

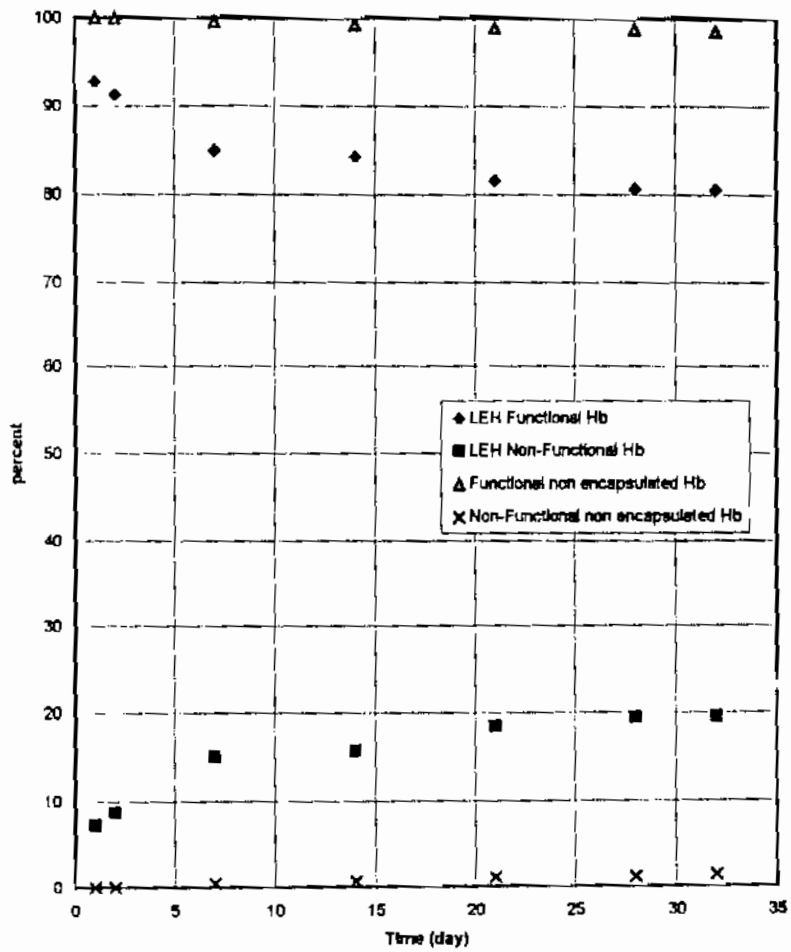


Figure 8: Effect of storage on encapsulated Hb quality as compared to non encapsulated Hb

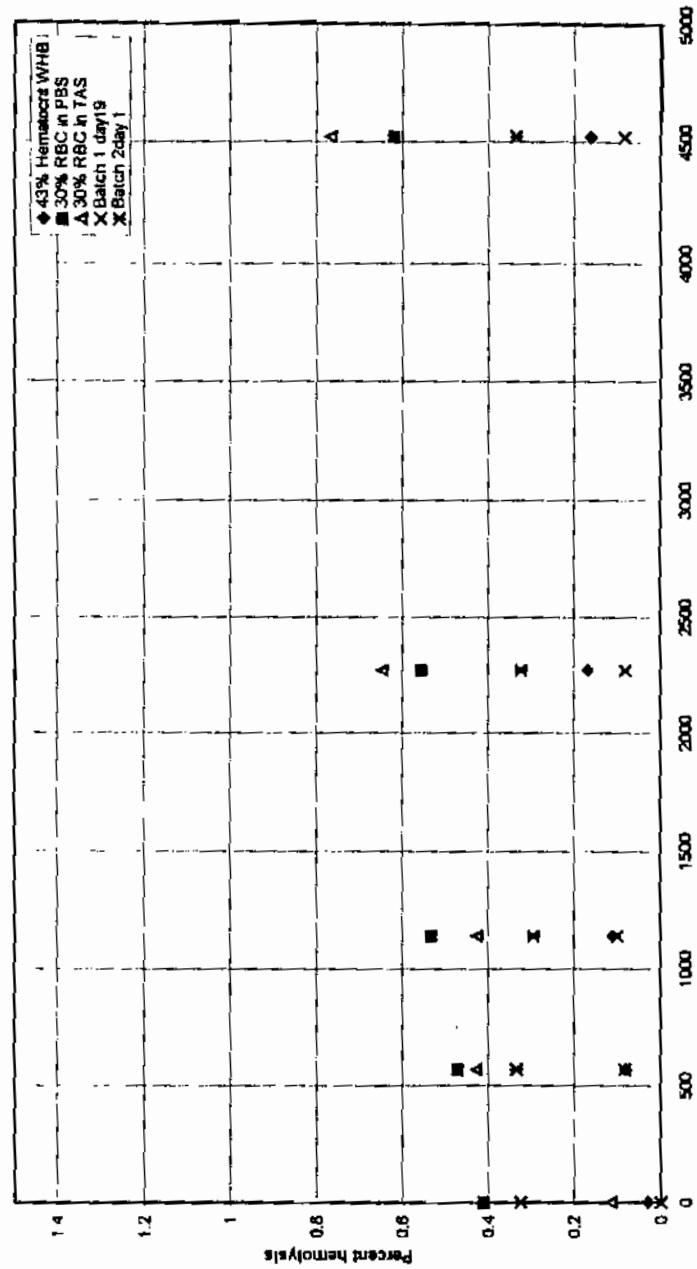


Figure 9: Percent hemolysis of LEH as compared to RBC suspensions

SAMPLE	PERCENT HEMOLYSIS
Batch 1 day 1	0
Batch 1 day 12	.042
Batch 1 day 19	.042
Batch 2 day 1	.22
Batch 2 day 8	.32
Batch 2 day 22	.32
43% WHB day 1	.02
30% RBC in PBS day 1	.312
30% RBC in TAS day 1	.07
43% WHB day 7	.02
43% WHB day 21	.09

Table 1: Initial percent hemolysis as a function of age for LEH samples as compared to blood

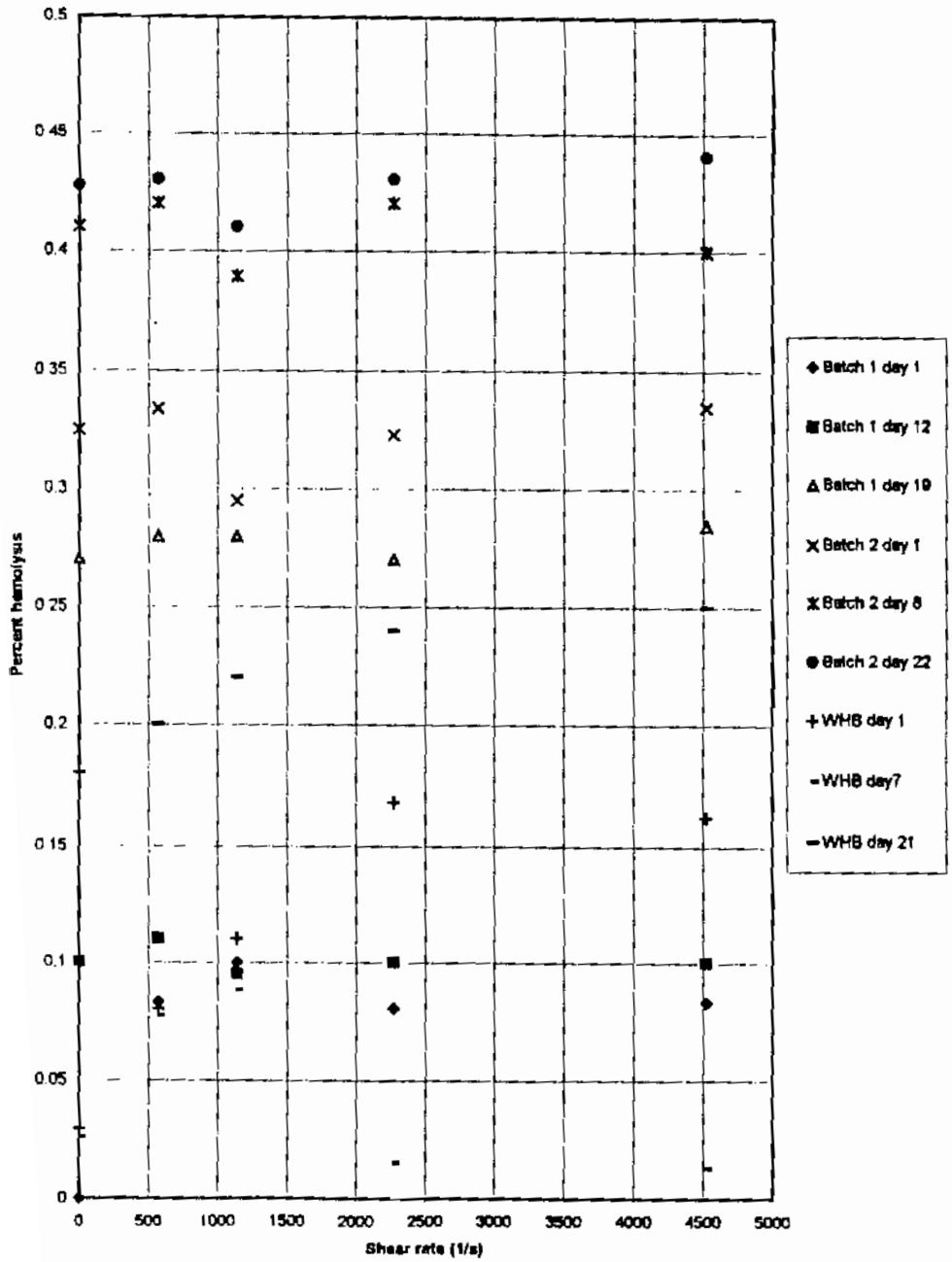


Figure 10: Percent hemolysis as a function of shear rate and age