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Stability of erythrocyte ghosts: A γ-ray perturbed angular correlation study

(drug delivery/enzyme replacement/γ-ray probe)

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ABSTRACT **The structural integrity of erythrocyte ghosts made by the preswell and slow-dialysis techniques has been studied *in vitro* by use of γ-ray perturbed angular correlation (PAC) techniques and also by standard *in vitro* leakage methods employing sequestered labeled markers. Complexes of $^{111}In^{3+}$ and nitrilotriacetate were encapsulated in ghosts made from human, rabbit, rat, and mouse erythrocytes, and their leakage was monitored by both methods. In addition, ^{125}I-labeled bovine serum albumin was encapsulated, and ghost integrity was monitored by conventional leakage measurements. With the PAC technique the percentage of material released from human ghosts was determined quantitatively, and the results were equivalent to those obtained by the conventional method. In addition, at various times after intravenous injection, tissue distribution of the ghosts in the mouse was studied. The percent injected dose per gram of tissue of the labeled surface proteins of erythrocyte ghosts in circulation approximated that of the entrapped labeled albumin. This suggests that the ghost membrane and contents are strongly associated *in vivo*. Large ^{125}I-labeled bovine serum albumin molecules and small $^{111}In^{3+}$–nitrilotriacetate complexes were delivered in high quantities to the lung initially, and to the liver and spleen. Because erythrocyte ghosts have the ability to entrap a wide range of substances and deliver them to specific organs, ghosts may be preferable to other drug carriers or drug therapy for treatment of certain disorders.**

An attractive strategy for the *in vivo* delivery of biologically active molecules is the encapsulation of drugs or enzymes in erythrocytes. As carriers, erythrocytes offer several advantages over other systems: they can entrap large volumes of concentrated loading material, and they can avoid host immunologic reactions if they are from isologous or autologous blood. Different enzymes and drugs have been successfully encapsulated by erythrocytes and delivered to specific cell and tissue sites in animal models (1–3). Methods of entrapment, such as the preswell, rapid-lysis, and slow-dialysis methods, involve variations of erythrocyte osmotic lysing and resealing (4, 5); methods such as applying an external pulsed electric field or drug treatment induce membrane permeability changes or endocytic activity in erythrocytes (1, 4, 6). The successful entrapment of various active enzymes, transport systems, and drugs has been demonstrated (1–9). To develop the erythrocyte ghosts as practical carrier systems, we have studied the leakage of entrapped substances from ghosts of different species prepared by various methods, and we have studied the fate of the erythrocyte ghosts after administration *in vivo*.

γ-Ray perturbed angular correlation (PAC) techniques have been used to monitor the changes of lipid vesicles of different compositions *in vitro* and *in vivo* (10, 11). With PAC, Hwang and Mauk (12) showed that the integrity of liposomes *in vivo* can be assessed by monitoring changes of rotational correlation time of $^{111}In^{3+}$ encapsulated in the carrier: a high tumbling rate is observed when the encapsulated $^{111}In^{3+}$ is chelated to small molecules; the tumbling rate decreases if $^{111}In^{3+}$ is released from the carrier and binds to proteins in circulation or to serum proteins *in vitro*. In this paper we describe the application of the PAC technique to characterize the integrity of annealed erythrocyte ghosts from mouse, rat, rabbit, and human sources made by the preswell and dialysis techniques. The strategy applied to monitor the integrity of lipid vesicles is thus extended to erythrocyte ghosts, alternative model membrane carriers for drugs and enzymes.

MATERIALS AND METHODS

Preparation of Labeled Erythrocyte Ghosts. Intact human erythrocytes were isolated from freshly drawn heparinized blood. Whole blood was centrifuged at 1,000 × *g* for 10 min at 4°C. The serum and buffy coat were removed and the packed cells were washed three times in 10 vol of phosphate-buffered saline (P_i/NaCl; 5 mM sodium phosphate buffer, pH 7.4/0.15 M NaCl). Freshly drawn blood from rabbits (New Zealand), rats (Fischer 433), or mice (Swiss–Webster) was obtained from LabPets (Pasadena, CA) and washed as described for the human erythrocytes.

Erythrocyte ghosts were made by either the preswell or the slow-dialysis techniques. The two techniques are variations of osmotic lysing that differ from each other by the volume of hypotonic saline used to hemolyze the cells and the rate at which the cells swell before lysis. With both techniques, conservation of the loading substance can be achieved because the ratio of erythrocytes to lysis buffer is small (5).

Preswell erythrocyte ghosts were loaded with a 10 mg/ml solution of ^{125}I-labeled bovine serum albumin (^{125}I-albumin) as described (5). The preparation of slow-dialysis erythrocyte ghosts (Fig. 1) loaded with ^{125}I-albumin follows that described by Kruse *et al.* (13) with modifications: (*i*) nitrilotriacetate (NTA; 1 mM) was present in the buffer solutions during the lysis and resealing steps so that chelator scavengers could be eliminated from the system, (*ii*) the annealing period was extended to 30 min because the membranes leaked less of the entrapped markers, (*iii*) ghosts were pelleted at 12,000 × *g* for 10 s with an Eppendorf microcentrifuge. Radioiodination of the albumin was performed with IODO-GEN (Pierce) (14). Typically, 0.5 mCi (1 Ci = 3.7 × 10^{10} Bq) of $Na^{125}I$ was used for iodination, and after desalting on Sephadex G-50, the ^{125}I-albumin was added to albumin in P_i/NaCl (10 mg/ml) and this solution was used for

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Abbreviations: PAC, perturbed angular correlation; P_i/NaCl, phosphate-buffered saline; NTA, nitrilotriacetate.

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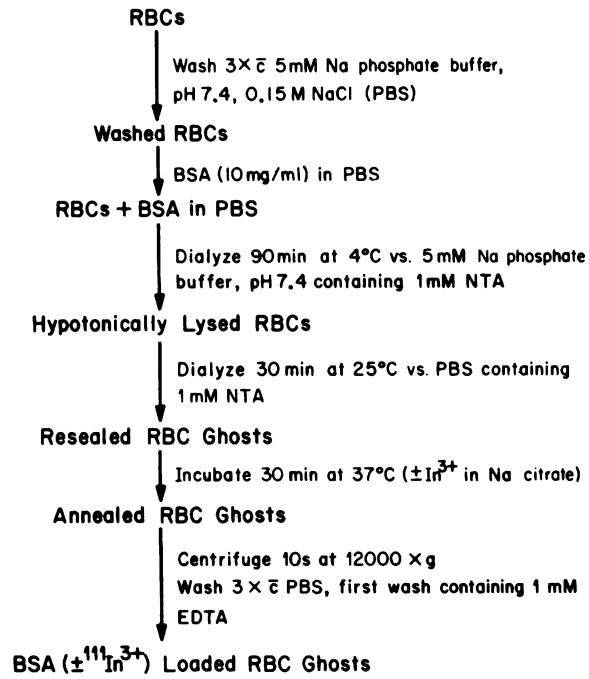

FIG. 1. Erythrocyte loading by the slow-dialysis method. RBCs, erythrocytes; BSA, bovine serum albumin; c̄, with; PBS, P_i/NaCl.

loading erythrocytes in a ratio of albumin to packed erythrocytes of 1:1.5. Erythrocyte surface proteins were labeled in the same manner, with 0.1 ml of washed, packed erythrocytes; free iodine was removed by dialysis for 1 hr at 4°C. A short dialysis time was preferable because it closely reflected the time used for ghost preparation and resulted in minimal crenation of the intact cells. $^{111}In^{3+}$-Loaded erythrocyte ghosts were prepared by a similar procedure. Unlabeled albumin was used as before to maintain the protein concentration; during the 37°C annealing step 50–100 μCi of $^{111}In^{3+}$ in 2 mM HCl plus 30 μl of 3.4 μM $InCl_3$ in 0.1 μM sodium citrate (pH 7.4) and 30 μl of 2× P_i/NaCl were added to 0.5 ml of packed erythrocyte ghosts. To obtain erythrocyte ghosts with a high specific activity the label was added at the annealing step. Carrier-free $^{111}InCl_3$ (Medi+Physics, Emeryville, CA) was chromatographed on a Bio-Rad AG 1X-8 column (0.7 × 10 cm) in 0.1 M HCl. The peak fraction was collected in a 10-ml disposable polystyrene beaker, dried under a heat lamp, and reconstituted in 0.1 ml of 0.002 M HCl (≈3 mCi/0.1 ml). Portions of this stock were taken for erythrocyte loading experiments. The calcium ionophore A23187 did not appreciably increase the uptake of $^{111}In^{3+}$ by the ghosts as it does for vesicles (15) in the concentration 0.04 μmol of A23187 per ml of packed erythrocytes; thus it was not used in the experiments reported here. The washing was as described (13) except the first wash was with 10 mM EDTA in P_i/NaCl to facilitate removal of free radioactive $^{111}In^{3+}$ and that which is nonspecifically bound to the cell surface. The erythrocytes were suspended in P_i/NaCl or in serum (1:1) for PAC measurements.

***In Vitro* Studies.** For *in vitro* leakage studies a suspension of erythrocyte ghosts in P_i/NaCl was made and equal portions were put into 1.5-ml microcentrifuge tubes. After incubation at 37°C for given periods, the tubes were centrifuged in the microcentrifuge for 10 s to pellet the cells. The supernatant and one wash of the ghost pellet with P_i/NaCl were collected along with the pellet. The samples were placed in 12 × 75 mm tubes and the γ-ray activity was measured.

***In Vivo* Studies.** A suspension of labeled mouse erythrocyte ghosts (0.2 ml) was administered to Swiss–Webster mice (18–25 g) by intravenous (tail vein) injection. The amount of $^{111}In^{3+}$ or ^{125}I-albumin administered ranged between 1 and 20 μCi per mouse. As controls, some mice were given 0.2-ml injections of free label (e.g., $^{111}In^{3+}$ or ^{125}I-albumin) in P_i/NaCl. Also, a full dose (0.2 ml) and an aliquot (0.1 ml) were obtained from the syringe and analyzed for radioactivity. The tissue distribution of injected radioactivity was determined by assaying washed and blotted tissues of the mouse in a γ-ray spectrometer. The tissue distribution of label was measured at various intervals after intravenous injection of radiolabeled ghosts. We assumed that blood made up 7.3% of the total body weight of the animal. Mice receiving an imperfect injection (e.g., >10% of the injected dose per gram of tissue for the tail) were eliminated from consideration. Typically, 3 or 4 mice were used for each group of time points.

PAC Spectroscopy. The PAC technique was developed to monitor the changes in the rotational correlation time of suitable radioactive nuclei bound to various molecules (16). The technique requires the use of a radioactive nucleus that decays by emitting two γ-rays in succession. The $^{111}In^{3+}$ we employ here undergoes electron capture and subsequently decays, emitting two γ-rays with energies of 173 and 247 keV. A correlation exists between the directions of propagation of the emitted γ-rays, which are detected by a coincidence spectrometer. The angular correlation can be perturbed if, in the time between emission of the first and second gamma rays, the nucleus interacts appreciably with its environment. PAC studies provide information concerning changes in the tumbling rate of $^{111}In^{3+}$ bound to molecules with different rotational correlation times. Carrier-entrapped $^{111}In^{3+}$ chelated to a small molecule has a short rotational correlation time. If the $^{111}In^{3+}$ probe is released from the carrier and binds to a macromolecule, its rotational correlation time increases substantially. Monitoring the average rotational correlation time of the $^{111}In^{3+}$ probe thus provides a measure of the integrity of the carrier *in vitro* or *in vivo*.

RESULTS

Slow-dialysis and preswell methods were used for loading the $^{111}In^{3+}$ cation into erythrocyte ghosts. When human and mouse erythrocyte ghosts were annealed for 30 min or longer, the membranes leaked less of the entrapped markers (e.g., $^{111}In^{3+}$ and ^{125}I-albumin). The preswell ghosts leaked more $^{111}In^{3+}$ than did the slow-dialysis ghosts, which suggests that the preswell procedure for making ghosts is more harsh.

Measurements of $\langle G_{22}(\infty)\rangle$, a quantity proportional to the rotational correlation time for $^{111}In^{3+}$ in various environments, were obtained by PAC spectroscopy (Table 1). The G_{22} values were about the same for the $^{111}In^{3+}$–NTA complex in the presence of albumin, intact human erythrocytes, and annealed erythrocyte ghosts. Thus, nonspecific binding of the $^{111}In^{3+}$ to these components was not significant; the rotational correlation time of $^{111}In^{3+}$–NTA entrapped within the erythrocyte ghosts was less than that for the free complex, indicating a somewhat higher viscosity inside the resealed ghosts. The slow-dialysis human ghosts were fairly stable as determined by PAC. When incubated with serum at 37°C, human ghost leakage determined from G_{22} values always was between 10% and 20% after 2 days. The leakage *in vitro* for ghosts of erythrocytes from other animal species could not be reliably determined by PAC measurements because of high initial leakage rates of entrapped markers.

In vitro leakage of entrapped markers from erythrocyte ghosts made from different species was also monitored by conventional techniques. The ghosts were suspended in P_i/NaCl and incu-

Table 1. Value of $\langle G_{22}(\infty)\rangle$ for $^{111}In^{3+}$ in various environments

	Sample	$\langle G_{22}(\infty)\rangle$
I	NTA–$^{111}In^{3+}$ complex in P_i/NaCl	0.75 ± 0.04
	With serum (1:1)	0.30 ± 0.01
	With albumin (1 mg/ml; 1:1)	0.78 ± 0.05
	With intact human erythrocytes (1:1)	0.70 ± 0.04
	With reannealed human ghosts (1:1)	0.77 ± 0.01
II	$^{111}In^{3+}$–albumin-loaded human ghosts, slow dialysis	0.61 ± 0.02
	With serum	0.56 ± 0.03
	With serum and isopropyl alcohol	0.30 ± 0.01
	$^{111}In^{3+}$–albumin-loaded human ghosts, preswell	0.64 ± 0.01
	With serum	0.57 ± 0.01
	With serum and isopropyl alcohol	0.32 ± 0.02

PAC measurements of $^{111}In^{3+}$ in the presence of components present in significant quantities during the loading of erythrocytes or entrapped within erythrocyte ghosts. The G_{22} values for the first group of results (I) are the mean ± SD calculated from values obtained from monitoring the sample for at least 2 hr. The G_{22} values for the second group of results (II) are initial values obtained approximately 15 min after the final wash step in the sample preparation, except for the samples with isopropyl alcohol, the values of which were obtained after monitoring the sample for at least 2 hr. The isopropyl alcohol is added to disrupt the integrity of the ghosts.

bated at 37°C for various periods. Fig. 2 shows the percent of labeled marker (cpm) remaining with the ghosts at given times. The human ghosts were remarkably stable over a 26-hr period under the given conditions. The erythrocyte ghosts of other animal species exhibited a much greater leakage of contents, whether determined by the very small $^{111}In^{3+}$ marker or the larger albumin marker. Rabbit and rat erythrocyte ghosts exhibited a high initial loss of contents followed by a slow release of entrapped markers over a 26-hr period. The integrity of the mouse ghosts was good initially; however, they steadily lost a major portion of the markers within the first 4 hr of incubation.

Two different labels ($^{111}In^{3+}$ and ^{125}I) were used to study the tissue distributions of mouse erythrocytes and ghosts. Both the $^{111}In^{3+}$–NTA complex and ^{125}I-albumin were used as internal aqueous markers. In addition, ^{125}I was used to label the surface proteins of annealed ghosts and intact erythrocytes from mice.

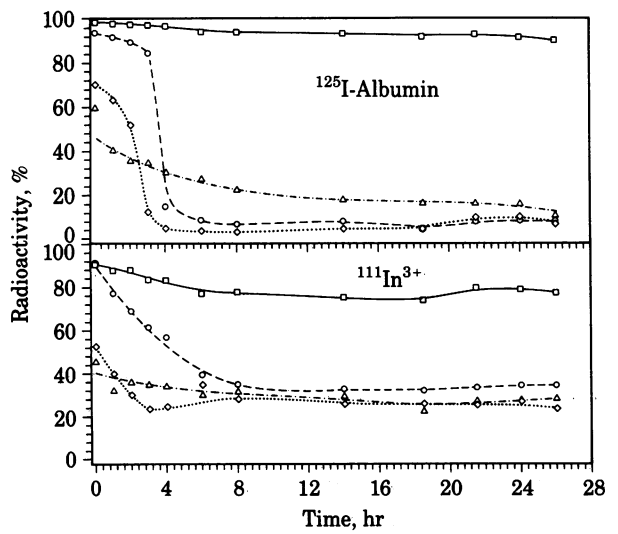

FIG. 2. Integrity of ^{125}I-albumin and $^{111}In^{3+}$-loaded erythrocyte ghosts, made by the slow dialysis technique. Human (□), rabbit (△), rat (◇), and mouse (○) ghost leakage was determined from the γ-ray activity of supernatant, wash, and ghost pellet at given times.

Table 2 contains data showing the disposition *in vivo* of labeled marker after intravenous injection of $^{111}In^{3+}$–loaded mouse ghosts, ^{125}I-albumin-loaded mouse ghosts, ^{125}I-labeled mouse ghosts, and ^{125}I-labeled mouse erythrocytes into mice after given times. $^{111}In^{3+}$-Loaded mouse ghosts rapidly cleared from circulation; a high percentage of the injected ghosts (per gram of tissue) initially was found in the lung. The liver and spleen, in 30 min, showed maximal accumulation of activity.

The ^{125}I-albumin-loaded mouse erythrocyte data closely paralleled the ^{125}I-labeled erythrocyte ghost protein data (e.g., label of carrier versus the carried). This parallelism, however, was not obtained for the ^{125}I-labeled ghosts and the ^{125}I-labeled erythrocytes. Similar to $^{111}In^{3+}$-loaded ghosts, high values (percent injected dose per gram of tissue) for the ^{125}I-labeled ghosts were found in the spleen and liver. Conversely, intact erythrocytes were maintained in circulation longer and not filtered by the spleen as dramatically as the ghosts. The free labels ($^{111}In^{3+}$–NTA complex and ^{125}I-albumin in P_i/NaCl) injected into mice appeared in the blood and kidney at higher levels than when entrapped in ghosts. These findings suggest that a sizeable quantity of loaded material was released from the mouse ghosts shortly after they were annealed. This initial loss of loaded material gave rise to the unexpectedly high activity found in the kidney and blood with respect to $^{111}In^{3+}$- and ^{125}I-albumin-loaded ghosts. These data are consistent with our *in vitro* findings, which also indicated a high initial loss of activity from the ghosts.

DISCUSSION

Previous PAC investigations have been performed on fairly homogeneous liposome preparations. The erythrocyte ghost preparations analyzed by PAC in this report are complex mixtures (17), containing components of varying stability. A heterogeneous population of ghosts may result from the loading procedures, and, in fact, there is evidence that irreversible alterations of the membrane occur, mainly due to the temporary exposure of the membrane to low ionic strength. In this study, the ratio of white ghosts to red ghosts increased with time. Thus, the white ghosts could represent the most stable population of loaded ghosts formed. We found that PAC measurements for human ghosts were reproducible; a sufficient differential in the G_{22} values allowed us to analyze reliably the leakage of entrapped markers. The greater stability of the human ghosts would make them useful for *in vivo* studies by PAC. However, the starting populations of ghosts made from rabbit, rat, and mouse erythrocytes had leakage rates so great that resulting G_{22} values were too low to allow reliable analysis by PAC. The *in vitro* leakage studies by the conventional method are consistent with this observation.

Fiddler *et al.* (4) entrapped β-glucuronidase, glucose, and inulin in human erythrocytes. After a 3-hr 37°C incubation in buffered whole blood, less than 1% leakage of the enzyme and less than 11% leakage of glucose or inulin occurred. Our findings with the albumin marker are in general agreement with their results. It was demonstrated recently that human erythrocyte ghosts with entrapped Ca^{2+} chelated to EGTA and dextran (M_r 80,000) did not leak Ca^{2+} after washing and subsequent incubation for up to 90 min at 37°C (18). This observation is consistent with the results reported here for human erythrocyte ghosts with entrapped In^{3+} chelated to NTA. Thus, human erythrocyte ghosts can act as stable containers of even very small components, especially when transport systems for those components are not present in the carrier membrane.

Erythrocytes from various animal species are basically of similar composition, yet significant differences exist for each kind that presumably improve their survival in their host. The mouse

Table 2. Disposition of $^{111}In^{3+}$ and ^{125}I-albumin in mouse tissues after intravenous injection of erythrocyte ghost-entrapped labels and free labels at given times

Sample	Time, min	% injected dose per gram of tissue				
		Blood	Lung	Liver	Spleen	Kidney
$^{111}In^{3+}$–Albumin-loaded ghosts	15	5.8 ± 1.9 (28.5)	29.4 ± 8.7 (13.2)	26.9 ± 10.6 (6.6)	36.3 ± 19.6 (6.6)	14.7 ± 6.2 (26.3)
	30	7.1	29.5	29.6	62.9	23.4
	60	4.8 ± 1.5 (11.4)	11.6 ± 2.0 (10.8)	30.7 ± 3.6 (5.6)	45.2 ± 9.1 (5.3)	19.7 ± 4.0 (24.5)
	180	2.9	7.06	25.43	46.28	23.15
^{125}I-Albumin-loaded ghosts	15	38.8 ± 2.8 (32.8)	15.5 ± 7.3 (13.1)	19.1 ± 2.8 (6.4)	115.9 ± 27.8 (4.7)	4.0 ± 1.2 (8.1)
	30	23.1 ± 1.3	8.1 ± 1.0	23.2 ± 3.5	238.0 ± 55.4	5.4 ± 1.1
	60	9.5 ± 5.4 (22.4)	5.7 ± 1.4 (10.4)	22.2 ± 2.4 (4.9)	158.9 ± 31.7 (3.1)	4.0 ± 1.6 (6.7)
	180	3.4	1.7	6.2	79.0	2.3
^{125}I-Labeled ghosts	15	28.9	19.0	26.2	114.5	5.2
	60	14.8	9.9	28.6	102.6	4.4
^{125}I-Labeled erythrocytes	15	27.9	49.8	17.2	7.8	57.1*
	60	28.8	34.1	14.1	6.2	9.4

Values are the mean (±SD, where given). Values in parentheses are for the respective free labels, $^{111}In^{3+}$–NTA complex or ^{125}I-albumin, in P_i/NaCl. For blood, the values were normalized to 7.3% of the total body weight.

*The high value for the kidney at this time probably reflects the presence of excess free label in the injected sample, which was dialyzed for 1 hr at 4°C.

erythrocyte has an average diameter of 5.9 μm (mean cell volume, 41–52 μm^3). The lifespan of mouse erythrocytes is between 20 and 30 days. The average diameter of erythrocytes from rat and rabbit is slightly larger (6.5–7.5 μm; mean cell volume, 60–68 μm^3) and the lifespan of these erythrocytes is between 60 and 70 days. The body temperatures of these animals are higher than the temperature in humans and their blood cell composition reflects a high reticulocyte count. Reticulocytes (immature erythrocytes) commonly make up 3–4% of erythrocytes in the blood of the mouse, rat, and rabbit, although values as high as 10% have been reported (19). In contrast, the human erythrocyte average diameter is 7–8.6 μm (mean cell volume, 100 μm^3); the life span is 120–127 days; the reticulocyte count is 0.1–1.5%. Erythrocytes from different animals show different stability in hypotonic solution. In general, increasing stability correlates with increasing mean erythrocyte volume (18). The results of the studies reported here show that the larger human ghosts are significantly more stable than the smaller ghosts from the other species tested. In addition, our results suggest that current methods used to load human erythrocytes are not adequate for loading erythrocytes from other animal species.

Our study shows a significant clearing of ghosts by the reticuloendothelial system in comparison to washed, intact erythrocytes. Fiddler *et al.* (4) observed morphologic changes of erythrocyte ghosts, made by several methods, to echinocytes or stomatocytes. In fact, simply washing erythrocytes can cause the cell shape to change to an echinocytic form, possibly because various plasma proteins are removed from the surface of the erythrocyte (20). These morphologic forms presumably are cleared by the reticuloendothelial system more rapidly than normal discoid erythrocytes. Ghosts made by the dialysis technique have been shown to retain their discoid shape to a greater extent (4). Thus, this method of preparing ghosts may prolong their lifetime *in vivo*. Beutler *et al.* (21) have demonstrated, in an asplenic patient, a half-life range of 14 hr to 10 days for ^{51}Cr-labeled ghosts with entrapped enzyme made by a dialysis procedure. With the advent of techniques to separate stable, highly loaded populations of ghosts from irreversibly lysed, unloaded, and unstable ghosts (17), delivery systems with the erythrocyte ghost as a biodegradable carrier may be developed to their full potential.

We have shown that PAC spectroscopy can be used to monitor the stability of human erythrocyte ghosts *in vitro*. In addition, these studies demonstrate that erythrocyte ghosts can entrap agents and offer a means to deliver these agents to reticuloendothelial cells. Organs with erythrophagocytic activity, including spleen, liver, and bone marrow, are natural targets for erythrocyte ghosts. We believe, as Ihler suggested (8), that drug-loaded erythrocytes could be used for treatment of cancers of these tissues, namely monocytic leukemia, reticulum cell sarcoma, and histiocytic medullary reticulosis. Possibly, surface-modified ghosts could be used for treatment of hepatic or other cancers.

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