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The Stabilization and Release of Hirudin from Liposomes or Lipid-Assemblies Coated with Hydrophobically Modified Dextran

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ABSTRACT Hirudin is a 65– amino acid peptide and the most potent and specific known inhibitor of thrombin (K_i = 0.2 pM). The short elimination half-life of hirudin from the body (1 hour) necessitates the use of a sustained and controlled delivery system.

A proliposome method was used to entrap hirudin in liposomes coated with palmitoyl dextran-coated liposomes and lipid-assemblies. In vitro release studies of hirudin were performed using the lipid systems enclosed in dialysis membranes or deposited in the pores of a vascular graft. The activity of hirudin and released hirudin was measured using a thrombin chromogenic substrate assay.

Entrapment efficiencies of hirudin in lipid-assemblies approached 100%, however, the release of hirudin from these systems was rapid with 90% released in 17 hours. Entrapment efficiencies of hirudin in coated-liposomes ranged from 5% to 55% and were dependent on several variables. Palmitoyl dextran– coated-liposomes showed a burst of 30% hirudin released in 5 hours with an additional 10% to 35% released over the next 600 hours. In all samples, 30-40% of the hirudin remained associated with the lipid-systems even after 600 hours. The released hirudin retained only 33% of its ability to inhibit thrombin when released from uncoated liposomes. However, hirudin retained 95% of its thrombin inhibitory activity when released from palmitoyl dextran– coated liposomes.

Coated liposomes were found to stabilize hirudin and result in greater retention of hirudin's ability to inhibit thrombin's enzymatic activity, although the mechanism is not yet understood.

KEYWORDS: hirudin, stabilization, in vitro release, liposomes, thrombin, dextran, vascular graft

INTRODUCTION

Hirudin, a 65– amino acid peptide (MW 7000), is the most potent and specific known inhibitor of thrombin (K_i = 0.2 pM). It was originally isolated from medicinal leeches (*Hirudo medicinalis*) in the late 1950s, but can now be made by recombinant techniques. Recombinant hirudin (REVASC™) has recently been approved in Europe for the prevention of deep-vein thrombosis following knee and hip replacement surgery. The short elimination half-life of hirudin from the body (1 hour) necessitates the use of a sustained and controlled delivery system. Hirudin has been considered as an alternative to heparin for a variety of reasons [1-3]. Other investigators have looked at ways to retard its rapid elimination from the blood, including coupling it to high molecular weight carriers such as dextran [4]. The chemical immobilization of hirudin on polymer surfaces for use as thromboresistant biomaterials has been investigated [5]. Oral delivery of hirudin via liposome systems has also been studied [6].

In this work, hirudin was encapsulated into two different lipid delivery systems: coated-liposomes and lipid-assemblies. The lipid delivery systems were composed of phospholipids combined with one of two hydrophobically modified polysaccharides, either palmitoyl-dextran (PalD) or palmitoyl-dextran phosphate (PalDP). In an aqueous environment, these hydrophobically modified polysaccharides (ie, conjugates) self-associate to form hydrophobic

domains via the interaction of their palmitate groups. Micelles and even liposomes can form if the concentration of hydrophobic groups reaches a critical concentration [7]. The term "lipid-assemblies" is used to describe the phenomenon whereby a relatively low concentration of lipids, such as phospholipids, assemble around the palmitate arms of the conjugates present in high concentration. The palmitate arms of these conjugates, in the presence of liposomes, will insert in the phospholipid bilayer while the dextran portion will associate with the aqueous phase at the surface of the liposome [8-11].

A sustained and controlled delivery system for hirudin could serve as an alternative to heparin therapy and prevent coronary thrombosis, or clot formation on implanted devices. Therefore, the objective of our work was to develop a sustained delivery system for hirudin that could be used systemically or deposited as a reservoir system within the pores of a small diameter vascular graft or both. Thrombus formation causes a high failure rate in small diameter vascular grafts due to occlusion [12-13]. It is thought that the sustained delivery of hirudin deposited in the pores of a vascular graft may aid in the natural "pacification" process of the graft and reduce the failure rate.

MATERIALS AND METHODS

Dextrans (T-40, T-70, and T-500) and Sepharose CL-4B were obtained from Pharmacia (Uppsula, Sweden). Palmitoyl chloride 98%, formamide 99+%, chloroform 99%, and tributylamine 99%, were purchased from Aldrich Chemical Co. (Milwaukee, WI). Polyphosphoric acid 83% was obtained from Eastman (Rochester, NY). Hirudin (Leach, Recombinant, [Lys47]-rHV2; 7110 units, mg), dioleoyl L-β-phosphatidylcholine (DOPC), dioleoyl L-α-phosphatidylethanolamine (DOPE), cholesterol, polyethylene glycol (PEG; MW = 8 kDa), tris(hydroxymethyl) aminomethane (TRIZMA base) were purchased from Sigma (St Louis, MO). Carrier-free $Na^{125}I$ was obtained from Amersham (Piscataway, N.J.) . α-Human thrombin (MW = 27.4 kDa) and Tosyl-Gly-Pro-Arg-4-nitranilid-acetate (Chromozym-TH) were obtained from Boehringer (Mannheim, Germany). Gore-Tex® microporous vascular grafts (ePTFE; 4-5 mm) were supplied by W.L. Gore Inc. (Flagstaff, AZ). Phosphate-buffered saline (PBS; 0.15 M), pH 7.4, was prepared as needed. All other chemicals were of reagent grade and were used without further purification.

Preparation of palmitate-modified dextrans

Palmitoyl-dextran phosphate (PalDP) and palmitoyl-dextran (PalD) were prepared and purified using a modified method of Suzuki et al [14]. Palmitate contents were determined by spectrophotometric determination of colored ferric hydroxamic acid complexes at 530 nm using methyl palmitate as a standard [15]. See **Table I** for reaction conditions, compositions, and physical properties of palmitate-modified dextrans.

Table I. Composition and Physical Properties of Palmitate-Modified Dextrans

Pal-Modified Dextran	Dextran (kDa)	Pal-Cl (g)	% Pal (w/w)	% Pal Substitution	Water Solubility
PalDP-1	70	0.1	9.8	6.6	soluble
PalDP-2	70	0.25	21.1	8.2	sl. soluble
PalDP-3	70	0.5	14	9.5	v. sl. soluble
PalDP-4	70	2	29.3	19.9	v. sl. soluble
PalDP-5	70	3.5	39.9	27.1	v. sl. soluble
PalDP-6	70	5	46.7	31.7	not soluble
PalD-1	40	0.5	3.2	2.1	soluble
PalD-2	500	0.5	1.5	1	soluble

Note: The reaction conditions for palmitoyl-dextran phosphate (PalDP) and palmitoyl-dextran (PalD) were 24 hours at 25°C and 2 hours at 25°C, respectively. For each modified dextran, the specified amount of Pal-Cl was reacted with 1.0 g Dextran (dried in vacuum for 24 hours at 105°C in the presence of P_2O_5) in warmed anhydrous formamide (100 ml) with anhydrous tributylamine (20 ml) as a catalyst. For PalDP preparations, polyphosphoric acid (5.0 g) was also added as a phosphorylating agent. For all PalDP preparations, the final % phosphorous was <2% (w/w) as determined by both ^{31}P-NMR and potentiometric titration. The % Pal (w/w) content for all preparations was determined by spectrophotometric determination as described in the Methods Section.

Preparation of liposomes or lipid-assemblies containing ^{125}I-hirudin

^{125}I-hirudin was prepared using the modified chloramine-T method of Tuong et al and stored at 4^{o}C until used [16]. The labeling efficiency of ^{125}I-hirudin was 70% to 80%, which agreed with the results of Tuong et al.

Coated-liposomes

Liposomes containing ^{125}I-hirudin were made using a modified proliposome method described by Perrett et al [17]. The process involves the formation of hydrated bilayers composed of selected weight ratios of lipid:ethanol:buffer followed by dilution to form liposomes. In these studies, the weight ratios of lipid:ethanol:buffer ranged from 20:80-160:20-200. Although many different lipid ratios were tested, for all lipid preparations described in this paper, the lipid portion consisted of DOPC:DOPE:cholesterol (50:25:25, w/w/w). Briefly, 20 mg lipids dissolved in 1.0 ml chloroform were transferred to a 15.0 ml glass test-tube. The chloroform was evaporated to dryness using a nitrogen gas stream, and 80 or 160 mg warmed ethanol was added to redissolve the lipids. The proliposome mixture was completed by the addition of phosphate-buffered saline (PBS) containing ^{125}I-hirudin (20-200 μg). Aliquots of PBS (100 μl) were added slowly while vortexing until the formed liposome suspension that formed was diluted to 10.0 ml. Alternatively, if palmitoyl-dextran coated-liposomes were being formed, the proliposome mixture was diluted with a solution of palmitoyl-dextran in PBS, pH 7.4. The liposomes or coated-liposomes were then sonicated for 30 min. Photon Correlation Spectroscopy (PCS) using a Brookhaven BI-90 Particle Sizer was used to determine the intensity averaged sizes and polydispersity of the liposomes.

Lipid-assemblies

Palmitoyl-dextran phosphates (100.0 mg) with various palmitate contents were dissolved/suspended in 2.0 ml chloroform and the following liposome-forming lipids were added: DOPC (20.0 mg/1.0 ml chloroform), DOPE (10.0 mg/1.0 ml chloroform), and cholesterol (5.0 mg/1.0 ml chloroform). The chloroform was evaporated to dryness with a nitrogen gas stream, and 100 μL warmed ethanol was added to redissolve the liposome-forming lipids and either redissolve or resuspend the palmitate-modified dextran. To this ethanolic mixture, 200 μl PBS, pH 7.4, containing ^{125}I-hirudin was added causing the immediate precipitation of a white fluffy material. Aliquots of PBS (100 μl) were added slowly while vortexing until the formed lipid-assemblies were diluted to 10.0 ml, and then sonicated for 30 min.

Separation of free ^{125}I-hirudin from lipid delivery systems

Unentrapped ^{125}I-hirudin were separated from liposomes or coated-liposomes by centrifugation at 16 000 rpm for 20 min. The entrapment efficiencies of ^{125}I-hirudin were calculated as the ratio of radioactivity in the centrifuged liposome pellet to the total radioactivity. However, due to the more fragile nature of the lipid-assemblies, gel filtration was needed to separate free hirudin from lipid-assemblies. Briefly, suspended lipid-assemblies (200 μl) were passed through 2.5 ml Sepharose CL-4B columns (Amersham Pharmacia Biotech (Piscataway, N.J.) (length/width = 14) using PBS, pH 7.4, as the eluting solvent. Fifty fractions (1 drop each) were collected and counted for radioactivity. The fraction number was then plotted against ^{125}I radioactivity. Also, the amount of residual ^{125}I radioactivity adhering to the column after elution was calculated as the ratio of ^{125}I retained on the column divided by the total ^{125}I activity eluded on the column.

In vitro release studies and vascular graft release studies

In vitro release studies at 37^{o}C: Liposome suspensions or lipid-assemblies containing ^{125}I-hirudin in 3.0 ml PBS were enclosed in Spectra/Por membranes (flat width = 2.8 mm) having a MW cut-off of 50 kDa. The membranes containing the suspensions were then transferred to 50 ml polypropylene tubes and 22.0 ml of additional PBS was added so that the membrane was completely submersed in media. The polypropylene tubes were gently shaken using an orbital shaker (200

rotations/minute). At selected times, 100 µl aliquots were taken and counted for radioactivity. Cumulative ^{125}I-hirudin released was calculated after correcting for volume changes. At the end of the release study, the membranes were counted for ^{125}I radioactivity before and after rinsing with PBS (to remove the lipid systems).

Loading and Release from Gore-Tex® Vascular Grant Pores

A vacuum pump was attached to a stainless steel syringe holder (13 mm). Circular sections of material were cut from Gore-Tex® microporous vascular grafts (ePTFE; 4-5 mm) and placed in the syringe holders with the lumen side facing the syringe. The coated-liposomes or lipid-assemblies were loaded into the grafts. Following initial wetting of the grant with PBS alone, a syringe containing a 125 µl suspension of lipid-assemblies with ^{125}I-hirudin was loaded into the graft using a vacuum pressure of 1600 Pa for 5 seconds. Selected membranes were then washed by soaking the loaded graft segment in 20 ml PBS for 10 seconds. Loading efficiencies for both washed and unwashed graft segments were calculated by the ratio of loaded ^{125}I activity to ^{125}I activity in the 125 µl suspension.

Spectrophotometric determination of hirudin and hirudin activity

To determine the amount of hirudin in solution and its activity (ability to inhibit thrombin), a chromogenic substrate assay was used [18]. To generate a standard curve, 10-125 µl of hirudin in PBS, pH 7.4 (9.667×10^{-8} M), was added to 100 µl of thrombin (1.21×10^{-7} M) in an assay buffer consisting of 0.1M Tris-HCl, pH 7.4, with 0.15M NaCl and 0.1% PEG (MW= 8 kDa). The volume of the reaction mixture was always adjusted to 0.9 ml by the addition of additional assay buffer. After 2 min at 25°C, 100 µl of Chromozym-TH (1.509×10^{-3} M) was added to bring the total volume 1.0 ml, and the absorbance of 4-nitroaniline was monitored at 405 nm for 3 min. The retention of hirudin activity was calculated as the ratio of hirudin from the chromogenic assay to the hirudin detected from the ^{125}I radioactivity. In addition, the standard curve was reproduced using radiolabelled hirudin in order to verify that the ^{125}I-hirudin had identical thrombin inhibitory activity. Statistical analysis of data was performed using a two-sample two-tailed *t*-test assuming equal variances.

RESULTS AND DISCUSSION

Palmitate modified-dextransThe PalDPs and PalDs used to form lipid-assemblies and coated-liposomes are listed in **Table I.** In all cases, the % palmitate substitution on the dextran chain was proportional to the amount of Pal-Cl reacted with dextran and the reaction time. Of the PalDPs, only PalDP-1 was soluble in water at 5.7% weight/volume (w/v). As the palmitate content increased in the PalDPs, the solubility ranged from partially to completely insoluble. All PalDPs had less than 2% weight/weight phosphate groups as measured by potentiometric titration.

Lipid-assemblies

PalDPs, having different palmitate contents, resulted in the formation of lipid-assemblies with very different properties as shown in **Figure 1.**

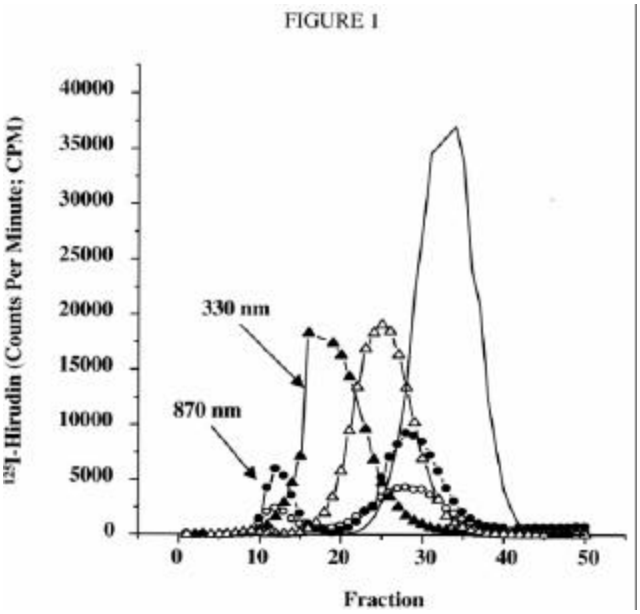

Figure 1. Gel-chromatography plots of fractions eluted versus ^{125}I-hirudin (CPM). Plots shown are free ^{125}I-hirudin (-), PalDP-1 lipid-assemblies (▲), PalDP-5 lipid-assemblies (Δ), free liposomes (●), and PalDP-6 liposomes (o). For reference, the sizes of selected lipid systems are shown on the plot.

The preparations were all passed through gel-filtration columns and plots of fraction number versus ^{125}I-hirudin were compared with those of liposomes (no PalDP added) and free ^{125} I-hirudin.

Free ^{125}I-hirudin eluted from the column in a broad peak in fractions 22-44. The gel-filtration data and particle size analysis confirmed the presence of intact lipid-assemblies (fractions 10-35) when either soluble or slightly soluble PalDPs were used. For clarity, the plots for PalDP-2 and PalDP-3 are not shown, but are similar to PalDP-1. Likewise, the plot for PalDP-4 is not shown, but is similar to PalDP-5. Due to the insolubility of the PalDP-6, no lipid-assemblies formed; only larger liposomes formed (fractions 10-20). It was also interesting to note the amount of residual ^{125}I-hirudin adhering to the Sepharose columns. Free liposomes and lipid-assemblies made with PalDP-6 had up to 40% residual radioactivity adhered to the column whereas lipid-assemblies made with PalDP (1-5), had less than 1% residual radioactivity adhered to the column. This low residual radioactivity confirmed the presence of a lipid-assembly having a hydrophilic surface, which was less likely to adhere to the hydrophobic Sepharose column. Interestingly, free hirudin also resulted in <1% residual radioactivity adhered to the column. This may be explained by hirudin's behavior in solution. Due to aggregation of its hydrophobic knots, hirudin does not dissolve in aqueous solution as a single entity. Instead, 3 to 8 hirudin molecules aggregate or even form micelles with their flexible ionic tails directed outward from a hydrophobic core [1].

Hirudin entrapment in free liposomes ranged from 5% to 55% depending on lipid and cholesterol content, and sonication time; whereas the broad single peak observed for PalDPs (1-5) suggested systems were achieved with 100% hirudin entrapment. In vitro release studies with lipid-assemblies revealed that they generally released ^{125} I-hirudin quickly with 90% released in 17 hours and 100% within 60 hours. This rapid release of hirudin was attributed to the relative instability of the lipid-assemblies, which were composed primarily of soluble PalDPs. The PalDPs may have significantly increased the critical micelle concentration of these systems. Although critical micelle concentrations are equilibrium values, perhaps what is more important to the micelle or assembly behavior of the systems is the rate of dissociation of the soluble PalDPs to free polymers. As a direct result, PalDP lipid-assemblies may form less stable liquid-like cores. It has also been reported that chain length of both the hydrophobic (Pal) and hydrophilic (Dextran Phosphate) constituents and the content of the hydrophobic segment determine micelle size and stability [19].

Loading and release from vascular grafts

The in vitro release profiles of ^{125}I-hirudin from PalDP-1 lipid-assemblies deposited in the pores of a Gore-Tex® Vascular Graft are shown in **Figure 2.**

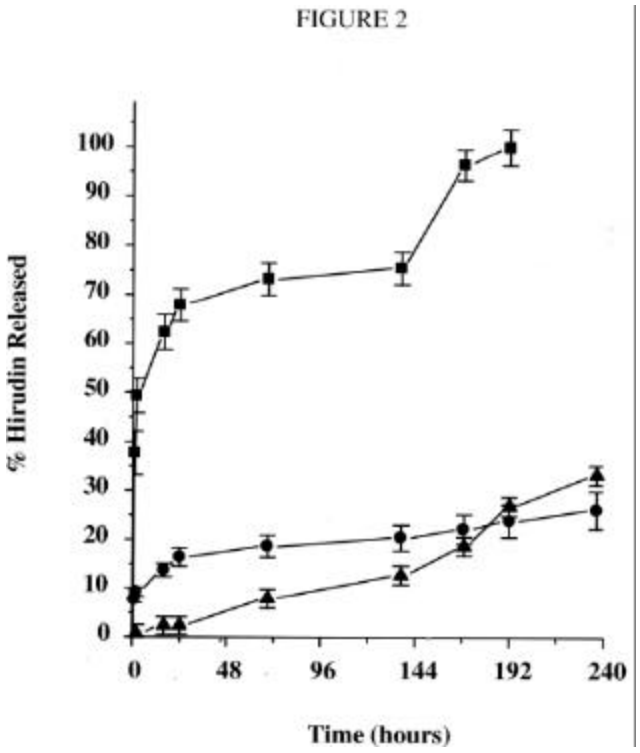

Figure 2. The in vitro release of ^{125}I-hirudin from PalDP-1 lipid-assemblies deposited in the pores of a Gore-Tex® Vascular Graft. The grafts were suspended in PBS, pH 7.4, at 37 °C. The release profiles from washed grafts containing free ^{125}I-hirudin (▲) and lipid-assemblies (●), and from unwashed grafts containing lipid-assemblies (■) are shown.

It is apparent that the technique employed to deposit the delivery system in the pores needs to be optimized. The unwashed and washed membranes contained only 29% and 3%, respectively, of the activity in the original 125 µl suspension used for deposition in the graft. Thus, a

significant portion of the delivery system was not truly deposited in the pores, but remained adhered to the surface of the graft. This portion was apparently removed during the washing step. Based on the exposed surface area of the graft (0.636 cm^2), the concentration of entrapped ^{125}I-hirudin deposited in the washed membrane was 0.2 ng/cm^2. Free hirudin was also deposited in the graft as a control. The rate of release of free hirudin from the pores was comparable to that of entrapped hirudin and suggested that the lipid-assemblies deposited in the pores of the graft released hirudin quickly. This implied that the rate-limiting step for the release of hirudin from the graft pores was its diffusion from the very hydrophobic pore environment and not its release from the lipid-assembly system. Due to the low deposition of both the delivery system and free hirudin in the graft, we were not able to assess the stability of released hirudin.

Coated-liposomes

The entrapment efficiencies of hirudin in liposomes formed by the proliposome method are shown in **Figure 3**.

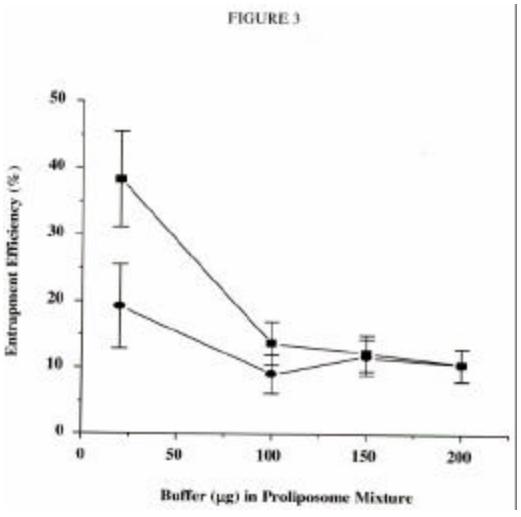

Figure 3. Entrapment efficiency of hirudin in PalD-1 coated-liposomes. The liposomes were made by the proliposome method (see Ref. 17) wherein rehydrated lipid (20 mg):ethanol:buffer mixtures are diluted. Entrapment efficiencies are shown as a function of buffer weight (20-200 mg) and ethanol added in the proliposome mixture. Ethanol amounts added were: 160 mg (■), and 80 mg (●).

The proliposome method was used to entrap hirudin for primarily two reasons: to avoid excessive vortexing and/or sonication, which may cause conformational or structural changes in hirudin, and because hirudin has excellent stability in a variety of conditions/solvents including ethanol (see **Table II**). In fact, our current work with hirudin demonstrates that hirudin retains 100% of its ability to inhibit thrombin even after exposure to 90% ethanol at 37°C for 1 hour (data not shown). The selection of a broad range of different ratios of DOPC, DOPE, and cholesterol had only a modest effect on the entrapment efficiency of hirudin. The most important parameters for hirudin entrapment were the ethanol and buffer compositions in the proliposome mixture. As expected, when the buffer portion of the lipid:ethanol:buffer proliposome mixture was increased from 20 mg to 200 mg, the entrapment efficiency of hirudin was reduced due to a dilution effect. The presence of cholesterol (in even very low amounts) in the proliposome mixture did increase the entrapment efficiency by about two-fold, presumably by sequestering hirudin in the lipid bilayers of the liposomes and/or by increasing the overall stability of the lipid systems.

The in vitro release profiles of PalD coated-liposomes and PalDP coated-liposomes are shown in**Figures 4A** and **4B**, respectively. PalD coated-liposomes and uncoated liposomes showed similar hirudin release rates with a burst of 30% in 5 hours, an additional release of 10% to 35% over the next 140 hours, and only a small release of hirudin up to 600 hours. In comparison, the PalDP coated-liposomes, especially with ratios of PalDP to lipids of 5 and 10, showed an enhanced burst of 40% in 3 hours, followed by additional release up to 150 hours. Presumably, the enhanced burst of PalDP coated-liposomes was due to the presence of the phosphate groups, and not due to the difference of dextran molecular weight. PalD-1 and PalD-2 coated-liposomes, consisting of 40 kDa and 500 kDa dextran, respectively, resulted in similar release rates of hirudin. Mass balance of hirudin was confirmed by radioanalysis of the membranes containing the lipid-systems after 600 hours. In all samples, 30-40% of the I^{125}-hirudin still remained associated with the lipid-systems in the membranes and not the membranes themselves.

FIGURE 4

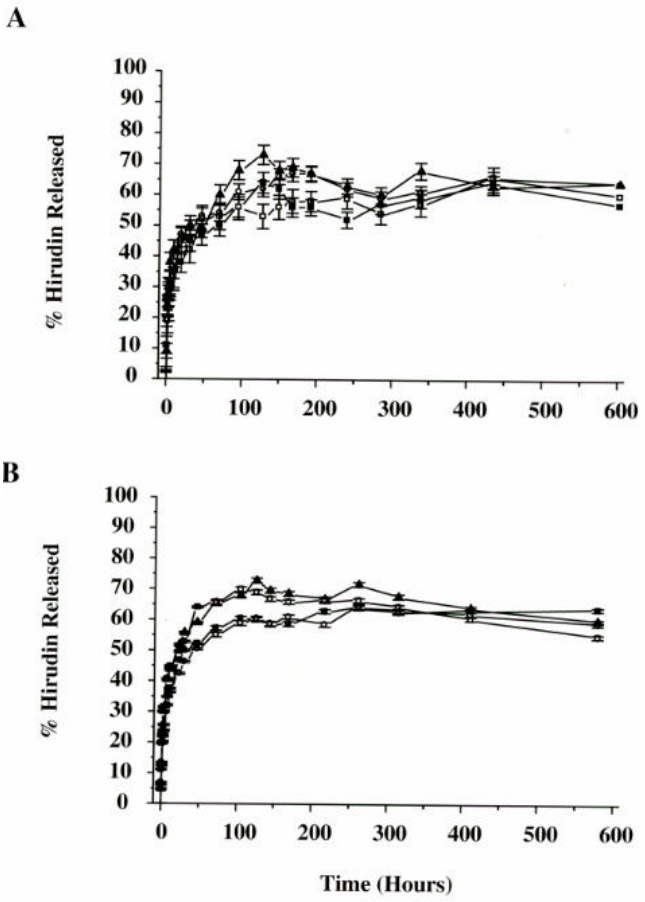

Figure 4. The in vitro release of hirudin in PBS, pH 7.4, at 37°C from A) PalD-1 coated-liposomes, or B) PalDP-1 coated-liposomes. The samples consist of free liposomes (■), 1:1 coated-liposomes (O), 5:1 coated-liposomes (▲), and 10:1 coated- liposomes (☐).

It was surprising that coated-liposomes did not release hirudin differently from uncoated liposomes. Previous investigators have shown that the exposure of liposomes to bile salts or phospholipase D greatly enhances the release of entrapped agents [8-9]. However, polymer-coated liposomes were stabilized and the stabilization apparently retarded the release of the entrapped agent. Likewise, Elferink et al demonstrated that liposomes coated with palmitoyl-dextran greatly retarded the rate of Triton-induced release of 5,6-carboxyfluorescein (CF) [11]. However, when liposomes and palmitoyl-dextran coated-liposomes containing CF were allowed to incubate in the absence of detergents, the dextran coating actually increased the initial leakage of CF, with only a few percent of CF released over the next few days.

Hirudin activity

Figure 5A demonstrates the ability of hirudin to inhibit thrombin.

FIGURE 5

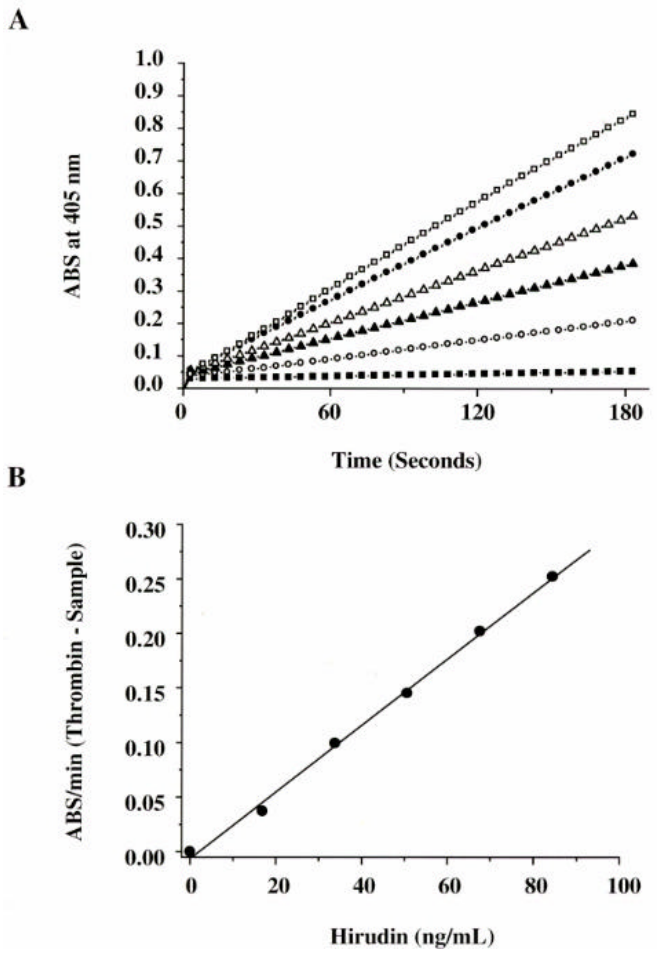

Figure 5.A) The reaction kinetics of Chromozym-TH and uncomplexed thrombin. Hirudin was added to excess thrombin and residual uncomplexed thrombin was assayed spectroscopically after the addition of Chromozym-TH. Hirudin was added to the 1.0 ml volume in the following amounts: 0 ng (☐), 16.9 ng (●), 33.8 ng ( ), 50.7 ng (▲), 67.6 ng (O), and 84.4 ng (■).

Figure 5.B) Calibration curve for released hirudin in solution. The figure was made by plotting the difference between the thrombin base value absorbance (thrombin in the presence of substrate only) and the hirudin sample values versus the concentration of hirudin standards from A.

At a hirudin concentration of 84.4 ng/ml, thrombin was entirely inhibited, the Chromozym-TH substrate remained uncleaved, and the absorbance value remained constant. This amount of hirudin

corresponded to 1.2 × x 10^{-11} moles which was the molar equivalent to thrombin in the assay. The data from Figure 5A was used to construct a calibration curve for hirudin as shown in Figure 5B. The difference in absorbance between the thrombin base value and the sample value were plotted against the concentrations of hirudin in the samples (0-84.4 ng). This linear plot was used to determine the retained activity of released hirudin in solution.

After 72 hours the ^{125}I-hirudin released from liposomes and PalD coated-liposomes (10:1) was assayed using the chromogenic substrate. The percent retention of hirudin's ability to complex thrombin was calculated using the calibration curve in **Figure 5B.** The results are shown in **Table II** and are compared with the results obtained when hirudin was incubated in various mixtures for 72 hours at 37°C.

Table II. The Retention of Hirudin Inhibitory Activity after 72 hours in PBS, pH 7.4, at 37°C

Sample	Retained Hirudin Activity (%)
Released hirudin (n = 3-4)	
Liposomes	33.0 + 6.0
PalD-1 coated liposomes (20:1)	95.0 + 3.0
Incubated hirudin (n = 3)a	
Hirudin control	96.6 + 1.4
Hirudin with 1.4% ethanol (v/v)	98.6 + 3.0
PalD-1 (0.05% w/w)	95.0 + 2.8
PalD-1 (0.10% w/w)	94.1 + 1.0
PalD-1 (0.5% w/w)	77.5 + 7.1
Dextran (0.05% w/w)	91.9 + 4.3
Dextran (0.10% w/w)	104.8 + 7.2
Dextran (0.50% w/w)	96.6 + 0.7

[a]Liposomes and PalD-1 coated liposomes samples all had identical lipid components, weight ratios, and overall lipid concentrations.

[b]All samples contained 338.3 ng hirudin per 5.0 ml buffer with 1.4% ethanol (v/v) and were sonicated for 5 min prior to incubation at 37 °C. The hirudin control sample contained no ethanol.

Hirudin retained only 33% of its ability to inhibit thrombin when released from uncoated liposomes. However, when released from PalD-1 coated-liposomes, hirudin retained 95% of its thrombin inhibitory activity. These results are very interesting and unexpected. For both the "liposomes" and the "PalD-1 coated liposomes," the amount of DOPC:DOPE:cholesterol in each and the amount of ethanol used in the proliposome method were identical. Further, it is unlikely that intact liposomes or lipid-assemblies could diffuse through the 50 kDa Mw cut-off dialysis membrane used for the studies. It is possible that the PalD-1 coating on the liposomes provided a stabilized environment for the entrapped hirudin in terms of chemical decomposition or conformational changes leading to a lose of activity. Staples et al reported on the phosphate-accelerated degradation of Hirulog, a negatively charged 20-residue fragment of hirudin [20]. It was shown that phosphate at pH>7 provided a poor environment for Hirulog, with rapid degradation occurring in only a few hours at 55°C. However, in our studies at 37°C, the hirudin control lost only 3% to 4% of its inhibitory activity when incubated for 72 hours in phosphate buffer. It was possible that hirudin entrapped in uncoated liposomes underwent degradation due to prolonged contact with DOPC and DOPE, whereas the dextran at the surface of the coated-liposomes reduced detrimental interaction of the ionic tail of hirudin with the phospholipids. More extensive compatibility studies will have to be performed to elucidate the mechanism and extent of apparent lipid induced degradation of hirudin. The incubation studies of hirudin with dextran in solution, especially dextran at 1 mg/ml, suggest that dextran may have a protective effect on hirudin in phosphate buffer at 37 °C. The enhanced retention of hirudin activity when incubated with dextran at 1 mg/ml was significant ($p<0.05$) when compared with the control.

It is apparent from the incubation data that the low amounts of residual ethanol have little if no effect on the inhibitory activity of entrapped hirudin. A PalD-1 concentration of 5 mg/ml the apparent retained hirudin activity was reduced to 77.5%. However, at this high concentration of PalD-1, micellularization of hirudin and the palmitate groups of PalD-1 could result in the inaccessibility of hirudin during the assay. In fact, Elferink et al [11] suggested that the increased accumulation of hydrophobic anchors in solution may be responsible for forming micelles.

The effect of PalD and hirudin on thrombin activity In an attempt to determine if PalD potentiates hirudin's

ability to inhibit thrombin or if PalD itself inhibits thrombin, several additional studies were completed. PalD-1 (0-20 mg/ml) was added to thrombin with and without a fixed concentration of hirudin in solution. As observed in **Figure 6** , PalD-1 concentrations greater than 1 mg/ml inhibited thrombin in a linear manner (r^2 = 0.99; slope = 3.1).

FIGURE 6

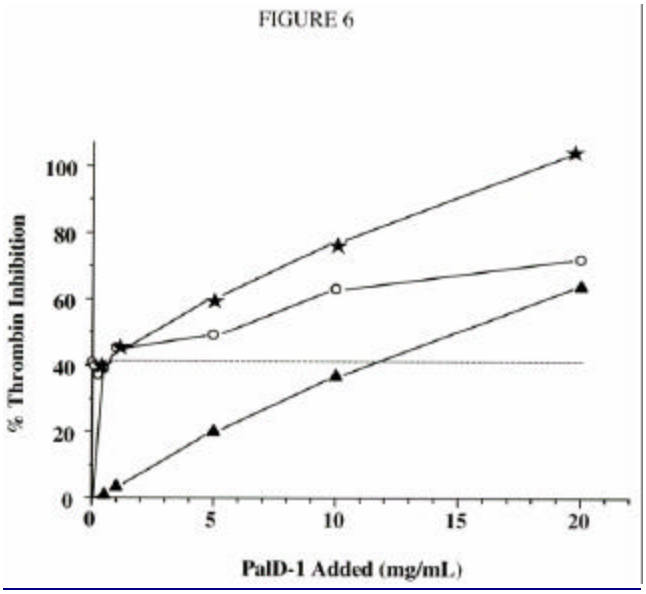

Figure 6. Thrombin inhibition by PalD-1 and hirudin. The following samples were added to the thrombin assay buffer and allowed to react for 2 min prior to the addition of Chromozym-TH: (▲) PalD-1, (O) PalD-1 with fixed hirudin of 33.8 ng corresponding to the hirudin baseline inhibition of 40%. The theoretical thrombin inhibition (★) was calculated by adding the hirudin baseline to the free PalD-1 inhibition values.

In addition, PalD-1 concentrations greater than 1 mg/ml, in the presence of 33.8 ng of hirudin, also linearly inhibited thrombin (r^2 = 0.94; slope = 1.5). However, the thrombin inhibition values by PalD-1 and hirudin were less than expected (theoretical) based on the inhibition plots of PalD-1 and hirudin individually, and suggested that PalD-1 and hirudin compete for thrombin binding sites. In fact, PalD-1 is similar in structure to previously reported active site-directed thrombin inhibitors having a hydrophobic residue and a non-hydrolyzable fraction [3]. From these results, it was shown that PalD-1 does not potentiate the ability of hirudin to inhibit thrombin. It does, however, have its own ability to inhibit thrombin, but only at concentrations greater than 1 mg/ml. Even at concentrations of 1 mg/ml, PalD-1 produces a milky solution. Although the concentration of PalD-1 was not determined in the release sample used for determining the activity of hirudin, the receiving solution outside of the membrane containing the coated-liposomes was a clear solution estimated to have PalD-1 concentrations far less than 1 mg/ml. Therefore, the retention of hirudin activity released from coated-liposomes was attributed to a stabilizing effect of the PalD-1 with hirudin, and not due to significant independent action of PalD-1 in the thrombin enzymatic assay. Additional studies are ongoing to better elucidate the mechanism of loss of hirudin activity.

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