

Platelets and Blood Cells

RGD-modified liposomes targeted to activated platelets as a potential vascular drug delivery system

Anirban Sen Gupta¹, Guofeng Huang¹, Brian J. Lestini¹, Sharon Sagnella¹, Kandice Kottke-Marchant², Roger E. Marchant¹

¹Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio, USA

²Department of Clinical Pathology, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Summary

Local drug delivery has become an important treatment modality for the prevention of thrombotic events following coronary angioplasty. In this study, we investigate the ability of liposomes bearing surface conjugated linear Arg-Gly-Asp (RGD) peptide (GSSSGRGDSPA) moieties to target and bind activated platelets, and the effect of such RGD-modified liposomes on platelet activation and aggregation. The binding of RGD-liposomes to human platelets was assessed by fluorescence microscopy, phase contrast microscopy and flow cytometry. The effect of RGD-modified liposomes on platelet activation and aggregation was investigated *in vitro*, with and without platelet agonists. RGD-li-

posomes were found to bind activated platelets at levels significantly greater than the control RGE-liposomes. The RGD-liposomes did not exhibit any statistically significant effect on platelet activation or aggregation. The results demonstrate the ability of the RGD-modified liposomes to target and bind activated platelets without causing significant platelet aggregation and suggests a feasible way for the development of a platelet-targeted anti-thrombogenic drug delivery system. Furthermore, the approach can be extended to the development of liposomes for other vascular targets, for application in drug delivery or gene therapy.

Keywords

Liposomes, RGD-modification, platelet-targeted, drug delivery

Thromb Haemost 2005; 93: 106-14

Introduction

Local drug delivery is used often to facilitate the management of thrombosis and re-occlusion following invasive cardiological procedures such as balloon angioplasty and intracoronary stent placement. Current strategies typically focus on the delivery of low-molecular weight thrombolytic, anticoagulant, antiplatelet, and anti-inflammatory agents in tandem with catheterization and balloon inflation. In order to facilitate local accumulation of a drug, delivery vehicles such as liposomes, nanoparticles, and impregnated balloons and stents have been investigated (1–3). These approaches passively increase the mean residency time of the therapeutic agent in the vicinity of the vascular lesion, but lack mechanisms for actively targeting the sites of vascular injury. In this report, we describe an alternative strategy for actively targeting liposomes to platelets localized to a site of vascular lesion, using a linear Arg-Gly-Asp (RGD) peptide incorporated on the liposome surface (Fig. 1).

Numerous studies have indicated that platelet activation and deposition immediately following vascular injury is a primary

thrombotic event leading to restenosis (3–8). Consequently, platelets are attractive candidates for exploiting the targeting of liposomes to sites of thrombus formation. In the event of thrombus formation following a vascular injury, bridging of adhered and activated platelets is mediated by the binding of fibrinogen to the activated platelet. This binding results from the specific interactions between the *RGDS*, *RGDF* and *KQAGDV* regions of the fibrinogen molecule with the docking regions of the GPIIb-IIIa receptor expressed on the activated platelet (9–13). Thus, the RGD sequence is an important tool for the manipulation of platelet targeting and binding. Previously, Gyongyossy-Issa et al. have demonstrated that RGD peptide-coupled liposomes can bind purified GPIIb-IIIa in solution (14). Nishiya and Sloan demonstrated that RGD liposomes show increased lipid mixing and mixing of aqueous contents with bovine platelets (15). In this study, we provide direct microscopy and flow cytometry evidence indicating that linear RGD peptides, surface-conjugated to liposomes through polyethylene oxide (PEO) spacers, retain the necessary conformation to bind specifically to activated platelets, in the presence of Ca^{++} . We have established the specifi-

Correspondence to:
Roger E. Marchant
Department of Biomedical Engineering
Wickenden Building
Case Western Reserve University
10900 Euclid Avenue
Cleveland, OH 44106-7207, USA
Tel.: + 1 (216)-368-3005, Fax: + 1 (216)-368-4969
E-mail: rxm4@po.cwru.edu

Received June 3, 2004
Accepted after resubmission November 1, 2004

Financial support:
This study was supported by a research grant: NIH HL-70263.
Prepublished online December 8, 2004 DOI: 10.1160/TH04-06-0340

city of platelet-binding of RGD-liposomes by comparative studies with liposomes without any peptidic surface modification and also with liposomes surface-modified by RGE peptide moieties, the latter having no specific binding to any platelet receptor. Such unmodified and RGE-modified liposomes were used as negative controls. Furthermore, we have established the apparent inability of RGD-modified liposomes to facilitate platelet aggregation *in vitro*, an important requirement for potential *in vivo* application as a drug delivery vehicle.

The scheme employed to attach the RGD peptide to the liposome surface draws upon a previously described strategy for lipidation of synthetic peptides, and extends its utility to liposomes (16). This method can be used to prepare liposomes incorporating any number of short synthetic peptides. Together, this work demonstrates the utility of such peptides for platelet-directed liposomal drug delivery and, by extension, for developing liposomes directed against other potential molecular targets within the coagulation system for intravascular liposomal drug delivery or gene therapy applications.

Materials and methods

Reagents

Amino acid derivatives, activator (1-hydroxy-1-azabenzotriazolium, HATU), and synthesis resin were purchased from Anaspec, Inc. (San Jose, CA). All solvents used were HPLC grade. An activated, purified synthetic polyethylene oxide (PEO) derivative of distearoylphosphatidylethanolamine (DSPE-PEO) containing a terminal N-hydroxysuccinimide (NHS) activated carboxyester was purchased from NOF America Corporation. The PEO component had a reported average molecular weight of 2000. For the synthesis of liposomes, cholesterol, distearoylphosphatidyl choline (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEO₂₀₀₀), 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzodiazol-4-yl)]amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (PDPC-NBD) and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Lissamine RhodamineB Sulfonyl) (ammonium salt) (DOPE-RhB) were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at -20°C prior to use. Bovine serum albumin (BSA) and sodium citrate were obtained from Sigma Aldrich (St. Louis, MO).

Synthesis of DSPE-PEO-peptide conjugate

An 11 residue, RGD-containing linear peptide (GSSSGRGDSPA) was synthesized by solid-phase peptide synthesis using standard 9-fluoromethoxycarbonyl (Fmoc) chemistry (17). Briefly, the RGD peptide was generated on a PEO-polystyrene resin support using an ABI433A automated peptide synthesizer and side chain protected amino acid derivatives. Activity of the free peptide was assessed by competition with ¹²⁵I-labeled fibrinogen for binding to ADP activated platelets, using a method described previously (18). For coupling peptide to lipid, a 1.1-times molar excess of DSPE-PEO-NHS was reacted with the RGD peptide while still attached to the synthesis resin. After reaction, the resin was washed extensively to remove unreacted lipid. Cleavage of DSPE-PEO-RGD from the synthesis resin and removal of side chain protecting groups were conducted in Reagent K without

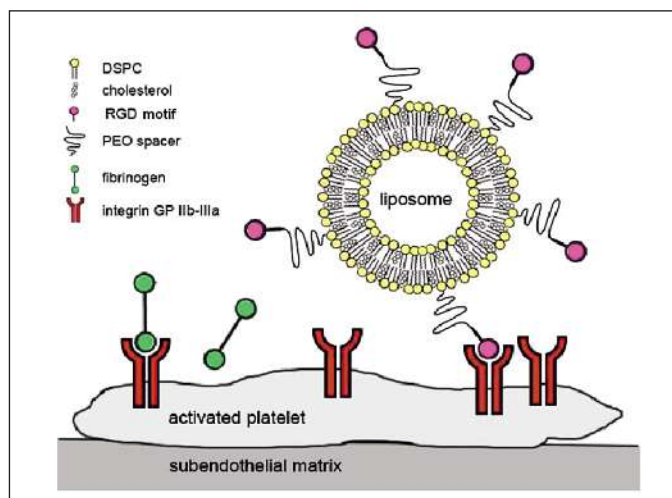


Figure 1: Schematic diagram of RGD-modified liposome targeting and binding to integrin GPIIb-IIIa expressed on activated platelet adhered onto subendothelial matrix in case of a vascular injury. The diagram also shows fibrinogen binding to similar integrin receptors. Optimization of the surface density and integrin affinity of the RGD motifs on the liposome surface will enable competition with native ligand fibrinogen and provide a delivery system for anti-thrombotic therapeutics.

thioanisole (17). The resulting solution was precipitated into ice-cold ether, and the precipitate extensively dialyzed against deionized water and lyophilized. The identity of the product was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager Biospectrometry Workstation, PerSeptive Biosystems), and also by Fourier transform infrared spectroscopy (FT-IR, Bio-Rad). For the negative control, a similar 11 residue peptide (GSSSGRGESPA) was synthesized using solid-phase chemistry and the RGE moiety was conjugated to the lipid in the same way as for the RGD peptide.

Liposome preparation

Liposomes containing the DSPE-PEO-peptide construct (RGD-modified and RGE-modified liposomes) were formed by reverse-phase evaporation followed by extrusion. Briefly, the peptide-lipid conjugate was dissolved in chloroform:methanol (1:1) and mixed into solution with DSPC, cholesterol and the fluorescent phospholipid (PDPC-NBD or DOPE-RhB) to achieve a final peptide content of 5 mole %. After solvent evaporation, the resulting lipid film was resuspended in fresh phosphate buffered saline (PBS), subjected to several repetitions of freeze/thaw, and extruded ~15 times through a 100 nm pore size Nucleopore polycarbonate membrane at 70°C. Unmodified control liposomes containing 5 mole % DSPE-PEO₂₀₀₀ in place of the peptide conjugate were prepared similarly. Liposome size and stability were characterized by light-scattering measurements using a Brookhaven Instruments Corporation model 90 Plus Particle Size Analyzer. The liposome suspensions were found to be stable (no aggregation) upon standing at 70 °C and for several weeks at room temperature following extrusion. The mean vesicle diameters for unmodified, as well as, peptide-modified liposomes remained in

the range of 100–140 nm over a period of several weeks, indicating the formation of stable vesicles.

Platelet adhesion to coverslips

Citrated whole blood (10 ml) was collected from an aspirin refraining human donor according to an IRB-approved protocol, after obtaining informed consent. Whole blood was centrifuged at 800 RPM for 15 min at 25°C, to obtain platelet rich plasma (PRP). The PRP was transferred to another tube and centrifuged at 1600 RPM for 15 min at 25°C to obtain a platelet pellet. The supernatant plasma was removed and the platelets were resuspended in PBS containing 1% BSA. The average platelet count in the suspension was about 250,000/μL. The platelets were adsorbed onto collagen III coated glass coverslips for 1 hr, and then gently washed 3 times with PBS. The adhesion of a platelet monolayer was confirmed by staining with fluorescein isothiocyanate (FITC)-tagged anti-CD41a (anti-GPIIb-IIIa) monoclonal antibody and observing the coverslips under a fluorescence microscope. RGD-liposomes or control RGE- and PEO-liposomes fluorescently labeled with PDPC-NBD were diluted to a final concentration of 25 μM and incubated with the adsorbed platelets for 1 hr at room temperature in the dark. For the liposome-incubated samples, platelet-staining with FITC-anti-CD41 was not performed, since the antibody and the liposomes would potentially compete for the same receptor sites. All incubations were carried out in the presence of 5 mM CaCl₂ solution (19, 20). Coverslips were again washed 3 times with PBS, fixed in 1% paraformaldehyde (PFA) for 30 min at 37° C, washed with PBS, and mounted onto glass slides with Crystallmount (Biomed) containing AntiFade (Molecular Probes). In a separate experiment, the platelets adhered on the collagen-III coated coverslips were stained first with fluorescein isothiocyanate (FITC)-tagged anti-CD42b (anti-GPIb) monoclonal antibody. Following this, the adhered platelets were incubated with DOPE-RhB labeled test and control liposomes. The washing and mounting steps were carried out as before.

Microscopy analysis

Platelets were imaged using a Nikon Diaphot epifluorescence inverted microscope fitted with a Photometrics chilled CCD camera. A 450–490 nm excitation filter, a 510 nm dichromatic mirror, and a 520–560 bandpass barrier filter were used for the detection of FITC or NBD fluorescence. A 510–560 nm excitation filter, a 575 nm dichromatic mirror and a 610 longpass barrier filter were used for detection of RhB fluorescence. Images were collected in MetaMorph™ (Universal Imaging Corp.), using an exposure time of 500 ms, and processed in Adobe Photoshop. Surface averaged intensity analysis was performed on unprocessed images using MetaMorph™. Platelets were first identified automatically by thresholding and then confirmed by visual inspection. Initially, a 40X objective (400X magnification) was used to locate fluorescent spots on the coverslip surface and subsequently 100X (1000X magnification) objective was used to zoom in on and further analyze the spots. Once fluorescent spots were identified, the imaging mode was changed to phase contrast and the same spots were observed to identify the location of adhered platelets. At least three images were averaged to determine the mean pixel intensities of labeled platelets. One to one cor-

respondence of fluorescent spots in the epifluorescent micrographs to activated platelets in phase contrast images provided confirmation of specific binding of the RGD-modified liposomes to the activated platelets. For the dual staining experiments, images were captured simultaneously for green fluorescence (from FITC-anti-CD42b) and red fluorescence (from liposome-DOPE-RhB). Use of a filter slider made it possible to capture both images of the same field, which could be then superimposed using Adobe Photoshop®.

Flow cytometry analysis

The effect of peptide modification of liposomes on platelet binding was further characterized using a FACScan flow cytometer (Becton-Dickinson) with a 488nm laser. For potential drug delivery applications it is necessary to assess the binding of RGD-liposomes to resting/ unactivated, as well as to activated platelets. However, the centrifugation and resuspension steps used to obtain washed platelets from whole blood results in significant artifactual activation of platelets (21). Hence suspensions of platelets isolated in this way, can be used only to assess liposome interaction with moderately activated or highly activated (agonist added) platelet populations. To assess biological characteristics of unactivated platelets, flow cytometry analysis of platelets in freshly drawn whole blood has been reported (22). Agonists can be added subsequently to activate the platelet populations to different levels for further study.

Citrated whole blood was collected from aspirin refraining human donors and the platelet number was assessed by Coulter Counter to be 280,000–300,000 μl⁻¹ for representative batches. 10 μl whole blood was used for each analysis run in the flow cytometer. The activation level of the platelets in the blood was assessed initially by appropriate gating in flow cytometry density plots using simultaneous fluorescence staining for GPIIb-IIIa (with FITC-anti-CD41a) and P-Selectin (with PE-anti-CD62). The platelet population that stained positive for CD41a and negative for CD62 was deemed unactivated. After assessment of the activation level, similar whole blood samples were incubated with NBD-labeled test or control liposomes for 30 min and then analyzed by flow cytometer. The volume of liposome-incubated blood sample in the flow cytometer tubes was diluted to ~600 μL with PBS/1% FBS solution, such that the final lipid concentration was 50 μM. For the liposome incubated samples, FITC-anti-CD41a staining was not used, since both liposome and antibody could potentially compete for the same receptor (GPIIb-IIIa) on the platelet. The PE-anti-CD62 staining was used to gain insight about the effect of liposomes on platelet activation. PE and NBD fluorescence intensities were recorded simultaneously for appropriately gated platelet populations, thereby providing insight into liposome interaction with predominantly unactivated platelets. In a separate set of experiments, blood samples were incubated with 10 μM ADP for 15 min to achieve moderate activation of the platelets. The activation level was again assessed by anti-CD41a and anti-CD62 staining and appropriate gating. The liposome incubation step was repeated for similar moderately activated samples without the anti-CD41 stain and flow cytometry analysis was performed to gain insight into the variation in fluorescence intensity for the gated platelet populations. In a third set of experiments, blood samples were incu-

bated with Thrombin Receptor Activating Peptide (TRAP, 5 μM) to achieve a high level of platelet activation. The previously described steps for activation level assessment, liposome incubation and liposome fluorescence intensity assessment in the gated platelet populations were repeated. A total of $\sim 100,000$ counts within the gated platelet population were collected for all samples run in the cytometer. Fluorescence histograms were plotted for the platelet populations at different levels of activation, which provided quantitative information about the interaction of RGD- and RGE-liposomes with platelets at different activation states.

In vitro platelet aggregation assay

Platelet aggregation in the presence of liposomes was assessed using a Bio/Data PAPS-4 aggregometer. PRP was obtained by centrifuging whole blood at 800 RPM for 15 min at 25°C. A portion of the PRP was centrifuged at 1600 RPM for another 15 min at 25°C to precipitate the platelets as a pellet and the supernatant liquid was collected as platelet-poor plasma (PPP). The adjusted PRP with a platelet count of $\sim 250,000 \mu\text{l}^{-1}$ was used to prepare the aggregometry samples. PPP was used as the “blank” for obtaining the baseline for the aggregometer. Pelleted platelets were resuspended in PBS/ 1% BSA as described previously. Samples were prepared by taking 450 μl of PRP or platelet suspension, in the absence or presence of an agonist (10 μM ADP or 5 μM TRAP) and RGE- or RGD-modified liposomes at a final lipid concentration of 500 μM . The final volume of each sample was made up to 500 μl using freshly filtered PBS. For PRP-based samples, due to inherent plasma concentration of Ca^{++} , external addition of calcium was not performed. For platelet suspension samples CaCl_2 was added externally at $\sim 2 \text{ mM}$ final Ca^{++} concentration. Initially, sample tubes containing PRP or platelet suspension were warmed at 37°C for 2 min and final % aggregation of platelets was assessed in the absence or presence of agonist, without addition of liposomes. Platelet aggregation was monitored for 10 min with stirring, as the % aggregation plots were found to reach saturation after only about 3 min. For the liposome incubated samples, the incubation period was varied from 0 to 60 min. Free RGD peptide also was assayed at 12.5 μM and 150 μM , corresponding to around 2 and 24 times the expected accessible RGD concentration in the 250 μM RGD-liposome samples, respectively. This is in accordance with the assumption that at least 50% of the RGD moiety incorporated in the liposomes are expressed on the surface and therefore accessible for receptor binding. The final % aggregation of platelets in PRP and in platelet suspension was recorded after the 60 min runs. All experiments were performed in triplicate.

Results

Activity of the RGD peptide, GSSSGRGDSPA

Table 1 shows the IC_{50} value for the linear RGD peptide, corresponding to the concentration of peptide required to reduce ^{125}I -labeled fibrinogen binding to activated platelets by 50%. Control peptides are shown for comparison. The IC_{50} values determined for linear sequences (125– 275 μM) are considerably higher than those previously measured for shorter RGD peptides and analogues (23). In addition, when compared with the IC_{50}

Table 1: Activity of the RGD-peptide GSSSGRGDSPA. IC_{50} values for test peptide and control peptides corresponding to the concentration of peptide required to inhibit ^{125}I -labeled fibrinogen binding to GPIIb-IIIa activated platelets by 50%. The test peptide (GSSSGRGDSPA) shows a higher IC_{50} compared to the shorter control peptide (GRGDSP) chain, indicating moderate affinity.

Peptide	IC_{50} (μM)
GSSSGRGDSPA	275
GRGDSP	125
GRGESP	n.i.
fibrinogen gamma chain	216

IC_{50} measured by competitive binding to platelets against ^{125}I -fibrinogen n.i. = no inhibition of fibrinogen binding observed

for GRGDSP (125 μM), the increase in the IC_{50} for GSSSGRGDSPA may be attributed, in part, to the GSSSG tag attached to the amino terminus of the peptide. The tag sequence may be partly altering the conformation of the RGD tripeptide,

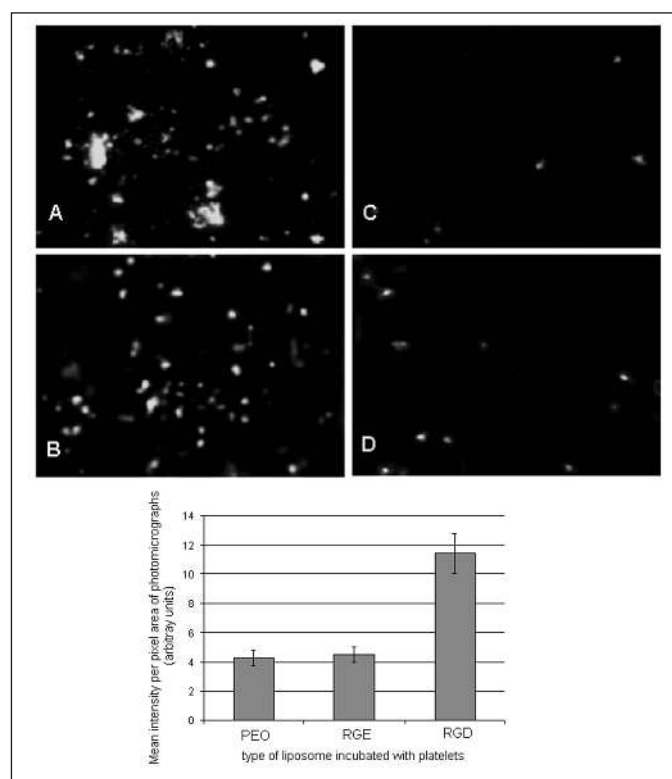


Figure 2: Representative fluorescence micrographs (40X objective) and mean fluorescence intensity (per pixel area) for platelets adhered onto collagen-III coated glass coverslips and incubated with test liposomes. “A” shows micrograph image for platelets labeled with FITC-anti-CD41 monoclonal antibody. “B” shows micrograph image for platelets incubated with NBD-labeled RGD-liposomes. “C” and “D” shows micrographs for platelets incubated with NBD-labeled PEO-liposomes and RGE-liposomes respectively. Comparing “B”, “C” and “D” it is evident that RGD-liposomes stain platelets more than PEO- and RGE-liposomes. The mean fluorescence intensity data in “E” shows that RGD-liposomes show about three-fold enhancement in binding activated platelets compared with control liposomes.

or interfering with peptide binding during docking with the receptor. Thus, the linear RGD peptide has only moderate affinity for binding.

Platelet binding studies

Binding of peptide-modified liposomes to activated platelets was observed by fluorescence microscopy. The presence of platelets on the collagen-coated surface was confirmed by staining with FITC-conjugated anti-CD41a, a monoclonal antibody specific to GPIIb-IIIa expressed on activated platelets (Fig. 2A). Surface adsorbed platelets incubated with RGD-modified liposomes (Fig. 2B) showed significantly greater staining than those labeled with PEO- or RGE-modified control liposomes (Fig. 2C and 2D). From these images, it is apparent that the entire platelet surface was labeled with fluorescent phospholipid.

RGE-modified and PEO-modified liposomes were able to minimally stain some platelets, indicating a low, but non zero level of nonspecific binding. This is consistent with flow cytometry results, in which comparatively lower levels of platelet staining were observed with control liposomes at various levels of platelet activation. RGE-modified liposomes stained platelets

more than PEO-modified liposomes. This may represent transient liposome binding with subsequent lipid exchange. Nonetheless, the microscopy images clearly demonstrate that the RGD peptide facilitates the “specific interaction” of liposomes with activated platelets by recognition of and binding to specific receptor sites. The mean intensities of platelets labeled with RGD-modified liposomes increased approximately threefold compared with control liposomes, as shown in Figure 2E.

In order to validate platelet integrin GPIIb-IIIa as the major receptor for RGD-liposomes, additional experiments were performed. FITC-tagged anti-GPIIb (anti-CD42b) monoclonal antibody was used to stain the platelets adhered onto collagen-III coated coverslips prior to incubating them with Rhodamine-B-labeled RGD-liposomes and control liposomes. This experiment enabled simultaneous observation of adhered platelets (green fluorescence from FITC-anti-GPIIb) and platelet-attached liposomes (red fluorescence from Rhodamine-B-lipid). This was important from the point of view that any effect from this receptor to binding of liposomes was minimized. The GPIIb receptor being thus blocked, GPIIb-IIIa remained as the most expressed receptor on the activated platelet surface. In Figure 3, A1 and B1

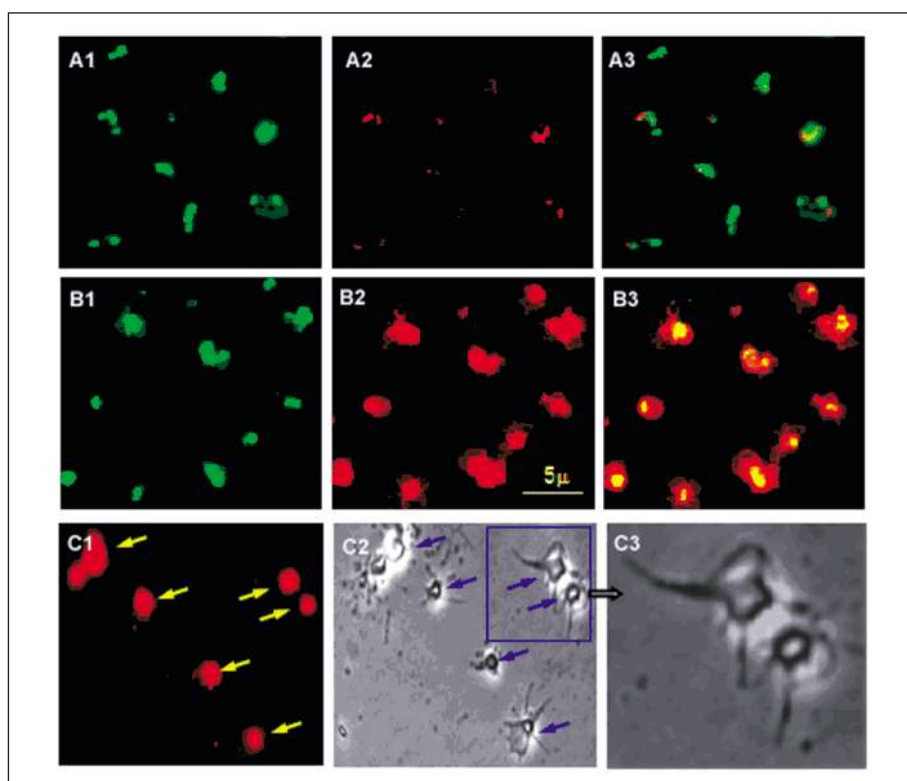


Figure 3: Representative dual-fluorescence and phase contrast micrographs showing enhanced specificity of platelet-binding for RGD-liposomes compared with RGE-liposomes. “A1” and “B1” show images of platelets adhered onto collagen-III coated glass coverslips, stained with FITC-anti-GPIIb monoclonal antibody. “A2” and “B2” show images of the same platelets incubated with Rhodamine B-labeled RGE-liposomes and RGD-liposomes respectively. “A1” and “A2” are superimposed in “A3” while “B1” and “B2” are superimposed in “B3”. The yellow color indicates super-imposed red and green staining. Comparison of “A1” and “A2” shows minimal staining of platelets by RGE-liposomes and this can be attributed to lipid-exchange based non-specific binding. Comparison of “B1” and “B2” shows significant en-

hanced staining of platelets with RGD-liposomes and this can be attributed to *enhanced specific binding* of these liposomes to activated platelets. The specificity of platelet binding for RGD-liposomes is further verified by observing fluorescent spots on samples incubated with Rhodamine B-labeled RGD-liposome and comparing them with phase contrast micrographs of the same field, as shown in “C1” and “C2”. The comparison showed one-to one correspondence of fluorescence spots with spread (activated) platelets. “C3” shows a magnified section of the phase contrast micrograph revealing the “spread” morphology of the adhered activated platelets.

show platelet staining by FITC-anti-CD42b, while A2 and B2 show the same platelets stained by RhB-liposomes. In A2, RGE-liposomes were used, while in B2 RGD-liposomes were used. A3 and B3 show superimposed representations of A1-B1 and A2-B2 combinations respectively. Interestingly, the green fluorescent spots (in A1 and B1 from anti-CD42b staining) did not show complete platelet morphology indicating that the surface density of GPIb per platelet was not very high. The liposome red fluorescence (from RhB) on the same platelets revealed distinct spread platelet morphology for the RGD-liposome incubated samples (B2), indicating numerous RGD-liposomes binding to a high surface density receptor (even higher than GPIb). The RGE-liposome incubated samples showed minimal RhB fluorescence (A2). From these results, it can be concluded that the RGD-liposomes predominantly and specifically bind to GPIIb-IIIa receptors, as the latter significantly outnumbers other potential RGD receptors. Although these results do not exclude the possibility of RGD-recognition and binding by other platelet receptors, they do establish GPIIb-IIIa to be the major receptor for RGD-liposomes and indicate successful platelet-targeting.

The fluorescence on the RGD-liposome incubated coverslips was observed simultaneously in epifluorescence and by phase contrast mode. Spread platelets were present in exactly the same positions as the fluorescent spots; thin platelet pseudopodial structures also were distinguishable (C3 in Fig. 3). Representative images of one-to-one correspondence of fluorescence spots with activated platelets on the RGD-liposome incubated coverslips are shown in C1-C3 in Figure 3. The number of RGD-modified liposomes within each fluorescent spot could not be determined because of the resolution limitations of the microscope. The platelet morphology suggests that platelets were moderately activated during or after adhesion, while the central intensification of the corresponding fluorescence spot reflects the possible presence of “pseudonucleus,” a previously described artifact of platelet topography.

Flow cytometry studies

Flow cytometry studies performed with liposome-incubated aliquots of whole blood at different platelet activation levels provided conclusive results regarding enhancement of RGD-liposome binding with increased platelet activation. Figure 4 shows representative data from flow cytometry experiments. Figure 4A shows the gated area in the forward scatter (FSC) vs. side scatter (SSC) density profile of whole blood components that was confirmed as platelets by simultaneous FITC-anti-CD41a and PE-anti-CD62 staining. Figures 4B, 4C and 4D show the change in activation level of this population in going from zero agonist to ADP to TRAP. Figures 4E, 4F and 4G show the relative NBD-fluorescence intensity histograms for gated platelet populations incubated with NBD-labeled RGD- and RGE- liposomes, at zero agonist, 10 μ M ADP and 5 μ M TRAP respectively. The histogram for unlabeled platelets is marked as (1), for RGE-liposome incubated platelets marked as (2) and RGD-liposome incubated platelets marked as (3), in each of “4E”, “4F” and “4G”.

As evident from 4B, freshly drawn blood showed about 74.2% unactivated platelets. It is evident from Figure 4E that such a population of platelets is minimally stained by both RGD- and RGE-liposomes to the same extent, over unlabeled platelets.

With the addition of ADP, ~89% of the platelet population is activated, as evident from the density plot in 4C. Incubation of an activated platelet population with NBD-labeled RGE-liposomes did not result in any enhancement of NBD-fluorescence intensity, while incubation of similar platelet population with RGD-liposomes resulted in a significant enhancement of NBD-fluorescence intensity (Fig. 4F). A similar effect (Fig. 4G) was observed in TRAP activated platelet population (~97.3% activated, Fig. 4D).

Comparing Figures 4E, 4F and 4G, shows that both RGE- and RGD-liposomes minimally attach to unactivated platelets in a non-specific fashion but only the binding of RGD-liposomes increases considerably with increasing platelet activation. Similar experiments with a fibrinogen-depleted

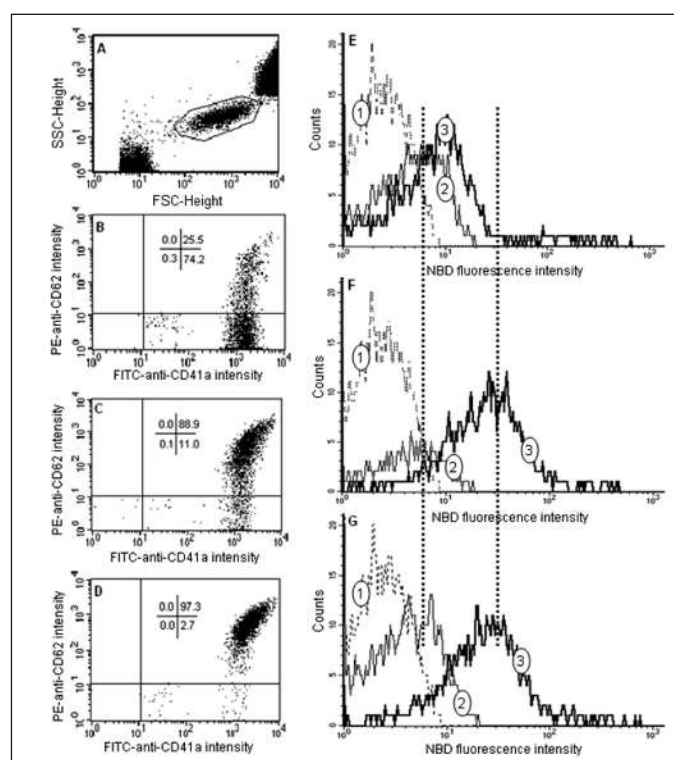


Figure 4: Representative flow cytometry results from analysis of human whole blood aliquots incubated with test liposomes at different levels of platelet activation. “A” shows the gated area in the FSC vs. SSC density plot for flow cytometry analysis of whole blood samples which corresponds to platelet population in the sample, as confirmed by simultaneous PE-anti-CD62 and FITC-anti-CD41a staining. “B”, “C” and “D” represent density plots for platelets at zero agonist, 10 μ M ADP and 5 μ M TRAP respectively. “E”, “F” and “G” represent fluorescence intensity histogram data for platelet populations incubated with NBD-labeled test liposomes at zero agonist, 10 μ M ADP and 5 μ M TRAP respectively. The histogram for unlabeled platelets is marked as (1), for RGE-liposome incubated platelets marked as (2) and RGD-liposome incubated platelets marked as (3), in each of “E”, “F” and “G”. Comparison of “E”, “F” and “G” shows enhanced specific binding of RGD-liposomes to platelets with progressive activation while non-specific binding of RGE-liposomes to platelets remains same irrespective of platelet activation state. The dotted vertical lines are drawn to show the shift of NBD-fluorescence histogram maxima towards higher intensity for RGD-liposome incubated platelets, once agonist (ADP or TRAP) was added, indicating enhancement in RGD-liposome attachment to activated platelets.

platelet suspension yielded comparable results for moderately and highly activated platelets in presence of externally added Ca^{++} (data not shown). This calcium dependence is consistent with the known calcium requirement for effective RGD binding (19, 20). In the suspension samples, liposome interaction with unactivated platelets could not be assessed properly as a significant percentage ($\geq 50\%$) of platelets were already activated, presumably due to the centrifugation and resuspension procedures.

The representative fluorescence histogram shown in Figure 4 demonstrates that RGD-modified liposomes *specifically* bind to activated platelets. The percentages of platelets labeled above background (unlabeled) were $\sim 10\text{--}30\%$ for RGE- and RGD-liposomes interacting with a predominantly unactivated platelet population. For a predominantly activated platelet population, the percentage of platelet labeling for RGE-liposomes remained the same while that for RGD-liposomes increased to $\sim 60\text{--}80\%$ above background. These results suggest a potential way for liposome-mediated localized drug delivery to activated platelets at sites of vascular injury.

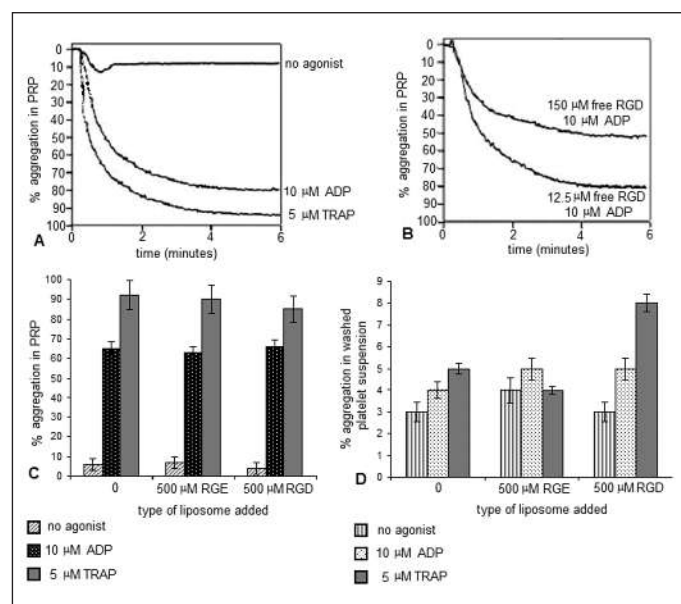


Figure 5: Representative data from aggregometry analysis of platelets in PRP and in fibrinogen-depleted suspension (in PBS/1% BSA) incubated with test liposomes at different activation states. “A” represents the baseline aggregation measurements in PRP at different activation levels of platelets, without liposomes. “B” represents aggregation profiles of platelets in PRP at $10\ \mu\text{M}$ ADP, in presence of $12.5\ \mu\text{M}$ free RGD-peptide and $150\ \mu\text{M}$ free RGD-peptide. As evident in “B” only very high concentration of free peptide ($150\ \mu\text{M}$) causes substantial inhibition of platelet aggregation. “C” represents the aggregation data for platelets in PRP in predominantly unactivated (zero agonist), moderately activated ($10\ \mu\text{M}$ ADP) and predominantly activated ($5\ \mu\text{M}$ TRAP) states, in absence and in presence of test liposomes. “D” shows the same for fibrinogen-depleted platelet suspension in PBS/1%BSA suspension. “C” and “D” suggest that the peptide-modified liposomes have no significant induction or inhibition effect on platelet aggregation. “D” consequently also suggests no effect of liposomes on platelet activation.

In vitro platelet aggregation studies

One potential limitation of liposomes targeting activated platelets for anti-thrombotic therapy is the possibility that the peptide multivalency on the liposome may induce aggregation within the circulating platelet population, or between circulating and previously adsorbed platelets. In order to test for this effect on platelet aggregation, the behavior of PRP and platelet suspensions (in PBS/1%BSA) in the presence of RGE- and RGD- liposomes was studied by aggregometry. Experiments were performed using no agonist, $10\ \mu\text{M}$ ADP and $5\ \mu\text{M}$ TRAP. Figures 5A and 5C show representative data for aggregometry with PRP, while Figure 5D shows data for studies with platelet suspension. As evident from 5A and 5C, without agonist, and in the absence of liposomes, the mean aggregation for PRP was $6\pm 2\%$. At $10\ \mu\text{M}$ ADP concentration, without liposomes, the aggregation was $65\pm 8\%$. At $5\ \mu\text{M}$ TRAP concentration, without liposomes, the aggregation was $92\pm 6\%$. All aggregation levels reached saturation after about 3 min.

Next, PRP aliquots were incubated with $500\ \mu\text{M}$ concentration of test liposomes without agonist, with $10\ \mu\text{M}$ ADP and with $5\ \mu\text{M}$ TRAP for time periods of 0, 20, 40 and 60 min and the corresponding aggregation profiles were recorded. Without agonist, as well as, with $10\ \mu\text{M}$ ADP and with $5\ \mu\text{M}$ TRAP, RGE- and RGD-liposome incubated PRP aliquots showed aggregation levels close to that for liposome-free PRP samples ($\sim 6\%$ without agonist, $\sim 70\%$ for ADP and $\sim 90\%$ for TRAP), even after 60 min. Thus, the liposomes showed negligible inhibition or enhancement of platelet aggregation.

For a fibrinogen-depleted washed platelet suspension, in absence of liposomes, negligible aggregation ($4\text{--}6\%$) was observed even at high agonist concentration ($10\ \mu\text{M}$ ADP or $5\ \mu\text{M}$ TRAP), as shown in Figure 5D. When the washed platelet suspensions were incubated with RGE- and RGD-modified liposomes, no significant change in aggregation was observed without agonist or with $10\ \mu\text{M}$ ADP. With $5\ \mu\text{M}$ TRAP, only RGD-liposomes incubated with washed platelets for 60 min showed slight increase in aggregation ($9\pm 5\%$).

To compare liposome-incubated sample data with that for free peptide-incubated sample, unconjugated free GSSGRGDSPA peptide was also incubated with PRP at concentrations of $12.5\ \mu\text{M}$ and $150\ \mu\text{M}$ (Fig. 5B), using similar aggregometry protocol. No effect on platelet aggregation was observed at lower concentration of the free peptide and maximal aggregation was recorded to be $\sim 80\%$. At $150\ \mu\text{M}$ of free peptide, aggregation decreased to $\sim 50\%$. These results are consistent with the measured IC_{50} of $\sim 275\ \mu\text{M}$, determined for the free peptide in the competitive binding assay (Table 1). This high concentration of free peptide is practically unattainable on a liposome surface.

Discussion

This work focuses on the development of a liposomal delivery system targeted to activated platelets for potential localized delivery of therapeutic agents to activated platelets at the site of a vascular injury. The results demonstrate a number of important attributes necessary for successfully attaining a delivery system for such a purpose. Firstly, the application of solid-phase chemistry to create a functional RGD peptide-lipid conjugate for de-

velopment of platelet-targeted liposomes, is shown. The images and intensity data obtained by fluorescence microscopy experiments indicate that liposomes containing DSPE-PEO-RGD conjugate (5 mole %) bind activated platelets at levels significantly greater than those modified with RGE peptide or PEO alone. The specificity of liposome-binding to activated platelets is confirmed by dual-staining experiments using simultaneous FITC-anti-GPIIb (green) and RhB-liposome (red) fluorescence. One-to-one correspondence of fluorescent spots imaged by epifluorescence microscopy, with spread platelets imaged by phase contrast microscopy also complements the specificity data. This implies that the RGD peptide retains an active conformation and accessible orientation for binding specifically to activated platelet receptors, mainly integrin GPIIb-IIIa. It is unclear whether the liposome staining represents lipid mixing with the platelet plasma membrane, endocytosis, or intact vesicles bound to the platelet surface. Other studies have shown that platelets have active uptake mechanisms, and with incubation times of one hr at room temperature, significant lipid mixing and liposome endocytosis may occur²⁵. This may have a significant impact on the choice of encapsulated therapeutic agent, whether intended for intracellular or extracellular delivery.

The incorporation of ligands at the molar percentages utilized in this study indicates that each liposome carries multiple copies of peptide on its surface. Assuming a cross-sectional diameter of 10 Å for peptide-conjugated phospholipid and a liposome mean vesicle diameter of 100 nm, incorporation of 5 mole% peptide leads to ~1000 copies of the RGD sequence accessible on the liposome surface, assuming that at least 50% of the RGD moiety is expressed on the exterior surface of the liposome. This peptide multivalency can be a concern in that RGD-liposomes may induce aggregation within the circulating platelet population, or between circulating and previously adsorbed platelets. This is of particular concern in the case of GPIIb-IIIa targeting, as this receptor is known to bind fibrinogen under low shear, leading to the recruitment of platelets from the circulation to a site of vascular injury. Therefore, the ability of multivalent RGD-modified liposomes to induce (or possibly inhibit) platelet aggregation was assessed using *in vitro* aggregation assays. At high concentrations of 500 µM total lipid, no discernible effect on aggregation was noted in PRP aliquots. This lipid concentration can correlate to an equivalent free GSSGRGDSPA concentration of 12.5 µM, by the approximation that at least half of the total concentration of peptide is present on the liposome outer surface. We also tested the ability of the free RGD sequence alone to affect aggregation under the same assay conditions, at concentrations of 12.5 µM and 150 µM. Only in the presence of 150 µM free RGD, did the platelet aggregation decrease to values consistent with the observed IC₅₀ value for the free peptide (275 µM). An equivalent RGD-peptide density on the liposome surface is theoretically unattainable, indicating that the effective RGD-density on the liposome surface is unable to affect platelet-platelet crosslinking and hence unable to facilitate aggregation. Although the RGD distribution in a liposome suspension is not homogeneous, as compared with free peptide in solution, the high concentration of free RGD required to inhibit aggregation would suggest that even at more reasonable compositions, the peptide density required for liposome binding

should be much less than that required for potentiation of aggregation.

During aggregometry analysis with washed platelet suspensions, the RGD-liposomes showed only a slight enhancing effect on platelet aggregation (9 ± 5%), in the presence of 5 µM TRAP. However, this is unlikely to be of any clinical significance as this high concentration of a potent agonist is above what would be likely *in vivo*. The inability of multiple RGD-motifs on the liposome surface to induce major platelet aggregation can be attributed to the moderate platelet receptor affinity of the GSSGRGDSPA sequence. Thus, the RGD-modified liposomes should be capable of targeting activated platelets *in vivo*, but unlikely to induce platelet aggregation. Also, the RGD-liposomes are incapable of activating platelets, as evident from their inability to augment platelet aggregation in PRP, without agonist. These results are consistent with the extent of platelet labeling observed by flow cytometry, and the absence of larger platelet aggregates observed by fluorescence microscopy.

From flow cytometry studies with whole blood aliquots, RGE- and RGD-liposomes were found to only minimally stain predominantly unactivated platelet populations in a non-specific fashion. With progressive activation of platelets using agonists (ADP and TRAP), the RGE-liposomes still showed minimal staining, while RGD-liposomes showed significant enhancement in staining activated platelets. This may be attributed to specific interaction of RGD-motifs on the liposomes with an increasing number of RGD-recognizing receptors expressed on the platelet surface with progressive platelet activation. The enhanced binding of RGD-modified liposomes to activated platelets makes it necessary to address the *specificity* of interaction between RGD motifs and various RGD-recognizing platelet surface receptors. An activated platelet surface can potentially express several RGD-recognizing and binding receptors, namely α2β1 (RGD-specificity debatable), αvβ3, α5β1, α6β1 and GPIIb-IIIa (αIIbβ3). Of these, the surface density of GPIIb-IIIa, however, is orders of magnitude higher (~50,000/platelet) compared with the others (~100–1000/platelet) (24). The next most expressed receptor is GPIb/IX/V (~25,000/platelet), the vWF receptor whose expression is dependent on high shear (11). The role of RGD-recognition and binding by this receptor is also unclear, since vWF interacts with this receptor not through RGD domain but an A1 domain (aa 497–716) containing a disulfide bond between Cys 509 and Cys 695. Nevertheless, the results from the dual staining microscopy experiments strongly suggest enhanced specific interaction of RGD-liposomes with a high surface density receptor other than GPIIb. Hence, GPIIb-IIIa can be considered as the predominant receptor for the RGD-liposomes. If any other receptors on activated platelets bind RGD-modified liposomes that would increase the liposome attachment and thus be considered as a beneficial effect in platelet targeting.

For flow cytometry studies with whole blood aliquots, the RGD-liposome binding seems to occur in spite of the presence of fibrinogen. This may suggest that the RGD-liposomes can compete effectively with fibrinogen in platelet-binding. Such competition may suggest potential inhibition of fibrinogen-mediated platelet-platelet bridging. However, this seems to contradict the results observed in aggregometry assays where platelet aggre-

gation in PRP was found to be unaffected by liposome incubation. The apparent discrepancy in liposome behaviour can be explained by consideration of the significant difference in experimental conditions for the two methods. Firstly, the aggregometer is calibrated to detect progressive platelet aggregation and not fibrinogen or liposome binding to platelets. In the aggregation assays in PRP, a finite number of liposomes are likely to bind to platelets but that competitive effect is negligible compared with the binding of the high concentration of fibrinogen present in 450 μL of PRP used for each reading. Similar liposome-binding takes place in the flow cytometry aliquots but in this case, the competitive effect seems more pronounced due to a much lower concentration of fibrinogen in 10 μL of whole blood aliquot used for each reading. The flow cytometry instrumentation detects fluorescence signal from any number of liposomes that may potentially bind to activated platelets even in presence of fibrinogen. The appropriate gating co-ordinates then enable the observation of the fluorescence enhancement over unlabeled platelets. For ADP or TRAP activated samples in flow cytometry, about 20–30% of the platelet population stained positive for PE-anti-CD62 (emission \sim 578 nm) but showed no fluorescence in the NBD (emission \sim 530 nm) region, indicating negligible liposome attachment. This may be the effect of fibrinogen binding potentially excluding liposome binding on that population of platelets.

In conclusion, the results demonstrate the feasibility of using peptide-modification of liposomes to develop drug delivery systems

directed to cardiovascular targets. The coagulation system presents a number of molecular candidates from which peptides could be designed and exploited for drug delivery within the vasculature. RGD sequence is recognized by specific integrins of several cell-types like platelets, endothelial cells and smooth muscle cells. The results have shown the successful exploitation of the RGD-recognizing ability of platelet integrins to develop a platelet-targeted delivery system. However, it is important to note, that, to make such a delivery system platelet-specific in presence of other cell types, further customization of peptide ligands will be necessary. Peptide amphiphiles generated prior to liposome formation can easily be incorporated into a heterogeneous lipid mixture, using virtually any hydrophilic spacer molecule to elevate the peptide above the liposome outer surface. The utility of peptides for targeted intravascular drug delivery, therefore, relies on the identification of specific receptor/ligand systems from which minimum length active sequences can be derived that retain specificity for particular cell surface receptor phenotypes. Such an approach holds significant promise in the development of a broad spectrum of cardiovascular therapeutic systems.

Acknowledgements

The authors would like to acknowledge Grace Lin, Coby Larsen, Victoria Vachon and Emily Link for providing technical assistance in microscopy, aggregometry and flow cytometry studies.

References

1. Fishbein I, Waltenberger J, Banai S, et al. Local delivery of platelet-derived growth factor receptor-specific tyrophostin inhibits neointimal formation in rats. *Arterioscler Thromb Vasc Biol* 2000; 20: 667–76.
2. Kandarpa K, Nakatsuka S, Bravo SM, et al. Mural delivery of iloprost with use of hydrogel-coated balloon catheters suppresses local platelet aggregation. *J Vasc Interv Radiol* 1997; 8: 997–1004.
3. Nugent HM, Edelman ER. Local drug delivery and tissue engineering regulate vascular injury. *Curr Pharm Des* 1997; 3: 529–44.
4. Mauro L, Borovicka M, Kline S. Introduction to coronary artery stents and their pharmacotherapeutic management. *Ann Pharmacother* 1997; 31: 1490–8.
5. Le Breton H, Plow EF, Topol EJ. Role of platelets in restenosis after percutaneous coronary revascularization. *J Am Coll Cardiol* 1996; 28: 1643–51.
6. Tchong JE. Glycoprotein IIb/IIIa receptor inhibitors: putting the EPIC, IMPACT II, RESTORE and EPILOG trials into perspective. *Am J Cardiol* 1996; 78: 35–40.
7. Jang Y, Lincoff A, Plow EF, et al. Cell adhesion molecules in coronary artery disease. *J Am Coll Cardiol* 1994; 24: 1591–601.
8. Barron M, Lake R, Buda A, et al. Intimal hyperplasia after balloon injury is attenuated by blocking selectins. *Circulation*. 1997; 96: 3587–92.
9. D'Souza SE, Ginsberg MH, Natsueda GR, et al. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature* 1991; 350: 66–68.
10. Cierniewski CS, Byzova T, Papierak M, et al. Peptide ligands can bind to distinct sites in Integrin $\alpha_{IIb}\beta_3$ and elicit different functional responses. *J Biol Chem* 1999; 274: 16923–32.
11. Deckmyn H, Ulrichts H, Van de Walle G, et al. Platelet antigens and their function. *Vox Sanguinis* 2004; 87: S105–S111.
12. Pytela R, Piersbacher MD, Ginsberg MH, et al. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science* 1986; 231: 1559–62.
13. Lam SC-T, Plow EF, Smith MA, et al. Evidence that arginyl-glycyl-aspartate peptides share a common binding site on platelets. *J Biol Chem* 1987; 262: 947–50.
14. Gyongyossy-Issa MI, Muller W, Devine DV. The covalent coupling of Arg-Gly-Asp-containing peptides to liposomes: purification and biochemical function of the lipopeptide. *Arch Biochem Biophys* 1998; 353: 101–8.
15. Nishiya T, Sloan S. Interaction of RGD liposomes with platelets. *Biochem Biophys Res Commun* 1996; 224: 242–5.
16. Berndt P, Fields GB, Tirrell M. Synthetic lipidation of peptides and amino acids: monolayer structure and properties. *J Am Chem Soc* 1995; 117: 9515–22.
17. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int Journal Peptide Protein Res* 1990; 35: 161–214.
18. Plow E, Pierschbacher M, Ruoslahti E, et al. Arginyl-glycyl-aspartic acid sequences and fibrinogen binding to platelets. *Blood* 1987; 70: 110–15.
19. D'Souza SE, Haas TA, Piotrowicz RS, et al. Ligand and cation binding are dual functions of a discrete segment of the integrin β_3 subunit: cation displacement is involved in ligand binding. *Cell* 1994; 79: 659–67.
20. Lestini BJ, Sagnella SM, XY Z, et al. Surface modification of liposomes for selective cell targeting in cardiovascular drug delivery. *J Control Rel* 2002; 78: 235–47.
21. Michelson AD. Flow cytometry: A clinical test of platelet function. *Blood* 1996; 87: 4925–36.
22. Schmitz G, Rothe G, Ruf A, et al. European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost* 1998; 79: 885–96.
23. Craig WS, Cheng S, Mullen DG, et al. Concept and progress in the development of RGD-containing peptide pharmaceuticals. *Biopolymers (Peptide Science)* 1995; 37: 157–75.
24. Beer JH, Springer KT, Collier BS. Immobilized Arg-Gly-Asp (RGD) peptides of varying lengths as structural probes of the platelet glycoprotein IIb/IIIa receptor. *Blood* 1992; 79: 117–28.
25. Male R, Vannier WE, Baldeschwieler JD. Phagocytosis of liposomes by human platelets. *Proc Natl Acad Sci U S A* 1992; 89: 9191–5.