Published in final edited form as:

Biomacromolecules. 2013 March 11; 14(3): 910–919. doi:10.1021/bm301996p.

A Platelet-Mimetic Paradigm for Metastasis-Targeted Nanomedicine Platforms

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Abstract

There is compelling evidence that beyond their traditional role in hemostasis and thrombosis, platelets play a significant role in mediating hematologic mechanisms of tumor metastasis by directly and indirectly interacting with pro-metastatic cancer cells. With this rationale, we hypothesized that platelets can be an effective paradigm to develop nanomedicine platforms that utilize platelet-mimetic interaction mechanisms for targeted diagnosis and therapy of metastatic cancer cells. Here we report on our investigation of the development of nanoconstructs that interact with metastatic cancer cells via platelet-mimetic heteromultivalent ligand-receptor pathways. For our studies, pro-metastatic human breast cancer cell line MDA-MB-231 was studied for its surface expression of platelet-interactive receptors, in comparison to another low-metastatic human breast cancer cell line, MCF-7. Certain platelet-interactive receptors were found to be significantly over-expressed on the MDA-MB-231 cells and these cells showed significantly enhanced binding interactions with active platelets compared to MCF-7 cells. Based upon these observations, two specific receptor interactions were selected and corresponding ligands were engineered onto the surface of liposomes as model nanoconstructs, to enable platelet-mimetic binding to the cancer cells. Our model platelet-mimetic liposomal constructs showed enhanced targeting and attachment of MDA-MB-231 cells compared to the MCF-7 cells. These results demonstrate the promise of utilizing platelet-mimetic constructs in modifying nanovehicle constructs for metastasis-targeted drug as well as modifying surfaces for ex-vivo cell enrichment diagnostic technologies.

Keywords

cell attachment; metastasis; platelet-cancer cell interaction; platelet mimicry; targeted delivery

Introduction

Tumor metastasis is a highly complicated pathological process involving multi-factorial pathways of cancer-to-host interactions. Elucidating the precise biomolecular pathways of metastasis is an area of major importance in current cancer research, and the insight gained therefrom can provide us with new avenues for potential therapeutic interventions. In this

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The authors declare no competing financial interests.

respect, there is compelling experimental and clinical evidence that dysregulated hemostatic components have a significant involvement in hematogenous mechanisms of metastasis.¹⁻³ It has been found that beyond their physiologic role in hemostasis and pathologic role in vascular inflammation and thrombosis, blood platelets may have crucial involvement in facilitating cancer aggression and spreading.⁴⁻⁷

The involvement of platelets in malignancy was first recognized in 1865 by the French clinician Armand Trousseau through the observation that migratory thrombophlebitis could be an indicator of occult malignancy, including the diagnosis of his own fatal pancreatic carcinoma.^{2,8} This clinical manifestation of the hypercoagulable state seen with advanced carcinomas is now known as Trousseau's Syndrome. About a decade later, German surgeon Theodor Billroth observed tumor thrombi within the blood vessels of cancer patients and conjectured that tumor-platelet microemboli played a critical role in metastatic disease.⁹ Almost a 100 years later in 1968, Gasic et al. provided evidence of the association between platelet number and metastatic cancer potential, by demonstrating that thrombocytopenia resulted in decreased metastasis in mice.¹⁰ Subsequently, in 1998 Pinedo and Folkman provided mechanistic evidence regarding the role of platelet-derived secretome in tumor angiogenesis, which is a major component of tumor metastasis.¹¹ In recent years, several research groups have extensively reported on the interaction between activated platelets and tumor cells as a functional component of metastatic mechanisms.¹²⁻¹⁷ Correlative analysis between the platelet-associated components of hemostasis and the biomolecular components in aggressive malignancies indicate several mechanistic possibilities as follows (Figure 1):

- i. Active platelets can promote epithelial-to-mesenchymal transition in cancer cells, for example, by secretion of TGF β and activation of TGF β /Smad and NF-kB pathways.¹⁸
- ii. Active platelets can secrete pro-angiogenic cytokines (e.g. VEGF-A, PDGF etc) and proteases (e.g. MMP-2, MMP-9 etc) to influence tumor vascularization, matrix and basement membrane degradation, cancer cell migration and intravasation.¹⁹⁻²¹
- iii. Following intravasation, tumor cells can induce further platelet activation (e.g. via tissue factor pathway) and aggregation around themselves to form a 'platelet cloak' to stay protected from immune surveillance in circulation.^{17,22}
- iv. Subsequently, active platelets can facilitate the arrest of circulating tumor cells under dynamic blood flow conditions onto the vascular wall at secondary sites, and mediate endothelial retraction, matrix degradation and extravasation.²³⁻²⁵
- v. Active platelet secretome and platelet-mediated recruitment of leukocytes can regulate inflammation, matrix remodeling and angiogenesis to promote and maintain the metastatic microenvironment.^{24,26,27}

Based upon the above descriptions, we rationalize that active platelets can be an effective paradigm to develop nanomedicine platforms that enable enhanced interaction with circulating and metastasized cancer cells utilizing platelet-mimetic binding mechanisms. Following this rationale, here we report on our investigation of the development of nanoconstructs that interact with pro-metastatic cancer cells via platelet-mimetic heteromultivalent ligand-receptor pathways.

For our studies, liposomes were chosen as a model nanomedicine vehicle platform because of their established clinical history in delivering a variety of anti-tumor agents and other therapeutics.²⁸⁻³² However the platelet-mimetic ligand-receptor technology reported here is adaptable to various other nanovehicles, utilizing appropriate particle surface bioconjugation chemistry tools. As a model aggressive pro-metastatic cancer cell line, the human breast cancer line MDA-MB-231 was chosen. This cell line, first obtained from a patient in 1973 at

M.D. Anderson Cancer Center in Texas, has a spindle-shaped epithelial-like morphology and has an invasive phenotype that has been confirmed by chemoinvasion and chemotaxis assays.³³ In vitro, the MDA-MB-231 cells can grow on agarose with high colony forming efficiency, indicating high tumorigenicity. Tail vein injection of these cells into nude mice has been reported to produce experimental metastasis.³⁴

We hypothesized that the pro-metastatic MDA-MB-231 cancer cells have high surface expression of multiple platelet-interactive receptor proteins that can be potentially exploited to engineer platelet-mimetic nanomedicine vehicles for actively targeting these metastatic cells. To test our hypothesis, we have compared these pro-metastatic cells against another human breast cancer cell line, MCF-7, which is known to have low metastatic potential.²³ Both cell lines were analyzed using immunofluorescence techniques for surface-expression of platelet-interactive moieties. In addition, both cell lines were allowed to interact with active versus inactive platelets under a flow environment, and the platelet-cancer cell binding was analyzed by fluorescence microscopy. Based upon these data, specific receptor interactions were selected and corresponding ligands were engineered onto the surface of liposomes to enable heteromultivalent platelet-mimetic binding to the cancer cells. Rhodamine was loaded into the liposomes as a readily available model hydrophobic payload and liposome binding and Rhodamine delivery to MDA-MB-231 cells versus MCF-7 cells was studied using fluorescence microscopy. In additional experiments, slide surfaces were coated with platelet-mimetic liposomes and exposed to MDA-MB-231 cells versus MCF-7 cells under flow, to determine whether the enhanced interaction of the pro-metastatic cells with the liposomes led to higher attachment of these cells on the slide surface as a proof-of-concept for potentially developing ex vivo diagnostic technologies.

Results

Surface expression of platelet-interactive moieties on cancer cells

Figure 2 shows the comparison of post-transcriptional surface expression of platelet-interactive receptor protein moieties on MDA-MB-231 cells versus MCF-7 cells, as obtained by the immunofluorescence staining analysis. As evident from the data, the pro-metastatic MDA-MB-231 cells showed significantly higher surface-expression of GPIIb-IIIa-like receptors (that can bind to platelets mediated by fibrinogen, ~2-fold higher expression), P-selectin (that can bind to platelets mediated by P-selectin Glycoprotein Ligand, PSGL-1, ~7-fold higher expression), and GPIa-IIa-like receptors (that can bind to platelets mediated by collagen-like molecules, ~5-fold higher expression), compared to MCF-7 cells. In addition, the MDA-MB-231 cells also showed comparatively higher expression of several other platelet and endothelial interaction relevant proteins like E-selectin (binds to sialyl Lewis moieties, ~2-fold higher expression), integrin $\alpha_v\beta_3$ (binds to fibronectin, vitronectin etc, ~2-fold higher expression) and GPIb α -like proteins (binds to von Willebrand factor, ~12-fold higher expression). These results are highly interesting as they suggest several possible ligand-receptor pathways by which active platelets can undergo enhanced binding to the pro-metastatic MDA-MB-231 cells, compared to the low metastatic MCF-7 cells.

Interaction of active platelets with cancer cells in a flow environment

Building on the results regarding high surface-expression of platelet-interactive moieties on MDA-MB-231 cells, the interaction of active platelets with the cancer cells in a flow environment was analyzed using fluorescence microscopy. As described in detail in the Experimental Section, two complimentary experimental set-ups were utilized for these studies. In one set-up slide surfaces were coated with a monolayer of active platelets and DAPI-stained cancer cells (MDA-MB-231 or MCF-7) were allowed to flow over at wall

shear of 5 dynes/cm². In the complimentary set-up, cancer cells (MDA-MB-231 or MCF-7) were cultured on surfaces, stained with DAPI (blue for nucleus) and Phalloidin (orange for microfilaments) and exposed to Calcein-stained (green) resting or active platelets in a gyratory shaker. Figure 3B shows representative fluorescence microscopy data for the first set-up, while Figure 4B shows the multi-color fluorescence microscopy data for the alternate set-up. From Figure 3C, it is evident that the MDA-MB-231 cells bind to active platelet-coated surface at significantly higher levels compared to MCF-7 cells (~2 fold higher binding). On non-specific surfaces (i.e. albumin) neither cancer cells show high binding. The higher platelet-interactive ability of the MDA-MB-231 cells is further confirmed from the results of Figure 4C, which show significantly (~12-fold) higher binding of the activated green platelets on the surface of MDA-MB-231 cells, compared to MCF-7 cells. The resting platelets show only minimal binding to either cancer cell type. These results confirmed our hypothesis and inspired a nanomedicine approach that utilizes platelet-mimetic mechanisms for targeting metastatic cells.

Interaction of platelet-mimetic liposomal constructs with cancer cells in a flow environment

Based upon the receptor-expression data and the confirmation of higher interaction of active platelets with the pro-metastatic MDA-MB-231 cells, liposomes were fabricated and surface-engineered to render platelet-mimetic binding to the cancer cells. As detailed in the Experimental Section, for the current studies only two significant platelet-interactive pathways were selected, namely, interaction via the GPIIb-IIIa-like integrin receptors and via the P-selectin motifs. Consequently, the liposomes were heteromultivalently surface-decorated with a GPIIb-IIIa-binding FMP and a P-selectin-binding PBP. The relative peptide ratios on the liposome surface were kept at 1:1, while the total peptide composition was kept at 5 mol%, and the liposomes were labeled with Rhodamine (red fluorescence). As evident from the representative results shown in Figures 5B-C, the red fluorescent liposomes were able to bind to the MDA-MB-231 cells at significantly (~3-fold) higher levels compared to the MCF-7 cells. The low level of binding to the MCF-7 cells is probably due to the low level presence of GPIIb-IIIa-like receptors and $\alpha_v\beta_3$ -like receptors on these cells that still allow for a limited extent of binding to fibrinogen-type of ligands (and hence to the FMPs on liposomes). However, the MCF-7 cells showed almost no expression of P-selectin while the MDA-MB-231 cells showed very high expression of P-selectin. Hence, possibly due to the combined binding mechanisms of FMP ligands to the GPIIb-IIIa-like receptors and the PBP ligands to P-selectins, the heteromultivalently surface-modified liposomes showed the high level of binding to the MDA-MB-231 cells. The specificity of FMP decorations to bind to cancer cell surface GPIIb-IIIa-like receptors, and that of PBP decorations to bind to P-selectin receptors, was further confirmed by the results from 'blocking' studies. Figure 5D shows the immunofluorescence analysis results from these studies, indicating that the pre-incubation with liposomes significantly blocked the subsequent receptor-specific binding of the antibodies (hence reduced associated fluorescence). Also, it should be noted that for the Rhodamine-labeled targeted liposome studies, following liposome binding to the MDA-MB-231 cells, the whole cell seemed to be stained with the red fluorescence, suggesting possible fusion of liposomes with the cell membrane and delivery of the Rhodamine. This further establishes the potential of delivering therapeutic or imaging agents to metastatic cells, using platelet-mimetic targeted binding of liposomes and potentially other nanovehicle platforms.

As described in the methods section, we also tested binding of DAPI-stained cancer cells to surfaces coated with these heteromultivalent liposomes, under flow at a shear value of 5 dyn/cm² in the PPFC set-up. Figure 6 shows the experimental set-up and the representative results from these experiments. From the images in Figure 6B and the quantitative analysis

of the DAPI-fluorescence of attached cells shown in 6C, it is evident that the MDA-MB-231 cells bind to the platelet-mimetic liposome-coated surface at significantly (~2-fold) higher levels compared to MCF-7 cells. On unmodified liposomes, neither cancer cells show high binding. These results demonstrate the promise of using platelet-mimetic particles for specific binding to circulating or metastasized tumor cells for anti-cancer drug delivery, as well as, cell-enrichment diagnostic technologies.

Discussion

Over the last couple decades, significant refinement in surgical procedures, chemotherapy, radiotherapy, and biological therapy approaches have become successful in reducing the burden of primary tumors, but metastasized tumor still remains as a principal cause of cancer-related morbidity and mortality. Consequently, a mechanistic understanding of the complex multifactorial phenomena that lead to tumor metastasis is an ongoing research area of great clinical significance. Based upon progressive understanding of the metastatic processes, a variety of therapeutic strategies are currently being investigated for anti-metastatic effects, for example, delivering anti-angiogenic agents to reduce pro-metastatic vascularization events, molecular inhibitors of cell-motility and cell-communication pathways, inhibitory agents for hypoxia and hypoxia-induced signal transduction pathways, and agents that can bind and destroy circulating tumor cells and cancer stem cells.³⁵⁻⁴⁵ In addition, the involvement of dysregulated hemostatic components (e.g. platelet hyperactivity, upregulation of coagulation factors etc.) in facilitating metastasis has been recognized, and studies are being conducted to evaluate the therapeutic benefit of systemically administered anti-coagulatory and anti-platelet agents in metastasis.³⁶ These strategies are being investigated in parallel to studies involving systemic delivery of traditional chemotherapy agents. The 'targeted nanomedicine' approach of packaging therapeutic agents within nanovehicles with capability of passively accumulating within and actively binding to tumor cells, that has revolutionized the delivery of traditional chemotherapy agents, may be of great benefit in delivering a variety of anti-metastatic agents to circulating and metastasized tumor cells.^{46,47} For this, it is necessary to engineer nanomedicine platforms that have enhanced capability of binding to metastatic cells and thus can enhance the selectivity of drug delivery.

To this end, only a limited number of research approaches have been reported so far that focus specifically on metastatic or migratory malignant phenotypes. Interesting examples include Epithelial Cell Adhesion Molecule (EpCAM)-coated, selectin-coated or anti-CD34 antibody-coated microfluidic or shunt devices for preferential capture of circulating tumor cells for ex vivo diagnostic or in vivo therapeutic applications, and nanoconstructs targeted to upregulated sites of $\alpha_v\beta_3$ expression as a surrogate indicator of metastasis-associated high angiogenesis.^{43,48-57} We present a unique alternative approach of actively targeting metastatic cells using the multi-receptor mediated interactions between active platelets and metastatic cancer cells as a paradigm.

The metastatic process involves tumor cell dissemination from the primary tumor site, migration through the stromal matrix to undergo direct intravasation or through the lymphatic system to ultimately enter the vasculature, remaining viable in circulation while avoiding immune surveillance, arrest on the vascular wall at a secondary site, and extravasation followed by angiogenesis and growth to form secondary colonies. As indicated previously, different components of these steps can be directly and indirectly facilitated by active platelets and their secretome. Therefore, mimicking the binding interactions between active platelets and metastatic cancer cells on delivery vehicle platforms can provide an effective way to bind to these cells in circulation, as well as at aggression sites and deliver imaging probes and therapeutic agents. An additional advantage

of this approach is that the approach is based on potentially exploiting platelet-cancer cell interactions to bind to metastatic cancer cells but does not systemically affect platelet functions (unlike systemic anti-platelet or anti-coagulant agents tested clinically). To test this hypothesis, we utilized two of the several possible mechanisms that could be useful for platelet-mimetic interaction with cancer cells. Also for our proof-of-concept study, we chose to utilize these two mechanisms with equal ratios of heteromultivalent ligand modification. Utilizing multiple ligand-receptor interactions simultaneously via heteromultivalent ligand decorations of nanovehicle platforms can enhance the cell selectivity, targeting specificity and binding efficacy of drug delivery vehicles, as demonstrated previously by several research groups including ours.⁵⁸⁻⁶⁷ Future studies will be directed at investigating the effects of other platelet-relevant interactions as well as modulating the relative ligand ratios in the heteromultivalent modification to maximize the binding to the target metastatic cells.

Our studies demonstrate that active platelets indeed have a significantly high extent of binding to model pro-metastatic MDA-MB-231 cells compared to the low metastatic MCF-7 cells. The analysis of platelet-interactive receptors and protein moieties on these cell types suggested several possible mechanisms of enhanced active platelet binding to the MDA-MB-231 cells. Mimicking two of these mechanisms on liposomes as a model delivery vehicle demonstrated the ability of enhanced liposome binding to the MDA-MB-231 cells compared to MCF-7 cells. It is interesting to note that there seemed to be a slight decrease in binding of cancer cells to active platelet-coated surfaces after 15 min of flow in the PPFC (Figure 3C), although this was not statistical. We speculate that this is an effect of loosely bound cancer cells getting removed under flow over time, leaving behind only the firmly adhered cells. The active platelet surface has a heterogenous folded morphology in nano-to-microscale dimensions. As a result of this, the platelet-coated surface that the cancer cells are exposed to may have some non-uniformity of interactions that can be reflected in cells initially binding at a high level and but then some loosely bound cells getting removed over time. Interestingly, we did not see this trend with the binding studies on liposome-coated surfaces (Figure 6), possibly because the liposomes are uniform in morphology and dimension, allowing cancer cells to have enhanced uniformity of interaction at the nanoscale. This may be result in enhanced binding and retention of cancer cells on them under flow, compared to platelet-coated surfaces. Nonetheless, in both cases the pro-metastatic cells show higher binding and retention on the platelet (or platelet-mimetic liposome)-coated surfaces, compared to the low metastatic cells.

The flow medium used in the parallel plate flow chamber experiments was cell culture medium (saline-like buffer with 10% serum proteins). We acknowledge that in a physiological environment the presence of natural platelets and other blood cells in the flow may affect the binding interactions between targeted vehicles and cancer cells. For example, the presence of activated platelets could render competitive inhibition of vehicle binding to the cancer cells via the same receptors. In such possibilities, the vehicle surface decoration characteristics may need to be tailored to ensure that the vehicles can efficiently out-compete active platelets in proximity. It is to be noted that most platelets in circulation are not activated but in a resting condition, and they only get activated in response to presence of disease-associated or injury-associated agonist molecules (e.g. ADP, thrombin, tissue factor etc). So the metastasis-targeted vehicles would possibly not face much competition from the bulk of resting platelets, but possibly from the smaller subset of active platelets. Furthermore, for the studies demonstrated here, only two representative binding interactions were tested and only one heteromultivalent peptide decoration ratio was tested. In progression of this research, we will study a variety of possible ligand-receptor interactions and determine the candidate ligands and the optimum decoration densities that maximize the target cell binding, especially in the presence of other blood cells.

In our current studies, the relative enhancement of binding of the platelet-mimetic liposomal vehicles to pro-metastatic cells (MDA-MB-231) is about 2-3 fold more compared to the low-metastatic cells (MCF-7). We rationalize that these smaller levels of enhancement is because MCF-7 itself also bears some levels of platelet-interactive receptors, as evident from Figure 2. The focus of the current work is to show that pro-metastatic cells have higher interactive capacity with platelets, compared to low-metastatic ones, which can be exploited for developing metastasis-targeted drug delivery systems. It is to be noted that in actual drug delivery applications, the targeting of metastatic cells by the platelet-mimetic vehicles will be in comparison to normal non-malignant healthy cells, which will have minimal platelet-interactive capacity. Hence, in such cases the level targeted binding to metastatic cells may turn out to be even higher, at levels sufficient for a useful drug delivery technology.

Also, in our study Rhodamine was loaded into the liposomes as a readily available model hydrophobic payload, adapting other reported studies.⁶⁸⁻⁷⁰ Rhodamine is a small molecule (MW 479.02), and many of the hydrophobic anti-cancer therapeutic molecules that are potential payload candidates for our nanovehicles are also similar small molecules, for example, Doxorubicin (MW 579.98), Daunorubicin (MW 563.99), and Paclitaxel (MW 853.91). These therapeutic molecules have all been shown to be readily incorporated into liposomes in clinically used applications (e.g. Doxil, DaunoXome, and Lipusu), and hence we rationalized that Rhodamine can be used as a comparative model payload. In future studies we will look into loading the hydrophobic anti-cancer drugs mentioned above and also study loading of bigger macromolecular agents like proteins and enzymes that can have anti-tumor capabilities.

Conclusion

Rationalizing from the mechanistic role of active platelets in facilitating metastatic spread of tumors, a liposome-based model nanomedicine platform was developed that can undergo enhanced interaction to pro-metastatic MDA-MB-231 cells via platelet-mimetic ligand-receptor mechanisms. The resultant liposomal nanovehicles demonstrated the capability of enhanced binding to the MDA-MB-231 cells in flow environment. Refinement of this platelet-mimetic nanomedicine paradigm through further in vitro and in vivo evaluation can lead to effective drug delivery and diagnostic technologies for targeted treatment of metastatic cancers.

Experimental Section

Materials

MCF-7 and MDA-MB-231 cells were purchased from American Type Culture Collection (Manassas, VA). RPMI 1640 media, penicillin, streptomycin, bovine serum albumin (BSA), 1× sterile Dulbecco's phosphate buffered saline (DPBS), phosphate buffered saline (PBS), 3.8% w/v sodium citrate, paraformaldehyde (PFA), 0.25% Trypsin were purchased from Fisher Scientific (Pittsburgh, PA). Calcein AM AlexaFluor® 488, 4',6-diamidino-2-phenylidole (DAPI), Phalloidin AlexaFluor® 647, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE)-Rhodamine B, and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). Fluorescein isothiocyanate (FITC)-conjugated anti-CD41a was purchased from BioLegend (San Diego, CA). Phycoerythrin (PE)-conjugated anti-CD62P, CD62E, CD62L, and CD42b and FITC-conjugated anti-CD51/61 and CD49b were purchased from BD Bioscience (San Jose, CA). Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA). The parallel plate flow chamber was purchased from Glycotech (Gaithersburg, MD). Distearyl phosphatidyl choline (DSPC), carboxy-polyethylene glycol-modified distearyl phosphatidyl ethanolamine (DSPE-PEG₂₀₀₀-COOH), and biotin-polyethylene glycol-modified DSPE (DSPE-PEG₂₀₀₀-biotin)

were purchased from Avanti Polar Lipids (Miami, FL). Cholesterol, Triton-X100, and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO). Resins and amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY).

Cell culture

Human breast cancer cell lines, MCF-7 (low metastatic) and MDA-MB-231 (high metastatic), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/mL) and streptomycin (50 µg/mL). All cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. In all experiments, ~80% confluent cell cultures were used.

Immunofluorescence analysis of platelet-interactive proteins on cancer cell surface

MDA-MB-231 and MCF-7 cells were cultured in black-walled 96 well plates until ~ 80% confluent, media was removed, and cells were gently washed with 1× DPBS. Cells were subsequently fixed with 4% PFA for 15 minutes at room temperature, and then washed with 1× DPBS to remove excess fixative. To block any non-specific antibody binding, cells were incubated with 10% bovine serum albumin in 1× DPBS for 1 hour at room temperature, and excess FBS was removed and washed from the surface with 1× DPBS. Cells were incubated with platelet-interactive receptor-specific, fluorescently-labeled antibodies as shown in Table 1 for 1 hour at room temperature in the dark. After any unbound antibody was removed and rinsed with 1× DPBS, antibody-associated fluorescence was measured using a fluorescence plate reader (FITC λ_{ex} = 490nm, λ_{em} = 525nm, PE λ_{ex} = 526nm λ_{em} = 575nm). The measured fluorescence intensity can be correlated to the extent of receptor expression, and levels were compared between MCF-7 and MDA-MB-231 cells. All statistical analysis was performed using ANOVA, and significance was considered to be $p < 0.05$.

Microscopy analysis of platelet-cancer cell interactions

For the experiments of cancer cell binding to platelets under flow conditions, PRP was incubated with ADP for 30 minutes at 37°C to activate platelets. Glass microscope slides were coated with two equal circular area regions with albumin and collagen. The ADP-incubated PRP was then added to the collagen substrate, and the platelets were allowed to adhere and form a monolayer, as we have shown via SEM images previously.^{67,71,72} Excess PRP was removed and any unbound platelets were washed with 1× PBS. Adhered platelets were subsequently fixed with 4% PFA for 1 hour at room temperature. Binding of MDA-MB-231 and MCF-7 cells to the platelet monolayer or albumin coating under flow was determined using a parallel plate flow chamber (PPFC) system (10 mm width × 0.01 in height) fitted to a flow pump and imaged using an inverted fluorescence microscope. The two cell types were cultured in T175 flasks until ~80% confluent, cells were removed from the flasks using 0.25% Trypsin and suspended in RPMI media at a concentration of 10⁶ cells/ml. The cancer cells were stained in suspension with 300 nM DAPI in 1× PBS for 5 minutes at 37°C, and then allowed to flow over the microscope slides containing activated platelets and an albumin control surface at 5 dynes/cm² shear stress. DAPI-stained cancer cell binding to the platelet monolayer or albumin coating was imaged using fluorescence microscopy after 5, 15, and 30 minutes of flow, and the fluorescence intensity of the images was quantified using a MATLAB script. Extent of binding of the cancer cells to platelet monolayer and to the albumin coating was compared between MCF-7 and MDA-MB-231 cells. All statistical analysis was performed using ANOVA, and significance was considered to be $p < 0.05$.

For the complementary experiments studying platelet binding to cancer cells, MDA-MB-231 and MCF-7 cells were cultured in black-walled 96 well plates until ~80% confluent, media was removed, and cells were gently washed with 1× DPBS. Cells were subsequently fixed

with 4% PFA for 15 minutes at room temperature, and then washed with 1× DPBS to remove excess fixative. To block any non-specific binding of fluorescent stains and platelets, cells were incubated with 10% bovine serum albumin in 1× DPBS for 1 hour at room temperature. The membrane was permeabilized by incubating cells with 0.1% Triton-X100 in 1× DPBS for 1 minute, and then Phalloidin at 6μM ($\lambda_{\text{ex}} = 650\text{nm}$, $\lambda_{\text{em}} = 668\text{nm}$) in 1× DPBS was used to stain the F-actin cytoskeleton of the cancer cells for 15 minutes at room temperature. The nucleus was then stained with DAPI at 300nM in 1× PBS ($\lambda_{\text{ex}} = 358\text{nm}$, $\lambda_{\text{em}} = 461\text{nm}$) for 5 minutes at room temperature. Human whole blood was obtained from healthy, aspirin-refraining donors by venipuncture following an IRB-approved protocol and collected in citrated tubes. Platelet rich plasma (PRP) was obtained from the whole blood by centrifugation ($150 \times g$, 15 minutes, room temperature). The platelet cytoplasm was stained with Calcein AM at 2μM in 1× DPBS ($\lambda_{\text{ex}} = 495\text{nm}$, $\lambda_{\text{em}} = 515\text{nm}$) for 30 minutes at 37°C. The Calcein-stained PRP was then centrifuged at $2500 \times g$ for 25 minutes at room temperature to obtain platelet poor plasma (PPP) and a platelet pellet. The PPP was removed to get rid of excess Calcein, and the platelet pellet was resuspended in fresh PPP. Half of this platelet suspension was activated using ADP at a concentration of $2 \times 10^{-5} \text{ M}$ for 30 minutes at 37°C. The Calcein-stained platelets, active or inactive, were allowed to incubate on the cancer cells at low shear using a gyratory shaker at 80rpm ($\sim 2 \text{ dynes/cm}^2$) for 30 minutes at 37°C.⁷³ Any unbound platelets were washed off with 1× DPBS, bound platelet-associated fluorescence was measured using a fluorescence plate reader, and levels were compared between MCF-7 and MDA-MB-231 cells. Platelet binding to the cells was also imaged using a Zeiss Axio Observer.D1 inverted fluorescence microscope fitted with a photometrics chilled CCD camera and a 10× objective, to obtain tricolor merged fluorescence images using suitable filters.

Fabrication of heteromultivalent liposomes with platelet-mimetic interaction capability

Based upon the immunofluorescence analysis of possible receptors on cancer cells that can mediate direct or indirect interaction with active platelets, two receptor interactions were chosen as our initial test metric for studying platelet-mimetic interactions with cancer cells. These were interactions with GPIIb-IIIa (can promote fibrinogen-mediated binding between cancer cells and platelets) and with P-selectin (can promote sialoprotein-based direct binding of cancer cells to platelets). To mimic these interactions, a fibrinogen-mimetic peptide (FMP), cyclo-CNPRGDY(OEt)RC, which binds to GPIIb-IIIa (and maybe $\alpha_v\beta_3$ to a much lower extent), and a sialoprotein-mimetic, P-selectin binding peptide (PBP), DAEWVDVS were synthesized using fluoroenylmethoxycarbonyl chloride (Fmoc)-based solid-phase chemistry on a Knorr resin and characterized using mass spectrometry^{67,74-76}. The peptides were then conjugated via their N-termini to the carboxyl terminus of DSPE-PEG₂₀₀₀-COOH via standard carbodiimide chemistry adapted from previously reported methods, resulting in DSPE-PEG-peptide molecules.^{67,71} These DSPE-PEG-peptide molecules, at 2.5 mole % total lipid each, were combined with 49 mole % DSPC, and 45 mole % cholesterol to fabricate peptide-decorated $\sim 150 \text{ nm}$ diameter liposomes via reverse phase evaporation and extrusion (RPEE) technique.⁷⁷ DHPE-Rhodamine B (red-orange fluorescence), at 1 mole %, was incorporated into the membrane of the liposomes during the lipid film formation step of the RPEE process, for use as a model hydrophobic payload and an imaging probe. Liposome size was characterized using dynamic light scattering (DLS).

Microscopy analysis of platelet-mimetic liposome binding to cancer cells

For studying liposome binding to cancer cells, MDA-MB-231 and MCF-7 cells were cultured in black-walled 96 well plates until $\sim 80\%$ confluent and fixed with 4% PFA for 15 minutes at room temperature. To block any non-specific receptor binding of liposome, cells were incubated with 10% bovine serum albumin in 1× DPBS for 1 hour at room temperature. The Rhodamine-labeled platelet-mimetic liposomes prepared above or

unmodified liposomes (containing no platelet interaction-mimetic peptides) were allowed to incubate with the cancer cells at low shear using a gyratory shaker at 80rpm for 30 minutes at room temperature. Any unbound liposomes were washed off with 1× DPBS, cell-bound liposome-associated fluorescence (Rhodamine B λ_{ex} = 540nm, λ_{em} = 625nm) was measured using a fluorescence plate reader, and levels were compared between MCF-7 and MDA-MB-231 cells. Liposome binding to the cells was also imaged using a Zeiss Axio Observer.D1 inverted fluorescence microscope with a 10× objective.

In an additional experiment, we wanted to confirm that the liposome binding to the MDA-MB-231 cells were via specific interactions with the desired cancer cell receptors (GPIIb-IIIa, $\alpha_v\beta_3$, and P-selectin). For this, receptor pre-blocking studies were performed in vitro. Cancer cells were cultured, fixed, and treated with BSA as before. The cells were then incubated with non-fluorescent (without Rhodamine) platelet-mimetic liposomes (decorated with FMP and PBP as before), for 30 minutes at room temperature, and subsequently washed with PBS to remove any unbound liposomes. Following this, the cells were incubated with fluorescent antibodies which bind to the GPIIb-IIIa, $\alpha_v\beta_3$ and P-selectin receptors (see Table 1) for 1 hour at room temperature in the dark. After 1 hour, unbound antibody was rinsed off with 1× DPBS and antibody-associated fluorescence was measured using a fluorescence plate reader (FITC λ_{ex} = 490nm, λ_{em} = 525nm, PE λ_{ex} = 526nm λ_{em} = 575nm). The working principle of this experiment was that if the pre-incubation with non-fluorescent liposomes resultant in liposomes binding to the cancer cells via interaction with the specific receptors, then these liposomes will block the subsequent binding of the fluorescent antibodies, resulting in a reduction of receptor-associated fluorescence intensity when compared to samples without liposome pre-incubation.

In complementary experiments to study cancer cell binding to liposomes under flow conditions, a monolayer of either platelet-mimetic or unmodified liposomes was created on glass microscope slides. For this, liposomes were fabricated as described in the previous section, however the 1 mole % of DHPE-Rhodamine was replaced with 1 mole % DSPE-PEG₂₀₀₀-biotin. Glass microscope slides were coated with 5mg/ml avidin in 1× PBS 0.01% sodium azide for 4 hours at room temperature, and then the biotinylated platelet-mimetic or unmodified liposomes were allowed to adhere to the avidin substrate for 1 hour at room temperature to form a monolayer coating of liposomes. Binding of MDA-MB-231 and MCF-7 cells to the platelet-mimetic or unmodified liposome-coated surfaces under flow was analyzed using a PPFC. As previously described, suspensions of the two cell types were prepared in RPMI media at a concentration of 10⁶ cells/ml. The cancer cells were stained in suspension with 300 nM DAPI in 1× PBS for 5 minutes at 37°C, and then allowed to flow over the microscope slides containing platelet-mimetic or unmodified liposomes at 5 dynes/cm² shear stress. Cancer cell-liposome interactions were imaged using fluorescence microscopy after 5, 15, and 30 minutes of flow, and the DAPI fluorescence intensity of the images was quantified using a MATLAB script. Levels of binding were compared between MCF-7 and MDA-MB-231 cells. All statistical analysis was performed using ANOVA, and significance was considered to be $p < 0.05$.

Acknowledgments

The research was partially supported by National Science Foundation Graduate Research Fellowship Program No. 10-604 (Recipient: Modery-Pawłowski), National Institute of Health F31-DE019998 (Recipient: Master), CWRU Support of Undergraduate Research & Creative Endeavors Summer Fellowship (Recipient: Pan), and NSF Research Experience for Undergraduates Summer Fellowship (Recipient: Howard). We would also like to thank Dr. Ann-Marie Broome (Medical University of South Carolina) for providing the cell lines (MDA-MB-231 and MCF-7) and Dr. Nicole Steinmetz (Case Western Reserve University) for the use of her cell culture facilities. Finally, we would like to acknowledge the facilities provided by the Center for Cardiovascular Biomaterials (CCB) in the Department of Biomedical Engineering at Case Western Reserve University.

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Figure 1.

Schematic of the possible mechanistic roles of platelets in cancer metastasis. 1) Active platelets can promote epithelial-to-mesenchymal transition in cancer cells by secretion of TGF β and activation of TGF β /Smad and NF- κ B pathways, 2) Active platelets can secrete pro-angiogenic cytokines (e.g. VEGF-A, PDGF etc) and proteases (e.g. MMP-2, MMP-9 etc) to influence tumor vascularization, matrix and basement membrane degradation, cancer cell migration and intravasation, 3) Following intravasation, tumor cells can induce further platelet activation (e.g. via tissue factor pathway) and aggregation around themselves to form a 'platelet cloak' to stay protected from immune surveillance in circulation, 4) Active platelets can facilitate the arrest of circulating tumor cells under dynamic blood flow conditions with the assist of leukocytes onto the vascular wall at secondary sites, and mediate endothelial retraction, matrix degradation and extravasation, and 5) Active platelet secretome and platelet-mediated recruitment of leukocytes can regulate inflammation, matrix remodeling and angiogenesis to promote metastatic microenvironment.

Figure 2.

Surface expression of platelet-interactive moieties on cancer cells. A) Schematic of experimental method for the immunofluorescence assay to determine receptor expression. B) Fluorescence intensity of bound antibodies depicts expression levels of the various platelet-interactive receptors tested. The MDA-MB-231 cells showed significantly high surface-expression of GPIIb-IIIa-like receptors (can bind to platelets mediated by fibrinogen), P-selectin (can bind to platelets mediated by sialoprotein ligands), GPIa-IIa-like receptors (can bind to platelets mediated by collagen-like molecules), E-selectin (can bind to sialyl Lewis moieties), integrin $\alpha_v\beta_3$ (can bind to fibronectin, vitronectin etc), and GPIIb- α -like proteins (can bind to von Willebrand factor) compared to MCF-7 cells, suggesting several possible ligand-receptor pathways for enhanced interaction with active platelets.

Figure 3. Representative results of cancer cell binding to a monolayer of active platelets. A) Schematic of experimental method for the cell-binding assay. B) Representative fluorescence images of DAPI-stained cancer cells bound to the platelet-coated surface or a BSA negative control surface. C) Fluorescence intensity analysis shows that MDA-MB-231 cells bind to the active platelet-coated at significantly higher levels compared to MCF-7 cells, while neither cancer cells bind to the non-specific BSA surface.

Figure 4.

Representative results of platelet binding to cancer cells. A) Schematic of the experimental method for the platelet-to-cancer cell binding assay. B) Representative fluorescence images of active or inactive calcein-stained platelets (green) bound to cancer cells co-stained with phalloidin (red for cytoskeleton) and DAPI (blue for nucleus). C) Fluorescence intensity analysis shows higher binding of the activated platelets on the MDA-MB-231 cells compared to MCF-7 cells, while resting platelets show only minimal binding to either cancer cell type.

Figure 5.

Representative results of the targeted binding of platelet-mimetic liposomes to cancer cells. A) Schematic of platelet-to-cancer cell binding mechanisms and their mimicry using heteromultivalently ligand-decorated liposomes; B) Representative fluorescence images of Rhodamine-labeled (red) liposomes binding to cancer cells, showing higher red staining (higher liposome binding) of MDA-MB-231 cells compared to MCF 7 cells; C) Fluorescence intensity analysis confirms significantly higher binding of the platelet-mimetic liposomes on the MDA-MB-231 cells compared to MCF-7 cells; D) Results from the liposome-based receptor blocking studies show that the MDA-MB-231 cells pre-incubated with non-fluorescent, platelet-mimetic liposomes exhibit significant reduction in receptor-specific antibody fluorescence, indicating binding specificity of the receptor-targeted liposomes.

Figure 6.

Representative results of cancer cells binding to surface coated with platelet-mimetic liposomes. A) Schematic of experimental method to study cancer cell attachment to liposome-coated surface; B) Representative fluorescence images of DAPI-stained cancer cells bound to surface coated with platelet-mimetic or unmodified liposomes, showing significantly higher attachment of MDA-MB-231 cells on platelet-mimetic liposome coated surface; C) Fluorescence intensity analysis confirms that MDA-MB-231 cells attach to the platelet-mimetic liposome-coated surface at significantly higher levels compared to MCF-7 cells, while neither cancer cells bind to the unmodified liposomes.

Table 1

Platelet-interactive receptors and the respective fluorescent antibodies used for their immunofluorescence analyses.

Receptor	Antibody
GPIIb-IIIa	FITC-conjugated anti-CD41a
P-selectin	PE-conjugated anti-CD62P
$\alpha_v\beta_3$	FITC-conjugated anti-CD51/CD61
E-selectin	PE-conjugated anti-CD62E
L-selectin	PE-conjugated anti-CD62L
GPIa-IIa	FITC-conjugated anti-CD49b
GPIb- α	PE-conjugated anti-CD42b