Quantification of heparin's antimetastatic effect by single-cell force spectroscopy

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Abstract
In circulation, cancer cells induce platelet activation, leading to the formation of a cancer cell-encircling platelet cloak which facilitates each step of the metastatic cascade. Since cancer patients treated with the anticoagulant heparin showed reduced metastasis rates and improved survival, it is supposed that heparin suppresses the cloak's formation by inhibiting the interaction between platelet's adhesion molecule P-selectin with its ligands on cancer cells. To quantify this heparin effect, we developed a single-cell force spectroscopy approach and quantified the adhesion (maximum adhesion force \(F_A\) and detachment work \(W_D\)) between platelets and human non-small cell lung cancer cells (A549). A configuration was used in which A549 cells were glued to tipless cantilevers and force-distance (F-D) curves were recorded on a layer of activated platelets. The concentration-response relationship was determined for heparin at concentrations between 1 and 100 U/mL. Sigmoid dose-response fit revealed half-maximal inhibitory concentration (IC50) values of 8.01 U/mL (\(F_A\)) and 6.46 U/mL (\(W_D\)) and a maximum decrease of the adhesion by 37.5% (\(F_A\)) and 38.42% (\(W_D\)). The effect of heparin on P-selectin was tested using anti-P-selectin antibodies alone and in combination with heparin. Adding heparin after antibody treatment resulted in an additional reduction of 9.52% (\(F_A\)) and 7.12% (\(W_D\)). Together, we quantified heparin's antimetastatic effect and proved that it predominantly is related to the blockage of P-selectin. Our approach represents a valuable method to investigate the adhesion of platelets to cancer cells and the efficiency of substances to block this interaction.

KEYWORDS
cancer cell, heparin, platelets, P-selectin, single-cell force spectroscopy

1 | INTRODUCTION
Cancer is still one of the most lethal diseases worldwide and the vast majority of cancer deaths can be attributed to metastatic spread. Metastases are formed by cancer cells that have been shed from the primary tumor and disseminate in distant organs through blood or lymphatic vessels.\(^1,2\) The interaction of cancer cells with platelets is crucial for hematogenous metastatic dissemination\(^3\) and is denoted as an unholy alliance.\(^4-6\) The connection between cancer and platelets has already been known for over 100 years. In the second half of the 19th century the French physician Armand Trousseau associated the sudden occurrence of migratory deep vein thrombosis and...
thrombophlebitis with concealed cancer.\textsuperscript{7} From this time on this connection has been reported for various types of cancer in several case reports and cohort studies.\textsuperscript{5} The platelet-cancer relationship is known for their deleterious action in all steps of cancer progression but there are also observations that indicate protective functions of platelets in cancer.\textsuperscript{6} However, it was shown that the metastatic activity correlates to the platelet count: The depletion of platelets inhibits metastasis,\textsuperscript{9} whereas platelet reconstitution restores metastatic abilities.\textsuperscript{10} It is undoubted that platelets contribute to metastasis in various ways.\textsuperscript{11}

One of the first steps of the metastatic cascade is the intravasation and a cancer cell that enters the blood stream is termed a circulating tumor cell (CTC). Only a very small fraction of CTCs survives the first minutes in blood vessels\textsuperscript{12} due to cell-degrading high shear forces and immune surveillance.\textsuperscript{13} Platelets protect CTCs by forming a CTC-encircling protective cloak. This platelet cloak then facilitates the shielded cancer cells in each following step of the metastatic cascade, starting with immune evasion and the tethering and arrest to the vessel wall, as well as the extravasation, colonization, angiogenesis, and tumor growth which, in the end, leads to the formation of a metastatic focus.\textsuperscript{4} Formation of this protective platelet cloak needs a stable adhesion between platelets and CTCs. Under physiological conditions, platelet surface receptors are stored in their granules and platelet activation leads to the redistribution of various adhesion molecules from membranes of secretory granules to the cell surface of platelets.\textsuperscript{11,14} CTCs release a variety of platelet-activating mediators such as thromboxan A\textsubscript{2}, thrombin, ADP, CD97, and high-mobility group box 1 (HMGB1).\textsuperscript{1,5} The ability of cancer cells to release factors inducing platelet activation is called tumor cell-induced platelet activation (TCIPA) and one of the reasons of a prothrombotic state in cancer.\textsuperscript{15}

Platelets in close vicinity to CTCs became activated, redistribute their adhesion molecules from secretory granules to the cell surface and release platelet-activating molecules like thromboxan A\textsubscript{2}, thrombin and ADP. These mediators, among others, in turn serve as amplifying signal in the activation of additional platelets.\textsuperscript{16} Upon activation platelets become “sticky” due to the membrane expression and activation of several adhesion molecules like six different integrins (α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{3}β\textsubscript{1}, α\textsubscript{9}β\textsubscript{1}, α\textsubscript{6}β\textsubscript{1}, α\textsubscript{Ib}β\textsubscript{2}, α\textsubscript{IIb}β\textsubscript{2}), GPIbα-IX-V, GPVI, CLEC-2, and P-selectin.\textsuperscript{17} Binding of platelets to CTCs and the formation of a protective cloak is mediated by these platelet surface receptors. In the first step of this physical interaction, P-selectin forms a weak bond with sialyl-Lewis X carbohydrates like P-selectin glycoprotein ligand-1 (PSGL-1, CD162)\textsuperscript{18} as well as with its other ligands like tethered mucins 1, 3, 4, 10 to 18\textsuperscript{3} on cancer cells. Binding of P-selectin triggers binding of the cytoplasmic tail of P-selectin to talin1 which in turn activate α\textsubscript{IIb}β\textsubscript{3}.\textsuperscript{19} It follows the firm adhesion of platelets to CTCs established by α\textsubscript{IIb}β\textsubscript{3} and other platelet integrins.\textsuperscript{20} However, the P-selectin induced integrin-mediated platelet adhesion to tumor cells is an important step of metastasis.\textsuperscript{21,22} The average rupture force (\textit{F}_{rupt}) of a P-selectin PSGL-1 ligand receptor bond was quantified on the single molecule level and found to be \textit{F}_{rupt} = 171 ± 56 pN.\textsuperscript{23} The present work aims on quantification of the platelet to cancer cell adhesion with a particular emphasis on the P-selectin-dependent adhesion using atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS). SCFS is the favorable technique for this purpose since it allows measuring the adhesion between two cells under physiological conditions with a high force resolution.\textsuperscript{24} Furthermore, SCFS allows adding inhibitors or antibodies during an experiment thus enhancing the validity of receptor-blocking maneuvers. Heparin was used to quantify the P-selectin dependent adhesion since it is a potent inhibitor of P-selectin\textsuperscript{25} and known for its antimetastatic potential.\textsuperscript{26} The ability of tumor cells to activate platelets (TCIPA) causes a sevenfold increased risk of venous thromboembolism (VTE)\textsuperscript{27} and need therefore an anticoagulant therapy. Heparins are the standard anticoagulation treatment for cancer-related VTE.\textsuperscript{28} It was shown that heparins not only reduce mortality and morbidity related to VTE but also improve the overall survival of cancer patients.\textsuperscript{27,29} It is supposed that heparin suppresses the adhesion of platelets to CTCs by inhibition of platelets P-selectin\textsuperscript{30,31} and thereby prevent the formation of the protective cloak. Following this logic, antiadhesive features of heparin would be the equivalent of its antimetastatic effect. Anti-P-selectin antibodies were used alone and in combination with heparin in order to test this hypothesis.

The aim of this project was to develop a SCFS approach that allows quantification of the platelet to cancer cell adhesion on single-cell level. This method is useful to test inhibitory effects of molecules on platelet-CTC interaction and determine their half-maximal inhibitory concentration (IC\textsubscript{50}) as shown with heparin in this work. It further allows identifying receptors involved in the platelet-CTC interaction, which can be helpful to develop antimetastatic pharmaceuticals and strategies.

\section*{2 \quad METHODS}

\subsection*{2.1 \quad Reagents}

All chemicals were purchased from Sigma Aldrich (Munich, Germany) unless stated otherwise.

\subsection*{2.2 \quad Cell culture}

A549 cells, a human non-small cell lung cancer cell line (ATCCCCCL-185), were cultured at 37°C, 5% CO\textsubscript{2} in Dulbecco’s modified eagle’s medium (DMEM medium-high glucose) supplemented with 10% Fetal Bovine Serum (SERVA Electrophoresis, Heidelberg, Germany) and 50 U/mL penicillin/streptomycin (Biochrom, Berlin, Germany). Cell passaging processes were carried out using EDTA buffer (in mM: 0.53 EDTA; 137 NaCl; 2.7 KCl; 10 Na\textsubscript{2}HPO\textsubscript{4}; 1.8 KH\textsubscript{2}PO\textsubscript{4}; pH 7.4) instead of trypsin to avoid any degradation of adhesive molecules.

\subsection*{2.3 \quad Platelet isolation from peripheral blood}

Venous blood was drawn from healthy volunteers, who gave their informed consent and did not take any anti-coagulative drug within
the last 2 weeks, using a 5 mL citrate S-Monovette (Sarstedt, Nümbrecht, Germany). In order to prevent platelet activation 500 μL acid-citrate-dextrose buffer (ACD-buffer: 39 mM citric acid; 75 mM sodium citrate; 135 mM dextrose; pH 7.4) was added (1:10 v/v)). Platelet rich plasma (PRP) was separated by a centrifugation at 270g for 20 minutes. PRP (approx. 2 mL) was transferred with a large orifice pipette into a 15 mL conical centrifuge tube (Fisher Scientific, Schwerte, Germany) and complemented with the same volume of calcium-free platelet buffer (in mM: 5 HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid); 140 NaCl; 4 KCl; 1 MgCl2; 1 D-Glucose; 3.5 g/L bovine serum albumin (BSA; SERVA Electrophoresis); 50 U/mL penicillin/streptomycin; pH 7.4) as well as 10% (v/v) ACD-buffer. The tube was inverted two times to mix the solution and platelets were pelletized at 500g for 10 minutes. After removal of the supernatant, the pellet was resuspended in 2 mL calcium-free platelet buffer. Remaining erythrocytes were removed by a further centrifugation step at 100g for 5 minutes. All isolation steps were performed at room temperature.

2.4 | Layer of activated platelets

Glass bottom dishes (FluoroDish; 23.5 mm diameter; World Precision Instruments, Friedberg, Germany) were cleaned twice with ethanol. One silicone culture insert (two well; 70 μL volume per well; 8.4 x 8.4 x 5 mm [w x l x h]; ibidi, Gräfelfing, Germany) was placed in the FluoroDish. The insert was filled with PBS (Dulbecco’s Phosphate Buffered Saline; low endotoxin; without calcium; without magnesium; Biochrom) containing 1% (w/v) BSA and 50 U/mL penicillin/streptomycin (BSA coating) to prevent collagen coating of this area. The free glass surface was coated with monomeric collagen at room temperature overnight using a solution of calf skin collagen A (Biochrom) mixed with the same volume of PBS (pH adjusted to 3-3.5 with HCl). Dishes were washed with PBS before use. About 400 μL of isolated platelet solution (containing approx. 5 x 10⁵ platelets/μL) was added to collagen-coated FluoroDishes and homogeneously distributed by tilting the dish gently. After 2 hours at room temperature a layer of activated platelets was developed. Dishes were washed three times with platelet buffer supplemented with 2 mM CaCl₂ (platelet buffer with calcium) and finally covered with 2 mL of the same buffer. Experiments with these layers were performed within 3 hours after preparation.

2.5 | PeakForce quantitative nanomechanical mapping

Atomic force microscopy was performed in HEPES-Ringer buffer at room temperature in PeakForce Tapping mode using a BioScope Resolve AFM (Bruker Nano Surfaces, Santa Barbara, CA). A PeakForce quantitative nanomechanical-Live Cell (PFQNM-LC) probe (Bruker AFM Probes, Camarillo, CA; tip length 17 μm, tip radius 65 nm, opening angle 15°) was used to image the cell surface. The spring constant of the cantilever was determined with a vibrometer (OFV-551; Polytec, Waldbronn, Germany) and found to be 0.064 N/m. The collagen-coated glass bottom Petri dish with living platelets were held down through the use of vacuum that has been incorporated into the AFM sample plate while still allowing optical access to the sample from below. Images were taken at 384 x 384 pixels with a PeakForce Tapping frequency of 1 kHz and amplitude of 300 nm. Probe-sample contact time was about 200 μs each cycle. Automatic gain control was used to improve the feedback for surface tracking. Height sensor signal was used to display the cell surface image using Nanoscope Analysis v1.90 (Bruker Nano Surfaces).

2.6 | Single-cell force spectroscopy

A CellHesion 200 AFM (JPK BioAFM, Berlin, Germany) mounted on an inverted microscope (Axiovert 135; Zeiss, Feldbach, Switzerland) was used to quantify the interaction of A549 cancer cells with platelets by SCFS. Tipless cantilever (Arrow TL1; NanoWorld, Neuchâtel, Switzerland) with a nominal spring constant of 30 pN/nm was incubated for 15 minutes in wheat germ agglutinin (WGA, 1 mg/mL). The lectin WGA binds to N-acetylglucosamine residues in the glyocalyx of A549 cells and served a glue to attach a cell to the cantilever. All further steps were performed in platelet buffer with calcium. The deflection sensitivity was determined by obtaining several force-distance (F-D) curves in the silicone culture insert region of the FluoroDish and linear fits of the resulting curves. The force constant was determined using the thermal fluctuations of the free cantilever. After successful calibration 10 μL of a diluted A549 cell suspension was added to the BSA coated region. The WGA-coated cantilever was approached to a single A549 cell under optical control in constant force mode using a contact force of 2 nN, a contact time of 10 seconds, a cantilever velocity of 5 μm/s and a pulling length of 80 μm. During contact time, the cantilever was gently moved to increase the contact area with the cell. After attachment, cell was allowed to rest for 5 minutes before experiment starts. The cantilever was moved to the area with the layer of activated platelets under optical control to check the integrity of the attached cell and the platelet layer (Figure 1). F-D curves were recorded in constant force mode using the following parameters: contact force of 1 nN, contact time of 1 second, cantilevers approach/retraction velocity of 12 μm/s, pulling length of 80 μm and 2000 Hz sample rate. The total separation was larger than the pulling length due to the return of the piezo to the height of the starting point of the measurement. This extra separation of approx. 5 to 10 μm was also recorded in the F-D curves. For each F-D curve the position of the cantilever was changed. Typically, 20 to 30 measurements were performed for each condition. At first, reference measurements were performed without heparin. Afterward, 40 μL of a diluted heparin solution was added to the dish using a pipette with a thin 200 μL gel loading tip (Kisker Biotech, Steinfurt, Germany). Unfractionated heparin (Heparin-Natrium-25000-ratiopharm; Ratiopharm, Ulm, Germany) was diluted with PBS prior to the addition. The final concentrations of heparin ranged between 1 and 100 U/mL in the
experiments. Measurements were performed at room temperature. F-D curves were processed using the JPK Data Processing Software (version spm-5.0.96). Data processing included conversion of force-distance into force-separation curves, baseline fit of the retraction curve and determination of the maximum adhesion force ($F_A$) and detachment work ($W_D$).

2.7 | Antibody experiments

To address the effects of heparin to a specific target molecule, anti-P-selectin antibodies known for blocking adhesion of platelets (BD Pharmingen Purified Mouse Anti-Human CD62P; clone AK-4; BD Biosciences, San Jose, CA) were added at a final concentration of 50 μg/mL after acquisition of the F-D curves under heparin free condition. Then, F-D curves were recorded under P-selectin blocking conditions and hereinafter heparin was added to a final concentration of 5 U/mL.

2.8 | Statistics

For statistical analysis, OriginPro 8 (OriginLab Corporation, Northampton, MA) was used. All data processing steps were performed for both parameters of interest, $F_A$ and $W_D$. Capitalized "N" stands for the number of A549 cells, lowercased "n" stands for the number of acquired F-D curves: $N = 4$, $n = 83$ means that four individual A549 cells were used to record 83 F-D curves. Since each F-D curve was recorded at a different position, 83 F-D curves means 83 different platelets. Mean values ($\bar{x}$) were calculated from the reference measurements of each experiment. The data ($d$) of one experiment were transformed into percentage values ($p$) of the corresponding means ($p = [d/\bar{x}] \times 100$). Separated into reference and heparin measurements, processed data of experiments with the same heparin concentration were merged. For normal distributed data sets (tested with Shapiro-Wilk test) two-sample t-test was performed, otherwise Mann-Whitney U test. The same procedure was employed for the analysis of the antibody experiments.

In order to visualize the results of all heparin experiments, for each concentration median-, Q1-, and Q3-values of the processed $F_A$- and $W_D$-data were combined in one graph with logarithmic x-scale for the heparin concentration followed by sigmoid dose-response fitting and determination of the half-maximal inhibitory concentrations (IC50-values). The equation of the applied fitting model DoseResp was: $y = A1 + (A2 – A1)/(1 + 10^{([LOGx0-x] \times p)})$ [A1: bottom asymptote; A2: top asymptote; LOGx0: center; p: hill slope]. Derived parameters were: span = abs(A1-A2); IC50 = 10 LOGx0. As iteration algorithm Levenberg-Marquardt was used. Q1- and Q3-values were plotted using the B-spline interpolation.

3 | RESULTS

PeakForce quantitative nanomechanical mapping scan revealed not only an irregular shape of living platelets but also clear mechanical variations within the platelet. The inhomogeneity among the platelet, especially globular structures and alterations within the plasma membrane are plainly apparent in the mechanical maps but not clearly visible in the height image. Figure 2 shows a PFQNM of an activated living platelet. Nonactivated platelets have a discoid shape with a diameter of approx. 2.5 μm which transforms into a large variety of different shapes and sizes upon activation (Figure 1). The activated platelet represents a typical phenotype with an irregular shape and patchy distributions of mechanical properties, for example, elasticity, adhesion, and deformability (Figure 2). Noteworthy, the elasticity (given as Youngs Moduli) is strongly overestimated due to the mapping technique (PFQNM) and bottom effect. Nevertheless, differences within the platelet membrane are clearly visible in elasticity, adhesion, and deformation. The globular structures represent most likely intact α-granules. A platelet has approximately 50 to 80 α-granules, ranging in size from 200 to 500 nm. It was shown with scanning transmission electron microscopy that not all α-granules fuse immediately upon activation. The observation in Figure 2 is therefore in good correlation with published results. The large variety of different shapes and sizes of activated platelets on the collagen-coated surface are clearly visible in Figure 1. This platelet shows the characteristics of activation and is representative for the platelets within the layer we used for SCFS. The SCFS configuration used for this project was a A549 cancer cell glued to a tipless cantilever (Figure 3). F-D curves were recorded on activated platelets on a collagen-coated surface (Figure 1). Optical resolution was sufficient to ensure an approach on the platelets with the A549 cell. Nevertheless, there were also approaches on the collagen-coated surface. Representative F-D curves of approaches on platelets and collagen-coated surface are shown in Figure 4. Adhesion between A549 cells and collagen are clearly distinguishable from interactions between A549 cells and platelets. That enables us to use only F-D curves of
cell-platelet interactions for data evaluation. The median values of A549 cell-platelet adhesion was $F_A = 1.018 \, \text{nN}$ and $W_D = 3.807 \, \text{fJ}$ (Figure 5). Shapiro-Wilk test showed that the data at the .05 significance level ($\alpha$) was not normally distributed. Adding heparin at final concentrations between 1 and 100 U/mL significantly ($\alpha$: .05) reduced the $F_A$ for all concentrations. The sigmoid dose-response fit (Figure 6; Table 1) revealed an IC$_{50}$ value of $8.01 \pm 1.08 \, \text{U/mL}$ (mean $\pm$ SE) and a maximum decrease (100% - bottom asymptote A1) of 37.50%. The span between both asymptotes (A1 and A2) of the fitting curve was $18.56\% \pm 4.85\%$ (mean $\pm$ SE). For each heparin concentration of this test series, even for the lowest (1 U/mL), a significant reduction (Mann-Whitney $U$ test, $\alpha$: .05) of the parameter was found, which resulted in a top asymptote of the fitting curve of the dose-response relationship lower than 100% and explains the difference between the maximum decrease and the span. For the heparin concentration of 1 U/mL (N: 4; n: 83) the median $F_A$ was 87.50% compared to the basis of the reference measurements for this concentration (n: 107).

Regarding $W_D$, heparin significantly reduced the detachment work for all concentrations $\geq 8 \, \text{U/mL}$. The sigmoid dose-response fit (Figure 7; Table 1) revealed an IC$_{50}$ value of $6.46 \pm 1.30 \, \text{U/mL}$ (mean $\pm$ SE) and a maximum decrease (100% - bottom asymptote A1) of 38.42%. For the effect of heparin on the $W_D$ the maximum decrease is equal to the span between both asymptotes of the fitting curve, as for the computation of the dose-response relationship the A2 parameter was fixed at 100%. This was justified by the finding that no significant reduction of the $W_D$ could be detected for heparin concentrations lower than 8 U/mL, which is the equivalent of a value of 100% for the top asymptote.

The effect of anti-P-selectin antibodies was tested in paired experiments: the A549-platelet adhesion was measured and followed by adding antibodies in-situ. The application of antibodies known to block P-selectin activity reduced the $F_A$ significantly to 68.05% (Mann-Whitney $U$ test, $\alpha$: .05; Figure 8). Herein after heparin was added in-situ and caused a significant further reduction of $F_A$ by 9.52% (median$_{\text{Antibody}}$ − median$_{\text{Heparin}}$).

$W_D$ was also significantly reduced to 48.63% upon application of the antibodies (Figure 9). Heparin application after antibody treatment additionally reduced $W_D$ significantly by 7.12% (median$_{\text{Antibody}}$ − median$_{\text{Heparin}}$).
DISCUSSION

The adhesion of platelets to cancer cells is reported to be a crucial step for the establishment of metastatic foci, as it leads to the formation of a protective cloak around the cancer cells which facilitates each step of the metastatic cascade.\textsuperscript{4,42} We used SCFS to investigate this early step of metastasis, the adhesion of platelets to cancer cells. The configuration of our approach comprised a layer of platelets immobilized to a collagen-coated glass slide and an A549 cancer cell glued to a tipless cantilever (Figure 1). The binding of platelets to collagen is mediated by integrin $\alpha_2\beta_1$ and glycoprotein VI (GP VI) paralleled by an outside-in signaling that initiates platelet activation through an increase of intracellular calcium concentration.\textsuperscript{43} Platelet activation includes a strong shape change (spreading) which is paralleled by a biomechanical transformation and the release of their $\alpha_\text{-}$ and dense granules content. These immobilized and activated platelets did not cover the surface completely but the density is sufficient to bring under optical control reliably the cantilever-mounted A549 cell in contact with a platelet-covered region. The contact of the cantilever-mounted A549 cell with the collagen-coated surface resulted in a completely different F-D curve and allowed therefore a clear distinction (Figure 4). The retraction curve shows characteristics

![Figure 3](image-url)  
**Figure 3** Principle of a SCFS measurement (left) with an exemplary F-D curve (right). Left: red box: diode laser; red line: laser beam; blue trapeze: detector; I: approach (black arrow) of the cantilever (blue bar) with the attached cancer cell (red sphere) to the layer of activated platelets (green structures); II: contact and adhesion between cancer cell and platelets; III: retraction (black arrow) of the cantilever and detachment of the adhesive bonds between the cancer cell and platelets (e.g., P-selectin—PSGL-1); IV: return of the cantilever to the basic position. Right: black line: approach; red line: retraction; The peak value of the retraction curve in respect to the zero force level (gray double arrow) represents the maximum adhesion force $F_A$. The area (blue region) between the zero force level and the retraction curve represents the detachment work $W_D$. I to IV: parts of the F-D curve corresponding to the SCFS scheme. F-D, force-distance; SCFS, single-cell force spectroscopy

![Figure 4](image-url)  
**Figure 4** Representative F-D curves acquired on collagen, A, and on platelets, B. F-D curve acquired on a collagen A coated surface, A, and on activated platelets, B, (black line: approach; red line: retraction). $F_A$: The peak value of the retraction curve in respect to the zero force level (gray double arrow) represents the maximum adhesion force; $W_D$: The area (blue region) between the zero force level and the retraction curve represents the detachment work. The explicit differences of adhesion allow to distinguish between approaches on platelets and on collagen-coated surface. F-D, force-distance
that are commonly used to quantify cell-cell adhesion. For this project, F-D curves were used to quantify the maximum adhesion force \( F_A \) and the detachment work \( W_D \). \( F_A \) is the peak value of adhesion in respect to the zero force level (Figure 4, gray double arrow) and \( W_D \) is the area between the zero force level and the retraction curve (Figure 4, blue region). \( W_D \) represents force times distance and therefore contains the work necessary to separate the A549 cell and the platelet completely. The parameters \( F_A \) and \( W_D \) obtained from SCFS measurements characterize the overall adhesion between cells without unconsidering the individual bonds involved in the detachment process.\(^{24}\) The mean values ± SEM of \( F_A \) are found to be 1.075 ± 0.016 nN and the \( W_D \) values are 4.721 ± 0.121 fJ (Figure 5). The large variation of \( W_D \) between individual measurements can be attributed to differences of the cell-platelet contact geometry. Adhesion of platelets to the surface is relatively strong due to the large contact area, whereas the interaction of an A549 cell and a platelet is limited by a small contact area and an interaction time of just 1 second. Even though we never observed the removal of a platelet from the collagen-coated surface during measurements, we could not exclude a contribution of the platelet-to-collagen binding to the adhesion force.

**FIGURE 5** \( F_A \) and \( W_D \) values of the reference measurements. Spheres: data points; box: first quartile (Q1), median, third quartile (Q3); whiskers: outlier; \( x \): mean. The following values (in nN) were identified for \( F_A \): Q1 = 0.753, median = 1.018, Q3 = 1.328, mean ± SEM = 1.075 ± 0.016. The following values (in fJ) were identified for \( W_D \): Q1 = 2.427, median = 3.807, Q3 = 6.019, mean ± SEM = 4.721 ± 0.121

**FIGURE 6** Effect of heparin on \( F_A \). \( F_A \) in percent of the reference; upper and lower gray line: first quartile (Q1) and third quartile (Q3); \( x \): median; red line: dose-response fit. Adding heparin at concentrations between 1 and 100 U/mL significantly reduced \( F_A \) for all concentrations. The sigmoid dose-response fit revealed an IC\textsubscript{50} value of 8.01 ± 1.08 U/mL (mean ± SE) and a maximum decrease (100% - bottom asymptote A1) of 37.50%. Detailed results of the \( F_A \) for all heparin concentrations are shown in Supplemental S1 and detailed results of the dose-response relationship are shown in Table 1.
detachment work completely. However, since heparin does not influence the adhesion of platelets to collagen, the observed effects of heparin reflect changes between the force/work of platelets and A549 cells.

The activation of a platelet leads not only to shape change but also to redistribution of adhesion proteins to the platelet surface, for example, glycoprotein IIb/IIIa (GPIIb/IIIa, also known as integrin αIIbβ3) and P-selectin (CD62P). P-selectin is a transmembrane glycoprotein involved in cell-cell interactions under different pathological conditions, including cancer metastasis and inflammation.42,30,45

Binding between platelets and other cell types is mediated primarily by P-selectin. The density of P-selectin is approximately 350 sites/μm² or 10 000 P-selectin molecules on each activated platelet.46 P-Selectin forms weak bonds with its ligand P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a transmembrane glycoprotein expressed on nearly all types of cancer cells and it was shown that platelets interact with A549 lung cancer cells via P-selectin–PSGL-1 bonds.18 Heparin, a highly sulfated glycosaminoglycan, is a potent inhibitor of P-selectin activity21 and inhibits the binding of tumor cells to immobilized P-selectin.47

The inhibitory effect of heparin on A549-platelet interaction was tested using SCFS and the dose-response relationship was determined in the range of 1 to 100 U/mL heparin. To improve comparability data are presented as relative values as it has been described in the methods section. Briefly, measurements without heparin mean 100% of adhesion and the difference to the remaining adhesion value (bottom asymptote) indicate the maximal heparin effect. None of the tested heparin concentrations was able to reduce the adhesion between platelets and A549 cancer cells completely. The maximal effect of heparin is a reduction of $F_A$ and $W_D$ by approx. 40% ($62.5 ± 3.2 [F_A]$ and $61.58 ± 3.87 [W_D]$). It was not surprising that heparin cannot block the binding completely since it is known that cancer cells and platelets interact through a great variety of adhesion molecules.5 Additionally, nonspecific adhesion forces contribute to the measured F-D curves.24 The dose-response fit for the $F_A$ does not add up to 100%. This could be explained by the finding that even for the lowest heparin concentration of our test series (1 U/mL) a significant reduction of $F_A$ was found leading to a remaining adhesion force of 81%. The half-maximal effective concentration $IC_{50}$ for $F_A$ was $8.01 ± 1.08$ U/mL and $6.46 ± 1.30$ U/mL for $W_D$ (Table 1).

**TABLE 1** Fitting results for the dose-response relationship

|       | $F_A$ | $W_D$ |
|-------|-------|-------|
|       | Mean  | SE    | Mean  | SE    |
| Red. chi-sqr. | 64.1113 | - | 134.44993 | - |
| Adj. R-sqr. | 0.49304 | - | 0.58359 | - |
| A1 | 62.50 | 3.21 | 61.58 | 3.87 |
| A2 | 81.06 | 3.50 | 100.00 | - |
| LOGx0 | 0.90 | 0.06 | 0.81 | 0.09 |
| p | −10.49 | 12.85 | −48.36 | 128.45 |
| span | 18.56 | 4.85 | 38.42 | 3.87 |
| $IC_{50}$ | 9.14 | 1.82 | 6.64 | 1.58 |
| $IC_{80}$ | 8.01 | 1.08 | 6.46 | 1.30 |
| $IC_{90}$ | 7.02 | 1.55 | 6.27 | 1.20 |

Note: Red. chi-sqr.: Reduced Chi-squared; Adj. R-sqr.: Adjusted R-squared; A1: Bottom asymptote; A2: Top asymptote; LOGx0: Center; p: Hill slope; span: abs(A1-A2); $IC_{50}$: 10 [LOGx0 + log(0.25)/p]; $IC_{80}$: 10 LOGx0; $IC_{90}$: 10 [LOGx0 + log(4)/p]; SE: Standard error.

**FIGURE 7** Effect of heparin on $W_D$. $W_D$ in percent of the reference. Upper and lower gray line: first quartile (Q1) and third quartile (Q3); x: median; red line: dose-response fit. Adding heparin at concentrations between 1 and 100 U/mL significantly reduced $W_D$ for all concentrations ≥ 8 U/mL. The sigmoid dose-response fit revealed an $IC_{50}$ value of 6.46 ± 1.30 U/mL (mean ± SE) and a maximum decrease (100% - Bottom asymptote A1) of 38.42%. Detailed results of the $W_D$ for all heparin concentrations are shown in supplemental S2 and detailed results of the dose-response relationship are shown in Table 1.
It is most likely that the P-selectin–PSGL-1 bonds contribute largely to the adhesion since it has already been reported that knockdown of PSGL-1 in A549 cells significantly reduces the aggregate formation of activated platelets and A549 cells. In order to test if the heparin effect on adhesion depends on P-selectin, anti-P-selectin antibodies, known for blocking adhesion of platelets, were added after...
acquisition of the F-D curves under heparin free condition. F-D curves were recorded in presents of P-selectin blocking antibodies and hereinafter heparin was added to a final concentration of 5 U/mL. Figures 8 and 9 show that anti-P-selectin antibodies reduce $F_A$ to 68.05% and $W_D$ to 48.63% (median values). Application of heparin (5 U/mL) reduced adhesion further by additional 9.52% ($F_A$) and 7.12% ($W_D$). The remaining adhesion upon antibodies and heparin treatment is 58.53% ($F_A$) and 41.51% ($W_D$) and therefore below the bottom asymptote values obtained from the fitting curve of the heparin experiments. The small but significant difference of heparin and anti-P-selectin antibodies on adhesion indicated that heparin blocks not exclusively the P-selectin-PSGL-1 bond. It is likely that heparin also inhibits the adhesion by blocking other, yet unidentified, receptors since heparin binds to a variety of membrane-anchored and extracellular proteins.

It was reported that binding of heparin and its derivatives to heparan sulfate (HS) binding proteins modulate the function of these HS-binding proteins with a pivotal role in cancer growth and progression. However, the antibody approach demonstrates that the P-selectin-PSGL-1 bond contributes largely to the adhesion between A549 cancer cells and platelets.

The meaning of platelets for metastasis is an undoubted case and the binding of platelets to circulating cancer cells a crucial step. It seems reasonable to conclude that blocking the binding of platelets to circulating cancer cells will cause a substantial reduction of metastasis and may explain the antimetastatic effect of heparin. Our approach aimed to quantify the inhibitory effect of unfractionated heparin by means of SCFS. Therapeutic levels of unfractionated heparin normally range from 0.3 to 0.7 U/mL (by factor Xa inhibition). However, the obtained IC$_{50}$ values from our experiments exceed this interval, which in practice would lead to an increased bleeding risk for patients if applied in such high concentrations. Nevertheless, the presented method provides a useful way to determine effective concentrations of other, therapeutically relevant heparins as well as other types of P-selectin or PSGL-1 inhibitors.

5 | CONCLUSION

With our method, we quantified the antimetastatic effect of heparin on single-cell level. For both parameters of the platelet-A549 cell interaction, $F_A$ and $W_D$, we were able to determine the IC$_{50}$ values of the anticoagulant. In our antibody experiments we showed that heparin’s antimetastatic effect predominantly is related to P-selectin but also to yet unidentified adhesion molecules. At long sight, this may support the development of new antimetastatic strategies.

ACKNOWLEDGEMENTS

This work was financially supported by Cells-in-Motion Cluster of Excellence EXC 1003-CIM (University of Münster) and the doctoral training program MedK Münster.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Liebsch AG, Schillers H. Quantification of heparin's antimetastatic effect by single-cell force spectroscopy. J Mol Recognit. 2021;34:e2854. https://doi.org/10.1002/jmr.2854