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RGDfK-functionalized gold nanorods bind only to activated platelets

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Abstract

Integrin-targeting peptide RGDfK-labeled gold nanorods (GNR) seek to improve hyperthermia targeted to solid tumors by exploiting the known up-regulation of integrin $\alpha v\beta 3$ cell membrane proteins on solid tumor vasculature surfaces. Tumor binding specificity might be expected since surrounding tissues and endothelial cells have limited numbers of these receptors. However, RGD peptide binding to many proteins is promiscuous, with known affinity to several families of cell integrin receptors, and also possible binding to platelets after intravenous infusion via a different integrin receptor, $\alpha IIb\beta 3$, on platelets. Binding of RGDfK-targeted GNR could considerably impact platelet function, ultimately leading to increased risk of bleeding or thrombosis depending on the degree of interaction. We sought to determine if RGDfK-labeled GNR could interact with platelets and alter platelet function. Targeted and untargeted nanorods exhibited little interaction with resting platelets in platelet rich plasma (PRP) preparations. However, upon platelet activation, peptide-targeted nanorods bound actively to platelets. Addition of RGDfK-GNR to unactivated platelets had little effect on markers of platelet activation, indicating that RGDfK-nanorods were incapable of inducing platelet activation. We next tested whether activated platelet function was altered in the presence of peptide-targeted nanorods. Platelet aggregation in whole blood and PRP in the presence of targeted nanorods had no significant effect on platelet aggregation. These data suggest that RGDfK-GNR alone have little impact on platelet function in plasma. However, nonspecific nanorod binding may occur in vascular beds where activated platelets are normally cleared, such as the spleen and liver, producing a possible toxicity risk for these nanomaterials.

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Additional Supporting Information may be found in the online version of this article.

Keywords

nanoparticles; coagulation; peptide ligand; blood clot; activation; integrin

INTRODUCTION

The vascular bed of solid tumor tissues is distinguished from that of normal tissues by the presence of over-expressed surface markers on vascular endothelium.^{1,2} Targeting of cancer treatments toward the angiogenic endothelium of solid tumors has frequently used monocyclic peptide RGDfK to target the cell membrane integrin of interest ($\alpha v\beta 3$) found in the neo-vasculature of developing tumors.^{3–6} Despite integrin peptide targeting,^{7,8} this approach often leads to dose deposition at nontarget sites for RGD-labeled soluble polymers,^{9,10} particles,^{11–13} and other bioactive compounds.^{14–17} In vitro affinities for RGD and RGDfK peptide ligands in serum-free media with endothelial cell lines are $12.22 \times 10^6 \text{ M}^{-1}$ and $6.33 \times 10^6 \text{ M}^{-1}$ ($K_d=818$ and 158 nM), respectively.¹⁸ *In vivo*, the integrin affinity constant for $^{99\text{m}}\text{Tc}$ -RGD was reported to be comparable ($7 \times 10^6 \text{ M}^{-1}$) in both human renal adenocarcinoma and human colon cancer cell line-based murine ectopic tumor models. Nonetheless, tumor accumulation of $^{99\text{m}}\text{Tc}$ -labeled RGD versus RGE controls exhibited no statistical differences, possibly due to limited numbers of $\alpha v\beta 3$ integrin receptors per tumor cell and the modest RGD binding affinity, limiting *in vivo* RGD-based targeting.¹⁹

The $\alpha v\beta 3$ integrin receptor targeted by RGD is also found on platelets²⁰ and as a receptor for phagocytosis on macrophages²¹ and dendritic cells.²² The binding promiscuity of the RGDfK peptide moiety allows it to not only target the $\alpha v\beta 3$ integrin, but also other integrins in the same family, such as $\alpha \text{IIb}\beta 3$,^{23,24} an integrin expressed on platelets.^{25,26} Platelet $\alpha \text{IIb}\beta 3$ integrin activation is the final step in platelet aggregation pathway, regardless of the initial activating stimulus. Recent studies of the biodistribution of intravenously administered RGDfK-targeted drug carriers indicate more rapid clearance and frequently less tumor accumulation than their untargeted counterparts.^{10,27} One hypothesized mechanism producing this RGD-mediated particle-targeting failure and gold nanorods (GNRs) rapid clearance is promiscuous RGD-mediated platelet binding and possible aggregation response during GNR intravenous infusion, attributed to the high concentration and abundant availability of alternative competing platelet integrin targets present in blood.

Platelets circulate in the blood at concentrations of $150 \times 10^9/\text{L}$ to $400 \times 10^9/\text{L}$ with a half-life of around ten days.²⁸ Platelets are activated by many different agonists, and upon activation provide a high concentration of exposed integrins on their activated membranes. These integrins are known to bind many different peptides²⁹ and may bind RGDfK off-target to reduce the fractional dosage of RGDfK-guided therapy reaching its intended target by binding integrin $\alpha v\beta 3$ in the tumor vasculature. In addition, any interference with the diverse platelet surface integrins by RGDfK-targeted therapies may have unintended hemostatic consequences by affecting the patient's ability to activate platelets normally, form stable clots and prevent bleeding. Despite the prevalence of RGD-targeted tumor studies using intravenously administered agents (liposomes, polymers, particles), few studies

detail RGD peptide binding and possible activation of platelets. We therefore have examined how RGDfK-labeled GNR might alter platelet function *ex vivo*.

MATERIALS AND METHODS

GNRs synthesis

GNRs were prepared as described previously.²⁷ Briefly, GNRs were fabricated by gold salt reduction in hexadecyltri-methylammonium bromide (CTAB) to exhibit a characteristic surface plasmon resonance peak for GNRs between 800–810 nm. After successive washing and centrifugation in deionized water to remove excess CTAB, GNRs were stored at 4°C in water until coated with thiolated polyethylene glycol (PEG 5000 MW, Creative PEGWorks) bearing terminal cyclic peptides RGDfK or control RGEfK (New England Peptide, Inc.). PEG-peptide conjugates were prepared as previously described²⁷. GNR PEGylation was performed by addition of either thiolated RGDfK-PEG conjugates or thiolated RGEfK conjugates (100 μ M) for 2 hr at room temperature under stirring. The PEGylated GNRs were then thoroughly dialyzed (3.5k MWCO, Spectrum Labs), sterile filtered, concentrated by centrifugation and stored at 4°C for a maximum of two months. Resulting GNR size and shape were determined by transmission electron microscopy (TEM) and GNR peptide content was found by amino acid analysis (University of Utah Core Research Facilities, Salt Lake City). GNRs were diluted to optical density of 1 or 10 at 810 nm, which is the equivalent of 10 or 100 μ g/mL, for use in all studies.

Whole blood collection and platelet preparation

Human peripheral venous blood from healthy, medication-free adult subjects ($n=10-15$ different donors) was drawn into acid-citrate-dextrose (1.4 mL of ACD/8.6 mL of blood) through a standard venipuncture technique and used immediately upon collection. Platelets were isolated from whole blood and purified platelets re-suspended in M199 medium (Lonza, Walkersville, USA) (1×10^8 platelets/mL, final) as previously described³⁰⁻³³ with minimal leukocyte contamination.³⁴⁻³⁷ In some cases, platelet-rich plasma (PRP) was obtained from whole blood after the first centrifugation step. PRP and purified platelet preparations were used within 4 h.

Flow cytometry

Platelet activation was evaluated in isolated platelet and whole blood systems. Isolated platelets treated with RGDfK-PEG- or RGEfK-PEG-bearing GNRs for indicated times were stained for P-selectin (CD62P, a marker of platelet alpha granule release) using phycoerythrin (PE) (BD Biosciences, San Jose, USA) or using PAC-1 (a marker of α IIb β 3 activation) using dye-labeled Fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, USA). Platelets were then analyzed by flow cytometry using a FACScan (BD Biosciences, USA). Whole blood was simultaneously analogously stained and stimulated with either RGDfK-PEG- or RGEfK-PEG-bearing GNRs for 15 min. In addition to CD62P and PAC-1, platelets were also identified using FITC or PE anti-human CD41a (BD Biosciences, USA) and analyzed by flow cytometry as above. Thrombin (0.1 U/mL, final) (Sigma-Aldrich, USA) or Ser-Phe-Leu-Leu-Arg-Asn-Pro-Lys-Tyr-Glu-Pro-Phe (TRAP, 10 μ M, final) (Sigma-Aldrich, USA) activation was used as a positive control in washed platelets or whole blood,

respectively. To assess if fibrinogen was still capable of binding platelets in the presence of RGDfK- or RGEfK-labeled GNRs, Alexa-fluor 488-labeled fibrinogen (Invitrogen, USA) (100 µg/mL, final) was added to platelets for 15 min and binding measured compared to platelets incubated with fibrinogen alone.

Dark field microscopy

Isolated platelets treated for indicated times with RGDfK- or RGEfK-coated GNRs and in some cases, activated with thrombin 0.1 U/mL (final concentration). In some experiments untargeted GNRs were used. No difference in untargeted or RGEfK-targeted GNRs was observed. Samples were then fixed and mounted to slides with mounting medium. Slides were then imaged as previously described²⁷ with an Olympus BX41 microscope coupled to the CytoViva 150 Ultra Resolution Imaging (URI) System (CytoViva Inc., Auburn, USA) using a 100× oil objective.³⁸ A DAGE XLM (DAGE-MTI, Michigan City, USA) digital camera and software were used to capture and store images.

Platelet aggregometry

RGDfK- or RGEfK-labeled GNRs were added to citrated human whole blood at 10 µg/mL final or PRP at 1 µg/mL final and platelet aggregation in whole blood was then measured by impedance in a whole blood aggregometer (Chronolog Model 490, Havertown, PA, USA) or by turbidity in a light transmission aggregometer (Chronolog Model 490) immediately after addition of type 1 fibrin collagen agonist (2.5 µg/mL final, Chronolog).

Statistical analysis

Data are representative as the mean±standard error of the mean. Significant was determined by Student's *t* test or by ANOVA. When significance was reached for multiple comparisons, a Tukey's post-hoc analysis was used. Significance was determined at a *p* < 0.05. All experiments were performed a minimum of three times (technical replicates).

RESULTS

Because GNRs intrinsically scatter light as metallic nanoparticles, GNR binding to the platelets was qualitatively visualized by high-resolution dark field microscopy (Figs. 1 and 2). Captured images show that targeted (RGDfK) GNRs associated with platelets only after activation with thrombin (Figs. 1 and 2). Untargeted GNRs demonstrated no significant or detectable platelet associations. Similar findings were observed with RGEfK GNRs (data not shown). This suggested the direct involvement of αIIbβ3, an RGDfK-binding integrin, in platelet-GNR associations.

To determine if different GNR dosages activate human platelets in a time-dependent manner, purified platelets were incubated with GNRs for varying times and expression of P-selectin (CD62P), a marker of alpha granule release, and PAC-1 binding, a marker of αIIbβ3 activation, were assessed via flow cytometry. Activation of platelets with thrombin demonstrated significant expression of P-selectin and PAC-1 binding, indicating platelets were capable of being activated as a positive control. However, no significant platelet activation with these markers was observed using either the RGDfK or RGEfK-GNRs at any

time point or GNR dosage (Fig. 3). GNRs alone did not induce platelet activation (Supporting Information Fig. S1).

Since GNRs also interact with other cells in the blood expressing integrin receptors, we next assessed if GNRs were capable of activating platelets in more complex milieu. Whole blood was isolated and incubated with different concentrations of RGDfK- or RGEfK-labeled GNRs and platelet activation markers were assessed by flow cytometry. Similar to our purified platelet PRP results, platelets were activated when incubated with a known platelet agonist TRAP but showed no activation when incubated with either GNR or GNRs alone (Fig. 4 and Supporting Information Fig. S2).

These initial data suggest that GNRs are unable to activate quiescent platelets and only interact with platelets when pre-activated by specific platelet agonists (for example, thrombin, collagen). Since activated platelets play a substantial role in preventing bleeding through integrin-dependent mechanisms,³⁹ RGDfK-labeled GNRs could interfere with platelet functions and increase risks for coagulopathies or bleeding. To test if platelet function is compromised in the presence of RGDfK-GNRs, platelet aggregation was assessed. Platelet aggregation depends on fibrinogen, a RGD-containing protein,^{40–42} binding to $\alpha\text{IIb}\beta\text{3}$; therefore, we first determined if fibrinogen binding to platelets was altered in the presence of RGDfK-labeled GNRs. Labeled fibrinogen bound to platelets and in an activated-dependent manner, and was not competed off by addition of RGDfK-labeled GNRs (data not shown). Light transmission aggregometry confirmed fibrinogen binding results by demonstrating no significant differences between platelets left untreated compared to RGDfK-GNRs or RGEfK GNRs [Fig. 5(A) and Supporting Information Fig. S3]. In addition, whole blood platelet aggregation showed no significant changes in the presence of RGDfK-GNRs [Fig. 5(B) and Supporting Information Fig. S4]. GNRs alone had no effect on platelet aggregation (data not shown).

DISCUSSION

Diagnostic, biomedical, and clinical applications for gold nanoparticles are diverse and increasing, central to new optical imaging-based contrast agents, delivery vehicles for drugs and infused media for photothermal therapy.^{43–47} Targeted approaches to elicit selective and specific binding and tissue biodistributions using specific ligands conjugated to gold nanoparticle surfaces are reported to increase their localization to specific tissues^{13,48,49} although substantial off-target accumulation in filtration organs (that is, RES/MPS system comprising liver, spleen, kidneys, lungs) is consistently reported. In targeting nanoparticles *in vitro* and *in vivo*, significant attention has been paid to the $\alpha\text{v}\beta\text{3}$ integrins, commonly targeted through the well-known cell adhesion peptide, Arg-Gly-Asp (RGD), and its increasingly diverse related peptide analogs.^{50,51} Interestingly, $\alpha\text{v}\beta\text{3}$ is highly expressed on endothelial cells lining tumor and tumor cells but poorly expressed in resting endothelial cells.^{52,53} Thus, selectively targeting the $\alpha\text{v}\beta\text{3}$ integrin with anti-tumor drugs or thermal stress is thought to provide an opportunity to deliver agents and destroy tumor vessels while leaving healthy tissue intact. However, previous efforts have had only moderate success in targeting such therapies using RGD ligands to these tumor-specific receptors.^{9–17} More specifically, a prior report describing intravenous infusion of the same RGDfK-labeled

GNRs used here to ectopic solid murine tumor models showed that the majority of the GNRs were sequestered by the liver and spleen, with little targeting specificity seen toward the tumor.^{10,27}

Given the significant high specific surface areas for GNRs ($\sim 100 \text{ m}^2/\text{g}$) and their stochastic encounters in blood after intravenous introduction with numerous cell membrane surfaces, some at high concentrations (that is, $\sim 10^8$ platelets/mL), blood compatibility and resulting coagulation stimulus is an important consideration. Introduction of RGD ligands on their surfaces produces concerns that these ligands interact adversely as pro-coagulant stimuli with platelet activation triggers such as integrins. Platelets play essential roles in bleeding control through binding to each other and to other cells through integrin receptors such as $\alpha\text{IIb}\beta_3$ and $\alpha\text{v}\beta_3$. Like $\alpha\text{v}\beta_3$, $\alpha\text{IIb}\beta_3$ avidly binds RGD⁵⁴ as well as RGD-containing proteins such as fibrinogen and von Willebrand factor at moderately high affinities (10^{-7} M).⁵⁵ However, cyclic RGDfK has been reported to bind with a much greater affinity (10^{-8} – 10^{-9} M),⁵⁶ suggesting that RGD-targeted agents against specific tumor-based sites may exert selective binding to tumor-based cells after systemic administration despite the high competitive platelet abundance. Platelet studies focus naturally more on platelet $\alpha\text{IIb}\beta_3$ ligand engagement, as only trace amounts of $\alpha\text{v}\beta_3$ are present on platelets (~ 100 $\alpha\text{v}\beta_3$ receptors per platelet vs. $\sim 100,000$ $\alpha\text{IIb}\beta_3$ receptors).⁵⁷ Based on these receptor densities, RGDfK-GNRs more than likely affect $\alpha\text{IIb}\beta_3$ on the platelet surface compared to $\alpha\text{v}\beta_3$.

Integrin priming has been proposed to describe integrin ligand affinity regulation, whereas integrin activation is differentiated as the process of ligand-induced propagation of intracellular signals resulting from the increase in binding affinity coupled to alterations in integrin conformation.⁵⁸ However, priming is also defined as the affinity- or valency-based regulatory events enhancing ligand-binding efficiency. Hence, the terms “priming” or “activation” might be confounding in their description of integrin-associated events unless carefully defined for a specific experimental system.⁵⁹

Platelet activation involving platelet integrins was shown in early studies of the platelet glycoprotein GPIIb/IIIa complex (that is, integrin $\alpha\text{IIb}\beta_3$). In these studies, platelet activation induced conformational changes in subsequent activation of the fibrinogen binding capabilities of GPIIb/IIIa.^{60–62}

Platelet “priming” is an altered platelet response to a secondary agonist that is influenced by prior exposure to a platelet mediator. Analogous examples of cell priming are recognized in neutrophils and myocytes.⁶³ Certain agents are shown to not fully activate platelets, but rather increase their ability to become activated when later exposed to a second agonist, essentially “priming” platelets for activation. Numerous molecules are now identified to prime platelets, including proteins, hormones, cytokines and chemokines.⁶⁴ *In vivo*, the vast majority of platelets encountering an injury site or biomaterial fail to adhere under flow, but continue to flow downstream within circulation despite transient contact with the wound or biomaterial.⁶⁵ Corum et al. recently demonstrated that the presence of a surface-adhered platelet agonist is sufficient to prime flowing platelets, producing enhanced adhesion at downstream locations.^{66,67} They propose that transient interactions between platelets and agonist-covered surfaces primes these platelet populations to produce a higher propensity to

activate and adhere downstream when exposed to a subsequent agonist or stimulus. Platelet $\alpha\text{IIb}\beta_3$ integrin activation is the final step in the platelet aggregation pathway, regardless of the initial priming activating stimulus. That nanoparticles, specifically RGD-bearing GNRs, might also either prime platelets, or react as agonists to activate pre-primed platelets is the related concept explored in this manuscript.

Addition of targeted RGDfK GNRs to resting platelets resulted in minimal binding of the GNRs to the platelets based on dark field microscopy. However, upon activation of the platelets through protease-activated receptors (PAR), a substantial increase in binding was observed in a dose-dependent manner. This binding appeared to be specific as untargeted GNRs did not bind to activated platelets. Taken together, these data suggest a significant portion of RGDfK targeted GNRs bind to platelets after activation through $\alpha\text{IIb}\beta_3$.

While dark field microscopy reveals little binding of RGDfK-labeled GNRs in the presence of unactivated platelets, it is possible that RGDfK-GNRs still alter normal platelet functions and induce platelet activation. When purified platelets were incubated with different concentrations of RGDfK GNRs, or with fixed concentrations of GNRs but over different incubation times, platelet activation markers did not change. Specifically, alpha granule release as measured by P-selectin (CD62P) expression and activation of $\alpha\text{IIb}\beta_3$ were similar to levels in resting platelets and in platelets incubated with RGEfK-labeled control GNRs. In addition, activation of $\alpha\text{IIb}\beta_3$ based PAC-1 binding appeared to be similar in the presence of RGDfK-GNRs. PAC-1 is a murine IgM-K MoAb that recognizes an epitope at or in very close proximity to the fibrinogen binding site. The binding of PAC-1 is inhibited by RGD-containing peptides, and PAC-1 and fibrinogen compete with each other for binding to GPIIb-IIIa.^{61,68,69}

To further examine RGDfK-labeled GNR influence on platelets function, a more complex test system using whole blood was employed. In whole blood, RGDfK-targeted GNRs exhibited little observable effects on platelet activation in terms of alpha granule release and $\alpha\text{IIb}\beta_3$ activation. Collectively, RGDfK-labeled GNRs appeared to exert little effect on resting platelets, while they readily interacted with thrombin-activated platelets (Figs. 3–5).

Since activated platelets use $\alpha\text{IIb}\beta_3$ to prevent bleeding through binding to other platelets and cells, we sought to determine if RGDfK-labeled GNRs altered platelet function. At the concentrations tested, RGDfK-bearing GNRs were unable to block fibrinogen binding to platelet surfaces, suggesting that $\alpha\text{IIb}\beta_3$ function remained intact after GNR binding. Platelet aggregation, another marker of altered function, appeared uninfluenced in both whole blood or in PRP with GNR exposure when stimulated with collagen, again suggesting platelets remained functional after interacting with RGDfK GNRs.

Significantly, RGDfK-bearing GNRs exhibited little adverse reactivity toward resting platelets. Besides their role in preventing bleeding, platelets can play significant roles in the development of blood clots and coagulopathies, leading to both myocardial infarction and stroke. The inability of RGDfK GNRs to interact with basal resting platelets and their failure to induce activation suggest that their risk for increasing thrombosis events is negligible. In addition, our functional studies demonstrate that risks for bleeding or altered coagulant

response upon exposure to RGDfK GNRs to blood is also minor. RGDfK-targeted GNRs significantly impacted thrombin-activated platelets in terms of their binding, but this GNR binding exhibited little functional effect on platelet aggregation and binding of substrates. Nonetheless, higher RGDfK densities higher GNR concentrations or different RGD peptide chemistries may produce dramatically different effects on platelet functions.

To reach steady state platelet concentrations in humans of $150\text{--}450 \times 10^9/\text{L}$, $\sim 100 \times 10^9$ are removed from the circulation each day while the same amount is created from mature megakaryocytes in the bone marrow. Platelet consumption and clearance occurs through hemostatic processes such as controlling bleeding and active, continual removal by the reticuloendothelial system in the liver and the spleen. Previous research shows that the spleen and liver play a significant role in the clearance of the majority of intravenously dosed RGD-labeled targeted agents^{9–17} and specifically, GNRs.^{10,27} With platelet turnover close to a 100 billion platelets a day, many which are activated,⁷⁰ and the ability of activated platelets to bind RGDfK-labeled GNRs, the observed increase in liver and splenic clearance of targeted RGDfK GNRs^{10,27} could very be attributed to increased platelet uptake of these GNRs and clearance through these organs. In addition, consumption of platelets through prevention of bleeding also contributes to decreased GNR targeting efficiency. GNRs that are cleared through platelet binding, consumption and clearance mechanisms increase the local concentration of GNRs in these filtration locations, with possibly significant consequences on hemostatic parameters and local tissue GNR-induced toxicities.

While these data demonstrate the ability of RGDfK-GNRs to bind to platelets through $\alpha\text{IIb}\beta_3$ -dependent mechanisms, significance is limited in scope by the *in vitro* nature of the assays. We did not observe any RGDfK-GNR-dependent platelet activation or inhibition of platelet function. The addition of RGDfK-GNRs to blood circulation *in vivo* may prompt additional effects we cannot account for based on our *in vitro* assays; therefore, *in vivo* examination of RGDfK-GNR effects on platelet function and risks for increased thrombosis or bleeding is necessary to capture the full dynamic.

Our data demonstrate that RGDfK-labeled targeted GNRs are unable to induce platelet activation in PRP or whole blood. However, upon platelet activation with thrombin, RGDfK-labeled GNRs appear to bind to platelets through $\alpha\text{IIb}\beta_3$ -dependent mechanisms. Thus GNR-platelet binding may have consequences to hemostatic functions of platelets depending on the GNRs and local pharmacology of bioactive agents it might be carrying.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

RGDfK-labeled GNRs do not bind unactivated platelets. Purified platelets were pre-incubated with the indicated concentration of RGDfK-labeled GNRs or untargeted GNRs and then imaged using dark field microscopy (intrinsic GNR light scattering detected). The platelets were then imaged using dark field microscopy. Experiments were performed at least three times.

FIGURE 2.

RGDfK-labeled GNRs bind activated platelets. Purified platelets were stimulated 0.1 U/mL thrombin in the presence of the indicated concentration of RGDfK-labeled GNRs or untargeted GNRs. Inset shows a higher magnification of unstimulated and thrombin stimulated platelets in the presence of 100 µg/mL RGDfK GNR. The platelets were then imaged using dark field microscopy. Experiments were performed at least three times.

FIGURE 3.

Purified platelets in PRP are not activated by RGDfK-GNRs in a time-dependent manner. RGDfK-labeled GNRs at 10 µg/mL or 100 µg/mL were added to purified platelets for 30 minutes or 1 hour. After these incubation periods, platelets were stained with CD41 antibody, and activation was assessed by flow cytometry for CD62P expression and conformational changes in integrin α IIb β 3 through PAC-1 binding. Positive staining was confirmed by activating platelets with the PAR1 agonist, thrombin (0.1 U/mL, final) compared to nontreated purified platelets (NT). Averages $\pm$ standard deviations are shown from an $n = 3$. * indicates significantly different compared to other conditions tested ($p < 0.05$).

FIGURE 4.

RGDfK-labeled GNRs do not induce platelet activation in whole blood. RGDfK-labeled GNRs at 10 µg/mL or 100 µg/mL were added to healthy human citrated blood and incubated for 30 min. After 30 min, platelets were stained with a CD41 antibody and activation was assessed by flow cytometry for CD62P expression and conformational changes in integrin α IIb β 3 (PAC-1 binding). Positive staining was confirmed by activating platelets with the PAR1 agonist, TRAP (10 µM, final) compared to nontreated whole blood (NT). Averages $\pm$ standard deviations are shown from an $n = 3$. * indicates significantly different compared to other conditions tested ($p < 0.05$).

FIGURE 5.

RGDfK-labeled GNRs do not alter platelet aggregation. Purified platelets in PRP were left untreated or treated with 1 $\mu\text{g/mL}$ RGDfK- or RGEfK-labeled (control) GNRs. The platelets were then stimulated with 2.5 $\mu\text{g/mL}$ collagen and platelet aggregation was monitored using Chrono-log platelet aggregator. A representative tracing for purified platelet PRP treated with RGDfK (blue) and RGEfK control (black) are shown above (A). Citrated whole blood was left untreated or treated with 10 $\mu\text{g/mL}$ RGDfK or RGEfK-labeled GNRs. Whole blood was then stimulated with 10 $\mu\text{g/mL}$ collagen and platelet aggregation was monitored using Chrono-log whole blood aggregometry. A representative tracing for whole blood treated with RGDfK (blue) and RGEfK (black) are shown above (B). $N > 3$ for each condition examined.