SUPPLEMENTARY MATERIAL

Materials and Methods

Materials

Poly(lactic-co-glycolic acid)(PLGA) 503H (Resomer 503H, 50:50 lactic to glycolic acid ratio and a M_n ~ 25 kDa) was from Boehringer Ingelheim. H signifies PLGA terminated with a carboxylic acid group. Poly(ε-carbobenzoxy-**L**-lysine) (molecular weight ~ 1000 Da) was from Sigma. Poly(ethylene glycol) (PEG)(molecular weight ~ 1500 and 4600 Da) was from Acros Organics and Sigma, respectively. Arg-Gly-Asp (RGD) peptide sequences were from EMD Biosciences. Peptide sequences include RGD, Arg-Gly-Asp-Ser (RGDS), Gly-Arg-Gly-Asp-Ser (GRGDS), and Gly-Arg-Ala-Asp-Ser-Pro (GRADSP). Collagen I (rat tail) was from BD Biosciences. Deuterated dimethyl sulfoxide (D_6 –DMSO) was from Cambridge Isotope Laboratories, Inc. Poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed) was purchased from Polysciences. CMFDA (5-chloromethylfluorescein diacetate) was from Molecular Probes. Recombinant human factor VIIa (rFVIIa) was from Innovative Research. Vectashield with DAPI was from Vector Laboratories. All other chemicals were A.C.S. reagent grade and other materials were used as received from Sigma.

Methods

PLGA-PLL synthesis

The copolymer PLGA-PLL was synthesized as follows (Fig. S1A). Briefly, PLGA 503H and poly(ε-carbobenzoxy-**L**-lysine) (1:1 molar ratio) were dissolved in anhydrous dimethyl formamide (DMF). Two molar equivalents (with respect to PLGA) of dicyclohexyl carbodiimide (DCC) and 0.1 molar equivalents of (dimethylamino-pyridine) DMAP were added. The reaction was allowed to run for 36 hours under argon. Following the conjugation, the polymer solution was diluted with chloroform and filtered to remove N,N'-dicyclohexylurea (DCU), an insoluble by-product of the reaction. The presence of DCU was indicative of successful conjugation. The block copolymer was then precipitated in methanol and vacuum filtered to remove unconjugated poly(ε-carbobenzoxy-**L**-lysine). The polymer was then redissolved in chloroform, precipitated in ether, vacuum filtered and lyophilized for at least 48 hours.

To expose (deprotect) the primary amines of the poly(ε-carbobenzoxy-**L**-lysine), ~1.5 g of the block copolymer was dissolved in hydrogen bromide (HBr), 30 wt% in acetic acid

(HBr/HOAc) and stirred. After 1.5 hours, ether was added to the solution and the precipitated polymer was removed. The polymer was washed with ether until an off-white, brittle mass was obtained. The mass was then dissolved in chloroform, re-precipitated in ether and lyophilized for 48 hours.

Conjugation/deprotection was verified with ultraviolet-visible spectroscopy (UV-vis) (Cary 50 Bio UV-Vis Spectrophometer, Varian). At 257 nm, the protecting carbobenzoxy (CBZ) group can be visualized (Fig. S1B). Presence of this CBZ group prior to deprotection verifies successful conjugation while its later absence is indicative of successful deprotection and thus amine exposure. ^{1}H NMR was also utilized for determining successful conjugation/deprotection of PLGA-PLL (Fig. S1C). ^{1}H NMR spectra were recorded at room temperature in D_6-DMSO on a 400 MHz Bruker spectrometer and referenced to tetramethylsilane (TMS) peak (δ = 0.0 ppm). The benzene ring peak associated with the CBZ protecting groups (δ = 7.3 ppm) is present in the protected PLGA-PLL but successfully removed in the deprotected form *(S1)*.

PEG conjugation to PLGA-PLL

PEG (molecular weight ~ 1500 and 4600 Da) was activated with CDI. Briefly, PEG was dissolved in dioxane at 37°C. An 8:1 (CDI:PEG) molar excess of CDI was added, and the resulting mixture was stirred under argon at 37°C for 2 hours. Unreacted CDI was removed by dialysis in deionized water for 12 hours. The dialysate was changed every hour. The resulting solution was frozen in liquid nitrogen and freeze-dried for 3 days. The resulting activated PEG was then stored at -20°C.

A mixture of excess activated PEG and PLGA-PLL (5:1 molar ratio PEG:PLGA-PLL) was dissolved in anhydrous DMF and allowed to stir under argon. An excess of PEG was used to ensure that only one imidazole end group reacted with the pendant amino groups of the PLGA-PLL, while the other imidazole group was available for later conjugation to the RGD moiety. After 48 hours, the polymer solution was diluted with chloroform and precipitated in methanol. Unconjugated PEG is soluble in methanol and easily removed. Polymer dissolution and precipitation was repeated two times to ensure the removal of unconjugated PEG.

^{1}H NMR was also utilized for determining conjugation of PEG to PLGA-PLL. The presence of the ether linkage associated with PEG (δ = 3.51 ppm) verified its successful incorporation (Fig. S1C) *(S1)*.

RGD conjugation to PLGA-PLL-PEG

Twenty-five milligrams of the peptide moiety RGD, RGDS, GRGDS, or GRADSP was mixed with 200 mg of PLGA-PLL-PEG in 3 ml of anhydrous DMSO and stirred. After 24 hours, the polymer solution was diluted with more DMSO, and dialyzed against deionized water for 12 hours to remove unconjugated peptide *(S1)*. The dialysate was changed every hour. The PLGA-PLL-PEG-RGD was then lyophilized for 3 days. Following freeze-drying, the polymer was redissolved in DMSO, and dialysis and lyophilization were repeated.

The successful conjugation of RGD was determined with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) *(S1, 2)*. Spectra were collected by a Perkin-Elmer Instruments Spectrum One FTIR equipped with a Universal ATR sampling accessory. Each sample was scanned 32 times at a resolution of 1 cm^{-1}. Although useful in validating the presence of amide linkages (Fig. S1D), further amino acid analysis was required to differentiate the PLL and RGD contributions (Fig. S2). For amino acid analysis, samples were submitted to the W.M. Keck Facility, Yale University. Analyses were carried out on a Beckman Model 7300 ion-exchange instrument. The molar ratio of arginine to lysine was defined as the conjugation efficiency of the RGD moiety to the PLGA-PLL-PEG.

RGD conjugation to nanospheres

Twenty-five milligrams of the RGD peptide was reconstituted in 3 ml of PBS. This peptide solution was then added to 200 mg of PLGA-PLL-PEG nanospheres and allowed to react for 3 hours. Following this conjugation of RGD to the PEG imidazole group on the nanosphere, the nanosphere/peptide mixture was diluted with deionized water and centrifuged. The supernatant with unconjugated RGD was discarded. Nanospheres were then reconstituted with deionized water and washed two more times by repeating this process. Nanospheres were then frozen and freeze-dried for 3 days. Successful conjugation of RGD was determined via amino acid analysis as described above. As a control, PLGA-PLL-PEG nanospheres without imidazole activated PEG were used. These nanospheres had undetectable levels of RGD, demonstrating the necessity of the PEG imidazole end groups for peptide incorporation.

Fluorescent labeling of rat platelets

Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine (80/10 mg/kg). Following induction of anesthesia, blood was obtained via cardiac puncture in a syringe

containing 1000 U sodium heparin/ml (in 0.9% saline) solution (anticoagulant solution:blood, 1:9 v/v). To prepare platelet rich plasma (PRP), the collected blood underwent a "soft spin" of 180 g for 10 minutes at 22°C. Platelets were then sedimented by centrifuging the PRP at 1600 g for 5 minutes. PPP was extracted and the remaining platelets were resuspended in Buffer A (140 mM NaCl, 3mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10mM glucose, and 10mM HEPES, pH = 7.4) *(S3)*. Reconstituted platelets were then stained with 10μM CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes) (2.5 mM stock in DMSO). Platelets were stained for 40 minutes at room temperature and then centrifuged at 1600 g for 5 minutes. Buffer A was extracted and platelets were reconstituted in platelet poor plasma (PPP) to a final concentration of $5x10^8$ platelets/ml. Platelet concentration was determined with a Beckman Coulter Multisizer 3 with a 50 μm diameter aperture based on a sample volume of 100 μl.

Validation of in vitro assay

For the initial validation of our in vitro assay, collagen I (rat tail) was used. Briefly, 96-well plates were coated by adding 100 μl of 500 μg/ml collagen to each well. Plates were then allowed to sit for 24 hour at 4°C. Wells were then washed three times with PBS to remove nonadherent collagen. Following the PBS rinse, 100 μl of PRP with CMFDA fluorescently labeled platelets ($5x10^8$ platelets/ml) was added to each well. This was followed by the addition of 10 μl of 100 μM ADP as a proaggregatory stimulus, or PBS as a control. Immediately following ADP/PBS addition, the 96-well plate was agitated for one minute on an orbital shaker (Barnstead International) at 180 rpm *(S4)*. After a 3 minute equilibration, plasma and non-aggregated platelets were gently extracted, and entire wells were imaged from the bottom with a 4x objective at 490nm /525nm (excitation/emission) (Olympus IX71 Fluorescent microscope). Area of fluorescence was then quantified to elucidate the differences in platelet adherance/aggregation *(S5)*.

Supplementary Figures and Legends

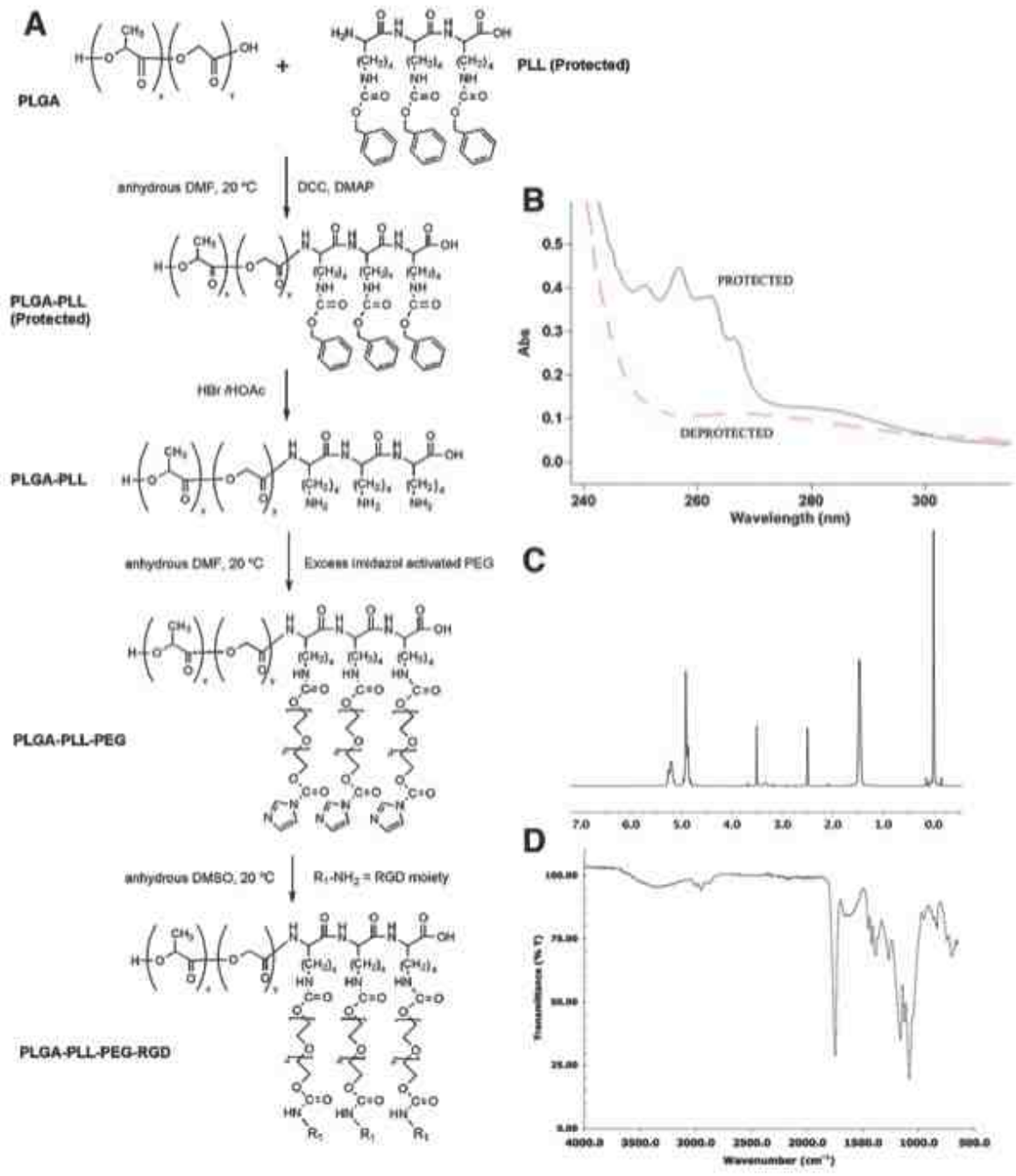

Fig. S1. Polymer synthesis and characterization. (A) Reaction scheme for PLGA-PLL-PEG-RGD polymer. (B) Conjugation/deprotection was verified with ultraviolet-visible spectroscopy (UV-vis). At 257 nm, the protecting carbobenzoxy (CBZ) group can be seen. Presence of this

CBZ group prior to deprotection verifies successful conjugation while its later absence is indicative of successful deprotection and thus amine exposure. (C) ^{1}H NMR was also utilized for determining conjugation of PEG to PLGA-PLL. The presence of the ether linkage associated with PEG (δ = 3.51 ppm) verified its successful conjugation. (D) The successful conjugation of RGD was partially determined with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Although useful in validating the presence of amide linkages (broad absorption at 3325 and peaks at 1632 and 1578 cm^{-1}), further amino acid analysis was required to differentiate the PLL and RGD contributions (Fig. S2A).

A

Polymer	Lysine Content (μmol/g)	Peptide Content (μmol/g)	Conjugation Efficiency (%)
1500-RGD	23.6	7.7	32.6
4600-RGD	22.7	6.2	27.2
1500-RGDS	20.9	6.5	31.0
4600-RGDS	16.7	5.2	31.3
1500-GRGDS	17.9	4.3	24.1
4600-GRGDS	19.4	5.4	27.7
4600-GRADSP	17.5	7.1	40.7

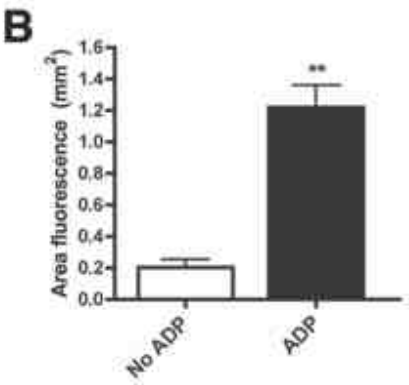

Fig. S2. Polymer characterization and in vitro assay with collagen. (A) Lysine and peptide concentrations of PLGA-PLL-PEG-RGD polymer as determined by amino acid analysis. Conjugation efficiency was defined as the peptide to lysine ratio multiplied by 100. PLGA-PLL-PEG-RGD polymer was used for in vitro studies. Polymer was fabricated as described in Fig. S1A. (B) Quantification of CMFDA-labeled platelet aggregation in wells coated with collagen I (rat tail). Comparison between wells with and without ADP. Experiments were performed in triplicate. Data are expressed as mean ± SEM (** $P < 0.01$).

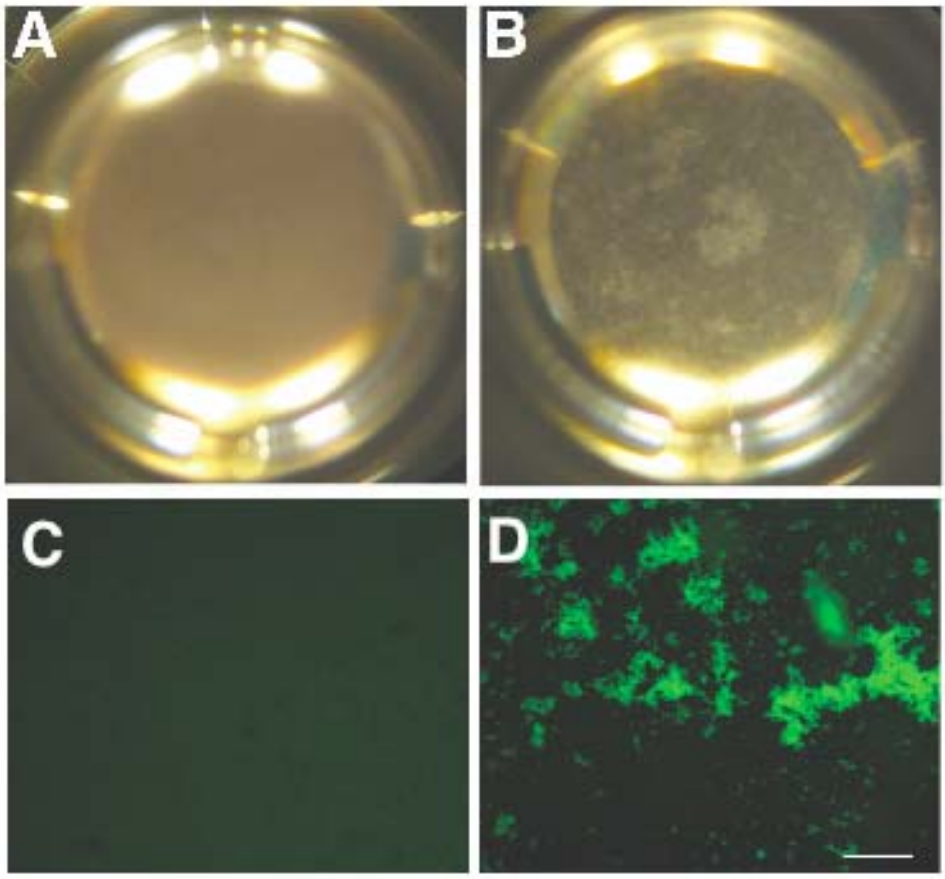

Fig. S3. In vitro analysis of PRP with synthetic platelets. (A) PRP with synthetic platelets plus PBS following agitation for 10 minutes. Note the lack of platelet activation/aggregation, resulting in a homogenous distribution of endogenous/synthetic platelets. (B) Platelet rich plasma with synthetic platelets plus ADP and agitation (1 minute). The addition of a proaggregatory stimulus (i.e. ADP) results in the formation of platelet aggregates. (C) PLGA-PLL-PEG-RGD coated well with CMFDA-labeled platelets. Even after agitation for 10 minutes, there are no signs of platelet adhesion or aggregation. (D) Aggregation of platelets on the polymer surface happens immediately following the addition of ADP. Scale bar, 500 μm.

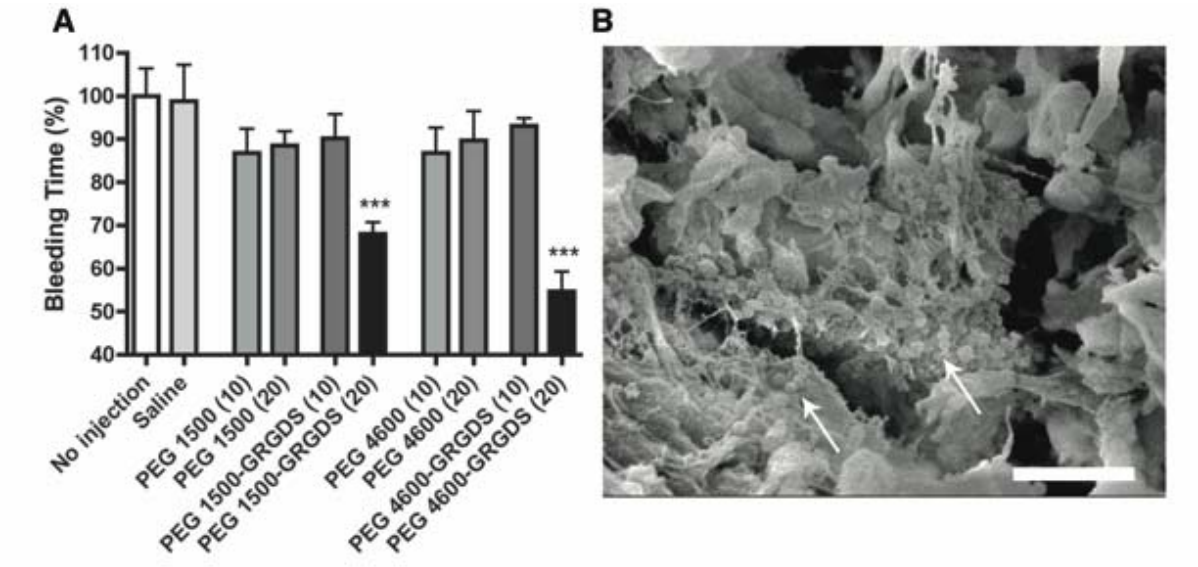

Fig. S4. In vivo analysis of bleeding times for different concentrations of synthetic platelets. (A) Bleeding times iafter femoral artery injury following intravenous administration of the synthetic platelets (n = 5). Data presented as % of the No injection mean (240 ± 15 seconds). Data are expressed as mean ± SEM (***P < 0.001 versus saline). The 10 and 20 are the concentration of synthetic platelet dose in mg/ml. A 40 mg/ml concentration was also attempted, however, cardiopulmonary complications were observed in some of the groups. Therefore, the 40 mg/ml concentration study was halted. (B) SEM micrograph of clot excised from injured artery after synthetic platelet administration (4600-GRGDS). Arrows, synthetic platelets in clot. Clot imaged from lumen side. Scale bar, 2 μm.

References

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