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<th>Citation</th>
<th>Chan, Juliana M. et al. “Spatiotemporal controlled delivery of nanoparticles to injured vasculature.” Proceedings of the National Academy of Sciences 107.5 (2010): 2213 -2218. Copyright ©2010 by the National Academy of Sciences</th>
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<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.0914585107">http://dx.doi.org/10.1073/pnas.0914585107</a></td>
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<tr>
<td>Publisher</td>
<td>National Academy of Sciences</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Wed Mar 16 19:08:37 EDT 2016</td>
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<td>Citable Link</td>
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Spatiotemporal controlled delivery of nanoparticles to injured vasculature

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Contributed by Robert Langer, December 17, 2009 (sent for review November 9, 2009)

There are a number of challenges associated with designing nanoparticles for medical applications. We define two challenges here: (i) conventional targeting against up-regulated cell surface antigens is limited by heterogeneity in expression, and (ii) previous studies suggest that the optimal size of nanoparticles designed for systemic delivery is approximately 50–150 nm, yet this size range confers a high surface area-to-volume ratio, which results in fast diffusive drug release. Here, we achieve spatial control by biopanning a phage library to discover materials that target abundant vascular antigens exposed in disease. Next, we achieve temporal control by designing 60-nm hybrid nanoparticles with a lipid shell interface surrounding a polymer core, which is loaded with slow-eluting conjugates of paclitaxel for controlled ester hydrolysis and drug release over approximately 12 days. The nanoparticles inhibited human aortic smooth muscle cell proliferation in vitro and showed greater in vivo vascular retention during percutaneous angioplasty over nontargeted controls. This nanoparticle technology may potentially be used to target the treatment of injured vasculature, a clinical problem of primary importance.

Results and Discussion

Selection and Characterization of Basement Membrane Targeting Peptides for Vascular Wall Targeting. To discover a functional vascular targeting peptide, a combinatorial library of random heptamers fused to the minor coat protein (pIII) of M13 filamentous phage was subjected to five rounds of biopanning against human collagen IV. Fifteen clones per round were randomly sequenced from Round 3–5 (R3–R5) (Fig. L4), and in R5, 100% of the clones were found to be C-8, HWGSLRA. To find similarities to resident basement membrane structures, we searched the nonredundant version of the current National Center for Biotechnology Information Homo sapiens sequence database using the pBLAST algorithm against peptides from the screen (24, 25). Sequences were classified into three groups. The first group consists of peptides with homology to resident basement membrane proteins such as nidogen, serum amyloid P component, gelsolin, and laminin (21). The second group of peptides was enriched in proline residues, such as Pro-Pro-Ser (PPS) and Pro-Pro-Pro (PPP) runs, which resemble the Gly-Pro-Pro (GPP) motif in the collagen triple helix (20). Finally, the third group consists of unique peptides with no identifiable relationship with the basement membrane.

It has been discussed in the literature that penultimate rounds of biopanning may be a rich source of phage binders suspected to be lost due to reduced fitness (26). Possible reasons include reduced infectivity rates of phages for their Escherichia coli hosts due to low pH elution, disulfide-bond formation between cysteine containing phages resulting in the rarity of cysteine-rich peptides | collagen | nanoparticle | paclitaxel | angioplasty

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0914585107/DCSupplemental.
sequences, faster growth rates of certain clones, or simply aounder effect when a fraction of amplified phages are input into the
next round of biopanning. In Fig. 1A, a binding experiment
was performed to determine the affinities of the sequenced clones
for Matrigel. 23 clones were incubated in triplicate against
Matrigel (22) and BSA (SI Materials and Methods). The phages
were ranked according to absorbance values indicating their
binding capacity to Matrigel. No reactivity was observed against
BSA compared with the random library (R0). Despite the simi-
larity of the PP motifs with collagen IV, Group B peptides
showed less binding affinity compared to Group A and C, and
showed no detectable binding affinity above the library to
Matrigel. Clones A-8, A-9, C-10, and C-11 were the best candi-
dates of the sequenced clones when compared to Group A and C,
and we noted that these four clones resembled each other.
The four clones were aligned pairwise using the CLUSTAL
multiple sequence alignment and gave a consensus
sequence of KIWVLPQ, or more generally, KZWXLPX, where
X is any amino acid (Fig. 1B).

In a sequence-specific competition assay, we analyzed the con-
text-dependence of the phage toward the peptide-collagen IV
binding interaction (SI Materials and Methods). Synthetic peptides modeled after phage clones A-8, A-9, C-10, and C-11 com-
petitively inhibited their cognate phage in a dose-dependent
manner on Matrigel-coated surfaces (Fig. 1C). Phage C-11 showed
the best peptide competition, which suggests that C-11 binding
affinity may represent a specific peptide-collagen IV interaction
independent of the phage context. We further examined the
binding of phage C-11 in three independent titer count analyses
(Fig. 1D). Phage titers of C-11 were compared against the library
(R0) for binding to Matrigel and collagen IV with an initial phage
input of $10^{12}$/mL pfu. C-11 showed approximately 300-fold
Matrigel binding and approximately 900-fold collagen IV binding
compared to the library ($n = 3, P < 0.001$).

**Synthesis and Characterization of the Nanoburr Drug Delivery System.** To investigate the targeting properties of the candidate
peptide against the basement membrane, we covalently conjugated
peptides onto hybrid NPs that have a hydrophobic drug-eluting
core, a hydrophilic polymeric shell, and a lipid monolayer (7). PEG
(1) covalently conjugated to 1,2-diesteroyl-sn-glycero-3-phos-
phoenolanamine (DSPE) was used to form the hydrophilic poly-
meric shell. To complete the lipid monolayer, soybean lecithin,
which is considered Generally Regarded As Safe (GRAS), was used
to form the core-shell interface. For the hydrophobic drug-eluting
core, we synthesized paclitaxel–poly(lactide) (Ptxl–PLA) conjugates
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phoenolanamine (DSPE) was used to form the hydrophilic poly-
meric shell. To complete the lipid monolayer, soybean lecithin,
increase and made the surface charge cationic (13.5 ± 5.9 mV, mean ± SD), presumably because the peptides were N-terminally exposed to retain their original phage-displayed orientation.

To characterize the nanoburrs physiochemically, we quantified their release kinetics by taking aliquots (n = 3) at scheduled time points for RP-HPLC analysis (Fig. 2G). Ptxl is released by diffusion when the Ptxl–PLA ester bond is hydrolyzed, and the amount of Ptxl released from Ptxl–LA25 was 43.4% on day 2, and 91.0% and 93.8% on day 10 and day 12, respectively. Drug elution rates can be further controlled by varying lactide/Ptxl ratios during ring-opening polymerization, resulting in different PLA chain lengths attached to the Ptxl drug (8). The use of polymers to control Ptxl release is also a significant feature of drug eluting stents (DES), however, 80–90% of the Ptxl fraction is never released (27).

The two parameters of drug loading and release are important for drug efficacy. Increased drug loading into the particle core tends to reduce overall stability, giving an undesired burst release effect and reduced efficacy (28). Larger particles tend to have slower in vitro release profiles, but when systemically delivered may be more readily detected and cleared from circulation, resulting in a lack of efficacy (29). For vascular targeting, because small particles show improved vessel adhesion and retention (16, 30–32), integrating slow-eluting conjugates into the nanoburr design allows for (i) improved drug loading; (ii) sub-100-nm NPs for vascular targeting; and (iii) sustained drug release over 2 weeks.

**Targeted Drug Release from Nanoburrs.** To validate the therapeutic efficacy of this treatment, a human aortic smooth muscle cell (haSMC) cytotoxicity study was used to assess nanoburr differential cellular cytotoxicity and binding affinity on Matrigel-coated wells with haSMC (Fig. 3). To test the sequence specificity of the KLWVLPK peptide (T), we designated two controls: scrambled PWKKLLV peptide (S) and nontargeted (B) NPs. In addition, we included a media-only control and fourfold dilutions of free Ptxl in DMSO (maximum 0.1% DMSO in media). The maximum free
Ptxl concentration used was 51 μM, exceeding by two log scales a suitable Ptxl dose range of 50–1,000 nM for haSMC cytotoxicity. An incubation time of 45 min was significantly shorter than typical incubation times with Ptxl (approximately 4–24 h). The wells were rinsed twice with complete media and further incubated with fresh media for 48 h. Unlike free Ptxl, which is removed during the washing step, the nanoburrs attached to the collagen IV matrix were retained for continued Ptxl release. Hence, lowered formazan product formation (absorbance at 490 nm) reflects increased haSMC cytotoxicity as a function of greater targeted-NP retention on Matrigel-coated plates (n = 5, P < 0.001).

### Binding Studies in Angioplasty Models of Injured Vasculature

We evaluated the targeting affinity of the nanoburr system toward injured vasculature. To create those vascular characteristics, a Fogarty 2-French balloon catheter was used to injure rat arteries by repeatedly advancing, inflating the balloon, and withdrawing to denude the endothelial monolayer and expose the basement membrane. This loosely mimics a percutaneous angioplasty procedure in human patients, the difference being that in human patients the catheter is inflated locally in a preexisting stenotic lesion. In Fig. 4.4, a representative H&E stained cross-section shows an injured aorta with the endothelial layer removed, and an uninjured aorta with an intact endothelial monolayer.

Our ex vivo study examined targeting of the nanoburr system to balloon-injured rat aortas. We used Alexa Fluor 647 fluorescent dye-poly(lactic-co-glycolic acid) (A647–PLGA) conjugates as a substitute for Ptxl–PLA drug conjugates to visualize the nanoburrs by fluorescence microscopy and optical imaging. This wavelength is beyond the autofluorescence range of typical endogenous tissue fluorophores such as collagen and elastin, which excite and emit maximally at approximately 300–500 nm. Therefore, any A647–PLGA fluorescence detected would be NP deposition. A647–PLGA encapsulated nanoburrs were incubated in the abdominal aorta for 5 min under constant pressure, followed by extensive washing using a syringe-pump to remove nonadsorbed samples (Fig. 4B). Subsequently, the abdominal aortas were harvested and viewed with whole vessel fluorescent optical imaging (SI Materials and Methods). In fluorescent imaging, the detected surface intensity depends on the illumination intensity, which varies according to the field-of-view and wavelength. To eliminate the effect of illumination intensity, images are normalized against a reference illumination image. The resulting “normalized” fluorescent efficiency image is unitless, and the value of each pixel represents the fractional ratio of emitted photons per incident excitation photon. We used the region-of-interest (ROI) function to quantify nanoburr retention (Fig. S1), and measurements are displayed as average fluorescent efficiency (relative fluorescence units, rfu). The nanoburrs bound to balloon-injured aortas (Fig. 4C) at 1.43 ± 0.48 × 10−6 (rfu), while scrambled-peptide and nontargeted NPs bound on average two-fold less at 48% (n = 3, P < 0.05) and 47% (n = 3, P < 0.05), respectively. To ensure that the nanoburrs would not target intact endothelial layers, they were also incubated with uninjured aortas and bound fourfold less at 3.39 ± 0.50 × 10−5 (rfu) (n = 3, P < 0.01) compared to injured vessels. Frozen histological sections were photographed to show nanoburr binding along the arterial cross-section (Fig. S2).

We next examined targeting in vivo via IA infusion using a left carotid injury model (Fig. 4D). The nanoburrs were injected into angioplastied left carotids through a catheter positioned in the aortic arch over the course of 1 min and allowed to circulate for 1 h. Fourfold more nanoburrs (8.71 ± 3.80 × 10−6 (rfu)) were found in the injured vessel compared to the uninjured vessel. Similar to the ex vivo model, the nanoburrs were retained maximally at approximately 300–500 nm. Therefore, any A647–PLGA fluorescence detected would be NP deposition. A647–PLGA encapsulated nanoburrs were incubated in the abdominal aorta for 5 min under constant pressure, followed by extensive washing using a syringe-pump to remove nonadsorbed samples (Fig. 4B). Subsequently, the abdominal aortas were harvested and viewed with whole vessel fluorescent optical imaging (SI Materials and Methods). In fluorescent imaging, the detected surface intensity depends on the illumination intensity, which varies according to the field-of-view and wavelength. To eliminate the effect of illumination intensity, images are normalized against a reference illumination image. The resulting “normalized” fluorescent efficiency image is unitless, and the value of each pixel represents the fractional ratio of emitted photons per incident excitation photon. We used the region-of-interest (ROI) function to quantify nanoburr retention (Fig. S1), and measurements are displayed as average fluorescent efficiency (relative fluorescence units, rfu). The nanoburrs bound to balloon-injured aortas (Fig. 4C) at 1.43 ± 0.48 × 10−6 (rfu), while scrambled-peptide and nontargeted NPs bound on average two-fold less at 48% (n = 3, P < 0.05) and 47% (n = 3, P < 0.05), respectively. To ensure that the nanoburrs would not target intact endothelial layers, they were also incubated with uninjured aortas and bound fourfold less at 3.39 ± 0.50 × 10−5 (rfu) (n = 3, P < 0.01) compared to injured vessels. Frozen histological sections were photographed to show nanoburr binding along the arterial cross-section (Fig. S2).

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the angioplastied left carotid arteries compared to healthy right carotids (Fig. 4E). Scrambled-peptide and nontargeted NPs were retained in the left carotids at 40% ($n = 3, P = 0.0818$) and 53% ($n = 3, P = 0.23716$) of nanoburr retention, respectively (Fig. S3). Representative frozen histological sections show fluorescence along the carotid arteries (Fig. S4).

The nanoburr system was studied for systemic delivery because repeat dosing may be helpful in the treatment of chronic vascular disease (30). Using a left carotid injury model, the nanoburrs were given as a 1 mL i.v. dose via tail-vein injection and allowed to circulate for 1 h (Fig. 4F). Nanoburr retention was 5.46 ± 1.02 × 10^-6 (rfu) in the angioplastied left carotids compared to scrambled-peptide and nontargeted NPs (Fig. 4G), which were 35% ($n = 5, P < 0.0001$) and 64% ($n = 5, P < 0.01$) of nanoburr retention, respectively. The nanoburrs bound to the left carotids twofold over healthy right carotids ($P < 0.0001$) (Fig. S5). Representative fluorescence images of nanoburr binding are shown in Fig. S6.

Our binding studies to sites of injured vasculature altogether show the successful targeting and retention of nanoburrs to injured carotid arteries in vivo and abdominal aortas ex vivo. Further in vivo tests of efficacy are necessary to expand on our findings.

**Conclusion**

In summary, we developed a spatiotemporally controlled delivery vehicle using clinically safe biomaterials that have the capacity to target injured vasculature. When these vehicles are administered IA or i.v., they demonstrate localization to sites of injured vasculature and exhibit controlled drug release over approximately 10–12 days in vitro. Our initial application of this technology was for vessel wall targeting in cardiovascular disease. The utility of the nanoburr system is broader and may include oncologic and regenerative medicine and exhibit controlled drug release over approximately 10–12 days in vitro. Our initial application of this technology was for vessel wall targeting in cardiovascular disease. The utility of the nanoburr system is broader and may include oncologic and regenerative diseases and indications where neoangiogenesis is commonly observed.

**Materials and Methods**

A detailed description of materials and methods is available in **SI Materials and Methods**.

**Screening of Phage Display Peptide Library**

The Ph.D.-7 phage library was obtained from New England Biolabs. Briefly, approximately 10 μg/mL human collagen IV in 0.1 M NaHCO3, pH 8.6 was coated onto a 96-well enzyme and radioimmunoassay (ELA/RIA) high binding plate (Corning Life Sciences) overnight at 4 °C for biopanning according to the manufacturer’s instructions. From R2 to R5, the Tween-20 concentration was raised to 0.5%, and the collagen IV enriched phage pool from R1 was subtractively panned against human collagen I for 1 h at room temperature (RT) to reduce collagen I binding interference before biopanning against collagen IV. In R5, 1-μg/mL collagen IV coated plates were used for increased stringency. Fifteen clones per round were randomly picked from R3 to R5 for DNA sequencing and further analysis (**SI Materials and Methods**).

**Pacitaxel-Poly-lactide Conjugation.** [(BDI)]2[Zn(TM5)], [(BDI =2-(2,6-diisopropylphenyl)amino)-4-((2,6-diisopropylphenyl)imino)-2-pentene, TMS = trime-thylsilyle]) (6.2 mg, 0.01 mmol) and Ptxl (8.5 mg, 0.01 mmol) were mixed in 0.5-M anhydrous THF. O-Lactide (36 mg, 0.25 mmol) in 2 mL anhydrous THF was added dropwise to initiate polymerization. Lactide was completely consumed within hours at RT and monitored by FTIR or 1H NMR spectroscopy. The polymerization solution was added to ethyl ether (25 mL) to precipitate out the Ptxl-PLA conjugate (approximately 25 dL-lactide monomer units, 19.2 wt% Ptxl).

**Synthesis and Characterization of Nanoburrs.** A 3-ml DSPE-PEG-lecithin mixture in 4% ethanol containing 0.17 mg DSPE-PEG-Maleimide/DSPE-PEG (1:4 molar ratio) and 0.08 mg lecithin was heated for 3 min above the lipid phase transition temperature to 68 °C under gentle stirring. After heating, 1 mg of Ptxl-PLA in acetone (1 mg/mL) was added dropwise at 1 mL/min. The solution was vortexed vigorously for 3 min followed by self-assembly under gentle stirring for 2 h at RT. The NPs were washed three times using an Amicon Ultra 4-centrifugal filter with 30,000 Da MWCO (Millipore). The NPs were reconstituted in pH 7.2 PBS buffer and 2 mM EDTA and incubated with peptides (MW = 1137.54 Da) at a 1/2 molar ratio to DSPE-PEG-Mal for 45 min at RT. The peptides were previously reduced using Bond-breaker TCEP solution. Neutral pH (Thermo Scientific) in PBS-EDTA at a 1/100 disulfide bond/TECP molar ratio. Free peptides were removed using a Sephadex PD-10 (G-25) column. For scale-up, multiple vials of NPs were made with concentrations and volumes kept constant to maintain small NP diameters. For animal studies, NPs were sterile filtered before IA or i.v. delivery. TEM images of the nanoburrs (1 mg/mL) were obtained by negative-staining with 3% w/v uranyl acetate. Size (diameter, nm) and surface charge (zeta potential, mV) were evaluated by quasi-elastic laser light scattering using a ZetaPALS dynamic light-scattering detector (15 mW laser, incident beam = 676 nm; Brookhaven Instruments).

**haSMC Cytotoxicity Studies.** Ninety-six-well plates were Matrigel-coated and BSA-blocked (as described in **SI Materials and Methods**) in PBS. haSMC were plated at 10,000 cells/well in a 37 °C, 5% CO2 incubator and grown for 24 h in Medium 231 supplemented with 10 μM gentamycin, 0.25 μg/mL amphotericin B, and smooth muscle growth supplement (all from Cascade Biologics, Invitrogen). Treatment groups (n = 5) included nanoburrs, scrambled-peptide NPs, nontargeted NPs, fourfold dilutions of Ptxl (in maximum 0.1% DMSO) in media and a media-only control. Samples were incubated with cells for 45 min. The wells were washed twice with complete media and replaced with fresh complete media for 48 h. Medium 231 was replaced with phenol red-free RPMI medium supplemented with 10% FBS (Invitrogen) containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTT)] [(BDI =2-((2,6-diisopropylphenyl)amino)-4-((2,6-diisopropylphenyl)imino)-2-pentene, TMS = trime-thylsilyle]) (6.2 mg, 0.01 mmol) and Ptxl (8.5 mg, 0.01 mmol) were mixed in 0.5-M anhydrous THF. O-Lactide (36 mg, 0.25 mmol) in 2 mL anhydrous THF was added dropwise to initiate polymerization. Lactide was completely consumed within hours at RT and monitored by FTIR or 1H NMR spectroscopy. The polymerization solution was added to ethyl ether (25 mL) to precipitate out the Ptxl-PLA conjugate (approximately 25 dL-lactide monomer units, 19.2 wt% Ptxl).

**Balloon-Angioplasty ex Vivo and in Vivo Studies.** Sprague–Dawley rats weighing approximately 450–500 g were obtained from Charles River Laboratories and fed a normal rodent diet. All animal procedures were conducted by a certified contract research organization using protocols consistent with local, state, and federal regulations as applicable and approved by the Institutional Animal Care and Use Committee.

For ex vivo studies, animals were killed for open abdominal cavity surgery in situ. Aortas were flushed with saline and injured by four passages of a Fogarty arterial embolectomy 2F balloon catheter (Model 120602P; Edwards Lifesciences) in a rotating fashion. AlexaFluor 647 (A647) dyes were covalently conjugated to PLGA (viscosity 0.19 dL/g) using EDC/NHS chemistry in DMF. A647-PLGA conjugates were precipitated in 2/1 ethanol/methanol by centrifugation, dried in vacuum, and resuspended in acetone for NP preparation. Fluorescence (rfu) was quantified using a GeminiXPS micro-plate spectrofluorometer (Molecular Devices), and samples were diluted accordingly in PBS for comparable NP delivery into the aortas. 0.4-ML samples (approximately 6 mg/mL) were incubated in the aorta for 5 min using metal clips to secure both ends of the aorta. Nonadsorbed samples were flushed away with saline using an Advance Infusion Pump Series 1200 syringe pump (Roboz Surgical Instrument Co.) programmed at 4 mL/min for 15 min.

For in vivo i.v. studies, animals were anesthetized intramuscularly with ketamine (60 mg/kg)/xylazine (10 mg/kg) and given buprenorphine as an analgesic. Left common carotids were injured by four passages of the 2F balloon-catheter, before a 30-gauge tubing was inserted via the external carotid into the common carotid and advanced beyond the angioplastied region into the aortic arch. Samples (approximately 10 mg/mL) were infused at 1 mL/min for 1 min. The external carotids were permanently ligated. The animals were killed 1 h after surgery, and the carotids were harvested.

For in vivo I.V. studies, animals were additionally given heparin (500 IU/kg) by i.v. injection immediately before surgery. The animals underwent left common carotid artery surgery and samples (approximately 15 mg/mL) were given by 1-ml i.v. tail vein injections. The animals were killed after 1 h and the carotids were harvested.

**ACKNOWLEDGMENTS.** We thank Angela Doye, Juan Deleon, and Dr. Judith Gwathmey for their assistance with the animal surgeries. We thank Drs. Elazer Edelman and Dr. Chester Drum for helpful discussions. We thank Dr. Richard Cook and Natalie Schiller of the Massachusetts Institute of Technology and Research, Singapore. Financial support from the B.S.-Ph.D. National Science Scholarship awarded by the Agency for Science, Technology and Research, Singapore.