DOI: 10.1002/cmdc.200800205

Enzymatic Release of a Surface-Adsorbed RGD Therapeutic from a Cleavable Peptide Anchor

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Implantation of a polymeric, ceramic, or metallic implant will unavoidably activate a response from the surrounding tissue.[1] Means to attenuate this inflammatory response at the implant site are of significant interest and currently include strategies based on surface morphology, chemical modification, or drug delivery. This response is particularly evident at the site of stent deployment, where the overproliferation of smooth muscle cells can lead to restenosis—a re-narrowing of the lumen.[2] Drug-eluting stents (DES) were introduced to decrease the rate and severity of this neointimal formation through passive diffusion of a drug physically entrapped in a nondegradable polymer coating over a metal framework. However, recent studies have expressed concern over the widespread use of DES owing to their increased late-thrombotic potential of two to three times the rates for a traditional bare metal stent.[3] This clinical outcome is likely due to delayed healing and endothelium regeneration as a result of the polymer coating (e.g., poly(styrene-*b*-isobutyl-*b*-styrene)) and the delivery of non-phenotype-specific antimitotic/antiproliferative drugs (e.g., sirolimus and paclitaxel). With the goal of improving implant performance through appropriate interactions with the surrounding biology, we previously reported the use of implant-specific peptide coatings to prevent nonspecific surface biofouling and to promote a pro-healing response through increasing cell adhesion and spreading.[4] Herein we report a third approach whereby a surface-adsorbed therapeutic is enzymatically released, resulting in drug elution (Figure 1).

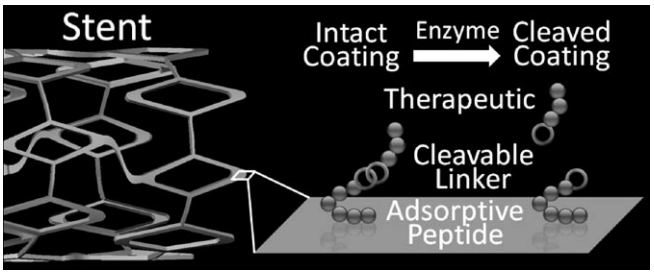

Figure 1. A drug elution mechanism whereby a tethered therapeutic can be actively released from a substrate, such as a stent, upon addition of an enzyme.

Engineering of an enzymatic recognition site into a material is an elegant approach to promote active degradation and has been used successfully with hydrogels, microspheres, bioplexes, and interpenetrating networks,[5] as well as for evaluating enzyme kinetics in the degradation of peptides on surfaces.[6] The enzymatic release of an adsorbed or tethered therapeutic from an implant surface is an exciting idea which would likely be of interest for many medical devices, including stents. Current stenting applications rely on passive drug entrapment and diffusion, and a wide variety of therapeutics are under investigation.[7] Some of these low-molecular-weight therapeutics include dexamethasone, methylprednisolone, 17-β-estradiol, angiopeptin, paclitaxel, actinomycin D, sirolimus, and arginine-glycine-aspartic acid (RGD).[8] The last example is particularly interesting, as clinical trials have shown that elution or local delivery of RGD decreased neointimal hyperplasia through the recruitment of circulating endothelial progenitor cells to the site of implantation and promoted arterial re-endothelialization.[8h,9] Building upon these observations, we designed a peptide-based coating that consists of three distinct peptide domains: an implant-adsorptive sequence, an enzymatically cleavable recognition site, and a therapeutic to be delivered (i.e., RGD). Medical devices such as stents coated with this peptide could then be implanted in vivo and remain stable and non-eluting against endogenous enzymes until systemic injection of the selected exogenous enzyme to catalyze the controlled local delivery of the therapeutic.

We selected the polystyrene (PS) binding sequence H_2N-FFSFFFPASAWGS-CO_2H, previously identified through phage display, as PS serves as a model polymeric substrate.[4a] To select an appropriate enzyme for use with this proof-of-concept, we exposed a solution of FFSFFFPASAWGSSGSSGK-(biotin) **1** to varied concentrations of trypsin, papain, chymotrypsin, enterokinase, thrombin, and factor Xa to determine the stability of the base sequence using both ELISA and MALDI-ToF MS. As shown in Figure 2, papain and chymotrypsin significantly cleave the base sequence; trypsin, thrombin, and factor Xa have a more moderate effect, and enterokinase did not show any activity. No additional degradation was observed after continued incubation with enterokinase for up to one week. We thus synthesized a cleavable peptide with an internal enterokinase recognition sequence (DDDDK) terminated with an RGD trimer (Table 1). The resulting peptide, FFSFFFPA-SAWGSSSGDDDDKSSGK-(biotin)-RGD **2** showed good stability in solution with no evidence of hydrolysis for at least one month.

To ensure the resultant peptide continued to express affinity for PS after inclusion of the hydrophilic DDDDK sequence,

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200800205.

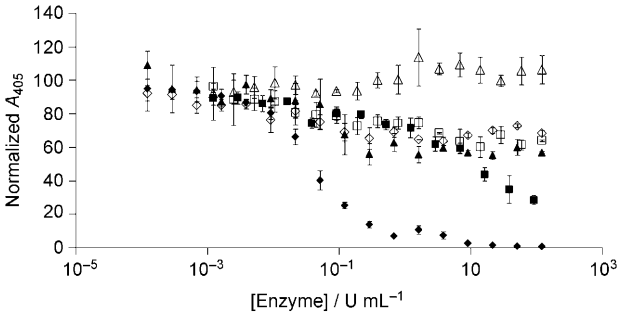

Figure 2. ELISA results for the enzymatic digestion of **1**, which serves as the base sequence for the cleavable peptides by papain (◆), chymotrypsin (■), thrombin (▲), trypsin (◇), factor Xa (□), and enterokinase (△). Values represent the mean ± SD; $n = 3$.

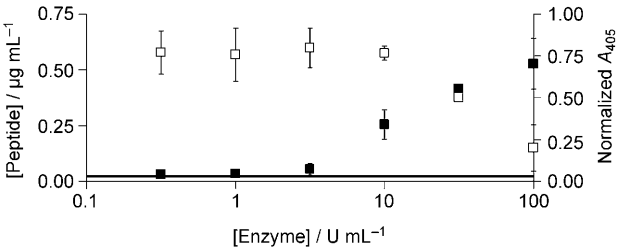

Figure 3. The amount of cleaved peptide in solution as a function of enzyme concentration, calculated from MALDI data with the solid line representing the zero-enzyme level (■; left axis). The amount of surface-bound peptide on the same plate calculated from ELISA (□; right axis). Values represent the mean ± SD; $n = 3$.

Table 1. Collated list of peptide sequences under investigation; biotin groups are attached to the lysine side chain.

No.	Sequence	Name
1	FFSFFFPASAWGSSGSSGK-(biotin)	PS-Biotin
2	FFSFFFPASAWGSSSGDDDDKSSGK-(biotin)-RGD	PS-DDDDK-RGD
3	FFSFFFPASAWGSSGSSGRGD	PS-RGD

ELISA was used to characterize the peptide–substrate interaction. This peptide was coated onto PS by exposing the surface to an aqueous solution of peptide (0.1 mg mL^{-1}) for 2 h followed by a rinse with phosphate-buffered saline (PBS). The dissociation constant for peptide **2** was calculated to be within a factor of 1.4 of the value calculated for the base sequence **1** (K_d for **1**: 6.34×10^{-6} M and **2**: 8.36×10^{-6} M), showing that the DDDDK motif does not affect the binding of the underlying base sequence (Figure S1, Supporting Information).

Next, to ensure enzyme cleavage occurred where expected, MALDI experiments were performed on cleavable peptide **2**. The peptide was dissolved in water followed by the addition of enterokinase (100 U mL^{-1}) for 2 h. The recorded and expected molecular weights of the cleaved portions and undigested peptide agreed within 0.2%, and cleavage was only observed at the C terminus of the inserted DDDDK recognition site (Table S1).

To determine the surface release kinetics of the peptide coating, which contains a lysine-linked biotin residue, **2** was dissolved in nanopure water and exposed to the wells of a PS plate. After a wash, varied amounts of enterokinase were added to the wells and left to interact for 24 h. The supernatant in the wells was removed, and the amount of peptide remaining on the surface was determined using a biotin–streptavidin–alkaline phosphatase assay (Figure 3). Concurrently, the supernatants removed in the earlier step were individually examined by MALDI-ToF MS by mixing in a known amount of FFSFFFPASAWGSSGSSGRGD **3**, which served as an internal standard. The peak corresponding to the cleaved therapeutic portion was integrated and normalized to the amount of peptide **3** in the same spectrum (Figure S2). As expected, an inverse relationship was observed between the amount of peptide remaining on the surface of the plate (uncleaved) and the amount of peptide observed in the supernatant (cleaved) (Figure 3). Increasing amounts of enzyme catalyzed the release of more RGD from the plate. A logarithmic equation can be fit to the release with high accuracy: $Y = 0.14 \ln(X) - 0.08$, for which Y is the supernatant peptide concentration (μg mL^{-1}) and X is the input of enzyme (U mL^{-1}) ($R^2 = 0.985$).

To determine the temporal release of the RGD therapeutic, the above experiment was repeated, but the enzyme concentration was kept constant (0, 10, or 31.6 U mL^{-1}). A trend was observed in which increased reaction time afforded a larger proportion of released RGD (Figure 4). The ability to determine

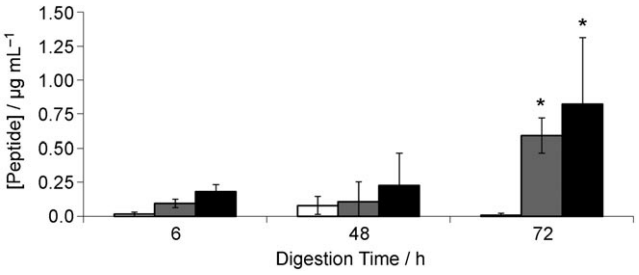

Figure 4. Temporal digestion of cleavable peptide **2** with 31.6 U mL^{-1} enterokinase (black) 10 U mL^{-1} enterokinase (gray) and no enzyme added (white). Values represent the mean ± SD; $n = 3$; *$p < 0.05$ over no enzyme control.

the solution concentrations at shorter time periods was hindered by the signal-to-noise ratio of the MALDI, but a rate of approximately 525 nM day^{-1} (23 ng mL^{-1} h^{-1}) can be estimated using the final time points for the 10 U mL^{-1} condition. As expected, increased amounts of enzyme produced increased elution at all time points, and only background signal was observed for the no-enzyme condition. Increases in elution concentration would be obtainable by using textured surfaces to increase the surface area available for adsorption. Although a fraction of the eluted drug will enter the bloodstream and be swept downstream from the implant site, another portion will be eluted into the arterial wall, where the therapeutic will accumulate over time.[10] RGD concentrations on surfaces as low as 1.05 μM have been shown to recruit endothelial progenitor cell invasion,[8h] a concentration that would be reached in the

tissues around the implant site in a matter of days with the system as it currently stands.

In summary, we have devised and evaluated a noncanonical enzymatic release system for therapeutic delivery from a surface such a stent. The enterokinase system is highly specific and shows no random peptide cleavage over extended time periods. The RGD is eluted in an enzyme-concentration-dependent manner with a rate on the order of tens of nanograms per hour on a smooth surface. Additionally, besides serving as the prototypical enzyme, enterokinase is exogenous to the bloodstream and so would only be present in the circulation if the patient is systemically dosed with the enzyme; this creates a mechanism for controlled local release. Because other proteases would be present in the circulation as well, a coating containing an implant-absorptive peptide domain containing D-amino acids is envisioned to determine if increases in stability can be obtained. We hypothesize, and are currently investigating the possibility, that by switching the handedness of the residues in the FFSFFFPASAWGSSG portion of the peptide, the coating will still readily adsorb but have increased stability against endogenous proteases. We can also easily vary the material domain used with our peptide coating beyond PS, as the coatings are modular. We have previously identified peptides via phage display that adsorb strongly to a variety of materials such as titanium, stainless steel, and poly(glycolic acid), among others. This modularity in the selection of the peptide components, the ease-of-application and tethering, as well as the active delivery method holds promise for the creation of new drug-eluting stents and localized drug-delivery coatings for a range of biomaterials.

Experimental Section

Peptide synthesis: Peptides were commercially synthesized by solid-phase peptide synthesis techniques. The resultant peptides were purified to at least 95% purity and included HPLC and MS analysis. The biotinylated peptides for affinity constant calculation were synthesized using a C-terminal long-chain biotin (biotin conjugated to aminocaproic acid) attached through the ε-NH_2 group of a lysine residue.

Base sequence digestion: The wells of a PS plate were treated for 1 h with a 1% nonfat milk solution. The wells were washed, and FFSFFFPASAWGSSGSSGK-(biotin) **1** (0.1 mg mL^{-1}) was added for 2 h at room temperature. The plates were washed, and a series of 17 logarithmically spaced enzyme concentrations (in U mL^{-1}) were added in triplicate along with controls of no enzyme and no peptide for 2 h at 37 °C. After 2 h, the wells were washed 3× with Dulbecco's phosphate buffered saline (DPBS), and streptavidin–alkaline phosphatase (SA–AP; USB Corporation, Cleveland, OH, USA) in DPBS (1:500 dilution) was added for 30 min at room temperature. After an additional wash, the chromogenic agent *p*-nitrophenyl phosphate (pNPP) (Sigma, St. Louis, MO, USA) was added to the wells, and the results were read after 3 min at 405 nm on a plate reader (AD340C; Beckman Coulter, Fullerton, CA, USA). The results were normalized so that the wells without enzyme were noted as 100% OD, and those without the peptide were represented as 0% OD. All enzyme activity units were estimated from the ranges given in the manufacturer's literature. Trypsin was purchased from the American Type Culture Collection (Manassas, VA, USA); papain, thrombin, and chymotrypsin were purchased from Sigma (St. Louis, MO, USA), and factor Xa and enterokinase were purchased from New England Biolabs (Ipswich, MA, USA).

Aliquots of a solution of **3** (0.1 mg mL^{-1}) in nanopure water were taken and added to trypsin, thrombin, factor Xa, and enterokinase at equal enzyme concentrations (100 U mL^{-1}). After 48 h on a 37 °C heat block, aliquots (2 μL) were removed from the mixture and combined with an α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (20 μL). The MALDI matrix was made by dissolving CHCA (Fluka, St. Louis, MO, USA) in acetonitrile (500 μL) and water containing 0.1% trifluoroacetic acid (500 μL). The samples were then spotted on a plate and analyzed using a Shimadzu AXIMA CFR MALDI-ToF mass spectrometer. Spectra were then examined for their peak values.

Affinity constant calculation: The wells of a PS plate were treated for 1 h with a 1% nonfat milk mixture. The wells were washed 3× with a solution of DPBS–Tween 20 (0.5%) (Sigma, St. Louis, MO, USA), followed by the addition of triplicate logarithmic dilutions of peptides **1** and **2** in PBS into the wells of a PS plate. The wells were washed again 3× with PBS–Tween 20 (0.5%) followed by 30 min treatment with SA–AP in PBS (1:500 dilution). After a through wash with PBS–Tween 20 (0.5%), pNPP tablets were dissolved in buffered saline and added to all the wells. The absorbance of each dilution was measured using a plate reader at 405 nm, and the absorbance versus log concentration was plotted to yield a sigmoidal binding curve. A four-parameter sigmoidal equation was fit to each curve using a custom-written MATLAB (MathWorks, Natick, MA, USA) routine, and the inverse of the concentration at the half-point of the sigmoidal model curve was extracted as the apparent affinity constant.

MALDI digest: The manufactured peptide containing the enterokinase DDDDK recognition sequence (0.1 mg mL^{-1}) was dissolved in nanopure water. Water was used as the solvent in these studies because MALDI analysis is sensitive to the presence of salts. Enterokinase (100 U mL^{-1}) was added, the peptide was heated at 37 °C for 2 h, and a small aliquot (2 μL) was removed and added to the CHCA matrix solution (20 μL) for MALDI analysis. Appropriate controls containing no enzyme were also run in parallel. The spectra were then analyzed, and the reported peptide weights are shown in Table S1. The peptide performed as expected.

Enzyme kinetics: Wells of a PS plate were treated with a solution of cleavable RGD-terminated peptide **2** (0.1 mg mL^{-1}) in nanopure water for 2 h at room temperature. The wells were then washed 3× with nanopure water, and varied amounts of enzyme in water were then added for 24 h and incubated at 37 °C in triplicate. After 24 h, the supernatant was removed and set aside for later MALDI analysis. The wells were then washed 3× with DPBS and blocked for 1 h with a 1% nonfat milk solution at room temperature. The plate was again washed, and SA–AP:DPBS (1:500) was added for 30 min, followed by a final 3× DPBS wash and the addition of pNPP. The results were then read on a plate reader at 405 nm. An aliquot of the supernatant (2 μL) was removed and mixed with the CHCA matrix solution (20 μL), and FFSFFFPASAWGSSGSSGRGD **3** (20 ng), which served as the internal standard. After collection of the MALDI spectra, the results were analyzed, and the integrations of the unknown peaks were then normalized to the integration of the internal standard. The signal-to-noise ratio of the MALDI system prevented reliable detection of concentrations below 0.1 μg mL^{-1}.

A similar experiment was conducted using the identical protocol as above, however a constant concentration of the enzyme (0, 10,

or 31.6 U mL^{-1}) was used, and the time of digestion was varied (0, 6, 48, and 72 h), with each time point in triplicate. The 48- and 72-hour time points had fresh enzyme added every 24 h, as previous experiments had suggested loss of enzyme activity after longer time periods.

Acknowledgements

M.W.G. and D.J.K. thank the NIH for funding (EB-000501). We thank the team at Affinergy Inc. for useful discussions (MWG and DJK are co-founders of Affinergy Inc.).

Keywords: adsorption · biomimetic device modification · drug delivery · enzymes · peptides

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Received: July 3, 2008
Revised: July 25, 2008
Published online on September 15, 2008

