Peptide Therapeutics

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The Plasma Membrane as a Reservoir, Protective Shield, and Light-Triggered Launch Pad for Peptide Therapeutics

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Abstract: *Although peptide-based therapeutics are finding increasing application in the clinic, extensive structural modification is typically required to prevent their rapid degradation by proteases in the blood. We have evaluated the ability of erythrocytes to serve as reservoirs, protective shields (against proteases), and light-triggered launch pads for peptides. We designed lipidated peptides that are anchored to the surface of red blood cells, which furnishes a protease-resistant environment. A photocleavable moiety is inserted between the lipid anchor and the peptide backbone, thereby enabling light-triggered peptide release from erythrocytes. We have shown that a cell-permeable peptide, a hormone (melanocyte stimulating hormone), and a blood-clotting agent can be anchored to erythrocytes, protected from proteases, and photolytically released to create the desired biological effect.*

Peptides enjoy a number of favorable attributes as drug candidates, including high potency, selectivity for their biochemical targets, and a modular framework that makes them amenable to library screening and structure–activity relationship (SAR) studies.[1] However, these favorable traits are offset by a limited circulation lifespan due, in large part, to rapid degradation by proteases in the blood plasma. Massive SAR optimization efforts, along with newer approaches (stapling and cyclization),[2] have been required to address the concerns of peptide bioavailability and stability.[1,3]

Can human cells be used as carriers of peptide therapeutics? Live cells have garnered intense interest as therapeutic agents, including cancer-seeking/killing T cells,[4] erythrocytes,[5a] and stem cells.[5b] Patient-derived cell-based therapeutics display a number of potentially useful properties, including relatively long in vivo persistence, evasion of immune surveillance, and access to specific tissues depending on cell type. These properties could potentially endow "hitchhiking" peptide drugs with analogous traits, but only if the peptide guest can be simultaneously protected from proteolytic degradation. Proteases are ubiquitous, inhabiting the intracellular environment of cells, the extracellular matrix of tissues, and the blood stream. Assuming that a protease-safe zone on the cell carrier can be identified, once the cell has reached the intended in vivo destination, the peptide must be released from the carrier. We have previously shown that fluorescent probes can be anchored to intracellular biological membranes and are rapidly released into the cytoplasm upon photolysis.[6] In this regard, the outer leaflet of the plasma membrane offers a number of promising features as a reservoir for peptide therapeutics. First, since the majority of peptides are not cell permeable, addition to and release from the plasma membrane sidesteps the issue of cell permeability. Second, the cell surface is covered by a glycoprotein/polysaccharide coat (glycocalyx) that could serve as a protective shield against extracellular proteases for peptides moored on the cell surface. Indeed, peptide carriers that lack a glycoprotein sheath (e.g., liposomes), do not protect peptides from their enzyme targets (e.g., protein kinases).[6] We chose erythrocytes as the carrier because of their long circulatory lifetime (up to 4 months), their easy acquisition from and introduction into patients, and their circulation throughout all regions of the body.[5a] The design of membrane-anchored photoreleasable peptides is illustrated in Figure 1. Lipidation should anchor the peptide to the cell surface and therefore under the protective umbrella of the glycocalyx, whereas insertion of a light-sensitive cleavage site between the lipid anchor and the peptide moiety should furnish the desired on-command release of the peptide from the cell carrier.

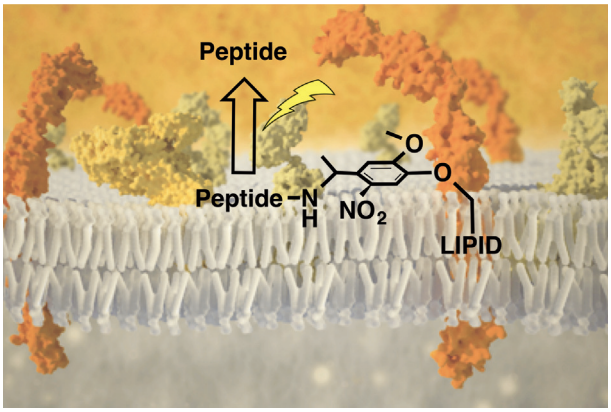

Figure 1. A lipidated photocleavable peptide anchored to the surface of a cell carrier and positioned within the protective shell of the glycoprotein/polysaccharide coat. Illumination at the appropriate wavelength releases the peptide, which is now able to act upon its biological target. Image used under license from Shutterstock.com

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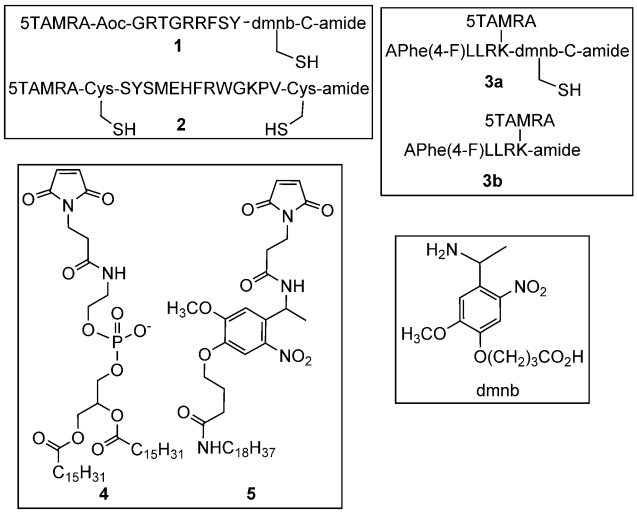
Our initial studies focused on 5TAMRA-Aoc-GRTGRRFSY-amide (where 5TAMRA = 5-carboxytetramethylrhodamine and Aoc = amino octanoic acid; Scheme 1), a fluorescent cell-permeable peptide (Figure S1



Scheme 1. Structures of the peptide (**1**–**3**) and linker/lipid anchor (**4**, **5**) modules used in this study, along with the photocleavable unit dmnb.

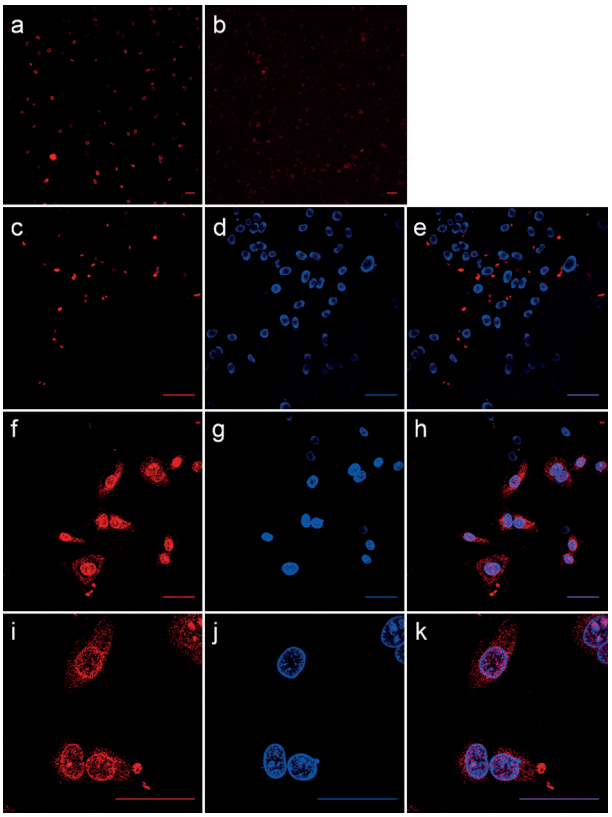


Figure 2. a, b) Erythrocytes/**6** imaged on a confocal microscope at 40x magnification before (a) and after (b) photolysis. c–k) Erythrocytes/**6** were co-cultured with HeLa cells in 1% serum in the dark (c–e) and exposed to 360 nm light (f–h, i–k). Hoechst nuclear stain was used to differentiate HeLa cells from erythrocytes. Fluorescence of **6** is shown in red (left), Hoechst stain in blue (center), and an overlay in purple (right). c–e) HeLa co-culture without photolysis. f–h) HeLa co-culture with photolysis; 40x magnification. i–k) HeLa co-culture with photolysis; 100x magnification. Scale bars = 50 μm.

in the Supporting Information). Analogues of the peptide were prepared that contain an *o*-nitrobenzyl light-cleavable amino acid (dmnb) inserted between the fluorescent peptide and a membrane-anchoring lipid moiety. For example, the C-terminal Cys residue in peptide **1** is easily attached to the lipidated maleimide **4** to furnish **6** (Figure 3). A key issue proved to be identifying a lipid moiety that stably anchors the peptide to the erythrocyte membrane. We addressed this by co-incubating erythrocytes bearing peptide–lipid conjugates with HeLa cells. Ideally, the fluorescent peptide should be retained on erythrocytes in the absence of photolytic cleavage. By contrast, peptide transfer from erythrocytes to HeLa cell membranes in the dark would suggest insufficient membrane retention. Indeed, although attachment of a C18 fatty acid to Aoc-GRTGRRFSY-amide furnishes a conjugate that readily binds to erythrocytes, this lipid–peptide migrates from erythrocytes to HeLa cells in the dark (Figure S2 in the Supporting Information). However, peptide–lipid conjugate **6**, derived from the reaction of **1** with the dipalmitoyl derivative **4**, is stably maintained on erythrocytes upon dark incubation with HeLa cells (Figure 2)

Peptide–lipid conjugate **6** was anchored to the surface of hemoglobin-depleted erythrocytes through brief incubation, followed by centrifugation and washing of the erythrocyte pellet. Approximately 2×10^5–2×10^6 molecules of **6** are bound per erythrocyte (see the Supporting Information). Erythrocytes containing **6** were separately exposed to trypsin and chymotrypsin. In both cases, erythrocytes afford nearly complete protection of the peptide against proteolysis (Figure 3). By contrast, the non-lipidated analogue of **6**, 5TAMRA-Aoc-GRTGRRFSY-amide (Figure 3), as well as **6** in the absence of erythrocytes (Figure S4), are digested by both proteases. Erythrocytes containing **6** were also co-incubated with HeLa cells. Illumination at 360 nm triggers the anticipated migration of 5TAMRA fluorescence from erythrocytes to the adherent HeLa cells (Figure 2 f–k), which is consistent with the photolytic conversion of **6** into the cell permeable 5TAMRA-Aoc-GRTGRRFSY-amide (Figure S3).

The melanocortins (MCs), such as α-melanocyte stimulating hormone (αMSH), elicit an array of biological effects, including pronounced anti-inflammatory activity.[7] Unlike currently employed therapeutics, the MCs do not abolish the immune response but rather modulate it, and thus have been described as having the "sledgehammer properties of a steroid, but without the side effects".[7a] However, the half-life of αMSH is only a few minutes due to the action of serum proteases.[8] Given the length of αMSH (where MSH = SYSMEHFRWGKPV), we were concerned that a single anchor (as in **8**; Figure 4) might not hold the entire peptide close enough to the cell surface to completely protect it from proteolysis. Consequently, we prepared both the double-lipid-anchored conjugate **7** (fluorophore-Cys-MSH-Cys-amide **2** + lipid **5**) and its single-anchored counterpart **8** (fluorophore-

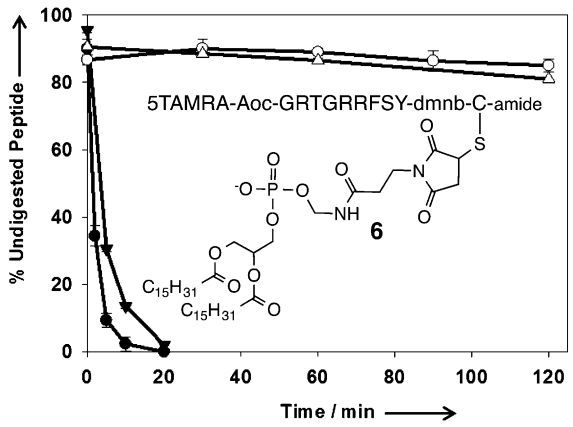


Figure 3. Erythrocytes were loaded with **6** (○, △) or exposed to a non-lipidated analogue (5TAMRA-Aoc-GRTGRRFSY-amide; ●, ▼). These mixtures were separately incubated with 5 nM trypsin (△, ▼) or chymotrypsin (○, ●). **6** anchored to erythrocytes is protected from proteolysis, but 5TAMRA-Aoc-GRTGRRFSY-amide is degraded by both proteases.

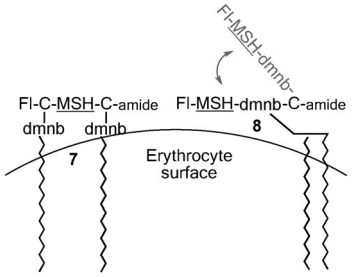


Figure 4. Peptide **7** contains lipids (**5**) positioned at both the N and C termini, which doubly anchors the MSH sequence to the cell membrane. Peptide **8** is singly anchored by a dipalmitoyl moiety (**4**) and may therefore display significant vertical mobility, thus potentially exposing the 13 amino acid MSH sequence to proteases. Fl = fluorophore.

MSH-dmnb-Cys-amide + **4**; Figure 4). As a control, we synthesized a non-lipidated MSH peptide, in which the two free Cys residues in **2** are modified with *N*-ethylmaleimide (fluorophore-C(NEM)-MSH-C(NEM)-amide).

As expected, non-lipidated fluorophore-C(NEM)-MSH-C(NEM)-amide is digested within minutes by both trypsin and chymotrypsin, whereas the erythrocyte-embedded double-lipidated peptide **7** remains fully intact after 2 h of incubation with the proteases (Figures S8, S9). By contrast, lipid–peptide conjugate **8** displays intermediate behavior, with approximately half of the peptide hydrolyzed after 1 h of incubation (Figure S10). Although it is tempting to ascribe this proteolytic sensitivity of **8** to the model illustrated in Figure 4, future studies will need to fully assess the effect of peptide length on proteolytic resistance. However, it is reassuring that two strategically positioned anchors offer full protection from proteolysis in the event that a single anchor is insufficient.

The action of MCs is mediated through the melanocortin receptors (MCRs), of which there are five. We chose to work with the MC1R receptor because of its selectivity for αMSH.[9] The fluorescent peptide **2** stains the MC1R protein expressed on the surface of HEK293T cells in the expected fashion (Figure 5). Two control experiments were performed to validate the selectivity of **2** for MC1R: 1) HEK293K cells

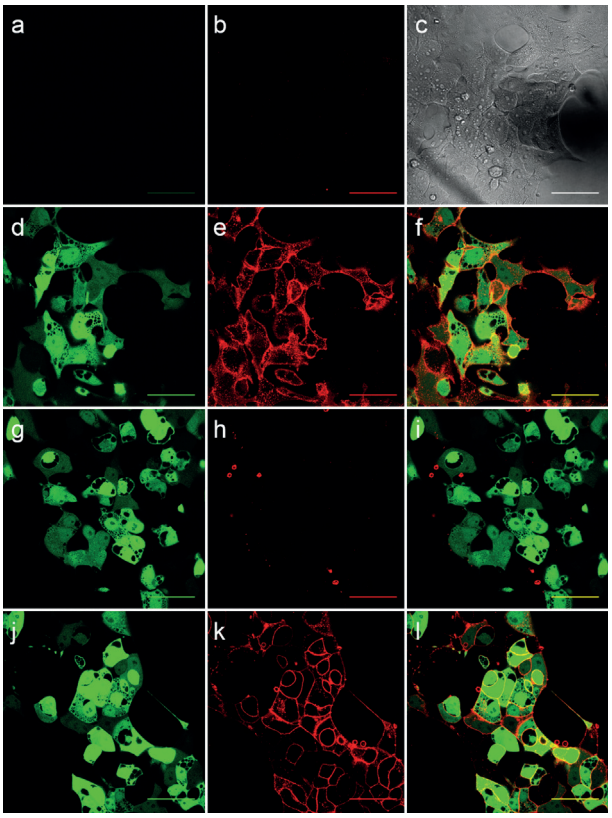
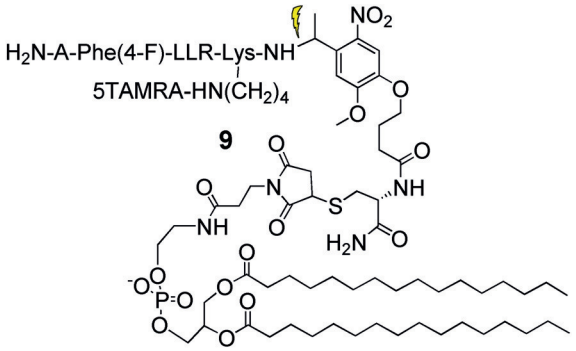


Figure 5. Binding of MSH peptides (red) to HEK293T cells co-expressing MC1R and enhanced green fluorescent protein (green). a–c) Non-transfected cells exposed to **2**. d–l) MC1R/EGFP-transfected cells exposed to **2** (d–f), erythrocytes/**7** without photolysis (g–i), or erythrocytes/**7** with photolysis (j–l). The left-hand panels show EGFP, the middle panels show fluorescent peptide, and the right-hand panels show an overlay. Scale bars = 50 μm.

not expressing MC1R were exposed to peptide **2**, and 2) HEK293K cells expressing MC1R were pretreated with non-fluorescent MSH and subsequently exposed to **2**. Under both conditions, no red fluorescence is associated with the HEK293K cells, thus implying a specific interaction between peptide **2** and MC1R (Figure S11). Erythrocytes loaded with peptide **7** were subsequently incubated with the HEK293Ks and, in the dark, the erythrocytes retained construct **7** (Figure 5). By contrast, upon illumination at 360 nm, the MC1R on HEK293Ks was labeled, thus demonstrating migration of the photolyzed product (Figure 5 and Figure S5) to the adherent HEK293s.

Blood clotting is mediated by platelet adhesion and aggregation, processes triggered by a multitude of surface receptors including protease-activated receptors (PARs). PARs are activated by pro-coagulant proteases (e.g., thrombin), the action of which exposes an N-terminal sequence on the PAR, which serves as an intramolecular ligand that

activates the receptor. Free peptide analogues of the N-terminal PAR sequence likewise act as PAR agonists, triggering platelet shape change and aggregation.[10] We constructed compound **9** (**3a** + **4**; Scheme 2), an analogue of a potent peptidic PAR agonist,[11] to create modified erythrocytes that promote aggregation upon exposure to light. When **9** is anchored to erythrocytes, it is nearly completely protected from proteolytic attack by trypsin or chymotrypsin, even after 2 h of incubation. By contrast, **3b**, a non-lipidated analogue of **9**, is digested within minutes under identical conditions (Figures S11, S12).



Scheme 2. Structure of the lipidated PAR agonist analogue **9**.

Aggregation assays were performed with platelets isolated from human volunteers and PAR-ligand-induced platelet aggregation was quantified through light transmission aggregometry (Figure 6).[12] In addition, aggregation is visually apparent since, as platelets begin to aggregate, there are fewer (albeit larger) particles and the solution becomes less turbid (Figure 6, insert). Illuminated erythrocytes/**9** (blue trace) induce the rapid (2 min) aggregation of platelets

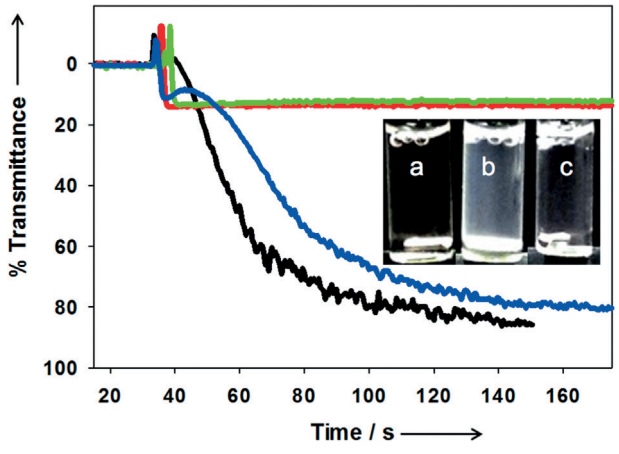


Figure 6. Representative kinetics traces for platelet aggregation upon treatment with non-lipidated PAR agonist **3b** (black), illuminated erythrocytes/**9** (blue), erythrocytes without **9** (red), or non-illuminated erythrocytes/**9** (green). Insert: Platelet aggregation in the presence of **3b** (a), non-illuminated erythrocytes/**9** (b), and illuminated erythrocytes/**9** (c).

(67.7 ± 3.8 %), an effect similar to that produced by the non-lipidated PAR agonist **3b** (black trace; 87.0 ± 7.0 %; Figure 6 and Figure S14). The moderately lower final transmittance of the former sample is likely due to the presence of erythrocytes. Finally, non-illuminated (green trace) samples of erythrocytes/**9** fail to induce any detectable platelet aggregation. The sensitivity of light-induced aggregation is notable since the erythrocyte hematocrit employed is 12.5 %, which is one quarter of the hematocrit of human blood.[13]

In conclusion, we have used erythrocytes as a reservoir for lipidated therapeutic peptides. The erythrocyte surface offers protection from proteolytic degradation while serving as a light-triggered launching pad. The photorelease of biologically potent proteolytically sensitive peptides recapitulates the endogenous behavior of natural peptide hormones (e.g., MSH). The powerful biological activity of hormones is elicited where and when needed, but activity is rapidly curtailed by subsequent proteolysis. We are currently extending these studies to other cell carriers, additional therapeutically active peptides, and at longer wavelengths for deeper tissue penetration.[14]

Keywords: targeted delivery · peptides · peptide drugs · photochemistry · proteolysis

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