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Cloaking cytolytic peptides for liposome-based detection and potential drug delivery

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Abstract

Potent cytolytic peptides with specific tethering and cloaking sites have been synthesised and used to release payload from liposomes in a quantitative manner. A functionally located cloaking site has been modified specifically by simple conjugation without adversely affecting the cytolytic properties of the peptide. The cytolytic activity of modified peptides was then efficiently (>98%) cloaked and uncloaked by ligand–protein or hapten–antibody interactions. The principle of a dual response peptide has been demonstrated using an avidin-cloaked pH-sensitive peptide. Biospecific cloaking/uncloaking provided a new sensitive (~12 pmol) homogeneous diagnostic and also appears potentially suited to bioresponsively targeted release of antimicrobial, anticancer and other drugs now delivered using liposomes.

Keywords: Cytolytic; Peptide; Liposome; Release; Drug; Analyte

1. Introduction

Potent cytolytic peptides are found widely from insects to mammals, particularly as antimicrobial peptides or defensins [1], where they are involved as venoms and in innate defence at mucosal membranes [2] and as cytolysins in lymphocytes [3]. In order to target and localise their otherwise catholic action, their biological delivery is tightly controlled at the cellular and molecular levels (e.g., activating synthesis, release from lysosomes, cleavage of propeptides). While inactive peptides such as prodefensins have now been cloned, proteolytic cleavage provides limited specificity. It has not proved possible to exploit directly the more sophisticated mechanisms available within cells. Mimics of such smart biological behaviour may provide new directions for targeted therapy and sensitive diagnostics, but despite extensive study of the structural basis of action of some cytolytic peptides [4,5], this has proved difficult and has limited their exploitation. In natural cytolytic peptide sequences the potential to adopt an amphipathic α-helical conformation and the presence of net positive charge are prominent features required to permeabilise membranes, while a number of other factors, including affinity for the phospholipids, ability to penetrate the lipid acyl chain region and the actual orientation within the bilayer, may influence the actual mode of action. Additional interactions come into play when phospholipid-associated peptides self-associate to produce larger aggregates resulting in pore formation. The precise details of mechanism are unclear, even for the most extensively studied peptide, melittin, as uncertainties remain concerning its membrane bound aggregation state [6]. Typically, as in the case of melittin, the positively charged end of the peptide makes an electrostatic contact with the phospholipid resulting in the amphiphilic alpha helical conformation embedding into the

Abbreviations: BOP, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, *N*,*N* dimethylformamide; DMSO, dimethylsulfoxide; DIPEA, N,*N*-diisopropylethylamine; HF, hydrogen fluoride; HOBT, hydroxybenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; Myr, myristoyl; PETN, pentaerythritol tetranitrate; TNP, 2,4,6-trinitrophenyl; TNT, 2,4,6-trinitrotoluene; TNBS, 2,4,6-trinitobenzene sulfonic acid

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lipid. This amphiphilic structure, with its flexible hinge in the central part of the molecule and unordered C-terminal end, has axis parallel to the plane of the membrane with polar face towards the lipid head group and hydrophobic face somewhat residing into the bilayer. In one model, this alone leads to lysis by disruption of the phospholipid structure, while in another model aqueous channels are formed by the peptide chains associating into oligomers that span the membrane in perpendicular orientation (Fig. 1). Liposomes have been more widely used in drug delivery [7,8] than diagnostic applications [9,10] or as in vivo imaging agents [11]. While there has been extensive study of lipid formulations [12], attempts to accomplish controlled and quantitative release of payload in response to biospecific triggers have been limited. Liposomes have been considered for homogenous immunoassay using complement-mediated lysis but such assays proved unreliable as any one of the many labile components of complement could become inactivated [10,12,13]. Homogeneous liposomal immunoassay using nonspecifically labelled melittin as the lytic agent was reported as an alternative to the complement assay [14–16]. However, the solubility of melittin–hapten conjugates and the preservation of their lytic activity posed problems that restricted consideration largely to digoxin assay [12,17–19]. Lysyl, found in the cationic site, critical for lytic activity [4,5,20], are the key residues affected by hapten conjugation. Owing to such difficulties of using natural peptides, others have used conjugates with the larger enzymic cytolysin, phospholipase C, nonspecifically labelled with analytes [19,21]. While such conjugates had superior solubility and greater retention of activity after modification, only 75% to 85% of their activity was specifically inhibited in the presence of antiserum.

Broad-ranging analyte-specific release of marker molecules with insignificant nonspecific background leakage has not been achieved by either approach. Consequently, only the heterogeneous liposomal assay configurations have approached the necessary sensitivity and reliability [22]. Time-resolved fluorimetry has improved the nonspecific background and sensitivity problems to some extent by encapsulating a larger molecular weight protein chelator conjugate in liposomes, providing more specific fluorescent detection upon cytolysin-mediated complexation with ions such as Eu^{3+} [17]. However, nonspecific lysis by uninhibited conjugates presented a variable nonspecific background, which, combined with the reduced activity of conjugates, would undermine the sensitivity and reliability of such assays.

In order to overcome these problems and to develop a simple to use and self-contained system, we report the design of potent unnatural cytolytic peptides, which can be cloaked effectively (to provide stable insignificant activity) and activated quantitatively, including by co-activation of cloaked peptides using both pH and ligand binding reactions. Structural and covalent modifications at such a cloaking site would prevent the cytolytic action of the peptides on biomembranes by one or more mechanisms (Fig. 1), including: pH and redox titration, ligand and steric hindrance (e.g., by antibodies, avidin). When this is reversed by back titration of the ionisable groups, and/or by release of the bound protein or enzymatic cleavage of the cloaking moiety, biomembranes would be permeabilised to both small and large molecules. The requirement for more than one biospecific response to activate such peptides would be expected to improve the fidelity or specificity of the process. In principle, any combination could be used to permeabilise cells or to control the release of diagnostic or therapeutic payloads from liposomes. Such biospecific release may be particularly suited to antimicrobial, anticancer and the increasing range of other drugs now delivered in liposomes.

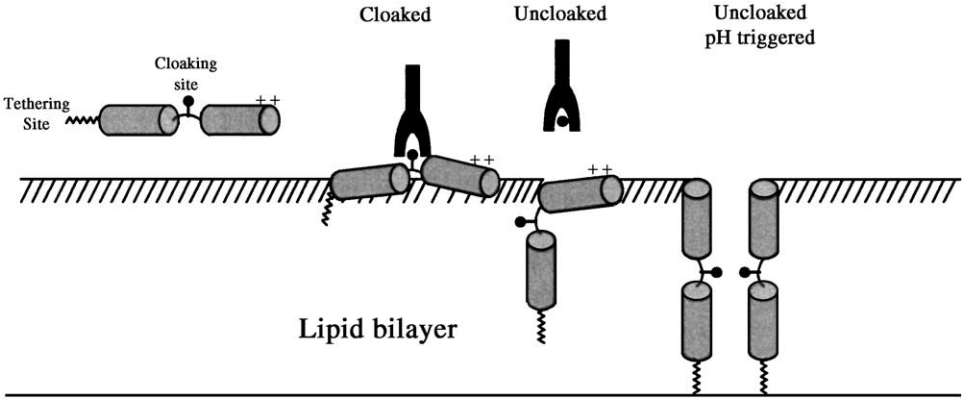


Fig. 1. Schematic illustration of the concept of synthetic cloaked peptides. Integration of peptide assemblies into lipid bilayer is effected by the introduction of an uncharged N terminus forming a tethering site. The cloaking site is regiospecifically located at a position, near the central region of the peptide, most likely to affect its function quantitatively by affinity binding reactions. A line has been drawn to show the polar–apolar interface. Mechanisms involved in activating such peptides could include steric, pH, redox and enzymatic cleavage reactions or a combination for multiple-activation with the result that dissociation of the cloaking molecule(s) frees and presence of activation conditions enables the cytolytic peptide to breach the membrane.

2. Materials and methods

2.1. Peptide synthesis

Peptides (Table 1) were manually synthesised by solid phase t-Boc chemistry using 0.5 mmol of *p*-methylbenzhydrylamine (MBHA) resin. Amino acid (BACHEM, UK) side chain protections were 2-chlorobenzyloxycarbonyl for lysine; *p*-toluenesulfonyl for arginine; and benzyl for threonine, serine and glutamic acid. Couplings were conducted over 40 min using 1.5 mmol of amino acid, 1.5 mmol of BOP and 4.5 mmol of DIPEA in *N*,*N* dimethylformamide (DMF). Repeat couplings were used, when necessary, to drive the reaction almost to completion (>99.8%). *N*-acetylation was carried out over 30 min using acetic anhydride (0.5 M) and pyridine (0.5 M) in DMF. Coupling of 2,4,6-trinitrophenyl (TNP) to the N terminus was achieved by treatment for 14 h with a 10-fold molar excess of 2,4,6-trinitobenzene sulfonic acid (TNBS) in DMF. Myristic acid was coupled in the same manner as an amino acid. At the end of synthesis, peptides were cleaved from the resin using hydrogen fluoride (HF) in the presence of 0.5 g *p*-thiocresol and 0.75 g *p*-cresol as scavengers. The peptides were purified on a C-4 reverse phase semi-preparative column (Vydac C-4, 250 × 4.6 mm) using an acetonitrile/0.1% TFA gradient. The purity of the peptides was estimated by analytical reverse phase high performance liquid chromatography (HPLC). No peptides below the 95% purity level were used. Peptides were characterised using electrospray or MALDI mass spectrometry. All methods were performed at room temperature.

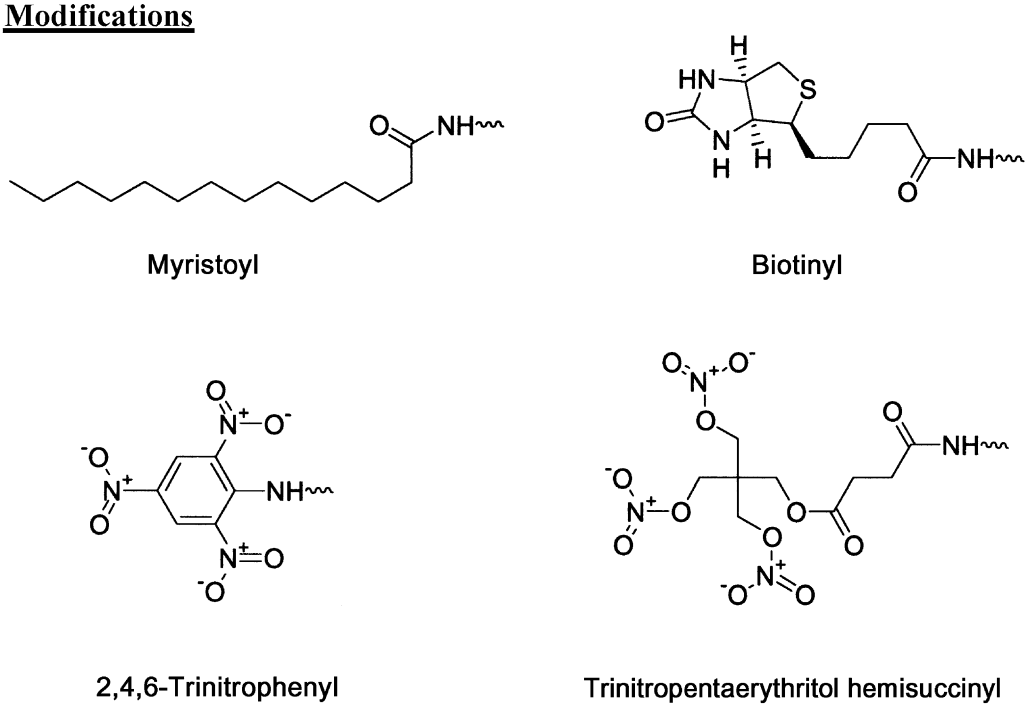
2.2. Chemically modified peptides

For biotinylation, peptides (20 μmol) were dissolved in 4 ml DMF, when biotin *N*-hydroxysuccinimide (100 μmol), followed by DIPEA (300 μmol), were added and reaction allowed to proceed with stirring for 3 h. TNP modification was achieved by dissolving peptide (20 μmol) in 4 ml of DMF containing TNBS (2 mmol) and stirring for 14 h. Generic peptide (5.3 μmol) to be modified with pentaerythritol tetranitrate (PETN) analogue (trinitropentaerythritol hemisuccinyl) was added to a total volume of 2.3 ml in DMF/dimethylsulfoxide (DMSO) (65:35 v/v ratio, respec-

Table 1
Primary sequences of synthetic peptides and structural modifications

Peptide	Sequence
(1) Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
(2) Generic	Acetyl-GIGAVLRVLTTGKPALISWIRRRRQQ-NH_2
(3) M-generic	Myr-GIGAVLRVLTTGKPALISWIRRRRQQ-NH_2
(4) pH hybrid	Myr-EAALAEALAEALAEGKPALISWIRRRLQQ-NH_2
(5) M16	Myr-LLRLLLKLLLLRLLR-NH_2

Peptides analogues were specifically modified on single Lys ε-amino located near the centre of the sequence, with the exception of natural melittin which has multiple labelling sites including the N-terminal amino.



tively) containing 3 equivalents of each of the following: trinitropentaerythritol hemisuccinate [23], hydroxybenzotriazole (HOBT) and diisopropylcarbodiimide (DIC) (Sigma). In all of these preparations, reactions were allowed to proceed to completion, as monitored by a decline of amine content using ninhydrin assays. The biotin and TNP reactions reached completion overnight, while the PETN analogue reaction proceeded to completion after 1 h. Residual solvent from the reaction mixtures was then removed under vacuum and the product was purified by reverse phase HPLC on a C-4 preparative column using acetonitrile and 0.1% TFA gradients. Peptides were characterised by mass spectrometry as above. All methods were performed at room temperature.

2.3. Liposomes

Liposomes encapsulating 120 mM calcein were prepared by extrusion (Avestin Liposofast 100) at room temperature using 2:1 molar ratio of phosphatidylcholine and cholesterol passed 10 times through 0.2-μm polycarbonate filters (Nucleopore). The nonencapsulated dye was removed by gel filtration, through a PD-10 column (Pharmacia), using iso-osmotic eluting buffer. The total lipid concentration of the preparation was measured by the Stewart assay and adjusted to 3 mg/ml. The average liposome diameter (typically 175 nm) and polydispersity (typically 0.07) was estimated by photon correlation spectroscopy (Malvern 4700).

2.4. Cloaking/uncloaking assays

Peptides were prepared at concentrations ranging from 1 to 0.01 mg/ml, depending upon the activity of the particular peptide. Melittin, nonspecifically labelled with TNP, was only sparingly soluble in water and was prepared in 25% (v/v) DMSO in water. Avidin (5 units per ml; 1 unit of avidin binding 1 μg of biotin) was prepared in PBS pH 7.4 buffer. Biotin, PETN and 2,4,6-trinitrotoluene (TNT) solutions (10 mg/ml in DMSO) were diluted using water to obtain working concentrations of 1 μg/ml. Typically, assays were performed at room temperature in a total volume of 2 ml containing calcein liposomes (5 μM lipid). Calcein release was monitored by fluorescence emission at 520 nm following excitation at 490 nm using a Perkin Elmer LS-50B fluorimeter. Avidin was incubated with biotin for 2 min prior to addition of biotinylated peptide (44 nM), when after further 2 min, liposomes (7 μM lipid) were added. No biotin was added when peptide (2.8 nM) was to be cloaked by a 1.2-fold excess of avidin, which was pre-incubated for a minimum of 3 min. TNT and PETN assays were conducted in a similar manner, replacing biotinylated peptide with trinitropentaerythritol hemisuccinyl and TNP-labelled peptides, and avidin with monoclonal (supplied by Defence Evaluation and Research Agency, Kent, UK) anti-TNT and anti-PETN antibodies [23] and incubating for 5 rather than 2 min. The antibody titre necessary was pre-determined by titration of the peptide concentration with antibody until insignificant (or no further reduction in) peptide activity could be achieved (e.g., for TNT, 30 μl antibody for 7.4 nM peptide; for PETN, 20 μl antibody for 7.7 nM peptide). To evaluate the dual response properties of biotinylated hybrid peptides, calcein liposomes (7 μM lipid) in 2 ml of buffer were treated with peptide (2.8 nM) and the fluorescence monitored as before.

2.5. Nitroavidin–peptide complex

Nitroavidin was prepared and nonmodified sites were blocked with biotin [24]. Biotinylated peptide was incubated with a threefold molar excess of nitroavidin in PBS pH 7.4 for 30 min, when excess biotinylated peptide was exhaustively dialysed against PBS for 24 h (retaining approximately 2 mg protein/ml as estimated by BCA assay, Pierce). As calcein release assays indicated residual contamination from free peptide, nitroavidin–peptide complexes were further purified by gel filtration on a Sephadex G-50 (Pharmacia) column (22×2 cm) equilibrated with PBS pH 7.4 buffer. Early fractions (0.5 ml), with activity in the presence of biotin but little or no activity in its absence, were pooled (recovering approximately 0.4 mg protein/ml as estimated by BCA assay). Biotin (0.4 μmol) displacement of nitroavidin was performed by adding 0.6 ml of the pooled nitroavidin–peptide complex fraction to 2 ml of PBS pH 7 buffer containing calcein liposomes (2 μM lipid). All methods were performed at room temperature.

3. Results

The sequences of peptides and structure of labels conjugated at cloaking sites via the single free ε-amino group are given in Table 1. The calculated and expected mass values were found to agree within 0.8 of a unit, within the accuracy of the mass spectrometer using external calibration. The HPLC purity was above 95%. The conjugates of the peptides were also purified to homogeneity and characterised in the same way with the exception of nonspecifically labelled TNP–melittin, which presents four primary amino groups as potential conjugation sites. Consequently, treatment of this peptide with 2,4,6-trinitobenzenesulfonic acid (TNBS) in solution phase produced a heterogeneous mixture of products with significantly reduced potency and low aqueous solubility. Using a molar extinction coefficient of 10550 cm^{-1} M^{-1} for the ε-amino TNP product [25], the nonspecifically labelled conjugate had on average 3.6 TNP groups per melittin monomer. TNP-labelled melittin exhibited lytic activity, which was many orders of magnitude lower than the parent peptide (Table 2). Due to the low solubility of the peptide, higher concentrations could not be tested; turbidity was apparent and light scattering interfered with fluorescent measurements. In contrast, regiospecific

Table 2
Relative cytolytic activities of synthetic peptides and their modified analogues

Peptide	EC_{50} [nM]
Melittin	22
N-terminal labelled TNP-melittin	16
Nonspecifically labelled TNP-melittin	$>10^3$
Generic	312
M-generic	18
Biotinylated M-generic	29
Biotinylated generic	72

The percentage lysis was measured using various peptide concentrations (ranging from 1 nM to 10 μM) in 2 ml of PBS pH 7.4 containing calcein liposomes at 5 μM lipid concentration. Lysis was calculated by measuring the fluorescent signal 3 min after addition of liposomes and expressing the value as a percentage of that observed upon addition of 0.1% Triton X-100. Replicate studies were within 2% variation. The EC_{50} value represents the peptide concentration required to cause 50% release of signal.

coupling of the TNP group to the N terminus by solid phase methods gave a homogeneous product, whose activity although largely retained (Table 2), could not be diminished significantly in the presence of anti-TNP (Fig. 4). Typically, more than 60% of liposome contents were released by the peptide in the presence of anti-TNP antibody. It did not prove possible to produce any melittin conjugate that retained sufficient activity, which was blocked in the presence of excess antibody. Consequently, various synthetic structures were prepared with a single modification site. The activity of such a 'generic' peptide (Table 2) was about 14-fold lower than melittin. However, this was not such a large loss in activity as when Leucine 13 residue was completely eliminated [26]. When Lysine 13 of the generic peptide was modified with a relatively nonpolar group, to produce biotinylated generic peptide, a significant increase in activity was observed (Table 2). 'M-generic' peptide with a N-terminal myristoyl (Myr) (Table 2) exhibited lytic activity near equivalent to melittin. M-generic peptide also retained adequate lytic activity following biotinylation. Furthermore, its activity was insignificant in the presence of avidin (Fig. 2). In the presence of biotin, however, quantitative recovery of lytic activity was observed (Figs. 2 and 3). In typical assays, 20 pmol of biotin could be rapidly and reliably detected in 2-ml volume (Fig. 3). The potential may exist to increase the assay sensitivity by using liposomes with lower cholesterol content, requiring lower peptide (to lipid ratios) to release liposome contents.

The ability to achieve further specificities was illustrated by coupling trinitropentaerythritol hemisuccinate and TNP to the ε amino group of Lysine 13 of the 'generic and M-generic' peptides (Fig. 3), respectively. In both cases, cloaking of the modified peptides with specific antibodies was almost complete with very little residual or background activity (Fig. 4). In the presence of analyte, however, quantitative recovery of lytic activity proved possible with detection sensitivity in the nM range.

Since in all cases, when using different labels (biotin, TNP and trinitropentaerythritol hemisuccinyl), the peptide remained active against liposomes and the activity could be almost completely switched off in the presence of the relevant binding protein; the same approach was applied to two further cytolytic peptide structures. Again, using avidin binding to biotinylated peptides 4 and 5 (Table 1), efficient cloaking could be achieved with low uncloaked background activity (Fig. 4). Comparing the residual activ-

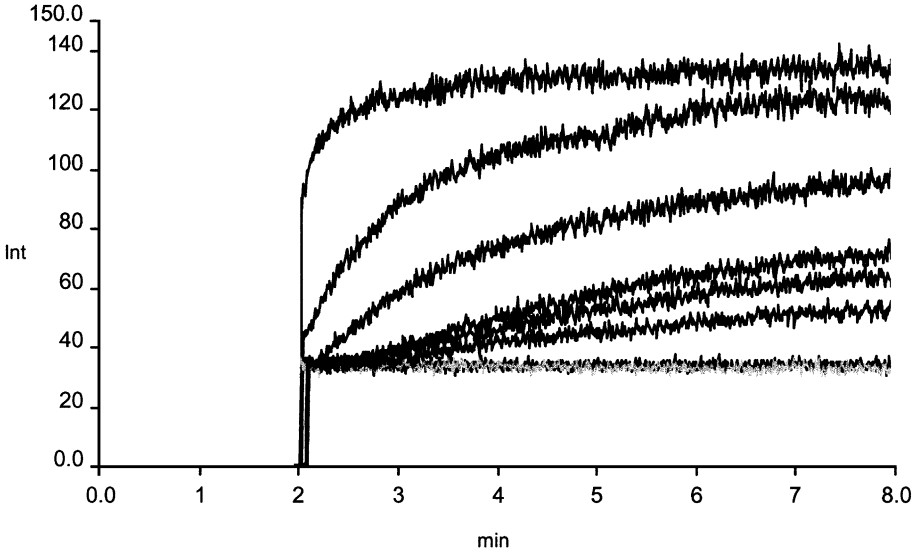


Fig. 2. Cloaking and uncloaking the activity of biotinylated M-generic peptide. Progress of lysis of calcein liposomes (7 μM lipid) upon addition (at approximately 2 min) to 2-ml peptide solution (44 nM) in PBS buffer pH 7.4 containing avidin (1.2-fold excess) with various concentrations of biotin. The top trace represents lysis by peptide while the bottom trace represents background fluorescence in the absence of any peptide. The remaining curves (bottom to top) represent biotin concentrations of 0, 10, 20, 30, 50 and 100 nM.

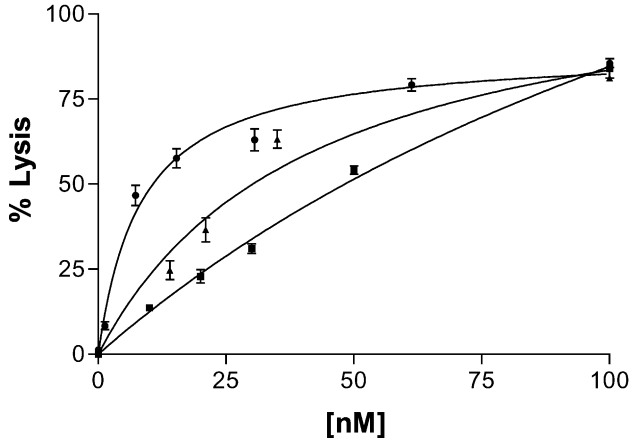


Fig. 3. Response curves for Biotin, TNT and PETN. For biotin curve (■) the data from Fig. 2 was used taking the top and bottom curves to represent 100% and 0% lysis, respectively. The percentage lysis was then calculated 5 min after addition of liposomes. For TNT and PETN the percentage lysis was estimated 3 min after the addition of calcein liposomes (1 μM lipid) to 2-ml PBS buffer pH 7.4 containing various concentrations of TNT (▲) incubated with 30 μl of monoclonal anti-TNT, and M-generic TNP peptide (7.4 nM); PETN (●) incubated with 20 μl of monoclonal anti-PETN and trinitropentaerythritol hemisuccinyl-generic peptide (7.4 nM). Each determination was conducted in triplicate.

ity of all the cloaked peptides in this study (Fig. 4), we note that the uncloaked background activity was generally low (<2% release of liposome contents in most cases) and, more importantly, the small initial release that does occur remained almost unchanged during the course of assays. In contrast, when the natural sequence of melittin, regiospecifically labelled at the N terminal, was used, there was a high level of residual activity, which approached 80% release of liposome contents within 6 min.

In order to develop a single peptide–liposome reagent, it is essential to integrate the cloaked peptide within each liposome particle, while retaining the payload, and demonstrate uncloaking of peptide activity by displacement of the bound protein to release the liposome contents. Nitroavidin has been shown to bind biotin in a reversible manner [24]. Biotinylated peptide 2 (Table 1) was cloaked by incubation with nitroavidin and integrated into liposomes. These nitroavidin-cloaked peptide–liposome complexes remained stable over long incubation times, when rapid uncloaking could be achieved by the addition of biotin (Fig. 5). In this manner, one-step and one-pot assay configurations could be achieved. Competitive reactions could also be considered, particularly when the affinity or immuno-specific trigger is armed by another mechanism (e.g., pH), which would also be expected to improve the practicality of homogeneous assays. A biotinylated pH-sensitive peptide was developed, which was a hybrid of cationic and anionic sequences (peptide 4 in Table 1). This peptide, in its unmodified form, exhibited pH-sensitive activity, depending upon the level (peptide-to-lipid ratio) used. Upon modification with biotin, the peptide retained its activity and pH-responsive properties, preferentially activating at mildly acidic pH values with only slight activity at pH 8 (Fig. 6). While the peptide could be efficiently cloaked upon avidin binding, liposomes could only be triggered to release at acidic pH in the presence of biotin (Fig. 6). The potential would now exist to prepare other dually responsive peptides, requiring both (not either) reactions to activate.

Bioaffinity reactions have proved to be particularly useful in biosensors and diagnostic tests [27]. In the above assays, affinity cloaking was performed competitively in the presence of various levels of analyte, and quantitative release of fluorescent signal from liposomes could be monitored in the presence of a few nanograms of analyte. A simple fluorescent test taking a few minutes to perform was developed to enable signal to be observed with the naked eye. To a glass vial containing 1.1 ml of calcein liposomes (4 μM lipid) in PBS pH 6.2 buffer was added a mixture of 12 pmol biotin, 17 pmol of peptide (peptide 4 in Table 1) and 1.2-fold excess of avidin premixed in 100 μl of PBS pH 8 buffer. The contents were rapidly mixed simply by inverting the vial three times. The vial was then illuminated from below by a 3-mm blue light-emitting diode. Within 5 min of mixing the analyte and reagent, green fluorescence became clearly visible compared to the reagent background signal (without biotin), which had no or negligible intensity that remained constant, allowing

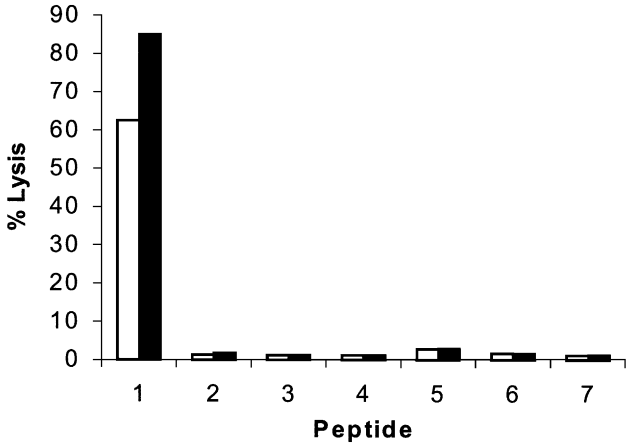


Fig. 4. Residual activity after cloaking of peptides. Liposomes were added to biotin-, TNP- and trinitropentaerythritol hemisuccinyl-labelled peptides pre-incubated with avidin, anti-TNT or anti-PETN antibody (respectively) in 2 ml of PBS buffer pH 7.4. Residual activity was estimated as a percentage of activity relative to uncloaked peptide in the absence of binding proteins at 3 (□) and 6 (■) min after addition of liposomes. In the case of biotinylated peptides, 1.2-fold excess units of avidin were used to block the peptides: (1) N-terminally labelled TNP melittin (7.4 nM) and anti-TNT 30 μl, 1 μM lipid; (2) biotinylated M-generic peptide (50 nM) and 7 μM lipid; (3) TNP-labelled M-generic peptide (7.4 nM), anti-TNT (30 μl) and 1 μM lipid; (4) biotinylated generic peptide (16 nM) and 2.1 μM lipid; (5) biotinylated M16 R peptide (300 nM) and 7 μM lipid; (6) trinitropentaerythritol hemisuccinyl-labelled generic peptide (7.7 nM), 20 μl anti-PETN and 1 μM lipid; (7) biotinylated-hybrid (2.8 nM) peptide and 7 μM lipid.

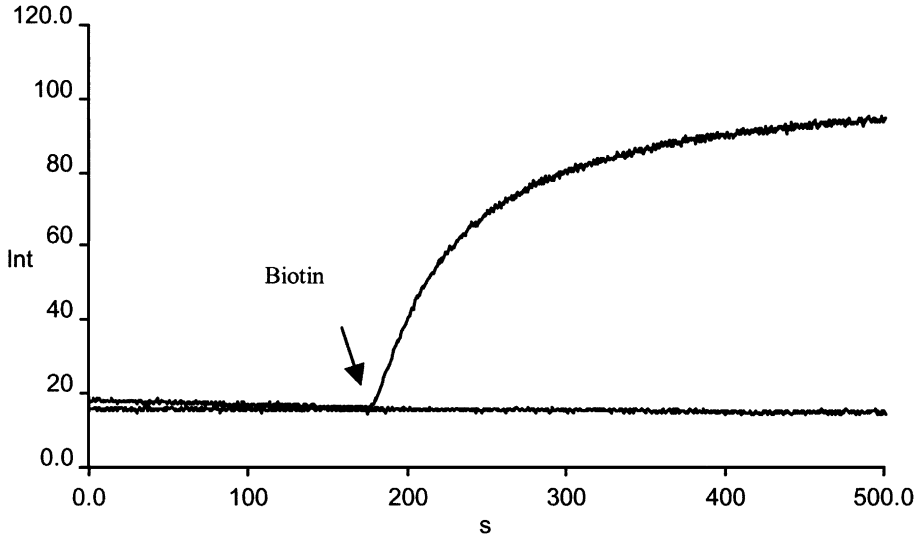


Fig. 5. Uncloaking of peptide by ligand displacement. Nitroavidin–biotinylated generic peptide complex (0.32 mg protein) incubated in 2 ml of PBS containing calcein liposomes (2 μM lipid) was treated with excess biotin (0.4 μmol). The fluorescent lower trace shows the equivalent level of liposomes alone to indicate the background fluorescence.

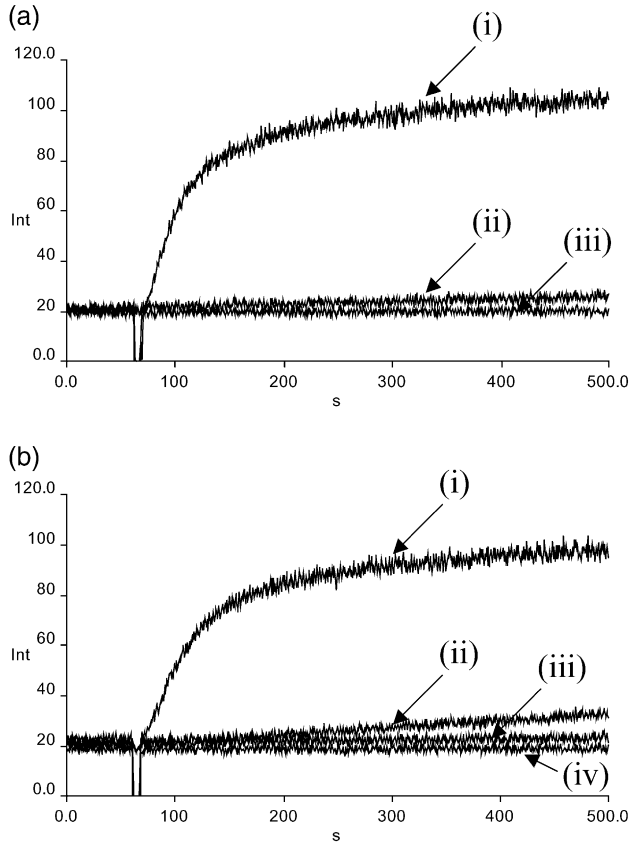


Fig. 6. Suppression of residual cloaked activity with pH. pH-dependent lysis by 2 ml of biotinylated hybrid peptide (2.8 nM) under acidic and alkaline conditions in PBS containing calcein liposomes (7 μM lipid): (a) traces from top to bottom represent (i) peptide at pH 6.6, (ii) peptide at pH 8 and (iii) background signal in the absence of any peptide; (b) traces from top to bottom represent peptide in the presence of (i) 11 pmol of biotin and 1.2-fold excess of avidin at pH 6.6, (ii) 1.2-fold excess of avidin at pH 6.6, (iii) 11 pmol of biotin and 1.2-fold excess of avidin at pH 8 and (iv) 1.2-fold excess of avidin at pH 8.

detection of 12 pmol of biotin rapidly and without any instrumentation.

4. Discussion

Many natural cytolysins are known to comprise the common feature of a cationic site [28] critical for lytic activity [4,5,20]. It is clear that such cytolysins could not be labelled nonspecifically at multiple sites and still retain activity, nor could efficient cloaking at those sites be achieved. Such modifications appear to impose major and variable alterations on the structure and hydrophobicity of the peptides. In the case of nonspecifically TNP-labelled melittin, the lytic activity became poor (Table 2). This appears to agree with earlier studies, particularly that the elimination of positive charge by chemical modification of melittin resulted in an almost total loss of lytic activity for succinylated melittin [29], a melittin analogue whose positively charged Lysine 7 was replaced with a neutral leucine residue [30], permethylated melittin [31] and dimethylated melittin showing 70% loss in activity [32]. The solubility of conjugates produced when melittin is nonspecifically labelled is typically very low, as found here for TNP–melittin. In the case of the earlier digoxin assay, this was overcome by conjugation of a more soluble oubain analogue [16]. Similar observations have been made for other commonly used labels such as 4-nitro-7-cholorobenzofurazan (NBD), fluorescamine and 2-chloro-3,5-dinitrobenzoic acid, where nonspecific labelling even at a 1:1 molar ratio produced insoluble products [33]. Although the nature of the labelling group was different, a similar trend was observed to that shown by Ius et al. [17], who showed that the conjugation of a 2:2 molar ratio of estriol rendered melittin 95% inactive, while at a 1:1 molar ratio, the loss in

activity was 32%. Specific formylation of melittin at its N terminus had little effect on the activity [5], in line with studies here for N-terminally labelled TNP–melittin. Unfortunately, N-terminally labelled melittin could not be efficiently prevented (Fig. 4) from lysing liposomes in the presence of anti-TNP, rendering this site unsuitable for cloaking. As the lytic activity is largely retained, the data does, however, suggest that this part of the molecule may be suitable for tethering the peptide. Fatty acid modification at this position also improved activity and favoured insertion of such peptides into membranes.

The major obstacles associated with use of natural cytolysins can be overcome by engineering generic peptide structures devoid of their natural primary amino groups and provided with specific cloaking and tethering sites. These peptides were designed to meet several criteria. (i) The cationic character was maintained, as it appears critical for activity and solubility. (ii) Multiple primary amino groups were eliminated to allow regiospecific conjugation of molecules by common chemical procedures, post-synthetically, in 1:1 stoichiometric ratio at sites suitable for cloaking and tethering. (iii) The cloaking site was located at a position most likely to render the peptide conjugate active prior to protein binding and insignificantly active when noncovalently bound to larger proteins. (iv) The tethering site was designed to facilitate integration of the cytolytic peptide with biomembranes in the cloaked state and enhance its potency.

In the case of melittin, and indeed many other natural peptides, contradictory mechanisms appear in the literature regarding the orientation of the peptide, with respect to the lipid bilayer, which can be ''wedgelike'' or transbilayer [20,34]. In the wedge model, both termini are located outside the lipid bilayer, while in the transbilayer model the N-terminal inserts. In all cases, however, the central peptide segment is unambiguously shown to reside in the interior of the bilayer, making this the most suitable part of the molecule for cloaking purposes. On the other hand, the relatively hydrophobic nature of the N terminus formed a suitable tethering site where fatty acid could be attached to allow formation of an integrated complex with the bilayer of liposomes [35]. The replacement of lysines by arginines, substitution of Leu 13 by lysine and introduction of Myr residue at the N terminus appeared to fulfil such criteria. Due to its importance to lytic activity and its occupancy near the polar apolar interface [36], Leu 13 appears to be a good choice for sensitive modification of bilayer partitioning of the peptide on protein binding. It is noteworthy though that this residue has been shown to be one of the key residues, along with Trp 19, with respect to lytic activity, indicating that any changes at such positions must be as subtle as possible. The conformation of melittin is often described as helix–bend–helix. To minimally interfere with either of the helices, and to avoid significant alterations in steric effects, the Leu 13 residue was substituted with Lys in the unstructured bend, which incorporates residues 11 to 14 (hinge region) with a 120–160° kink near Threonine 11 and Glycine 12 [36–38]. In the absence of substantial structural changes, the lower activity observed for the generic peptide may simply be a result of introducing a charged residue into an apolar face of the molecule, thereby affecting its overall amphiphilicity. When this residue was modified with a relatively nonpolar group to produce biotinylated generic peptide, a modest increase in activity was observed. Similar modification with other haptens (TNP and trinitropentaerythritol succinyl) resulted in conjugates, which were active and could be inhibited efficiently by antibody binding (Fig. 4) with quantitative recovery of activity in the presence of respective analytes (Fig. 3). For these conjugates, the sensitivity levels were in the nanomolar range (Fig. 3). Further improvements can be expected by increasing lytic activity of peptides, as was the case with M-generic peptide, which detected biotin in the picomole range. Transport of unmodified Lysine 13 across the bilayer without deprotonation would be energetically unfavourable. The free energy gain by fatty acid-assisted partitioning of N terminus in the case of M-generic peptide may be large enough to compensate for the deprotonation of Lysine 13. The binding and insertion of the N-terminal region of melittin appears primarily driven by hydrophobic effects [39]. This may explain the enhanced activity of the M-generic structure, which was equivalent to that of melittin, and the ability to cloak and uncloak the peptide. Despite the high level of inhibition of the biotinylated M-generic peptide activity with avidin and no discernible activity of the cloaked peptide after lengthy incubation with liposomes, its activity was quantitatively and rapidly recovered in the presence of biotin (Fig. 2) by competitive and displacement reactions.

With the dually responsive peptide, both affinity reaction and low pH were necessary to release liposomal contents (Fig. 6). Reliance on any one step, with a small but nonetheless significant level of nonspecific triggering, would result in greater nonspecific release. Particularly in the case of amplified cascade mechanisms or peptide–liposome complexes, the dual response offers potential benefits in both diagnostic and potential drug delivery applications, where low levels of release would be more significant. This approach enabled a simpler assay with well-controlled background. Peptides cloaked in this way may be useful for producing biosensing assemblies to operate at the submicron scale and also for the bioresponsive release of drugs.

In conclusion, the major reason why cytolytic peptides have found very limited applications in controlling the release of liposome contents is because it has not been possible to control and target their activity in a specific and facile manner. Consequently, despite a wide range of studies on their properties and action, application of cytolytic peptides has been limited to topical and disinfection treatments and a liposomal immunoassay—almost forgotten because of its unreliability and restricted specificity. Using peptides specifically designed for cloaking, the range of specificities as well as fidelity of responsive release from

liposomes may be improved and diversified for: (a) sensitive and simple diagnostic tests and rapid screening procedures; (b) in vivo diagnostics and therapeutics, for the active targeting of specific regions or cells (e.g., cancer, micro-organism, cellular immune system); and (c) the bioresponsive delivery of drugs, where their scope for smart materials to mimic biological behaviour has been recently discussed [40]. Particularly by their integration into liposomes, to form stable and responsive sub-micron biosensor assemblies, the biospecific uncloaking of lipopeptide assemblies appears to provide a new direction for in vitro and in vivo diagnostics. Bioresponsive peptide–liposomes are small assemblies, which could be more easily and effectively introduced than any present biosensor concepts to sense locally and operate at the microscopic level. Studies are currently underway to release liposome-encapsulated anticancer drugs in the vicinity of tumours using cloaked peptides.

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