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The rational design and synthesis of a Trp-BODIPY cyclic peptide for the fluorescent labelling of apoptotic bodies is described. Affinity assays, confocal microscopy and flow cytometry analysis confirmed the binding of the peptide to negatively-charged phospholipids associated with apoptosis, and its applicability for the detection and characterisation of subcellular structures released by apoptotic cells.

Apoptosis is a process of programmed cell death taking place after severe cellular damage following specific signalling events. In early stages of apoptosis, molecules of phosphatidylserine (PS) are translocated to the outer face of the plasma membrane. Recent studies provide evidence that apoptotic cells release subcellular structures to the extracellular space in order to communicate with other cells.[1-5] These structures are membranous components that can be classified as exosomes, apoptotic vesicles and apoptotic bodies. Whereas exosomes, which range in size from 20-100 nm, are secreted by multivesicular endosomes upon fusion with the plasma membrane, apoptotic vesicles (100-1000 nm) and apoptotic bodies (> 1000 nm) are expelled directly from the plasma membrane to the extracellular space. Although their precise mechanisms of action remain elusive, these structures play critical roles in many biological processes, such as coagulation, inflammation, tumour progression, cell adhesion or transfer of signalling components and genetic information. For instance, recent studies have validated tumour exosomes as important mediators in defining pre-metastatic niches.[6,7] As a result, subcellular vesicles secreted by apoptotic cells might become valuable biomarkers for monitoring and diagnosing several pathological conditions, which has prompted the development of novel methods for their detection and characterisation.[8] One defining characteristic of apoptotic bodies is the presentation of negatively-charged phospholipids, such as PS, in high amounts. This feature has been exploited to design chemical reagents to monitor apoptosis in cells.[9-11] Annexin V, a 36 kDa protein with high binding affinity to PS, has been widely used to monitor cell death in vitro.[12] However, Annexin V-based labelling is limited by its high molecular weight, the formation of membrane lattices and its dependence on high concentrations of Ca^{2+}, which are incompatible with many physiological environments. We envisaged that the development of probes for labelling apoptotic bodies in a Ca^{2+}-independent manner would represent a significant advancement in the field.

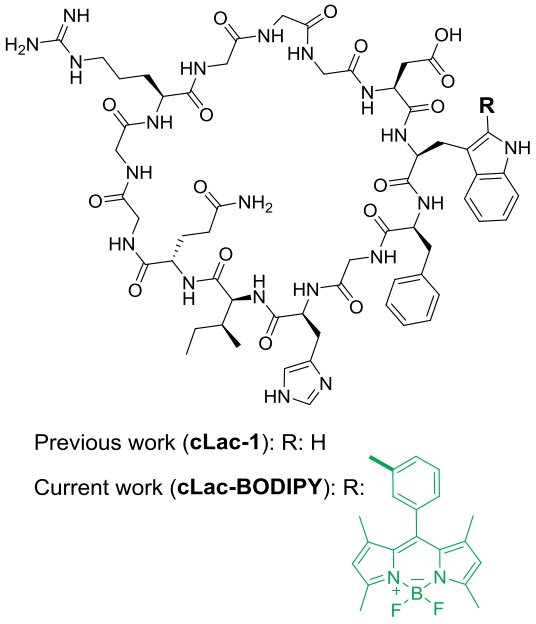

Figure 1 Chemical structures of peptide-based lactadherin mimics related to previous and the current work.

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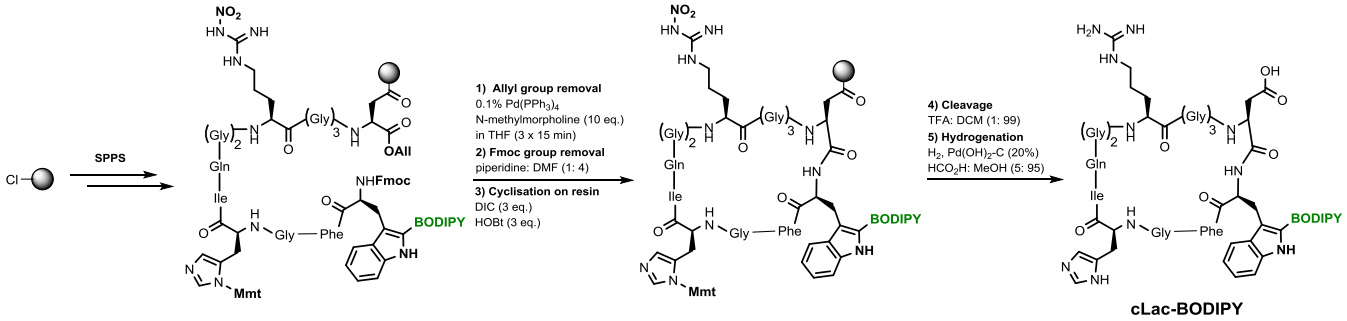
Scheme 1 Chemical synthetic approach for the solid-phase synthesis of the **cLac-BODIPY**.



Lactadherin is a β sheet-rich glycoprotein (43 kDa) that acts as a bridge between phagocytic receptors (e.g. $\alpha_V\beta_5$) and apoptotic cells by binding at externalised PS in a Ca^{2+}-independent manner. Based on the disposition of lactadherin residues upon binding to PS, Zheng and co-workers condensed its fundamental amino acids to prepare lactadherin-like small cyclic peptides (**cLac-1**, Fig 1).[13] We envisioned that fluorescent probes of the cLac family might be useful tools for labelling PS-rich apoptotic bodies, given their high sensitivity and suitability for imaging assays.[14-15] However, most labelling methods alter the molecular properties of small peptides. Our group has optimized C-H activation methodologies[16-19] and pioneered the Trp-BODIPY fluorogenic amino acid for non-perturbative labelling of peptide sequences.[20] The Trp-BODIPY amino acid emits fluorescence once labelled peptides bind to their corresponding targets, which leads to the rigidification and fluorescence enhancement of the Trp-BODIPY fluorophore (Figure S1 in Electronic Supplementary Information).[20] Since the **cLac-1** peptide contains one Trp in its original sequence, we prepared a fluorescent cyclic peptide (**cLac-BODIPY**) for binding to PS-rich vesicles by replacing the Trp of **cLac-1** with the Trp-BODIPY amino acid (Fig 1). Using this approach, we examined **cLac-BODIPY** as a novel probe to fluorescently label apoptotic bodies in a Ca^{2+}-independent manner.

We designed the synthesis of **cLac-BODIPY** in solid-phase by selecting appropriate orthogonal protecting groups and using 2-chlorotritylchloride polystyrene resin as the solid support, in order to cleave the peptide under mild acidic conditions without affecting the BODIPY core (Scheme 1).[21,22] We used Mmt as the protective group for His and the NO_2 group for Arg because they can be removed by mild acidic treatment and hydrogenation respectively,[23] and Gln was introduced without any side-chain protection. Furthermore, in order to maximize the efficiency of the cyclisation, we designed a head-to-tail solid-phase approach by anchoring the peptide through the side chain of the Asp residue, while protecting the C-terminal carboxylic acid as an allylic ester. The peptide elongation was performed using standard conditions,[24-26] followed by on-bead cyclisation. The partially-protected peptide was isolated after treatment with TFA: DCM (1: 99), and **cLac-BODIPY** was obtained in very high purities (> 99 %) after catalytic hydrogenation and reverse-phase HPLC purification (Figures S2-S3 in ESI).

In order to assess the properties of **cLac-BODIPY** and its affinity for PS, we measured the fluorescence spectra of **cLac-BODIPY** upon incubation with layers of constant lipid content and different ratios of PS and phosphatidylcholine (PC). As shown in Figure 2, **cLac-BODIPY** displayed a dose-dependent increase of the fluorescence emission after interaction with PS, with low emission in films containing only PC.

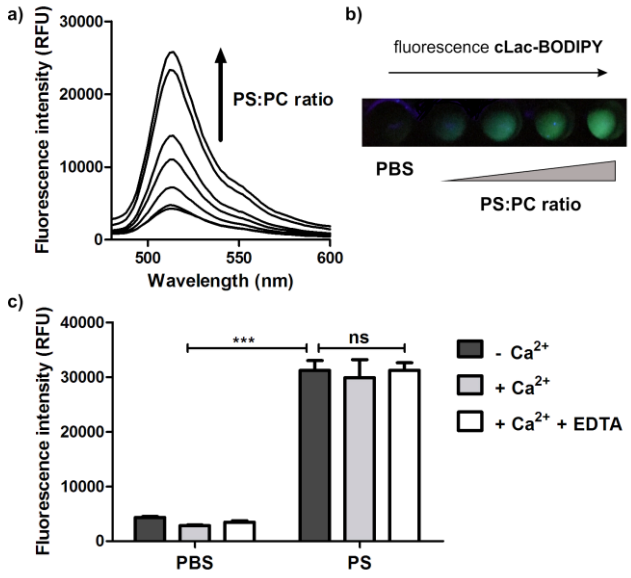


Figure 2 a) Fluorescence spectra of **cLac-BODIPY** in films with constant lipid content and increasing PS: PC ratios. QY (100% PS): 0.46, QY (100% PC): 0.08. b) Fluorescence emission of **cLac-BODIPY** under 365 nm in solutions with different PS: PC ratios and PBS as a blank. c) Ca^{2+}-independent binding between **cLac-BODIPY** and PS ([$CaCl_2$]: 2 mM, [EDTA]: 2.5 mM). *** for $p < 0.001$, ns for $p > 0.05$.

cLac-BODIPY showed a remarkable fluorescence quantum yield upon binding to PS (0.46) due to the fluorogenic behaviour of the Trp-BODIPY reporter. Furthermore, we assessed the fluorescence emission of **cLac-BODIPY** after incubation with PS-containing lipid layers in the presence and absence of Ca^{2+}. Unlike Annexin V, the interaction between **cLac-BODIPY** and PS did not show any dependence on Ca^{2+}, which confirms the applicability of **cLac-BODIPY** to recognise PS-rich vesicles under most physiological conditions (Figure 2 and Figure S4 in ESI).

In view of the excellent features of **cLac-BODIPY** as a fluorescent reporter for PS-rich environments, we examined its physicochemical properties in lipid-aqueous interfaces. First, we determined the tensioactive potential of **cLac-BODIPY** by measuring the surface pressure of lipid-aqueous mixtures at different concentrations of the probe. **cLac-BODIPY** displayed remarkable tensioactivity and was able to form monolayers in the air-water interphase with a saturation pressure (π_s) of 20.4 mN m^{-1} (Table 1 and Fig S5 in ESI). We compared the behaviour of **cLac-BODIPY** with the non-labelled peptide **cLac-1** under the same experimental conditions, and observed that **cLac-BODIPY** displayed significantly stronger tensioactivity (Fig S5 in ESI), suggesting its suitability to monitor biomolecular changes in these interphases. These results also confirm that the BODIPY fluorophore is an optimal scaffold for reporting molecular events associated with lipid-rich environments.[27-29]

Table 1 Critical surface pressure quantification assays in lipid monolayers.

	π_s (mN m^{-1})	π_c (mN m^{-1})	Decrease π_c vs PS [a]	
		PS	PC	PG
cLac-BODIPY	20.4	42.0	15%	<1%
cLac-1	5.0	30.3	16%	<1%

[a] Relative decrease in π_c values when comparing PC:PS (7:3) monolayers (PS) to PC monolayers (PC) or PC:PG (7:3) monolayers (PG: phosphatidylglycine).

Next we quantified the binding affinity of **cLac-BODIPY** for different phospholipids by determining the critical surface pressure of **cLac-BODIPY** in lipid monolayers with different composition. **cLac-BODIPY** incorporated into PS-containing monolayers (PC: PS, 7: 3) with a critical pressure of 42 mN m^{-1}, with 15% lower affinity (36 mM m^{-1}) in PC-only monolayers (Table 1 and Fig S6 in ESI). We extended the analysis to other negatively-charged phospholipids (e.g. phosphatidylglycine (PG), cardiolipin) that are associated with apoptotic processes,[30] and observed the increased binding of **cLac-BODIPY** for monolayers containing these lipids over PC-only monolayers (Figures S7 in ESI). This pattern suggests the preferential binding of **cLac-BODIPY** to labelling subcellular structures derived from apoptotic cells. We also examined the specificity of these interactions by analysing the behaviour of the non-labelled peptide **cLac-1**. Notably, the relative binding pattern of **cLac-1** to the different phospholipids closely correlated with that observed for **cLac-BODIPY** (Table 1 and Figure S7 in ESI). This minimal impairment in the lipid recognition ability of the **cLac-1** peptide indicates that the BODIPY fluorogen is optimally positioned within the cLac peptide sequence. Altogether, these results confirm the preferential binding of **cLac-BODIPY** to lipid domains found in structures released by apoptotic cells.

We employed **cLac-BODIPY** in fluorescence microscopy experiments with PS-containing vesicles as well as other lipidated vesicles. We prepared giant unilamellar vesicles (GUVs) containing PC-only or PC: PS (7: 3), and incubated them with the same concentration of **cLac-BODIPY** and lissamine-rhodamine-PE as a generic lipid stain. As shown in Figure 3, **cLac-BODIPY** displayed significantly brighter staining in vesicles containing PC: PS (7: 3) than in vesicles with only PC. This observation is in agreement with our results from the in vitro assays in lipid monolayers and confirms the applicability of **cLac-BODIPY** for the fluorescence labelling of apoptotic bodies.

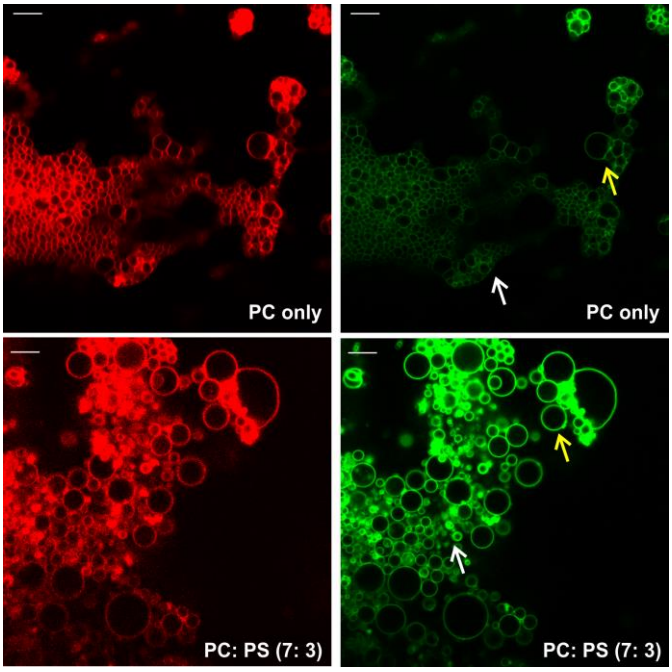


Figure 3 Confocal fluorescence microscopy images of giant unilamellar vesicles (GUVs) containing PC-only or PC: PS (7: 3) after co-staining with lissamine-rhodamine-PE (red, lipid stain) and **cLac-BODIPY** (green, 2 μM). White and yellow arrows point at vesicles of similar size in PC only and PC: PS (7: 3) GUVs. Scale bar: 10 μm.

Finally, we examined **cLac-BODIPY** as a fluorescent probe for labelling subcellular structures derived from apoptotic human cells. We induced apoptosis in Burkitt's lymphoma (BL2) cells by irradiation with UV light and subsequent culture, and isolated the apoptotic bodies. First we incubated the apoptotic bodies with different concentrations of **cLac-BODIPY** and analysed the fluorescence labelling by flow cytometry (Figures 4a and b). **cLac-BODIPY** showed dose-dependent staining, with significant labelling even at low concentrations (Figure S8 in ESI). Flow cytometry assays confirmed that most apoptotic bodies stained with **cLac-BODIPY** were also stained with Annexin V, suggesting the interaction with PS as the responsible for the fluorescence labelling of apoptotic bodies using **cLac-BODIPY** (Figure 4c). Furthermore, we employed

fluorescence microscopy to visualize subcellular bodies derived from apoptotic human A549 cells (Figure S9 in ESI).

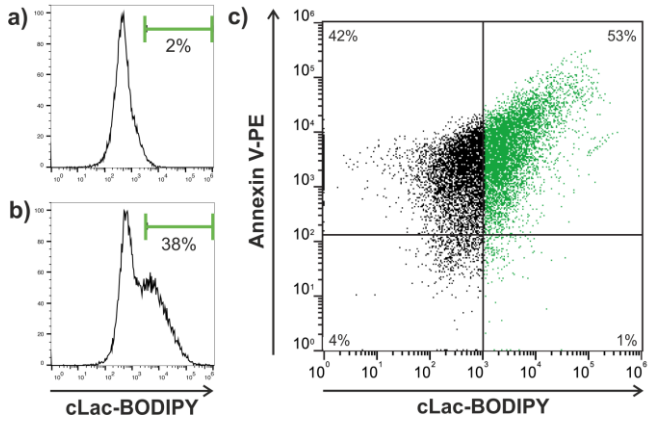


Figure 4 Flow cytometry analysis of apoptotic bodies from BL2 cells. Histograms of apoptotic bodies incubated without (a) and with **cLac-BODIPY** (2.5 μM) (b). c) Dot plot of apoptotic bodies treated with **cLac-BODIPY** (X-axis, green) and Annexin V-PE (Y-axis) showing the double staining.

In summary, we have synthesized a fluorescent cyclic peptide (**cLac-BODIPY**) to label apoptotic bodies by including the fluorogenic Trp-BODIPY amino acid into a short lactadherin-like peptide sequence. **cLac-BODIPY** shows remarkable fluorescence emission only after binding to phosphatidylserine and, unlike Annexin V, displays strong binding in a Ca^{2+} independent-manner. In vitro assays in lipid monolayers indicate the suitability of the Trp-BODIPY fluorogen to report changes in the composition of lipid-aqueous interphases, and validated the selectivity of **cLac-BODIPY** for PS-rich vesicles using fluorescence confocal microscopy. Flow cytometry experiments using **cLac-BODIPY** confirmed the labelling of apoptotic bodies from BL2 human lymphoma cells, creating new opportunities to monitor, profile and characterise these subcellular structures in multiple biological contexts.

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