**Dual colorimetric and fluorogenic probes for visualizing tyrosine phosphatase activity**


**Abstract**

Protein tyrosine phosphorylation plays a crucial role in cellular signalling pathways. Aberrant activity of the enzymes that control tyrosine phosphorylation, the protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), has been implicated in numerous human diseases. While inhibition of the PTKs is a well-established approach for the treatment of infections,7–10 there has been a resurgence of interest in PTPs as drug targets in recent years, due to an increased understanding of the biological roles of these enzymes fuelled in part by the development of new chemical tools for studying PTPs.17 Colorimetric and fluorogenic substrates have proven particularly useful for studying PTP activities *in vitro* and in cells.18–20 The prototypical assays for PTP activity are the Biomol Green assay and *para*-nitrophenylphosphate assay.29 Although commonly used *in vitro*, these substrates are unsuitable for cellular studies because they are used as discontinuous colorimetric assays. Fluorogenic substrates including 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)18,21 phosphocoumaryl amino propionic acid (pCAP)19,22 and fluorescein 3,6-diphosphate (FDP)18,23 offer excellent sensitivity for *in vitro* assays and pCAP and FDP have been used to monitor PTP activities in cells.26 However, DiFMUP and pCAP are UV active probes (λex ~ 360 nm) which can cause significant photo-damage to biological samples and the emission profiles of all three of these probes overlap with cellular autofluorescence, limiting their utility.24,25 As highlighted in Table 1, all of the existing probes are useful *in vitro*, but they have limitations in cellular studies. A red-shifted fluorogenic probe with good cellular permeability would be useful for monitoring PTP activity *in vitro* and in cells.

Resorufin has significantly red-shifted fluorogenic properties, making it a potential candidate for a new fluorescent PTP probe.26,27 Modified resorufins are suitable substrates for monitoring glucosidase and alkaline phosphatase activity and a probe with a self-immolative linker separating the phosphate and resorufin was recently reported as an efficient alkaline phosphatase substrate.29 Although alkaline phosphatases are structurally and mechanistically distinct from PTPs, utilizing a metal-bound water molecule to catalyse the hydrolysis of a range of phosphocarbohydrate, sulfocarbohydrate and phosphonocarbohydrate substrates as compared to the cysteine-dependent, substrate selective hydrolysis of phosphotyrosine by PTPs,30 we hypothesized that a resorufin-based substrate could be useful for monitoring PTP activity *in vitro* and in cells. We anticipated that a simple phosphorylated resorufin would be advantageous as compared to the previously reported, self-immolative alkaline phosphatase probe due to the easier synthetic route and kinetic analysis it should afford.

To this end, a phosphorylated version of resorufin (pRes, Fig. 1) was synthesized by phosphorylation of resorufin using diethyl chlorophosphate followed by removal of the ethyl protecting group using trimethylsilyliodide (TMSI) as reported previously (Scheme S1, ESI†).27,29 As expected, the pRes exhibited only weak background fluorescence. Upon dephosphorylation,
the probe displays a significant increase in fluorescence suggesting that it could be useful for quantifying PTP activity in vitro and in cells. In addition, when pRes was treated with a PTP, a color change from light orange to pink was observed and quantified by absorption spectroscopy (Fig. S1, ESI†).

 Gratifyingly, when tested with a panel of enzymes consisting of two classical PTPs (PTP1B and TCPTP), one receptor-type PTP (VHR) and one bacterial PTP (YopH), pRes proved to be an excellent substrate for the entire panel in vitro as shown in Fig. S2 (ESI†). An enzyme-free control showed no increase in fluorescence, indicating no detectable levels of auto-hydrolysis on the timescale of the experiment (Fig. S3, ESI†). The kinetic constants $K_M$, $k_{cat}$, and $k_{cat}/K_M$ are listed in Table 2. The $K_M$ values of pRes are slightly higher on average than those of DiFMUP and FDP for these enzymes (DiFMUP has an average $K_M$ of 14 and FDP has an average $K_M$ of 22 for PTP1B, CD45 and TCPTP). The $k_{cat}$ values for pRes are lower than those of DiFMUP but are similar to FDP. As highlighted in Table S1 (ESI†), the $K_M$ values of pRes against alkaline phosphatase and acid phosphatase are comparable (28 and 31 μM, respectively) while the $k_{cat}$ values differ significantly (400 vs. 0.085 s⁻¹). These data are consistent with the known catalytic activities of the different families of phosphatases.

One common use of fluorogenic probes is the screening and evaluation of potential enzyme inhibitors in high throughput screening (HTS) assays. The utility of pRes for inhibitor screening was validated using two known PTP inhibitors, sodium orthovanadate and 1,2-naphthoquinone. The IC₅₀ values (110 ± 20 μM and 8 ± 3 μM for sodium orthovanadate and 1,2-naphthoquinone, respectively, Fig. S4, ESI†) matched well with literature values for reported inhibition of PTP1B. The robustness and suitability of the assay for HTS was investigated. The average signal window (SW, defined as the distance of the upper limit of the noise for the mid-point signal to the lower limit of the noise for the maximum signal in an inhibition assay) and $Z'$ values (a statistical characteristic of an assay that can be used for quality assessment) for the assay were 10.42 and 0.72, respectively, indicating a robust assay (criteria for an acceptable assay are a SW > 2 and $Z'$ factor > 0.4) (Fig. S5, ESI†).

In addition to its properties as a fluorogenic PTP substrate, pRes displays a visible color change upon dephosphorylation, which can provide a fast and easy method for detecting PTP activities in bacterial samples. The human pathogen Staphylococcus aureus secretes an active tyrosine phosphatase while most other bacteria do not and this unique activity can be used to diagnose S. aureus infections. We incorporated 100 μM pRes into LB agar and grew four different human pathogens on the medium overnight. As shown in Fig. 2, S. aureus (Sa) growth in the bottom quadrant of the plate is accompanied by a significant color change from orange to magenta, indicating that the bacteria are secreting active phosphatase. Enterococcus faealcis (Ef) growth in the top quadrant is marked by a slight color change, consistent with literature indicating that this species produces an alkaline phosphatase.

Encouraged by the utility of pRes as a fluorogenic and colorimetric reporter of phosphatase activity in vitro, we decided to investigate the utility of pRes as a fluorogenic substrate for PTP activity in living cells. In these experiments, HeLa cells were incubated with pRes in the absence or presence of the pan-specific PTP inhibitor pervanadate. As shown in Fig. 3, the cells show no auto-fluorescence in the red region of the spectrum while the addition of pRes results in development of an intense red fluorescence throughout the cell. Pre-incubation of the cells with pervanadate results in an almost complete inhibition of cellular PTP activity and thus very little development of the red fluorescence. These results indicate that pRes is a useful substrate for monitoring phosphatase activities in living cells. An additional advantage of pRes is that it required no additional cell permeabilization techniques for incorporation into the cell.

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**Table 1.** Advantages and limitations of selected PTP substrates

<table>
<thead>
<tr>
<th>Probes</th>
<th>Excitation</th>
<th>Advantage/limitation</th>
</tr>
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<tbody>
<tr>
<td>pNPP¹⁸</td>
<td>Non-fluorescent</td>
<td>Colorimetric, discontinuous assay</td>
</tr>
<tr>
<td>MUP &amp; DiFMUP¹⁸,²¹</td>
<td>λₑₓ 360 nm</td>
<td>UV excitation, cell permeable</td>
</tr>
<tr>
<td>FDP¹⁸,²³</td>
<td>λₑₓ 495 nm</td>
<td>Emission overlaps with cellular auto-fluorescence, not freely cell permeable</td>
</tr>
<tr>
<td>pRes &amp; F₂pRes</td>
<td>λₑₓ 560–570 nm</td>
<td>Dual colorimetric and fluorogenic assay, cell permeable, red excitation &amp; emission</td>
</tr>
</tbody>
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**Table 2.** Kinetic constants ($K_M$, $k_{cat}$, and $k_{cat}/K_M$) for PTP assays using pRes as the substrate

<table>
<thead>
<tr>
<th>PTP</th>
<th>CD45</th>
<th>VHR</th>
<th>TCPTP</th>
<th>YopH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$</td>
<td>68</td>
<td>160</td>
<td>170</td>
<td>42</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>3.9</td>
<td>5.3</td>
<td>1.1</td>
<td>5.8</td>
</tr>
<tr>
<td>$k_{cat}/K_M$</td>
<td>$5.8 \times 10^4$</td>
<td>$3.4 \times 10^4$</td>
<td>$6.6 \times 10^4$</td>
<td>$1.4 \times 10^5$</td>
</tr>
</tbody>
</table>

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[Fig. 1] Structures of pRes and F₂pRes.

[Fig. 2] LB agar containing 100 μM pRes was the growth medium for A. baumannii (Ab, left), E. faealcis (Ef, top), S. saprophyticus (Ss, right) and S. aureus (Sa, bottom).
The commonly used PTP substrate DiF-MUP was designed as a derivative of MUP with the aim of lowering the $pK_a$ and showed an increased sensitivity for monitoring the activity of phosphatases active at acidic pH.\textsuperscript{18,21} Encouraged by the success of fluorination of the resorufin scaffold resulted in $k_{\text{cat}}/K_M$ at 40% yield by condensation of 4-nitrosoobenzene-1,3-diol and 2,4-difluorobenzene-1,3-diol in methanesulfonic acid. Phosphorylation production $F_2p$Res (Scheme S2, ESI\textsuperscript{†}). Like pRes, $F_2p$Res undergoes a color change upon addition of PTP enzymes from pale pink to magenta, corresponding with a shift in $\lambda_{\text{max}}$ from 495 nm to 571 nm. $F_2p$Res was hydrolysed by a broad range of PTPs (Table 3 and Fig. S7, ESI\textsuperscript{†}).

As expected, fluorination of the resorufin scaffold resulted in a reduction of $pK_a$ from 5.8 to 4.6. (Fig. S8, ESI\textsuperscript{†}). Thus, $F_2p$Res should be a better substrate for phosphatases active at acidic pH. We compared the activity of YopH, a bacterial tyrosine phosphatase with activity at both neutral and acidic pH, using pRes and $F_2p$Res as substrates. Consistent with the kinetic data shown above, pRes and $F_2p$Res are approximately equally effective substrates at pH 6.5. At pH 5.0, $F_2p$Res provides a much larger increase in fluorescence than pRes, making it the preferred substrate for applications requiring lower pH (Fig. S9, ESI\textsuperscript{†}). We further verified the utility of $F_2p$Res as a substrate for acid phosphatases by monitoring the activity of acid phosphatase 1 (ACP1, also known as low molecular weight protein tyrosine phosphatase, LMW-PTP) and acid phosphatase (from wheat germ) (Fig. S10, ESI\textsuperscript{†}).

In summary, we have validated pRes as an excellent substrate for the PTP family of enzymes. The red-shifted excitation and emission profiles of pRes render it useful for monitoring PTP activity in cells. The fluorogenic assay using pRes as the substrate is suitable for inhibitor screening with excellent HTS assay parameters. Furthermore, by using pRes as the substrate in a colorimetric assay for PTP activity, we are able to visually detect the presence of bacterial PTP activity, which is indicative of the presence of pathogenic \textit{Staphylococcus aureus} bacteria. Finally, a fluorinated version of pRes with improved utility for monitoring the activity of phosphatases active at acidic pH has been synthesized and validated.

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### Notes and references


