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Simultaneous detection of alkaline phosphatase and β-galactosidase activity using SERRS

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ABSTRACT

Surface enhanced resonance Raman scattering (SERRS) is an alternative to fluorescence for use in bioanalysis however due to the different optical mechanism it requires specifically designed reporters. Recently we have reported the use of 8-hydroxyquinolinyl azo dyes and their ester derivatives as reporters of lipase activity using SERRS. Acylation of the 8-hydroxy moiety significantly reduces surface enhancement of the Raman response and subsequent lipase catalysed ester hydrolysis enables the analyte to bind to silver nanoparticles, thus providing surface enhancement and the SERRS signal is 'switched on'. By following this principle, phosphorylated and galactosylated analogues of 8-hydroxyquinolinylazo dyes were prepared and shown to act as reporters of enzymatic activity for alkaline phosphatase and β-galactosidase respectively when using SERRS.

Masked SERRS substrates have been synthesised that allow for simultaneous analysis of alkaline phosphatase and β-galactosidase activity.

Both alkaline phosphatase and β-galactosidase are commonly used in a wide range of biological assays. Probes for the detection of phosphatases have been used in a number of elegant applications, including detection of alkaline phosphatase immobilised on microspheres in an optical sensor array,[1] microfluidic assays,[2] and in microplate assays for shellfish toxins.[3] β-Galactosidase is used to characterise strains of micro-organisms and is often useful as a reporter gene marker.[4–6] One of the most important reporter genes is *lac Z* from *Escherichia coli* which is extensively used as a reporter gene in molecular biology, and β-galactosidase is used to monitor its expression.[7–9] Alkaline phosphatase and, to a lesser extent, β-galactosidase are also used as enzyme conjugates for antibody quantitation in ELISA's.[10–12] The substrates are commonly phosphorylated or galactosylated phenols, where the 'free' phenol is fluorescent, coloured or strongly absorbs, but the spectroscopic response is diminished upon phenolic derivatisation. Phenyl phosphate derivatives have been used for spectrophotometric alkaline phosphatase detection since their introduction by Brandenberger and Hanson.[13] One of the earliest reported activity probes for any enzymatic substrate was an indolyl substrate, X-gal.[14] Upon treatment with β-galactosidase, X-gal is hydrolysed, and undergoes in situ oxidation to an indigo dye species which can be monitored by UV–vis spectroscopy. Probes for spectroscopic detection of enzyme activity have traditionally been reliant on a fluorescent or colorimetric response, but a number of recent publications have reported the potential use of surface enhanced resonance Raman scattering (SERRS).[15,16] Invariably fluorescence and colorimetry both provide broad electronic spectra and as such identification of multiple probes within a sample is not straightforward, yet the information-rich vibrational spectra obtained by SERRS lends the technique favourably to identifying multiple components in a mixture.[17] Recently we have reported the use of 8-hydroxyquinolinyl azo dyes and their ester derivatives as reporters of lipase activity. Acylation of the 8-hydroxy moiety significantly reduces surface enhancement of the Raman response, subsequent lipase-catalysed ester hydrolysis enables the analyte to bind to silver colloid, thus providing surface enhancement and the SERRS signal is 'switched on', as illustrated in Figure 1.[18] By following this principle, phosphorylated and galactosylated analogues of 8-hydroxyquinolinylazo dyes were prepared (Scheme 1), with the intended purpose

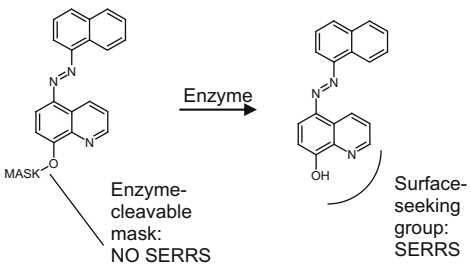

Figure 1. Conceptual diagram for principle of enzyme-cleavable masked 8-hydroxyquinolinylazo SERRS dyes.

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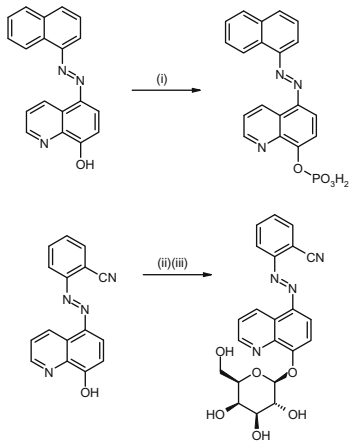

Scheme 1. Reagents and conditions: (i) $POCl_3$, 75% (ii) tetraacetyl α-bromogalactopyranose, Cs_2CO_3, acetone, reflux, 14 h, 21% (iii) MeOH, 5% NaOMe, rt, 2 h, 80%.

of using them as reporters of enzymatic activity for alkaline phosphatase and β-galactosidase respectively. **1** and **3** were prepared as previously reported,[18] phosphate **2** was prepared by treatment of **1** with phosphorous oxychloride, and the product isolated by reverse phase silica gel chromatography. A number of conditions for glycosyl transfer to **3** were explored, but apparent low nucleophilicity of both the phenol and its conjugate phenolate anion hindered reaction with tetraacetyl α-bromogalactopyranose. Two possible reasons for low reactivity of the phenolate anion are proposed: firstly a resonance effect via the electron-withdrawing azo group and secondly base-catalysed conversion to hydrazo species. Addition of base will shift the hydrazo-azo tautomerisation towards the hydrazo species, which will minimise availability of phenolate anion for reaction. In the first instance tetraacetyl α-bromogalactopyranose with silver oxide as a heavy metal promoter was attempted but did not afford any alkylated product. Phase transfer conditions were attempted,[19,20] but again no alkylation was observed. Finally galactose derivative **4** was prepared by reaction of tetraacetyl α-bromogalactopyranose with phenolic dye **2** in the presence of cesium carbonate in acetone at reflux. Cesium carbonate has been reported as exhibiting modified reactivity in phenolate alkylations in comparison to carbonate salts of sodium and potassium, Ouyang et al. found that reaction rate and nucleophile regioselectivity could be manipulated by variation of alkali metal.[21] It has been suggested that O-alkylations using Cs_2CO_3 in non-aqueous solvents occur via the naked phenolate anion, which will be a stronger nucleophile and more reactive, which may explain the modified reactivity seen when using cesium carbonate.[22] A subsequent Zemplen de-acetylation procedure[23] afforded the unprotected galactopyranoside **4** in good yield. Dyes **1** and **3**, the unmasked analogues of **2** and **4** respectively, can be easily discriminated using SERRS, as can be seen from the superimposition of their spectra (Fig. 2). Dye **3** has a peak at 1267 cm^{-1} which is not present in **1**, dye **1** has a peak at 1340 cm^{-1} which is not present in **3**. These bands have been previously assigned to in-plane C–H bending, and N=N-aryl stretching respectively.[18]

To facilitate simultaneous reactions of multiple enzymes, a mutually compatible medium for satisfactory activity of all enzyme components was sought. It has been reported that alkaline phosphatase and β-galactosidase have an optimum reaction pH of 8–9[24] and 6–8,[25] respectively. Accordingly, all reactions were buffered at pH 8, in Tris buffer. To maintain consistency the SERRS spectra of **1** and **3** were also recorded in the same buffer. A mixture of 10^{-6} M galactosidase substrate **4** and 10^{-6} M phosphatase substrate **2** was treated solely with β-galactosidase and left to react

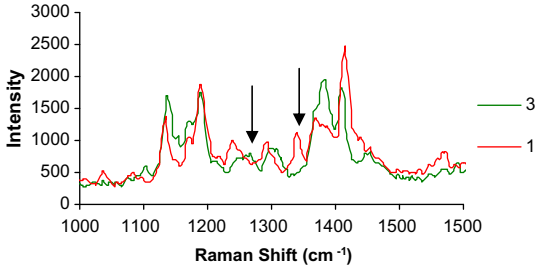

Figure 2. Superimposed SERRS spectra for unmasked dyes **1** and **3**. The peaks used for discrimination, at 1267 cm^{-1} and 1340 cm^{-1} are highlighted.

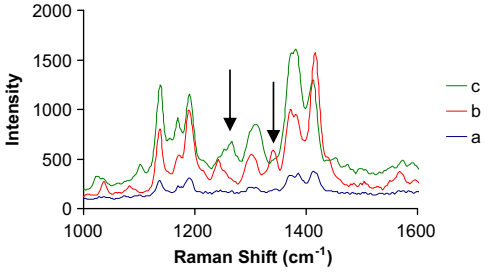

Figure 3. SERRS spectra obtained from a mixture of **2** and **4**. (a) without enzyme added (b) with alkaline phosphatase added (c) with β-galactosidase added.

at room temperature for 30 min. A clearly distinguishable increase in SERRS response was found (Fig. 3), and due to the emergence of a peak at 1267 cm^{-1}, and lack of signal at 1340 cm^{-1}, this increase in SERRS can be attributed to the emergence of dye **3** only. Thus, the β-galactosidase had only hydrolysed **4** and masked substrate **2** remained intact. In each case a blank was also run, where water was added in place of the relevant enzyme, to investigate whether non-enzymatic hydrolysis was occurring. The reverse experiment was conducted, with alkaline phosphatase replaced with β-galactosidase. This time the peak at 1340 cm^{-1} was emergent, but no trace of the peak at 1267 cm^{-1} was observed. Thus the alkaline phosphatase has hydrolysed **2** selectively (Fig. 3).

In conclusion, we have demonstrated that 8-hydroxy quinolinyl azo dye derivatives can act as SERRS reporters of both alkaline phosphatase and β-galactosidase. Furthermore we have shown that it is possible to simultaneously detect the presence of either of these enzymes using a mixture of the substrates. To our knowledge, this is the first time a simultaneous assay for the detection of alkaline phosphatase or galactosidase using SERRS has been demonstrated. By demonstrating simultaneous enzyme detection in this manner, the technique allows for one-pot detection of multiple analytes, offering more expeditious analysis times, plus the prospect of multiplexing ELISA's.

Supplementary data

Supplementary data (solvents and reagents) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.030.

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