# A chemiluminescence nanosensor to monitor lipid peroxidation

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#### **Experimental/Materials**

5 Chemicals were purchased from the sources indicated and were used as supplied; thionyl chloride (99.0 %, Fluka), coumarin 343 (97.0 %, Aldrich), triethylamine (99.5 %, Aldrich), 15 nm silicon dioxide nanopowder (Aldrich), silica gel 60 (Fluka), L-α-phosphatidylcholine (~60.0 % from egg-yolk, Sigma), boric acid (98.0 %, Aldrich), cumene hydroperoxide (99.0 %, Aldrich), Triton X-100 (Sigma) and tetraethyl orthosilicate (TEOS, + 99.0 %, Aldrich). All solvents used, 30.0 % aqueous ammonium hydroxide and conc. hydrochloric acid were of reagent grade and purchased from Fisher Scientific.

## 10 Preparation of aminopropyl-functionalised silica nanoparticles 1:

15 nm Silicon dioxide nanopowder (2.0 g) was added to a stirred solution of 3-aminopropyltriethoxysilane (0.47 g, 2.0 mmol) in anhydrous toluene and heated to reflux, under  $N_2$ , for 24 hr. The resulting reaction mixture was centrifuged (3000 rpm), the supernatant decanted off and the resulting pellet of nanoparticles redispersed in toluene. The nanoparticles were again centrifuged and the procedure repeated using DCM, ethanol and acetone, affording the aminopropyl-functionalised nanoparticles **1** as a free flowing white powder (1.95 g, 92.2 % yield).

#### 15 Preparation of Silica- conjugated Coumarin C343 6:

Thionyl chloride **2** (0.18 ml, 2.42 mmol, 3 eq.) was added to a stirred solution of coumarin 343 **3** (0.23 g, 0.81 mmol) in DCM, under N<sub>2</sub> and heated to reflux for 3 h. The reaction mixture was subsequently concentrated in vacuo, to afford the acid chloride  $4^{\uparrow}$ , redissolved in anhydrous toluene (10 ml) and added dropwise to a stirred solution of aminopropyl-functionalised silica nanoparticles **1** (0.80 g, 1.0 mmol g<sup>-1</sup>) and triethylamine (Et<sub>3</sub>N) **5** (0.18 ml, 3 eq.) in toluene (10 ml) and the reaction mixture stirred overnight at room temperature. The reaction mixture was centrifuged (3000 rpm),

20 the supernatant decanted off and the nanoparticle pellet redispersed in DCM. The nanoparticles were again centrifuged and the procedure repeated using deionised water, ethanol and finally acetone, to afford a free flowing orange powder 6 (0.984 g, 95.2 % yield). Nanosensor Fabrication-Emulsion Formation:

Into a stirred flask 8 ml Triton X-100 was pipetted, followed in order by 33.0 ml cyclohexane, 8.0 ml hexanol and 1232 µl dye suspension in 100% dimethylsulfoxide. After allowing 15 min for complete mixing a 40 ml portion was taken and 1500 µl tetraethyl orthosilicate (TEOS) and 944 µl 28%

25 aqueous ammonium hydroxide were added under constant stirring, which was continued for 24 h. Quantities were scaled up to produce larger amounts but all other conditions used were the same.
Nanosensor Collection:

The nanoparticles were precipitated by addition of 20 ml acetone followed by centrifugation and decanting of the supernatant; after redispersing in acetone, the process was repeated 4 times. The resulting precipitate was washed 4 times with 10 ml 50% aqueous ethanol and then passed through a

30 200 nm membrane filter which was washed with three 10 ml aliquots of deionised water, which were collected. The filtrate was then refiltered at 20 nm over a vacuum; the residue was washed in the filter with 10 ml portions of ethanol, deionised water and acetone left to dry under suction. Scanning Electron Microscopy (SEM):

A 1 mg ml<sup>-1</sup> suspension of blank nanosensors (containing no dye) was prepared in 1 mmol dm<sup>-3</sup> aqueous sodium chloride, which had been filtered at 200 nm and deaerated with nitrogen for 15 min; homogeneity was obtained by 30min sonication at 120W. The sample was dried onto a glass 35 substrate and coated with a ~1 nm film of gold/palladium and images taken of random areas at magnifications of x50000 and x100000.

# pH Investigation:

The effect of pH on chemiluminescence was investigated at and around physiological conditions. A set of phosphate buffers was prepared covering the pH range 5.8 to 8.0 and used to prepare five buffered 10 mg ml<sup>-1</sup> suspensions in 20 mg ml<sup>-1</sup> PC of nanosensors fabricated using a 10mM suspension of conjugated C343. Using a two-channel flow-injection manifold with 20 mg ml<sup>-1</sup> PC flowing in channel A and 0.04 mol dm<sup>-3</sup> cumene

40 hydroperoxide in channel B, the total flow-rate being 4 ml min<sup>-1</sup>. Five injections of each nanosensor suspension were made into channel A and the enhancement (in mV) of the chemiluminescence was calculated from the peak heights.
 Variation of Nanosensor Suspension Concentration:

This investigation was carried out in pH-regulated conditions similar to those observed inside cells and organisms. Sensors made from 50 mM conjugated dye were suspended in phosphate buffer, pH 7.4, containing 2.29 mM sodium chloride and 2.58 mM potassium chloride at concentrations

45 of 2, 5, 8, 10 and 15 mg ml<sup>-1</sup>. The two-channel flow-injection manifold was employed as before. 20 mg ml<sup>-1</sup> PC in the phosphate buffered electrolyte flowed in channel A and 0.04 mol dm<sup>-3</sup> cumene hydroperoxide in channel B, the total flow-rate being 4 ml min<sup>-1</sup>. Six injections of each nanosensor suspension were made into channel A and the percentage enhancement of the chemiluminescence was calculated from the peak heights. Effect of Varying Dye Concentration used in Nanosensor Fabrication:

Nanosensors fabricated using different suspensions of conjugated C343 were suspended at 10 mg ml<sup>-1</sup> in the same phosphate buffered electrolyte as in 50 the last experiment. Using the two-channel flow-injection manifold with 20 mg ml<sup>-1</sup> PC in the phosphate buffered electrolyte flowing in channel A and 0.04 mol dm<sup>-3</sup> cumene hydroperoxide in channel B, the total flow-rate being 4 ml min<sup>-1</sup>. Six injections of each nanosensor suspension were made into channel A and the percentage enhancement of the chemiluminescence was calculated from the peak heights. Leaching Experiments:

Leaching experiments were carried out to measure the retention of the conjugated dye within the porous nanosensor. Nanosensors made from 10mM 55 silica conjugated dye prepared at 5.1mg ml<sup>-1</sup> and the initial absorbance was measured using spectrophotometry. The suspension was filtered through a 20nm filter and the filtrate was collected and assayed. The total leaching was determined over a 24 hour period but to indicate the mechanism by which leaching was occurring the filtrate was assayed at more frequent time intervals (see Table 5).

## 60 Evaluation of the Nanosensors:

To evaluate the nanosensors it is important to be able to compare dye loadings. Comparisons between dyes must be made at equal concentrations; relative molecular mass and hence molar concentration changes when the dye is conjugated. Because the absorption spectrum of a conjugated dye differs from the that of the free dye, calibration against a free dye cannot be used for spectrophotometric determinations of conjugated dyes. However dye loading of **3** can be calculated from the proportion of non-conjugated dye **2** recovered after the conjugation reaction. This was found to be 9% of

65 0.1 g coumarin C343 2 which yielded 0.98 g silica-conjugated dye 3 implying a loading of 26%. This calculation assumes, as a best estimate, that all dye is either conjugated or recovered and that none is lost or destroyed during the immobilisation reaction.

<sup>&</sup>lt;sup>↑</sup> Preparation of the acid chloride **4** was confirmed by quenching a portion of the acid chloride **4** with methanol, to afford the respective methyl ester followed by NMR analysis; δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>) 1.59 (1H, br s, OH), 2.00 (4H, m, 2 x CH<sub>2</sub>), 2.79 (2H, t, *J* = 6.3, CH<sub>2</sub>), 2.90 (2H, t, *J* 6.3, CH<sub>2</sub>), 3.41 (4H, m, 2 x CH<sub>2</sub>), 3.90 (3H, s, OCH<sub>3</sub>), 7.03 (1H, s, CH) and 8.54 (1H, s, CH).