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Efficient Functionalisation of Dextran-Aldehyde with Catechin: Potential Applications in the treatment of Cancer

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Abstract:

Here, we report a simple and efficient method to functionalise dextran with catechin - a polyphenol present in red wine, red berries, and green tea. Aldehyde functionalised dextran was reacted with catechin in the presence of acetic acid to yield dextran-aldehyde-catechin conjugates via a condensation reaction. 1D and 2D (DOSY) ^{1}H NMR and UV-Vis analyses were employed to characterise the polymers. The degree of catechin conjugation was easily manipulated by varying the aldehyde amount in the dextran, the catechin concentration and the reaction temperature and conjugates with up to 38 wt% catechin functionalisation were prepared. In particular, the conjugation reaction was found to be especially sensitive to temperature and this was shown to be partially due to the equilibrium between the aldehyde and the hemiacetal in dextran aldehyde shifting to a greater proportion of aldehyde groups as the temperature increased. The stability of the dextran-aldehyde-catechin conjugates was enhanced in comparison with native catechin in both deionised water and phosphate buffer solution. Finally, the anticancer properties of the dextran-aldehyde-catechin conjugates were investigated *in vitro*, demonstrating cytotoxic efficacy against neuroblastoma cells whilst showing no cytotoxicity towards normal cells.

Introduction

Well-known for their health benefits and antioxidant properties,[1] and more recently as potential engineering materials,[2, 3] polyphenols are naturally occurring compounds containing one or more phenol groups. Ubiquitous in the plant kingdom, over 8000 phenolic structures have been identified[4] with polyphenols broadly classified into four main groups – flavonoids, stilbenes, lignans, and phenolic acids.[5] Catechin, a component of red wine, red berries and green tea, is a polyphenol from the flavonoid group, which has demonstrated potential in both the prevention and treatment of a wide range of diseases, including cancer,[6, 7] cardiovascular disease,[8] osteoporosis,[9] Alzhiemer's,[10] and Parkinson's disease.[10] Despite its impressive therapeutic effects, catechin suffers from a number of drawbacks, including instability in dilute solutions[11] and when exposed to light, heat and basic conditions.[12] To overcome its poor stability, catechin has been successfully conjugated to a number of polymers, including chitosan,[13-16] poly(ε-lysine),[17] poly(allylamine),[18-20] gelatin,[21-23] inulin,[24, 25] and alginate,[24, 25] conferring enhanced stability and antioxidant activity.[1] However, the typical degree of functionalisation is less than 10 wt%. For instance, catechin has been conjugated to dextran realising a maximum 2 wt% degree of functionalisation with the conjugate exhibiting anticancer activity against pancreatic ductal adenocarcinoma.[26, 27] This low level of functionalisation could necessitate the administration of high concentrations of polymers to have the desired therapeutic effect, thereby potentially limiting clinical applications.

Dextran aldehyde (dex-ald) has demonstrated considerable potential for therapeutic use, both as a hydrogel component for surgical applications[28, 29] and as a drug carrier.[30] Dex-ald is typically conjugated to target drugs via Schiff base reactions between the aldehyde groups and amino groups on the target drug.[31] To the best of our knowledge, dex-ald has not previously been conjugated to a polyphenol. Herein we describe, for the first time, the conjugation of a polyphenol, catechin, to dex-ald via a condensation reaction; achieving degrees of functionalisation from 5 to 38 wt% catechin (almost 20 times higher than has been previously achieved for dextran catechin conjugations[26]). This degree of functionalisation was easily tuned by varying the reaction temperature, the concentration of catechin or the amount of aldehyde in the dextran. The conjugates were tested *in vitro* against two neuroblastoma cell lines (IMR-32 and BE(2)-C) as well as in non-malignant (MRC-5) cells to assess both their cytotoxicity in cancer cells and their safety in normal cells.

Materials and methods

Materials: Dextran from *Leuconostoc mesenteroides* (average mol wt 9,000-11,000), (+)-catechin hydrate, sodium periodate, sodium carbonate, Folin & Ciocalteu's phenol reagent, dimethyl sulfoxide (DMSO), glacial acetic acid, sodium borohydride and Cellu Sep regenerated cellulose tubular membrane (MWCO-3500 g/mol) were purchased from Sigma Aldrich. Deionised (DI) water was produced by a Mili-Q water purification system and had a resistivity of 17.9 mΩ/cm. DMEM medium and alfa-MEM medium were purchased from Life Technologies. AlamarBlue reagent was purchased from ThermoFisher.

Synthesis of dextran aldehyde (dex-ald): Using a slight modification of the method described by Sokolsky-Papkov et al.,[32] typically, 2.0 g of dextran was dissolved in 40 mL of DI water and 1.76 g, 2.67 g or 3.52 g of sodium periodate was dissolved in 40 mL of DI water to achieve approximately 33%, 50% and 66% degree of oxidation, respectively. The solutions were mixed in a flask, protected from light and stirred for 6 h at room temperature. The dex-ald formed was subsequently dialysed (MWCO-3500 g/mol) for 72 h in the dark against DI water (12 changes of water) and then lyophilised. Dex-ald was stored at 4 ^{o}C until required.

Synthesis of dextran aldehyde-catechin (dex-ald-cat) conjugate: Typically, 250 mg of dextran aldehyde was dissolved in 10 mL of DI water. Varying amounts of catechin (see **Table 1**) were dissolved in 3.4 mL DI water / 3.0 mL DMSO / 3.6 mL acetic acid. The solutions were mixed in a 25 mL vial and, in some cases, purged with nitrogen for 30 min and sealed (see **Table 1**). The reactants were protected from light and stirred for 48 h at various temperatures (see **Table 1**). The conjugate formed was dialysed (MWCO-3500 g/mol) for 96 h in the dark against 15% DMSO/85% DI water, then against DI water (16 changes of solvent in total) and then lyophilised. UV-Vis spectroscopy was used to confirm the absence of catechin in the final dialysis water. Dex-ald-cat was stored at 4 ^{o}Cprotected from light until required.

Synthesis of reduced dextran-catechin (dex-cat-red) conjugate: Generally, 100 mg of dextran aldehyde-catechin conjugate was dissolved in 3.5 mL DI water / 1.5 mL DMSO in a 25 mL vial. 132 mg of sodium borohydride was dissolved in 5 mL DI water and added to the dextran aldehyde-catechin conjugate solution and it was stirred for 24 h at room temperature, protected from light. Acetic acid was then added dropwise until pH=3. The reduced dextran-catechin conjugate was

dialysed (MWCO-3500 g/mol) against DI water for 72 h in the dark (12 changes of water) and then lyophilised. Dex-cat-red was stored at 4 ^{o}C protected from light until required.

Measurement of catechin content: Typically, 5 mg of catechin conjugate was dissolved in 10 mL of DI water. 1 mL of this solution was added to a vial and diluted with 5 mL of DI water. 1 mL of Folin & Ciocalteu's phenol reagent was added and the mixture was shaken. After 3 min, 3 mL of 10.75 wt% sodium carbonate was added and the mixture was stored in the dark for 3 h with intermittent shaking. The absorbance of the final solution was measured at 760 nm against a solution prepared from a blank polymer. The degree of functionalisation with catechin was determined by comparing the absorbance with a calibration curve developed from native catechin.

Stability testing: Stability testing was performed in both DI water (pH ~ 5-6) and phosphate buffered saline (PBS) (pH – 7.4). 1 mg of catechin control and catechin conjugate with an equivalent amount of catechin were dissolved in either 10 mL of DI water or 10 mL of PBS in 20mL vials. The vials were then sealed with parafilm and placed in an incubator for 72 h at 37 ^{0}C. The solutions were then compared with fresh solutions via the Folin & Ciocalteu method described above and UV/Vis spectroscopy.

Analytical instruments

^{1}H-NMR Spectroscopy. All experiments were performed on a Bruker Avance 500 MHz NMR spectrometer, equipped with a 5mm TBI probe. All experiments were run with a gas flow across the probes of 535 L/hr, with sample spinning, and at a temperature of 25 ^{o}C unless otherwise specified. Samples were dissolved in deuterated NMR solvents supplied by Cambridge Isotopes (15% DMSO-d_6 / 85% D_2O). Spectra were referenced to residual protons in the NMR solvent (D_2O: δ 4.70). A presaturation water suppression experiment was employed to eliminate the residual non deuterated signal from the solvent.[33] Diffusion Ordered SpectroscopY (DOSY) was performed using a stimulated echo pulse program, which included bi-polar gradients and a watergate element for water suppression.[34] A linear sequence of 16 steps with gradient strengths from 2% to 95%, where the gradient is 50 G/cm. DOSY analysis was correlated to peak heights (i.e. Imode = Intensities).

UV Vis Spectroscopy. UV-Vis spectra were recorded in a quartz cuvette using a CARY 3000 spectrometer from Bruker at 25 ^{o}C.

Size Exclusion Chromatography (SEC). SEC analysis was performed in *N,N'*-dimethylacetamide (DMAc with 0.03% w/v LiBr and 0.05% 2,6-di-butyl-4-methylphenol (BHT)) at 50 ^{O}C at flow rate of 1 mL/min) with a Shimadzu modular system comprising an SIL-10AD automatic injector, a Polymer Laboratories 5.0 μL bead-size guard column (50 x 7.8 mm) followed by four linear PL (Styragel) columns (10^5, 10^4, 10^3 and 500 Å) and an RID-10A differential refractive-index detector. The SEC calibration was performed with narrow-dispersity poly(methyl methacrylate) standards ranging between 200 to 10^6 g/mol. Polymer solutions of 3 mg/mL were prepared in the eluent and filtered through 0.45 μm filters prior to injection.

Cell culture: Neuroblastoma IMR-32 cells were obtained from the ATCC and BE(2)-C were obtained from Prof June Biedler's lab, Fordham University, NY. Upon receipt, cell master stocks were prepared and validated by PCR-STR profiling and stored in the Children's Cancer Institute Cell Bank. Cells for experiments were passaged for less than 6 months. All neuroblastoma cells were grown in DMEM medium supplemented with 10% FBS, and 1% L-glutamate. Non-malignant lung fibroblast MRC-5 cells were cultured in alfa-MEM medium supplemented with 10% FCS, 2% sodium bicarbonate, 1% NEAA, 1% sodium pyruvate, and 1% L-glutamine. Cell lines were grown in a humidified atmosphere at 37 °C and in 5% CO_2 as monolayers. Cell lines were screened and tested negative for mycoplasma contamination using the MycoAlert MycoPlasma Detection Kit (Lonza, Switzerland).

Measurement of cell viability: Cells were plated in clear transparent 96-well plates at densities of 5-10x10^3 cells per well for neuroblastoma cells (BE(2)-C and IMR-32) and 2x10^4 cells per well for MRC-5 cells. Treatment was started after 24 or 48 h, dependent on the cell line, to allow for cell attachment. Cells were either treated with dex-ald, dex-ald-cat or dex-cat-red and incubated for 72 h. Treatment effects on cell viability were determined based on the metabolic activity of cells using the Alamar Blue assay and spectrophotometric analysis as previously described.[35]

Statistical analysis: All cell viability assays were undertaken in quadruplicate. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software) using two-way ANOVA followed by Tukey's multiple comparisons test comparing treatments with control as well as comparing between treatments.

Results and Discussion

Synthesis and characterisation of dextran aldehyde-catechin conjugate

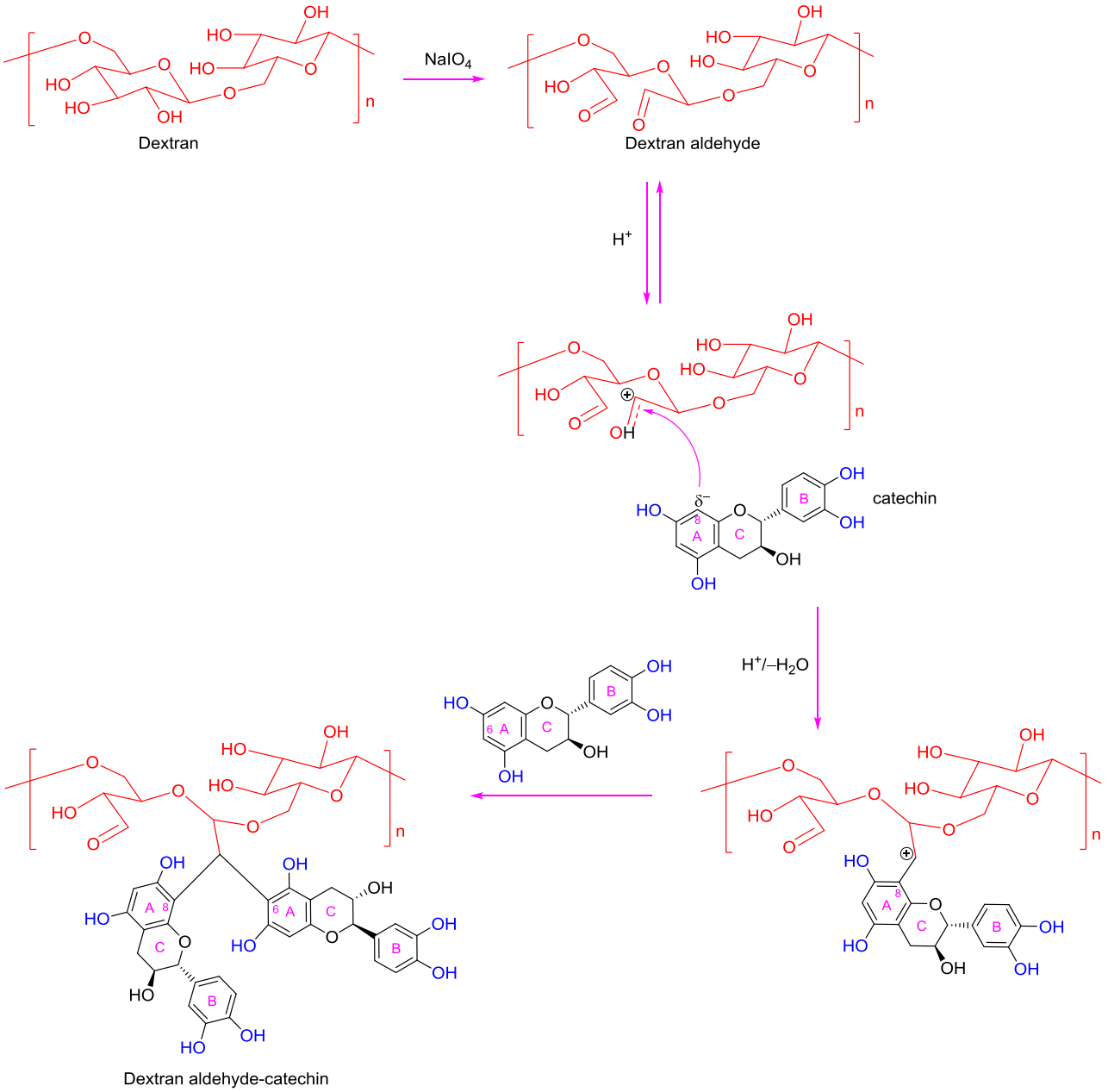
The acid catalysed condensation polymerisation of catechin with excess acetaldehyde is a well-known reaction that occurs in wines.[36] If excess catechin is employed instead of excess aldehyde, catechin can be conjugated to an aldehyde group. Indeed, Chung et al.[37] have demonstrated that excess (−)-epigallocatechin-3-O-gallate (EGCG) can be conjugated to aldehyde terminated poly(ethylene glycol). By using excess catechin with dex-ald, we conjugated catechin to the polymer with a degree of functionalisation ranging from 5% to 38% catechin by weight (see **Table 1**) in yields of 74% to 88% based on dex-ald.

Table 1: Functionalisation degree (wt% catechin) of conjugate under varying experimental conditions

Dextran degree of oxidation (percent of glucose units oxidised to aldehydes)	Concentration of catechin (mol/L) / cat units per aldehyde unit	Temperature (^{O}C)	Atmosphere	Functionalisation degree (wt% catechin)
0	0.18	25	air	0
0	0.18	40	air	0
33%	0.09 / 2	25	nitrogen	5
33%	0.09 / 2	25	air	5
33%	0.18 / 4	25	air	9
33%	0.09 / 2	40	air	13
33%	0.18 / 4	40	air	17
50%	0.14 / 2	25	air	11
50%	0.18 / 2.6	25	air	14
50% / 0*	0.18 / 2.6	25	air	12
50%	0.18 / 2.6	40	air	22
66%	0.18 / 2	25	air	14
66% / 0*	0.18 / 2	25	air	11
66%	0.18 / 2	25	nitrogen	13
66%	0.09 / 1	40	air	16
66%	0.18 / 2	40	air	22
66%	0.18 / 2	40	nitrogen	20
66%	0.27 / 3	40	air	31
66%	0.36 / 4	40	air	37
66%	0.18 / 2	55	air	31
66% / 0*	0.18 / 2	55	air	33
66%	0.18 / 2	55	nitrogen	32
66%	0.18 / 2	70	air	38
66%	0.18 / 2	70	nitrogen	38

*Note: * Samples reduced with sodium borohydride after conjugation*

Dextran was initially oxidised to dex-ald with sodium periodate. The ratio of dextran to sodium periodate was varied to afford different degrees of oxidation.[32] Following purification and lyophilisation, the dex-ald was reacted with excess catechin in the presence of acetic acid to afford dextran aldehyde-catechin (dex-ald-cat) conjugate (see **Scheme 1**). The reaction likely proceeds in a similar manner to the condensation polymerisation of catechin.[36] The first step is the protonation of an aldehyde group to yield a carbocation, which subsequently undergoes nucleophilic attack by catechin at C-6 or C-8 of the A ring. A water molecule is then lost from the adduct to yield a new carbocation, which is then attacked by a second catechin molecule to yield the conjugate. Although catechin could potentially also bond via the ortho-carbons of the B ring, NMR studies with dimers from catechin-acetaldehyde condensations show that bonding occurs exclusively via the A ring[38] so it is likely that conjugation is also primarily via the A ring.



Scheme 1: Schematic representation of oxidation of dextran to dextran aldehyde and proposed mechanism of subsequent conjugation with catechin

The successful bonding of catechin to dex-ald was confirmed by UV-Vis and ^{1}H NMR spectroscopy. The UV-Vis spectra of dex-ald-cat showed a peak at 280 nm, characteristic of catechin (see **Figure S1)**. No appreciable absorption in this region was seen for dex-ald. A broad peak from 6.6-7.2 ppm corresponding to the aromatic protons of catechin was observed in the ^{1}H NMR spectra for all dex-ald-cat conjugates (see **Figure 1**), indicating covalent bonding of catechin to the polymer. In addition, sharp peaks were noted in the same region, suggesting free or hydrogen bonded catechin. As the final dialysis wash water showed no catechin when analysed by UV-Vis spectrometry, we postulated that the catechin was hydrogen bonded to the polymer.

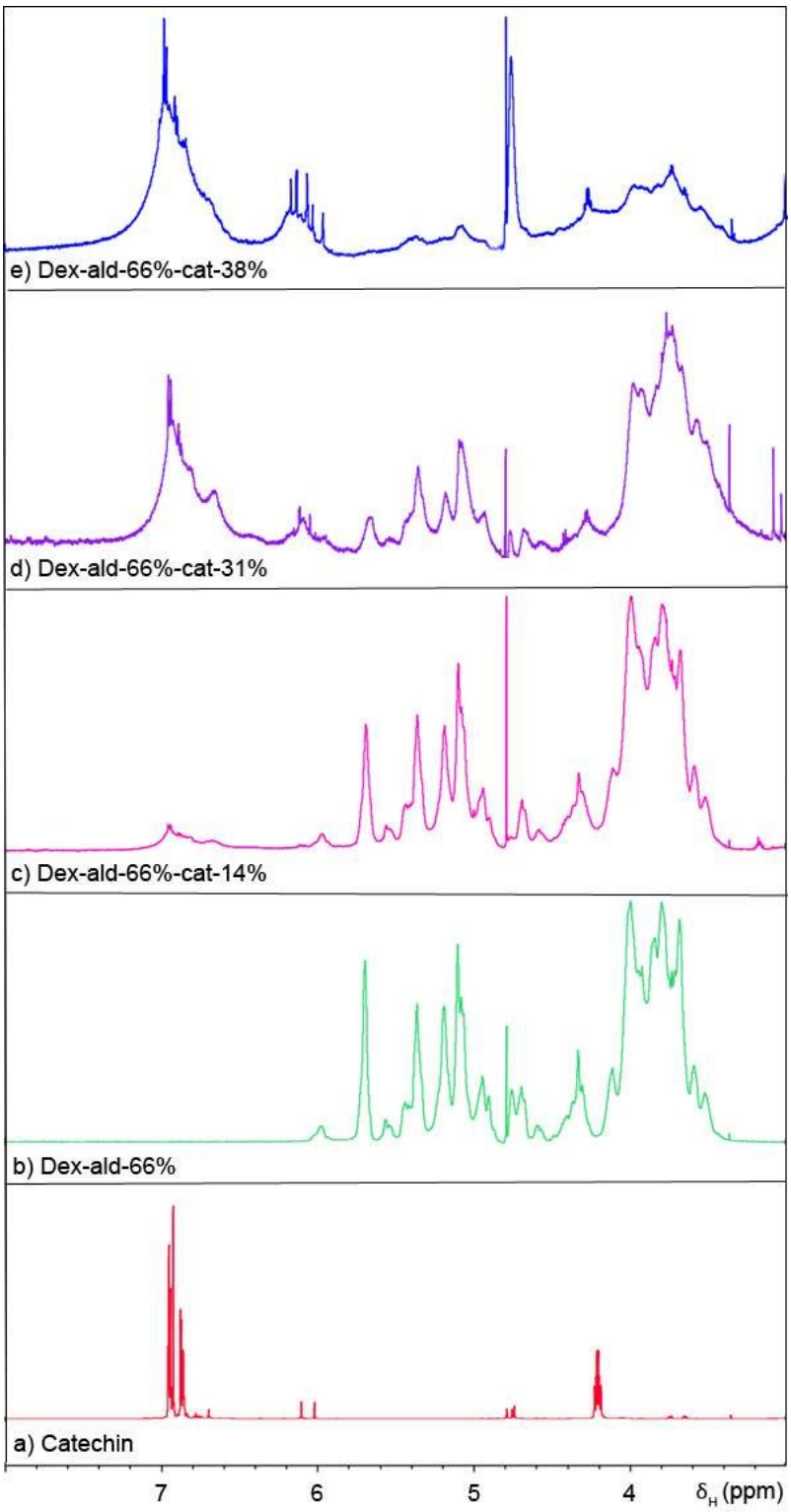


Figure 1: Overlay of ^{1}H NMR spectra of catechin (a), dex-ald with 66% oxidation (b), and dex-ald-cat conjugate with 66% oxidation and with 14 wt% (c), 31 wt% (d) and 38 wt% (e) catechin, respectively in 15% DMSO-d_6 / 85% D_2O. Note: (c) was conjugated at 25 ^{o}C, (d) was conjugated at 55 ^{o}C and (e) was conjugated at 70 ^{o}C.

To confirm that catechin was hydrogen bonded to dex-ald, in addition to being covalently bound, Diffusion Ordered SpectroscopY (DOSY) NMR was performed. The fitted intensities were correlated to peak height rather than integrals so the results would be more sensitive to the sharp peaks. DOSY NMR is commonly used to identify compounds in mixtures by separating the NMR signal based on differing diffusion coefficients. **Figure 2** shows an example of a DOSY spectrum of

a physical mixture of dex-ald-cat and unbound (free) catechin. As can be seen, the signals from the aromatic catechin protons between 6 ppm and 7 ppm stretch down from the lower diffusion co-efficient corresponding to dex-ald-cat to the higher diffusion coefficient corresponding to catechin. If free catechin was present in our samples, as well as covalently bound catechin, we would expect to see similar DOSY spectra for our conjugates.

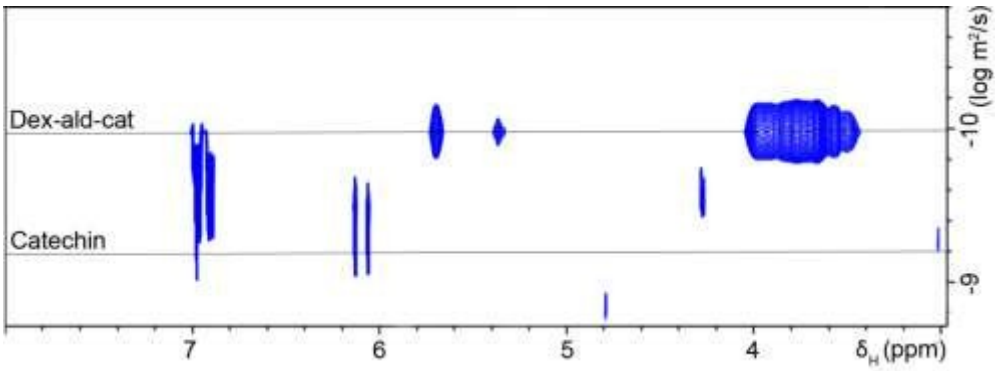


Figure 2: DOSY spectrum of physical mixture of dex-ald-cat and unbound catechin.

Figure 3 shows DOSY spectra for free catechin, dex-ald and the dex-ald-cat conjugates whose ^{1}H NMR spectra are shown in **Figure 1**. The free catechin DOSY signal is centred on -9.2 log (m^2/s) whereas the dex-ald-cat signals are centred at lower diffusion co-efficients of -9.9 to -10.0 log (m^2/s). No signal is seen stretching down to the level of the free catechin signal on the DOSY spectra of any of the dex-ald-cat conjugates, therefore, it is likely that the sharp catechin peaks are the result of hydrogen bonded catechin.

The integrals of the sharp catechin peaks were compared with the integral of the broad covalently bonded catechin peak to estimate the proportion of hydrogen bonded catechin. Regardless of the total catechin concentration, the proportion of hydrogen bonded catechin was found to be 11-12% of the total catechin content.

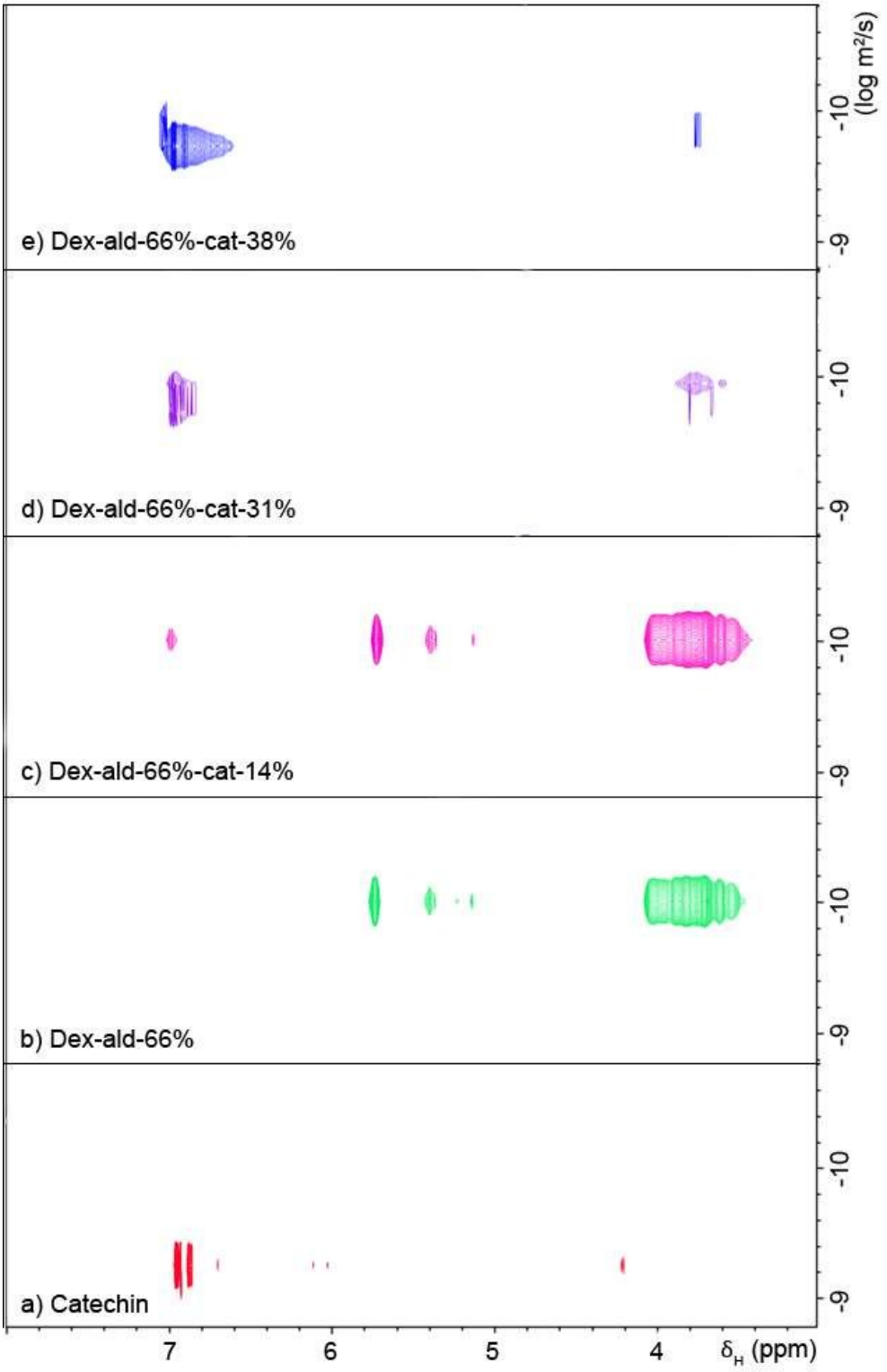


Figure 3: Overlay of DOSY spectra of catechin (a), dex-ald with 66% oxidation (b), and dex-ald-cat conjugate with 66% oxidation and with 14 wt% (c), 31 wt% (d) and 38 wt% (e) catechin, respectively in 15% DMSO-d_6 / 85% D_2O. Note: (c) was conjugated at 25 ^{o}C, (d) was conjugated at 55 ^{o}C and (e) was conjugated at 70 ^{o}C.

Catechin comprises five hydroxyl groups, which can participate in hydrogen bonding, and dextran contains a number of oxygen atoms, which are potential recipients of this bonding. To determine if catechin was hydrogen bonding to the oxygens in the dextran chain, dextran was reacted with catechin under the same conditions as for the synthesis of dex-ald-cat at 25 ^{o}C and 40

^{O}C. Following purification by dialysis, the samples were analysed by ^{1}H NMR (see **Figure S2b**) and Folin & Ciocalteu's phenol reagent (see **Table 1**) and were found, in fact, to contain no catechin. However, dex-ald-cat also contains a number of carbonyl bonds – aldehyde groups from dex-ald as well as some quinone groups from partially oxidised conjugated catechin, particularly in the case of conjugations performed at higher reaction temperatures. As carbonyl bonds are more polar than the other oxygen bonds in dex-ald, it was surmised that catechin was hydrogen bonding with aldehyde groups and/or quinone groups.

The nature of the hydrogen bonding was further investigated by analysis of the products from the reduction of dex-ald-cat with sodium borohydride (dex-cat-red). Sodium borohydride is known to reduce the aldehyde groups in dex-ald to alcohol groups[39] and reduce quinones to catechols (phenolic groups).[40] For the conjugates that were originally conjugated at 25 ^{O}C, the reaction mixture faded in colour from brown to almost colourless during the reaction, suggesting that the majority of quinones[41] were reduced back to phenolic groups. Pure catechin is colourless but usually presents as light brown due to partial oxidation of the phenolic groups. Despite the likely reduction of most quinones back to phenolic groups, analysis by Folin & Ciocalteu's phenol reagent showed a reduction in catechin content for the reduced conjugate (see **Table 1**), indicating that some catechin was removed as a result of the reduction process. Furthermore, ^{1}H NMR analysis (see **Figure S3a, b**) showed that all hydrogen bonded catechin was removed following the reduction reaction.

During the reduction reaction of the dex-ald-cat that was originally conjugated at 55 ^{O}C, the reaction mixture also faded in colour. However, some colour still remained at the end of the reaction, suggesting some quinone groups were still present on the conjugate. A greater density of quinone groups would be expected on this conjugate compared with those conjugated at 25 ^{O}C owing to the higher degree of catechin functionalisation as well as higher levels of oxidation during the conjugation (a deepening in colour of the reaction mixture was noted during the conjugation reaction). The ^{1}H NMR spectrum of the reduced conjugate (see **Figure S3d**) showed a substantial decrease in the height of the peaks associated with hydrogen bonded catechin compared with the original dex-ald-cat (see **Figure S3c**). No peaks remained that were associated with dex-ald so it is likely that the catechin was hydrogen bonded with the remaining quinone groups. Interestingly, although the ^{1}H NMR spectra revealed a decline in the amount of catechin that was hydrogen bonded to the conjugate after reduction with sodium borohydride, a small

increase in the weight percentage of catechin was measured by the Folin & Ciocalteu assay (33 wt% vs. 31 wt%).

Reaction conditions were varied to determine the effect of dex-ald degree of oxidation, catechin concentration, atmosphere (air vs. nitrogen), and temperature on the degree of functionalisation of dex-ald-cat (see **Table 1**). **Figure 4a** shows the effect of varying the degree of oxidation of dex-ald on the functionalisation of the conjugate at temperatures of 25 ^{O}C and 40 ^{O}C. As previously mentioned, no catechin was conjugated at either temperature when the dextran was unoxidised. A substantial rise in the catechin functionalisation of the conjugate was observed at both temperatures when the degree of dex-ald oxidation was increased from 33% to 50% (9 wt% to 14 wt% at 25 ^{O}C and 17 wt% to 22 wt% at 40 ^{O}C) but no further increase in catechin content was observed following a further rise to 66% degree of oxidation.

The effect of varying catechin concentration for a reaction with 66% oxidation dex-ald at 40 ^{O}C is shown in **Figure 4b**. Not surprisingly, as the concentration of catechin in the reaction mixture was increased, the level of catechin functionalisation of the conjugate was also increased with the catechin content rising from 16 wt% at 0.09 mol/L to 37 wt% at 0.36 mol/L catechin concentration. The effect of increasing catechin concentration was also explored at other temperatures and degrees of oxidation (see **Table 1**) and a positive effect on final catechin content was also observed under these conditions.

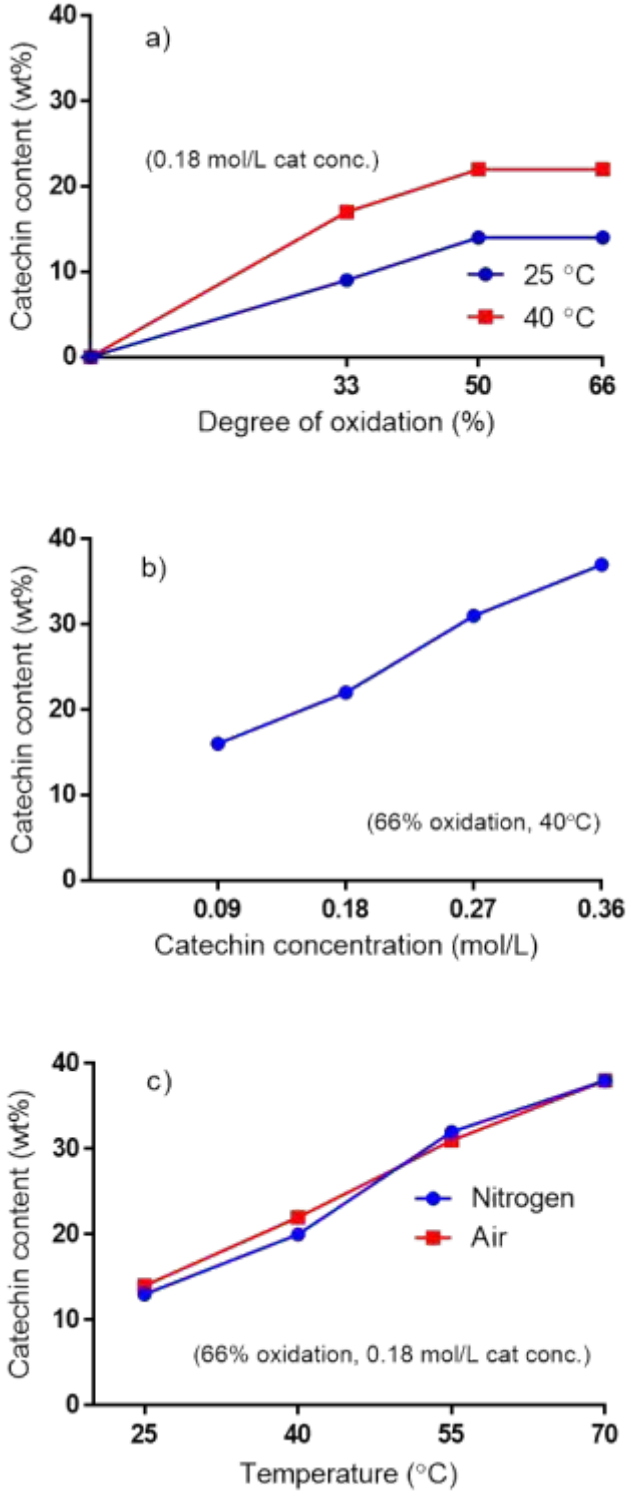


Figure 4: Catechin content of Dex-ald-cat as a function of degree of oxidation of dex-ald (a), catechin concentration (b) and temperature (c).

Figure 4c shows the effect of reaction atmosphere and temperature on the degree of catechin functionalisation of the final conjugate. The Folin & Ciocalteu assay measures the reduction potential of the catechin added to the conjugate[42] and is therefore dependent on the degree of oxidation of the conjugated catechin. Reactions were undertaken at low pH and protected from light to minimise catechin oxidation.[12] It was also considered that purging the reaction mixture

with nitrogen and subsequently using a nitrogen atmosphere during the reaction could protect the catechin from oxidation, particularly at higher temperatures, and therefore lead to a higher measured catechin content for the conjugate. However, purging and changing the atmosphere did not significantly alter the measured catechin content of the final conjugate. In contrast, reaction temperature had a marked effect on the degree of catechin functionalisation with an elevation in measured catechin content from 13-14 wt% at 25 ^{O}C to 38 wt% at 70 ^{O}C. This increase was almost linear (r^2 = 0.9979) up to 70 ^{O}C despite a deepening in colour of the reaction mixture at 55 ^{O}C and 70 ^{O}C, suggestive of some catechin oxidation.

Although an increase in the degree of catechin functionalisation with an increase in reaction temperature was expected owing to faster reaction kinetics at elevated temperatures, the substantial increase seen suggested the influence of other factors as well. The aldehyde groups in dex-ald are believed to exist primarily as hemiacetals at room temperature[43, 44] (see **Scheme S1**). We hypothesised that, as the temperature increased, the equilibrium shifted to a greater proportion of aldehyde groups thereby providing more reaction sites for catechin. To test this hypothesis, dex-ald was dissolved in an equivalent reaction mixture (water, DMSO and acetic acid) using deuterated solvents and no catechin. This solution was then analysed by ^{1}H NMR spectroscopy at each reaction temperature (see **Figure 5**). At 25 ^{O}C the aldehyde peak was barely visible but it increased markedly at each temperature rise, thereby confirming our hypothesis.

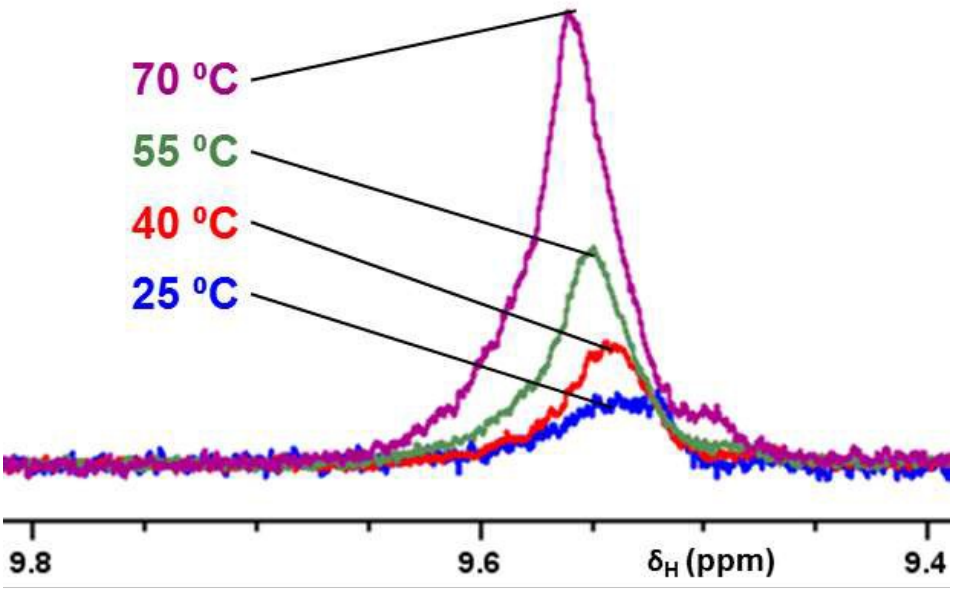


Figure 5: ^{1}H NMR spectra of dex-ald-66% aldehyde peak at different temperatures. Spectra were scaled so non-aldehyde peaks were consistent heights.

Some evidence of polymer breakdown was noted in the ^{1}H NMR spectrum of the dex-ald-cat conjugate reacted at 70 ^{O}C (see **Figure 1e**). This was explored further by performing size exclusion chromatography on samples of dextran, dex-ald-66% and dex-ald-66%-cat conjugates, which had been reacted at 25 ^{O}C, 40 ^{O}C, 55 ^{O}C, and 70 ^{O}C (see **Table S1** and **Figure S4**). Owing to no suitable reference standards being available and considerable differences in the solubility of dextran (sparingly), dex-ald (highly) and dex-ald-cat (slightly) in DMAc likely affecting hydrodynamic volume, no valid molecular weight values could be determined. However, some information could still be gleaned from the SEC traces and dispersities.

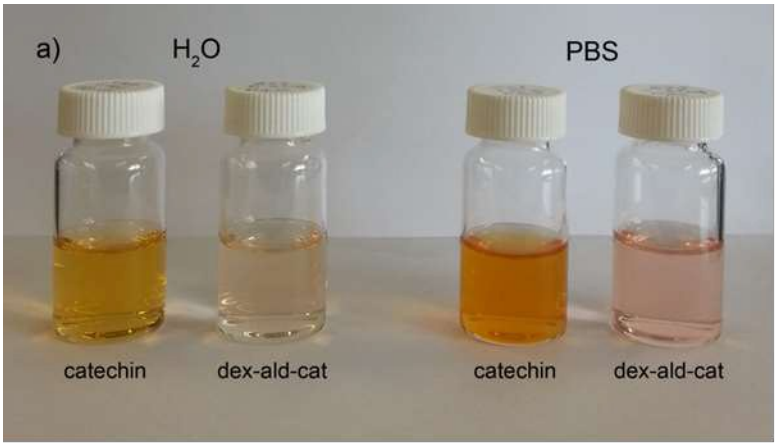
Firstly, consistent with the literature,[32] an increase in dispersity was noted when dextran was oxidised to dex-ald (1.25 to 1.49) but the SEC trace remained smooth and unimodal. As expected, dispersity increased further when catechin was conjugated to dex-ald (1.65 and 1.68 when functionalised with 14 wt% catechin at 25 ^{O}C and 22 wt% catechin at 40 ^{O}C, respectively) but decreased to 1.45 when functionalised with 31 wt% catechin at 55 ^{O}C, possibly due to more chain to chain consistency at higher levels of functionalisation. Consistent with the evidence of breakdown noted in the ^{1}H NMR spectrum, the SEC trace for dex-ald-cat reacted at 70 ^{O}C was multimodal and a dramatic drop in molecular weight was noted.

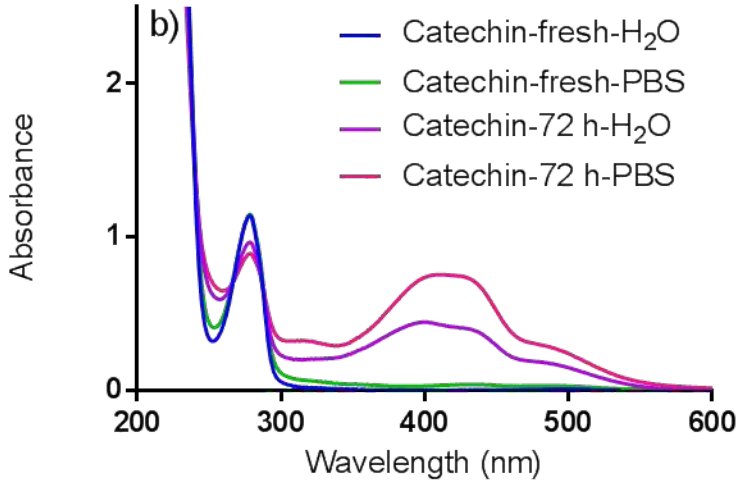
Confirmation of enhanced catechin stability

Poor serum stability is one of the major drawbacks of catechin and limits its clinical use. Catechin is particularly unstable in dilute solutions,[11] pHs above 6 and when exposed to heat.[12] The instability of catechin is primarily due to the oxidation of the two phenol groups on the catechol moiety (B ring) to a semiquinone and, subsequently, a quinone.[45] Evidence of this oxidation is readily observable from visual inspection of oxidised solutions, which show a characteristic yellow-brown colour; UV-Vis spectra, which show the appearance of new peaks with the largest around 430 nm;[46] and the Folin-Ciocalteu assay, which measures the reducing capacity of the solution.[42]

A key expectation from conjugating catechin with dex-ald was to afford an improvement in stability. To confirm that we had, in fact, enhanced its stability, native catechin and dex-ald-cat with an equivalent concentration of catechin were dissolved in both DI water and PBS and the solutions incubated for 72 h at 37 ^{O}C. Owing to the absorption of CO_2 from the atmosphere, DI

water typically has a pH between 5 and 6. We therefore expected some protection of the catechin from the mildly acidic conditions.[12] Despite this, a definite yellowing of the catechin in DI water solution was noted (see **Figure 6**a) but no discernible colour change was seen for the dex-ald-cat solution. Moreover, the UV-Vis spectra showed a clear peak around 430 nm for the catechin solution after incubation (see **Figure 6b**), confirming oxidation, but no peak was seen for the dex-ald-cat solution (see **Figure 6c**). Finally, the Folin-Ciocalteu assay showed a 15% decrease in reducing power for the catechin solution but only a 2% decrease (within error margin) for the dex-ald-cat solution.





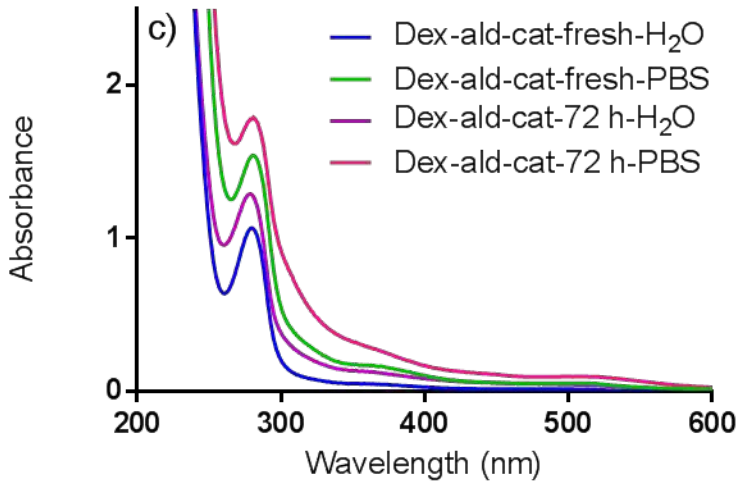


Figure 6: Effect of incubation of catechin and dex-ald-cat in H_2O and PBS for 72 h at 37 ^{0}C. (a) Samples after incubation. (b) UV-Vis spectra of catechin before and after incubation. (c) UV-Vis spectra of dex-ald-cat before and after incubation.

In PBS, with a pH of 7.4, the difference was even more dramatic with the catechin solution showing a greater deepening in colour (see **Figure 6**a) and a higher peak at 430 nm (see **Figure 6b**). A 25% decrease in reducing power was measured for the catechin in PBS solution whilst the dex-ald-cat solution showed no decrease in reducing power. No peak was visible at 430 nm in the

UV-Vis spectrum of the incubated dex-ald-cat in PBS solution, confirming no significant oxidation had occurred. Interesting, an increase in the intensity of the peak at 280 nm was noted in both the fresh and incubated samples of dex-ald-cat in PBS solutions but not in the catechin in PBS solutions. It is possible that this is a result of the ionic PBS solution breaking hydrogen bonds leading to an increase in resonance of the aromatic rings and hence a hyperchromic shift.

Confirmation of anticancer activity

Neuroblastoma is an aggressive tumour, which primarily affects infants and children. Despite intensive therapy, survival rates are less than 50%[47] and survivors frequently have lifelong health issues stemming from their treatment.[48] Therefore, less toxic treatments are urgently required. The anticancer activity of dex-ald-cat was evaluated in two independent neuroblastoma cell lines (IMR-32 and BE(2)-C) as well as in non-malignant (MRC-5) cells (see **Figure 7**).

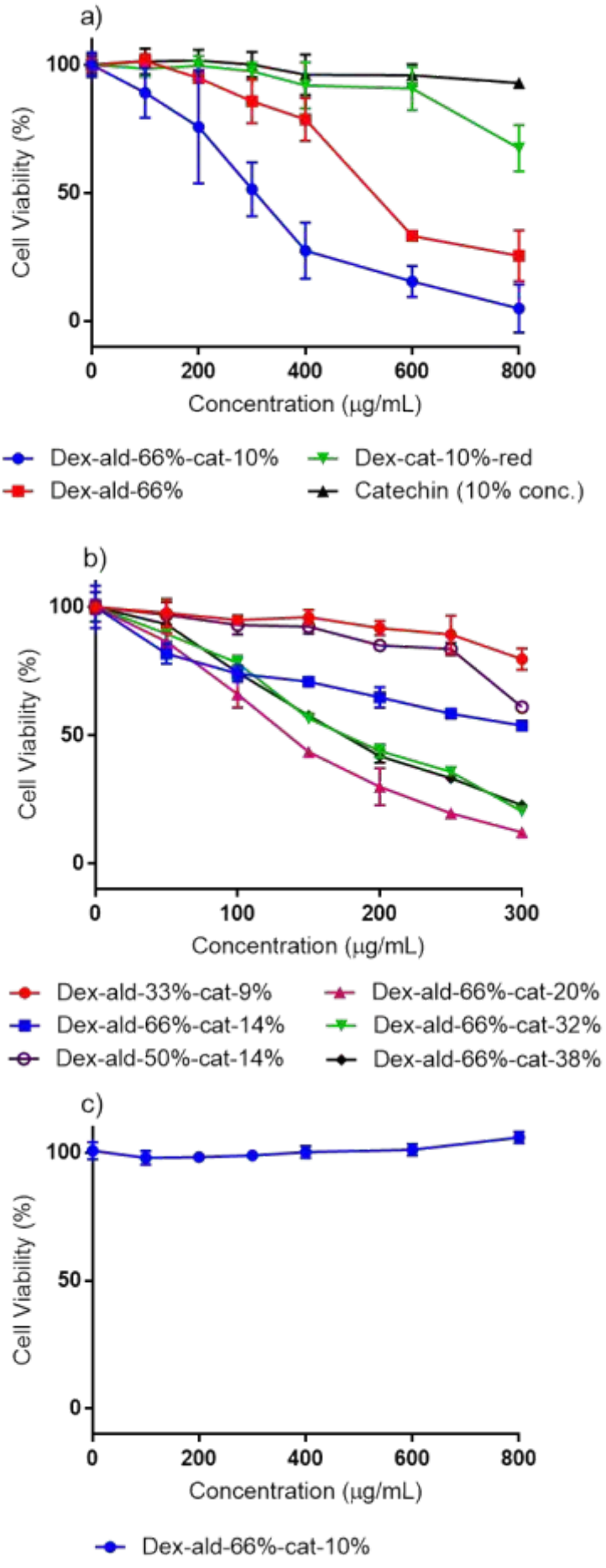


Figure 7: Effect of treatment on cell viability for neuroblastoma cells, IMR-32 (a) and BE(2)-C (b) and normal cells, MRC-5 (c).

Dex-ald has been shown to be cytotoxic to normal cells[28, 32, 49] so we expected to see some activity against neuroblastoma cells from this compound alone. Treatment of neuroblastoma cells, IMR-32, with dex-ald with a 66% degree of oxidation for 72 h decreased cell viability at concentrations above 600 μg/mL ($P < 0.0001$) (see **Figure 7a**). More importantly, when these cells were treated with dex-ald-cat containing 10 wt% of catechin, a further decrease in cell viability was observed with activity demonstrated for concentrations as low as 200 μg/mL ($P=0.0111$) (see **Figure 7a**). In contrast, free catechin, when tested at equivalent concentrations to catechin in the conjugate (i.e. 10% of conjugate concentration), showed no significant effect on IMR-32 cells even at the highest equivalent concentration tested (see **Figure 7a**), indicating improved anticancer activity of conjugated catechin compared with free catechin. It should be noted that, unlike imine bonds with dex-ald, the covalent bond between dex-ald and catechin is not acid labile and hence the anticancer activity can be attributed to the conjugate and not released catechin.

The effects on cell viability of different degrees of oxidation and catechin functionality of dex-ald-cat were assessed on the BE(2)-C neuroblastoma cell line (see **Figure 7b**). Dex-ald-cat with 66% oxidation and 20 wt% functionalisation with catechin was found to be the most efficacious conjugate tested, demonstrating a significant cytotoxic effect on cells at 50 μg/mL ($P<0.0001$) and showing a significantly greater effect than all other conjugates at concentrations of 100 μg/mL and above ($P < 0.0001$ to $P= 0.0464$). Interestingly, conjugates with the same degree of oxidation but a greater degree of catechin functionalisation (30 wt% and 38 wt%) demonstrated slightly lower cytotoxicity, possibly due to the decrease in aldehyde groups from catechin conjugation. It is worth noting that the conjugate tested with 38 wt% catechin was the dex-ald-cat that was reacted at 70 ^{O}C so although the polymer was degraded, it still maintained anticancer efficacy. Not surprisingly, conjugates with lower degrees of oxidation and catechin functionalisation showed lower levels of efficacy. Dex-ald-cat with 33% oxidation and 9 wt% catechin functionalisation showed cytotoxicity at 200 μg/mL ($P= 0.0248$) whereas dex-ald-cat with 50% oxidation and 14 wt% catechin functionalisation showed cytotoxicity at 150 μg/mL ($P= 0.0463$). In contrast, dex-ald-cat with 66% oxidation and 14 wt% catechin functionalisation showed cytotoxicity at 50 μg/mL ($P<0.0001$) – again demonstrating the importance of both aldehyde groups and catechin functionality in the cytotoxic efficacy of dex-ald-cat.

As dextran aldehyde has known cytotoxicity in normal cells, it was critical that dex-ald-cat was assessed in non-malignant cells as well. MRC-5 cells were treated for 72 h with dex-ald-cat-10%

and no effects on cell viability were seen for any of the concentrations tested (up to 800 μg/mL) (see **Figure 7c**).

A number of mechanisms are believed to be responsible for the cytotoxic effects of aldehydes, including the promotion of reactive oxygen species (ROS)[50]. Polyphenols, like catechin, whilst behaving as antioxidants under normal physiological conditions, act as pro-oxidants in the presence of high levels of transition metal ions[51, 52] as is the case in malignant tumour cells.[53, 54] Therefore, it is possible that catechin is potentiating the pro-oxidant effects of dex-ald in tumour cells where it also has pro-oxidant effects but attenuating the effects of dex-ald in normal cells where it has antioxidant effects. Further evidence that catechin is potentiating the effects of dex-ald in neuroblastoma cells is seen in the decreased cytotoxic effect when the aldehyde groups in dex-ald-cat are reduced to alcohol groups (dex-ald-red). Dex-cat-red with 10 wt% degree of catechin functionalisation did not show a significant reduction in cell viability until concentrations of 800 μg/mL were reached (P=0.0003) (see **Figure 7a**). Collectively this data shows that Dex-ald-cat is active against the neuroblastoma cells but has minimal effects on the non-malignant cells.

Conclusion:

We have developed a method for the functionalisation of dextran aldehyde with 5 to 38 wt% catechin, conferring enhanced stability compared with native catechin. The catechin functionalised dextran aldehyde (dex-ald-cat) demonstrated superior cytotoxic efficacy against neuroblastoma cells compared with either dextran aldehyde (dex-ald) or reduced catechin conjugated dextran (dex-cat-red). Importantly, dex-ald-cat was not cytotoxic against normal cells at the highest dose tested. Dex-ald-cat therefore, shows promise as a potential treatment for neuroblastoma. We are currently investigating adapting this chemistry for use with other polyphenols and polysaccharides.

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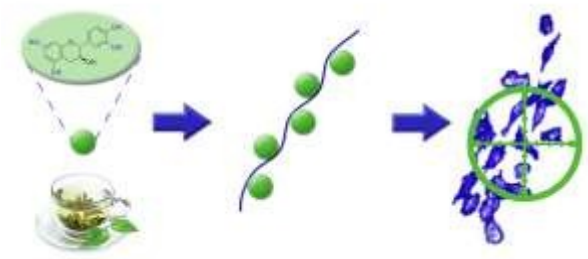
Table of Content for Use Only

Efficient Functionalisation of Dextran-Aldehyde with Catechin: Potential Applications in the treatment of Cancer

Susan Oliver,[a,b] Donald S. Thomas,[c] Maria Kavallaris,[d,e] Orazio Vittorio,[d,e*] and Cyrille Boyer[a,b*]



Dextran aldehyde was functionalised with up to 38 wt% catechin and the resulting conjugate demonstrated cytotoxic efficacy against neuroblastoma cells.