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COMMUNICATION

Imaging in Living Cells by use of ν B-H Raman Spectroscopy: Monitoring COSAN UptakeMàrius Tarrés^a, Elisabetta Canetta^{b,c}, Clara Viñas^{a*}, Francesc Teixidor^a and Adrian J. Harwood^{b*}Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
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The boron-rich cobaltabis-dicarbollide (COSAN) and its 8,8'-I2 derivative (I2-COSAN), both of purely inorganic nature, are shown to accumulate within living cells, where they can be detected by ν B-H Raman Microspectroscopy. This demonstrates an alternative method for cell labelling and detection.

The inorganic, boron-based molecule cobaltabisdicarbollide, [3,3'-Co(1,2-C₂B₉H₁₁)₂]⁻, commonly known as COSAN, comprises of a cobalt atom sandwiched by two carboranyl clusters.¹ This structure exhibits both electrostatic interactions, via a dispersed negative ionic charge covering the whole molecule,² and non-bonding intermolecular interactions between its weakly polarized B-H and C-H bonds.³ This duality imparts a molecular property of being simultaneously hydrophobic and hydrophilic, and makes COSAN soluble in both water and oils. The polarized lipid molecules that make up biological membranes also possess amphiphilic properties, and can assemble into membranes and vesicles formed from lipid bilayers. COSAN has also been shown to form small nano-vesicles and above a critical aggregation concentration ($cac_{vesicle} \approx 0.01$ mM), begin to form micelles.⁴ However, unlike lipid bilayer membranes, the membranes of COSAN vesicles are monolayer. Recently, [3,3'-Co(8-I-1,2-C₂B₉H₁₀)₂]⁻, I2-COSAN, has also been found to self-assemble into a lyotropic lamellar phase with sufficient curvature to create closed vesicles.⁵ These similarities in physicochemical properties to biological lipids suggest that COSAN molecules may be compatible with living cells, and could be used to label cells in a similar manner to lipophilic dyes. Here, we use Raman Microspectroscopy to show accumulation of COSAN and I2-COSAN in living cells.

As a first step in this work, COSAN or I2-COSAN was added directly to the Dulbecco's Modified Eagle medium (DMEM) with Human Embryonic Kidney (HEK293) cells. When the cells were concentrated by low speed centrifugation, washed and incubated in new DMEM medium, visual inspection showed accumulation of COSAN or I2-COSAN (orange in colour) within cell pellets (Fig. 1). This observation indicated that these COSAN molecules can be taken up by living cells.

Raman offers a useful tool for visualising COSAN uptake, as carboranyl clusters are rich in B-H bonds, which are not present in cells. Particularly convenient, the B-H stretching frequency band at 2570 cm⁻¹ is at a region of the vibrational spectrum in which no other frequencies seen in organic molecules can be found.⁶ To establish the conditions for COSAN and I2-COSAN detection, Raman spectra were acquired for COSAN in solutions in a concentration range 2-100 mM. Both COSAN and

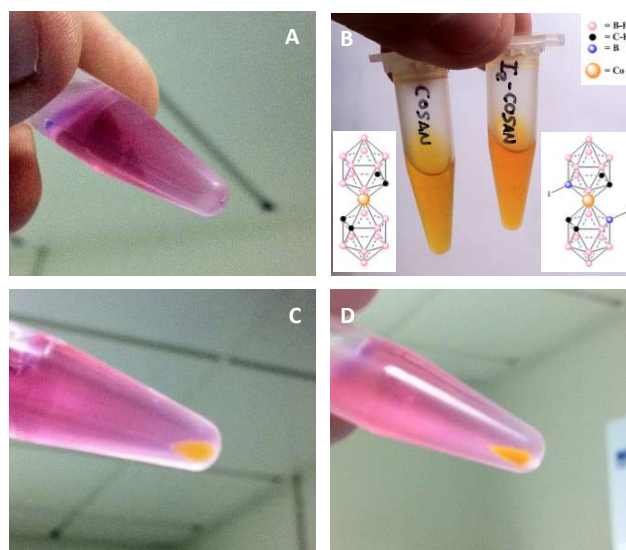


Figure 1.- (A) Untreated HEK293 cells (white dot at the bottom) in DMEM medium. (B) 25 mM solution of COSAN and I2-COSAN in PBS. (C) COSAN treated HEK293 cells after COSAN-DMEM removal, washing with DMEM medium and incubating in new DMEM medium. (D) The same as (C) but using I2-COSAN instead of COSAN.

I2-COSAN produced a distinct spectral peak at a frequency near 2570 cm⁻¹ (Figure 2), which was detectable above a concentration of 10 mM.

Cells were incubated in DMEM that contained COSAN (25 mM) (or I2-COSAN) on poly-L-lysine coated glass microscope coverslips for 1 hour. Cells were then washed with Phosphate-Buffered Saline (PBS) and imaged using Raman Microspectroscopy. For visualisation, cell samples were stimulated with a diode pumped solid-state (DPSS) laser operating at 532 nm and visualised with a Raman Microscope system (Eclipse Ti-U, Nikon).⁷ As a control, living HEK293 cells were analysed without COSAN (or I2-COSAN) by performing Raman imaging on the untreated cells (Figure 3). A uniform distribution across the cell of C-H bond stretching, corresponding to an intense peak at 2950 cm⁻¹, but no COSAN signal at 2570 cm⁻¹ were observed. In contrast, cells treated with 25 mM COSAN, or I2-COSAN, exhibited the distinctive B-H peak at 2570 cm⁻¹ (Figure 4). No such strong peak was observed outside the cell (Figure 4A) indicating a high accumulation of COSAN inside de cells.

If cells that had been treated with COSAN (Figure 4B) or I2-

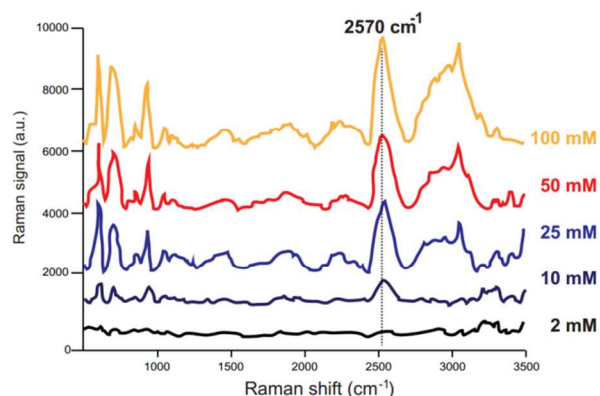
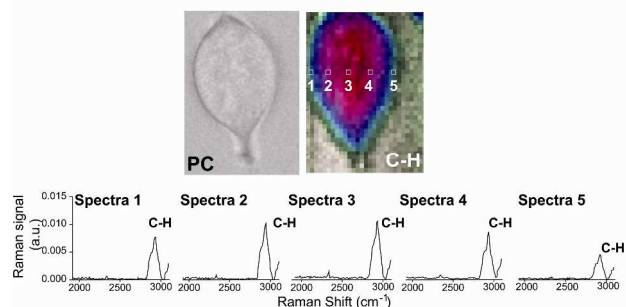


Figure 2.- Raman spectra calibration at different concentrations of I2-COSAN. The absorption at 2570 cm^{-1} that corresponds to ν B-H is detectable above 10 mM.

5 COSAN were washed and then re-incubated in absence of the compounds, the ν B-H band was still observed inside the cell, but was then gradually lost over times. Raman imaging at points across the cell showed a loss of uniformity in the C-H distribution when the cells were exposed to COSAN, the presence of COSAN
10 within the cytoplasm and to a lesser extent in the nucleus. Although dispersed throughout the cell, there appeared to be local subcellular COSAN accumulation within the cytoplasm. The identity of these regions is currently unclear, however areas of high ν B-H signal match the areas of high C-H content (Figure
15 4C), indicating that COSAN-rich areas are associated with cell components, and are unlikely to exclude major proportions of the cytoplasm contents.

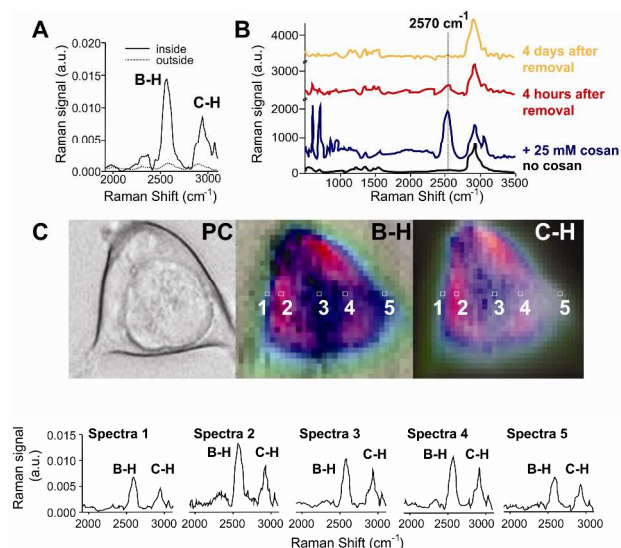


20 **Figure 3.-** Cellular imaging of COSANs untreated control HEK293. Images show phase contrast image (PC) and Raman chemical images at 2950 cm^{-1} (ν C-H peak). Five Raman spectra (Spectra 1-5 below) show the spectral fingerprint of the cell at different positions across the cell.
25 Pink zones in the C-H Raman image display high C-H content. The two weaker signals are at sites 1 and 5 at the cell, whereas the more intense are at 2-4 sites. No Raman signal at 2570 cm^{-1} (ν B-H peak) was present.

Conclusions

30 This report demonstrates a novel means of cell labelling that uses the B-H rich molecules COSAN and I2-COSAN, both of purely inorganic nature, and their direct imaging by Raman Microspectroscopy. We show that COSAN and I2-COSAN readily accumulate inside cells and are retained for at least four hours post-labelling, providing the opportunity to monitor
35 labelled cells for prolonged periods. In general, this indicates that COSANs, but perhaps also other boron cluster molecules, offer a means of cell labelling and unequivocal detection against the

background of all other cellular molecules, be they proteins, lipids, DNA or other biopolymers.



40 **Figure 4.-** (A) Comparison of spectral fingerprints of HEK293 cells treated with COSAN 25 mM for 1 hour. Spectra are normalised to the area under the ν C-H peak. The amount of I2-COSAN (based on B-H as the reference peak) was $\sim 85\%$ higher inside the cell (solid line) than in the external medium (dotted line). (B) Spectra were obtained both inside
45 and outside cells and 4 hours and 4 days after COSAN had been removed, the cells washed and then replaced in fresh medium. (C) Chemical imaging of HEK293 cell treated with 25mM COSAN. PC shows phase contrast image while B-H and C-H display Raman chemical images at 2570 cm^{-1} (B-H peak) and 2950 cm^{-1} (C-H peak). Pink zones in the B-H Raman image show I2-COSAN accumulation inside the cell, whereas pink zones in the C-H Raman image display high C-H content. Five Raman spectra (Spectra 1-5 below) show the spectral fingerprint of the cell at different positions across the cell.
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55 In the present study, we have examined the behaviour of COSAN and I2-COSAN alone, however improvements in derivatization chemistry of boron clusters makes it possible to functionalize molecules of biological interest.⁸ For example,
60 COSAN, and other borane/heteroborane clusters can be coupled to bioactive molecules, such as antibodies and protein ligands, and these protein-conjugated heteroboranes can be targeted to specific cell types.⁹ Given the apparent ease in which COSAN molecules enter cells, it may be possible to tag molecules of
65 biological interest and image them within subcellular localisations.

Medicinal boron chemistry is now attracting growing research interest¹⁰ and investment from the pharmaceutical industry in the quest of novel drugs to tackle cancer and infectious diseases,
70 potentially overcoming limitations and side effects of current products. These observations offer a direct approach to monitor and track Boron-based drugs at the cellular level.

Notes and references

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