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COMMUNICATION

Encapsulation of *closo*-dodecaiododododecaborate in 2-hydroxypropyl-γ-cyclodextrin prevents hemolysis†

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$Na_2B_{12}I_{12}$ has many of the properties desired by an X-ray contrast agent but is lethal at the concentrations needed for medical imaging. We demonstrate here that PBS solutions with > 50 mM $Na_2B_{12}I_{12}$ induce hemolysis, consistent with the previously reported superchaotropic nature of the anion. The presence of < 1 equiv of 2-hydroxypropyl-γ-cyclodextrin prevents hemolysis and suggests a strategy for exploiting $B_{12}I_{12}^{2-}$ as an X-ray contrast agent.

Although recent developments in magnetic resonance and nuclear imaging are driving advancement in medical diagnosis, X-ray imaging remains the most common form of medical imaging, allowing for rapid, non-invasive diagnosis.[1] X-ray tubes used in medical imaging typically contain a tungsten anode and, when operated between 50 and 150 kV, emit radiation with wavelengths ranging from 50 to 9 nm.[2] Radiographic contrast depends on the differences in the extent to which the imaged materials absorb the X-rays used (i.e., radiopacity), which in turn generally scales with the atomic numbers of the elements in those materials. As a result, Ca-containing bone material contrasts well with soft tissues that predominantly contain C, H, N, and O. Contrast between soft tissues can be obtained by introducing an X-ray contrast agent (XCA) of greater or lesser radiopacity.[3] An example of the former is the use of suspended $BaSO_4$ to image the gastrointestinal tract and an example of the latter is the injection of air into a joint to visualize the articular space. In many cases, e.g., when blockage or pressure is a concern, a soluble XCA is used. The most common soluble XCAs feature iodoarene rings functionalized with water-solubilizing groups. Although these iodinated species are generally well tolerated, some individuals reportedly suffer from contrast-induced nephropathy or disruption of thyroid function.[4, 5]

The similarities between arenes and *closo*-boranes have led to the investigation of the latter as a motif in drug design.[6] In particular, the icosahedral *closo*-dodecaborate scaffold, which occupies a volume approximately the same as that of an adamantyl group and roughly 50% larger than that of the sphere described by a rotating phenyl ring,[7] provides a rigid and biostable icosahedral framework upon which to construct functional molecules.[8] In the context of XCA design, it is noteworthy that modern iodine-containing XCAs feature 1,3,5-triiodophenyl groups (Fig. 1).[3] Although periodinated rings would afford greater contrast, they severely impair solubility. In contrast, the periodinated $B_{12}I_{12}^{2-}$ forms salts that are highly water-soluble. For example, $Na_2B_{12}I_{12}$ is 90% iodine by mass and can be prepared as solutions with > 200 mM concentration (>300 mg iodine mL^{-1}).

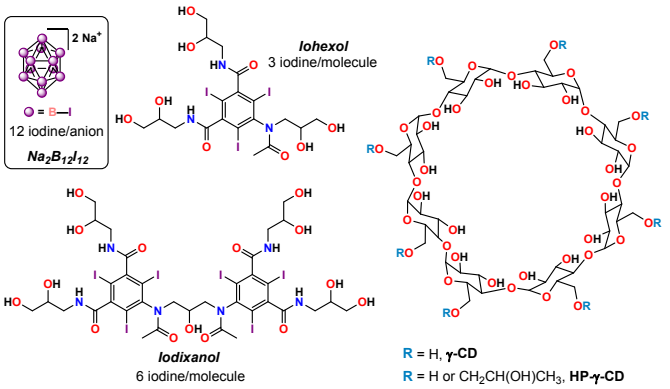

Fig. 1 Chemical structures of $Na_2B_{12}I_{12}$, two examples of commercially used iodinated XCAs (iohexol and iodixanol), as well as γ-cyclodextrin (γ-CD) and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD).

Given the high iodine content and water solubility of $Na_2B_{12}I_{12}$, it is unsurprising that in the same year that the synthesis of this salt was first reported,[9] it was investigated as an intravascular XCA.[10] This study found $Na_2B_{12}I_{12}$ to be toxic to mice and cats and the investigators concluded that further development of the compound for this application would be inappropriate and, indeed, no further reports appeared.[‡] The origin of the toxicity of $Na_2B_{12}I_{12}$ remained unexplained and seemed unlikely to stem from its chemical reactivity. Indeed,

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part of the original interest in using $B_{12}I_{12}^{2-}$ salts for biological applications stemmed from the stability of the B—B and B—I bonds and the distinct *lack* of reactivity of the cluster: earlier reports describe the $B_{12}I_{12}^{2-}$ ion being unchanged after treatment with $Cl_{2\,(g)}$, heating to 85 °C in 5 M NaOH, or heating to 150 °C in neat H_2SO_4.[9] We confirmed the inertness of the cluster under biologically relevant conditions by demonstrating a lack of any new ^{11}B NMR signals after refluxing $Na_2B_{12}I_{12}$ in phosphate-buffered saline (PBS, pH 7.4) for 1 h. There was also an absence of new signals after 24 h incubation at room temperature with a suspension of human red blood cells (RBCs), bovine serum, or defibrinated bovine blood (Fig. S1).

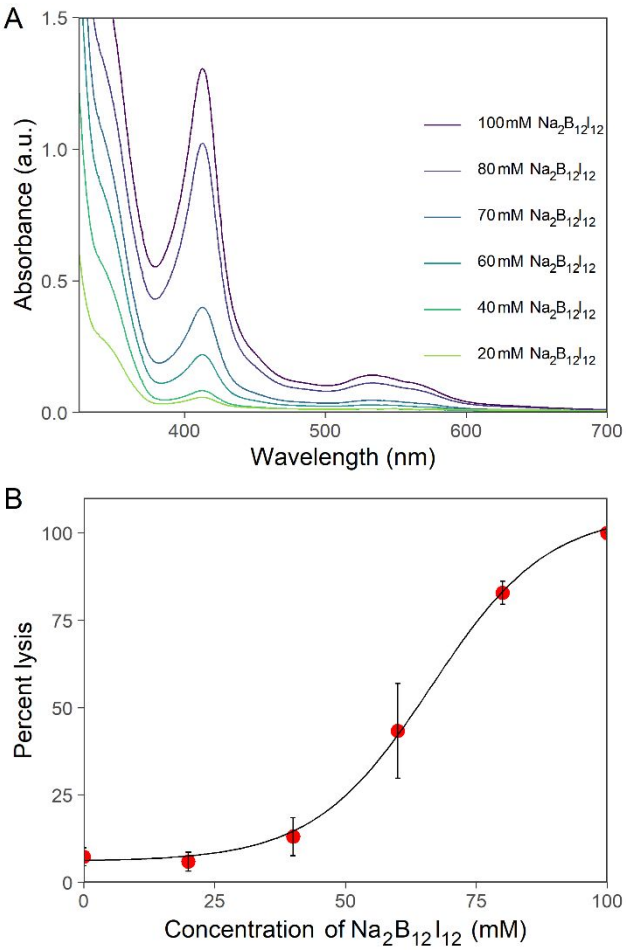

Fig. 2 A) Hb release from RBCs suspended in PBS (pH 7.4) containing increasing concentrations of $Na_2B_{12}I_{12}$. B) Proportion of RBCs lysed by $Na_2B_{12}I_{12}$ on the basis of the absorption of the supernatant at 413 nm (Soret band) after pelleting. Error bars reflect ± SEM for three independent replicates and the fitted curve was obtained by logistic regression (IC_{50} = 66.33 mM).

Insight into the biological effects of $B_{12}I_{12}^{2-}$ can be gleaned from more recent physical inorganic studies of the $B_{12}X_{12}^{2-}$ anions, where X = F, Cl, Br, or I. In aqueous solution, these ions are readily encapsulated in an appropriately sized supramolecular host molecule (e.g., cucurbituril, cyclodextrin, calixarene).[11, 12] Interestingly, the process is not governed by the entropy-driven hydrophobic effect but rather the enthalpy-driven chaotropic effect. In fact, because $B_{12}X_{12}^{2-}$ ions exhibit a chaotropism that far outstrips that of the classical Hofmeister series chaotropes, they are termed *superchaotropes*.[13] One manifestation of this chaotropism is the ability of $B_{12}X_{12}^{2-}$ ions to enhance the release of cargo from liposomes.[14-16] We reasoned that, at the high concentrations needed to function as an XCA, the superchaotropic activity of $B_{12}I_{12}^{2-}$ was disrupting the integrity of cell membranes. We observed that exposure of human RBCs to 100 mM $Na_2B_{12}I_{12}$ resulted in rapid hemolysis, as judged by clarification of the suspension. The dose dependence of this hemolytic effect was explored by suspending RBCs in a PBS solution of $Na_2B_{12}I_{12}$ for 10 s, followed by rapid centrifugation to pellet the cells, and removal of the supernatant. The extent of hemolysis was determined by measuring the absorbance of the hemoglobin (Hb) in the supernatant (Fig. 2).

If this hemolytic activity does indeed stem from the chaotropic activity of the borate anions, we hypothesized that encapsulation within a supramolecular host, which is driven by the same chaotropism, would prevent them from damaging cells. The interaction of $B_{12}I_{12}^{2-}$ with the most common biologically produced cyclodextrins (i.e., α-, β-, and γ-CD) has been previously explored by isothermal calorimetry and it interacts most strongly with γ-CD (Fig. 1; $K_a = 6.7 \times 10^4$ L mol^{-1}).[13] Single-crystal X-ray diffraction studies of $B_{12}Br_{12}^{2-}$/γ-CD show the perbrominated cluster to form a 2:1 complex with the cyclic oligosaccharide in the solid state.[13] We succeeded in growing crystals from a 2:1 mixture of γ-CD and $Na_2B_{12}I_{12}$ by allowing diethyl ether to diffuse into a DMF solution of the two species. ^1H and ^{11}B NMR spectra of solutions prepared from isolated crystals suggest that they contained both γ-CD and $B_{12}I_{12}^{2-}$, along with 4 equiv of DMF with respect to γ-CD (Fig. S5, S6). To confirm that the sample was not a mixture of γ-CD crystals and $Na_2B_{12}I_{12}$ crystals, isopycnic flotation density measurements were performed. The crystals all exhibited the same isopycnic point in a mixture of bromoform and hexanes. The measured density (ρ_{exp} = 1.6 g mL^{-1}) was greater than the calculated density of γ-CD (1.41 g mL^{-1} for the tetradecahydrate)[17] and below the calculated density of $Na_2B_{12}I_{12}$·6DMF·H_2O (2.19 g mL^{-1}, see ESI). This result is consistent with the present crystals containing both substances. Unfortunately, the crystals did not diffract beyond 1.5 Å (Fig. 3A), and no solution could be obtained by direct methods, Patterson methods, intrinsic phasing, or charge flipping.

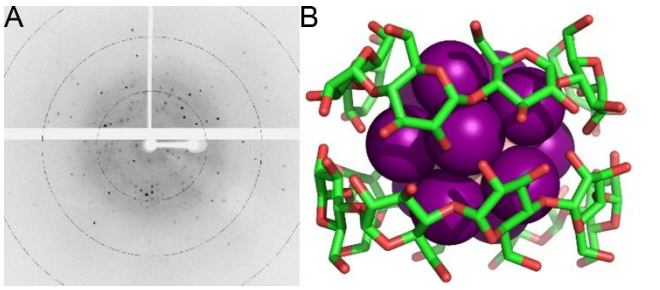

Fig. 3 A) X-ray diffraction (Cu Kα) from a crystal containing γ-CD, $Na_2B_{12}I_{12}$, and DMF showing clean, but low-resolution reflections. Circles are drawn at resolution levels of 3.66, 2.15, and 1.71 Å. B) Semi-empirically (PM6) optimized structure of a putative $[(\gamma\text{-CD})_2(B_{12}I_{12})]^{2-}$ complex based on cumulative crystallographic data and previous work with

$B_{12}Br_{12}^{2-}$.[13] $B_{12}I_{12}^{2-}$ is shown as spheres and γ-CD as sticks. Color code: I purple, B pink, C green, O red.

Nevertheless, the diffraction pattern was indexed and exhibited cubic metric symmetry (a = 60.0168(13) Å), which was consistent with the fact that the crystals were perpetually extinguished when viewed between crossed polarizers. The volume of the unit cell (216,181 Å^3) is consistent with the composition (γ-CD)$_2$·$Na_2B_{12}I_{12}$·8DMF and Z = 48, if the non-H atoms have the chemically reasonable average volume of 19 Å^3;[18] the number of DMF molecules is consistent with the NMR data obtained from the crystals. The systematic absences and enantiomeric purity of the γ-CD narrow the possible space groups to F23 and F432. With Z = 48, the complex would reside on a general position in the former and on a 2-fold axis in the latter. In summary, although the structure could not be solved, the aggregate crystallographic data suggest that the crystals may contain a 2:1 complex of the type observed for $B_{12}Br_{12}^{2-}$.[13] A semi-empirical (PM6) geometry optimization confirms that such a host-guest complex is a minimum on the potential energy surface of this supramolecular system (Fig. 3B). We stress that the structure depicted in Fig. 3B is a theoretically optimized structure that, although consistent with the data collected from the crystals, was not obtained by refinement of a full crystal structure against the observed structure factors.

Although the X-ray diffraction data support the interaction of γ-CD with $B_{12}I_{12}^{2-}$, and suggest that they may form a 2:1 complex in the solid state, gas-phase experiments and solution-phase thermodynamic measurements indicate that the 1:1 complex predominates in solution.[13, 19, 20] To appropriately avoid deviation from ideal-solution behavior, the prior thermodynamic measurements were performed on relatively dilute solutions (< 0.5 mM).[11, 13, 21, 22] Although $Na_2B_{12}I_{12}$ and γ-CD are both highly water-soluble, we have observed that PBS solutions containing > 6 mM of each species form an insoluble gel. Addition of PBS to dilute the mixture below this concentration produces fluid solutions. Unfortunately, as depicted in Fig. 2, $Na_2B_{12}I_{12}$ alone does not induce hemolysis at concentrations below 6 mM. Although the chaotropism-driven complexation of $B_{12}I_{12}^{2-}$ by γ-CD may well prevent the physical interaction with cells that leads to hemolysis, the low solubility of the complex prevents us from investigating this effect.

We turned, therefore, to the more water-soluble 2-hydroxypropyl-γ-CD (HP-γ-CD).[§] Semi-empirical (PM6) geometry optimization calculations predicted that the hydroxypropyl groups would not significantly perturb the host-guest interaction, as compared to unfunctionalized γ-CD (Fig. 4). ^{11}B NMR spectroscopic experiments show that addition of HP-γ-CD to solutions of $Na_2B_{12}I_{12}$ produces a shift in the resonance for the cluster (Fig. S2). The method of continuous variation permitted the stoichiometry of the complexation between HP-γ-CD and $B_{12}I_{12}^{2-}$ to be determined, confirming that the two form a 1:1 complex in PBS (Fig. S3). Although the ^{11}B resonance broadens in addition to shifting downfield as the molar fraction of HP-γ-CD is increased, the sharp nature of the Job plot speaks to the strength of the interaction. Further detailed studies are needed to quantify the thermodynamic parameters characterizing the binding interaction.

As intended, the complex formed upon combination of $Na_2B_{12}I_{12}$ and HP-γ-CD is significantly more soluble than the complex with unfunctionalized γ-CD; no precipitation is observed upon combination of equivalent volumes of 200 mM $Na_2B_{12}I_{12}$ and 200 mM HP-γ-CD (affording a 100 mM solution of the complex).

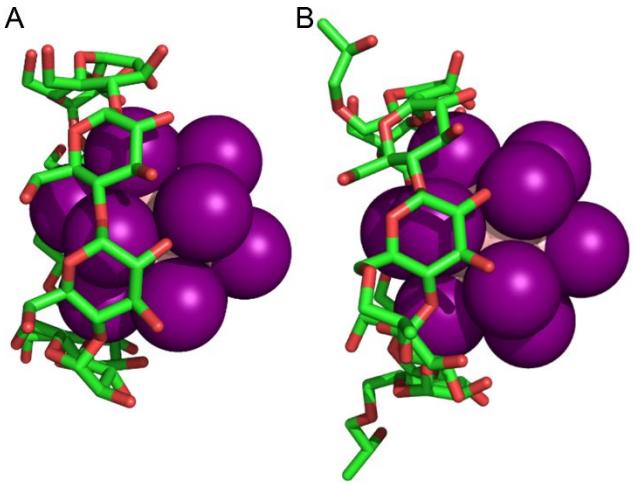

Fig. 4 Semi-empirically (PM6) optimized structures of (A) $[(\gamma\text{-CD})(B_{12}I_{12})]^{2-}$ and (B) $[(\text{HP-}\gamma\text{-CD})(B_{12}I_{12})]^{2-}$ highlighting that the 2-hydroxypropyl groups in the latter do not impact the binding of $B_{12}I_{12}^{2-}$. Note that the HP-γ-CD used in this work featured an average of between four and five 2-hydroxypropyl groups. The calculations were performed with four HP groups on alternating glucose units. $B_{12}I_{12}^{2-}$ is shown as spheres and γ-CD as sticks. Color code: I purple, B pink, C green, O red.

We next performed the Hb-release hemolysis assay by suspending RBCs in solutions that featured a consistent concentration of $Na_2B_{12}I_{12}$ (100 mM) but a systematic increase in the concentration of HP-γ-CD. As was also demonstrated in Fig. 2, these experiments confirm that in the absence of cyclodextrin, 100 mM $Na_2B_{12}I_{12}$ results in complete hemolysis. Strikingly, the presence of even small amounts of HP-γ-CD results in a drastic decrease in hemolysis (Fig. 5).

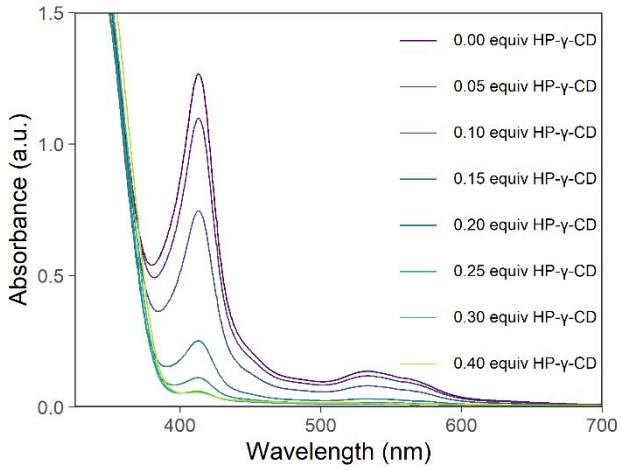

Fig. 5 Hb release from RBCs suspended in PBS (pH 7.4) containing 100 mM $Na_2B_{12}I_{12}$ with addition of increasing amounts of HP-γ-CD.

This attenuation of hemolysis increases with increasing HP-γ-CD concentration until it falls to near-baseline levels upon the addition of 0.4 equiv (Fig. 5). This protection was also maintained over periods reflective of the time over which an XCA would remain in circulation:[23] RBC incubation with 100 mM $Na_2B_{12}I_{12}$ and 50 mM HP-γ-CD (0.5 equiv) for either 4 h or 24 h resulted in no hemolysis (Fig. S7).

These results show that $Na_2B_{12}I_{12}$ induces rapid hemolysis at the concentrations used in medical imaging. This disruption of cell structure may be the origin of the previously reported toxicity of $Na_2B_{12}I_{12}$ and likely arises from the superchaotropic nature of the $B_{12}I_{12}^{2-}$ anions. This hypothesis is corroborated by the ability of γ-CD, which binds to $B_{12}I_{12}^{2-}$ because of its superchaotropic nature, to inhibit hemolysis. X-ray crystallography suggests that, in the solid state, $B_{12}I_{12}^{2-}$ may interact with γ-CD in the same manner as previously reported for $B_{12}Br_{12}^{2-}$: formation of a 2:1 γ-CD:borate complex. Prior solution-phase data support the formation of a 1:1 complex in solution. Unfortunately, the complex formed upon addition of $Na_2B_{12}I_{12}$ to γ-CD exhibits water solubility that is too low to observe any hemolysis-protective effect. The derivatized cyclic oligosaccharide HP-γ-CD forms a 1:1 complex with $B_{12}I_{12}^{2-}$ that is much more water-soluble. The protective effect of HP-γ-CD can be observed in hemolysis assays, where it can prevent cell destruction when added at substoichiometric levels. The 100 mM solutions of $Na_2B_{12}I_{12}$ with 0.4 equiv of HP-γ-CD feature an iodine concentration of 153 mg iodine mL^{-1}; further studies are underway to determine whether concentrations reaching those used in medical imaging (e.g., 300 mg iodine mL^{-1}) can be achieved with this strategy.

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There are no conflicts to declare.

Notes and references

‡ The chemistry of other iodoboranes and iodocarboranes has been explored,[24-28] and potential XCA applications were mentioned as motivations for this work but, to the best of our knowledge, none of these compounds have been subsequently explored as imaging agents.

§ In the work presented here, HP-γ-CD refers to a material in which 60% of O6-positions of the γ-CDs are functionalized with 2-hydroxypropyl groups and the average M_W is ≈ 1580 Da.

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