

Advancing glioblastoma imaging: Exploring the potential of organic fluorophore-based red emissive carbon dots

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(Article begins on next page)

1 **Advancing Glioblastoma Imaging: Exploring the** 2 **Potential of Organic Fluorophore-based Red** 3 **Emissive Carbon Dots**

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19 **Keywords**

20 carbon dots, red emission, cell imaging, glioblastoma, blood brain barrier

21 **Abstract**

22 Over time, the interest in developing stable photosensitizers (PS) which both absorb and
23 emit light in the red region (650 and 950 nm) has gained noticeable interest. Recently, carbon
24 dots (CDs) have become the material of focus to act as a PS due to their high extinction
25 coefficient, low cytotoxicity, and both high photo and thermal stability. In this work, a Federal
26 and Drug Association (FDA) approved Near Infra-Red (NIR) organic fluorophore used for
27 photo-imaging, indocyanine green (ICG), has been explored as a precursor to develop water-
28 soluble red emissive CDs which possess red emission at 697 nm. Furthermore, our material was
29 found to yield favorable red-imaging capabilities of glioblastoma stem-like cells (GSCs)
30 meanwhile boasting low toxicity. Additionally with post modifications, our CDs have been
31 found to have selectivity towards tumors over healthy tissue as well as crossing the blood-brain
32 barrier (BBB) in zebrafish models.

33 **1. Introduction**

34 The utilization of red emission in the phototherapeutic window (650-950 nm) for bio-imaging
35 and photo-therapeutics involves the exploitation of exciting chromophores located on the PS¹. A
36 key feature of the far-red range of excitation stems from the fact that there is an increase of light
37 penetration in tissues. Unfortunately, current commercial PS which use excitation wavelengths
38 outside the phototherapeutic window have their light absorption dampened by light scattering or

39 reflection of light by the host's tissue²⁻⁴. To achieve optimal efficiency, PS used in theranostics
40 techniques require the utilization of light within the red region to (NIR-I) window ranging
41 between 650-950 nm, respectively⁵. A direct consequence of this requirement results in the
42 degradation of the PS such as photobleaching or thermal degradation^{6,7}. Additionally, modern
43 PS have the disadvantage of low blood clearance from the host which limits biomedical
44 applications⁸. Significantly, CDs have attracted increasing attention in various biomedical areas
45 of application such as *in vitro/in vivo* imaging, biosensor, drug delivery, photodynamic therapy,
46 and photothermal therapy⁹⁻¹⁷. This interest is due to their characteristic of being free of metals,
47 having relatively low cytotoxicity in animal models, and boasting both high photo and thermal
48 stability^{18,19,28,29,20-27}. Up to date, there has been a scarcity of literature that focuses on water-
49 soluble, red emissive carbons dots past 650 nm suitable for biological studies. Xiong and
50 coworkers have explored the synthesis of red emissive CDs through a bottom-up approach using
51 a hydrothermal one-pot synthesis of red emissive CDs using urea and p-phenylenediamine as
52 precursors in 50 mL of water at 160°C for 10 hrs³⁰. The CDs, when purified via silica column
53 chromatography resulted in products with a wide emission range from 440 to 625 nm. The
54 rationale behind this characteristic was attributed to the increase of oxygen species on the CD's
55 surface which effectively lowers the band gap of the CDs causing a bathochromic shift towards
56 the lower red region at 625 nm. The data demonstrated that the surface chemistry of CDs plays a
57 pivotal role in elucidating red emission. Further works by Zheng et al. suggest that complex
58 surface modifications of produced CDs with cyanine-based dyes are of interest to unlock the
59 potential route to develop red emissive CDs³¹. Although promising, the previous work
60 mentioned fails to produce red emissive CDs which are also water-soluble. In our work, we
61 posit a variety of synthetic pathways for the formation of CDs derived from ICG, citric acid, and

62 PEG which leads to an enhanced bathochromic shift to produce amphiphilic CDs with highly
63 valuable emission in the red region. Our materials are ideal candidates for study as a NIR PS in
64 systems both *in vivo* and *in vitro*.

65 Glioblastoma (GBM) is a grade IV astrocytoma and is the most common malignant brain
66 in adults. Accounting for 1.35% of all cancer incidents, GBM is responsible for 4% of cancer
67 related deaths yearly with a survival median of 15 months³²⁻³⁴. Therapy can fail in treating GBM
68 due to various reasons. These include the difficulty of surgically removing the entire tumor,
69 limitations in the effectiveness of anti-cancer drugs in reaching the brain, and the presence of
70 treatment-resistant GSCs. This subpopulation of GBM tumor cells exhibit early tumor
71 progression, aggressive invasion into the surrounding brain parenchyma, and chemo-resistance
72 due in part to the overexpression of multidrug resistance pumps (P-glycoproteins), which leads to
73 increased efflux of drugs as well as imaging agents from the tumor cells³²⁻⁴⁰. It has been well-
74 documented that the extent of tumor resection directly correlates with patient outcome⁴¹. To
75 optimize tumor resection and patient outcome, surgeons are exploring fluorescence guided
76 surgery in which the tumor cells are fluorescently labeled in order for the surgeon to visualize
77 tumor cells especially at the leading edge of the tumor. The use of 5-aminolevulinic acid (5-
78 ALA) is increasing in the clinical area^{42,43}. 5-ALA is a non-fluorescent precursor to heme and is
79 converted to the fluorescent product protoporphyrin IX (PpIX) in tumor cells. However, PpIX
80 has its limitations as both photobleaching and variations of the concentration levels within the
81 tumor. The tumor core normally presents much brighter fluorescence when compared to the
82 leading edge as well as between patients with very low fluorescence observed in lower grade
83 astrocytomas⁴⁴. Therefore, the development of novel photo-stable imaging agents that can target
84 both bulk tumor cells and GSCs is necessary to achieve the most efficient success rate⁴⁵⁻⁴⁷. The

85 ideal fluorescent agent would demonstrate high selectivity toward tumor cells, high sensitivity to
86 improve contrast between tumor and normal tissue and low toxicity.

87 In this work, an (FDA) approved hydrophilic cyanine-based dye, namely ICG, was explored as
88 a model precursor to develop red emissive CDs (R-CD). ICG was selected as the precursor of
89 choice due to the wide scope of clinically excellent properties such as low toxicity, efficient
90 binding to blood lipo-proteins, short lifetime in blood circulation, and usability in the tissue
91 optical window (NIR) region⁴⁸⁻⁵¹. Herein, we hypothesize that it is possible to develop
92 hydrophilic CDs from ICG. Furthermore, it is expected that the CDs will retain most of the
93 functionality of the precursor such as favorable optical behavior in the far-red region to
94 effectively image tumor cells. CDs have several advantages over other red-emissive organic
95 dyes. They demonstrate high resistance to photobleaching, strong absorption in the red/NIR
96 region and are biodegradable. In comparison, ICG, another red-emissive material, suffers from
97 poor chemical and optical stability, as well as mediocre photothermal properties. Recent studies
98 found that CDs derived from NIR dyes such as ICG exhibit significantly improved thermal
99 stability and resistance to photobleaching compared to ICG⁵². Moreover, their photothermal
100 properties were greatly enhanced. The CDs showed a wider functional pH range, a 50%
101 improvement in photothermal conversion efficiency, and superior photothermal cyclability.
102 These findings indicate that CDs have the potential for more effective and stable applications in
103 various fields. In our work, R-CDs derived from ICG modulate BBB penetration and tumor
104 homing capabilities depending on peptide-surface functionalization. To our knowledge, this
105 work is the first account of far-red emissive CDs in purely water-based application.

106

107 **2. Experimental Section**

108 *2.1 Materials*

109 ICG (>99% purity) and polyethylene glycol 1000 (PEG1000, >99% purity) were procured
110 from Sigma-Aldrich (St. Louis, MO). Anhydrous citric acid (BDH, 99.5% purity) was obtained
111 from VWR (West Chester, PA). Nine residue peptide LINTT1 (AKRGARSTA) 94.5% purity
112 was obtained from LifeTein (Somerset, NJ). Transferrin (>98% purity) from human blood
113 plasma was obtained from Sigma-Aldrich (St. Louis, MO). Dialysis tubing with molecular
114 weight cutoff (MWCO) of 3500 was bought from Thermo Scientific (Rockford, IL). Deionized
115 (DI) water used was ultrapure (type I) water which was purified using a Millipore Direct-Q 3
116 water purification system acquired from EMD Millipore Corp. The purified water displayed a
117 surface tension of 72.6 mN m⁻¹, a resistivity of 18.2 MΩ cm and a pH value of 7.0 ± 0.3 at 20.0 ±
118 0.5 °C. All the chemicals were used as received. The human pediatric glioblastoma cell line (SJ-
119 GBM2) was obtained from Children's Oncology Group (COG, Texas Tech University, Health
120 Science Center, TX, USA) and maintained in RPMI media supplemented with 10% Fetal bovine
121 serum (FBS) and 1% penicillin-streptomycin (P/S). Human mesenchymal stem cells (MSCs)
122 were maintained in MEM alpha supplemented with 20% FBS and 1% P/S. Glio3, Glio9 and
123 Glio38 are GSCs derived from patient's resected tumors following patient consent and
124 Institutional Review Board (IRB) approval⁵³⁻⁵⁵. GSCs were maintained in neural stem-cell media
125 consisting of DMEM/F12 3:1 supplemented with 20 ng/mL each of epidermal growth factor
126 (EGF) and fibroblast growth factor (FGF), 2% Gem21 and 1% P/S. All cell culture reagents were
127 obtained from ThermoFisher Scientific except for EGF, FGF and Gem21 which were obtained
128 from Gemini Biosciences. All cell lines were routinely tested for mycoplasma using LookOut

129 mycoplasma PCR detection kit (Sigma Aldrich, St. Louis, MO, USA) according to the
130 manufacturer's instructions and maintained at 37 °C in a humidified 5% CO₂ incubator.

131 *2.2 Synthesis of R-CDs*

132 The R-CDs were obtained by means of microwave pyrolysis of citric acid (0.200 g), ICG
133 (0.005 g), and PEG1000 (0.050 g). To preface, the precursors were dispersed in a beaker
134 containing 15 mL of DI water ultrapure (type I) and sonicated for 1 min to ensure a homogenous
135 mixture which was observed to be a vivid light-green color. The solution of starting material
136 was then placed in the microwave and set for 150 s at a power setting of 700 W. Post pyrolysis,
137 it was observed that the solution remained with roughly 5 mL of water with a dispersion of dark
138 green colored solubilized product. The solution was then collected and centrifuged for 30 min at
139 10,000 RPM for three cycles to remove large, suspended particles. The remaining supernatant
140 was then placed within a 3500 MWCO Snakeskin dialysis for 72 hrs in deionized (DI) water
141 ultrapure (type I), with the water changed every 12 hrs. The purified solution of R-CDs was then
142 frozen at -40°C for 24 hrs and sequentially set for lyophilization for 72 hrs, producing a free-
143 flowing dark green powder. These freeze-dried R-CDs were then used for study.

144

145

146 *2.3 R-CD-Ligand conjugations via carbodiimide (EDC/NHS) coupling*

147 Both AKRGARSTA and transferrin (R-CD-AKRGARSTA and R-CD-Transferrin, R-
148 CD-Tf) conjugates were synthesized by carbodiimide coupling which was feasible due to the
149 abundance of carboxylic acids functional groups on the surface of the CD. This conjugation

150 involves the primary amine group of either the nine-residue sort peptide AKRGARSTA or
151 transferrin, which can form a stable amide bond. First, 50.0 mg of R-CD was solubilized in 10
152 mL of Phosphate-buffered saline (PBS, pH 7.4) and allowed to stir for 30 min. To proceed with
153 the activation of -COOH groups on the surface of R-CD, 63.9 mg of 1-ethyl-3-(3-
154 dimethylaminopropyl) carbodiimide (EDC) was transferred into 5 mL of the same buffer within
155 a vial and mixed to ensure a homogeneous mixture. Additionally, 193.2 mg of N-hydroxy
156 succinimide (NHS) was also transferred into another vial containing 5 mL of the same buffer and
157 mixed. Sequentially, both EDC and NHS were aliquoted in 1 mL increments to the solution
158 containing the R-CDs and were allowed to stir for 30 min. To synthesize the conjugate of
159 interest (R-CD-AKRGARSTA or R-CD-Tf), either 7.2 mg of AKRGARSTA or 20 mg of
160 transferrin was solubilized in a separate vial containing 5 mL PBS buffer and was aliquoted in
161 1mL increments of the mixture of R-CD, EDC, and NHS. The resultant mixture was then
162 allowed to stir for 48 hrs while protected from light. This process allows the conjugation from
163 the -COOH group on the surface of the R-CD and the -NH₂ of the ligand of interest. The newly
164 formed CD conjugate was then purified by dialysis against ultra pure water for 72 hrs to allow
165 unreacted small molecules to exit from the dialysis membrane with 3500 Da MWCO. Finally,
166 the purified R-CD-AKRGARSTA was lyophilized to yield a dark green powdered product.
167 Successful conjugation was confirmed by circular dichroism spectroscopy (CDS), zeta potential
168 (ZP), and mass spectrometry (MS) (**Figure S1**). Whereas the R-CD-Tf conjugate was
169 lyophilized to yield a turquoise powdered product with its conjugation confirmed by
170 photoluminescence (PL), transmission electron microscopy (TEM), Atomic force microscopy
171 AFM, MS and ZP (**Figure S2-3**).

172

173

174 *2.4 Characterization*

175 UV-vis spectra were obtained from an Agilent Cary 100 UV-vis spectrophotometer. PL
176 characterization was performed on a Fluorolog HORIBA Jobin Yvon fluorometer with a slit
177 width of 5 nm for excitation and emission. All optical characterization spectra were obtained
178 with quartz cells possessing an optical pathlength of 1 cm. Fourier-transform infrared (FTIR)
179 spectroscopy data were obtained with a PerkinElmer FTIR (Frontier) spectrometer (Waltham,
180 MA, USA) by using the attenuated total reflection (ATR) technique with air as background.
181 AFM images of CDs were obtained with an Agilent 5420 AFM (Santa Clara, CA, USA). To
182 perform AFM measurement, a drop of diluted CDs aqueous solution was applied on a clean
183 silica mica slide and air dried, which then was transferred to do the screening using tapping
184 mode. The tips used were silicon tips (length: 225 μm ; thickness: 5 μm) manufactured from
185 Nanosensors with a force constant of 15 N/m. TEM was performed by using a JEOL 1200X
186 TEM (Peabody, MA, USA). For TEM measurements, a drop of the R-CDs solution was placed
187 on a carbon coated copper grid and air dried prior to examination. The ZP was recorded on a
188 Malvern Zetasizer nano-series. The thermogravimetric analysis (TGA) and derivative
189 thermogravimetric analysis (DTG) of R-CDs and some precursors were conducted using a
190 thermo-microbalance (TG 209 F3 Tarsus, Netzsch, USA) while heating under a flow of nitrogen
191 gas from 40 to 1000 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C}/\text{min}$. Raman spectra were recorded using a Renishaw
192 Invia Spectrometer equipped with a
193 blue (457 nm), green (514.5 nm), and red (wavelength 785 nm) laser source and a 50x objective.
194 Applied power using green laser source was in the range of 10 to 100 μW , with the red laser line
195 from 50 to 500 μW and for the blue laser line from 100 μW to 1 mW. X-ray photoelectron

196 spectroscopy (XPS) spectra were recorded by using a PHI 50 0 0 Versaprobe (Physical
197 Electronics, Chanhassen, MN, USA) scanning X-ray photo electron spectrometer
198 (monochromatic Al K-alpha X-ray source with 1486.6 eV energy, 15 kV voltage, and 1 mA
199 anode current). The circular dichroism experiment was run under room temperature on the Jasco
200 (J-810) Circular Dichroism Spectropolarimeter. To obtain a good S/N ratio during the CDS
201 analyses, 100 µg/mL was used as the concentration of all samples (R-CD, RCD-AKRGARSTA,
202 and R-CD-Tf) in water as solvent; standard sensitivity; 100 nm/min scan speed; 1 nm band
203 width; 1 s response time; 0.5 nm data pinch and 5 accumulation scans.

204 *2.5 Cell Viability*

205 Cell viability was determined using the CellTiter 96® Aqueous One Solution Cell
206 Proliferation Assay (MTS) (Promega Madison, WI, USA). R-CDs were dispersed in phenol red-
207 free DMEM cell culture media at a concentration of 10 mg/ml. SJ-GBM2 cells and MSCs were
208 plated at a density of 1×10^5 and 0.5×10^5 cells/ml, respectively. GSC cell lines were plated in
209 neural stem cell proliferation media supplemented with 5% FBS at a density of 1.5×10^5
210 cells/ml. The following day cells were treated with 1-500 µg/ml and viability determined 72 hrs
211 later. Data is presented as percent viability compared to non-treated controls.

212 *2.6 Cell Imaging*

213 To facilitate cell adhesion, Glio3, Glio9 and Glio38 cells were plated in a media
214 supplemented with 5% FBS at a concentration of 1×10^6 cells/ml. At 24 hrs later, cells were
215 exposed to increasing concentrations of R-CDs (50-1000 µg/ml) for 6 hrs. To determine the
216 cellular location of R-CDs, Glio3 and Glio38 cells were treated with 500 µg/ml for 1 hour. Cells
217 were washed with PBS and treated with 4% paraformaldehyde for 30 min, washed again in PBS

218 and cover slips mounted using Prolong Gold Antifade reagent with and without 4',6-diamidino-
 219 2-phenylindole (DAPI). Images were obtained using a Fluid Cell Fluorescent Microscope
 220 (Thermo Fisher Scientific, Waltham, MA). For R-CD washout experiment, Glio 3 and Glio 38
 221 neurospheres were treated with 100 ug/ml R-CDs for 4 hrs. For R-CD washout, cell culture
 222 media was removed and neurospheres washed with PBS to remove any remaining R-CDs and
 223 incubated for an additional 48 hrs in fresh media. Alternatively, the neurospheres were washed
 224 and fixed immediately following treatment (0 hrs). For R-CD peptide experiments, GSCs and
 225 MSCs were treated with equimolar concentrations of R-CDs or R-CD- AKRGARSTA for 1 hour
 226 and imaged following PBS washes and fixation as described above.

227 2.7 Image analysis

228 ImageJ was used to quantify the fluorescence intensities of to evaluate CD uptake
 229 according to literature⁵⁶. First, images of GSC and MSCs incubated with the respective CD were
 230 median filtered to calculate an overall background value of each image, respectively. The region
 231 of interests (ROIs) for each image was detected by binarizing the images using an adaptive
 232 threshold value determined by a fixed percentile value of fluorescence intensities. Then, a
 233 weighted-fluorescence intensity was calculated for each image as follows in **Eq.1**.

$$\text{Mean fluorescence} = \frac{\sum_1^N [(FI_{ROI,i} - Bg) A_{ROI,i}]}{\sum_1^N A_{ROI,i}} \quad (\text{Eq.1})$$

234 FI_{ROI} is the average fluorescence intensity from a given ROI, Bg is the background fluorescence
 235 intensity, A_{ROI} is the area of a given ROI. Mean fluorescence intensity was calculated for all
 236 control and treatment groups.

237 To further quantify the fluorescence to as an increase in fold, the corrected total cell
238 fluorescence (CTCF) was calculated. The CTCF was obtained by first outlining the cell of
239 interest and their respective background. These acquired measurements enable the generation of
240 values for both the selected area and integrated density for the cell as well as the mean intensity
241 for the background. To measure the amount of CD fluorescence within cells, the calculation of
242 the CTCF was performed by subtracting the product of the average fluorescence in the
243 background and the area outside the cell from the total amount of fluorescence inside the cell.
244 This method has been described in previous literature⁵⁷⁻⁵⁹.

245 *2.8 Statistical Analysis*

246 Significance was determined using Student's t-tests for all pairwise comparisons of the
247 different treatments that were tested. All the results are presented as the mean±standard error of
248 the mean (SEM). Significance was set at $p \leq 0.05$.

249 *2.9 Zebrafish injection and bioimaging*

250 Wild-type zebrafish at 5 days post fertilization were obtained from the Zebrafish Core
251 Facility at University of Miami and anesthetized by 0.02% tricaine. Then, 20 mg/mL of CDs
252 aqueous solution were intravascularly injected into the heart of zebrafish. After 10 min, the
253 zebrafish were mounted with low-melting agar and observed under the Leica SP5 confocal
254 microscope under bright field and fluorescence at excitation of 405 nm. The animal care protocol
255 for all procedures used in this study was approved by the University of Miami Animal Care and
256 Use Committee and complies with the guidelines of the National Science Foundation. Zebrafish
257 studies were conducted under the procedures related to the IACUC 21-182 for the use of

258 zebrafish and meets the criteria for review under Section III F in accordance with the *NIH*
259 *Guidelines*

260

261

262

263 3. Results and Discussion

264 3.1 Characterizations of R-CDs

265 The analysis of the UV-vis spectrum of R-CDs reveals typical absorption bands attributed to
266 the $\pi\text{-}\pi^*$ transitions of the C=C bond and the $n\text{-}\pi^*$ transition of the C=O bond at 210 and 397
267 nm, respectively (**Figure 1**)⁶⁰. Further bands found at 770 and 890 nm are reminiscent of the
268 bands usually observed from ICG at 710 and 790 nm, respectively. These bands are normally
269 related to nitrogen-containing heterocyclic structures which the dye is composed of. The

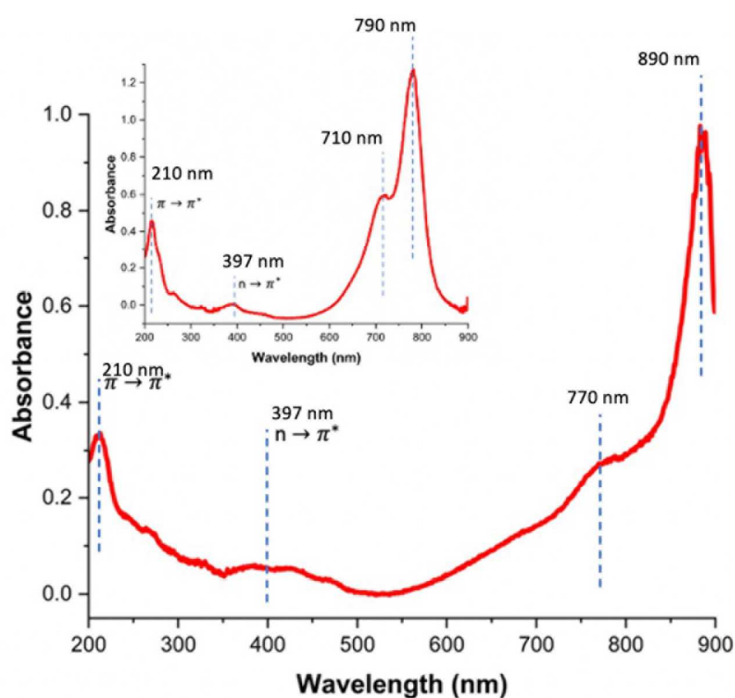


Figure 1. UV-vis analysis R-CDs, (inset) UV-vis spectrum of ICG.

270 bathochromic shift of the absorbance of R-CDs is hypothesized to be attributed to the increase of
271 oxygenated groups as confirmed by FTIR due to the implementation of citric acid and PEG1000
272 during the synthetic process.

273 The PL of R-CDs was studied to assess the viability of the material in fluorescent imaging
274 techniques across a wide range of excitations. The expected result was to produce CDs that
275 retained optical properties similar to ICG which has notable emission peaks at 599 and 815 nm,
276 respectively. It was hypothesized that there may be degradation of the dye during the synthetic
277 process resulting in slight loss of conjugation, leading to an increase of the energy band gap
278 producing a slight hypsochromic shift⁶¹. It is noteworthy that this behavior was observed in the
279 PL of the R-CDs when they were stimulated with light between 360 to 700 nm, at 20 nm
280 intervals (**Figure 2A**). R-CDs were found to possess red emission at 697 nm as well as a 599 nm
281 emissive peak similar to the ICG dye (**Figure 2B**). Interestingly, R-CDs were found to have a
282 clear isosbestic point at both 575 and 665 nm. These findings support a uniformity on the PL
283 profile across a long range of the visible spectrum. This discovery holds significant implications
284 as it suggests that the optical characteristics of the R-CDs will remain consistent across a specific
285 range of wavelengths owing to their uniformity. We hypothesize, during the synthesis of R-CD,
286 the electronic structure of the ICG molecule is partially preserved and is interconnected via PEG
287 chains. This results in the production of CDs that have similar excitation and emission properties
288 as the dye precursors.

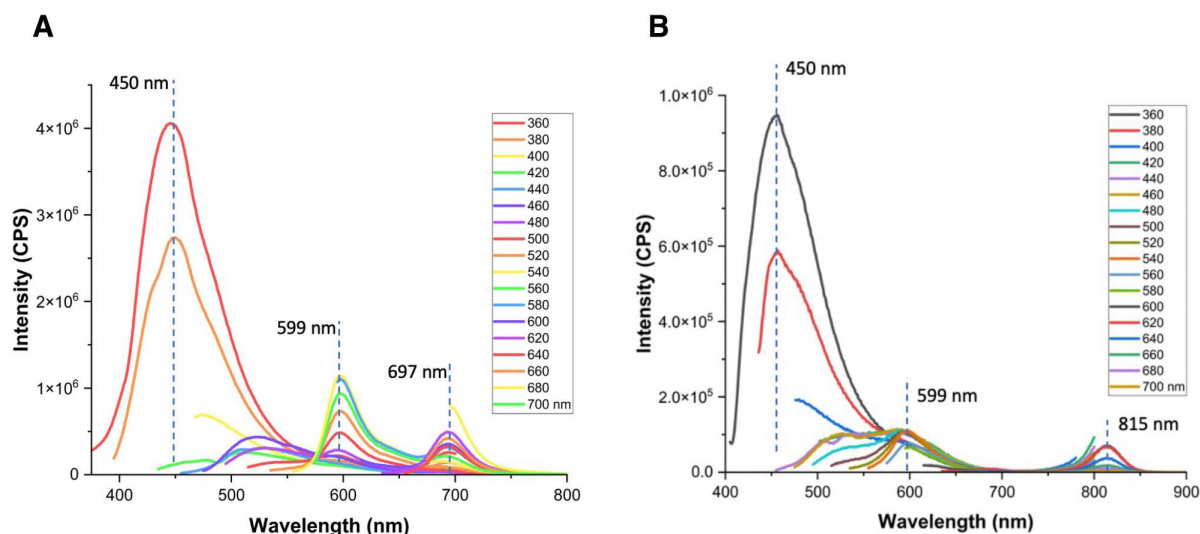
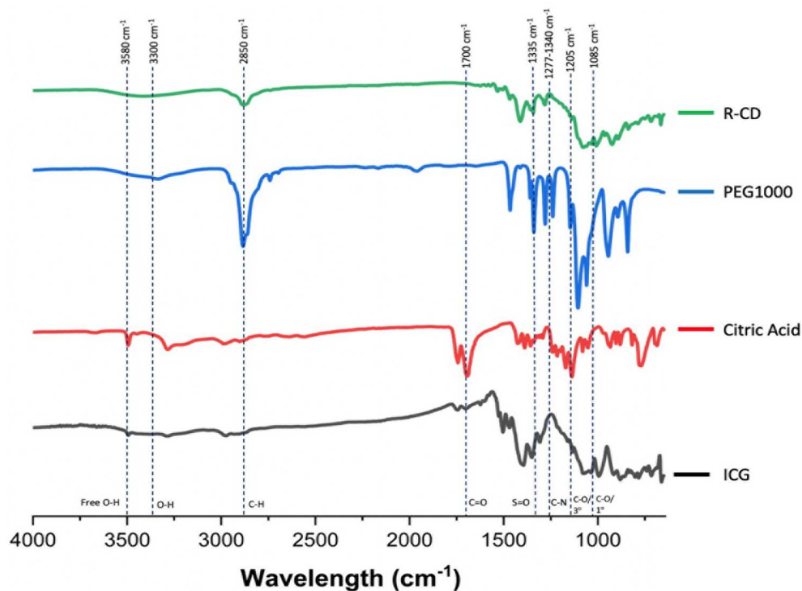


Figure 2. Optical analysis of R-CDs. (A) R-CD PL spectrum, (B) ICG PL spectrum.

289

290 FTIR is a suitable technique for revealing the surface functionality of CDs, which typically
 291 consists of functional groups containing oxygen and nitrogen. Moreover, FTIR has the unique
 292 ability to penetrate to a depth of 2 μm , making it a powerful tool for qualitative analysis of both
 293 the surface and core of CDs. A comparative analysis of R-CDs and the starting material was
 294 performed to reveal a glimpse into the surface of the CD (**Figure 3**). Stretching vibrations are
 295 1700 cm^{-1} are due to presence of C=O bonds on the surface of the CD. Upon deeper analysis of
 296 R-CD, the intense peaks at 1335 and 1277 cm^{-1} are due to the asymmetric S=O and C-N
 297 stretching vibrations, respectively. This characteristic supports the presence of moieties from the
 298 ICG dye, which have the same respective peaks. Between 3000 and 3100 cm^{-1} one would expect
 299 to observe the C-H stretching of the alkenes which make up the carbonized network of R-CDs,
 300 though increased spectral broadening from the carbonized network diminishes an observable
 301 peak. Additionally, the broad peak in between 3300 and 3580 cm^{-1} are attributed to stretching
 302 vibrations of O-H groups present as well as the medium peak at 2850 cm^{-1} indicative of C-H
 303 stretching vibrations of alkane groups due to the PEG1000 present during the synthetic process,

304 confirming the availability of these groups on the surface of the R-CDs, respectively. The peaks
305 at 1085 and 1205 cm^{-1} can be ascribed to $-\text{C}-\text{O}-$ and $\text{C}-\text{OH}$ groups on the surface of the R-CDs,
306 respectively. These results suggest that R-CD retains most functionality from ICG which
307 passivates the surface⁶². Additionally, through the implementation of PEG1000 during the
308 synthetic procedure, increased $-\text{OH}$ functional groups are to be found on the surface of the CD.
309 It is hypothesized the abundance of these groups is responsible for the high solubility of R-CDs
310 in water⁶³.



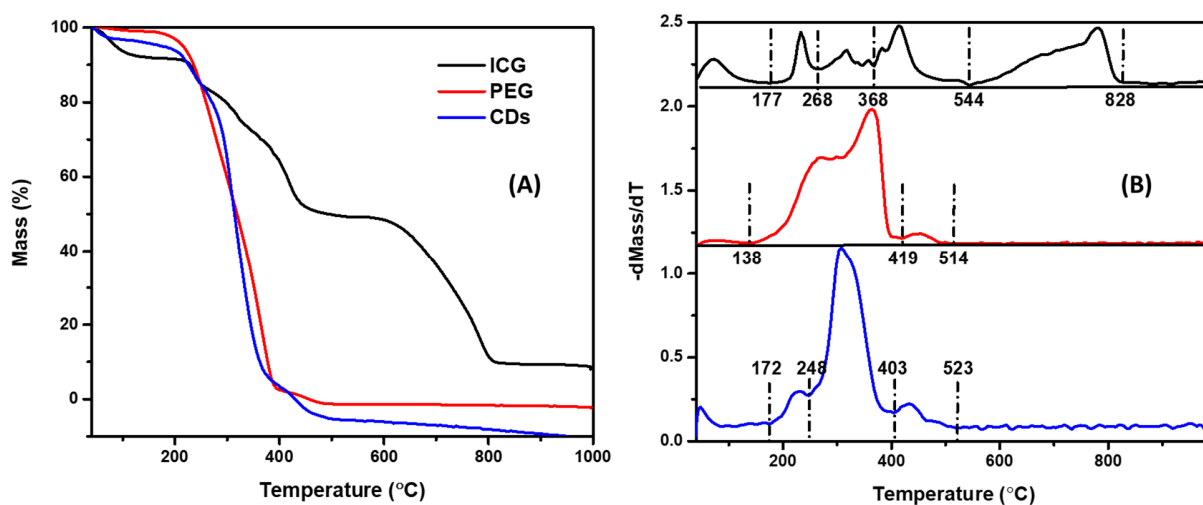
311 **Figure 3.** FTIR spectral analysis of R-CD, PEG1000, Citric Acid, and ICG.

312 Functional moieties can be identified and quantified by TGA and DTG measurements by their
313 different and discernible decomposition temperatures. TGA and DTG of citric acid were
314 previously provided, so they are not shown in **Figure 4**⁶⁴. In general, the TGA of R-CDs and
315 PEG (**Figure 4A**) show a similar thermal decomposition trend across different temperatures,
316 which demonstrates a structural similarity between R-CDs and PEG, as a result of the use of
317 PEG during the synthesis of R-CDs. Also, it is worth noting that an estimated 10% residue

318 remained after the decomposition of ICG. Considering the structure of ICG containing Na^+ and -
319 SO_3^- , the remnant is likely to be Na_2SO_3 (8%) or Na_2SO_4 (9%), which basically matches the 10%
320 residue mass percentage. Upon dialysis, the R-CDs no longer retained Na^+ ions, resulting in the
321 absence of any residual signal in the TGA analysis of the R-CDs. In addition, the DTG results
322 display noticeable differences and similarities between R-CDs and their precursors (**Figure 4B**):
323 (1) ICG has five decomposition stages: 40-177, 177-268, 268-368, 368-544 and 544-828 °C; (2)
324 PEG has three decomposition steps: 40-138, 138-419, and 419-514 °C; (3) the R-CDs possess
325 five decomposition periods: 40-172, 172-248, 248-403, 403-523 and 523-1000 °C; (4) the DTG
326 of PEG and R-CDs are somewhat similar; (5) the thermal stability of R-CDs is higher than ICG,
327 PEG and citric acid considering an enhanced highest decomposition temperature, which might
328 benefit from a higher carbonization degree that resulted from the hydrothermal treatment during
329 the CD synthesis; (6) with the DTG of a well-established CD species as a reference,⁶⁵ any peaks
330 between 40-122 °C indicate the loss of water molecules (moisture). The mass loss at 122-168 °C
331 suggests the loss of water molecules formed through intramolecular dehydration condensation
332 reactions⁶⁶. The stage between 168-250 °C is ascribed by the decomposition of edge-plane
333 oxygen-containing functional groups such as epoxy, carboxyl and carbonyl groups⁶⁷. The stage
334 at 250-338 °C is due to the decomposition of some relatively stable oxygen-containing functional
335 groups and sublimation of small carbon frameworks. The stage at 338-448 °C indicates the
336 decomposition of amines^{68,69}. Eventually, the stage at 448-1000 °C indicates the decomposition
337 of graphene-like structures⁷⁰⁻⁷²; (7) thus, in the first stage, PEG (40-138 °C) showed a desorption
338 of moisture while ICG (40-177 °C) and R-CDs (40-172 °C) exhibited the loss of water molecules
339 from moisture and formed through intramolecular dehydration condensation reactions. However,
340 ICG doesn't contain hydroxyl groups so the mass loss for ICG is hypothesized to be primarily

341 moisture desorption; (8) the second decomposition stages of ICG (177-268 °C) and R-CDs (172-
342 248 °C) are similar with a mass loss of 9 and 10%, respectively, due to the decomposition of
343 edge-plane oxygen-containing functional groups such as epoxy, carboxyl, and carbonyl groups.
344 Nevertheless, the only edge-plane oxygen-containing functional group in ICG is -SO₃, so this
345 DTG peak is likely to indicate the loss of -SO₃, which can be confirmed by the mass percentage
346 (10%) of -SO₃ in ICG (*Note:* one -SO₃ will be left to form Na₂SO₃ or Na₂SO₄). Additionally, in
347 this temperature range, citric acid is transformed into aconitic acid, itaconic acid and anhydrous
348 itaconic acid while releasing CO₂ and water molecules, so it is hard to identify that the mass loss
349 of R-CDs is due to the release of CO₂ or SO₂ solely based on the DTG measurement. In
350 comparison, the second decomposition stage of PEG was extended to 419 °C. Given the simple
351 unit-repeated structure of PEG, the dramatic mass loss (97%) during the second stage of 138-419
352 °C shows the breakdown of the unit-repeated oxygen-containing backbone; (9) the
353 decompositions of relatively stable oxygen-containing functional groups and carbon frameworks
354 were observed in the third decomposition stage (248-403 °C) of R-CDs and the corresponding
355 mass percentage in R-CDs is 82%. On the contrary, due to the lack of oxygen-containing
356 functional groups in ICG, the mass loss (13%) at 268-368 °C was hypothesized to occur due to
357 the decompositions of short alkane and alkene chains. A similar mass loss at this stage between
358 R-CDs and PEG also suggests a similarity in the structure; (10) due to the presence of amines,
359 ICG (368-544 °C) and R-CDs (403-523 °C) possess a fourth decomposition stage with a mass
360 loss of 21 and 9%, respectively. However, since amines are incorporated in aromatic structures,
361 the highest decomposition temperatures of at least ICG were raised together with the mass loss
362 compared to the theoretical amine contents; (11) compared to PEG and citric acid, given the
363 aromatic rings in the structure of ICG, the dramatic decomposition of ICG occurred at the last

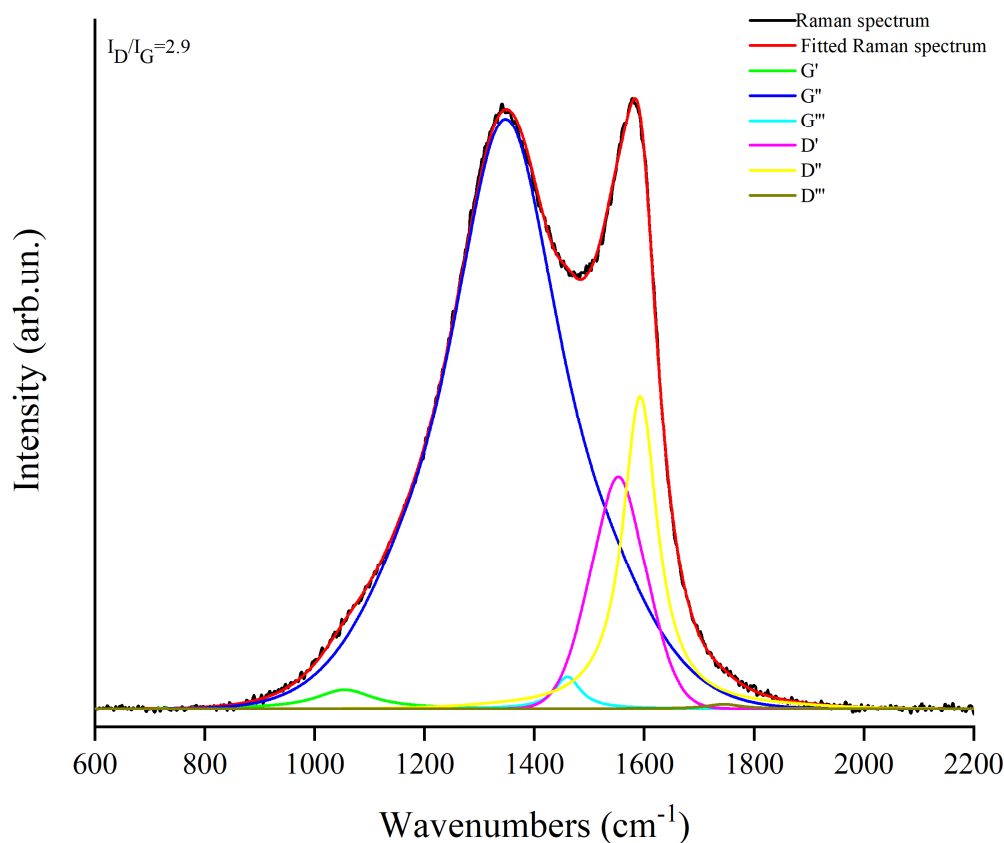
364 stage of 544-828 °C with a mass loss of 39%. Meanwhile, R-CDs also showed a mass loss of 5%
365 corresponding to the decomposition of graphene-like structures in the core; (12) according to the
366 contents of oxygen-containing carbon frameworks (82%) and aromatic rings (5%) inherited from
367 PEG (MW: 1000 Da) and ICG (MW: 775 Da), respectively, the structure of 1 mole R-CDs
368 (MW: 966 Da) is hypothesized to be composed of 1 mole PEG (incomplete), 1 mole ICG
369 (incomplete) and an unknown amount of citric acid (incomplete).



370 **Figure 4.** TGA (A) and DTG (B) of R-CDs and some precursors including ICG and PEG.

371 Raman spectroscopy is a facile and non-destructive technique that can be used to characterize
372 the crystallinity and chemical functionality of graphitic materials, as well as to elucidate defects.
373 As a powerful tool for investigating the disorder and crystallinity of CDs, Raman spectroscopy
374 can help to determine the level of disorder present in CDs, which plays a crucial role in their
375 electronic and optical properties. By using Raman spectroscopy to understand the degree of
376 disorder and crystallinity of CDs, researchers can optimize their performance for various
377 applications such as bioimaging. For our system, we are interested in both the position and
378 intensity of two prevalent spectral bands (D band and the G band). The G-band denotes the

379 amount of graphitization (sp^2 hybridization) R-CDs has and the D-band represents the degree of
380 defects and functionalization of R-CDs such as sp^3 hybridization^{73,74}. The Raman spectrum was
381 reported in the range from 600 to 2200 cm^{-1} and fitted according to Tagliaferro et al.⁷⁵ as shown
382 in **Figure 5**. The spectrum of R-CDs reveals two distinct peaks at 1375 (D-band) and 1585 cm^{-1}
383 (G-band), respectively⁷⁶⁻⁷⁸. Additionally, the baselined fitted Raman spectrum of R-CDs shows
384 the presence of highly disordered systems with a large D peak and broad 2D region. The
385 intensity ratio of the peak of the G band to the D band (I_D/I_G) was up to 2.9. In graphitic
386 material, a higher in I_D/I_G ratio indicates that the synthesized R-CDs have an increased amount
387 sp^2 clusters in its structure⁷⁹⁻⁸¹.



388 **Figure 5:** Raman spectra R-CDs

389 The ZP of the R-CDs was obtained to further understand the surface and the surface charge. In
390 brief, ZP is an essential technique for characterizing the surface properties of CDs such as
391 surface charge, stability, and functionalization. By measuring the ZP, researchers can gain
392 valuable insights into the electrostatic interactions between the CDs and their surrounding
393 environment. This information is crucial for understanding the behavior of CDs in various
394 applications such as drug delivery, imaging, and sensing. Furthermore for our system, this
395 technique is important to understand the colloidal stability of CDs in solution. Hence, a large
396 absolute value above 20 mV has been observed to result in CDs that participate in interparticle
397 repulsion leading to a well-dispersed colloidal solution^{82,83}. The R-CDs were found to have a
398 negative potential of -29.2 mV suggesting the presence of surface functional groups such as
399 carboxylic and amides possibly attributed to the functionality left by ICG (**Figure S3**).
400 Furthermore, the ZP data supports CDs high dispersibility/solubility when in solution since TEM
401 and AFM measurements confirmed a lower percentage of agglomerations. The ZP of R-CD-
402 AKRGARSTA was found to be -18.5 mV. This discovery is a good indication for aiding in the
403 confirmation of the conjugation taking place as we hypothesize that the peptide shields the
404 highly negative moieties on the surface of the R-CDs causing there to be a slightly less negative
405 charged CD. Additionally, the ZP of R-CD-Tf reinforces the same rationale as the ZP was
406 observed to be -18.2 mV.

407 TEM images were studied regarding R-CDs, R-CD-AKRGARSTA, and R-CD-Tf to
408 understand the X–Y plane size distribution. The sample was sonicated for 15 min prior to
409 measurements to breakdown any aggregate formation. R-CDs showed a narrow Poisson size
410 distribution of 1.0-6.0 nm with a mean size of 2 nm (**Figure 6A**). The TEM of R-CD-
411 AKRGARSTA had a Poisson size distribution of 1.0-6.0 nm with a slightly bigger mean size of

412 2.5 nm which is attributed to the peptide bound on the surface (**Figure 6B**). In following, the
413 TEM of R-CD-Tf was found to have had a Poisson size distribution of 1.0-7.5 nm with the
414 largest mean size of 3.0 nm (**Figure 6C**). We hypothesize this change is due to the bulky
415 transferrin which is bound to the surface of R-CD. Each histogram for all three types of CDs
416 comprises of a particle count of over 300 particles with a high degree of uniformity. AFM was
417 also performed on the R-CDs to understand the height profile of the CDs in the z-axis. The
418 AFM images display R-CD particles height within 2.0–2.8 nm, which is consistent with the

419 previous TEM diameter distribution confirming the quasi-spherical structure of the R-CDs. The
 420 AFM of R-CD-AKRGARSTA was found to have a particle height of 2.5-3.5 nm in agreement
 421 with the TEM. Likewise, the AFM of R-CD-Tf revealed a particle height of 2.5-4.0 nm which
 422 conforms with its TEM.

423 To further understand the surface chemistry, R-CDs were analyzed by using XPS (**Figure S4**)
 424 and high-resolution spectra for each relevant element (**Figure 7**) are reported together with table
 425 on the elemental composition (**Table 1**) and functionalities distribution (**Table S1**). As shown in

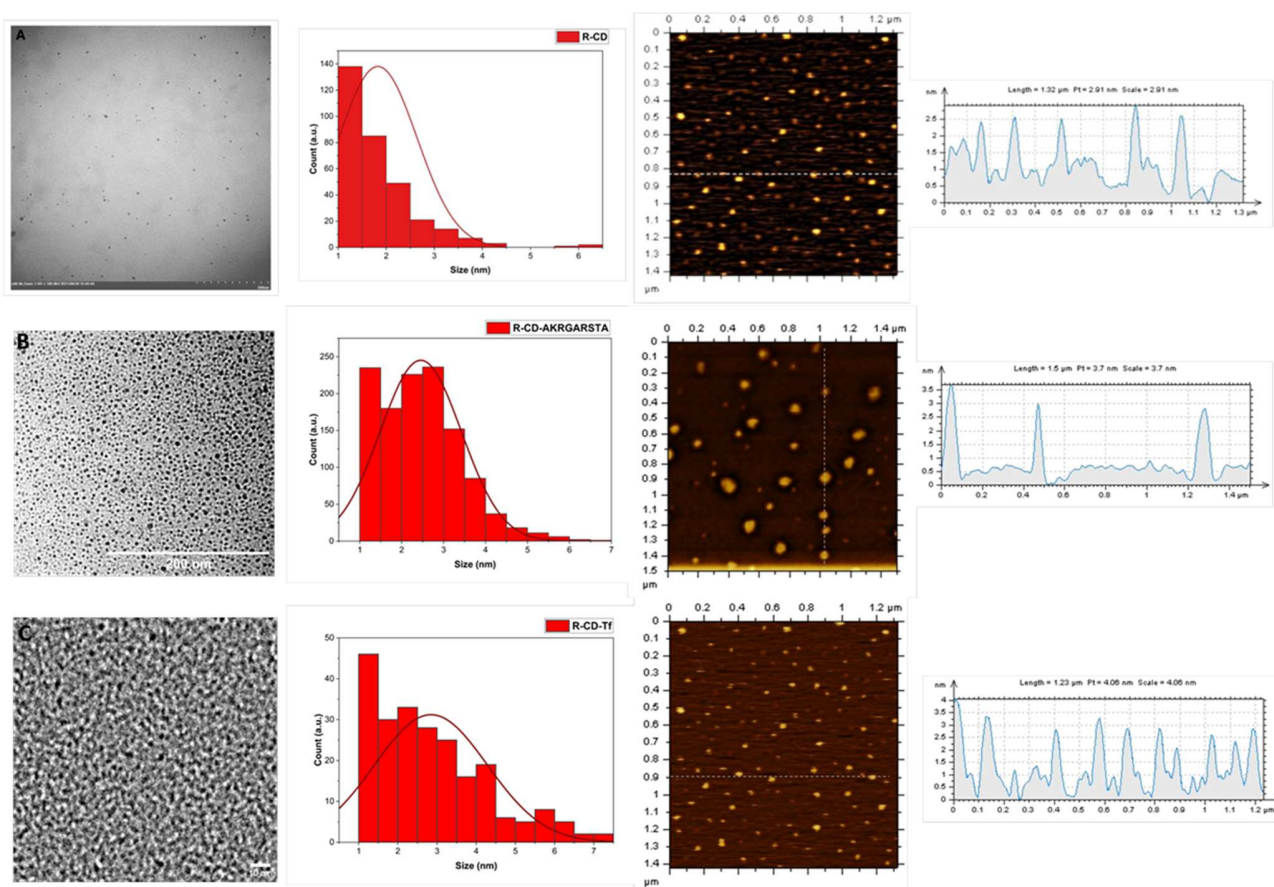


Figure 6. Morphology of R-CDs and R-CD conjugations. (A) R-CD TEM image with a scale bar of 200 nm, particle size histogram, AFM image and scale; (B) R-CD-AKRGARSTA TEM image with a scale bar of 200 nm, particle size histogram, AFM image and scale; (C) R-CD-Tf TEM image with a scale bar of 10 nm, particle size histogram, AFM image and scale.

Sample	Element (atom %)	C	N	S
R-CDs	28.9	1.1	0.5	

426 table 1, the preliminary results of elemental composition of R-CDs show a high amount of
427 oxygen up to 28.9 % a low content of both nitrogen (1.1 %) and sulfur (0.5 %). This was
428 reasonably due to the massive presence of PEG chain fragments. In **Figure 7A**, R-CDs C1s
429 spectrum showed the presence of relevant components due to sp^3 hybridized carbon (283.5 eV)
430 and to sp^2 hybridized carbon (peaked at 284.7 eV) with an intensity of 30.1 and 52.0 %,
431 respectively. Furthermore, C-X (X = N, O, S; 285.8 eV) is present with an intensity of up to 12.7
432 % while COOH (289.1 eV) reaches up to 5.1 %. O1s spectrum is significantly simple with a
433 major component due to C-O (531.3 eV) up to 90.2 % while sulphonate (532.2 eV) and COOH
434 (533.3 eV) represent only the 6.0 and the 3.5 % respectively (**Figure 7B**). This is in good
435 agreement with the model based on ICG fragments connected through PEG chains and residual
436 fragments of citric acid. N1's spectrum is considerably simple (**Figure 7C**), and it is composed
437 by one signal due to N5 (399.9 eV) as the sp^2 one that is composed by only one component due
438 to sulphonates (166.6 eV, **Figure7D**).

Table 1: : Elemental composition of R-CDs as calculated from XPS survey spectra
(average uncertainty of up $\pm 1\%$).

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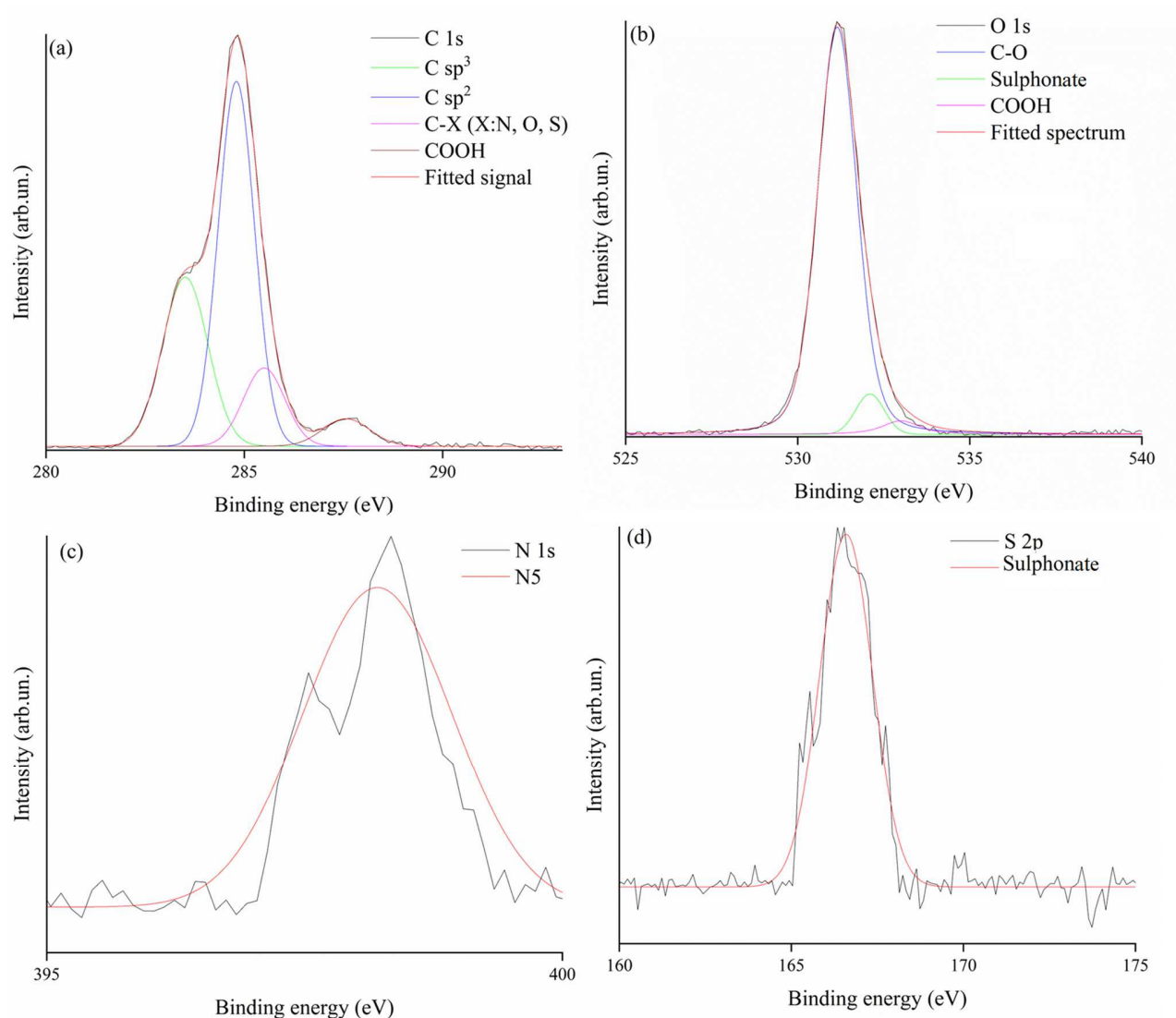


Figure 7: XPS spectra of relevant element for R-CDs.

440

441 3.2 Formation Mechanism of R-CDs

442 Preliminary consideration of the structure should be based on both TGA and FTIR data. As
 443 shown by TGA data, the graphitic domains are limited to only 5 wt.% of the total amount of R-
 444 CDs and there is unneglectable similarity with neat PEG suggesting the persistence of polyether
 445 chains into the final structure of R-CDs. Similarly, the FTIR spectrum of R-CDs suggests the

446 presence of alkyl chains together with the persistence of sulphonyl residues and the presence of
447 small amounts of aromatic and nitrogen containing aromatic systems. Additionally, Inductively
448 Coupled Plasma (ICP) analysis excludes the presence of sodium and Raman spectrum clearly
449 supports the presence of highly disorganized aromatic domains. Considering the characterization
450 data and the chemical formula of each reactant, we propose a mechanism for the formation of R-
451 CDs that is composed by several pathways as shown in **Figure 8**.

452 Firstly, we can considered the hemolytic cleavage of **1** in the polyenes chain (C=C energy
453 bond up to 255 kcal/mol⁸⁴) producing unsaturated species **2-4**. These species could undergo
454 through a Diels-Alder cycloaddition condensation forming chemical families like compound **5**.
455 Another modification of **1** is the isomerization of polyunsaturated chain that connects its two 1H-
456 benzo[g]indole units forming **6**. This species represents an interesting platform diene able to self-
457 condense forming species such **10** that could further condense producing small condensed
458 aromatic systems. Alternatively, **6** could undergo inter-molecular condensation with several
459 species such as **2-4** or unsaturated citric acid derivatives (**7-8**) formed under microwave
460 irradiation. Interestingly, cyclic anhydrides such as **13** could evolve into 4H-pyran derivatives
461 such as **14** and **15** accounted for the aromatic oxygen moieties detected by TGA analysis.
462 Similarly by TGA, the evolution of **8** could produce species with a naphthalenic (**21**) or biphenyl
463 (**23**) cores. The condensed ICG derivatives formed under microwave irradiation are highly
464 functionalized and hardly evolved to proper layered supramolecular structure as proved by the
465 residual functionalities detected by analytical by FTIR and MS. Additionally, **24** can degrade
466 under microwave irradiation forming low molecular weight species hydroxyl (**25**) or/and vinyl
467 (**26**) terminated species. Hydroxyl ended chains could easily react with acidic functions, both

468 carboxylic and sulphonyl, forming **27** while **26** could react with both dienes forming species
469 such as **28** and with acidic moieties forming **29**.

470 The PEG linked condensed ICG fragments could be schematized with a structure such the one
471 reported in **Figure 9**. The scattering and small size of aromatic domains is in good agreement
472 with both Raman and TGA analysis suggesting a hybrid structured in between a polymeric and
473 layered CDs. Accordingly, aromatic clusters are surrounded by a polyether environment bonded
474 through ester and saturated carbon-carbon bond. This could boost their local interaction through
475 $\pi - \pi$ stacking and could be accounted for the fluorescence emission at 697 as reported for
476 similar systems by Wang et al⁸⁵.

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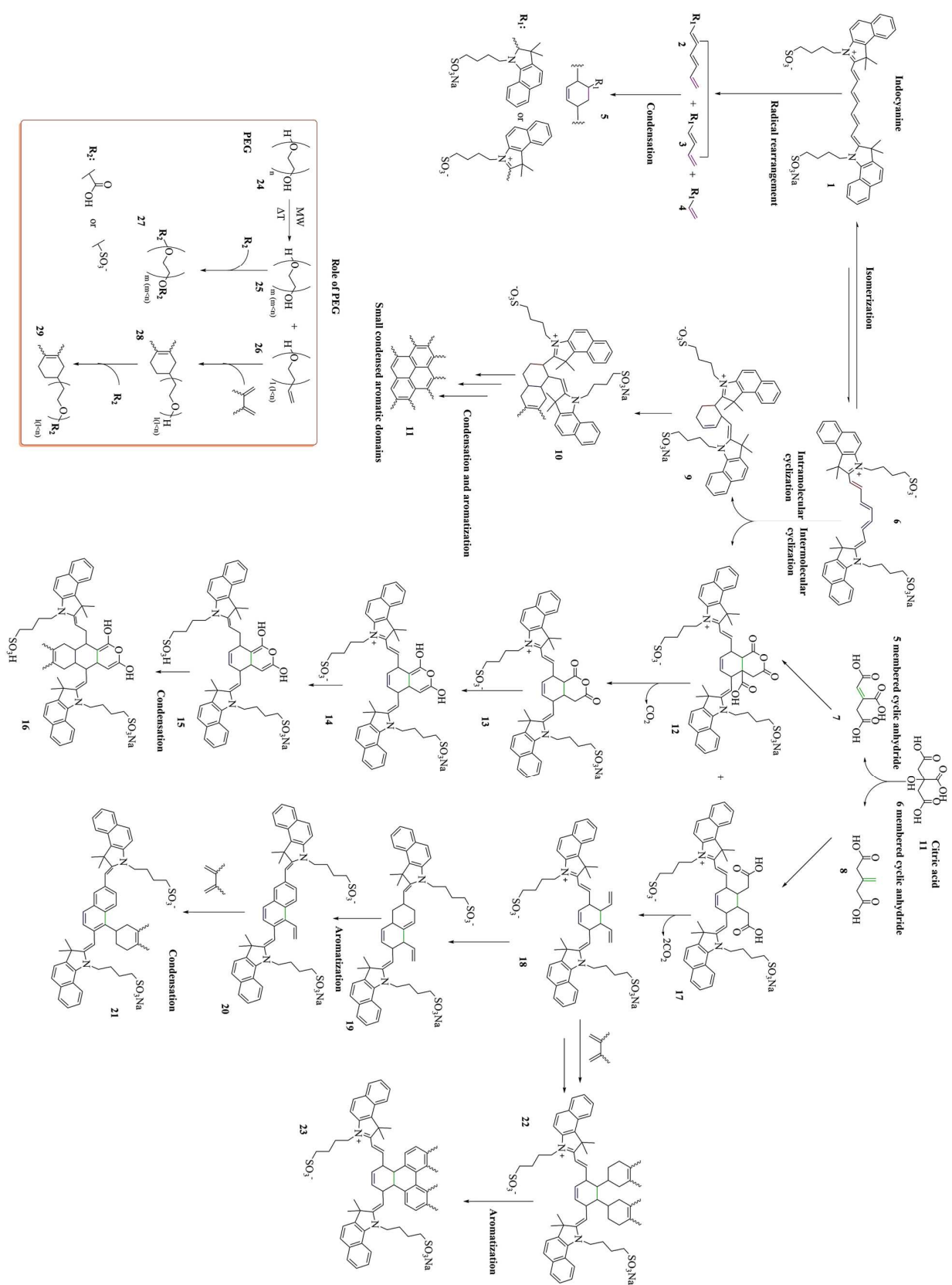
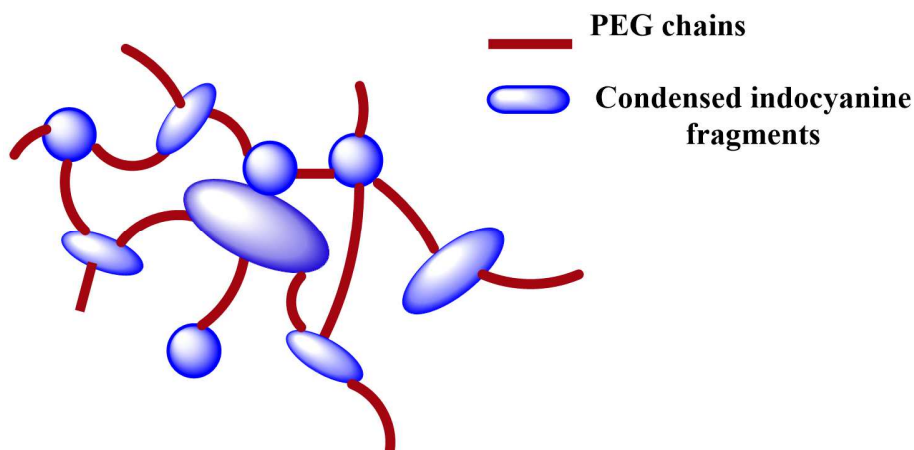


Figure 8: R-CDs formation mechanism.



479 **Figure 9:** R-CDs structural model.

480 *3.3 Cell Viability of R-CDs*

481 GSCs have been shown to be resistant to current treatment modalities and are responsible for
 482 tumor regrowth following surgery. Hence, it is important to develop an imaging material that
 483 can readily undergo cellular uptake. Additionally, it is necessary that the material is non-toxic
 484 towards cells before using it for imaging applications. We anticipate that our R-CDs will have
 485 high cell viability due to the abundance of PEG on the surface, which has been proven to have
 486 excellent biocompatibility with cells⁸⁶⁻⁸⁹. To determine the potential biocompatibility of R-
 487 CDs, the cell viability was determined using non-tumor, and tumor cell lines: normal MSCs, SJ-
 488 GBM2 and GSCs Gli03, Gli09, and Gli038, respectively. Cells were treated with increasing
 489 concentrations (1-500 $\mu\text{g/mL}$) and cell viability was determined following 72 hrs of exposure.
 490 The R-CDs were relatively non-toxic to all cell lines especially at concentrations of 100 $\mu\text{g/mL}$
 491 or below. At drastically high concentrations of 500 $\mu\text{g/mL}$, the cell viability of MSC and SJ-
 492 GBM2 were 65 and 83% ($p > 0.05$), respectively (**Figure 10A**). This finding is important as the
 493 upper limits of concentrations for cell viability are usually presented as 100 μM ⁹⁰⁻⁹². Suggesting
 494 that within normal standards, R-CDs are highly non-toxic which is a feature of PEGylated

495 nanoparticles⁹³. At the upper limit concentration, the cell viability for Glio3, Glio9 and Glio38
 496 were 100, 85 and 108% ($p > 0.05$), respectively (**Figure 10B**). As expected, the R-CDs were
 497 highly bio-compatible which further reinforced the need to apply these CDs for bioimaging.

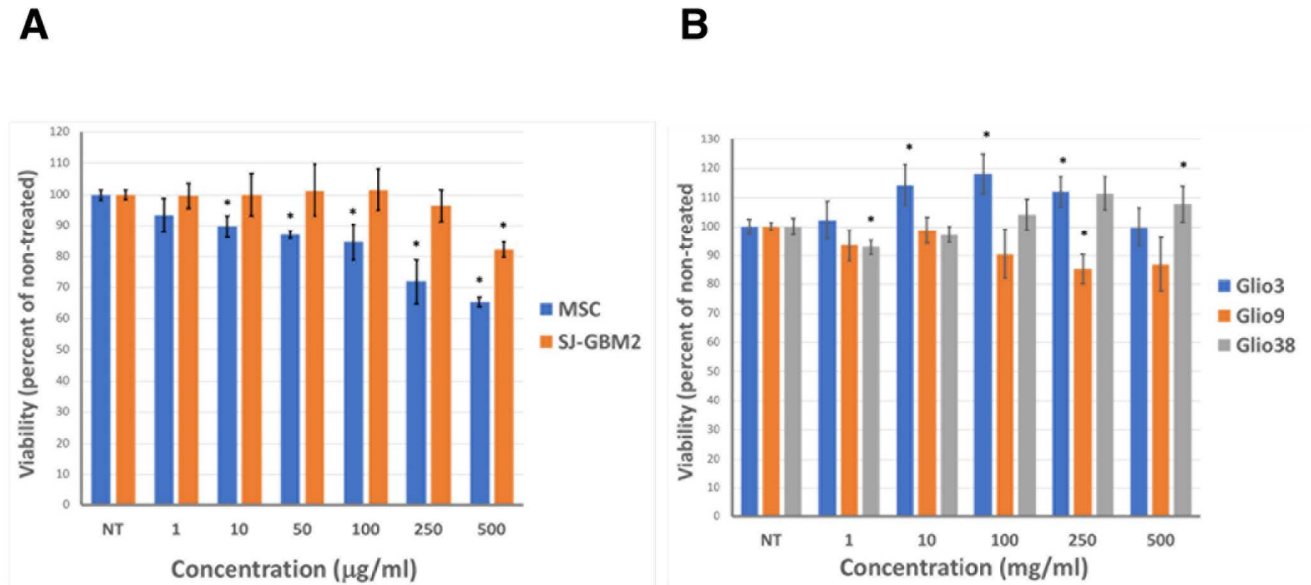


Figure 10. SJ-GBM2 and MSCs (A) and GSC lines, Glio3, Glio9 and Glio38 (B) were exposed to R-CDs at concentrations shown and viability determined by MTS assay 72 hrs later. * $p < 0.05$ compared to non-treated controls.

499 3.4 Red Fluorescent Imaging Capabilities of R-CDs

500 Given the relatively low cytotoxicity observed, especially in the GBM cells, the fluorescent
 501 imaging capabilities of R-CDs were assessed. For these studies we chose to use GSCs. Cancer
 502 stem cells (CSCs) including GSCs express high levels of ATP-binding cassette (ABC)
 503 transporters which not only pump anti-cancer drugs out of the cell but many fluorescent dyes as
 504 well. In fact, CSCs are also referred to as the side population and can be isolated from tumors or

505 cancer cell lines by their ability to rapidly efflux fluorescent dyes such as Hoechst 33342. The
506 ability to fluorescently label the CSCs as well as the bulk tumor cells is necessary for maximal
507 tumor removal by fluorescence-guided tumor resection techniques. Therefore, we sought to
508 determine the potential of R-CDs to effectively label our GSC lines. Glio3, Glio9, and Glio38
509 were treated with increasing concentrations (50-1000 $\mu\text{g/mL}$) of R-CDs for 6 hrs and visualized
510 by fluorescent microscopy. Upon observation it is evident that the fluorescent intensity increases
511 in a concentration dependent manner. **Figure 11A** displays the comparison between the
512 fluorescence microscope bright field (Bf) and the red channel for non-fluorescent and fluorescent
513 cellular visualization. While no fluorescence was observed in the non-treated controls,
514 fluorescence was observed in all 3 cell lines at 100 $\mu\text{g/mL}$. As the concentration is increased to
515 an upwards of 1000 $\mu\text{g/mL}$, the fluorescence is notably stronger. Furthermore, while R-CDs
516 dispersed throughout the cell, they appeared to be localizing mainly in the cytoplasm (**Figure**
517 **11B**) since DAPI staining normally targets and highlights only the cell nucleus. Based on these
518 results, R-CDs may be well-suited for cell/tumor imaging in the red region. Additionally, the
519 favorable cell viability and imaging serves as motivation to address the challenge presented by
520 GBM, which is increased drug efflux of theranostic materials.

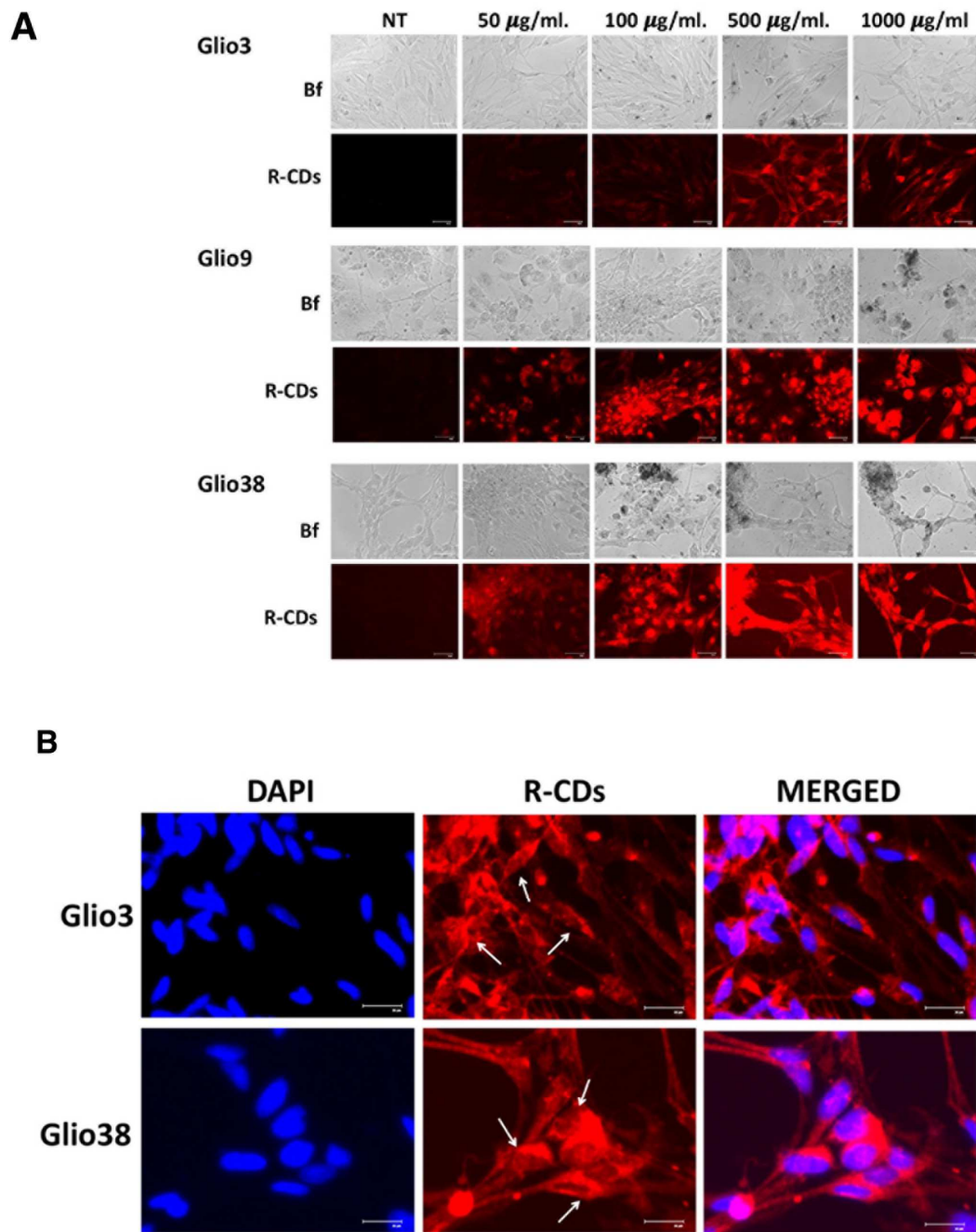


Figure 11. (A) GSCs were exposed to R-CDs as concentrations shown for 6 hrs and imaged with Bf and using the red channel. Scale bar is 50 μm . (B) GSCs were exposed to 500 $\mu\text{g/mL}$ for 1 h and the fluorescent intensity observed. DAPI was used to stain the cell nucleus. Arrows indicate reduced fluorescent intensity in cell nucleus. Scale bar is 25 μm .

522 3.5 Drug Efflux Assessment of R-CDs in Glio3 and Glio38

523 The R-CDs were assessed for their ability to resist drug efflux by both Glio3 and Glio38
524 neurospheres (**Figure 12A, 12B**). The neurospheres were exposed to R-CDs for 4 hrs and
525 imaged immediately (0 hr) or at 48 hrs following the removal of the CDs from the cell culture
526 medium. The neurospheres exhibited robust fluorescence both immediately following exposure
527 as well as 48 hrs later despite the removal of the R-CDs from the cell culture media. This
528 indicates that the R-CDs are not readily effluxed from the GBM neurosphere cells. Overall, these
529 favorable results make sense due to the presence of the PEG-linked network of R-CDs.
530 Literature confirms that PEG plays a key role in efflux pump inhibitions as well as PEGylated
531 compounds generally having a high cellular uptake^{94,95}. With these results in mind, we sought
532 to develop tumor selective R-CDs which possess low drug efflux.

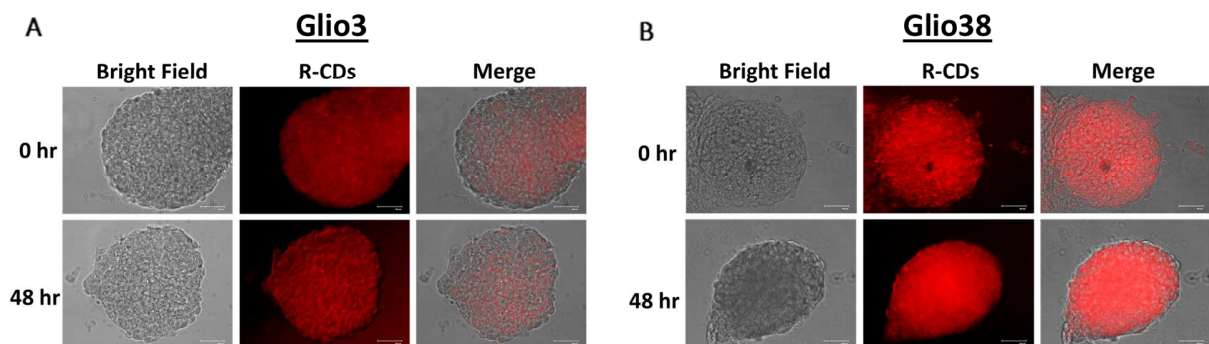


Figure 12. (A) Glio3 and (B) Glio38 neurospheres were treated with 100 ug/ml R-
CDs for 4 hrs and imaged immediately (0 hr) for R-CDs removed, neurospheres
washed 2 times in cell culture media and incubated an additional 48 hrs prior to

533

534 3.7 Cellular Uptake Enhancement by Homing Peptide Conjugation of AKRGARSTA and R-CD

535 To enhance the cellular uptake of R-CD, the conjugation between the CD and short homing
536 peptide AKRGARSTA was described in the **Experimental Section** with the scheme provided in

537 **Figure 13.** This particular peptide is known to provide higher cellular uptake towards GBM cell
538 lines since its mechanism of action relies on the interaction with key cell receptors. Based on our
539 PCR results (**Figure S5-6**), GBM cells overexpress neuropilin1 (NRP1) and p32 (C1QBP) which
540 normally interacts with AKRGASTA peptide for cellular intake. The rationale of why we are
541 interested in this specific homing peptide is due to the fact that there is a significantly higher
542 expression of both neuropilin1 (NRP1) and p32 (C1QBP) in GBM than in the normal brain as
543 provided in **Figure S5-6**. Furthermore, the justification of this reaction stems from
544 AKRGARSTA peptide's mode of action regarding GBM. The initial interaction of
545 AKRGARSTA begins at the p32 receptor of GBM tumor cells which are overexpressed on the
546 surface of vascular and malignant cells followed by proteolytic processing. This process then
547 exposes the C-end Rule (CendR) motif of the peptide (AKRGAR) to enable neuropilin-1 (NRP-
548 1) binding⁹⁶. Thus, activating a specialized tumor penetration pathway. AKRGARSTA has been
549 established as a conjugate for nanoparticles to deliver targeted treatment of breast cancer and
550 glioblastoma^{97,98}. Therefore, we posit that through this conjugation, the R-CD conjugates will
551 effectively enhance cellular uptake as provided in **Figure 14**.

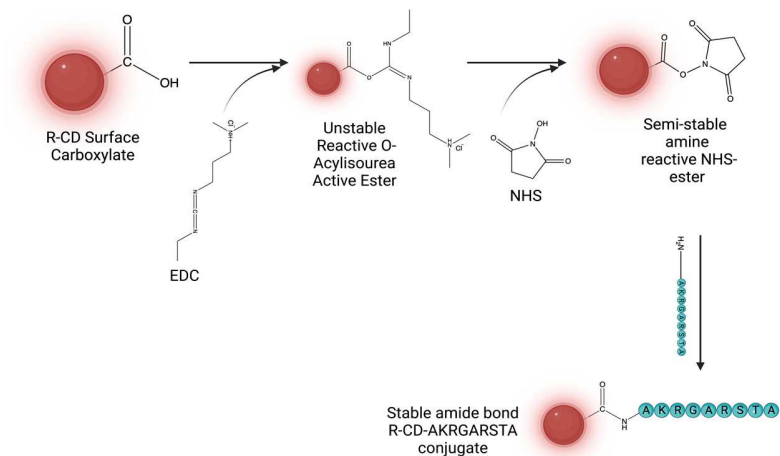


Figure 13. Schematic representation of the conjugation of AKRGARSTA to R-CD (R-CD-AKRGARSTA) using

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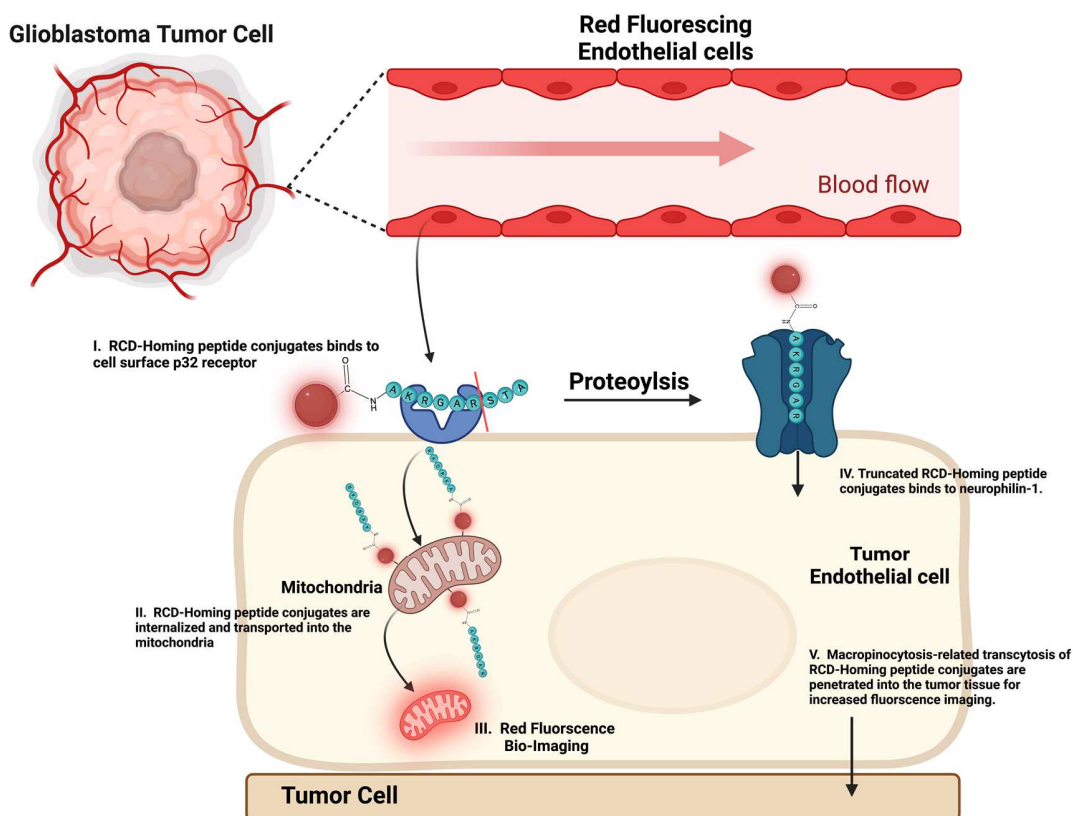


Figure 14. Schematic representation of the R-CD-AKRGARSTA penetration pathway with

553

GBM. Created with BioRender.com

554 Regarding the characterization of the conjugate to ensure the success of the reaction, CDS
555 was implemented to detect the presence of the peptide on the purified product. CDS is one of the
556 main techniques used to determine the secondary structure of a protein or peptide, making it
557 possible to obtain its ellipticity signal^{99,100}. A protein's structure mainly depends on the amino
558 acid sequence and its length. The longer the peptide chain, the more complex the structure can
559 be. Furthermore, the amino acid within the peptide tends to intramolecularly interact with one
560 another via dipole-dipole (H-bonding), π -related and hydrophobic interactions, alongside
561 covalent bond as disulfide bridges between cysteine residues^{101,102}. In addition to these
562 interactions, solvents also influence the final structure of the proteins which dictates their
563 functionality according to their stabilization¹⁰³. Therefore, investigation of its architecture is a
564 must to understand how it interacts and behaves with other compounds. When proteins or
565 peptides are studied using CDS, it is possible to obtain insights about their structure^{104,105}. Based
566 on the implementation of this methodology to our system, the data displayed on **Figure 15A**
567 indicates that the R-CDs do not present any significant signal on the CDS which concludes that
568 R-CDs are identified as non-chiral nanoparticles. In contrast, when the conjugated sample (R-
569 CD-AKRGARSTA) was analyzed, the ellipticity signal within the range of 190 – 250 nm
570 suggests that the coupling reaction was successful. Due to the small number of amino acids
571 residues (9), the peptide cannot form a more complex structure than a random coil based on
572 comparisons with the literature. In addition, to further sustain the random coil as the structure of
573 this peptide, we utilized the Beta Structure Selection (BeStSel) algorithm on the CDS spectrum
574 which didn't present any predominant beta-sheet structure (80 % or higher) as shown in **Figure**
575 **15B**¹⁰⁶⁻¹⁰⁸.

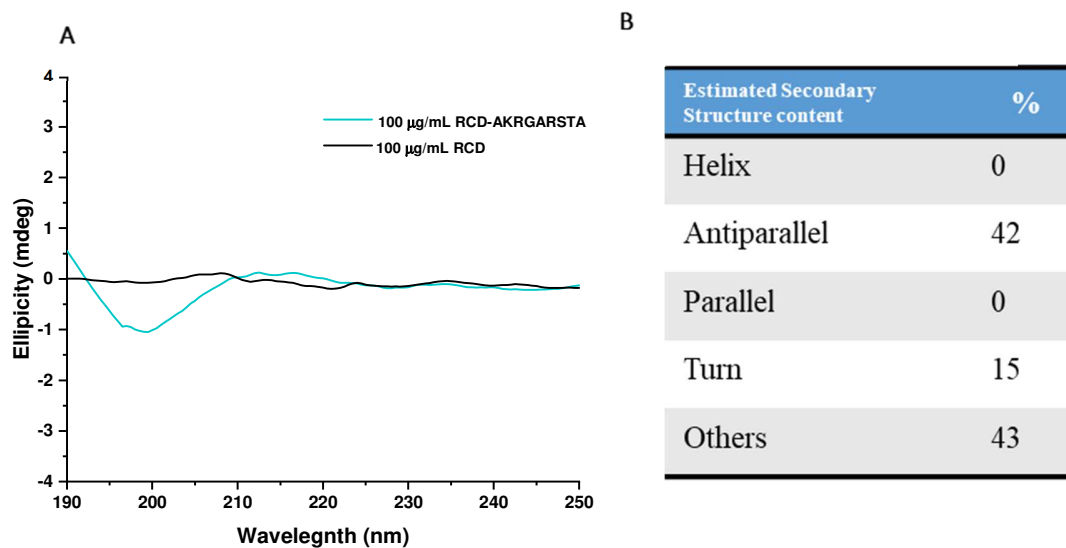


Figure 15. (A) Circular Dichroism spectra of naked R-CD and R-CD conjugated to AKRGARSTA; (B) BeStSel estimated secondary structure based on the circular dichroism data. The analysis of the BeStSel was repeated three times for each CD with an experimental uncertainty of $\mp 0.10\%$.

576

577 To assess the discreet differences of cellular uptake between cell lines, Glio3, Glio9, Glio38
 578 and non-cancer MSCs were treated with 100 μM of either R-CD or R-CD peptide conjugate for
 579 1 hr and evaluated by fluorescent microscopy (**Figure 16**). Non-treated (NT) cells were included
 580 as a control. Remarkably, the images show a vast difference in fluorescence intensity between
 581 both R-CD and R-CD-AKRGARSTA. Although the red channel imaging of R-CD is favorable,
 582 clearly the conjugate boasts superiority in terms of imaging capabilities for Glio3, Glio9, and
 583 Glio38. We can ascribe this result due to the enhanced selectivity of tumor cells over non-tumor
 584 cells via the short homing peptide AKRGARSTA as described previously. This specificity is
 585 evident when observing the red channel of the non-tumor MSC cell line as there are less
 586 significant changes between the CDs and conjugate.

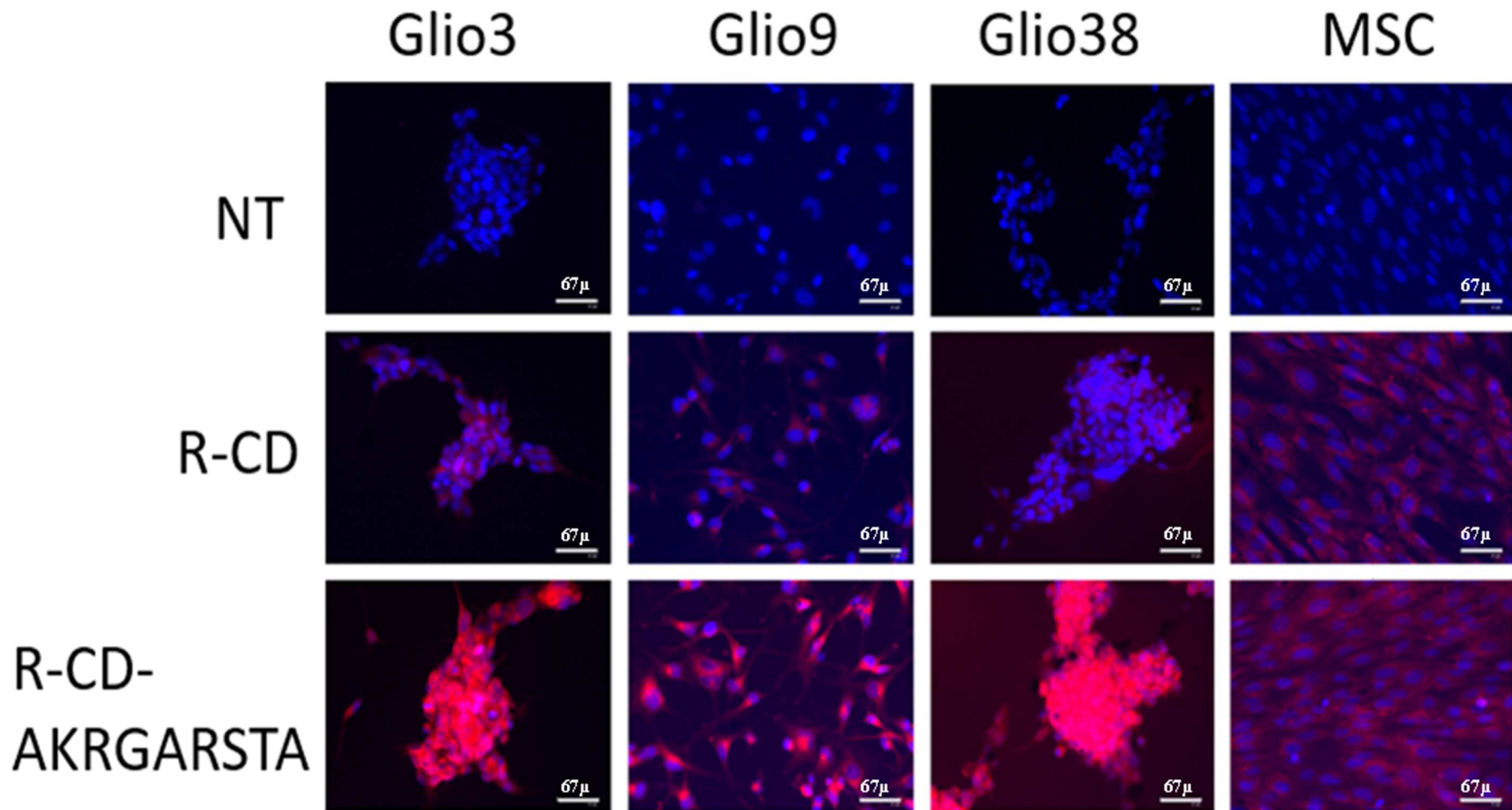


Figure 16. Effect of peptide targeting of R-CDs on cell cancer and non-cancer cells. A. GSC lines Glio3, Glio9, Glio38 and non-cancer MSCs were treated with 100 μ M of either R-CDs or R-CD- peptide conjugate (Conjugate) for 1 hr and evaluated by fluorescent microscopy. Non-treated (NT) cells were included as a control. Cell nuclei were stained with DAPI (blue channel) and CD fluorescence. Scale bar represents 67 microns and was visualized using the red channel. Merged red and blue channels are shown.

588 To elucidate the degree of fluorescent enhancement between the CD and its conjugate, both
589 mean fluorescence intensity and percent increase of corrected total cell fluorescence (CTCF)
590 were determined as described in **Experimental Section**. The observation of the mean
591 fluorescence in both GSC and MSCs are again noted to have noticeable differences which
592 correspond to nanoparticle uptake between the CD and CD conjugate (**Figure 17A**). With
593 regards to the results on R-CDs, whereas the mean fluorescence in MSC is 64, in Glio3, Glio9,
594 and Glio38 the values are 61, 39 and 66, respectively. Yet to our satisfaction, R-CD-
595 AKRGARSTA yielded much higher mean fluorescence with Glio3, Glio9, and Glio38 having
596 values of 156, 87 and 186, respectively. This data not only corroborates the earlier suggestion
597 that the conjugate has an effective mechanism for cellular uptake, but also indicates that there
598 was minimal alteration in the material intake by MSC. The mean fluorescence of conjugated R-
599 CDs in MSC provided a value of 77, which is only slightly higher than that of bare R-CDs. To
600 provide clearer insight into the increase of fluorescence due to the enhancement of cellular
601 uptake of the CDs, the CTCF values between R-CD and R-CD-AKRGARSTA were calculated
602 to determine the percent increase of fluorescence (**Figure 17B**). As expected, the data yielded
603 highly valuable information which supports the qualitative observation of the initial images. In
604 Glio3, Glio9, and Glio38, R-CD-AKRGARSTA has shown to increase the CTCF by 2.56, 2.23,
605 and 2.82-fold, respectively. In MSC, the conjugate only resulted in a CTCF increase of 1.2-fold.
606 The specificity of R-CD-pep for the tumor cells can also be demonstrated in **Figure S7** in which
607 Glio3, Glio9, Glio38 and MSCs were exposed to only 50 μ M R-CD-Pep and imaged by
608 fluorescent microscopy. While the GBM stem cell lines, Glio3, Glio9 and Glio38 clearly
609 demonstrated pronounced fluorescence, the fluorescence of the non-tumorous MSCs is barely

610 visibly. These results support our hypothesis that the peptide bound onto the surface of R-CD
 611 has specificity to mainly target GBM tumor cells.

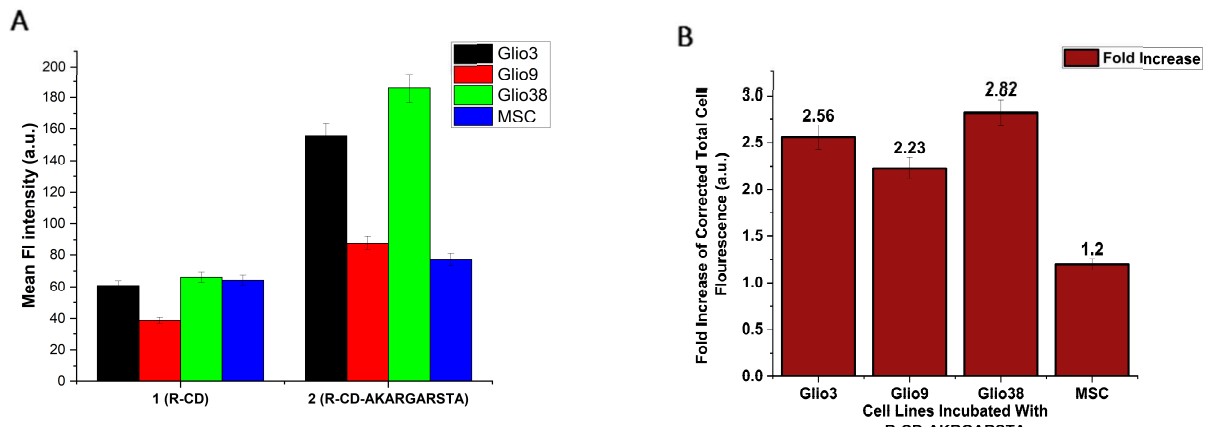


Figure 17. Fluorescence assessment of 100 μ M CDs incubated for 1 hr of CDs on GSC cell lines Glio3, Glio9, Glio38 and non-cancer MSC. (A) Mean fluorescence intensity. (B) Fold increase of CTCF from R-CD-AKRGARSTA when compared to R-CD.

612

613 *3.7 Crossing the BBB via R-CD-Tf Conjugate in zebrafish model*

614 Given the nature of GBM, it is imperative to address the challenge to cross the BBB. To be
 615 specific, the CNS comprises of both the brain and spinal cord which is protected by the intricate
 616 mechanisms that are regulated within the BBB³⁴. Moreso, the BBB serves as a physiological
 617 point of entry that has high specificity towards what molecules are permitted entry from the
 618 blood circulation of the host into the CNS. This feature coupled with physical characteristics of
 619 the BBB being composed of capillary endothelial cells that are interconnected by tight junctions
 620 leads to the difficult transport of materials such as drugs or CDs into the CNS¹⁰⁹. In fact, the vast
 621 majority of both large molecules and nanoparticles are unable to pass the BBB without surface
 622 modifications¹¹⁰. To remedy this limitation, our group has successfully conjugated R-CDs (R-
 623 CD-Tf) with the ligand transferrin with a scheme provided in **Figure 18A**. Transferrin was

624 selected as the ligand of choice due to the BBB's brain capillary endothelial cells having a high
625 degree of expressed transferrin receptors. These transferrin receptors have been proven to permit
626 the entrance of transferrin-conjugated nanoparticles according to literature¹¹¹.

627 To investigate the prospect of transporting R-CD-Tf into the CNS the zebra model was due to
628 their physiological homology to humans. This complex vertebrae species possesses the ideal
629 hormones, receptors, and major neurotransmitters that allow researchers to develop therapeutic
630 agents *in vivo* that can be extended to human disease¹¹². Furthermore, given their physical
631 attributes such as small size, transparent body, and conditions to breed, the zebrafish is a more
632 facile model to both simulate and follow non-invasive imaging techniques in the CNS than that
633 of mice¹¹³. The larval zebra fish were used at 5 days post fertilization as studies conclude that
634 the maturation of the BBB of zebrafish is completed within 3 days comparable to mammals¹¹⁴.
635 As indicated in **Figure 18B**, the CDs of choice were injected into the heart of the larval zebrafish
636 to allow the pumping of the material through the blood stream. We predict that, in comparison
637 to R-CD, R-CD-Tf will successfully be received by the transferrin receptor and transported
638 through the BBB and into the CNS.

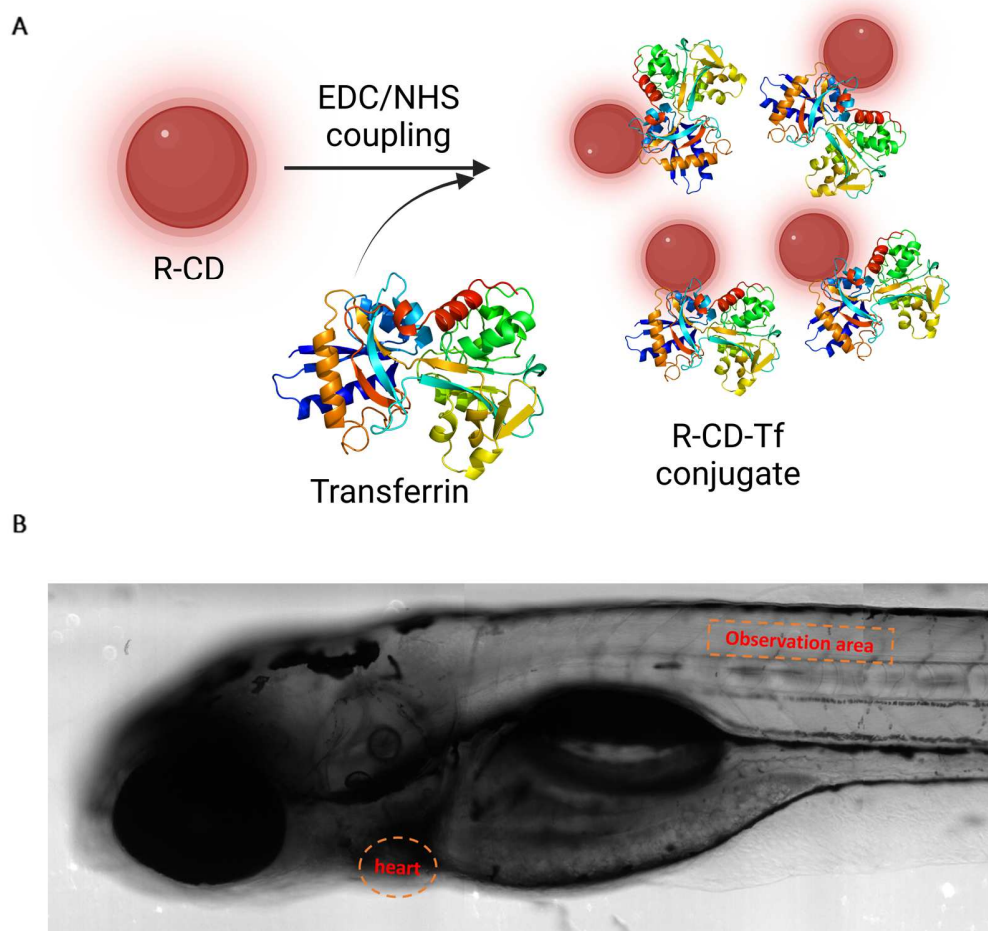


Figure 18. (A) Schematic representation of the conjugation of transferrin to R-CD (R-CD-Tf). (B) Image of zebrafish with the highlighting of both the heart (injection site) and central canal (observation area).

639

640 The confocal images of the larval zebrafish were overlapped with the bright field images which
 641 yielded quite interesting results (**Figure 19**). As expected, due to the absence of transferrin on
 642 the surface, the R-CDs when visualized under 405 nm excitation did not appear to be in the
 643 central canal suggesting that the R-CDs did not penetrate the BBB. Although it is worthwhile to
 644 note that the R-CDs were able to be clearly observed throughout the image. Upon analysis of the
 645 overlapped images containing R-CD-Tf, red fluorescence of the central canal was observed

646 indicating crossing of the BBB by R-CD-Tf. In following, we hypothesize that the R-CD-Tf
647 were successfully transported into the CNS across the BBB as provided in **Figure 20**. The
648 finding of this result has strong impact in the field of imaging as this is the first time to our
649 knowledge to observe stable, water-soluble and far-red emitting CDs to cross the BBB
650 successfully.

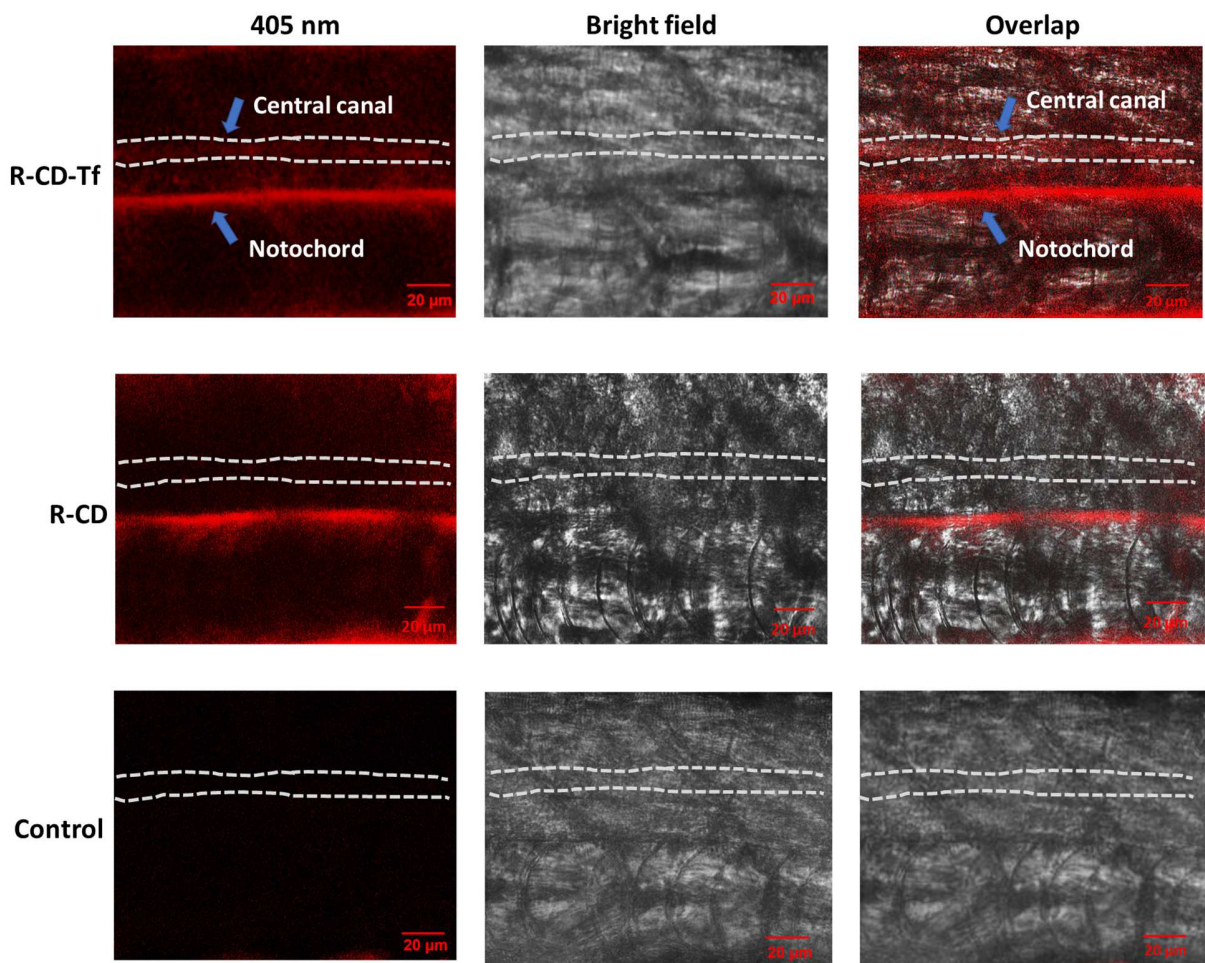


Figure 19. Confocal image of R-CD-Tf versus R-CDs in the CNS of zebrafish under 405 nm excitation. The blue arrow indicates the central canal of spiral cord of zebrafish.

651

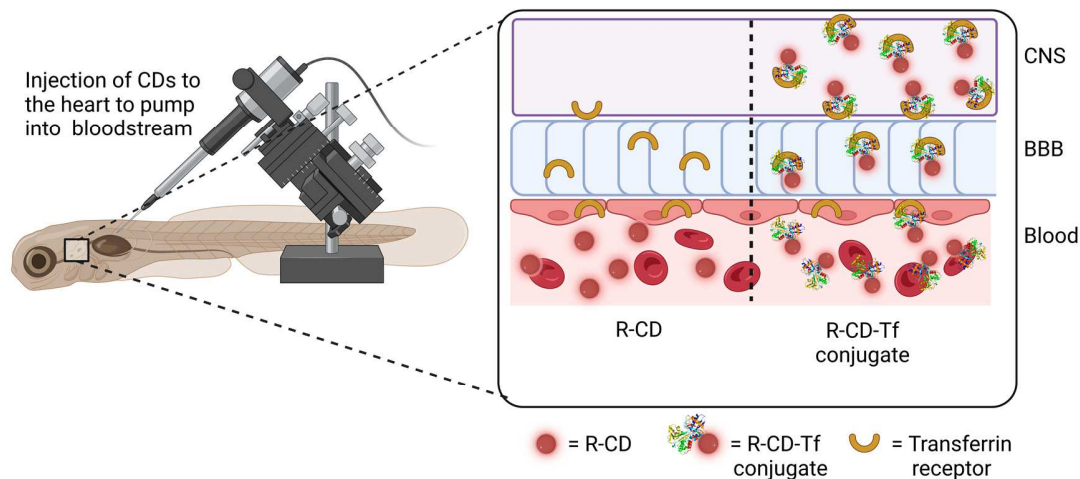


Figure 20. Graphic visualization of the transit of R-CD-Tf via the BBB into the CNS as compared to R-CD in zebrafish. Created with BioRender.com

652

653 **4. Conclusion**

654 For the first time, we have synthesized a new type of red-emitting CDs derived from
 655 mixture of the organic fluorophore ICG, citric acid, and PEG. Ultimately, the red emission
 656 was attributed due to the decrease of conjugation of the fluorophore that passivates the CD.
 657 Additionally, this is the first report of far-red emission CDs whose applications are favorable
 658 in water leading to excellent biocompatibility in comparison to previous reports^{115,116}. This
 659 type of CD was proven to exhibit low toxicity in both GBM and MSCs and was showcased
 660 as suitable materials for enhanced fluorescent imaging of cancer cells including GSCs.
 661 Furthermore, post-conjugation we were able to both selectively increase the cellular uptake
 662 of CDs into GSC as well as cross the BBB in the zebrafish model. To our knowledge, the
 663 this is the first account of CDs bound with AKRGARSTA to increase tumor selectivity. In

664 further application, we believe the potential of both drug delivery and drug tracking is the
665 next step field of study for these types of CDs in the field of theranostics^{117,118}.

666 **Credit authorship contribution statement**

667 **Justin Domena:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation,
668 Writing- original draft, Writing- review & editing, Project administration.

669 **Braulio C.L.B. Ferreira:** Formal analysis, Methodology, Writing-review & editing.

670 **Emel K. Cilingir:** Methodology, Formal analysis

671 **Yiqun Zhou:** Methodology, Formal analysis, Writing-review & editing

672 **Jiuyan Chen:** Methodology, Formal analysis

673 **Qiaxian R. Johnson:** Formal analysis.

674 **Bhanu P.S. Chauhan:** Formal analysis

675 **Mattia. Bartoli:** Methodology, Formal analysis, Writing-review & editing

676 **Alberto Tagliaferro:** Methodology, Formal analysis, Writing-review & editing

677 **Steven Vanni:** Methodology, Formal analysis

678 **Regina M. Graham:** Methodology, Validation, Formal Analysis, Investigation, Writing-
679 original draft, Writing- review & editing.

680 **Roger M. Leblanc:** Conceptualization, Methodology, Validation, Writing-review & editing.

681 **Declaration of Competing Interest**

682 The authors declare that they have no known competing financial interests or personal
683 relationships that could have appeared to influence the work reported in this paper.

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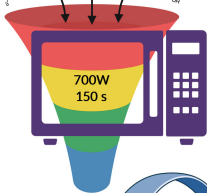
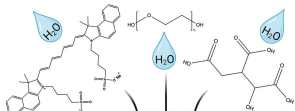
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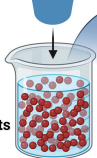
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Water Soluble Carbon Dots



697 nm
Red Emission

Red-Imaging
of Tumor Cell

