

1 **Biodegradable harmonophores for targeted high-**  
2 **resolution *in vivo* tumor imaging**

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24 **Abstract**

25           Optical imaging probes have played a major role in detecting and  
26 monitoring of a variety of diseases<sup>1</sup>. In particular, nonlinear optical imaging  
27 probes, such as second harmonic generating (SHG) nanoprobes, hold great  
28 promise as clinical contrast agents, as they can be imaged with little  
29 background signal and unmatched long-term photostability<sup>2</sup>. As their chemical  
30 composition often includes transition metals, the use of inorganic SHG  
31 nanoprobes can raise long-term health concerns. Ideally, contrast agents for  
32 biomedical applications should be degraded *in vivo* without any long-term  
33 toxicological consequences to the organism. Here, we developed  
34 biodegradable harmonophores (bioharmonophores) that consist of polymer-  
35 encapsulated, self-assembling peptides that generate a strong SHG signal.  
36 When functionalized with tumor cell surface markers, these reporters can target  
37 single cancer cells with high detection sensitivity in zebrafish embryos *in vivo*.  
38 Thus, bioharmonophores will enable an innovative approach to cancer  
39 treatment using targeted high-resolution optical imaging for diagnostics and  
40 therapy.

## 41 **Main Text**

42 Clinical and preclinical imaging holds great potential in mapping disease  
43 progression and can provide diagnostic information that may guide the choice  
44 of treatment strategies for disease<sup>3, 4</sup>. Optical techniques using bioluminescent  
45 and fluorescent probes have emerged as promising modalities for molecular  
46 imaging in disease and therapy due to their ease of use and improved cellular  
47 resolution, capable of distinguishing boundaries between malignant and normal  
48 tissue<sup>5</sup>. A key challenge for optical imaging probes and instrumentation,  
49 particularly those aimed at eventual clinical applications, is to overcome the  
50 limited depth penetration of excitation light, which often result in a low signal-  
51 to-noise ratio (SNR)<sup>6</sup>. The relatively poor photostability of most imaging probes  
52 pose another challenge to provide reliable and sensitive imaging of tumors.

53 Previously, we introduced inorganic second harmonic generating (SHG)  
54 nanocrystals, SHG nanoprobess<sup>2</sup>, as a new class of imaging probes that can be  
55 used for *in vivo* imaging. Given that SHG imaging employs near infrared (NIR)  
56 incident light for contrast generation, SHG nanoprobess can be utilized for deep  
57 tissue imaging. Unlike commonly used fluorescent probes, SHG nanoprobess  
58 neither bleach nor blink, and their signal does not saturate with increasing  
59 illumination intensity, ensuring high probe sensitivity<sup>7</sup>. Since their signal profile  
60 is very narrow, they can be imaged with high SNR by excluding the broad  
61 emission of typical autofluorescence background<sup>2</sup>. Robust functionalization  
62 allows targeting to a wide variety of cells and proteins of interest<sup>8</sup>, allowing  
63 these imaging probes to be promising tools for both clinical and preclinical  
64 imaging applications<sup>9</sup>. Despite these advantages, the chemical structure of  
65 inorganic SHG nanoprobess makes them stable in the body, which may cause

66 concerns for the long-term health of an organism that has been imaged with  
67 these reporters.

68 To create a foundation for safe SHG nanoprobe-based clinical imaging,  
69 we set out to generate a nanoprobe that consists of biodegradable materials,  
70 capable of generating sufficient SHG signal that can be detected with high SNR.  
71 Our efforts were guided by the observation that peptides with a variable number  
72 of amino acid units can self-assemble into large, solid nanostructures of  
73 different morphologies and symmetries<sup>10</sup> (**Fig. 1a**). It has been previously  
74 shown that such nanostructures can be ferroelectric and give nonlinear optical  
75 contrast such as SHG<sup>11, 12</sup> (**Fig. 1c**).

76 To render these nanostructures suitable for biological applications, we  
77 evaluated methods for the encapsulation of self-assembling peptides in order  
78 i) to hinder their macroscopic aggregation by confining their self-assembly in  
79 nanodroplets without affecting their ability to generate a strong SHG signal, and  
80 ii) to generate a nanoparticle that can be further functionalized without  
81 influencing the peptide assembly. To this end, we subjected several peptides  
82 that have been reported to self-assemble into complex nanostructures to the  
83 emulsion-solvent evaporation method<sup>13</sup>, a widely-used procedure for the  
84 fabrication of monolithic and core-shell nanoparticles (**Fig. 1b, see Methods**).

85 We identified three peptides with different self-assembling properties  
86 (pentaalanine<sup>14</sup>, trileucine<sup>15</sup>, and triphenylalanine<sup>16</sup>) that could generate  
87 detectable SHG signal when encapsulated in the biodegradable polymer (**Fig.**  
88 **1d and Supplementary Fig. 1**). Transmission electron microscopy (TEM)  
89 analysis of the predominantly spherical nanoparticles, hereinafter referred to as

90 bioharmonophores, revealed a diameter ranging from 50-150 nm, which was  
91 confirmed by dynamic light scattering (DLS) measurements (**Fig. 1e**).

92 SHG signal from bioharmonophores can stem from i) the bulk of the self-  
93 assembling peptides that form noncentrosymmetric crystalline structures or ii)  
94 the surface of the bioharmonophores where there is no inversion symmetry. To  
95 ascertain that the SHG signal originates from the crystalline peptide core, we  
96 performed X-ray diffraction (XRD) analysis of bioharmonophores with different  
97 peptide contents. In all cases, the peptides showed a high degree of internal  
98 order with distinct diffraction patterns associated with their individual crystalline  
99 phases and self-assembling behavior (**Supplementary Fig. 2a-d**).

100 Because bioharmonophores based on triphenylalanine (FFF) peptides  
101 yielded the strongest SHG signal compared to pentaalanine and trileucine, we  
102 subjected these bioharmonophores to detailed optical characterizations. The  
103 SHG signal of FFF-based bioharmonophores was spectrally well-defined (**Fig.**  
104 **2a**). Additionally, the SHG emission patterns of FFF-based bioharmonophores  
105 displayed a broad opening: one seemingly isotropic, and the other one  
106 displaying one lobe over 60° in the forward direction (**Fig. 2b**). These results  
107 indicate that bioharmonophores emit SHG signal in multiple directions (unlike  
108 the predominantly forward-directed SHG of large protein arrays)<sup>7</sup>, which  
109 allowed illumination and collection of SHG signal using the same microscope  
110 objective lens. Moreover, the presence of a single lobe demonstrates that the  
111 observed SHG signal originates from the bulk of the bioharmonophores and not  
112 from its surface, as described by Mie theory<sup>17</sup>.

113 Because SHG involves only virtual energy transitions,  
114 bioharmonophores did not display blinking, remained stable over extended

115 periods of illumination, and their SHG signal intensity rose quadratically when  
116 the laser intensity shone on them was linearly increased (**Supplementary Fig.**  
117 **3a,b**). The measured polarimetric diagrams (**Fig 2c and Supplementary Fig.**  
118 **3c-f**) were consistent with the hypothesis that bioharmonophores have a self-  
119 assembling peptide core with a monoclinic (C<sub>2</sub>) symmetry. Indeed, the  
120 experimental curves were well fitted with the analytical expression calculated  
121 for this symmetry (see **Supplementary Note 1 – Optical Characterization of**  
122 **Bioharmonophores**). Taken together, bioharmonophores have the same  
123 photophysical advantages for biomedical imaging applications that have been  
124 previously described for inorganic SHG nanoprobes<sup>2</sup>.

125 To gain insight into the parameters influencing the bioharmonophore  
126 stability and signal intensity, we tested several reaction conditions to generate  
127 bioharmonophores. Given that the SHG signal originating from  
128 bioharmonophores is dependent on the amount of encapsulated peptide, we  
129 first tested whether varying the FFF peptide concentration during production  
130 would improve the SHG signal intensity of generated bioharmonophores (**Fig.**  
131 **2d and Supplementary Fig. 4**). We found that an amount of 15 mg FFF (i.e.  
132 33wt%) peptide provided an optimal combination of intense SHG signal and  
133 bioharmonophore stability. Interestingly, while an FFF peptide amount of 20 mg  
134 (40wt%) increased the overall SHG signal, it also led to bioharmonophore  
135 aggregation and decreased colloidal stability. Conversely, 10 mg (25wt%) FFF  
136 peptide generated little SHG signal.

137 Because surfactant concentration plays a crucial role in emulsification of  
138 chloroform droplets<sup>13</sup>, we reasoned that altering the surfactant concentration  
139 during the preparation of bioharmonophores would have a profound effect on

140 their stability and signal strength (**Fig. 2e and Supplementary Fig. 5**).  
141 Bioharmonophores emulsified in an aqueous solution with 0.3% sodium  
142 dodecyl sulfate (SDS) (i.e. 40wt% of dispersed phase) yielded stable  
143 bioharmonophores with intense SHG signal, whereas compositions employing  
144 0.1% SDS (18wt%) yielded aggregated nanoparticles. Increasing the SDS  
145 concentration to 0.6% (57wt%) diminished the SHG signal intensity, suggesting  
146 that the bioharmonophore size and hence the number of enclosed peptide  
147 molecules within each bioharmonophore is influenced by the concentration of  
148 surfactant.

149 Finally, we varied the polymer quantity that encapsulates and shields  
150 peptides from environmental changes, and assessed its role in both SHG signal  
151 intensity and nanoparticle morphology (**Fig. 2f and Supplementary Fig. 6**).  
152 We identified that an amount of 30 mg of poly(*L*-lactic acid) (PLLA) (66wt%)  
153 resulted in an optimal combination of intense SHG signal and bioharmonophore  
154 stability. Lower polymer amount of 10 mg (40 wt%) yielded weaker SHG signal,  
155 whereas higher polymer amount (90 mg, 86 wt%) led to elongated  
156 bioharmonophore morphologies. Taken together, we identified optimal  
157 experimental conditions to generate bioharmonophores providing a high SNR  
158 along with an excellent stability and size distribution for biological applications.

159 Clinical imaging probes that are biodegradable provide the significant  
160 advantage of being able to be broken down in the body and removed after they  
161 have served their function. To demonstrate that bioharmonophores are indeed  
162 biodegradable, we utilized the highly effective serine protease, proteinase K,  
163 which exhibits a broad cleavage specificity<sup>18</sup>. We incubated bioharmonophores  
164 with a proteinase K concentration that is routinely used for dissolving tissue

165 structures<sup>19</sup> and probed the extent of degradation by monitoring the SHG signal  
166 at different time intervals (**Fig. 3a**). We observed a decrease of SHG signal  
167 within 2 hours of protease incubation. After 10 hours, the SHG signal  
168 disappeared and the turbid bioharmonophore suspension became transparent  
169 (**Fig. 3b and Supplementary Fig. 7**), indicating a successful biodegradation of  
170 the bioharmonophore.

171 To evaluate bioharmonophore degradation under physiological  
172 conditions (**Fig. 3c**), we functionalized bioharmonophores with Tat-derived cell  
173 penetrating peptides<sup>20</sup> using bioorthogonal click chemistry, (**Supplementary**  
174 **Fig. 8**), and incubated them with a model cancer cell line (see below) overnight.  
175 Adherent cells were then detached by trypsinization, and centrifuged to remove  
176 excess bioharmonophores that did not enter the cancer cells. Following this  
177 procedure, cells were reseeded and fixed at specific time periods to monitor  
178 bioharmonophores degradation (i.e. the intracellular presence of SHG signal  
179 per cell) using nonlinear optical imaging. 30 hours after cell reseeded, a  
180 pronounced decrease of intracellular SHG signal per cell was noticeable (**Fig.**  
181 **3c**). As bioharmonophores displayed long-term photostability even at low pH  
182 values (**Supplementary Fig. 9**), the drop of signal was not due to their potential  
183 accumulation in acidic endolysosomal compartments over time. In order to  
184 show that bioharmonophores can be degraded using intracellular proteolytic  
185 degradation, we tested whether the bioharmonophores could be degraded  
186 using a cell-free lysate system based on an established cell free degradation  
187 assays<sup>21</sup>. We also observed reduced SHG signal, indicating that intracellular  
188 enzymatic degradation of bioharmonophores might account for the signal loss  
189 (**Fig. 3e and Supplementary Fig. 10**). Importantly, bioharmonophores did not



190 exhibit any short-term toxicity *in vitro* and *in vivo* (**Supplementary Fig. 11**) and  
191 did not induce protein aggregation<sup>22</sup> (**Supplementary Fig. 12**), rendering them  
192 safe imaging probes.

193 Among various diagnostic applications, bioharmonophores could be  
194 ideal imaging probes for single-cell cancer detection due to their high SNR and  
195 photostability, which other intravital imaging modalities cannot achieve<sup>3</sup>. To  
196 demonstrate the unique optical features of bioharmonophores for cancer  
197 targeting and imaging, we employed xenograft zebrafish cancer models, which  
198 offer speed, cellular resolution, and the ability to perform large numbers of  
199 transplants for obtaining valuable information about several cancer types<sup>23, 24</sup>.

200 To generate a highly aggressive cancer model that can be tracked over  
201 time, we injected a DsRed-expressing metastatic human melanoma cells  
202 (MDA-MB-435-DsRed) into the Duct of Cuvier (DoC) of zebrafish embryos at 2  
203 dpf (days post fertilization)<sup>23, 24</sup> (**Fig. 4a**). By 3 days after the injection, the  
204 resulting tumors spread to various locations in the body and were found next to  
205 blood vessels, which likely support the tumors with nutrients<sup>25</sup> (**Fig. 4b**).

206 To demonstrate the specificity and efficiency of bioharmonophores as  
207 novel contrast agents that can accomplish resolution down to the single cell *in*  
208 *vivo*, we targeted bioharmonophores to tumor sites by taking advantage of the  
209 surface protein p32/gC1qR as a unique molecular marker for MDA-MB-435-  
210 DsRed cells<sup>26</sup>. To this end, we functionalized bioharmonophores with a p32  
211 targeting peptide, injected them into the DoC of zebrafish embryos at 3 dpf, one  
212 day after the embryos were injected with MDA-MB-435-DsRed cancer cells,  
213 (**Fig. 4c**) and assessed colocalization between cancer cells and  
214 bioharmonophore signal at 5 dpf (see **Supplementary Note 2** - Determining

215 the fraction of bioharmonophore-labeled tumors in a zebrafish cancer model).

216 In the absence of tumors, functionalized bioharmonophores did not cause

217 clustering at the site of injection and were localized at different parts of injected

218 zebrafish embryos (**Supplementary Fig. 13d-i**), indicating good biodistribution.

219 Without bioharmonophore injection, zebrafish as well as tumor sites did not

220 produce any SHG background signal (**Supplementary Fig. 13a-c, 14a-c**) with

221 the exception of minimal endogenous SHG signal localized at the zebrafish

222 tail<sup>27</sup>, which was excluded from assessing specificity of tumor targeting

223 (**Supplementary Fig. 15**). In the case of passive targeting, zebrafish injected

224 with PEG-coated bioharmonophores revealed limited tumor labeling

225 (**Supplementary Fig. 14d-f**), stemming from leaky blood vessels and

226 enhanced permeability and retention effect (EPR)<sup>28</sup>. In contrast, we observed

227 an increased accumulation of p32 peptide-targeted bioharmonophores within

228 individual cancer cells at tumor sites throughout the zebrafish embryos (**Fig.**

229 **4c1-c4'**), indicating that the tumor-labeling specificity and efficiency is highly

230 dependent on the p32 targeting peptide. While p32 peptide-targeted

231 bioharmonophores can extravasate to different tumor sites, not all the cancer

232 cells were labeled (**Fig. 4c**). This observation is potentially due to limited

233 accessibility within densely packed solid tumors<sup>29</sup> and the continued

234 proliferation and metastasis of cancer cells between bioharmonophore

235 administration and imaging (see **Supplementary Note 2** - Determining the

236 fraction of bioharmonophore-labeled tumors in a zebrafish cancer model).

237 In order to determine the extent of labeling of targeted

238 bioharmonophores in the xenograft zebrafish cancer model, we measured the

239 colocalization of cancer cells with bioharmonophores at each tumor site for non-

240 injected zebrafish as well as for zebrafish that were injected with p32 peptide-  
241 targeted and PEG-coated bioharmonophores, respectively (see  
242 **Supplementary Note 2** - Determining the fraction of bioharmonophore-labeled  
243 tumors in a zebrafish cancer model, **Fig. 4c,d**). The number of tumors were not  
244 significantly different between datasets (**Supplementary Fig. 16**). The  
245 zebrafish cancer model injected with p32 peptide-targeted bioharmonophores  
246 had a significantly higher fraction of labeled tumors compared with non-injected  
247 and PEG-coated bioharmonophores (**Fig. 4d**) due to our active targeting  
248 strategy. Overall, these results demonstrate that bioharmonophores exhibit  
249 high SNR and outstanding photostability for efficient labeling of individual  
250 cancer cells at multiple tumor sites *in vivo*.

251 In summary, we introduced bioharmonophores as a novel class of  
252 imaging probes that retain all the photophysical advantages of previously  
253 introduced inorganic SHG nanoprobcs. Because bioharmonophores consist of  
254 a biodegradable peptide core and a polymer shell, they can be metabolized  
255 within cells, which could render them the ideal contrast agent for clinical  
256 imaging applications. The straightforward implementation of robust  
257 functionalization strategies and a sufficiently high metabolic stability *in vivo*  
258 allowed us to target bioharmonophores with high detection sensitivity to  
259 individual tumor cells in live zebrafish embryos. With the recent development of  
260 nonlinear microendoscopes<sup>30, 31</sup>, bioharmonophores have the potential to  
261 emerge as superior contrast agents during image-guided surgery to help  
262 surgeons perform safer and highly precise tumor removal procedures.  
263 Moreover, their unique ability to target single cells could be exploited for  
264 detecting cancer stem cells, a subpopulation of cells responsible for

265 tumorigenicity, invasion, and metastasis<sup>32</sup>. Once successfully identified, the  
266 nonlinear signal of bioharmonophores could be used for light induced drug  
267 delivery or photodynamic therapy<sup>33</sup>. By employing pulsed lasers in the infrared  
268 wavelength range that permit deep tissue penetration, targeted  
269 bioharmonophore signal could trigger highly localized cancer stem cell death<sup>34</sup>.

270 Finally, as the SHG signal intensity of bioharmonophore relies on the  
271 unique self-assembly behavior of each peptide<sup>35</sup>, we anticipate that a screen  
272 for alternative peptide sequences may yield even brighter bioharmonophores  
273 that will potentially permit diagnosis with deep-tissue single-molecule detection  
274 sensitivity.

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## 289 **Author contribution**

290 A.Y.S. conceived and A.Y.S. and P.P refined the idea. A.Y.S. produced  
291 and characterized bioharmonophores with the help of S.J., S.B., D.C., and K.L..  
292 A.Y.S., C.T., and S.R., performed optical characterization. S.Y. and A.Y.S.  
293 performed cell culture experiments. A.Y.S. and S.Y. generated *in vitro* and *in*  
294 *vivo* imaging data. M.K. and S.S. generated the zebrafish cancer model,  
295 performed bioharmonophore injections with the help of C.L.. A.Y.S. and P.P.  
296 wrote the manuscript and all authors contributed to editing the manuscript. P.P.  
297 supervised the project.

### 298 **Competing financial interests**

299 A patent application has been filed relating to aspects of the work  
300 described in this manuscript. Authors listed on the patent: P.P., A.Y.S., K.L.,  
301 and D.C.

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410  
411

## 412 **Figure Legends**

### 413 **Figure 1. Synthesis and analysis of bioharmonophores.**

414 **a**, Schematic of the self-assembling reaction of diphenylalanine peptides (FF)  
415 into large-scale nanotube structures from a concentrated solution. **b**, Schematic



416 of the emulsion-solvent evaporation method for the synthesis of  
417 bioharmonophores. Self-assembling peptides are dissolved in chloroform along  
418 with biodegradable poly(L-lactic acid) (PLLA) and emulsified with the surfactant  
419 sodium dodecyl sulfate (SDS) using sonication, followed by evaporation of  
420 chloroform. **c**, SHG signal from diphenylalanine peptide nanotubes aggregated  
421 on top of the imaging chamber. Peptide nanotubes were illuminated with a  
422 850 nm pulsed laser. Image composite of multiple stitched images. **d**, SHG  
423 signal from encapsulated triphenylalanine peptides (FFF) bioharmonophores  
424 immobilized in 1% low melting agarose illuminated with 850 nm pulsed laser.  
425 **e**, TEM image of synthesized FFF-based bioharmonophores showing uniform  
426 spherical nanoparticles. **Inset**: DLS data showing the size distribution of  
427 synthesized bioharmonophores. Scale bar, 100  $\mu\text{m}$  (**c**); Scale bar, 10  $\mu\text{m}$  (**d**);  
428 Scale bar, 500 nm (**e**).

429

430 **Figure 2. Optical characterization of bioharmonophores and analysis of**  
431 **parameters influencing harmonophore formation.** **a**, Normalized SHG  
432 signal spectrum of FFF-based bioharmonophores (signal ranging from 400 to  
433 600 nm) illuminated with 850 nm pulsed laser. The characteristic SHG peak is  
434 centered around 425 nm. **b**, SHG emission pattern of Triphenylalanine based  
435 bioharmonophores. Orange arrow indicates excitation beam direction. Green  
436 arrow shows SHG collection direction, which rotates between  $-90^\circ$  and  $90^\circ$ . The  
437 detected polarization is in the beams plane (P, black arrow). Red pattern shows  
438 PPP polarization configuration (excitation and detection polarizations in the  
439 beams plane), and blue pattern shows PSS (excitation with a perpendicular  
440 polarization). **c**, SHG intensity vs. incident polarization angle for a

441 bioharmonophore, highlighted by the solid white circle in **Supplementary Fig.**  
442 **3**. Red color shows detection along the X axis while green color shows  
443 detection along the Y axis. The experimental curve is a dotted line, the  
444 corresponding fitted curve, assuming C<sub>2</sub> symmetry, is a solid line. **d**, Influence  
445 of using different amounts of FFF peptide during bioharmonophore production  
446 on the SHG signal intensity. The optimal condition (33 wt%) is marked in green.  
447 The use of higher FFF peptide amount leads to aggregates (n=5). **e**, Influence  
448 of SDS concentration (wt% of disperse phase) on SHG intensity of generated  
449 bioharmonophores. The optimal condition (40 wt% SDS) with high  
450 bioharmonophore stability and less aggregation is marked in green (n=5). **f**,  
451 Influence of using different amounts of PLLA during bioharmonophore  
452 production on the SHG intensity of the generated bioharmonophores. The  
453 optimal condition (66 wt% PLLA) is marked in green (n=5). Mean ± s.d. \*\*\*\*, P  
454 < 0.0001, \*\*, P < 0.005, \*, P < 0.05 (Ordinary one-way ANOVA with Tukey's  
455 multiple comparisons).

456

457 **Figure 3. Bioharmonophores can be degraded by proteases, cells, and**  
458 **cell-free lysate systems.**

459 **a**, Schematic showing different degradation methods utilized to assess  
460 biodegradability of the bioharmonophores. **b**, Graph displaying the change of  
461 SHG signal intensity over time of bioharmonophores incubating with proteinase  
462 K (n=5). Mean values of data points were fitted for one phase exponential  
463 decay. **c**, Quantification of SHG signal/cell after overnight incubation of Tat-  
464 peptide functionalized bioharmonophores over time. SHG signal/cell is  
465 significantly reduced 30 hours after reseeding. Mean ± s.d. \*\*\*\*, P < 0.0001,

466 \*\*,  $P < 0.005$ , \*,  $P < 0.05$  (non-parametric Kruskal-Wallis test with Dunn's post  
467 hoc multiple comparison). **d**, Graph showing the loss of SHG signal intensity  
468 when bioharmonophores are subjected to the cell-free reticulate lysate  
469 degradation system ( $n=5$ ). Mean values of data points were fitted using a one  
470 phase exponential decay. Scale bar, 10  $\mu\text{m}$  (**c**); Scale bar, 10  $\mu\text{m}$  (**d**).

471

472 **Figure 4. Bioharmonophores can be specifically targeted to single cancer**  
473 **cells *in vivo*.** **a**, Schematic showing the generation of a zebrafish cancer model  
474 by injecting MDA-MB-435-DsRed cancer cells into the Duct of Cuvier (DoC) at  
475 2 dpf, resulting in tumors spread to multiple locations of the zebrafish body at  
476 5 dpf. **b**, Composite image of the cancer model (left) in a 5 dpf old zebrafish  
477 embryo. Close-up image of one of the tumor sites (right) reveals DsRed-labeled  
478 tumors (magenta), adjacent to the eGFP-labeled vasculature (green). **c**,  
479 Schematic showing cancer cell injection of 2 dpf zebrafish embryos followed by  
480 bioharmonophore injection into DoC of 3 dpf zebrafish embryos and  
481 subsequent fluorescence and SHG imaging at 5 dpf. Red rectangles labeled as  
482 **c1-4** denote the regions of interest that are illustrated in more detail. Individual  
483 panels showing the images of labeled cancer cells with the details of  
484 bioharmonophore (white) labeling down to single cancer cells (magenta) in  
485 solid tumors (**c1-4**). Colored cell boundary reconstruction of targeted cancer  
486 cells using the bioharmonophore SHG signal (**c1'-4'**). Note that cellular  
487 bioharmonophore distribution can in most cases predict cell morphologies.  
488 Scale bar, left panel 200  $\mu\text{m}$ , right panel 20  $\mu\text{m}$  (**b**); Scale bar, 15  $\mu\text{m}$  (**c**). **d**,  
489 Quantification of the fraction of SHG-labeled tumors as the ratio of labeled  
490 tumors to all tumors in a given zebrafish embryo after PEG- and p32 peptide-

491 coated bioharmonophore injection, respectively. Each data point signifies one  
492 zebrafish. Note that active targeting with p32-coated bioharmonophores  
493 significantly increases the labelling efficiency (approx. 4-fold). Mean  $\pm$  s.d. \*\*\*\*,  
494  $P < 0.0001$ , \*\*,  $P = 0.0063$ , \*,  $P = 0.0470$  (non-parametric Kruskal-Wallis test  
495 with Dunn's post hoc multiple comparison). N=12, pooled from 3 independent  
496 experiments.

## 497 **Methods**

### 498 **Formation of large-scale peptide nanotubes**

499 Diphenylalanine (FF) and triphenylalanine (FFF) (Bachem) peptide  
500 assemblies were prepared as previously described<sup>36</sup>. Briefly, peptides were  
501 freshly dissolved in hexafluoroisopropanol (Sigma) at 100 mg/ml concentration  
502 prior to experiments and diluted to 5 mg/ml final concentration in deionized  
503 water.

### 504 **Encapsulation of SHG active peptide assemblies**

505 For the evaluation of different peptides and their SHG capabilities, 30 mg  
506 PLLA was dissolved in 3 ml chloroform (Sigma) along with 15 mg  
507 triphenylalanine, 30 mg pentalanine (Bachem), and 30 mg trileucine (Sigma)  
508 peptides in separate glass vials. Resulting suspension was mixed with aqueous  
509 SDS (Sigma) solution with a final 0.3% SDS concentration. A macroemulsion  
510 was obtained by stirring the samples at 1000 rpm for 1 hour. Afterwards, the  
511 samples were sonicated (Branson Sonifier) with a 1,5 inch tip at 70% power in  
512 a pulsed mode (30 seconds ON and 10 seconds OFF) under ice cooling. The  
513 chloroform was evaporated from the obtained emulsions by stirring the samples  
514 at 500 rpm at 40 °C overnight. For the remaining experiments with  
515 triphenylalanine peptide containing bioharmonophores, the same protocol was  
516 followed unless stated otherwise. For probing the optimal conditions for  
517 nanoparticle formation, FFF peptide, PLLA, and SDS concentrations were  
518 varied as described in Supplementary Figures 2, 3, and 4.

### 519 **Characterization of encapsulated SHG active peptide assemblies**

520 Produced samples were characterized using Dynamic Light Scattering.  
521 Nanoparticle morphology, aggregation tendency along with the SHG signal

522 intensity were evaluated using nonlinear microscopy. XRD patterns were  
523 obtained using a PANalytical X'PERT Pro powder diffractometer in Bragg-  
524 Brentano geometry and with Cu K-alpha1 radiation in grazing incidence  
525 geometry between 2–60 using a step size of 0.0167. The samples were air-  
526 dried on silicon single crystals and four identical scans are obtained from each  
527 sample and summed up.

### 528 **SHG polarimetry**

529 The SHG polarimetry was performed on a wide-field SHG microscope  
530 (See Supplementary Info). A 1030nm laser, pulse width 190fs, and 200kHz  
531 repetition rate (Pharos, LightConversion), delivered 36mW on the sample over  
532 a 150um FWHM diameter field-of-view ( $1 \text{ mJ.cm}^{-2}$ ). Two noncolinear beams  
533 are incident on the sample, with an angle 30 degrees in between the two. SHG  
534 signal was detected in the phase matching condition (transmission). The image  
535 was recorded with an electron-multiplying intensified charge-coupled device  
536 (EM-ICCD) camera. Nonlinear polarimetry was performed by controlling and  
537 analyzing the polarization state of the illuminating and emitted beams. A  
538 polarization state generator, comprising a half- and a quarter-wave plate, was  
539 used. The polarization state of the emitted light was analyzed with a half-wave  
540 plate placed in the emission path, followed by a polarizing beam splitter.

### 541 **Second Harmonic Spectroscopy Patterns**

542 SHG emission pattern measurement was performed on a custom-build  
543 setup for this purpose (See Supplementary Info). Excitation was performed with  
544 a 1030nm laser, pulse width 190fs, and 200kHz repetition rate, which delivered  
545 60mW on the sample, a cylindrical cuvette containing the solution, over a 36um  
546 focal spot ( $30 \text{ mJ.cm}^{-2}$ ). The signal was detected with a rotating PMT and a

547 filter (515+10, Chroma) at angles between -90 and 90. Both incident and  
548 detection polarizations can be controlled.

#### 549 **Stability of biodegradable bioharmonophores at different pH values**

550 To evaluate how different pH values might influence the PLLA coated  
551 peptide assemblies and their signal intensity, synthesized bioharmonophores  
552 were centrifuged for 3 minutes at 13500 rpm and resuspended in citric  
553 acid/ $\text{Na}_2\text{HPO}_4$  buffer ranging from 4 to 7 pH values. The bioharmonophores  
554 were incubated for 72 hours in the buffers containing 1% Tween 80 to prevent  
555 aggregation and the signal intensity was monitored using nonlinear microscopy.

#### 556 **Biodegradation of bioharmonophores *in vitro***

557 Bioharmonophores were centrifuged for 3 minutes at 13500 rpm and  
558 resuspended in 1% Tween 80 containing PBS. In order to assess proteinase K  
559 (Sigma) degradation, 1 ml bioharmonophore suspension was incubated with  
560 100  $\mu\text{g}/\text{ml}$  final proteinase K concentration at 37 °C and the SHG signal  
561 intensity was measured every 2 hours. Similarly, in order to assess how  
562 bioharmonophores were degraded using cellular content, an *ex vivo*  
563 biodegradation protocol was adapted based on the Rabbit Reticulocyte Lysate  
564 system (Promega). In a typical setup, 1 ml of bioharmonophore in 1%  
565 Tween 80 containing PBS was mixed with 25 mM phosphocreatine (Sigma),  
566 10  $\mu\text{g}/\text{ml}$  phosphocreatine kinase (Sigma), 1 mM ATP (Sigma), and 50  $\mu\text{l}$   
567 Rabbit Reticulocyte Lysate. The mixture was incubated at 37 °C and the SHG  
568 signal intensity was monitored every 2 hours.

#### 569 **Biodegradable bioharmonophore functionalization**

570 1 ml of 1.5 mg/ml bioharmonophores were incubated with 1 mg Candida  
571 Antarctica Lipase B (Sigma) for 2 hours, which hydrolyzes the PLLA polymer to

572 increase the number of carboxyl groups. Bioharmonophore suspension was  
573 centrifuged at 13500 rpm for 3 minutes and resuspended in 1% Tween 80  
574 containing PBS, and mixed with 10 mg *N*-(3-dimethylaminopropyl)-*N'*-  
575 ethylcarbodiimide hydrochloride (EDC) (Sigma), 10 mg *N*-hydroxysuccinimide  
576 (NHS) (Sigma), and 10 mg methoxypolyethylene glycol amine 5000 Da (mPEG  
577 Amine) (Sigma) for 2 hours. The suspension was centrifuged and resuspended  
578 in 1% Tween 80 containing PBS and stored at 4 °C prior to use.

579 For further functionalization experiments thiol-PEG-amine 2000 Da (SH-  
580 PEG-NH<sub>2</sub>) (Sigma) was used as a platform for bioorthogonal click chemistry. In  
581 a similar setup, 10 mg EDC, 10 mg NHS, and 10 mg thiol-PEG-NH<sub>2</sub> were  
582 incubated for 2 hours. The suspension was centrifuged and resuspended in 1%  
583 Tween 80 containing PBS with methyltetrazine-PEG4-Maleimide (Click  
584 Chemistry Tools) of 200 µM final concentration. The mixture was incubated for  
585 2 hours at room temperature, centrifuged, and resuspended in 1% Tween 80  
586 containing PBS.

587 The other click chemistry pair trans-cyclooctene (TCO)-PEG3-  
588 Maleimide (Click Chemistry Tools) (3 mM in 200 µl PBS) was incubated for  
589 2 hours with cysteine-containing Tat or P32 targeting peptides (1 mM final  
590 concentration) depending on the application. The peptides were passed  
591 through Illustra Microspin G25 columns (GE Healthcare) to remove TCO-  
592 PEG3-maleimide.

593 200 µl tetrazine modified bioharmonophores were incubated with 20 µl  
594 TCO modified peptide for 2 hours. The bioharmonophore suspension was  
595 washed with 1% Tween 80 containing PBS to remove unbound peptides and  
596 resuspended in PBS to be immediately used for cell culture experiments.



597 **Cellular degradation and toxicity**

598 MDA-MB-435-DsRed cancer cells were kindly gifted by Prof. R. Klemke.  
599 The cells were cultured at 37°C, 5% CO<sub>2</sub>, in high glucose DMEM with  
600 GlutaMAX (10569010, Thermo Fisher), supplemented with 10% FBS (P40-  
601 37500, Pan Biotech) and 1X Penicillin-Streptomycin solution (15140122,  
602 Thermo Fisher).). The cells were cultured on 6-well plates (140675, Thermo  
603 Fisher) until they reached ~80% confluency and were incubated with 400 µl  
604 Tat-derived cell penetrating peptide coated bioharmonophores overnight. The  
605 cells were washed with 1X PBS twice and detached using 0.05% Trypsin-EDTA  
606 (25300054, Thermo Fisher) in order to remove bioharmonophores that did not  
607 enter the cancer cells. Detached cells were centrifuged for 5 minutes at 500xg  
608 to remove excess bioharmonophores that were not taken up, reseeded or  
609 ibitreat coated 8-well slides (80826, Ibidi GmbH), and fixed after 6, 30, 54 and  
610 78 hours to monitor bioharmonophores degradation. The samples were then  
611 washed 3 times with 1X PBS and stained with the CellMask Orange Membrane  
612 dye (Invitrogen). The samples were washed again and imaged subsequently.  
613 To determine cell viability after treatment with functionalized  
614 bioharmonophores, trypan blue exclusion method was used. Briefly, cells in  
615 triplicates seeded in 96-well tissue culture plates (167008, Thermo Fisher) were  
616 exposed to varying concentrations of functionalized bioharmonophores for 48  
617 or 72 hours. After incubation, cells were washed with 1X PBS twice and  
618 detached as described above. 10 µl of cell suspension was then mixed with  
619 10 µl 0.4% Trypan Blue, and 4 µl of this mixture was added to the cell counting  
620 slide (C10228, Thermo Fisher) and measured using Countess II Automated cell

621 counter (Thermo Fisher). The viability was expressed as a fold difference of the  
622 untreated samples for each time point.

### 623 **Toxicity Assay and Thioflavin T staining**

624 For toxicity assay, cells were grown in 96 well plates and were incubated  
625 with bioharmonophores at different concentrations for 48 and 72 hours. After  
626 the incubation period, the cells were detached with trypsinization and their  
627 viability was analyzed using Trypan Blue (Sigma) staining.

628 For Thioflavin Staining cells were seeded in an 8-well chamber (ibidi)  
629 with 50% confluency. The cells were treated with either Amyloid Beta Peptide  
630 (Bachem) or 5  $\mu$ l of bioharmonophores for 24 hours and extensively washed  
631 with PBS to remove excess peptides and bioharmonophores. To evaluate  
632 whether bioharmonophores induce fibril formation the cells were fixed with 4%  
633 paraformaldehyde for 10 minutes and washed with PBS three times.  
634 Afterwards, 0.05% Thioflavin T (Sigma) solution was added to the sample for  
635 8 minutes and excess dye was washed with 80% ethanol for 5 minutes. The  
636 washing step was repeated three times and the samples were imaged using  
637 confocal microscopy.

### 638 **Zebrafish Cancer Model and bioharmonophore Targeting**

639 Animal experiments and zebrafish husbandry were approved by the  
640 "Kantonaes Veterinaeramt Basel-Stadt". MDA-MB-435-DsRed cancer cells  
641 were injected into the Duct of Cuvier of *Tg(fli1:egfp)* zebrafish embryos at  
642 2 days post fertilization (dpf). After injection, embryos were incubated for 1 hour  
643 at 29°C for recovery and cell transfer then verified by fluorescence microscopy.  
644 Fish harboring red cells were incubated at 35°C essentially as described  
645 before<sup>23, 24</sup>. Fish were anesthetized and embedded in low melting agarose as

646 described previously<sup>37</sup> and were imaged at 5 dpf for assessing cancer cell  
647 localization.

648 For targeting experiments, p32/gC1qR ligand-functionalized  
649 bioharmonophores were injected into the zebrafish embryos 24 hours after  
650 cancer cell injection following the same procedure. *In vivo* bioharmonophores  
651 targeting was evaluated at 5 dpf using nonlinear laser scanning microscopy.

## 652 **Transmission Electron Microscopy**

653 Bioharmonophore samples were spun down to remove aggregated  
654 nanoparticles at 3000 rpm for 3 minutes and the bioharmonophores (i.e., the  
655 supernatant of the centrifuged solution). 5  $\mu$ l of the sample was placed on a  
656 carbon coated grid (Quantifoil, D) previously glow-discharged for 30 seconds  
657 (Emitech K100X, GB). The drop was allowed to remain for 60 seconds; after  
658 this interval, excess fluid was drained along the periphery using a piece of filter  
659 paper followed by staining with 2% uranyl acetate for 1 second and 15 seconds,  
660 respectively. Excess moisture was drained after each step and when dry the  
661 grid was examined in an FEI Morgagni 268 TEM operated at 100 kV.

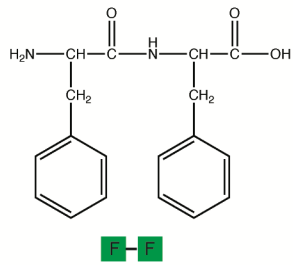
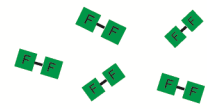
## 662 **Nonlinear and Confocal Light Microscopy**

663 Bioharmonophores were immobilized in low melting agarose by mixing  
664 200  $\mu$ l bioharmonophore with 100  $\mu$ l 1% SeaPlaque low melting agarose  
665 (Lonza) solution in 8-well imaging chambers (Lab-Tek). Imaging experiments  
666 were performed on a Zeiss LSM 780 microscope (Carl Zeiss AG) equipped with  
667 a spectral GaAsP detector and a tunable two-photon laser source (Chameleon  
668 Ultra II, Coherent Inc.), using an LD C-Apochromat 40x/1.1 water immersion  
669 objective lens (Carl Zeiss AG). Throughout the imaging experiments,  
670 bioharmonophores were illuminated with 850 nm incident wavelength and the

671 SHG signal was collected between 405 and 435 nm or with a GaAsP spectral  
672 wavelength detector for spectral measurements.

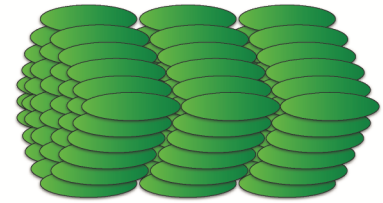
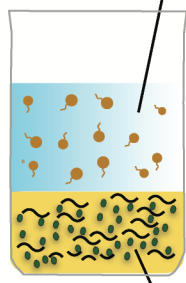
### 673 **Statistical analysis**

674 All numerical values represent mean  $\pm$  s.d. Sample sizes (n) were given  
675 in the figure legends for each experiment. Each experiment was repeated at  
676 least 3 times. Normal distribution of datasets were established using  
677 D'Agostino & Pearson omnibus normality test where  $P > 0.05$  indicated  
678 Gaussian distribution. When all the datasets had Gaussian distribution, one-  
679 way Anova was used for multiple comparisons followed by Tukey's multiple  
680 comparisons. When one or more datasets showed a non-Gaussian distribution  
681 or high degree of variance as in the case of zebrafish tumor models, Kruskal-  
682 Wallis test was applied along with Dunn's multiple comparisons. For all  
683 statistical tests, P value was reported, n.s.,  $P > 0.05$ , \*,  $P < 0.05$ , \*\*,  $P < 0.005$ ,  
684 \*\*\*,  $P < 0.001$ , \*\*\*\*,  $P < 0.0001$ . Second order polynomial fit and one phase  
685 exponential decay values were calculated and graphs were drawn using  
686 GraphPad Prism 6.

**a**Concentrate  
in Solution

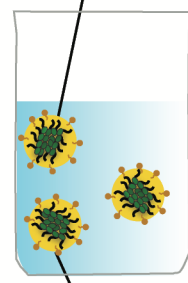
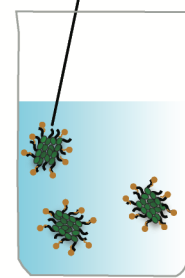
Individual Peptides

Self-assembly

Aggregated Peptide  
Nanotubes**b**Continuous Phase  
(Water and SDS)Disperse Phase  
(Chloroform, PLLA and Peptide)

Sonication

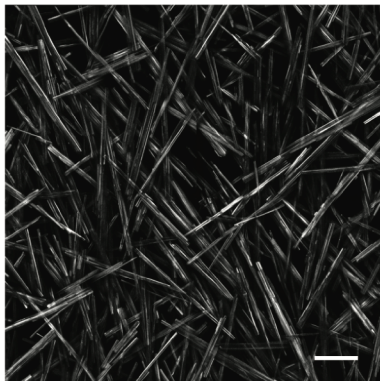
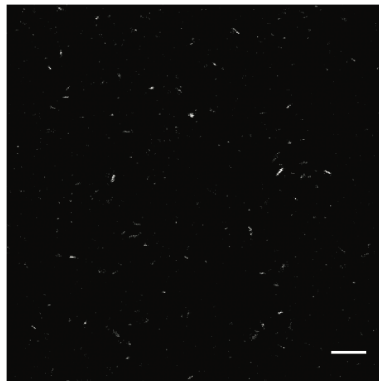
Chloroform Droplet

SHG-positive  
Self-assembled PeptidesEvaporation of  
ChloroformEncapsulated Biodegradable  
Harmonophores

○ SDS

● Peptide

~ PLLA

**c****d****e**