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1 **Title page**

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3 **Title:** Photoactive imaging and therapy for colorectal cancer using a CEA-Affimer

4 conjugated Foslip nanoparticle.

5

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36 Antibody mimetic; cancer therapeutics; carcinoembryonic antigen; silica nanoparticle;
37 photodynamic therapy; colorectal cancer; *in-vivo* targeted delivery.

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51
52 Theranostic nanoparticles hold a promising strategy for simultaneous imaging and
53 therapy in colorectal cancer. Carcinoembryonic antigen can be used as a target for
54 these nanoparticles because it is overexpressed in most colorectal cancers. Affimers
55 reagents are synthetic proteins capable to binding specific targets, with additional
56 advantages over antibodies for targeting. We fabricated silica nanoparticles using a
57 water-in-oil microemulsion technique, loaded them with the photosensitiser Foslip, and
58 functionalised the surface with anti-CEA Affimers to facilitate fluorescent imaging and
59 photodynamic therapy of colorectal cancer. CEA-specific fluorescent imaging and
60 phototoxicity was quantified in colorectal cancer cell lines and a LS174T murine
61 xenograft colorectal cancer model. Anti-CEA targeted nanoparticles exhibited CEA-
62 specific fluorescence in LoVo, LS174T and HCT116 cell lines when compared to
63 control particles ($p < 0.0001$). No toxicity was observed in LS174T cancer mouse
64 xenografts or other organs. Following photo-irradiation, anti-CEA targeted particles
65 produced significant cell death in LoVo (60%), LS174T (90%) and HCT116 (70%)
66 compared to controls ($p < 0.0001$). Photodynamic therapy (PDT) at 24 h *in vivo* showed
67 a 4-fold reduction in tumour volume compared to control mouse xenografts
68 ($p < 0.0001$). This study demonstrates the efficacy of targeted fluorescent imaging and
69 PDT using Foslip nanoparticles conjugated to anti-CEA Affimer nanoparticles in *in vitro*
70 and *in vivo* colorectal cancer models.

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Introduction

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77 Personalised surgery involves a tailored approach to the individual patient and the
78 underlying disease. Up to 30% of colorectal cancer (CRC) patients undergoing
79 curative surgical resection develop locoregional recurrence or distant metastases (1-
80 3). Lymph node micrometastases and residual tumour cells are thought to be the main
81 contributing factors. They are not detectable at surgery and can be easily missed
82 during routine histopathological examination (4, 5). An accurate means of identifying
83 positive lymph nodes (LN) intraoperatively would allow the radicality of surgery to be
84 tailored to the biology of the primary cancer; lymph node positive cancers would
85 undergo radical D3 lymphadenectomy, whereas lymph node negative cancers could
86 be effectively treated by limited segmental resection. Reducing the radicality of
87 surgery, whilst maintaining oncological efficacy, is important as the incidence of CRC
88 is rising, particularly amongst the elderly population (6, 7).

89 Theranostics has emerged as a promising route for personalised cancer treatment,
90 allowing real-time imaging of cancers and cytotoxic cell killing (8, 9). A theranostic
91 photo-active nanoparticle would enable surgeons to visualise positive lymph nodes,
92 tumour margins and distant metastasis intra-operatively, facilitating complete cancer
93 eradication. Selective uptake of a photosensitiser (PS) by cancer cells allows
94 fluorescent visualisation, whilst irradiation with a specific wavelength of light triggers
95 cancer cell death due to generation of cytotoxic reactive oxygen species (ROS) (10-
96 12). Photodynamic therapy is particularly attractive in cancer surgery, which is now
97 mostly undertaken using laparoscopy. Changing the light wavelength delivered to the
98 abdominal cavity to activate a PS is relatively straight forward.

99 One of few photosensitisers that is clinically approved for treatment of different
100 cancers in Europe is meta-tetra(hydroxyphenyl)chlorin (mTHPC); commercially known



101 as Foscan® (13). mTHPC is characterised by its favourable absorption wavelength in
102 the near infra-red region (652 nm) and high singlet oxygen quantum yield (14). In
103 preclinical studies, the liposomal formulation of mTHPC, known as Foslip®, gave
104 enhanced PDT efficacy and offered several advantages such as being non-
105 immunogenic and biodegradable in addition to increasing drug solubility and tumour
106 selectivity while reducing unwanted skin accumulation (15, 16). However, a lack of
107 stability, with 60% of liposome destruction after 24 hours, is an obvious shortcoming
108 (17-19). Leakage of the phospholipid membrane can be halted by coating the
109 liposomes with a polymer net (20) or silica shell (21) and is widely used to stabilise the
110 lipid bilayer (22). Silica based nanoparticles are attractive because of their
111 compatibility with biological systems and transparency to light. Their degradation is
112 enhanced by the increased ROS levels observed in the tumour microenvironment,
113 facilitating delivery of the payload. Silica-based 'C dots' have recently been approved
114 for Phase 2 clinical trials (23, 24). We have shown previously that carcinoembryonic
115 antigen is a reliable tissue biomarker for colorectal cancer (25) and that our CEA
116 antibody targeted NIR664 dye-doped silica nanoparticles allowed specific *in vivo*
117 fluorescent imaging of colorectal cancer in a mouse model (26). However, antibody
118 based drug targeting has its limitations, including high cost of production, stability, and
119 batch-to-batch variation, which limit clinical translation (27, 28). Affimers are an
120 attractive alternative with equivalent biorecognition characteristics to antibodies (29).
121 The absence of cysteine residues in the Affimer scaffold allows the introduction of
122 cysteine for site-specific conjugation to nanoparticles. Affimers are thermo- and pH-
123 stable and easily expressed in prokaryotic cells (*E.coli*), thereby reducing the cost of
124 production. We have recently shown that CEA-Affimers bind to cancer cells expressing
125 CEA with high affinity and with K_D values in the nM range (30, 31).



126 The aim of this study was to develop a photoactive, theranostic nanoparticle for
127 fluorescence tumour imaging and ablation. We report the first successful use of
128 Affimer-targeted, silica-coated Foslip nanoparticles for fluorescent imaging and
129 photodynamic therapy *in vitro* and in an animal CRC model.

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Results

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152 **Synthesis and characterisation of Affimer tagged silica coated Foslip** 153 **nanoparticles**

154 We aimed to synthesise silica coated Foslip nanoparticles to target colorectal cancer
155 cells using anti-CEA Affimers as bioreceptors. Silica coating formation was achieved
156 by a hydrolysis process of TEOS, according to protocols published in the literature (32,
157 33). The precipitation of silica on the surface of Foslip resulted in the formation of a
158 spherical core-shell-like structure, as visualised by scanning electron microscopy
159 (**Figure 1A**), with a mean diameter of 140 nm (± 1 nm SEM) (**Figure 1B**).
160 Encapsulation of Foslip was demonstrated by absorption and fluorescence emission
161 spectra of the synthesised nanoparticles. Fluorescence of the nanoparticles was
162 measured and recorded using a Cary Eclipse spectrofluorometer in water suspension,
163 using specific Foslip excitation and emission wavelengths of 420 and 652 nm
164 respectively (34). **Figure 1C** shows a typical spectrum for silica coated Foslip particles,
165 along with a spectrum for Foslip alone, demonstrating that encapsulation does not
166 alter the spectral properties. The efficiency of the encapsulation (EE) process was
167 quantified by measuring the absorbance of Foslip with reference to a dose standard
168 curve using a microplate reader (**Figure S1**). A typical nanoparticle sample containing
169 1 mg/ml of nanoparticles and 110 nM of Foslip correlated to Foslip EE of $\sim 82.2 \pm 2.1\%$
170 ($n=3$). We also assessed the stability of nanoparticles in different conditions by
171 measuring their fluorescence using a plate reader, as shown in **Figure 1D**. Particles
172 remained highly fluorescent either in stock PBS at 4 °C (98.5%) or in PBS containing
173 10% (v/v) FBS at 37 °C (97.0%) for 48 h, when compared to freshly synthesised NPs,
174 after which the signal reduced most likely due to leakage of mTHPC; **Figure S2**.



175 Despite this limitation, the NPs were able to achieve their target imaging and
176 cytotoxicity in less than 48 h as shown in following results.

177 Next, we selected two different anti-CEA Affimers (based on protein yield) to provide
178 polyclonal targeting of the CRC-antigen CEA, and as a control, an anti-myoglobin
179 (Myo) Affimer. The anti-myoglobin Affimer was used as a negative control because
180 colorectal cancer cells do not express this human cardiac muscle-related protein. Tris
181 (2-carboxyethyl) phosphine (TCEP) reduced anti-CEA and anti-Myo Affimers were
182 purified using Ni²⁺-NTA resin (**Figure S3, Table ST1**) and prepared for conjugation.
183 The hetero bifunctional cross-linker sSMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)
184 cyclohexane-1-carboxylate) was then used to link the free Affimer sulfhydryl group to
185 the aminated nanoparticle surface (provided by the salinisation agent, 3-aminopropyl
186 triethoxysilane (APTES)) as shown in **Figure S4**.

187 Quantification of Affimer amount bound to NPs comprised two main parts; (i), cleavage
188 of disulfide bond crosslink between Affimer and NPs, and (ii), quantification of the
189 Affimers concentration. The Affimer-tagged NPs were prepared at 1 mg/mL
190 concentration and reducing agent 2-mercaptoethanol was used to break the thiol-
191 maleimide conjugation and free the Affimers. Free Affimers were recovered from the
192 supernatant and the concentration was measured using a calibration curve for
193 NanoOrange[®] protein; **Figure 1E**. Knowing the estimate number of NPs per mL (~ 8.5
194 x 10⁷), the number of Affimers immobilised on each NP was estimated at 570 +/- 110
195 Affimer/NP.

196 Dynamic light scattering (DLS), showed monodispersed particle peaks at 148 nm (±
197 11 nm) and reassuringly, CEA-Fos-NPs and Myo-Fos-NPs showed almost identical
198 size distributions; **Figure 1F**. The mean zeta potential of the silica coated Foslip
199 exhibited a negative surface charge (-15.6 mV) whereas aminated NPs exhibited a



200 positive surface charge (27.9 mV). The surface charge of the Affimer-tagged NPs
201 shifted back to a more neutral charge state (2.8 mV) indicating successful conjugation.
202 The size distribution, zeta potential and polydispersity index (PDI) of NPs derivatives
203 is shown in **Table 1**.

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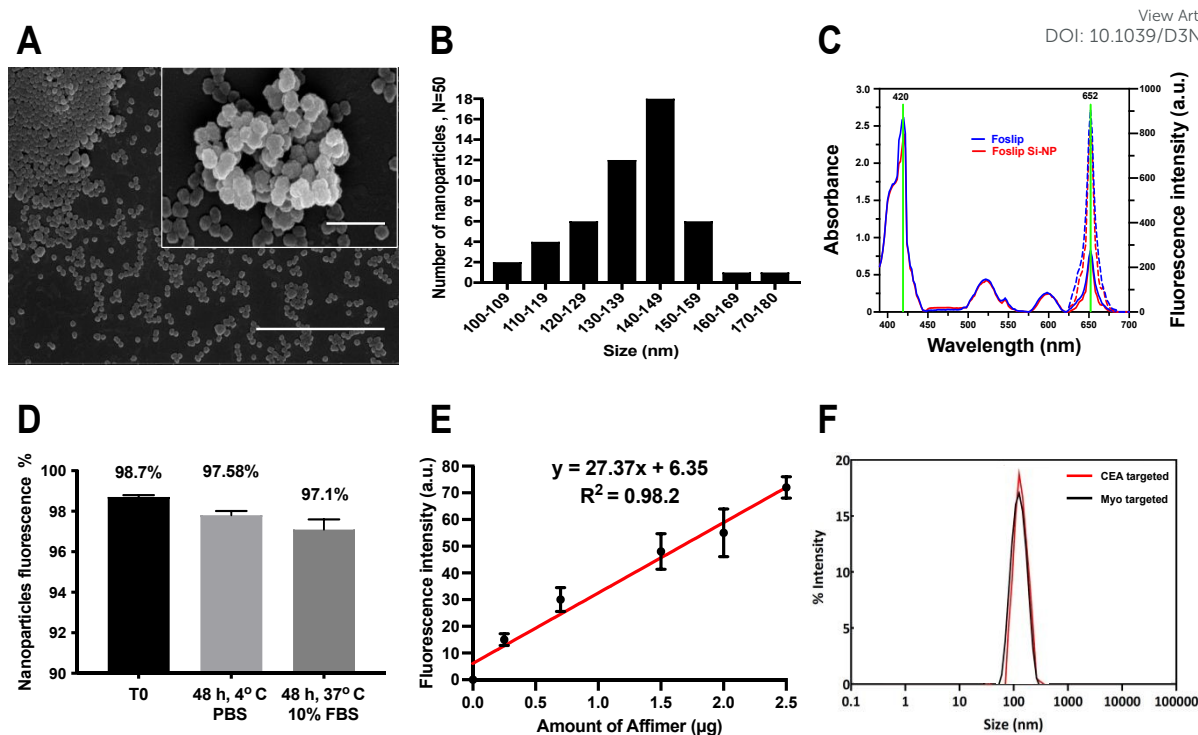
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Figure 1. *Characterisation of silica coated Foslip NPs.* (A), SEM image shows a spherical structure of NPs with size around 140 nm. The scale bars represent 1 μ m and 500 nm respectively for the whole view and the magnified view. (B), The size distribution is shown with particle diameters being binned into 10 nm intervals. (C), UV absorption spectra for Foslip alone (—), silica coated Foslip NPs (—) and fluorescence spectra for Foslip alone (---) and silica coated Foslip NPs (---). (D), Stability of Affimer tagged NPs monitored by fluorescence intensity under different condition using spectrofluorometer: the freshly prepared particles (T0); the sample stored for 48 h at 4 $^{\circ}$ C in PBS; and the sample incubated at 37 $^{\circ}$ C in 10% FBS for 48 h. Data show mean from 3 biological experiments (SEM, n=3). (E), A calibration curve of fluorescence intensity for NanoOrange dye with increasing dose of Affimer concentration. Data show mean from 3 biological experiments (SEM, n=3). (F), Affimer tagged NPs size as determined by DLS.



239 ***CEA-Fos-NPs enabled selective fluorescent imaging and cytotoxicity in*** View Article Online
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240 ***colorectal cancer cells lines.***

241 We aimed to assess the fluorescence and PDT effect on three colorectal cancer cell
242 lines (LoVo, LS174T and HCT116) and a control, CEA-negative non-cancer cell line
243 (HEK293) when incubated with CEA-Fos-NPs. We have previously reported that LoVo
244 cells show high CEA expression, LS174T cells moderate to high CEA expression,
245 HCT116 cells low CEA expression, and HEK293 cells no expression of CEA (26). Anti-
246 CEA or Myo- Affimer tagged nanoparticles (1 mg/mL) were incubated with colorectal
247 cancer and control cell lines for 24 h then imaged using confocal microscopy and cell-
248 specific fluorescence was quantified. CEA-Fos-NPs produced more intense tumour-
249 specific targeting, with anti-CEA targeted nanoparticles showing 9.5-, 10.2- and 3.5-
250 fold greater fluorescence than Myo-Affimer targeted nanoparticles in LoVo, LS174T
251 and HCT116 cells respectively ($p < 0.0001$) as shown in **Figure 2A**. Importantly, CEA-
252 Fos-NPs did not produce any significant fluorescence intensity in the control cell line
253 HEK293, suggesting that the anti-CEA Affimer targeted silica nanoparticles were
254 specific to CEA expressing cells and likely to prevent unwanted accumulation in
255 normal tissues, thereby reducing side effects. Representative confocal microscopy
256 images showed fluorescence in tumour cells at 24 h that correlates with CEA
257 expression data in the literature (**Figure 2B**). In order to assess the dose- and time-
258 effect on cellular fluorescence, cells were incubated with 1 or 2 mg/mL nanoparticles
259 for 4 and 24 h. After incubation, the cells were washed and fresh nanoparticle-free
260 media was added for an additional 20 h (4+20 h) or 24 h (24+24 h). Spectrofluorometer
261 evaluation showed that LoVo cells had significantly higher fluorescent signal with CEA-
262 Fos-NPs than other cell lines ($p < 0.02$), followed by LS174T and HCT116 cells, in a
263 dose and time dependent manner (**Figure 2C**). Cellular uptake was seen as early as



264 4 h but the difference between the single time points was most obvious at 24 and
265 24+24 h in all cancer cell lines. Although fluorescence was still present in cells after
266 24 h incubation, the mean fluorescence intensity at 24+24 h was greater than 24 h
267 ($p=0.01$), indicating that cellular uptake was also time dependent. The mean
268 fluorescence in HEK293 cell lines was almost identical at 4+20 h and 24 h ($p>0.9$)
269 whilst in the colorectal cell lines a significant difference was observed between these
270 two time points ($p<0.001$), highlighting that the anti-CEA Affimer increased the
271 selectivity for cancer cells.

272 Next, we assessed the internalisation and co-localisation characteristics of NPs in
273 LoVo and LS174T cells. For the purpose of this experiment, we synthesised silica NPs
274 tagged with fluorescein isothiocyanate (FITC) and CEA or Myo-Affimer. NPs (1
275 mg/mL, 150 nm (± 12 nm)) were incubated with cells for 1, 4 and 24 hours then
276 imaged by fluorescent microscopy to track internalisation. To determine the subcellular
277 localisation of NPs, we subsequently stained cells with lysotracker deep red. Based
278 on fluorescent microscope images, CEA-FITC-NPs were internalised in LoVo and
279 LS174T as early as 1 h whereas Myo-FITC-NPs were negligibly internalised. The
280 CEA-targeted NPs predominantly accumulated into the cytoplasm, with some
281 lysosomal localisation as shown in **Figure 2D**, where the lysotracker (red) and the
282 nanoparticles (green) were co-localised (yellow).

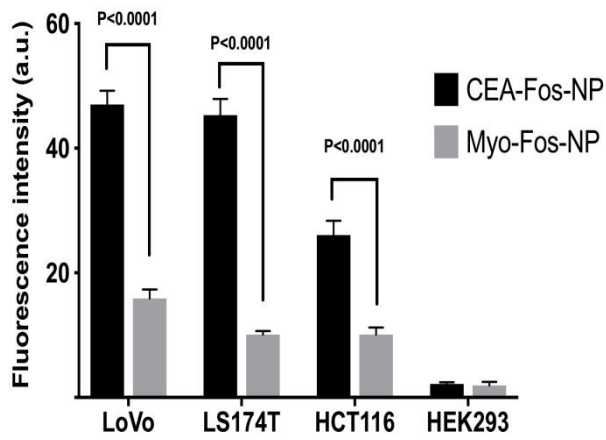
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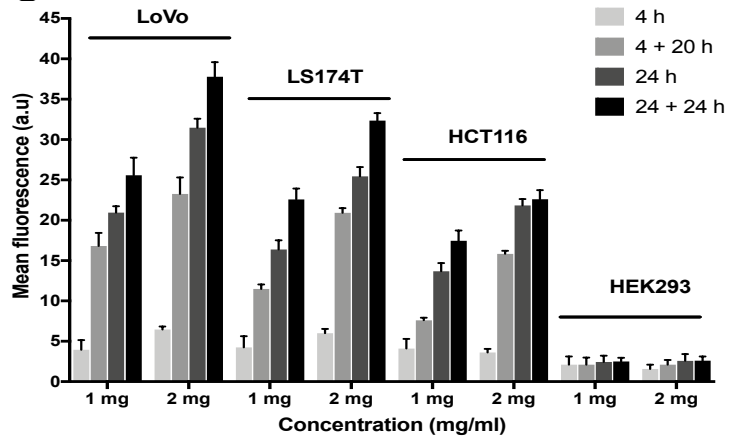
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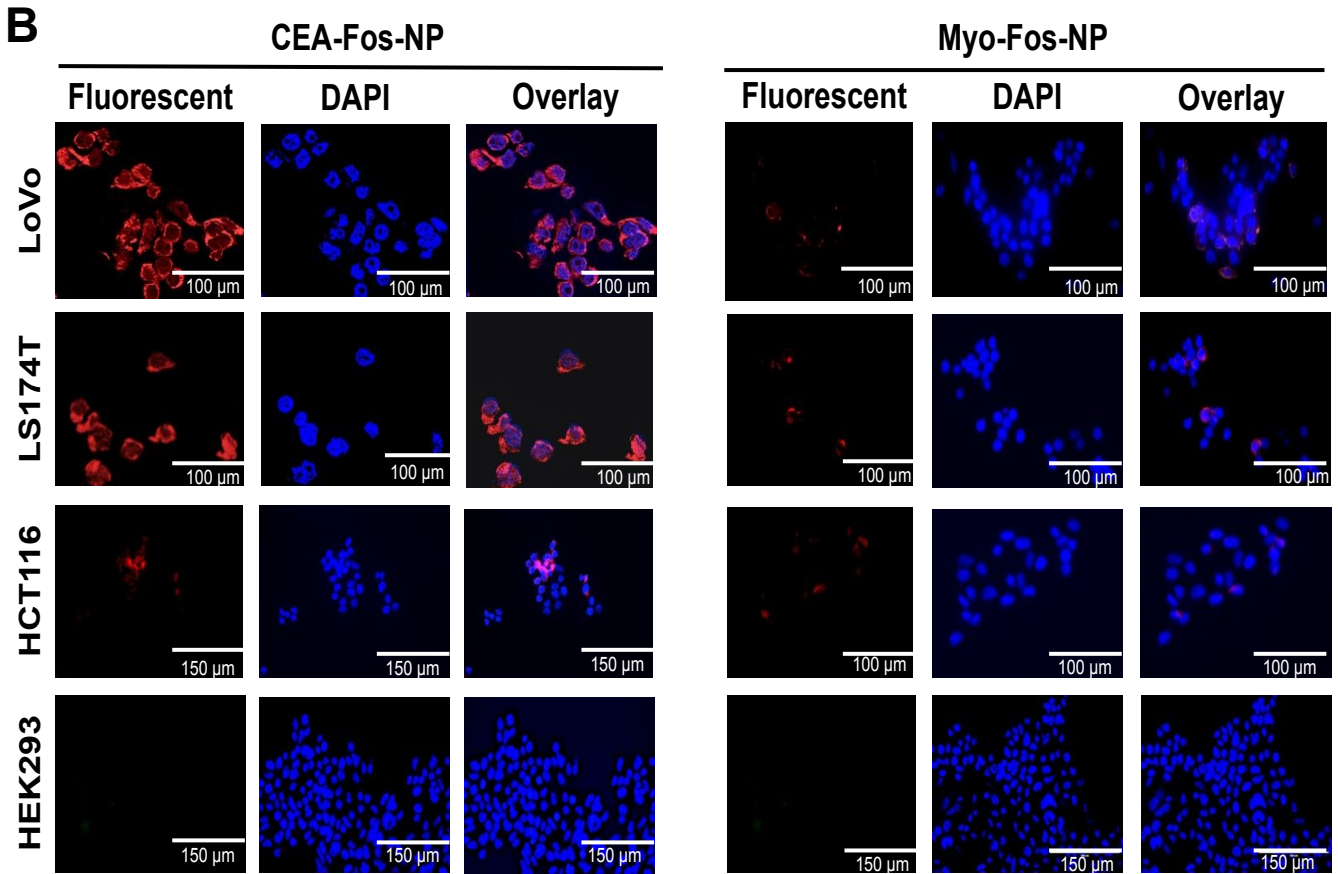


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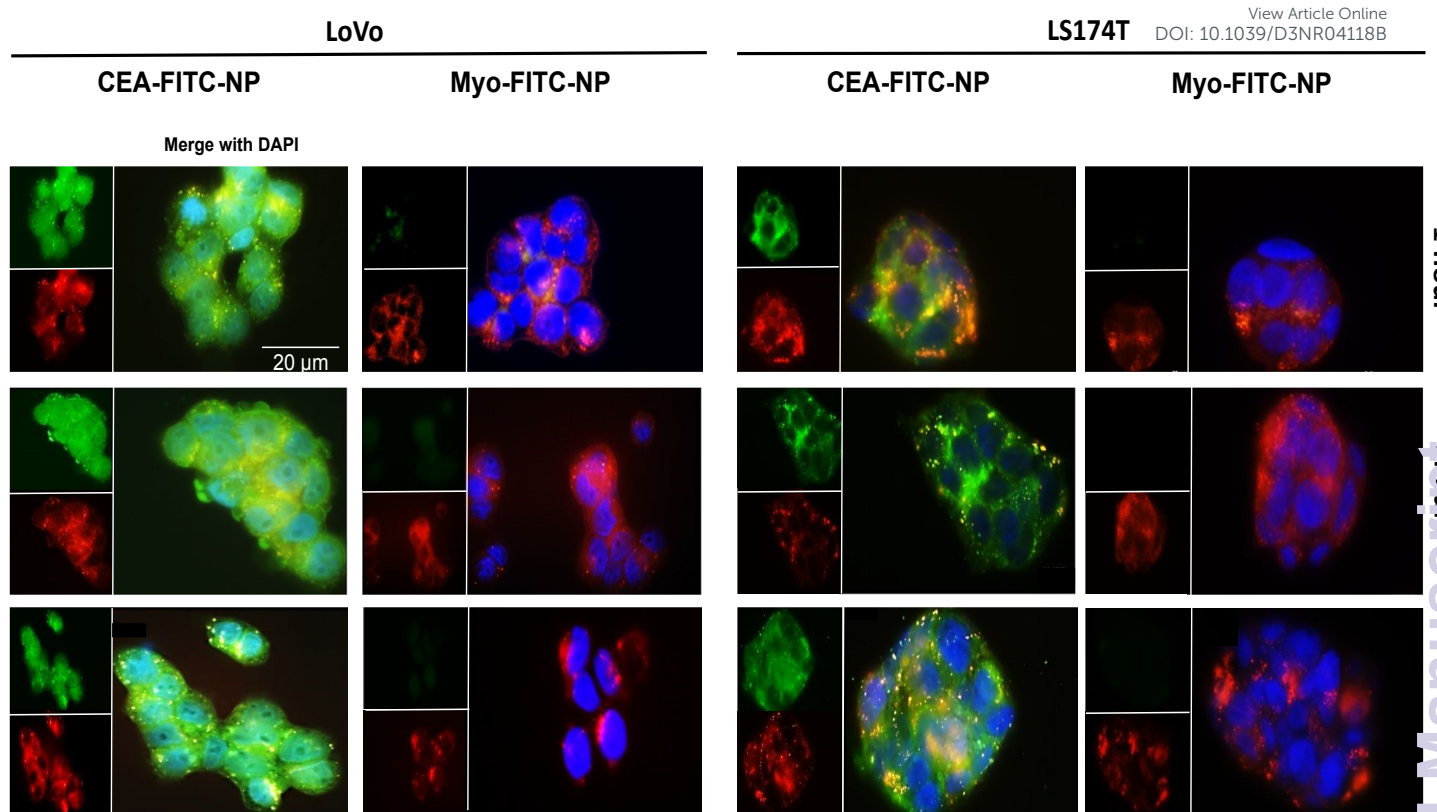
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296 Figure 2. CEA-Fos-NPs enabled targeted fluorescent imaging of CRC cells. (A)

297 Comparing the fluorescence intensity in CRC cancer cells when incubated with either

298 nanoparticles conjugated to CEA-Fos-NPs or Myo-Fos-NPs for 24 h. Data denote

299 fluorescence mean from 3 biological experiments (SEM, n=3). Significance was tested

300 using unpaired t-tests. (B) Representative confocal images of LoVo, LS174T, HCT116

301 and HEK293 cells right after 24 h incubation with CEA-Fos-NPs or Myo-Fos-NPs. (C)

302 Cellular uptake of CEA-Fos-NPs in CRC and control cell lines at different time points

303 and nanoparticle concentrations. Cells were incubated with 1 or 2 mg/mL of anti-CEA

304 targeted nanoparticles for 4 and 24 h. After incubation, the cells were washed and

305 fresh nanoparticle-free media was added for an additional 20 h (4+ 20 h) and 24 h

306 (24+24 h). Data denote fluorescence mean from 3 biological experiments (SEM, n=3).

307 (D) Fluorescence images of CEA-FITC-NPs internalised into cytoplasm and

308 lysosomes of LoVo and LS174T. Images show FITC from NPs (green, top left),

309 lysotracker staining of lysosomes (Red, bottom left) and merged DAPI fluorescence

310 of NPs and lysosomes (yellow, magnified). Scale bar is 20 μ m for all images.

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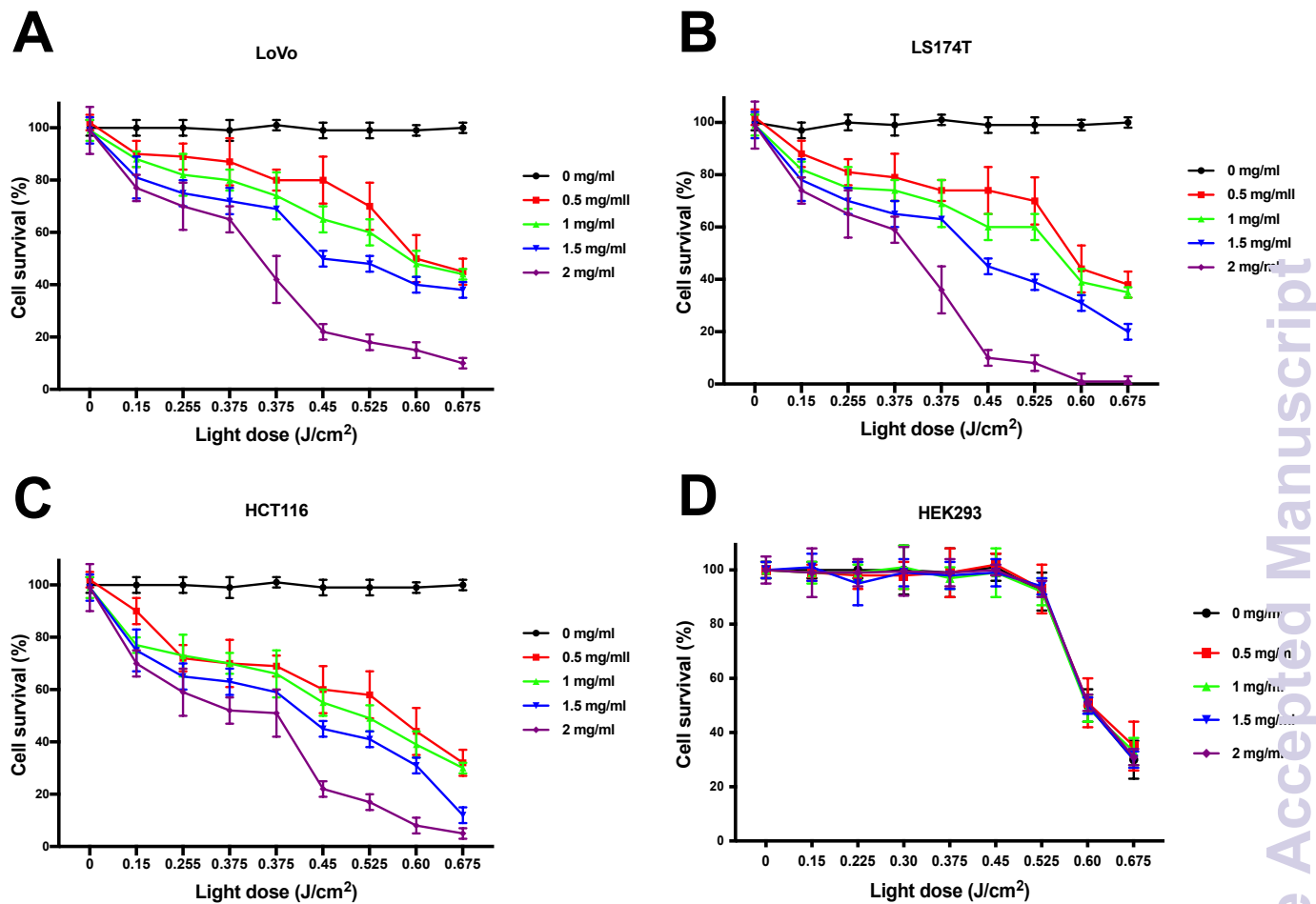
312 Next, we assessed the dark cytotoxicity of CEA-Fos-NPs against colorectal cancer
313 cells. Cells were incubated with CEA- or Myo-Fos-NPs at high concentration of 3
314 mg/mL for 24 h and 24+24 h then washed and kept in nanoparticle-free media followed
315 by MTT assay quantification of cellular viability. Cells were kept in the dark during
316 incubation periods. Affimer tagged Fos-NPs did not affect the survival of all cell lines
317 when exposed at high concentrations of 3 mg/mL for 24 h, which is much higher than
318 that used to achieve cell-specific fluorescence and cellular uptake in previous
319 experiments. Similarly, the MTT assay showed that the number of metabolically active
320 cells at 24+24 h after exposure to nanoparticles was not reduced relative to controls
321 (**Figure S5**).

322 We assessed the light dose effect on cell survival to ensure that any cytotoxic effect
323 was Foslip-mediated only. Cells were incubated with CEA-Fos-NPs at various
324 concentrations for 24 h, washed and incubated with fresh media, and immediately
325 incubated in the dark (0 J/cm^2) or photo-irradiated with light doses from 0.15 to 0.675
326 J/cm^2 . Cells were then kept in nanoparticle-free media for an additional 24 h followed
327 by assessment of cell viability by MTT assay.

328 In all the cancer cells, significantly reduced viability of cells was observed that was
329 dependent on light dose, and on nanoparticle dose ($p < 0.0001$; **Figure 3A-C**), with no
330 reduction in viability in the absence of nanoparticles at any light dose. For example,
331 more than 80% reduced viability was seen at the highest doses of nanoparticles after
332 0.45 J/cm^2 irradiation. By contrast, HEK293 cells showed no reduction in viability at
333 any dose of nanoparticles below 0.6 J/cm^2 (**Figure 3D**); at 0.6 J/cm^2 and above,
334 HEK293 cells showed light-induced toxicity that was independent of the presence of
335 nanoparticles suggesting that these cells were more sensitive to the light alone than



336 the cancer cells. Therefore, light dose at 0.45 J/cm^2 was considered as the cut-off
 337 point for safe photo-irradiation of cells in the next experiment.



338

339 Figure 3. Light dose and nanoparticles concentration effect in CRC cells. (A-D) Cells

340 were incubated with various concentrations of CEA-Fos-NPs for 24 h then photo-

341 irradiated with $0.15 - 0.675 \text{ J/cm}^2$ of 600-700 nm. Cells viability was quantified using

342 MTT assay. Data show mean cells viability from 3 biological experiments (SEM, $n=3$).

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348 Next, we assessed the phototoxicity efficacy of CEA-Fos-NPs in killing cancer cells
349 when photo-irradiated with the optimum light dose of 0.45 J/cm². Cells were incubated
350 with CEA-Fos-NPs or Myo-Fos-NPs at various concentrations for 24 h, washed and
351 incubated with fresh media, and immediately photo-irradiated with light doses of 0.45
352 J/cm² followed by MTT assay assessment as described previously. As shown in
353 **Figure 4A**, at 0.45 J/cm² light dose a significant reduction in cell survival was observed
354 in LoVo, LS174T and HCT116 cells when compared to control HEK293 cells
355 (p<0.0001). The reduction in cell survival measured at 24 h after irradiation with an
356 optimum light dose of 0.45 J/cm² was dose dependent. At 2 mg/mL CEA-Fos-NPs
357 concentration, significant cell death was observed in LoVo (60%), LS174T (90%) and
358 HCT116 (70%) when compared to HEK293 (0%); p<0.0001. Importantly, no cellular
359 toxicity was observed when cells were treated with increasing dose of the control anti-
360 myoglobin Affimer nanoparticles at 0.45 J/cm² (**Figure 4B**). Interestingly, the PDT
361 induced cellular toxicity did not correlate with the fluorescence intensity seen in the
362 respective cell lines as shown earlier in **Figure 2**. As cell density per well may impact
363 the overall PDT efficacy, we attempted to standardise this variable by measuring cell
364 viability per 1000 cells per well. Following PDT, cells were trypsinised and stained with
365 trypan blue and the number of viable cells per 1000 cells per well was calculated
366 (**Figure S6**). The data show when cell numbers was standardised per well, LoVo cells
367 viability dropped to ~ 30 %. Importantly, the variation in response to PDT appeared to
368 correspond to the degree of differentiation of the cell line suggesting that tumour cell
369 biology and genetic differences may be associated with variations in cellular pathways
370 and overall sensitivity to PDT.

371 The DCFDA assay was performed to study the cell death mechanism following PDT
372 to mimic the experiments in which cell viability was assessed using the MTT assay.

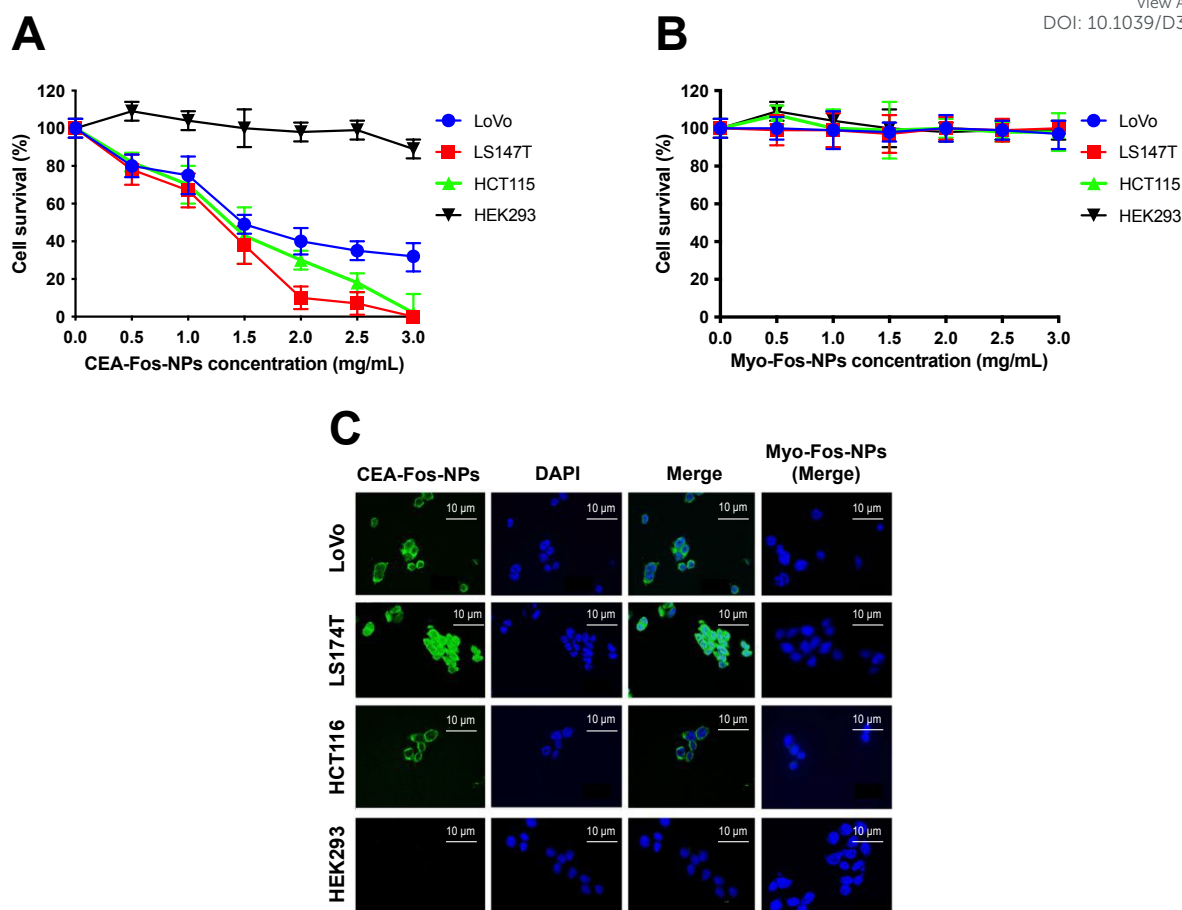


373 DCF fluorescence was observed using confocal microscope. The assay showed
374 strong fluorescence in all cancer cells treated with CEA-Fos-NPs but not with Myo-
375 Fos-NPs or in control cells (**Figure 4C**). The results support the hypothesis that the
376 cytotoxic effect seen in the PDT experiment was Foslip mediated via ROS generation.
377 Collectively, Foslip-loaded silica nanoparticles conjugated to anti-CEA Affimers
378 allowed tumour cell-specific fluorescence and photodynamic therapy *in vitro*.

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399 Figure 4. *CEA-Fos-NPs enabled targeted PDT in CRC cells.* Cells were incubated with

400 various concentrations of CEA-Fos-NPs or Myo-Fos-NPs respectively for 24 h then

401 photo-irradiated with 0.45 J/cm² of 600-700 nm. Cells viability was quantified using

402 MTT assay. Data show mean cells viability from 3 biological experiments (SEM, n=3).

403 (C), Representative confocal images of ROS detection in cells following PDT post-

404 incubation with nanoparticles at 2 mg/mL concentration and 0.45 J/cm² light dose are

405 shown. Cells were incubated with PBS containing 10 mM DCFDA for 30 minutes in a

406 CO₂ incubator then washed with PBS. DCF fluorescence observed in cells and their

407 respective DAPI staining are shown. Confocal images of ROS detection showed DCF

408 fluorescence in cancer cells treated CEA-Fos-NPs but not with Myo-Fos-NPs.



409 **Theranostic application of CEA-Fos-NPs in LS174T xenograft model of**
410 **colorectal cancer**

411 We next assessed the theranostic potential of CEA-Fos-NPs in a clinically relevant
412 mouse xenograft model of colorectal cancer. The tumour growth pattern of LS174T
413 xenograft is shown in **Figure S7**. Nanoparticles were suspended in sterile PBS at 2
414 mg/mL concentration and 150 μ L of nanoparticles injected into the tail vein of mice.
415 Two groups of five mice were injected with either CEA-Fos-NPs (n=5) or control Myo-
416 Fos-NPs (n=5) and imaged at 6, 24, 30 and 48 h. For better understanding of the
417 biodistribution and fate of the nanoparticles, one mouse from each group was
418 sacrificed after imaging at each time point and organs were harvested then imaged *ex*
419 *vivo*. The background fluorescence point was set high to eliminate the hepato-biliary
420 fluorescence and ensure that any fluorescence seen in the xenograft was a real signal.
421 Tumour-specific fluorescence was seen in the xenografts of mice that were injected
422 with CEA-Fos-NPs as shown in **Figure 5A**. No fluorescent signal was seen in any of
423 the mice that were injected with Myo-Fos-NPs. The fluorescent signal was seen as
424 early as 6 h, peaked at 24-30 h and remained in the xenograft at 48 h. When
425 background fluorescence was set to a lower point ($\sim 50 \times 10^6$ (p/s/cm²/sr) / (μ W/cm²)),
426 Foslip loaded nanoparticles exhibited a similar biodistribution to our previously
427 published report on NIR664-dye-doped silica nanoparticles (26); **Figure 5B and 5C**.
428 Liver fluorescence was evident at 6 h in all mice (mean 59.1×10^6 (p/s/cm²/sr) /
429 (μ W/cm²)) and increased at 24 h (85.8×10^6 (p/s/cm²/sr) / (μ W/cm²)). Hepatic
430 localisation was confirmed by *ex vivo* imaging of isolated organs. There was no
431 significant difference in liver fluorescence between mice injected with control particles
432 and those injected with anti-CEA Affimer targeted particles at any time point; **Figure**
433 **S8**.



434 Fluorescence in the CEA-targeted tumours was significantly greater than Myo-
435 targeted tumours at all time points ($p < 0.0001$). Mean tumour fluorescence increased
436 from 6 h (mean 0.55×10^7 (p/s/cm²/sr) / ($\mu\text{W}/\text{cm}^2$)) to 30 h (mean 9.415×10^7
437 (p/s/cm²/sr) / ($\mu\text{W}/\text{cm}^2$)); **Figure 5D**. The fluorescence ratio, which was defined as the
438 fluorescence of the tumour site over the fluorescence of normal tissue, at 6, 24, 30
439 and 48 h was 21, 88, 95 and 85 respectively. No tumour fluorescence, above
440 background, was seen in mice injected with Myo-Fos-NPs.
441 Tumour tissue, and other organs, were harvested and imaged *ex vivo*. Tumour
442 fluorescence was only detected in xenografts from mice injected with CEA-Fos-NPs,
443 as shown in **Figure 5E**. Importantly, the *ex vivo* imaging of the CEA-targeted
444 xenografts showed fluorescence within the core of the xenograft, suggesting that the
445 nanoparticles accumulated within the tumour microenvironment. To confirm this,
446 confocal imaging was performed on histological sections from a xenograft of a mouse
447 injected with CEA-Fos-NPs and Myo-Fos-NPs; **Figure 5F**. The xenograft showed
448 fluorescent signal within the tumour (section taken through the middle of the xenograft)
449 whilst no fluorescence was seen in the control xenograft, suggesting directed-
450 nanoparticle delivery to the tumour site.

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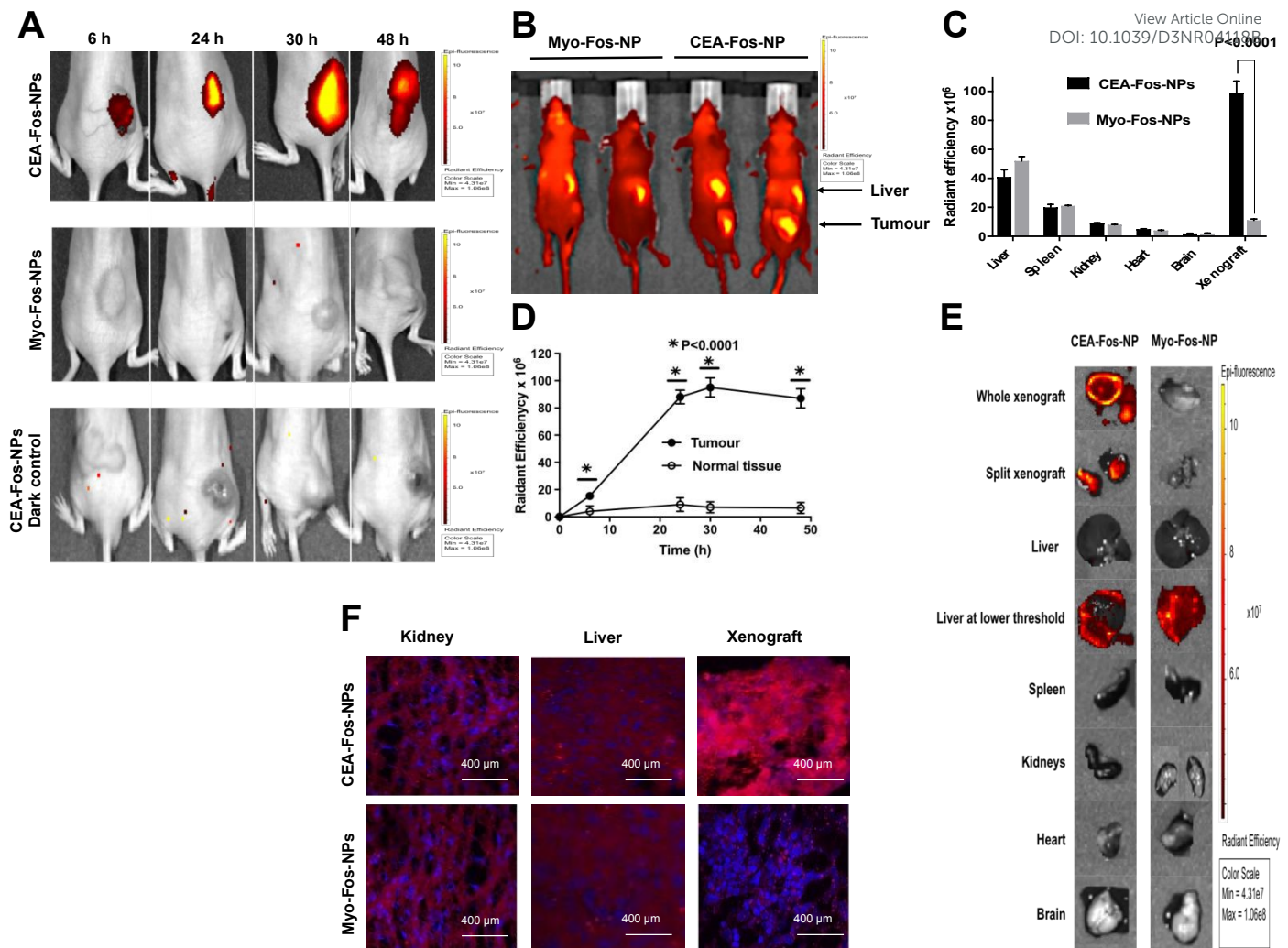
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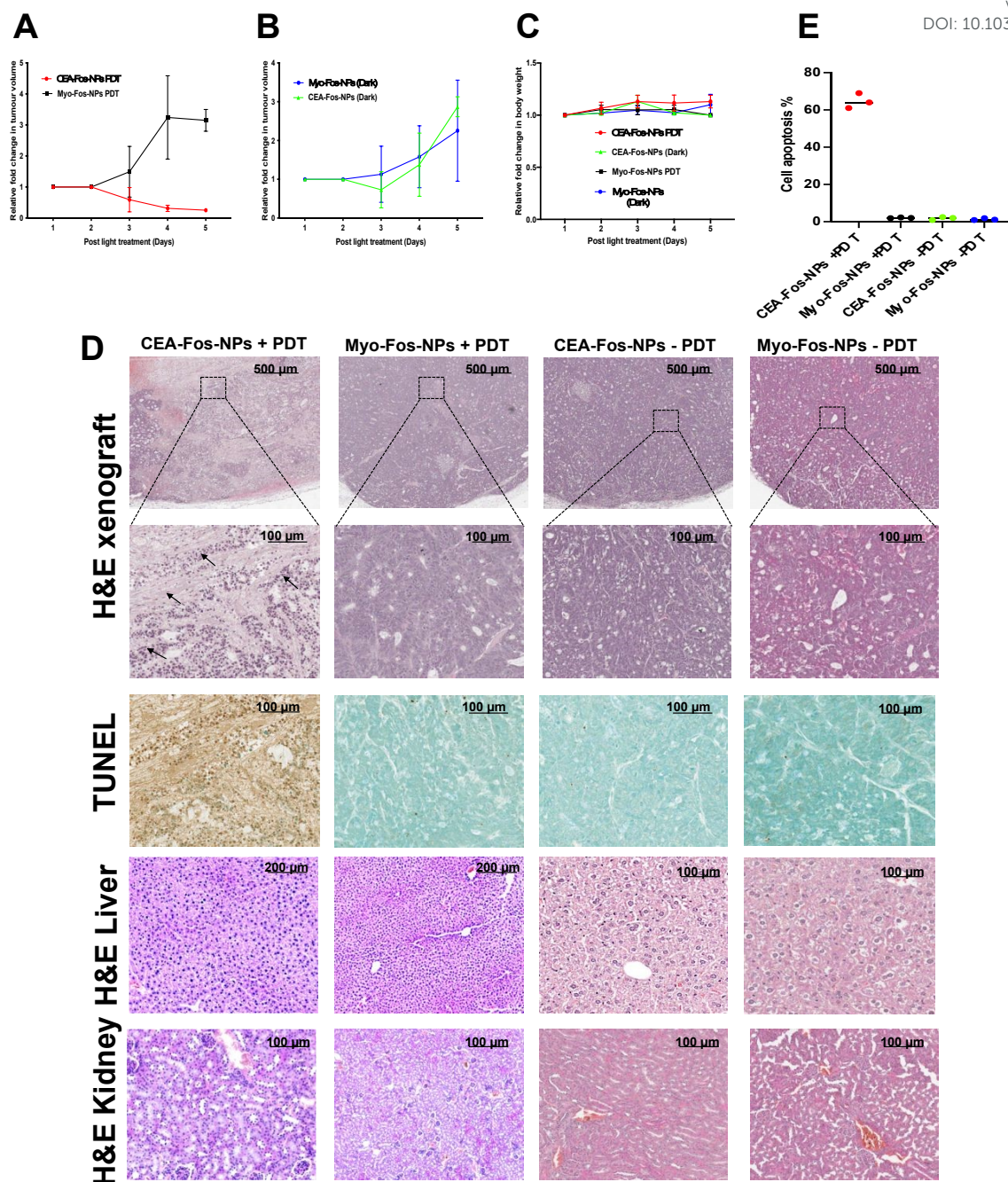
460 Figure 5. CEA-Fos-NPs enabled targeted fluorescent imaging of CRC in vivo. (A)
461 Representative *in vivo* fluorescence activation of CEA-targeted versus control NPs in
462 LS174T xenograft model at 6, 24, 30 and 48 h after intravenous injection with 150 μ L
463 (2 mg/mL), n=5. Colour scale bar: minimum = 4.31×10^7 and maximum = 1.06×10^8
464 (p/s/cm²/sr) / (μ W/cm²). (B) Fluorescence *in vivo* biodistribution of CEA-targeted and
465 control NPs at 24 h post NPs injection. (C), NPs biodistribution quantified using IVIS
466 with excitation filters at 615 - 665 nm and 8 s exposure time. Data show fluorescence
467 mean (SEM, n=5). (D) Data are mean tumour fluorescence for *in vivo* xenografts for
468 mice injected with CEA-Fos-NPs and Myo-Fos-NPs (SEM, n=5). Normal tissue
469 represents skin. (E) Representative *ex vivo* fluorescence images of the excised organs
470 and xenografts from CEA-targeted and control dosed mice at 24 h post injection. Liver
471 at lower threshold = ($\sim 50 \times 10^6$ (p/s/cm²/sr) / (μ W/cm²)). Colour scale bar: minimum =
472 4.31×10^7 and maximum = 1.06×10^8 (p/s/cm²/sr) / (μ W/cm²). (F) Fluorescence
473 histology images of kidney, liver and tumour xenograft from mice injected with CEA-
474 Fos-NPs and Myo-Fos-NPs 48 h after injection using confocal microscopy.



475 Having observed significant accumulation of CEA-Fos-NPs in colorectal tumours, we
476 next wished to test whether they could mediate efficient PDT activity *in vivo*. A further
477 *in vivo* experiment was performed: xenograft tumours were established as before and
478 animals were split randomly into four groups (each n=5). Two groups were treated with
479 CEA-Fos-NPs and with Myo-Fos-NP as previously then subjected to PDT. Two control
480 groups were treated with CEA-Fos-NPs or Myo-Fos-NP and received no PDT. PDT,
481 given 24 h post-delivery of NPs, consisted of trans-cutaneous laser irradiation (650
482 nm, 60 mW/cm², 50 J/cm², 14 min). The PDT efficacy was evaluated by tumour volume
483 measurements and postmortem histopathological analysis. To eliminate the possibility
484 of laser-induced thermal ablation and cell death in xenografts, thermal imaging videos
485 were recorded for 1 min at 0 min, 7 mins and 14 mins during treatment for each mouse
486 and we found no noticeable increase in surface temperature during laser treatment
487 (**Figure S9**). The CEA-targeted PDT group displayed ~ 4-fold decrease in tumour
488 volume when compared to Myo-targeted PDT group at day 5 (0.24 vs 3.15 median;
489 P<0.001) whilst mice weights remained unchanged in all groups; **Figure 6A-C**.
490 Importantly, there was no reduction in tumour volume in any of the dark control groups.
491 Histological analysis showed condensed nuclei and loss of cell structure in tumour
492 xenografts of CEA-targeted PDT, which was not observed in control groups; **Figure**
493 **6D**. TUNEL assay revealed dense staining (brown) at the site of DNA fragmentation
494 in CEA-Fos-NPs PDT xenografts in keeping with significant tumour cell apoptosis (64
495 ± 2.3%), whilst the controls showed methyl green of normal cells indicating no tumour
496 apoptosis (2 ± 0.1% for Myo-Fos-NPs, 1.8 ± 0.5% and 1.3 ± 0.3% for PDT-negative
497 controls) (**Figure 6E**). Overall, the results demonstrate the high selectivity and
498 accuracy of CEA-targeted PDT to colorectal tumour xenografts.

499





500 Figure 6. *CEA-Fos-NPs enabled targeted PDT of CRC in vivo.* (A) Tumour
 501 growth curves of PDT groups over the treatment period until mouse sacrifice (SEM,
 502 n=5). (B), Tumour growth curves of control dark groups over the treatment period until
 503 mouse sacrifice (SEM, n=5). (C) Body weight curves of different groups over the
 504 treatment period until mouse sacrifice, (SEM, n=5). (D) Representative images of
 505 histological analyses of tumour sections (H&E and TUNEL staining), liver and kidney
 506 (H&E) at day 5 post treatment. Black arrows point to condensed nuclei and loss of
 507 cells structure. (E) Quantitative analysis of TUNEL positivity out of whole tumour
 508 region in the four groups (n = 3).



509

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510 Developing a targeted nanoparticle against a specific tissue to produce a reliable
511 molecular probe remains challenging. Several studies have demonstrated improved
512 delivery when a nanoparticle is actively targeted to CEA in colorectal cancer cells
513 using site specific reagents such as antibodies, antibody-fragments, aptamers,
514 peptides and nanobodies (35-39). To date, anti-CEA antibody has shown the most
515 promising targeting bioreceptor in colorectal murine models but translation to clinical
516 application was complicated by the immunogenicity and the clearance from
517 bloodstream, both owing to binding of Fc receptor-containing entities to the antibody
518 Fc region (40). In addition, antibody size (~150 kDa) makes cell penetration difficult.
519 Tiernan *et al* (2015) were the first to show specific tumour fluorescent imaging using
520 NIR669-doped silica nanoparticles (mean diameter of 65 nm) in LS174T murine
521 xenograft mouse model (26). They immobilised monoclonal anti-CEA antibody to the
522 surface of the nanoparticle using PAMAM dendrimer. Conjugation of anti-CEA Affimer
523 carried out with sSMCC, as used here, showed strong tumour-specific targeting in the
524 same animal model. However, anti-CEA Affimer targeted xenografts exhibited higher
525 fluorescence mean 9.415×10^7 (p/s/cm²/sr) / (μ W/cm²) vs 4.74×10^7 (p/s/cm²/sr) /
526 (μ W/cm²) and a similar biodistribution but with notably lower liver uptake. In keeping
527 with our findings, Pramanik *et al* (2022) have shown that anti-CEA Affimer tagged
528 cubosomes, loaded with copper acetylacetonate as a model drug, actively targeted
529 LS174T colorectal cancer cells *in vivo* (41). The authors showed preferential
530 accumulation in colorectal cancer mouse xenografts, while maintaining low
531 nonspecific absorption and toxicity in other organs. Owing to their small size (~12
532 kDa), controlled orientation on the surface of nanoparticles and their high affinity to
533 CEA expressing cells ($K_D = 15.3 \pm 0.37$ nM and 34.4 ± 16 nM for the two Affimers



534 tested) (30), anti-CEA Affimers are expected to achieve important advances in CEA-
535 targeting nano technologies. Despite the huge potential of new tools, targeting the
536 CEA biomarker, uptake of research into CEA-targeting systems to enhance the
537 efficiency of colorectal cancer targeting has been modest.

538 High accumulation and penetration of anti-cancer drugs into the inner parts of the
539 tumour tissues are required to efficiently eradicate malignancies. Our data showed
540 that anti-CEA Affimer targeted nanoparticles allowed significant Foslip-mediated
541 localisation in tumour cells and photodynamic therapy *in vitro* and *in vivo* when
542 compared to control nanoparticles. Reports on targeted delivery of mTHPC
543 nanoparticulate formation to colorectal cancer cells for photodynamic therapy are
544 limited. Millard *et al* (2020) used ~203 nm mTHPC-loaded extracellular vesicles (EV)
545 and compared them with Foslip in a colorectal HT29 murine xenograft mouse model
546 (42). They showed that in 3D cancer cell models, mTHPC-EV uptake produced deeper
547 penetration after 24 h incubation as compared to Foslip, whilst *in vivo* results showed
548 a 33% increase in tumour killing with PDT treatment applied 24 h after injection but
549 0% was observed after Foslip-mediated PDT. However, a concerning finding was the
550 big difference between liposomal and EV formulations in mTHPC-EV accumulation in
551 the lung (five to seven times higher than Foslip) and liver. In sharp contrast, our data
552 showed that significant fluorescence was only observed in the hepatobiliary system,
553 which peaked at 24 h and reduced by 48 h post-injection. Other studies that
554 investigated the biodistribution and excretion of silica nanoparticles have reported
555 similar findings (43-45). Bretin *et al.* (2019) demonstrated the usefulness of
556 nanoparticle encapsulation for PDT tumour targeting efficacy in CRC (46). They used
557 80 nm silica nanoparticles coated with xylan to encapsulate 5-(4-hydroxyphenyl)-
558 10,15,20-triphenylporphyrin (TPPOH) and tested it in a colorectal HT-29 murine



559 xenograft mouse model. They showed significant phototoxic effects of TPPOH
560 SNPs mediated by ROS generation and stronger cell uptake in human colorectal
561 cancer compared to free TPPOH. Abdelghany *et al* (2013) successfully encapsulated
562 meso-tetra(N-methyl-4-pyridyl) porphine tetra tosylate (TMP) photosensitiser in a
563 hydrogel-based chitosan/ alginate nanosystem with an anti-death-receptor-5 (DR5)
564 antibody tagged onto the surface (47). Although their nanoparticle elicited a more
565 potent phototoxic effect than free drug, the nanoparticle diameter was prohibitively
566 large at 560 nm. In addition, DR5 is not specific to colorectal cancer and is not highly
567 expressed. Others have reported successful encapsulation of mTHPC in
568 nanoparticles for photodynamic therapy in colorectal cancer cells, but without a
569 surface targeting molecule (48). To date, Foslip has been intensively tested in different
570 *in vitro* preclinical models (2D and 3D tumour cell cultures) (34, 49, 50), whilst PDT
571 studies, including biodistribution, pharmacokinetics, and PDT efficacy in tumour-
572 bearing *in vivo* animal models are limited (51, 52).

573 The surgical management of colorectal cancer is often limited by difficulty in
574 delineating tumour margins and an inability to visualise occult nodal metastasis. This
575 predisposes to tumour recurrence and decreased survival. Although several studies
576 have investigated the efficacy of PSs for PDT regimens in CRC, only few have
577 meticulously explored their application for fluorescence imaging. We have shown that
578 CEA-Fos-NPs enabled real-time fluorescence imaging of colorectal tumours,
579 accurately distinguishing tumour from normal tissue. Gavrina *et al.* (2018),
580 investigated Chlorin e6 (Ce6) conjugated to polyvinyl alcohol (PVA) nanoparticles for
581 *in vivo* fluorescence imaging in CT26 xenograft model (53). The authors found a higher
582 tumour-to-normal signal in mice treated with Ce6–PVA nanoparticles when compared
583 to Ce6 alone. Xu *et al* (2018) fabricated a H₂S-responsive NIR-fluorescent silica based

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584 NPs which allowed fluorescent imaging in HCT116 CRC cells, both *in vitro* and *in vivo*
585 (54). The study design lacked control NPs and control cell lines while for the *in vivo*
586 experiment the NPs were injected into the core of the tumour xenograft and not
587 systemically. Soster *et al* (2012), used PEG-conjugated dye-doped silica
588 nanoparticles, via systemic delivery, to image CRC metastases in murine xenograft
589 models (55). They used 'bare' nanoparticles as controls and imaged fluorescence only
590 in *ex vivo* organs, raising concerns for antigen-specific targeting.

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Conclusion

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We have successfully developed a unique targeting strategy to deliver Foslip to colorectal cancer in an animal model using a novel Affimer protein. We have shown that Affimer tagged silica coated Foslip nanoparticles are effective theranostic agents. The nanoparticle design enables stable assembly of the components within a small sized structure, with favourable pharmacokinetic profile and biodistribution, and superior cellular uptake. Our nanoparticle is potentially applicable to targeting other solid tumours by changing the surface Affimer and provided that there is a specific tissue biomarker.



634

Materials and Methods

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635 **Synthesis of silica coated Foslip nanoparticles**

636 All experiments were performed at room temperature using Sigma-Aldrich (USA)
637 reagents unless otherwise stated. Nanoparticles synthesis was modified from previous
638 publications (22, 26).

639 Water soluble meta-tetra (hydroxyphenyl) chlorin (mTHPC) Foslip® photosensitiser
640 (20 mg/mL DPPC/DPPG, 2.2 mM mTHPC, 50 mg/mL glucose) was provided by
641 Biolitec AG (Jena, Germany) with molecular weight of 680.764 g/mol. The powder was
642 dissolved in PBS to make a stock solution of 100 μ M and filtered through a syringe
643 filter (0.1 μ m pore size; TPP, Trasadingen, Switzerland). Tetraethyl orthosilicate
644 (TEOS) was added (12 μ L) into 1 mL of deionised water and stirred at 200 rpm for 24
645 h at room temperature. Next, 20 μ L of the Foslip suspension was added to the TEOS
646 solution and the mixture was stirred at 200 rpm for 1 h. Two mL of PBS (1x) buffer
647 solution was added to the mixture and stirred for 30 min then 24 μ L of fresh TEOS
648 were added and the mixture was stirred at 200 rpm for 48 h. The mixture was then
649 transferred into Corex centrifuge tubes (Corning) with equal volumes. Particles were
650 pelleted by centrifugation (15,000 \times g, 25 min), resuspended in wash solution using
651 ultrasound sonication, repelleted and the supernatant discarded. This wash step was
652 repeated three times before the liquid was discarded and the particles were
653 resuspended in 0.1 M PBS at a concentration of 1 mg/mL using sonication then stored
654 at 4 °C.

655 **APTES amination**

656 Freshly synthesised nanoparticles were suspended in 1 mL of ethanol plus 4% [v/v]
657 (3-aminopropyl) triethoxysilane (APTES) and stirred at 200 rpm for 3 h at room
658 temperature while stirring in a Falcon tube. The aminated particles were then



659 transferred to a centrifuge tube (Corex) followed by 2x washes with ethanol and
660 centrifuged at 11,000 xg for 25 min. The contents were then washed once using 2-(*N*-
661 morpholino) ethanesulfonic acid (MES) buffer (pH 7.0) then resuspended in MES
662 buffer at final concentration of 1 mg/mL.

663 **Affimer production**

664 Anti CEA specific Affimer clones were identified using a 'phage display library' method
665 as recently published by Shamsuddin *et al* (30, 31). Out of the three CEA binding
666 Affimers identified, clone II and III (molecular weight 12.5 and 12.6 kDa respectively)
667 were chosen for this study having 9 and 10 distinct amino acid residues at the variable
668 region respectively. Anti-human cardiac myoglobin Affimer was used as a control. Anti-
669 CEA (II and III) and control Affimer clone (molecular weight 12.5 kDa) DNA were
670 isolated as previously described and the Affimer protein were expressed from a
671 pET11a vector in BL21 (DE3) *E.coli* cells. The *E.coli* cells were grown in Luria-Bertani
672 broth medium containing 100 µg/mL of carbinicillin until the growth was 0.8 at A₆₀₀.
673 Then cells were induced with 0.1 mM IPTG and incubated at 25 °C for 6 hours. The
674 cells were harvested by centrifugation, lysed and the His₆ tagged Affimers were
675 purified on Ni²⁺-NTA affinity chromatography (Merck, New Jersey, USA). Pierce®
676 Immobilised tris (2-carboxyethyl) phosphine (TCEP) reducing gel was used to reduce
677 Affimer disulphide bonds to free all thiol groups for subsequent maleimide coupling
678 chemistry. TCEP gel (150 µL) was washed with PBS containing 1 mM edetate
679 disodium (EDTA) three times followed by 4 µL of PBS containing 50 mM EDTA,
680 followed by adding of 150 µL of 0.5 mg/mL Affimer. The mixture was stirred at 20 rpm
681 for 1 h then centrifuged at 1,000 xg for 1 min and finally reduced Affimers were
682 recovered from the supernatant.

683



684 **Silica nanoparticle Affimer conjugation**

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685 Fresh sulfo-succinimidyl 4-(maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (6
686 mg) was mixed with 60 μg (1 mg/mL) of polyclonal anti-CEA or anti-myoglobin Affimers
687 and stirred gently at room temperature for 20 minutes. The reaction mixture was then
688 added to 4 mL of 1 mg/mL aminated nanoparticles and stirred at room temperature for
689 2 h then washed twice with PBS (6,000 $\times\text{g}$ for 15 min) to remove unbound sulfo-SMCC.
690 The nanoparticles were resuspended at 2 mg/mL and finally 0.1% (w/v) BSA was
691 added. The nanoparticles were either stored in the dark at 4 °C or used immediately
692 for *in vitro* experiments.

693 **Scanning electron microscopy (SEM)**

694 SEM images were obtained with a field emission gun scanning electron microscopy
695 (FEG-SEM, LEO 1530 Gemini FEGSEM) fitted with an Oxford Instruments 80 mm X-
696 Max SDD detector, Carl Zeiss.

697 **Spectrofluorometer measurements of silica nanoparticles**

698 The fluorescence intensity of silica coated nanoparticles was quantified on a
699 spectrofluorometer (Berthold Technologies Mithras LB 940 multimode microplate
700 reader with Mikro Win 2000 software) with a halogen lamp intensity of 23,000 and
701 excitation and emission spectra of 645 nm \pm 30 nm.

702 **Affimer per nanoparticle quantification assay**

703 Affimer tagged NPs were suspended in PBS at 1 mg/mL then mixed with 5 mL of 2-
704 mercaptoethanol 1% (v/v) and incubated for 1 h at 37 °C. The suspension was then
705 centrifuged at at 12,500 $\times\text{g}$ for 30 min. The supernatant was recovered; then desalted
706 using a Zepa spin desalting column (7K MWCO) to remove any remnants that might
707 interfere with the fluorescent dye NanoOrange®. The released Affimers were
708 quantified using a NanoOrange® protein quantitation kit.



709 Standard solutions of Affimer (0 – 2.5 μg) were prepared in 1X NanoOrange® reagent
710 working solution from 10 $\mu\text{g}/\text{mL}$ stock solutions. For sample analysis, 10 μl of each
711 desalted solution was mixed with 240 μl of 1X NanoOrange® working solution. All
712 standard and sample solutions were prepared in 500 μL tubes and incubated at 95 $^{\circ}\text{C}$
713 in a water bath for 10 min. All processes were carried out protected from light. The
714 samples were allowed to cool down at RT for 20 min before 200 μL of each solution
715 was transferred to a 96-well plate for fluorescence intensity measurement. The
716 measurement was carried out with excitation and emission wavelengths of 485 nm
717 and 590 nm, respectively. The fluorescence values of the standards and samples were
718 subtracted from the value of reagent blank. The corrected values were used in
719 generating calibration curves using Graphprism and linear fitting was performed.

720 **Dynamic light scattering (DLS)**

721 The DLS measurement for nanoparticles was made using a Zetasizer Nano series,
722 Nano-ZS DLS system with a red (633 nm) laser (Malvern Instruments Ltd) at room
723 temperature and in a small volume disposable cuvette. The polydispersity index (PDI)
724 of the colloidal solutions was measured using DLS with a particle size analyser. The
725 zeta potential or overall surface charge of each nanoparticle sample in solution (~ 1
726 mg/mL in millipore water) was determined using a Zeta Plus, zeta potential analyser
727 (Brookhaven Instruments Corp. Holtsville, NY).

728 **Nanoparticle-mediated fluorescent imaging *in vitro***

729 HEK293 epithelial cell line, as a control, and the human colorectal cancer cell lines,
730 LoVo, HCT116, and LS174T, were obtained from the American Type Culture
731 Collection (ATCC). HEK293 cells were maintained in DMEM (1X) with GlutaMAX™-I
732 (Gibco®) and 10% (v/v) FBS (Sigma life Science). Cancer cells were maintained in
733 Advanced MEM (ATCC) for LoVo, F12K Nutrimix (Invitrogen, USA) for LS174T and



734 RPMI 1640 (Invitrogen, USA) for HCT116. All cells were supplemented with 10% FCS
735 and 1% L-glutamine at 37 °C in 5% CO₂. Cells (9x10⁴) were seeded onto sterile glass
736 coverslips (Cellpath, Newtown Powys, UK) in a six-well plate (Corning) and incubated
737 at 37 °C in 5% CO₂ for 24 h. Culture media was discarded and cells were washed 2x
738 with PBS followed by addition of paraformaldehyde (4%, v/v) for fixation. Following
739 incubation for 30 min at room temperature, the fixative was removed and cells were
740 washed 3x times with PBS. BSA (0.1% (w/v), (EMD chemicals, San Diego, USA))
741 was added for 30 min then followed by 3x washes with PBS. Anti-CEA or anti-
742 myoglobin Affimer tagged nanoparticles (1 mg/mL) were added to the wells and
743 incubated for 24 h in the dark at room temperature. The nanoparticle suspension was
744 discarded and the cells were washed 3x times with PBS then coverslips were mounted
745 onto glass slides using Depex (Waltham, Massachusetts, USA). The slides were left
746 to cure overnight then stored at 4 °C in the dark and imaged using confocal
747 microscopy. Images were captured using a Nikon A1R-A1 confocal microscope
748 (Nikon, Japan) with NHS Elements software (v 4.0). ImageJ v1.42q (NIH Freeware,
749 USA) was used to quantify fluorescence.

750 **Nanoparticles internalisation and co-localisation *in vitro***

751 LoVo and LS174T cells (2x10⁴) were seeded onto sterile coverslips and allowed to
752 adhere overnight. The following day, cells were treated with fluorescein isothiocyanate
753 (FITC) and CEA- or Myo-Affimer tagged-nanoparticles (1 mg/mL) for 1, 4 and 24
754 hours. Cells were washed three times with PBS to remove nanoparticle suspension
755 and incubated with LysoTracker™ deep red (Thermofisher) at 50 nM for 1 h. Following
756 three washes with PBS, cells were fixed with 4% PFA. Following routine wash, nucleus
757 was counterstained with DAPI and mounted onto slides using mounting media



758 (Fluoroshield, Sigma). To monitor NPs uptake, imaging was performed at 100x
759 magnification using a fluorescent microscope.

760 **Photodynamic therapy and cell cytotoxicity *in vitro***

761 LoVo, LS174T, HCT116 and HEK293 cells were grown in two identical 6 well plates.
762 Anti-CEA Affimer tagged nanoparticles and their respective controls with various
763 concentrations (1-5 mg/mL) were added to the wells and incubated for 24 h in the dark
764 at room temperature. The nanoparticle suspension was removed after 24 h and the
765 cells were washed 3x times with PBS the incubated with fresh media. The plates were
766 then immediately placed on top of a light-radiating device (Avago Technologies,
767 California, USA). Cells were treated with a light dose of 0.225 - 0.675 J/cm², peak
768 wavelength of 600-700 nm and a spectral half-width of 12 nm, then kept in the dark.
769 Light dose was calculated based on treatments which lasted for 10-45 min at
770 0.25 mW/cm². Control plates were kept in the dark with no light irradiation.

771 Stock solution of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)
772 tetrazolium salt MTT (Sigma) was prepared at 5 mg/mL in PBS and wrapped in foil to
773 protect from light. The media, in which the seeded cells were grown, was replaced
774 with 50 μ L of 1 mg/mL working MTT solution and incubated in the dark for 3 hours.
775 MTT solution was then removed and the dark blue formazan dye formed was dissolved
776 in 100 μ L of propan-1-ol. Optical density was measured using a microplate reader
777 (Opsys MRTM, Dynex technologies ltd, UK) at 570 nm.

778 **Cellular reactive oxygen species detection assay**

779 Cells were seeded on a 96 well plate 2.5 x 10⁴ cells/well and incubated for 24 h. Cells
780 were then washed once using 1X Buffer then stained with 25 μ M 2',7' -
781 dichlorofluorescein diacetate (DCFDA) in 1X Buffer for 45 min at 37 °C. Cells were then
782 washed once with PBS then incubated with functionalised silica nanoparticles for 24



783 h in the dark then illuminated for 30 min. Immediately after illumination, nanoparticles
784 suspension was then discarded and 10 μM DCF-DA (Merck, New Jersey, USA) in
785 Hank's balance salt solution (Merck, New Jersey, USA) was added for 30 min and
786 incubated in a CO_2 incubator then washed with PBS. DCF fluorescence was observed
787 using confocal microscope.

788 **Fluorescent imaging *in vivo***

789 The *in vivo* experiments were conducted in a UK Home Office designated animal
790 facility at Leeds Institute of Medical Research (University of Leeds, UK). The study
791 was conducted in line with UK Home Office regulations and in accordance with The
792 Animals (Scientific Procedures) Act 1986, under a personal animal licence (Licence
793 number: P93AOF172). BALB/c nu/nu female mice (4-6 weeks old) (Charles River, UK)
794 were injected subcutaneously with 1.5×10^6 LS174T cells to the right flank. Tumour
795 xenografts were developed to nearly 10 mm in diameter within ~ 10 days, then mice
796 were randomised to either CEA-targeted or control Affimer tagged-nanoparticles. Mice
797 were injected with nanoparticles at 150 μL (suspended in sterile PBS at 2 mg/mL
798 concentration) via the tail vein under general anaesthesia. Fluorescent images were
799 captured using IVIS imaging (filters: excitation 672 nm, emission 694 nm; Perkin
800 Elmer, USA) under anaesthesia then imaged at different time points. Living Image
801 (v4.3.1, Caliper Life Sciences, USA); was used for fluorescence measurements
802 (radiant efficiency in $(\text{p/s/cm}^2/\text{sr}) / (\mu\text{W/cm}^2)$) after calibration to background. *Ex vivo*
803 fluorescence imaging was performed on all the resected tumour xenografts and the
804 remaining organs.

805 **Photodynamic Therapy *in vivo***

806 The PDT efficacy of Foslip encapsulated silica nanoparticles was evaluated in LS174T
807 CRC xenograft models *in vivo*. Animal models were categorised into four treatment



808 groups: i) anti-CEA Affimer targeted NPs plus PDT laser treatment, ii) anti-myoglobin
809 targeted NPs plus PDT laser treatment, iii) anti-CEA targeted NPs alone and iv) anti-
810 myoglobin targeted NPs alone. Tumour xenograft volumes, mice weights and Body
811 Conditioning Scoring were recorded before and after PDT experiment. Mice were
812 anaesthetised then immobilised in plexi-glass holders 24 h post intravenous injection
813 then irradiated at the xenografts using 650 nm fibre optic laser. The laser was
814 positioned 18 mm directly above the skin, delivering a total light dose of 50 J/cm², at
815 a fluence rate of 60 mW/cm² resulting in a total irradiation time of 14 min. Thermal
816 imaging videos were recorded for 1 min at 0, 7 and 14 min during treatment for each
817 mouse. Mice were maintained and monitored for 5 days post PDT treatment. Following
818 completion of the experiment, mice were euthanised in accordance with Schedule 1
819 of the Animals (Scientific Procedures) Act 1986 and the tumour xenografts and organs
820 were harvested. The efficacy of PDT was evaluated by histological analysis in
821 harvested tissue.

822 **Statistical analysis**

823 GraphPad Prism Version 9.0 (GraphPad Software, California, USA) was used for the
824 statistical analysis of all the data. The difference between the groups were evaluated
825 using Student's t-test and Wilcoxon Signed Rank Test.

826 **Financial & competing interests disclosure**

827 This work was supported by a MRC fellowship and AMS grant awarded to YSK. The
828 authors have no other relevant affiliations or financial involvement with any
829 organisation or entity with a financial interest in or financial conflict with the subject
830 matter or materials discussed in the manuscript apart from those disclosed.

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833 **Author Contributions**View Article Online
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834 Y.S.K, P.A.M, T.A.H and D.G.J conceived and designed the experiments. E.A and
835 S.H.S performed the Affimer expression and purification. M.I.K, T.M, N.L, A.P and
836 Y.S.K performed the *in vivo* experiments and analysed the data. R.A-M performed
837 fluorescent microscopy experiments. Y.S.K performed all the other experiments and
838 analysed the data. L.C. contributed to design of the *in vivo* experiments. A.P, D.T, L.C,
839 D.G.J, T.A.H, P.A.M and J.T contributed to study design. All authors interpreted the
840 results. Y.S.K, P.A.M, T.A.H and D.G.J co-wrote the manuscript. All authors discussed
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