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1 **Novel fluorescent nanoprobe based on hyaluronic acid and polyethyleneimine functionalized**
2 **graphene oxide for detecting hyaluronidase as tumor marker**

3

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17

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21

22

23 **Keywords:** Nanoprobe; Hyaluronic acid; Polyethyleneimine; Graphene oxide; Tumor marker.

24

25

26 **Abstract**

27 A novel nanoprobe was developed for the detection of hyaluronidase as cancer marker, using
28 fluorescein 5-isothiocyanate (FITC) as fluorescence indicator, hyaluronic acid (HA) and polyeth-
29 yleneimine (PEI) functionalized graphene oxide (GO) as quencher. PEI was attached to GO
30 through amide bonding, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hy-
31 droxysuccinimide (NHS) as coupling agents. Aminated hyaluronic acid (HA) was then linked to
32 GO-PEI (GOP) via the same route. NMR, UV-Vis-NIR, FT-IR, SEM and TGA analyses confirmed
33 the successful synthesis of GOP-HA. Finally, FITC was attached to GOP-HA through reaction
34 between the isothiocyanate group of FITC and the amine group of aminated HA. The fluorescence
35 of FITC is quenched in the resulted GOP-HA-FITC nanoprobe. Addition of hyaluronidase can
36 cleave HA chains into small fragments, releasing FITC to emit fluorescence. Importantly, the na-
37 noprobe is highly sensitive and selective, and can enter cells by specifically binding to the CD44
38 receptor on the surface of Hela cells. Therefore, GOP-HA-FITC nanoprobe could be used in tar-
39 geted tumor cell imaging for the early diagnosis of cancers.

40

41

43 **1. Introduction**

44 Cancer is one of the leading causes of human death in the world. Nearly 10 million people died
45 of cancers in 2020 according to the World Health Organization (WHO). The early diagnosis of
46 cancer is thus of particular importance.¹ The occurrence of cancer is often accompanied by the rise
47 of a variety of indicators, among which enzyme determination has been used to diagnose cancers
48 for half a century. Studies have shown that the increase of Regan isoenzyme of alkaline phosphatase
49 is closely related to the occurrence of occult renal cell carcinoma. The continuous increase
50 after operation indicates the persistence or recurrence of the disease.² Leucine aminopeptidase
51 (LAP) is also an important marker for cancer diagnosis as it is overexpressed in many cancers. Chai
52 et al. developed a water-soluble near infrared (NIR) fluorescent probe (CHMC-M-Leu). The probe
53 exhibited high sensitivity and responsiveness to LAP, showed good cell uptake, and was successfully
54 used to monitor endogenous LAP in living cells.³

55 The occurrence, proliferation and metastasis of cancer are also closely related to extracellular
56 matrix (ECM).⁴ Hyaluronic acid (HA) is a linear polysaccharide composed of D-glucuronic acid
57 and N-acetyl-D-glucosamine. HA is largely present in the human body as the main component of
58 the ECM. Hyaluronidases are specific hydrolases which can cleave HA chains into small fragments.
59 Many recent studies have shown hyaluronidases are overexpressed in cancers, such as
60 breast cancer, colon cancer and Laryngeal carcinoma.⁶⁻⁸ A variety of hyaluronidase detection methods
61 have been developed for cancer diagnosis, including viscosity method, turbidimetry, zymography,
62 colorimetry, spectrophotometry, etc.⁹⁻¹² However, these methods present some disadvantages
63 such as complex operation, low sensitivity and low selectivity. Therefore, it is necessary to develop

64 a fast, highly sensitive and selective method for the detection of hyaluronidase, and nanoprobe are
65 one of the possible solutions.

66 It is well known that HA can specifically bind to the CD44 receptor on the surface of cancer
67 cells. Different nanoprobe for the detection of hyaluronidase have been reported. For example,
68 silicon nanoparticles (SiNPs) and molybdenum disulfide quantum dots (MoS₂ QDs) are assembled
69 with HA-functionalized gold nanoparticles (HA-AuNPs) to form a nanoprobe.^{13,14} A novel upcon-
70 version luminescent nanoprobe was developed by coupling HA upconversion fluorescent nanopar-
71 ticles (HA-UCNPs) with poly(*m*-phenylenediamine) (PMPD) nanospheres through covalent
72 bonds.¹⁵ There are also diagnostic and therapeutic probes made from carbon dots (P-CDs) and
73 HA.¹⁶ The fluorescence in nanoprobe is quenched by Förster resonance energy transfer (FRET).
74 With the addition of hyaluronidase, HA chains are cleaved and SiNPs, MoS₂ QDs, UCNPs, or P-
75 CDs are released, thereby restoring fluorescence. However, the preparation process of these nano-
76 probe is tedious and requires high temperature reaction.

77 Fluorescent nanoparticles have also been developed for the detection or imaging of other cells.
78 Yildiz et al. prepared doxorubicin-loaded NPs from Alexa Fluor 750-labeled poly(lactic-*co*-gly-
79 colic acid)-*b*-poly-L-lysine (PLGA-PLL-AF750) and poly(lactic acid)-*b*-poly(ethylene glycol)
80 (PLA-PEG) block copolymers. In vitro studies showed that NPs are able to deliver doxorubicin
81 over a long period of time and have fluorescent activation after interaction with proteolytic en-
82 zymes. Cell studies demonstrated the theranostic properties of the NPs as contrast agents for fluo-
83 rescence imaging and anticancer drugs.¹⁷ Hernandez et al. developed fluorescein isothiocyanate-
84 conjugated poly-D-lysine (PDL-FITC) coated gold nanoparticles (AuNP) which are sensitive to

85 reactive oxygen species (ROS), and can be used to identify pro-inflammatory macrophages in dif-
86 ferent cell populations.¹⁸

87 Graphene oxide (GO) has been widely studied for biomedical applications because of its unique
88 properties, including two-dimensional planar structure, large surface area, chemical and mechani-
89 cal stability, excellent conductivity and biocompatibility. Moreover, abundant oxygen-containing
90 functional groups are present on the surface of GO, which is conducive to modification.¹⁹ GO has
91 been widely used in optoelectronics, biomedicine, drug release, tumor treatment, etc.^{20,21} A novel
92 drug carrier was designed by modifying GO with polyetheramine (PEA) and hyaluronic acid (HA)
93 to load quercetin.²² The results showed that the anti-tumor efficacy was enhanced and the effect
94 was longer lasting compared to the use of quercetin alone, suggesting that GO-based materials
95 present good potential for clinical applications.

96 Polyethyleneimine (PEI) is a cationic polymer containing readily protonated primary, secondary
97 and tertiary amines.²³ It can interact with negatively charged DNA or drugs through electrostatic
98 interaction to form nanocomplexes, and has been widely investigated for uses as a gene or drug
99 carrier. Different from poly(L-lysine) (PLL), PEI is only partially amino protonated at physiologi-
100 cal pH, allowing the polymer to act as an effective "proton sponge". However, the cytotoxicity of
101 PEI considerably limits its applications. It has been shown that PEI with a molecular weight (MW)
102 below 2000 Da displays a low cytotoxicity.²⁴ PEI can also form a compact nanostructure with
103 negatively charged HA through electrostatic adsorption. Rodrigues et al. developed a tumor-tar-
104 geted chemo-photothermal nanomedicine through functionalization of acridine orange (AO)-
105 loaded gold-core mesoporous silica shell (AuMSS) nanorods with PEI and HA. Functionalization

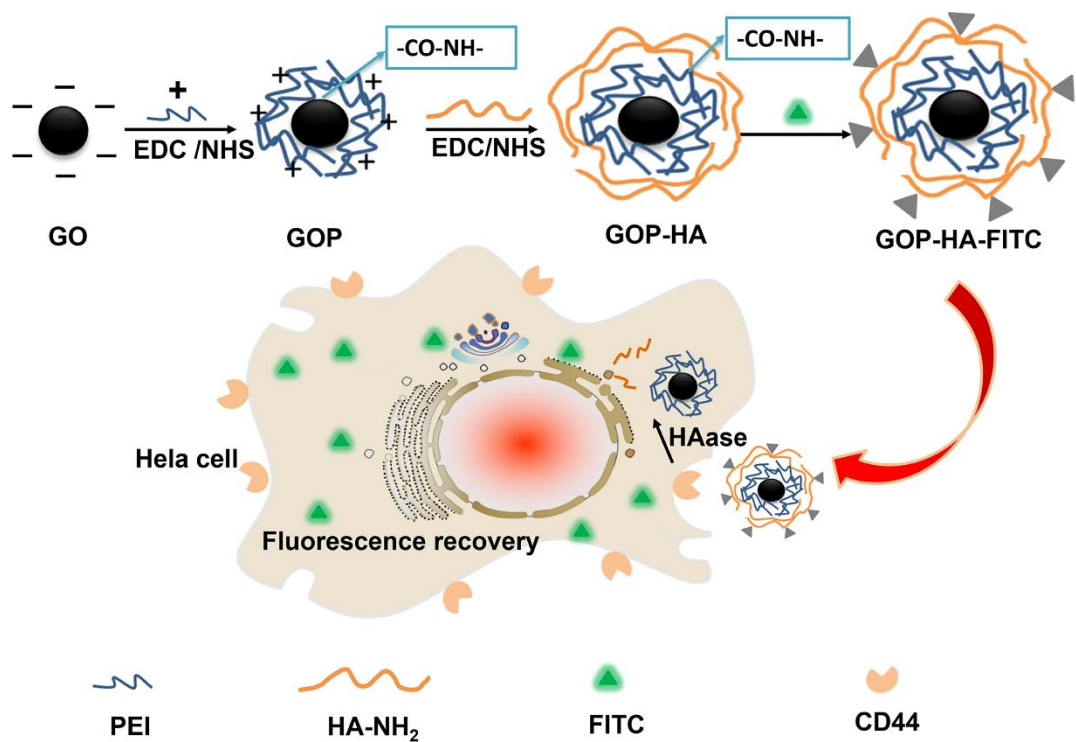
106 of the AuMSS nanorods was achieved through the chemical linkage of PEI followed by electro-
107 static adsorption of HA.²⁵

108 Fluorescein isothiocyanate (FITC) is a derivative of fluorescein widely used to label proteins by
109 reaction of isothiocyanate with amino groups. Hsu et al. synthesized FITC-labeled, HA-coated
110 nanogold (NP-FITC) to carry plasmid or siRNA into mesenchymal stem cells.²⁶ Cheng et al. pre-
111 pared FITC-labeled HA (FITC-HA) functionalized fluorescent gold nanoparticles (AuNPs) based
112 nanoprobe (FITC-HA-AuNPs) via chemical reduction of HAuCl₄ by using FITC-HA as both a
113 reducing and stabilizing agent.²⁷ The FITC-HA-AuNPs nanoprobe showed high detection sensi-
114 tivity and allowed for rapid hyaluronidase detection in complex biological specimens. Neverthe-
115 less, AuNPs are unstable in high salinity environment.²⁸

116 The aim of this work was to develop a new FITC labeled nanoprobe. PEI was attached to GO
117 through amide bonding, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hy-
118 droxysuccinimide (NHS) as coupling agents. Aminated HA was then linked to GO-PEI (GOP) via
119 the same route. Finally, FITC was attached to GOP-HA via reaction between the isothiocyanate
120 group of FITC and the amine groups of aminated HA. The successful synthesis of the fluorescent
121 probe was evidenced by UV-Vis-NIR spectroscopy, nuclear magnetic resonance (NMR), Fourier
122 transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), thermogravi-
123 metric analysis (TGA), and transmission electron microscopy (TEM). The sensitivity of the fluo-
124 rescent probe to hyaluronidase was determined by detecting the fluorescence characteristics in
125 phosphate buffered saline (PBS) and cancer cells. This probe presents three main advantages. First,
126 GOP-HA is obtained by covalent amide bonding. The preparation process is simple and the amide
127 bond is stable. Secondly, the detection of hyaluronidase has high sensitivity and selectivity. Third,

128 electrostatic interactions between negatively charged GO and HA with positively charged PEI con-
 129 tribute to stabilize a compact structure of GOP-HA. Scheme 1 illustrates the preparation of nano-
 130 probe and detection of hyaluronidase. FRET leads to fluorescence quenching in FITC labeled GOP-
 131 HA probe. Once entered the cancer cells, HA chains are cleaved into small fragments by hyaluron-
 132 idases. FITC is consequently released, thus restoring fluorescence which indicates detection of hy-
 133 aluronidase.

134



135

136

137 **Scheme 1.** Synthesis route of GOP-HA-FITC nanoprobe and schematic illustration of hyaluronidase detection.

138

139 **2. Materials and Methods**

140 **2.1 Materials**

141 GO was purchased from Xianfeng NANO Material Tech Co. HA (Mw = 10000) was purchased
142 from Xi'an Lande Biotechnology Co. PEI (Mw = 1800) and cysteine were purchased from McLean
143 reagent network. Ethylenediamine (EDA) monohydrate was purchased from Aladdin Reagent Net-
144 work. Hyaluronidase (from bovine testis) and protease K were purchased from Beijing Solarbio
145 Science & Technology Co. EDC and NHS were supplied by Shanghai Aladdin Biochemical Tech-
146 nology Co. Bovine serum albumin (BSA) and lysozyme were obtained from Roche Life Science
147 Products. Trypsin was obtained from Thermo Fisher Technology. Collagenase was supplied by
148 Sigma Aldrich. Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂),
149 calcium chloride (CaCl₂), and glucose and vitamin B1 were purchased from Sinopharm Chemical
150 Reagent Co. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (98% purity)
151 was purchased from Sigma–Aldrich Co., Ltd. (St. Louis, MO, USA).

152 **2.2 Synthesis of GO-PEI (GOP)**

153 PEI modified GO was prepared by condensation reaction between carboxyl groups of GO and
154 amine groups of PEI, as described in Scheme 2. 20 mg GO was dispersed in 20 mL distilled water,
155 and sonicated for 1 h to obtain a clear suspension. EDC (50 mg) and NHS (75 mg) were added
156 under stirring, and the mixture was stirred for 3 h at room temperature in the dark. 5 mL of 2% PEI
157 solution were then added dropwise under stirring, and the pH of the reaction system was adjusted
158 to 6 with 5% HCl. The reaction proceeded for 48 h at 30 °C under stirring, followed by 4 h ultra-
159 sonication. Afterwards, the solution was dialyzed against deionized water for 48 h. Finally, PEI
160 modified GO was obtained by centrifugation.

161 **2.3 Synthesis of GO-PEI-HA (GOP-HA)**

162 Aminated HA (HA-NH₂) was synthesized according to a previous report with slight modifica-
163 tions.²⁹ Briefly, HA (100 mg) was dissolved in 20 mL deionized water. 30 mg EDC and 18 mg
164 NHS were then added. After 30 min activation, 8.4 μL EDA was added, and the mixture was stirred
165 at room temperature for 24 h. Finally the mixture was dialyzed (SpectraPor, MWCO 3.5 kDa) for
166 48 h, and freeze-dried to yield aminated HA.

167 HA-NH₂ (10 mg) was dissolved in 10 mL deionized water. EDC (2.5 mg) and NHS (1.5 mg)
168 were then added. After 30 min activation, GOP (20 mg) was added, and the reaction proceeded
169 under stirring at room temperature for 24 h. The reaction mixture was then centrifuged to yield
170 GOP-HA (Scheme 2).

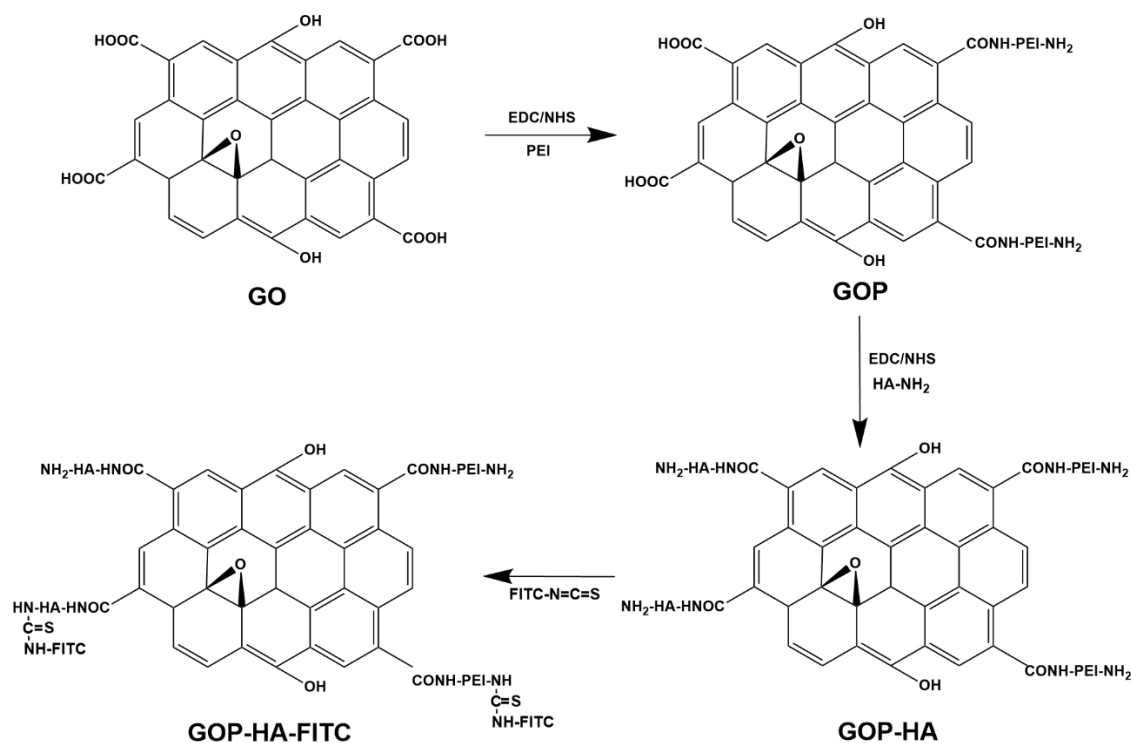
171 **2.4 Characterization**

172 FTIR spectroscopy was performed using a PerkinElmer Spectrum 100 spectrometer in the wave-
173 number range of 4000-400 cm⁻¹, with a resolution of 1 cm⁻¹. UV-Vis-NIR spectroscopy was carried
174 out with Shimadzu UV-2450 in the detection wavelength range of 200-800 nm. ¹H-NMR spectra
175 were registered on an AVANCE 500MHZ NMR spectrometer, using D₂O as solvent. TEM was
176 performed by using JEM-2100 electron microscope. One drop of 1 mg/mL GOP or GOP-HA sus-
177 pension was placed on a copper grid covered with nitrocellulose membrane, and air dried before
178 measurement. Zeta potential of samples in distilled water was measured by using Malvern Zetasizer
179 Nano ZS. XPS was performed using a Thermo ESCALAB 250 photoelectron spectrometer. Meas-
180 urements were made with a dual anode aluminum K α X-ray source (1486 eV, 300 watts). TGA
181 was carried out using NETZSCH STA 449F3 thermogravimetric analyzer. Measurements were
182 made in the temperature range from 50 to 800°C at a heating rate of 5 °C/min under air flow at 100
183 mL/min. Fluorescence intensity was measured by using an F-4600 FL spectrofluorometer in the

184 wavelength range of 400-800 nm. Cell imaging was achieved on an Olympus CKX41 fluorescence
185 microscope by the B excitation path, using a 50W mercury lamp, a DM500 beamsplitter, a BP460-
186 490C excitation filter and a BA520 IF absorption filter, respectively.

187 2.5 Preparation of GOP-HA-FITC

188 10 mg GOP-HA was dispersed in 2 mL distilled water. 0.1 mg FITC was then added. The reaction
189 proceeded for 24 h under magnetic stirring. The reaction mixture was then dialyzed against distilled
190 water for three days (MWCO = 3500) to remove unconnected fluorescent molecules. After dialysis,
191 the suspension was irradiated with ultraviolet lamp to ensure that there was no residual fluores-
192 cence, and finally lyophilized for 48 h in a freeze dryer to yield GOP-HA-FITC in the form of a
193 powder (Scheme 2).



194
195 **Scheme 2.** Synthesis route of GOP-HA-FITC synthesis in 3 steps: 1) synthesis of GO-PEI (GOP)
196 by attachment of PEI to GO via amide bonding, using EDC/NHS as coupling agents, 2) synthesis

197 of GOP-HA via amide bonding of aminated HA to GOP, 3) synthesis of GOP-HA-FITC via reac-
198 tion between the isothiocyanate group of FITC and the amine groups of aminated HA.

199

200 **2.6 Fluorescence quenching test**

201 FITC, GOP-HA-FITC (in duplicate), and mixture of GOP-HA-FITC with hyaluronidase were
202 separately dispersed in 2 mL pH 7.4 PBS. The concentration of FITC, GOP-HA-FITC and hyalu-
203 ronidase was 0.05 mg/mL, 0.05 mg/mL and 2.5 U/mL, respectively. One of the two GOP-HA-
204 FITC solutions and the mixture solution were incubated at 37 °C for 3 h. The fluorescence spectra
205 of the solutions were recorded by using fluorescence spectrophotometer under 490 nm excitation.

206 **2.7 Effect of enzyme concentration**

207 0.1 mg GOP-HA-FITC nanoprobe and various amounts of hyaluronidase were added to 2 mL
208 pH 7.4 PBS. The hyaluronidase concentration was 0, 0.05, 0.1, 0.25, 1, 2.5, 5, 7.5, 10, 12.5 and 15
209 U/mL, respectively. The solutions were incubated for 3 h at 37°C in the dark. The fluorescence
210 intensity of the various solutions was then recorded at an excitation wavelength of 490 nm.

211 **2.8 Effect of incubation time**

212 0.1 mg of GOP-HA-FITC nanoprobe was dispersed in 2 mL pH 7.4 PBS. Hyaluronidase is then
213 added at a concentration of 10.0 U/mL. The fluorescence intensity of the solutions was measured
214 after incubation at 37°C in the dark for 0, 30, 60, 90, 120, 180 min. Three parallel measurements
215 were made for each data point.

216 **2.9 Stability tests of GOP-HA-FITC nanoprobe**

217 A series of NaCl solutions at different concentrations (0, 10, 100, 500, 750, 1000 mM) were
218 prepared. Complete media for cell culture were prepared from DMEM and 1640 medium purchased

219 from Solebao Reagent Network with 10% fetal bovine serum. 0.1 mg nanoprobe was added into 2
220 mL of NaCl solutions or cell culture media, and the final probe concentration was 0.05 mg/mL.
221 After 3 h incubation in the dark with hyaluronidase (2.5 U/mL), the fluorescence of the various
222 solutions was measured by using a fluorescence spectrophotometer under 490 nm excitation.

223 0.1 mg of fluorescent probe was added into 2 mL of PBS solutions of different pH values (3, 4,
224 5, 6, 7, 8, 9). Hyaluronidase (2.5 U/mL) was then added. After 3 h incubation at 37°C in the dark,
225 the fluorescence of the solutions was determined.

226 0.1 mg of GOP-HA-FITC nanoprobe was suspended in 2 mL of pH 7.4 PBS. The solutions were
227 exposed to UV light for 30, 60, 90, 120, 150, and 180 min. Hyaluronidase (2.5 U/mL) was then
228 added. The various solutions were incubated in the dark at 37°C for 3 h, and the fluorescence was
229 measured.

230 All stability measurements were made in triplicate.

231 **2.10 Selectivity evaluation**

232 Different competing species were used to evaluate the selectivity of the nanoprobe, including
233 thrombin, bovine serum albumin, KCl, sodium chloride, lysozyme, cysteine, alkaline phosphatase,
234 glucose, cytochrome c, trypsin and glutathione. 0.1 mg of GOP-HA-FITC nanoprobe was added in
235 2 mL solution of the competitor species. The concentration of both the nanoprobe and competing
236 species was 0.05 mg/mL. The mixture was incubated in a shaker for 3 h in the dark. Then the
237 fluorescence intensity was recorded. Triplicate measurements were made for all competing species.

238 **2.11 MTT, hemolysis and coagulation assays**

239 L929 mouse fibroblasts were diluted to a concentration of 1×10^5 cells/mL with DMEM (10%
240 calf serum, 100 µg/mL Penicillin, 100 µg/mL streptomycin). 100 µL cell suspension was added to

241 each well of 96-well plates. The cells were cultured in a CO₂ incubator for 24 h to allow cell adhe-
242 sion. The nanoprobe was sterilized by exposure to UV light for 30 min and then dispersed in
243 DMEM. The solution was then removed and 100 μL of fresh medium containing GOP-HA-FITC
244 at various concentrations (10, 30, 50, 70, 100 μg/mL) were added to each well. Cells in pure me-
245 dium and in 0.5% phenol solution were used as negative and positive controls, respectively. After
246 24 and 72 h incubation, the solution was removed, and 100 μL medium and 20 μL MTT solution
247 (5 mg/mL) were added to each well. The solution was removed after 4 h incubation, and 150 μL
248 dimethyl sulfoxide (DMSO) was added. After 5 min homogenization, the OD value was measured
249 at 490 nm by using microplate reader (Elx800; BioTek, USA). All experiments were performed in
250 triplicate. The cell viability was calculated according to the following formula:

$$251 \quad \text{Cell viability (\%)} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{negative}}} \times 100\% \quad (\text{eq. 1})$$

252 Whole blood was obtained from four week old new Zealand rabbit by phlebotomy, and collected
253 in an anticoagulant tube containing EDTA. After homogenization, the mixture was centrifuged at
254 3000 rpm for 15 min to obtain blood cells. 20 μL of blood cells were separately mixed with 1 mL
255 of nanoprobe solution at different concentrations (25, 50, and 100 μg/mL). Physiological saline and
256 distilled water were used as negative and positive controls, respectively. The mixtures were incu-
257 bated at 37°C for 4 h, and then centrifuged at 3000 rpm for 15 min. The supernatant was transferred
258 into a 96-well plate, and the absorbance was measured at 540 nm with a microplate reader. Three
259 parallel measurements were made for each group. The hemolysis rate was calculated according to
260 the following formula:

$$261 \quad \text{Hemolysis rate (\%)} = \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{negative}}}{\text{OD}_{\text{positive}} - \text{OD}_{\text{negative}}} \times 100\% \quad (\text{eq. 2})$$

262 Whole blood was obtained from four week old new zealand rabbit by phlebotomy. It was col-
263 lected in an anticoagulant tube containing EDTA. At the same time, 0.2 M CaCl₂ solution was
264 prepared and sterilized at high temperature. Lyophilized nanoprobe was added in a CaCl₂ solution
265 to prepare suspensions of different concentrations (25, 50, 100µg/mL), and 25 µl of nanoprobe
266 suspension were pipetted into a siliconized glass tube. Siliconized glass tube was selected as the
267 negative control, and unsilanized glass tube as the positive control. After 5 min incubation at 37°C,
268 0.2 mL of fresh anticoagulant were added to each test sample and homogenized. At predetermined
269 time points, 20 mL of distilled water were added to the test tube, and the OD value of the superna-
270 tant was measured at 540 nm on a microplate reader. The relative clotting time of each sample was
271 determined from time *vs.* OD plots. Three parallel experiments were performed for each sample.

272

273 **2.12 Fluorescent nanoprobe in cell imaging**

274 HeLa cells were cultured as a model cell line to test the cancer targeting ability of the nanoprobe.
275 L929 cells were cultured under the same conditions as control. Cells were cultured in DMEM sup-
276 plemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 µg/mL penicillin
277 at 37°C in an incubator containing 5% CO₂. The nanoprobe was sterilized by exposure to UV light
278 for 30 min and then dispersed in DMEM. HeLa and L929 cells were cultured for 24 h to allow cell
279 adhesion. HeLa cells were then treated by culture with 1 mL HA solution (0.05 mg/mL) in carbon
280 dioxide incubator for 2 h. Afterwards, the nanoprobe suspension in DMEM (0.05 mg/mL) was co-
281 cultured with pretreated HeLa cells, normal HeLa cells and L929 cells for 6 h. The cells were
282 washed 3 times using PBS (10 mM, pH 7.4). Fluorescence images of the nanoprobe were per-
283 formed using a fluorescence microscope after excitation by a green excitation source.

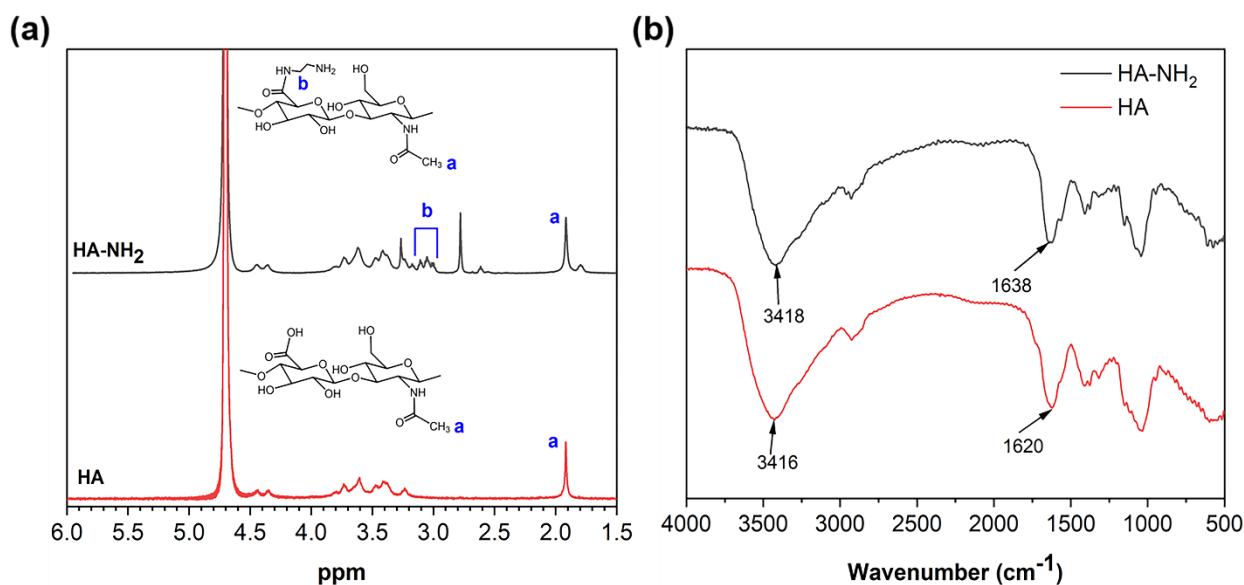
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285 3. Results and Discussion

286 3.1 Material characterization

287 3.1.1 Characterization of HA-NH₂

288 The structure of HA and HA-NH₂ was characterized by NMR spectroscopy. As shown in Fig.
289 1a, the peak at 1.9 ppm corresponds to N-acetyl group of HA. New peaks belonging to the meth-
290 ylene group of EDA are detected at 3.0-3.2 ppm in the spectrum of aminated HA, indicating the
291 successful modification of HA by EDA.³⁰ In contrast, the peaks appearing in the range of 2.7-3.0
292 ppm are those of ethylenediamine. Based on the peak areas at 3.0-3.2 and 1.9 ppm, the degree of
293 functionalization in aminated HA was estimated to be 66%.



294

295 **Figure 1.** (a) ¹H-NMR spectra of HA and HA-NH₂ in D₂O, and (b) FT-IR spectra of HA and HA-
296 NH₂.

297

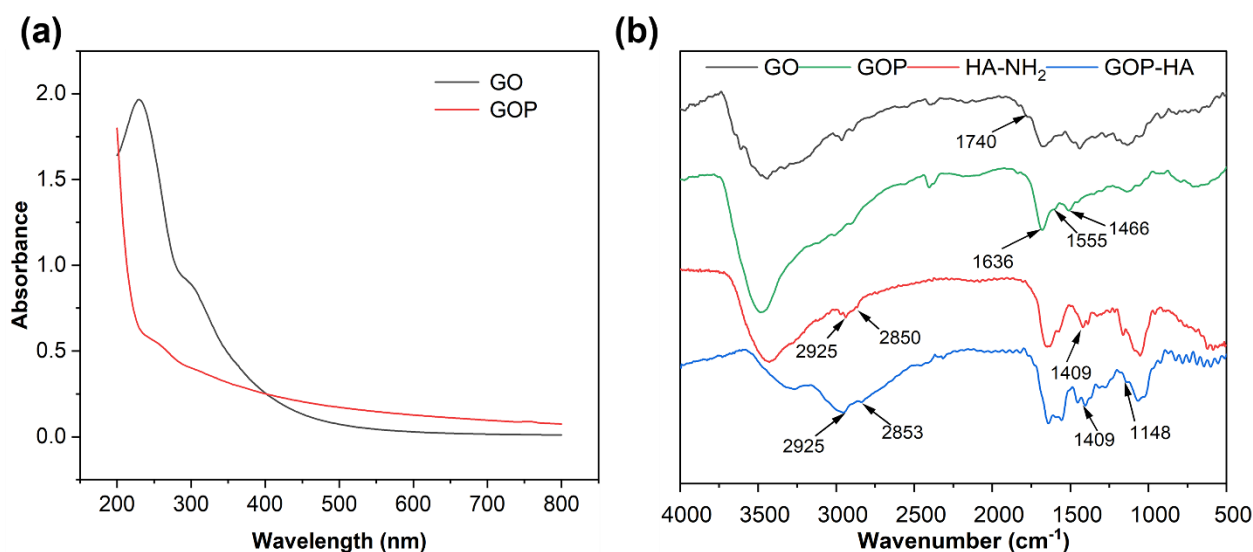
298 The FTIR spectra of HA and HA-NH₂ are shown in Fig. 1b. HA is a linear polysaccharide with
299 a large number of OH and NH groups which are observed at 3416 cm⁻¹. Another characteristic peak

300 detected at 1620 cm^{-1} belongs to the asymmetric carbonyl stretching vibration. The spectrum of
301 HA-NH₂ is almost identical to that of HA. However, the characteristic absorption at 1620 cm^{-1} of
302 HA slightly shifts to 1638 cm^{-1} , suggesting the functionalization of HA by EDA.³¹

303 3.1.2 Characterization of GOP and GOP-HA

304 It is well known that oxygen containing groups such as hydroxyl, carboxyl and epoxy are present
305 on the surface and edge of GO,³² while PEI and HA contain large number of amine and carboxyl
306 groups,³³ respectively. GO-PEI (GOP) was synthesized by reaction of the carboxyl groups of GO
307 and amine groups of PEI using EDC/NHS as coupling agents. PEI with Mw of 1800 was use be-
308 cause of its lower toxicity as compared to high Mw PEI.²⁴ HA was then attached to GOP by reaction
309 of the carboxyl groups of HA and amine groups of PEI.

310 Fig. 2a presents the UV-Vis-NIR spectra of GO and GOP. GO exhibits characteristic absorption
311 peaks at 230 and 305 nm which are attributed to the transition of C=C bonds on aromatic ring and
312 C=O bond in carboxyl group, respectively. In contrast, the UV-Vis-NIR curve of GOP has a char-
313 acteristic absorption at 260 nm, indicating that the reaction between GO and PEI leads to a red shift
314 of absorption of C=C bonds.³⁴



315

316 **Figure 2.** (a) UV-Vis-NIR spectra of GO and GOP, and (b) FT-IR spectra of GO, HA-NH₂, GOP
317 and GOP-HA.

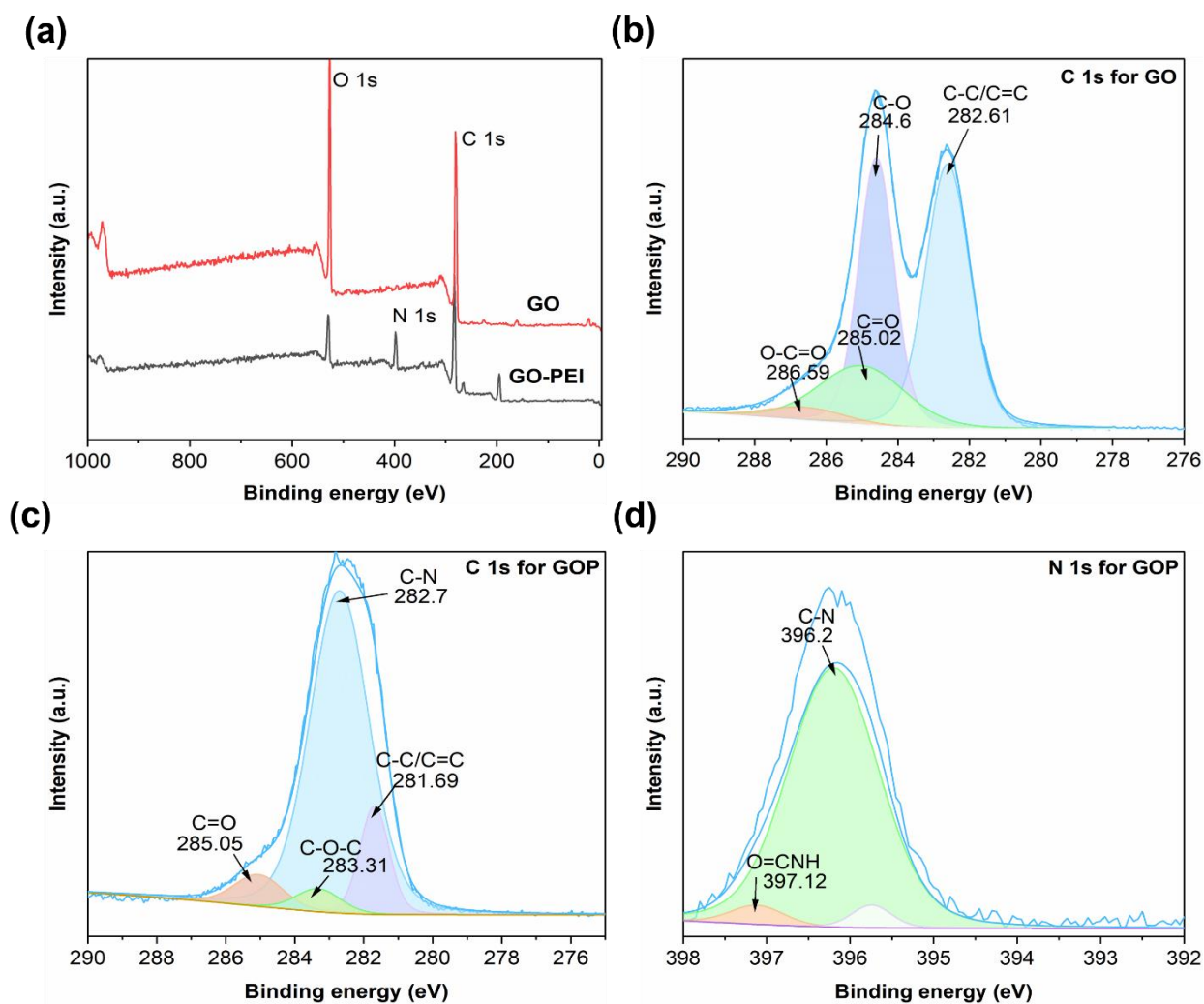
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319 Fig. 2b shows the FT-IR spectra of GO, GOP, HA-NH₂ and GOP-HA. GO surface is rich in
320 oxygen-containing functional groups, and stretching vibration bands of C-O, C-O-C, C-OH, C=O
321 and OH are observed at 1096, 1233, 1402, 1740, and 3450 cm⁻¹, respectively.³⁵ The absorption
322 peak at 1636 cm⁻¹ belongs to the bending vibration of the aromatic C=C bond. The small peak at
323 1740 cm⁻¹ disappears on the spectrum of GOP. At the same time, new absorption peaks appear at
324 1555 and 1466 cm⁻¹, corresponding to bending vibration of primary amine (-NH₂) and C-N stretch-
325 ing vibration, respectively. These findings suggest that GO has been successfully connected to PEI
326 via amide formation. GOP-HA has characteristic absorption peaks of HA-NH₂ at 1409, 2925 and
327 2850 cm⁻¹ belonging to C-O stretching vibration, antisymmetric absorption peaks of CH₃ and CH₂,
328 respectively.³⁶ Due to the formation of the amide group, the N-H in-plane bending vibration peak
329 of GOP at 1095 cm⁻¹ exhibits a red shift to 1148 cm⁻¹, indicating a reaction between GOP and HA.
330 Therefore, infrared spectroscopy confirmed the successful synthesis of GOP and GOP-HA.

331 The surface chemical composition of GO and GOP was further analyzed by XPS as shown in
332 Fig. 3. The main difference between the spectra of GO and GOP is the presence of N 1s peak (394-
333 398 eV) from GOP (Fig. 3a), suggesting the attachment of PEI on GO surface. In addition, the
334 oxygen peak intensity of GOP is lower than that of GO, in agreement with the partial reduction of
335 GO by PEI. The carbon and nitrogen elements in GO and GOP were further analyzed from high-
336 resolution C 1s and N 1s spectra. The C 1s spectrum of GO shows the characteristic peaks of C-C,
337 C-O, C=O and O-C=O at 282.61, 284.6, 285.02 and 286.59 eV (Fig. 3b), respectively, whereas the

338 C 1s spectrum of GOP shows a new peak of C-N at 282.7 eV (Fig. 3c). On the other hand, the N
 339 1s spectrum of GOP shows a peak at 397.12 eV attributed to the amide groups, and a peak at 396.2
 340 eV to the amine groups in PEI (Fig. 3d). Therefore, XPS results further confirmed the formation of
 341 amide bond between GO and PEI.³⁷⁻³⁹

342



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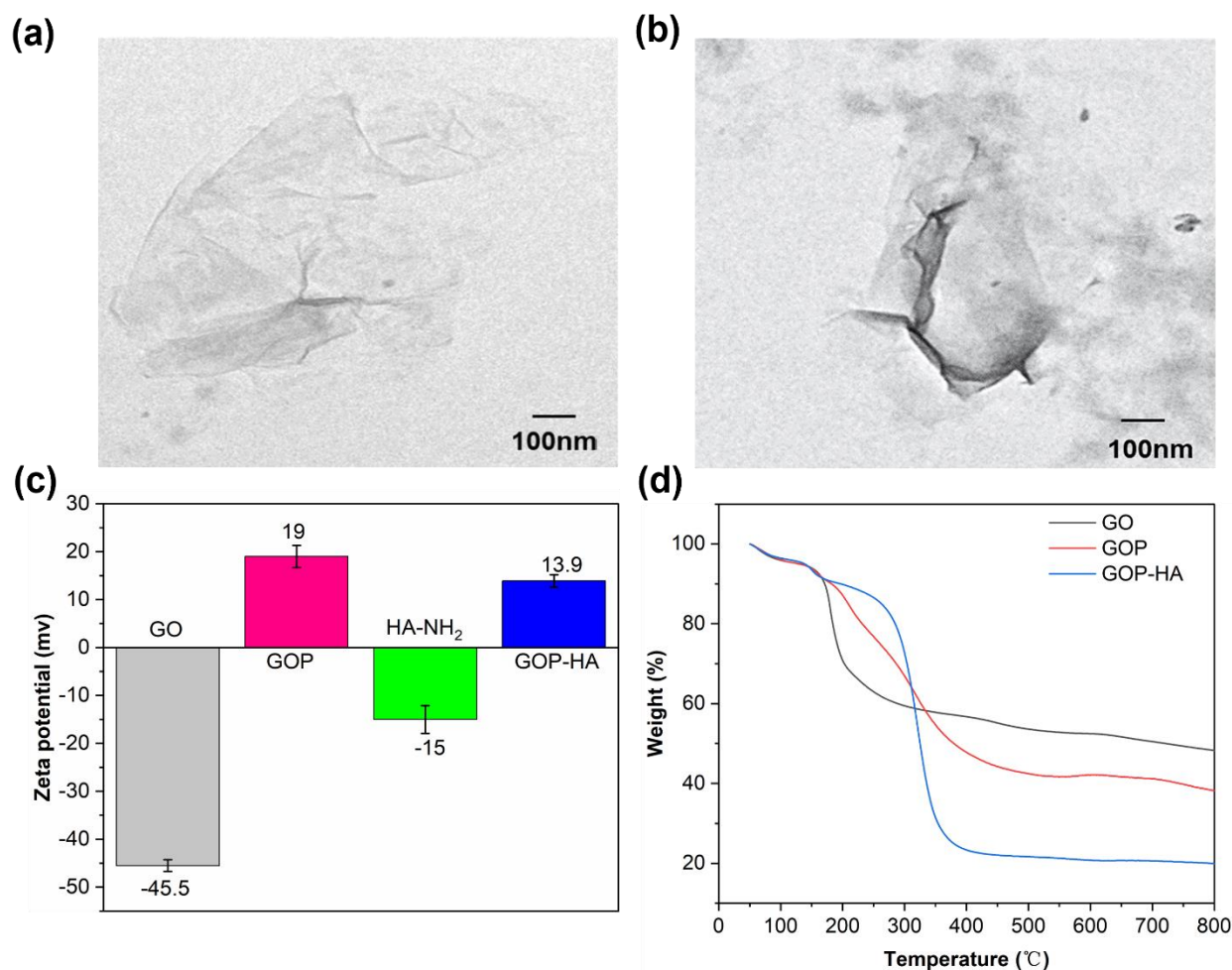
344 **Figure 3.** (a) XPS survey spectra of GO and GOP, (b) XPS spectrum of GO-C 1s, (c) XPS spectrum
 345 of GOP-C 1s, and (d) XPS spectrum of GOP-N 1s.

346

347 The morphology of GOP and GOP-HA was examined by TEM as shown in Fig. 4a and Fig. 4b.
348 Both GOP and GOP-HA exhibit a layered structure, which is characteristic of the structure of gra-
349 phene. The particle size is about 500 nm. No significant difference is observed between the surface
350 morphologies of GOP and GOP-HA.

351 The zeta potential of GO, GOP and GOP-HA was measured under neutral conditions, as shown
352 in Fig. 4c. The zeta potential of GO is -45.5 mV due to the presence of -COOH groups. In contrast,
353 the zeta potential of GO-PEI is 19.0 mV, in agreement with the attachment of positively charged
354 PEI ^{40,41}. After modification with HA-NH₂ whose zeta potential is -15 mV, the zeta potential of
355 GOP-HA slightly decreases to 13.9 mV because the reaction between the -COOH of HA and -NH₂
356 of GOP decreases the number of -NH₂ functional groups at the particle surface and that the presence
357 of HA shields part of the effect of -NH₂.

358



359

360 **Figure 4.** TEM images of (a) GOP and (b) GOP-HA; (c) Zeta potential of GO, GOP, HA-NH₂ and
 361 GOP-HA (n=3); (d) TGA thermograms of GO, GOP and GOP-HA.

362

363 Fig. 4d presents the TGA thermograms of GO, GO-PEI and GOP-HA. All three materials exhibit
 364 an initial weight loss of about 7% at 120 °C, which is attributed to the presence of water. The GO
 365 curve shows a second weight loss beyond 150°C, which reaches 45% at 450°C. This is caused by
 366 the cleavage of oxygen-containing groups. Compared with the GO curve, GOP exhibits a more
 367 progressive second weight loss phase from 150°C to 450°C, with a weight loss of about 55%. The
 368 weight loss of GOP is 10% higher than that of GO due to grafted PEI. The thermal stability of
 369 GOP-HA appears higher than that of GO and GOP as the second weight loss begins beyond 250°C,

370 which could be assigned to the higher thermal stability of PEI and HA as compared to GO. Weight
371 loss of GOP-HA reaches 80% at 450°C, which is 25% higher than that of GOP due to the decom-
372 position of PEI/HA grafted polymers.

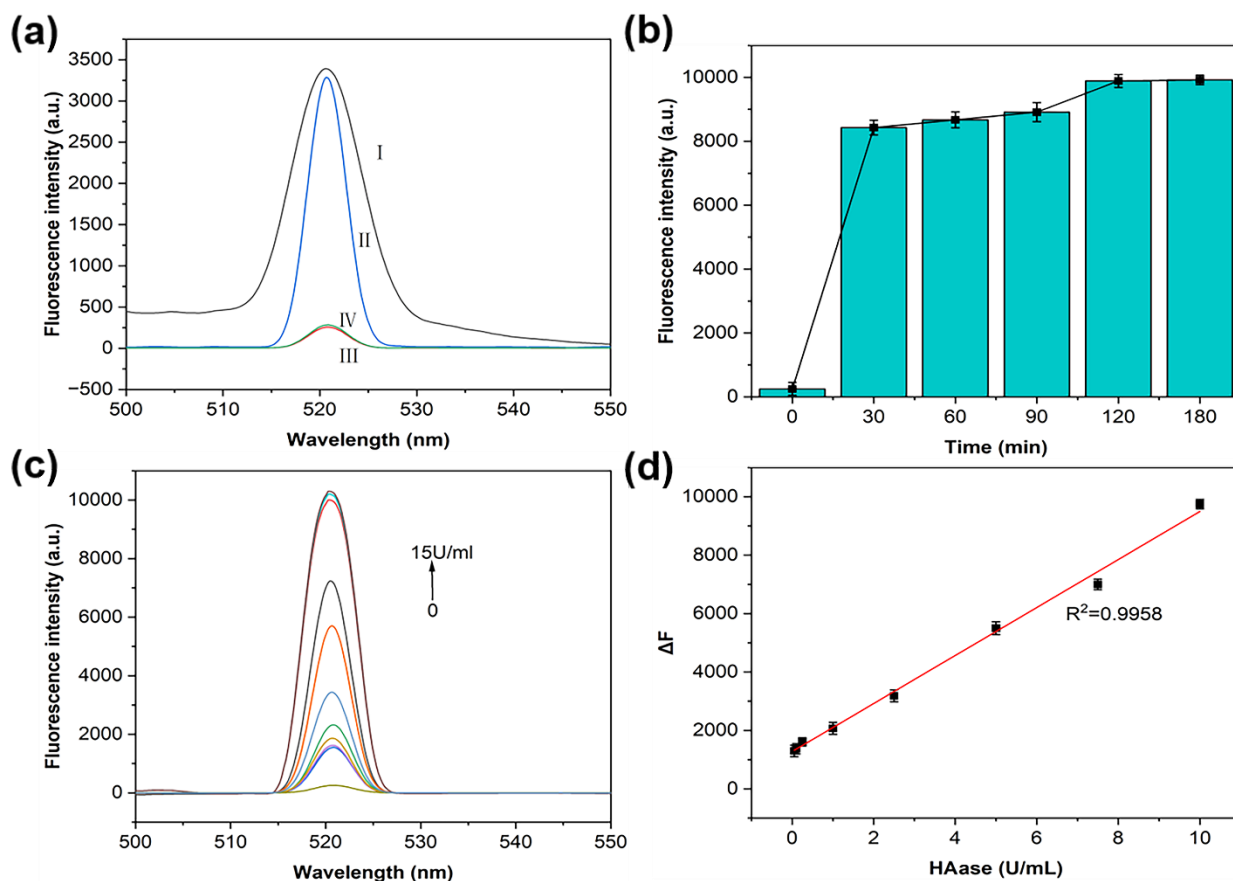
373 **3.2 Characterization of GOP-HA-FITC nanoprobe**

374 FITC is widely used to label proteins by reaction of its isothiocyanate group with amino termini
375 and primary amines in proteins ⁴². In this work, amino-functionalized HA was first synthesized,
376 HA and GOP were then linked, and finally the isothiocyanate group of FITC was used to react with
377 the amino group in HA to generate GOP-HA-FITC nanoprobe. The nanoprobe was characterized
378 from various aspects, including colloidal stability, effect of incubation time and enzyme concen-
379 tration, fluorescence quenching, and selectivity in the presence of potential interfering substances.

380 **3.2.1 Fluorescence quenching tests**

381 Fluorescence quenching of the probe was confirmed from fluorescence measurements. Fig. 5a
382 shows the fluorescence emission spectra of FITC, GOP-HA-FITC probe with hyaluronidase after
383 3 h incubation, GOP-HA-FITC probe, and GOP-HA-FITC after 3 h. FITC shows strong fluores-
384 cence emission at 520 nm. In contrast, GOP-HA-FITC nanoprobe showed very weak fluorescence
385 due to fluorescence quenching. After 3 h incubation with hyaluronidase, HA is cleaved into small
386 fragments by the enzyme, resulting in the release of FITC and the recovery of fluorescence. In
387 contrast, the fluorescence intensity of the GOP-HA-FITC nanoprobe remains almost the same after
388 3 h incubation without enzyme, indicating that the fluorescence of FITC was successfully quenched
389 in the probe.

390



391

392 **Figure 5.** (a) Fluorescence emission spectra ($\lambda_{ex} = 490$ nm): (I) FITC (0.05 mg/mL), (II) GOP-
 393 HA-FITC (0.05 mg/mL) and hyaluronidase (2.5 U/mL) after 3 h incubation, (III) GOP-HA-FITC
 394 (0.05 mg/mL), (IV) GOP-HA-FITC (0.05 mg/mL) after 3 h; (b) Fluorescence intensity detected by
 395 reaction of hyaluronidase (10 U/mL) and GOP-HA-FITC probe (0.05 mg/mL) at different times;
 396 (c) Fluorescence emission spectra ($\lambda_{ex} = 490$ nm) of nanoprobe after 3 h incubation with hyalu-
 397 ronidase at various concentrations (0, 0.05, 0.1, 0.25, 1, 2.5, 5, 7.5, 10, 12.5 and 15 U / mL from
 398 bottom to top); (d) ΔF vs. HAase concentration plot in the 0.05 to 10 U/mL range (ΔF is the differ-
 399 ence of fluorescence intensity in the presence and absence of nanoprobe). Data are expressed as
 400 the mean \pm standard deviation of three measurements.

401

402 3.2.2 Effect of incubation time

403 The GOP-HA-FITC probe was incubated in the presence of hyaluronidase for various times up
404 to 180 min in order to elucidate the effect of incubation time on the fluorescence intensity. As
405 shown in Fig. 5b, the fluorescence intensity strongly increases from 260 initially to 8500 at 30 min.
406 Thereafter, the fluorescence intensity slightly increases to 8600 at 90 min, and to 10000 at 120 min.
407 The intensity at 180 min is almost the same as that at 120 min, indicating that a maximum of
408 fluorescence intensity is reached beyond 120 min. An incubation time of 180 min was used for
409 further analyses.

410 3.2.3 Effect of hyaluronidase concentration

411 GOP-HA-FITC nanoprobe (0.05 mg/mL) was incubated with hyaluronidase at different concen-
412 trations in the dark for 3 h to evaluate its sensitivity. As shown in Fig. 5c, the fluorescence intensity
413 measured at the emission wavelength of 520 nm gradually increases with increasing enzyme con-
414 centration up to 10 U/mL, indicating that the nanoprobe has a good sensitivity in this concentration
415 range. However, beyond 10 U/mL, the fluorescence intensity remains almost unchanged. As de-
416 scribed above, FITC is released after hydrolysis of HA chains by hyaluronidase, resulting in fluo-
417 rescence recovery. These findings indicate that the fluorescence intensity reaches a maximum at
418 an enzyme concentration of 10 U/mL as all FITC molecules are released.

419 Fig. 5d shows the ΔF ($\Delta F = F - F_0$) vs. HAase concentration plot in the range of 0.05 to 10 U/mL.
420 F and F_0 are the fluorescence intensities in the presence and absence of HAase, respectively. A
421 good linearity is obtained for the plot (regression coefficient $R^2 = 0.99452$). The limit of detection
422 (LOD) was estimated to be 0.04 U/mL, which was calculated based on the signal-to-noise ratio
423 $S/N = 3$. Table 1 summarizes comparatively the performances (LOD, linear range, response time)

424 of GOP-HA-FITC and other fluorescent probes for hyaluronidase detection. The overall perfor-
 425 mance of GOP-HA-FITC is comparable to, or even higher than that some of reported HAase de-
 426 tection strategies, thus showing its potential in bioanalysis and intracellular bioimaging assay.

427 **Table 1.** Comparison of the performances between GOP-HA-FITC nanoprobe and other fluores-
 428 cent probes for hyaluronidase detection

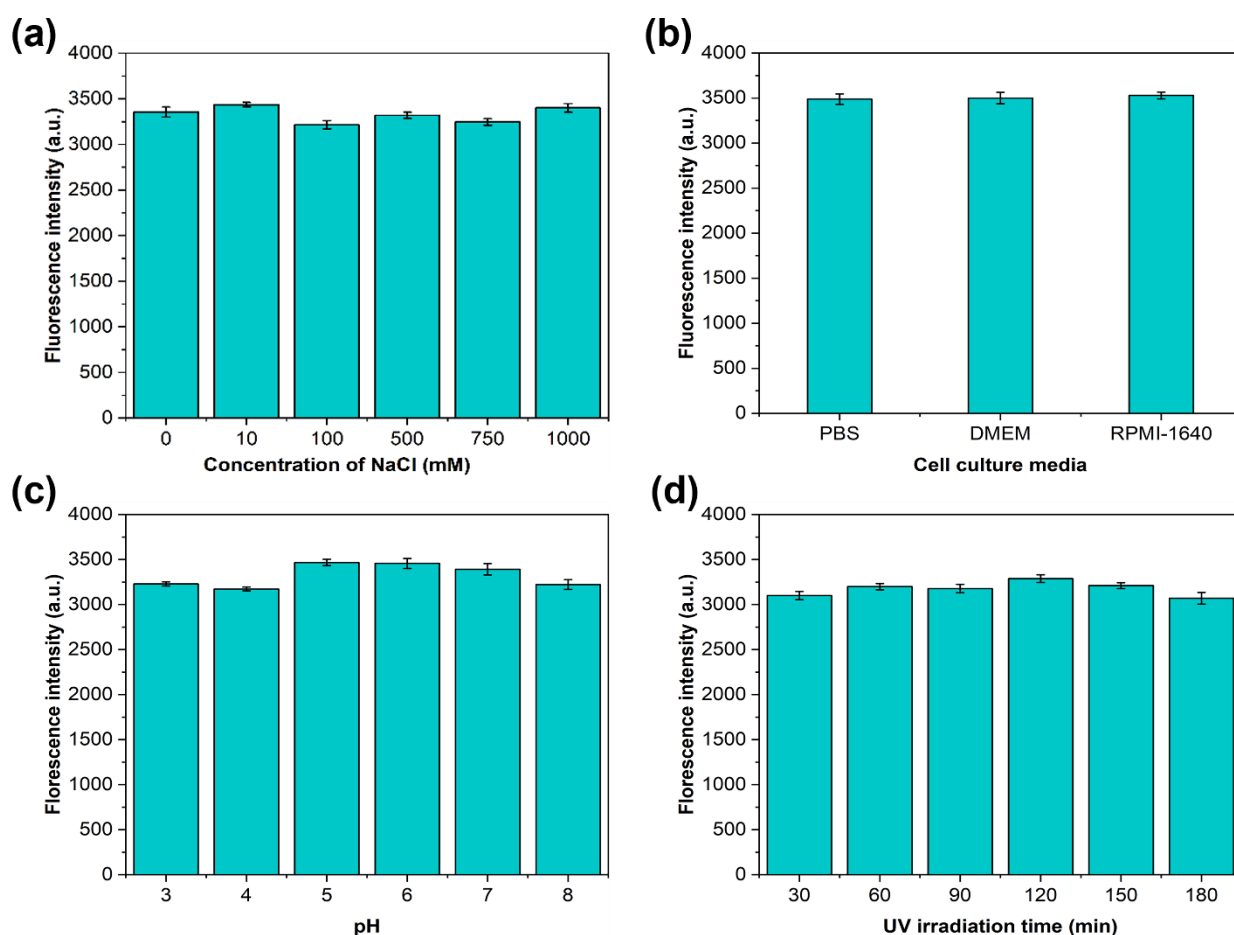
Fluorescent probe	Linear range (U/mL)	Response time (min)	Limit of detection (U/mL)	Reference
(TPE-2N, AN-N)-HA	0.005-10	100	0.0017	43
P-CD/HA-Dox	0-400	40	0.65	16
HA-UCNPs-PMPD	0-300	50	0.6	15
MoS ₂ QDs-(HA-AuNPs)	1-50	-	0.7	13
SiNPs-(HA-AuNPs)	0.01-10	45	0.004	14
FITC-HA-AuNPs	1.25-50	180	0.625	27
HA-ADH-FITC/HA-CHO@AuNPs	0.5-100	-	0.14	44
CQD/Naphthalimide/HA	0.1-16	80	0.09	45
Polycation1-HA/ Polycation1-QDS	0-0.1	40	0.01	46
N-Py-HA	0-3	90	0.007	47
GOP-HA-FITC	0.05-10	30	0.04	This work

429

430 3.2.4 GOP-HA-FITC stability tests

431 The colloidal stability of GOP-HA-FITC nanoprobe is of key importance as it is intended for
 432 intravenous applications. Stability tests were made by fluorescence intensity measurements of na-
 433 noprobe solutions (0.05 mg/mL) after 3 h incubation with hyaluronidase under different conditions,
 434 i.e. presence of salts, culture media, pH and UV light irradiation time. As shown in Fig. 6a, after 3
 435 h incubation with hyaluronidase, the fluorescence intensity of GOP-HA-FITC probe is almost the
 436 same in the presence of NaCl at different concentrations up to 1000 mM. Similar fluorescence
 437 intensity values were also found for different complete media, i.e., PBS, DMEM, and RPMI-1640

438 medium (Fig. 6b). The effect of pH was considered using PBS solutions in the pH range from pH
439 3 to 8. The fluorescence intensity of GOP-HA-FITC probe in PBS of different pH has little differ-
440 ence (Fig. 6c), indicating that the effect of pH on the probe is very small in the studied pH range.
441 Finally Fig. 6d shows that UV irradiation for different times up to 180 min has little effect on the
442 nanoprobe. Therefore, data shown in Fig. 6 well illustrate the colloidal stability of the nanoprobe.
443 The addition of the enzyme can produce approximately the same intensity of fluorescence under
444 different concentrations of salt solutions, different media, different pH and different UV light irra-
445 diation times.



446

447 **Figure 6.** Fluorescence intensity of GOP-HA-FITC probe (0.05 mg/mL) after 3 h incubation with
448 hyaluronidase (2.5 U/mL): (a) NaCl solutions at different concentrations, (b) different complete

449 culture media, (c) PBS solutions with different pH values, (d) UV light irradiation for different
450 times. Data are expressed as the mean \pm standard deviation of three measurements.

451

452 3.2.5 Selectivity of the nanoprobe

453 The nanoprobe (0.05 mg/mL) was incubated with various potential interfering substances at 37
454 °C for 3 h, including inorganic salts (NaCl, KCl, MgCl₂, CaCl₂), cysteine, glucose, vitamin B1,
455 BSA and some enzymes (trypsin, collagenase, lysozyme, protease K) in order to demonstrate the
456 selectivity of nanoprobe for hyaluronidase. As shown in Table 2, the fluorescence intensity of the
457 nanoprobe is 3118.1 after incubation with hyaluronidase, which is much higher than the values
458 obtained for potential interfering substances. These findings suggest that the nanoprobe has a good
459 selectivity for hyaluronidase which is capable of cleaving the HA chain of the substrate.

460 **Table 2.** Comparison of the fluorescence intensity GOP-HA-FITC probe after
461 incubation with hyaluronidase and potential interfering substances

Interfering substances	Amount added	Fluorescence intensity *
None	0	152.2 \pm 7.1
NaCl	10 mM	99.1 \pm 5.7
KCl	10 mM	93.2 \pm 6.6
MgCl ₂	10 mM	167.6 \pm 16.3
CaCl ₂	10 mM	122.8 \pm 15.0
Cysteine	30 μ M	107.3 \pm 11.6
Glucose	30 μ M	110.2 \pm 11.7
Vitamin B1	10 mM	130.8 \pm 2.9
BSA	10 mM	203.6 \pm 3.8
Trypsin	2.5 U/mL	188.8 \pm 78.2
Collagenase	2.5 U/mL	173.2 \pm 6.8
Lysozyme	2.5 U/mL	149.6 \pm 12.9
Protease K	2.5 U/mL	168.6 \pm 25.6
HAase	2.5 U/mL	3118.1 \pm 11.8

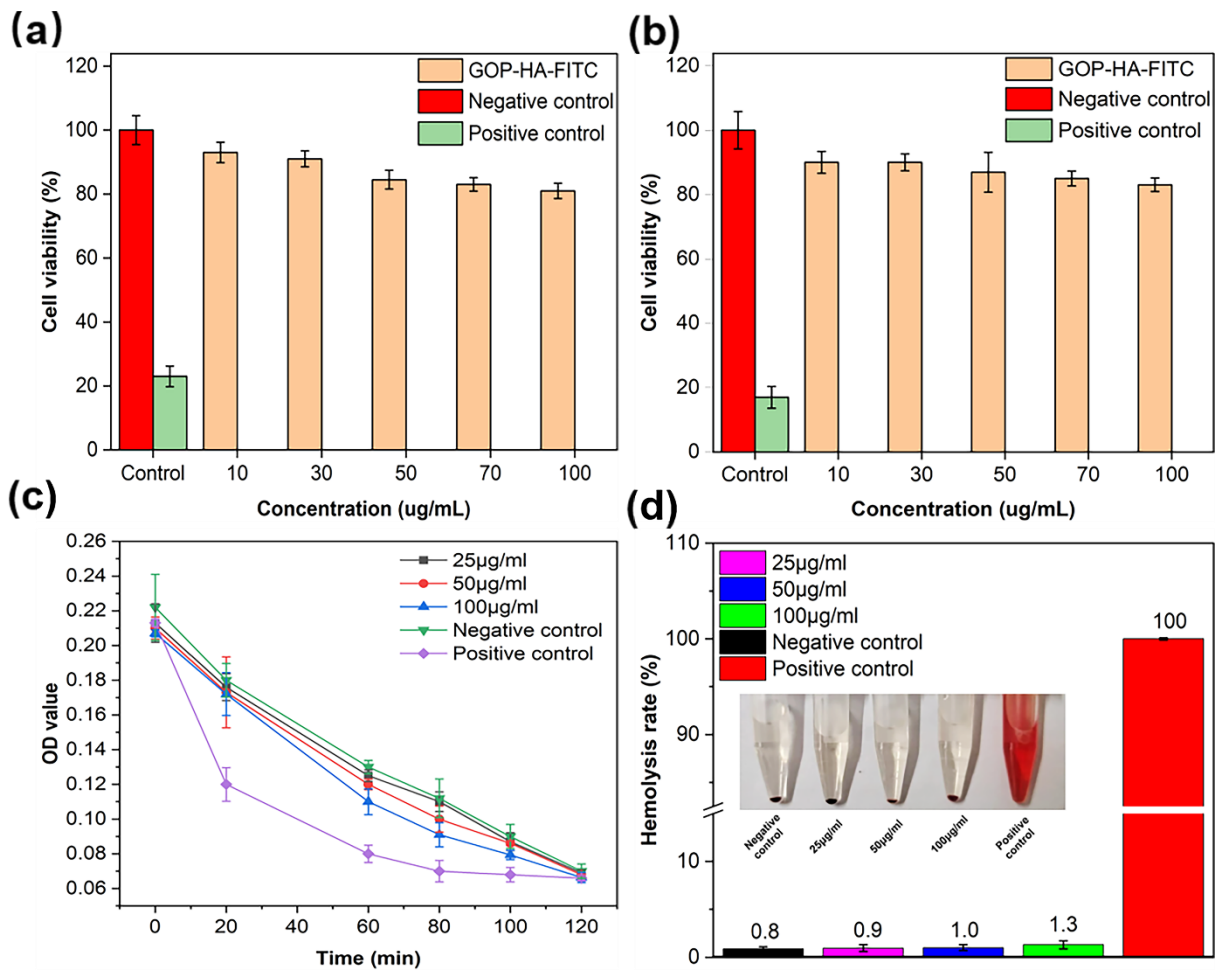
* Data are expressed as the mean \pm standard deviation of three measurements.

462

463

3.3 Cytocompatibility and haemocompatibility

465 MTT assay was used to assess the cytotoxicity of GOP-HA-FITC nanoprobe using L929 cell
466 line. The cell viability was evaluated from the ratio of cells incubated with GOP-HA-FITC nano-
467 probe to those with the negative control. As shown in Fig. 7a-b, GOP-HA-FITC nanoprobe exhibits
468 little cytotoxicity. In the concentration range of 10 to 100 $\mu\text{g}/\text{mL}$ up to 72 h incubation, the cell
469 viability is higher than 83%. These findings show that the nanoprobe presents good cytocompati-
470 bility, and thus has great potential in biological imaging applications under physiological condi-
471 tions.



472

473 **Figure 7.** Cell viability of L929 cells after co-culture with GOP-HA-FITC probes at different con-
474 centrations after (a) 24 h, and (b) 72 h; (c) absorbance changes of the probe at different concentra-
475 tions in comparison with the controls; (d) hemolysis rates (HR) of the probe at different concentra-
476 tions in comparison with the controls. Data are expressed as the mean \pm standard deviation of three
477 measurements.

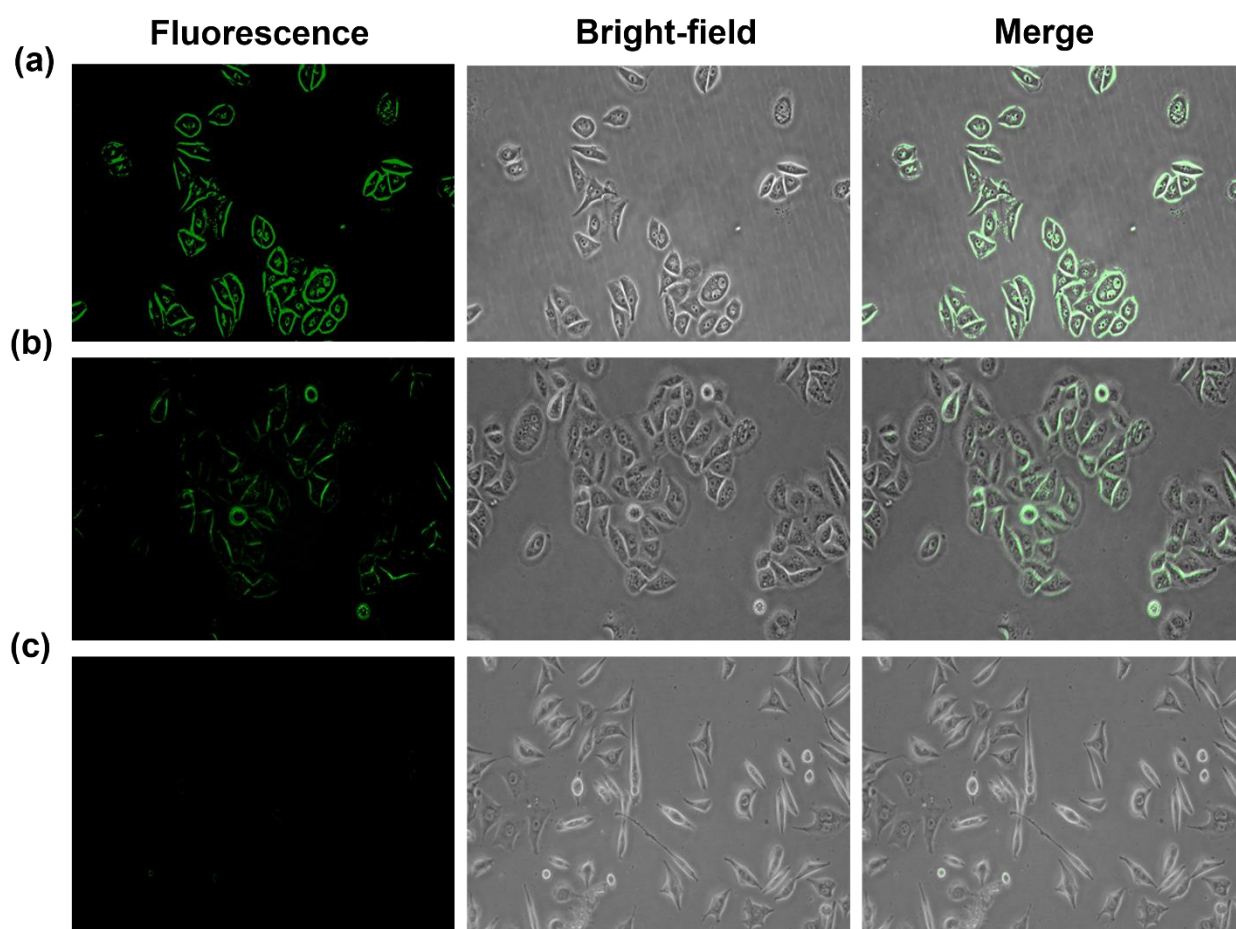
478

479 The haemo-compatibility of the GOP-HA-FITC probe was of key importance as the probe is
480 intended to be applied by intravenous injection. Dynamic coagulation and hemolysis experiments
481 were performed using rabbit blood. Fig. 7c shows the blood absorbance changes of the probe at
482 different concentrations in comparison with the controls. The absorbance of the positive control
483 decreases rapidly within the first 20 min, followed by slower decrease up to 120 min. The absorb-
484 ance values at 60 to 120 min are almost the same, i.e. the total blood clotting time is 60 min. The
485 absorbance changes of the probe are similar to those of the negative result. The absorbance de-
486 creases slowly, and reaches coagulation at 120 min. The above results show that the probe does not
487 cause blood coagulation, indicating that it could be used as a biomaterial. ⁴⁸

488 Fig. 7d shows the hemolysis rates (HR) of the probe at different concentrations in comparison
489 with the negative control. The HR values are in the range of 0.9 to 1.3. It is commonly admitted
490 that the hemolytic property of biomaterials is acceptable for medical applications if the HR value
491 is below 5%. Therefore, the HR values of the probe are all well below 5%, in agreement with the
492 excellent anti-hemolysis properties.

493 **3.4 Cell imaging**

494 CD44 is a type I transmembrane glycoprotein overexpressed on the cytoplasmic membrane of a
495 variety of tumors.⁴⁹ HA is known for its high affinity to tumor cells overexpressing CD44 receptor.
496⁵⁰ HeLa cells were used as a model cell line to examine the cancer targeting ability of GOP-HA-
497 FITC nanoprobe. HeLa cells were first treated with a HA solution. Reaction of HA with CD44
498 receptor on the surface of HeLa cells allowed subsequent comparison with HeLa cells that were
499 not pretreated with HA.



500
501 **Figure 8.** (a) Fluorescence images of HeLa cells after incubation with GOP-HA-FITC probe, (b)
502 fluorescence images of HeLa cells pretreated with HA after incubation with GOP-HA-FITC nano-
503 probe, (c) fluorescence images of L929 cells after incubation with GOP-HA-FITC probe. The first
504 column represents the fluorescence image of cells, the second column represents the image of cells
505 under the bright field of view, and the third column represents the combined image of both.

506 As shown in Fig. 8, the nanoprobe can target cancer cells thanks to the specific affinity of HA
507 for CD44 receptor. After incubation with GOP-HA-FITC nanoprobe, HeLa cells clearly showed
508 significant green fluorescence under fluorescence microscope due to the fluorescence emitted by
509 FITC (Fig. 8a). Green fluorescence is clearly visible in the cell membrane and nucleus, indicating
510 that the nanoprobe was internalized into HeLa cells and FITC was released after HA chain cleavage
511 by hyaluronidase. In fact, two homologous hyaluronidase isoforms, namely hyaluronidase-1 (Hyal-
512 1) and hyaluronidase-2 (Hyal-2), are predominately involved in the cellular HA catabolism in the
513 human body.⁵¹ Hyal-2 is mainly located on the external surface of the cell membrane, whereas
514 Hyal-1 is identified as the major tumor-derived hyaluronidase expressed in some cancer tissues.
515 Thus this finding should not affect the sensitivity and selectivity of the nanoprobe as both Hyal-1
516 and Hyal-2 can be detected. On the other hand, the fluorescence of HeLa cells pretreated with HA
517 is weaker (Fig. 8b), suggesting that pretreatment of HeLa cells with HA reduced the nanoprobe's
518 targeting efficiency because of less CD44 receptors and hyaluronidase remaining on the cell sur-
519 face. In L929 cells, there is only very weak fluorescence on the outer membrane, but there is no
520 fluorescence inside (Fig. 8c), indicating that the nanoprobe has no effect on normal cells. These
521 results suggest that GOP-HA-FITC nanoprobe can selectively bind to CD44 receptor and be inter-
522 nalized into cancer cells through receptor targeted endocytosis. Therefore, GOP-HA-FITC nano-
523 probe could present great potential for uses in targeted tumor cell imaging.

524 **4. Conclusion**

525 A novel fluorescence-quenched GOP-HA-FITC nanoprobe was successfully synthesized by suc-
526 cessive attachment of PEI, HA and FITC to GO nanoparticles via covalent linkage. The nanoprobe
527 exhibits high sensitivity and selectivity, and can enter cancer cells through the specific binding of

528 HA to cancer cells. After hydrolytic cleavage of HA chains by the overexpressed hyaluronidase,
529 FITC is released from the probe and its fluorescence recovered, showing strong fluorescence in-
530 tensity in cancer cells. Moreover, the probe presents outstanding cyto- and haemo-compatibility.
531 Therefore, it is concluded that the developed GOP-HA-FITC nanoprobe can be used for tumor-
532 targeted cell imaging, and is thus promising for use in early tumor diagnosis.

533

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547 J. Song; formal analysis, S. Wang; investigation, P. Zhang; curation, P. Zhang; writing—original
548 draft preparation, P. Zhang; writing—review and editing, S. Li; funding acquisition, F. Su, S. Li.
549 All authors have read and agreed to the published version of the manuscript.

550 **Notes**

551 **The authors declare no competing financial interest.**

552 **Data Availability Statements**

553 The data of our findings are available from the corresponding author upon reasonable request.

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