1 **Red fluorescent chitosan nanoparticles grafted with**
2 **poly(2-methacryloyloxyethyl phosphorylcholine) for live**
3 **cell imaging**

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11

1 **Abstract**

2 Poly(2-methacryloyloxyethyl phosphorylcholine) conjugated red fluorescent chitosan
3 nanoparticles (GCC-pMPC) were facilely fabricated by “grafting from” method via
4 surface initiated atom transfer radical polymerization (ATRP). Firstly, glutaraldehyde
5 crosslinked red fluorescent chitosan nanoparticles (GCC NPs) with many amino groups
6 and hydroxyl groups on their surface were prepared, which were then reacted with
7 2-bromoisobutryl bromide to form GCC-Br; subsequently, poly(MPC) (pMPC) brushes
8 were grafted onto GCC NPs surface using GCC-Br as initiator via ATRP. Compared with
9 PEGylated nanoparticles, zwitterionic polymers modified nanoparticles demonstrated
10 better performance in their cellular uptake. Moreover, the obtained GCC-pMPC
11 demonstrated excellent water-dispersibility, biocompatibility, and photostability, which
12 made them highly potential for long-term tracing applications. Importantly, the successful
13 live cell imaging of GCC-pMPC would remarkably advance the research of their further
14 bioapplications.

15 **Keywords:** red fluorescent nanoparticles; poly(2-methacryloyloxyethyl
16 phosphorylcholine); ATRP; live cell imaging

17

1. Introduction

Compared with small organic fluorescent molecules, fluorescent nanoparticles as bioprobes for imaging and medical applications have attracted more booming attention owing to their nanometer size scales, which would not only help them passively accumulating into tumor tissues due to the enhanced permeation and retention (EPR) effect, but also can facilitate the further functionalization of these nanoparticles thanks to their large surface area to volume ratios.[1-3] Specially, the red fluorescent nanoparticles are outstanding among these nanoprobe with emissions of different colors, since they can avoid the interference from biological autofluorescence and have great potential for deep tracing of biological systems.[4-9] As a result, various kinds of red fluorescent nanoprobe have been fabricated increasingly, including fluorescent proteins, dye-doped silicon nanoparticles, semiconductor quantum dots, upconversion nanoparticles, and polymeric nanoparticles.[10-17] Nevertheless, the high cost and low photobleaching thresholds of fluorescent proteins, potential cytotoxicity of inorganic nanoparticles, together with the notorious aggregation-caused quenching (ACQ) problems of most dye-doped silicon nanoparticles and polymeric nanoparticles, all restrict their further applications. Therefore, fabrication of photostable and biocompatible nanoparticles with low cost and high luminescence was of great significance for the research of nanoprobe.[18-20] It should be noted that the development and introduction of aggregation induced emission (AIE) dyes into nanoparticles solved the ACQ problem and greatly improved their fluorescent properties, since then a variety of AIE-based nanoparticles were constructed for bioimaging and biomedical applications.[21-29] However, more red fluorescent nanoprobe with excellent biocompatibility, photostability and water dispersibility are still in highly demand.

Different from dye-doped nanoparticles, fluorescent organic nanoparticles can also be prepared directly from nonluminous materials, which seem to be promising candidates as bioprobes since most commercial red fluorescence dyes were very expensive.[30, 31] Fluorescent nanoparticles with deep red emission of 670 nm from chitosan and glutaraldehyde (glutaraldehyde

1 cross-linked chitosan nanoparticles, GCC NPs) were successfully fabricated in our group, which
2 revealed huge superiorities in their facile preparation strategy and outstanding anti-photobleaching
3 property.[32] Compared with other fluorescent chitosan nanoparticles, no fluorescent dyes or
4 quantum dots were introduced during the preparation of GCC NPs. [33, 34] These GCC NPs can
5 emit red fluorescence due to their newly-formed pyridinium structures, yet which also reduced
6 their biocompatibility and thus hindered their further bioapplications owing to the positive
7 charges.[35-37] In addition, the size of these GCC NPs was only about 5.6 nm, which was too
8 small that it was not the proper size for cellular uptake.[38, 39] In this case, surface modification
9 of GCC NPs would powerfully boost the further development and biomedical applications of this
10 new type of red fluorescent nanoparticles. PEGylation was a common method to realize the
11 function, since it can shield the charges and prolong their blood circulation time.[40]
12 Unfortunately, it was reported recently that PEGylation would interfere with the cellular uptake
13 and then impair their tumor retention.[41] Interestingly, some zwitterionic polymers modified
14 nanoparticles demonstrated much better performance in their cellular uptake.
15 Poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC) was well known of their
16 superhydrophilicity and excellent biocompatibility, which was also a kind of zwitterionic
17 polymers. Therefore, construction of pMPC brushes conjugated GCC NPs was expected to be able
18 to demonstrate great cell uptake and cell imaging performance, and would greatly advance the
19 research of this red fluorescent bioprobes.

20 Herein we presented the construction and live cell imaging performance of biocompatible
21 and photostable red fluorescent nanoparticles (GCC-pMPC) for the first time, which were
22 prepared by “grafting from” method through surface initiated atom transfer radical polymerization
23 (ATRP) of 2-methacryloyloxyethyl phosphorylcholine (MPC) (Scheme 1). Compared with
24 PEGylated nanoparticles, zwitterionic polymers modified nanoparticles demonstrated
25 better performance in their cellular uptake. Thanks to the great superhydrophilicity character
26 of MPC and deep red fluorescent feature of GCC NPs, the resulted GCC-pMPC revealed high
27 water-dispersibility, excellent biocompatibility, biofavorable red fluorescence, and outstanding
28 anti-photobleaching ability. Moreover, their superb live cell imaging performance was also
29 determined.

2. Experimental

2.1 Materials and Method

Chitosan (Mv of ca. 50 kDa and deacetylation degree of 95.37%) was obtained from Jinan Haidebei Marine Bioengineering Co., Ltd., Shandong, China. Glutaraldehyde, 2-bromoisobutyryl bromide (98%), N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA, 98%), and 2-methacryloyloxyethyl phosphorylcholine (MPC) were purchased from J&K Scientific Ltd. All other solvents and chemicals were purchased from commercial sources and used as received directly.

The products were characterized by FTIR spectra and X-ray photoelectron spectra (XPS), which were obtained on a Perkin-Elmer Spectrum 100 spectrometer and a VGESCALAB 220-IXL spectrometer, respectively. Their optical properties was determined through UV-Visible absorption spectra and fluorescence spectra, using a UV/Vis/NIR spectrometer (lambda750, Perkin-Elmer, USA) and a fluorophotometer (RF-5301PC, Shimadzu, Japan), respectively. The detailed conditions were the same with those described in our previous reports.[42, 43] Their morphology was measured by transmission electron microscopy (TEM) on a JEM-1200EX microscope operated, and the size distribution were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, USA).

2.2 Preparation of GCC NPs

The GCC NPs were prepared through water in oil microemulsion method based on our earlier report.[32] Typically, chitosan (2 wt%) and glutaraldehyde solutions were mixed together as aqueous phase (1 equiv) in an ice/water bath under stirring, then oil phase (4 equiv) of Triton X-100, cyclohexane and octanol was quickly added. After sonication, the mixture was kept still at room temperature for the further reaction of about 20 h. Then the microemulsion was destroyed, and the GCC NPs can be finally obtained after thorough dialysis and lyophilization.

2.3 Preparation of GCC-initiator (GCC-Br)

GCC NPs (500 mg), triethylamine (1.0 mL), and anhydrous toluene (9 mL) together with anhydrous DMSO (1 mL) were mixed together for sonication of about 20 min, then 2-bromoisobutyryl bromide (1.0 mL) dissolved in 5 mL of anhydrous toluene was added dropwise at 0 °C. After stirring of 24 h at room temperature, the solid product can be separated from the mixture by dialysis against menthol. GCC-Br was finally harvested through further drying at 40 °C overnight under vacuum.

2.4 Preparation of GCC-pMPC

As indicated in Scheme 1, GCC-pMPC was prepared by “grafting from” method through surface-initiated ATRP. Firstly GCC NPs were prepared through microemulsion method and then reacted with 2-bromoisobutyryl bromide to form the nanoparticles initiator (GCC-Br), which had been described above in detail; after that, pMPC chains were covalently linked on the GCC NPs surface via ATRP utilizing Cu(Br)/PMDETA as the catalyst/ligand, which was demonstrated as follows.

GCC-Br (50 mg) was dispersed in 5 mL of anhydrous toluene with 0.5 mL of anhydrous DMSO to improve their dispersibility under ultrasonication. Then, CuBr (10 mg), PMDETA (10 mg) and MPC (200 mg) were added into above GCC-Br solution, which was set in a 20 mL dry Schlenk flask and sealed with a rubber plug. After freeze-pump-thaw circle for three times under N₂ atmosphere, it was maintained in a 70 °C oil bath for polymerization of 20 h, which was then stopped by exposure to the air and cooling. The mixture was centrifuged and washed with deionized water containing 50 mM Na₂EDTA and methanol. The product was finally obtained after being dried at 40 °C in vacuum overnight.

2.5 Cytotoxicity of GCC-pMPC

The cell viability of GCC-pMPC on HeLa cells was evaluated by cell counting kit-8 (CCK-8) assay, which was similar with our previous reports. HeLa cells were cultured at 37 °C under 5% CO₂ in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin to keep their exponential

growth. During the test, HeLa cells were first seeded in 96-well microplates overnight in order to get attached on the plate. Then, GCC-pMPC in DMEM solution of different concentrations were added to each well of the plate for further incubation of 12 and 24 h, respectively. Subsequently, all the wells were carefully washed with PBS for three times to remove the uninternalized GCC-pMPC, and 10 μ L of CCK-8 dye together with 100 μ L of DMEM culture medium were added to each well. After another incubation of 2 h, the absorbance at 450 nm of each well was measured by a microplate reader (VictorIII, Perkin-Elmer).

2.6 Cellular Imaging of GCC-pMPC

In order to clearly locate GCC-pMPC in cells after the internalization, fixed cell imaging of GCC-pMPC was first conducted using Hoechst 33258 as a reference which would stain the cell nuclei. More specifically, GCC-pMPC of 20 μ g mL⁻¹ were incubated with HeLa cells at 37 °C under 5% CO₂ for a period of time. Subsequently, the cells were washed with PBS carefully and fixed with 4% paraformaldehyde for 20 min at room temperature. Then HeLa cells were washed twice again, and stained with Hoechst 33258 (5 μ g mL⁻¹) for about 10 min in the dark. After extensive washes and addition of fresh DMEM, the cells were imaged by a confocal laser scanning microscope (CLSM) Zeiss 710 3-channel (Zeiss, Germany). The laser of 405 nm was employed for the excitation of Hoechst 33258, using 440–490 nm bandpass filter to collect their fluorescence. The laser of 633 nm and 650–700 nm bandpass filter were used to obtain the fluorescence images of GCC-pMPC. After the identification of their location in cells, the live cell imaging performance of GCC-pMPC was determined by CLSM Zeiss 710 META (Zeiss, Germany). The detailed process was similar with fixed cell imaging except for the removal of fixation and Hoechst 33258 staining procedures.

2.7 Photostability of GCC-pMPC

The photostability of GCC-pMPC in live cells was determined by examining the fluorescence intensity changes of GCC-pMPC in HeLa cells under continuous laser irradiation. Firstly, GCC-pMPC of 20 μ g mL⁻¹ were incubated with HeLa cells at

1 37 °C under 5% CO₂ for 5 h, then the cells were fixed with 4% paraformaldehyde as
2 described before. After extensive washes and addition of fresh DMEM, the cells were
3 imaged by Zeiss 710 3-channel under continuous laser irradiation of 633 nm for 20
4 min. The laser intensity in the experiment was set as 10%, which was about 40 μW.
5 The fluorescence intensities and corresponding confocal images of HeLa cells were
6 recorded every two minutes.

7 **3. Results and discussion**

8 **3.1 Preparation of GCC-pMPC**

9 In order to fabricate pMPC functionalized GCC NPs, firstly GCC NPs should be
10 prepared from chitosan and glutaraldehyde by water in oil microemulsion method
11 following the procedure described in our earlier literature. Thanks to the hydroxy
12 groups and amino groups on the surface of GCC NPs, MPC could be facilely
13 polymerized on the surface of GCC NPs to obtain GCC-pMPC by “grafting from”
14 methods through surface-initiated ATRP as illustrated in Scheme 1. In detail, the
15 amino groups and hydroxyl groups on the surface of GCC NPs reacted with
16 2-bromoisobutryl bromide to form GCC-Br in anhydrous toluene mixed with
17 anhydrous DMSO solution to improve the dispersibility of GCC NPs. Then MPC
18 chains were conjugated onto the GCC NPs through surface initiated ATRP using
19 GCC-Br as initiator. The construction strategy of GCC-pMPC revealed great
20 advantages compared with other ways of surface functionalization. For examples,
21 pMPC chains were covalently polymerized on the surface of GCC NPs, which was
22 more stable than those prepared by physically absorption and “grafting to” methods.
23 Moreover, the size of GCC-pMPC can be well adjusted by controlling monomer
24 concentration or polymerization time.[44-47] Therefore, the obtained GCC-pMPC
25 have their positive charges successfully being covered, and their size can also be tuned
26 as required.

27 **3.2 Characterization of GCC-pMPC**

1 In order to characterize the structure of obtained GCC-Br and GCC-pMPC, their FTIR
2 spectra were acquired and shown in Fig. 1. Compared with GCC, it can be obviously
3 identified from the spectrum of GCC-Br that 2-bromoisobutyryl bromide was
4 successfully linked on GCC NPs, because the stretching vibration of C=O band shown
5 a clear absorption at 1731 cm^{-1} . This absorption can also be observed in the spectrum
6 of GCC-pMPC, which meant pMPC had already been grafted on the surface of GCC
7 NPs as designed. The peak at 1100 cm^{-1} could be ascribed to the stretching vibration
8 band of C-O band, whose intensity increased remarkably in GCC-pMPC, further
9 proving the successful polymerization of MPC on the surface of GCC NPs. What's
10 more, the distinctive broad absorption at around 3400 cm^{-1} can be assigned to the
11 stretching vibration of O-H band. The two characteristic peaks located at 2950 and
12 2871 cm^{-1} could be seen from all the spectra of GCC, GCC-Br, and GCC-pMPC,
13 which was attributed to the saturated C-H bands from $-\text{CH}_2$ and $-\text{CH}_3$. While the
14 intensity of C-H bands in GCC and GCC-pMPC was larger than that in GCC-Br,
15 which was in that the amount of $-\text{CH}_2$ and $-\text{CH}_3$ bands on the surface of GCC-Br was
16 comparatively smaller. The wavenumber around 1470 cm^{-1} in GCC-pMPC was due to
17 C-H stretching of the methyl substituent of quaternary ammonium groups, which was
18 demonstrated in Fig. S2. All these results confirmed the successful preparation of
19 GCC-pMPC.

20 The chemical compositions of GCC, GCC-Br, and GCC-pMPC were tested by
21 X-ray photoelectron spectra (XPS) studies to further verify their elemental
22 information. From XPS spectrum of GCC as revealed in Fig. 2A, it can be figured out
23 that carbon and oxygen were two major components while nitrogen was a minor
24 component, which was consistent with earlier results. In the raw XPS data of GCC-Br,
25 a new element bromine shown up, which evidently proved the successful preparation
26 of Br- containing initiator. However, this Br signal disappeared in GCC-pMPC with
27 another new element P emerging. This gave a strong confirmation that pMPC was
28 successfully conjugated on the GCC NPs surface via surface initiated ATRP.
29 Furthermore, the high resolution XPS spectra for the elements of C, O, N, P and Br
30 were also obtained. The Br3d XPS spectrum of GCC-Br in Fig. 2B and the P2p XPS

spectrum of GCC-pMPC in Fig. 2C evidently proved the formation of initiator and the further polymerization of MPC on GCC surface. The peaks of C1s with the binding energies at 284.6 and 285.5 eV can be assigned to the sp² and sp³ hybridized carbon atoms, and the peak at a higher binding energy of ca. 288 eV was ascribed to the carbon atoms bound to oxygen (Fig. 2D). The O1s XPS spectra and N1s spectra were also determined as shown in Fig. 2E and Fig. 2F. These XPS peaks were also fitted by XPS PEAK software (Fig. S3). All these information further verified the surface polymerization of MPC on GCC NPs.

The specific elements content of GCC, GCC-Br, and GCC-pMPC samples was calculated based on XPS analysis and listed in Table S1. The two major components in GCC were C atom (63.11%) and O atom (35.09%), and the percent of N atom was 1.8%. After reaction with 2-bromoisobutyryl bromide, the resulted GCC-Br demonstrated a new element Br with percentage of 1.87%, and the percent of C, O, and N was detected to be 62.61%, 32.27%, and 3.25%, respectively. It was further confirmed that pMPC had been successfully grafted on GCC, in that a clear percent of P was determined in GCC-pMPC, which was 2.48%. Moreover, none Br was detected any more owing to the consumption of polymerization. Meanwhile, after the functionalization of GCC NPs, the percentage of N and C in GCC-pMPC increased to 4.79% and 65.76%, while the percentages of O were decreased to 26.97%. These results also indicated the successful modification of GCC with pMPC.

3.3 Morphology Analysis

The morphology of resulted GCC-pMPC was figured out through transmission electron microscopy (TEM) as demonstrated in Fig. 3A. It can be obviously identified that these NPs had a spherical shape and their size range was from 60 to 100 nm. Specially, it was also noticed that each of these NPs composed several GCC NPs, since these GCC NPs were quite small with an average size of only 5.6 nm.[32] After the functionalization of GCC NPs, the resulted GCC-pMPC demonstrated not only an increased diameter, but also great water dispersibility owing to the surface polymerization of superhydrophilic MPC. These characters were expected to be very

1 advantageous in their further bioapplications. Moreover, the size distribution of
2 GCC-pMPC NPs in PBS (pH 7.4) was also determined by a zetaPlus particle size
3 analyzer, and the result was shown as 195.9 ± 1.7 nm with a polydispersity index (PDI)
4 of 0.322 (Fig. 3B), which was larger than PEGylated GCC NPs. Probably, it was the
5 hydrodynamic effects and stretch of pMPC chains in PBS that caused this size
6 augment.

7 **3.4 Fluorescence Property**

8 It has been clearly stated that GCC NPs could emit intense red fluorescent at around
9 670 nm, which can be ascribed to the intramolecular charge transfer effect between
10 their hexahydropyridine ring and pyridinium structures (Fig. S1).[32] The biofavorable
11 red fluorescence property was believed to be maintained in resulted GCC-pMPC even
12 after the polymerization of MPC on the surface. In this case, the luminescent property
13 of GCC-pMPC was determined by checking their UV-Vis absorption and fluorescence
14 spectrum (Fig. 4). As expected, GCC-pMPC revealed strong red luminescence with
15 their maximum emission being located at around 667 nm, and they also demonstrated
16 similar absorption character with the maximum absorption wavelength of around 645
17 nm. Furthermore, in order to quantitatively evaluate their fluorescence, the quantum
18 yield value of GCC-pMPC in water was determined using Cy5 as the reference
19 dye.[48, 49] The final quantum yield of GCC-pMPC was calculated as $6.2 \pm 1.1\%$
20 (average $\pm$ standard deviation, from 5 independent experiments), which was similar
21 with PEGylated GCC NPs and a little weaker than that of GCC NPs (with the quantum
22 yield value of 8%). This was properly caused by the modification procedures. The
23 photostability of GCC-pMPC in long term were also determined (Fig. S4). This stable
24 red fluorescence property of GCC-pMPC would greatly benefit their bioimaging and
25 biomedical applications.

26 **3.5 Biocompatibility and photostability of GCC-pMPC**

1 Before their cellular imaging test, the biocompatibility of GCC-pMPC was determined
2 since it played significant roles in their further biomedical applications. Owing to the
3 surface polymerization of superhydrophilic PMC on GCC NPs, it was expected that
4 the biocompatibility of resulted GCC-pMPC would be prominently improved. In this
5 case, cell counting kit-8 (CCK-8) assay was employed to evaluate the biocompatibility
6 of GCC-pMPC through testing the cell viability of HeLa cells against GCC-pMPC of
7 different concentrations for different incubation time. As indicated in Fig. 5A, it can be
8 found out that there was no evident cell viability decrease after HeLa cells were
9 incubated with GCC-pMPC for 12 and 24 hours, respectively. Concentration of
10 GCC-pMPC was chosen as another important parameter to evaluate their
11 biocompatibility. Interestingly, even the concentration of GCC-pMPC was adjusted
12 up to $100\text{ }\mu\text{g mL}^{-1}$, the cell viability value was still greater than 90% no matter after
13 incubation time of 12 h or 24 h. These results demonstrated that the GCC-pMPC
14 possessed outstanding biocompatibility to HeLa cells, which endowed them great
15 potential for further bioapplications.

16 In order to apply GCC-pMPC for live cell imaging applications, their
17 photostability in cells was also a very important character for durable observation.
18 Therefore, we tested the fluorescence intensity changes of GCC-pMPC in HeLa cells
19 under continuous laser irradiation of 633 nm for 20 min (Fig. 5B). The laser intensity
20 in the experiment was set as 10%, which was about $40\text{ }\mu\text{W}$. After the violent
21 photobleaching, the results were demonstrated in Fig. 5B. Amazingly, GCC-pMPC
22 could still emit luminescence with about 90% of their initial intensity, which verified
23 their outstanding photostability and would greatly advance their durable cell tracing
24 applications.

25 **3.6 Cellular imaging of GCC-pMPC**

26 Thanks to their excellent biocompatibility, biofavorable red fluorescence, outstanding
27 photostability, and high water dispersibility, it was anticipated that GCC-pMPC should
28 be an ideal candidate for tracing and biomedical applications. Thus Confocal Laser

1 Scanning Microscope (CLSM) observation was employed to investigate their cellular
2 imaging performance. First of all, the imaging region of GCC-pMPC in cells was
3 determined by staining fixed HeLa cells with both GCC-pMPC and Hoechst 33258. As
4 a comparison, Hoechst 33258 can light the cell nuclei under the excitation wavelength
5 of 405 nm. From Fig. 6A-D, it can be evidently identified that most GCC-pMPC
6 localized in the cytoplasm of HeLa cells with intense red fluorescence upon the
7 excitation of 633 nm. It was speculated that GCC-pMPC were taken up by cells via
8 endocytosis, which would be facilitated by their size range and surface groups.

9 Prior to live cell imaging test, the cellular uptake time of GCC-pMPC were also
10 studied by examining the fluorescent intensity of GCC-pMPC in HeLa cells after
11 different incubation time. As observed in Fig. S5A-C, if the incubation time was three
12 hours using GCC-pMPC of $20 \mu\text{g mL}^{-1}$, only a small amount of GCC-pMPC was
13 internalized with weak fluorescence emission. However, if the incubation time
14 increased to five hours using GCC-pMPC of the same concentration, HeLa cells can be
15 lightened with bright fluorescence under the same laser irradiation (Fig. S5G-I).
16 Thereupon, five hours was suggested to be a better incubation time to obtain
17 satisfactory cellular imaging effect with GCC-pMPC of $20 \mu\text{g mL}^{-1}$. This incubation
18 time was shorter than that of PEGylated GCC NPs reported in our previous report.[40]
19 We also measured the fluorescence intensities of cells incubated with pMPC modified
20 NPs and PEGylated NPs using ImageJ (v1.48, NIH), after they were uptaken by HeLa
21 cells with the same concentration in 4 h. It can be clearly observed that the cell
22 fluorescence intensity of pMPC modified NPs was much stronger than that with
23 PEGylated NPs (Fig. S6). [50, 51] As a result, their live HeLa cell imaging was
24 conducted based on above discussions, which were demonstrated in Fig. 6E-G. It can
25 be found that these live HeLa cells could still grow well and maintain good
26 morphology even after being incubated with GCC-pMPC for such a long time.
27 Moreover, the obtained live HeLa cell imaging pictures were very clear with intense
28 red fluorescence. These cellular imaging experiments obviously verified the excellent
29 tracing ability of GCC-pMPC.

1 **4. Conclusions**

2 In summary, pMPC functionalized red fluorescent nanoparticles were facily prepared
3 from nonluminous materials via surface initiated ATRP for the first time, and their
4 excellent live cell imaging performance demonstrated their high potential in tracing
5 and biomedical applications. Their construction strategy method demonstrated superb
6 advantages in its simplicity and economic values without employment of any
7 expensive fluorescent dyes, since the red emission was originated from GCC NPs
8 which were directly prepared from chitosan and glutaraldehyde. The deep red
9 luminescence from resulted GCC-pMPC was very favorable for bioimaging
10 applications, in that the red emission would greatly facilitate the acquisition of
11 fluorescence images with high contrast and quality from complicated biosystem.
12 Furthermore, this red fluorescence of GCC-pMPC was also very stable and
13 anti-photobleaching, which can remarkably benefit their durable imaging and potential
14 biomedical applications. More importantly, GCC-pMPC demonstrated outstanding
15 biocompatibility owing to the superhydrophilic MPC chains on their surface, and they
16 can successfully light the cytoplasm of live HeLa cells with intense fluorescence. This
17 work not only can provide a new construction strategy for preparing biocompatible red
18 fluorescent nanoparticles, but also would advance the research of their further
19 bioapplications.

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25

1 **Figure Captions**

2 **Scheme 1.** Schematic showing the preparation of GCC-pMPC.

3 **Fig. 1** FTIR spectra of GCC, GCC-Br, and GCC-pMPC.

4 **Fig. 2** (A) Raw XPS spectra of GCC, GCC-Br, and GCC-pMPC. High resolution scan
5 of (B) Br3d, (C) P2p, (D) C1s, (E) O1s, and (F) N1s.

6 **Fig. 3** (A) TEM image of GCC-pMPC, scale bar = 200 nm. (B) Dynamic light
7 scattering results of GCC-pMPC in PBS.

8 **Fig. 4** The UV-Vis absorption spectrum (A) and fluorescence spectrum (B) of
9 GCC-pMPC dispersed in water.

10 **Fig. 5** (A) Cell viability of GCC-pMPC with HeLa cells for 12 and 24 h, respectively.
11 (B) Photostability of GCC-pMPC in HeLa cells under continuous scanning at 633 nm.
12 Insets demonstrated the confocal images of GCC-pMPC stained cells before (0 min,
13 left) and after the laser irradiation for 20 min (right). Scale bar = 20 μ m.

14 **Fig. 6** CLSM images of fixed HeLa cells: (A) the cell nuclei were stained by Hoechst
15 33258 with the excitation wavelength of 405 nm; (B) the cytoplasm were lightened by
16 GCC-pMPC under the excitation wavelength of 633 nm; (C) differential interference
17 contrast image; (D) the corresponding overlay image. CLSM images of live HeLa
18 cells: (E) fluorescent image which were excited with 633 nm laser; (F) differential
19 interference contrast image; (G) the corresponding overlay image. Scale bar = 20 μ m.

20

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