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Performance Study of TiO_2-Au Photoelectric Nanocomposites for Novel Neuromodulation

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Abstract: A novel noninvasive yet highly efficient neuromodulation method *via* photoelectric nanocomposites (NCs) was proposed. The NCs were constructed by Au nanoparticles-attached TiO_2 nanocrystals. Upon 405 nm light exposure, the photocurrent was enhanced when the pure TiO_2 nanocrystals were modified with Au nanoparticles. It is evidenced that effective depolarization of NCs-treated neurons takes place upon 405 nm light illumination, certified by fluorescent dye test on PC12 cells. Furthermore, the NCs could remarkably remit the epilepsy seizures of zebrafishes, indicating that the NCs are potential candidates for curing neurological disorders.

Key words: nanocomposites; photoelectric; neuromodulation; epilepsy

Neuromodulation plays an important role in neuroscience researches for studying cell functions[1] and curing neurological disorders[2-3]. Generally, the following methods are used for neuromodulation: electrode implanting and optogenetics. Yet these methods are believed to be invasive and complicated[4-5]. Recently developed functional nanoparticles for neuromodulation, such as magnetothermal Fe_3O_4 nanoparticles[6], photothermal carbon nanohorns[7] and gold nanoparticles[8-9], only take effect in neurons which express temperature-sensitive ion channels.

An improved neuromodulation method may be obtained by combining the advantages of electrical and optical stimulation together. As well known, current could be generated by semiconductor nanoparticles such as TiO_2 when irradiated by light with energy over its bandgap energy. Accordingly, in the present study, nanocomposites of TiO_2 nanocrystals modified with Au nanoparticles are constructed and used for neuromodulation by generating photocurrent (Fig. 1). Upon exposure to 405 nm light, the electron-hole pairs are generated in TiO_2 nanocrystals, followed by the electrons transferring from TiO_2 nanocrystals to the outer Au nanoparticles owning to the difference in work functions of semiconductors and noble metal nanoparticles. Subsequently, the electron flow interacts with the charges of neurons to make the neurons depolarized. The following objects were systemically studied in this study: 1) the ability of NCs to generate current upon 405 nm light exposure; 2) the biocompatibility of NCs and feasibility in triggering the depolarization of neurons; and 3) anti-epileptic performance of NCs on epileptic zebrafishes.

1 Experimental procedure

1.1 Materials preparation

The TiO_2 nanocrystals were prepared by pyrolysis method according to Gordon, *et al*[10]. The as-prepared TiO_2 nanocrystals were mixed with DSPE-PEG_{2000}-SH chloroform solution, followed by vacuum-rotary

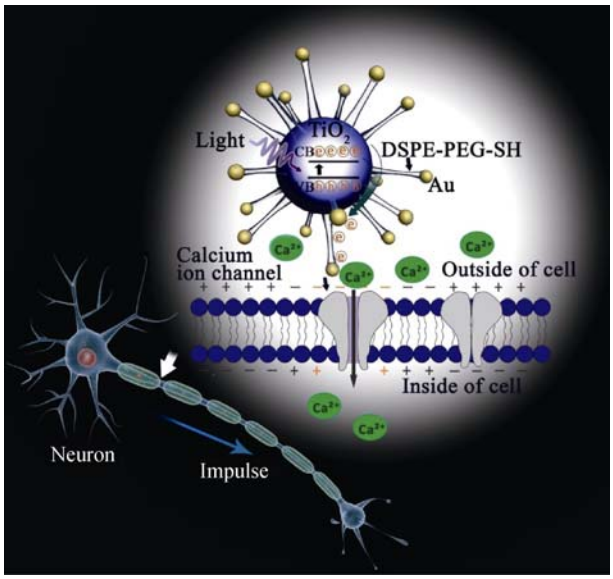

Fig. 1 Schematic illustration of NCs depolarizing neurons upon 405 nm light exposure

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evaporation in oil bath at 60℃ for 1 h. After washed for several times, the product was dispersed in deionized water. Au nanoparticles were prepared according to a previously reported method[11]. DSPE-PEG_{2000}-SH modified TiO_2 nanocrystals aqueous solution was blended with Au nanoparticles. Following ultrasonicating for 20 min, the product was washed with deionized water for several times before yielding NCs.

Seeded into a CLSM-special dish, the rat pheochromocytoma PC12 cells were stained with membrane potential indicator Di-8-ANEPPS. Afterwards, HHBS solution of NCs was added to the dish. The cells were observed with a fluorescence microscope. Tracking calcium ions influx by calcium indicator Cal-520 was carried out with similar procedure.

Wild type AB zebrafishes were induced epilepsy by bath application of pentylenetetrazol. The brains of epileptic zebrafishes were injected with 1 nL PBS solution of NCs and illuminated under 405 nm light for 2 min. The motion trails of zebrafishes in 1 h were recorded. More details about the experiment were reported in the Supporting Information section.

1.2 Characterization

Transmission electron microscopy (TEM) images were recorded on a JEOL 200CX microscope with an accelerating voltage of 200 kV. Standard TEM samples were prepared by dropping dilute products onto carbon coated copper grids. Dynamic light scattering (DLS) measurement was conducted on Nano-Zetesizer (Malvern Instruments Ltd). UV-Vis spectra were recorded on a UV-3101PC Shimadzu spectroscope. ICP determinations were carried out using a Varian Vista-MPX ICP instrument. CLSM images were recorded on FV1000, Olympus, Japan. The motion trails of zebrafishes were recorded by behavior analysis system V3, Viewpoint Life Sciences Inc.

2 Results and Discussion

2.1 Synthesis and characterization of NCs

Monodispersed TiO_2 nanocrystals with an average diameter of 17 nm were obtained by pyrolysis method (Fig. 2(a))[10]. The as-prepared TiO_2 nanocrystals could be well- dispersed in chloroform and showed blue in color. XRD result indicated the TiO_2 nanocrystals possessed an anatase crystalline phase (Fig. S2). The oleate-coated TiO_2 nanocrystals were surface-modified with DSPE-PEG_{2000}- SH for improving hydrophilicity. The prepared Au nanoparticles (Fig. 2(b)) were easily conjugated with the DSPE-PEG_{2000}-SH functionalized TiO_2 through covalent bonding between Au and –SH[12], which finally constructed NCs (Fig. 2(c)). The mass loading of the Au nanoparticles on the NCs was 5.46wt%, as assessed by ICP-MS. The UV-Vis spectrum of NCs displayed a sharp absorption edge at 380 nm and broad absorption throughout the visible region (Fig. 2(d)). To reduce the biological tissue damage from ultraviolet light of shorter wavelength, 405 nm light was chosen to excite NCs for photocurrent. In the amperometric *i-t* curves (Fig. 2(e)), evident switchable photocurrent of the NCs electrode was presented responding to a cyclic "on-off" illumination, with the intensity of photocurrent being significantly enhanced compared with pure TiO_2 nanocrystals. The attached Au nanoparticles contributed to the increase of photocurrent intensity by promoting separation of photoinduced electrons and holes in TiO_2 nanocrystals[13].

2.2 Depolarization of NCs-treated neurons

For the safe applications in cells as well as in living organisms, low cytotoxicity of NCs is of significant essence. As shown in Fig. S4, PC12 cells pre-incubated with NCs showed high viability in the cytotoxicity test, so did the PC12 cells pre-treated with NCs and illuminated by 405 nm light for 2 min, indicating good biocompatibility of NCs. To comfirm whether the neurons could be effectively depolarized by NCs, dye Di-8-ANEPPS was used to track the change of membrane potential. Compared with other control groups, remarkably enhanced fluorescence intensity was observed only in PC12 cells treated with both NCs (125 μg/mL) and 405 nm light (10 mW/cm^2) illumination (Fig. 3(a) and 3(b)), which certified the depolarization of cells. Furthermore, influx of calcium ions is accomplished during the process of neuron depolarization. It was demonstrated in the process of calcium indicator dye Cal-520 tracking calcium ions influx, as depicted in Fig. 3c and 3d, the fluorescence intensity of Cal-520 in PC12 cells treated with both NCs and illumination showed a prominent enhancement that could be distinguished by naked eye (Supporting video 1). It deemed to be another sound evidence that the current generated in NCs was capable to depolarize neurons.

2.3 Anti-epileptic performance evaluation of NCs

Epilepsy arising from excessive synchronous discharge of disordered brain neurons could be vastly ameliorated by electrical stimulation in related brain region[14-15]. Epilepsy-induced zebrafishes exhibited seizure-like activities and faster movement features in the experiment. Brains of epileptic zebrafishes were injected with 1 nL NCs PBS solution (200 μg/mL), followed by being illuminated with 405 nm light (10 mW/cm^2) for 2 min (Fig. 4(a)). An obvious difference in diversely treated zebrafishes was detected by observing their motion (Supporting video 2). It was apparent that epileptic zebrafishes treated with both NCs and illumination showed a remission of seizure and a slower movement. As clearly shown in Fig. 4(b), the

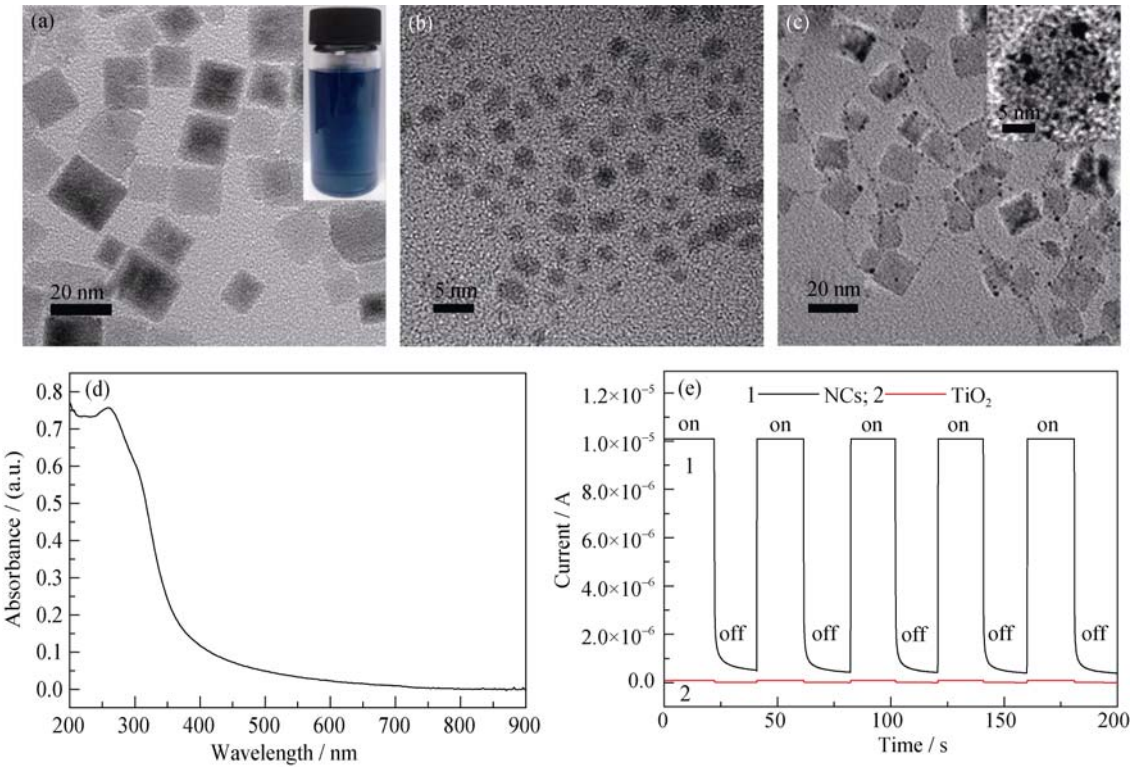

Fig. 2　Representative TEM images of (a) TiO_2 nanocrystals, (b) Au nanoparticles, and (c) NCs. Inset of (a) optical image of TiO_2 nanocrystals dispersed in chloroform, (c) magnifying TEM image of NCs. (d) UV/Vis absorption spectrum of NCs; (e) photocurrent responses of the NCs and TiO_2 electrode to a cyclic "on-off" illumination of 405 nm light. Measurements were performed on electrochemical workstation with Pt as counter electrode, Ag/AgCl as reference electrode, and 0.2 mol/L Na_2SO_4 as electrolyte 405 nm light from Xenon lamp (300 W) was used as the excitation source

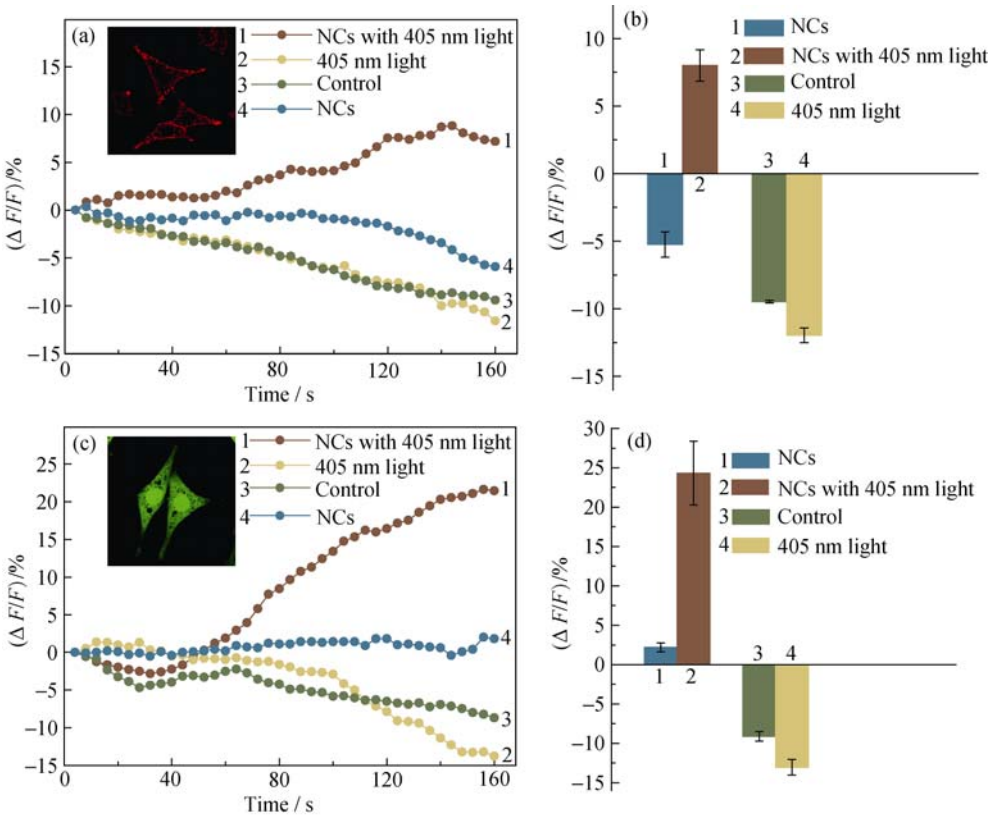

Fig. 3　Real-time fluorescence intensity variation of Di-8-ANEPPS (a) and Cal-520 (c) in PC12 cells observed by CLSM. Max fluorescence intensity variation of Di-8-ANEPPS (b) and Cal-520 (d) in PC12 cells; data represents mean ± SD (n = 3)

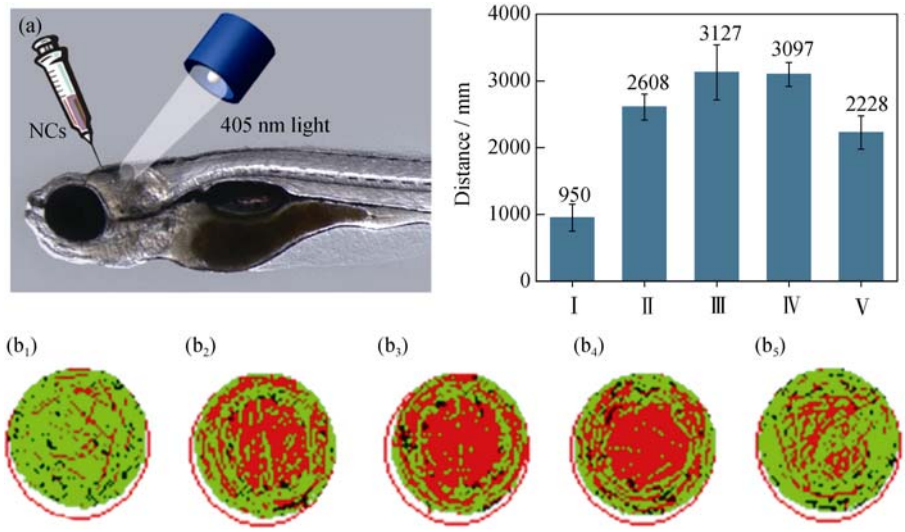

Fig. 4　(a) Schematic illustration of NCs anti-epileptic performance investigation on zebrafishes. The motion trails in 1 h of (b_1) normal zebrafishes, epileptic zebrafishes treated with PBS (b_2), NCs (b_3), illumination (b_4), both NCs and illumination (b_5). The red, green and black lines represent motion trails with speed of >20 mm/s, 4-20 mm/s, and <4 mm/s, respectively. (c) Distance of zebrafishes moving with speed of >20 mm/s; normal zebrafishes (I), epileptic zebrafishes treated with PBS (II), NCs (III), illumination (IV), both NCs and illumination (V). Data represents mean ± SD (n = 30)

epileptic zebrafishes only treated with either NCs or 405 nm light illumination preferred to act in a faster speed of >20 mm/s, while NCs and illumination-treated epileptic zebrafishes tended to move with a speed of <20 mm/s, the action of which was similar with their normal counterparts. Because moving speed of epileptic zebrafishes increased remarkably during epilepsy seizures, the moving distance with speed of >20 mm/s was utilized to estimate the degree of epilepsy (Fig. 4(c)). Thus the anti-epileptic efficiency could be figured by the formula:

$$\text{Anti-epileptic efficiency (\%)} = (D_1 - D)/(D_1 - D_0) \times 100\%$$

Where D was the moving distance of epileptic zebrafishes treated with both NCs and illumination. D_1 and D_0 represented moving distance of epileptic zebrafishes treated with illumination and normal zebrafishes, respectively. The anti-epileptic efficiency of NCs was calculated to be 40.5% according to the formula.

3　Conclusion

In the present study, photoelectric nanocomposites composed of Au nanoparticles-modified TiO_2 nanocrystals have been successfully constructed for the application in neuromodulation. The NCs could efficiently trigger the depolarization of neurons by generating current under 405 nm light illumination. Compared with the current neuromodulation method of electrode implanting and optogenetics, the light- controlled electrical stimulation pattern seems to be more universal and secure for broad applications. Moreover, the NCs showed an exciting anti-epileptic performance on zebrafishes. This novel nanocomposite for neuromodulation provides a feasible access to sophisticated experimental designs in neuroscience as well as the development of innovative therapies for neurologic disorders.

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新型神经调控用 TiO_2-Au 光电转换纳米复合物性能研究

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摘 要: 本文提出了一种基于光电转换纳米复合物(NCs)的新型神经调控方式。NCs 是 TiO_2 纳米晶表面连接金纳米粒子的复合物, 可产生光电流并有效引发神经细胞去极化。在 405 nm 光照射下, NCs 产生的光电流比单一 TiO_2 纳米晶产生的光电流强度显著提高。PC12 细胞上的膜电位荧光探针和钙离子荧光探针测试结果显示, 在 405 nm 光照下, 经 NCs 处理的细胞发生去极化。在活体抗癫痫实验中进一步证明, NCs 产生的光电流可明显减轻斑马鱼的癫痫发作。本研究结果表明 NCs 可进行神经调控, 对神经疾病的治疗具有重要意义。

关 键 词: 纳米复合物; 光电转换; 神经调控; 癫痫

中图分类号: TQ174 **文献标识码:** A

Supporting Information

Performance Study of TiO_2-Au Photoelectric Nanocomposites for Novel Neuromodulation

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Part 1: Experimental procedures

1.1 Materials

The following chemicals were used as received without further purification. Titanium(IV) fluoride (TiF_4, 99%), chloroauric acid hydrate (Au≥48%), 2-propanol, methanol, toluene, acetone, chloroform, anddimethylsulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. Tetrakis (hydroxylmethyl) phosphonium chloride solution (THPC, 80% in H_2O), oleylamine(OLAM, 70%), oleic acid (OLAC, 90%), 1-octadecene (90%) (1-ODE), and pentylenetetrazol (PTZ) were obtained from Sigma Aldrich. 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[sulfydryl (polyethylene glycol)-2000] (DSPE-PEG_{2000}-SH) was obtained from Shanghai Yare Biotech, Inc. Fluorescent indicator dye Di-8-ANEPPS and Cal-520 were purchased from AAT Bioquest, Inc. PBS solution (pH=7.4) and HHBS were obtained from Shanghai Runcheng Biomedical Co., Ltd.

1.2 Synthesis of NCs

1.2.1 Synthesis of TiO_2 nanocrystals

The TiO_2 nanocrystals were prepared by thermal decomposition according to Gordon, *et al*[1].Nitrogen atmosphere was needed during the preparation of stock solutions and TiO_2 nanoparticles. The TiF_4 stock solution consisting of 0.2 mol/L TiF_4 and 1.0 mol/L OLAC in 1-ODE was heated to 80℃ with stirring to promote TiF_4 dissolution. To prepare TiO_2 nanocrystals, 30 mmol of cosurfactant OLAM, 10.2 mL of 1-ODE, and 1.5 mmol of OLAC were mixed in a 100 mL flask and degassed at 120℃ for 1 h, followed by adding 1.5 mL of the stock solution at 60℃. The solution was quickly heated to 290℃ and held for 10 min, after which 8 mL stock solution was pumped into the flask at 0.3 mL/min using a syringe pump. Afterward, the flask was left to cool naturally to ambient temperature. The reaction contents were washed by toluene for several times and the mixture of 2-propanol and methanol was added to precipitate the NCs. Subsequently the as-prepared oleic acid capped TiO_2 nanocrystals were dispersed in 40 mL chloroform.

1.2.2 Surface modification of TiO_2 nanocrystals

In order to obtain good biocompatibility and introduce functional group for further connection of Au nanoparticles, DSPE-PEG_{2000}-SH was used to modify the surface of hydrophobic TiO_2 nanocrystals. 2 mL TiO_2 chloroform solution was mixed with 150 μL DSPE-PEG_{2000}-SH chloroform solution (100 mg/mL) in a stand-up flask followed by vacuum-rotary evaporation in oil bath under 60℃ for 1 h. Then the TiO_2 nanocrystals were washed three times with deionized water and acetone, and finally dispersed in 5 ml deionized water.

1.2.3 Synthesis of NCs

Au nanoparticles were prepared according to a previously reported method using THPC as reductant[2]. Then 5 mL DSPE-PEG_{2000}-SH modified TiO_2 nanocrystals aqueous solution was mixed with 2 mL Au nanoparticles solution followed by ultrasonication for 20 min. The Au nanoparticles could be attached onto the TiO_2 surface by the covalent bonding between Au and –SH. The product was collected through centrifugation, and washed for several times with deionized water until the supernatant was colorless.

1.3 Cytotoxicity tests of NCs

PC12 cells were seeded into a 96-well plate and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained at 37℃ under 21% O_2/5% CO_2/74% N_2 for 24 h. NCs in RPMI 1640 medium with different concentrations (0, 7.5, 15, 31.5, 62.5, 125, and 250 μg/mL) were added into the wells and co-incubated for 24 h, followed by addition of 10 μL Cell Counting Kit-8 (CCK-8) solutions. After incubation for another 4 h, the absorbance of each well was monitored by a microplate reader at the wavelength of 450 nm. Another plate was placed under 405 nm light (10 mW/cm^2) illumination for 2 min after the addition of

NCs, and co-incubated for 24 h before detecting absorbance at 450 nm wavelength. The cytotoxicity was finally expressed as the percentage of cell viability in contrast to the untreated cells.

1.4 Depolarization of NCs-treated neurons

PC12 cells were seeded into a CLSM-special dish and cultivated for 24 h. The cells were stained with membrane potential indicator dye Di-8-ANEPPS and washed for three times with HHBS, followed by addition of NCs HHBS solution (200 μL, 125 μg/mL). CLSM experiments were carried out on an Olympus FV1000 laser-scanning microscope. A 100× oilimmersion objective lens was used and 480 nm light and 405 nm light were applied to excite Di-8-ANEPPS and NCs respectively. Visible luminescent signals were detected in the wavelength regions of 700–730 nm. Time Series of the microscope was used to record florescence intensity variation with time interval of 0 s and frames of 40.For luminescent observation of calcium ions influx using Cal-520, similar procedures were carried out.

The images were analyzed with ImageJ by choosing the region of interest which corresponded to the membrane, then measuring the average florescence intensities along this region. The values of relative fluorescence intensityΔF were calculated to get a more reliable measurement according to the formula:

$$\Delta F = (F - F_0) / F_0 \times 100\%$$

Where the florescence intensity of the first frame was F_0, the florescence intensities of later frames were F.

1.5 Anti-epileptic performance evaluation of NCs

Wild type AB zebrafishes (six days post fertilization) were induced epilepsy by bath application of PTZ (10 mmol/L). 1 nL NCs PBS solution(200 μg/mL) was injected into the epileptic zebrafish brains and illuminated under 405 nm light for 2 min. The motion trails of zebrafishes in 1 h were recorded by zebrafish behavior analysis system. As control experiments, the motion trails of normal zebrafishes, epileptic zebrafishes injected with 1 mL PBS, epileptic zebrafishes injected with NCs and epileptic zebrafishes illuminated by 405 nm light for 2 min were also recorded by zebrafish behavior analysis system. Each group contained 30 zebrafishes.

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Supporting videos:

Supporting video 1. Real-time observation of Cal-520 fluorescence intensity variation on PC12 treated by NCs and 405 nm light illumination.

Supporting video 2. Motor behaviors of differently treated zebrafishes.

Part 2: Supplementary figures

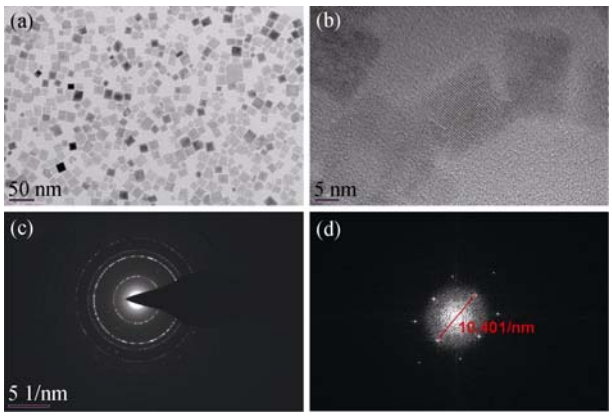

Fig. S1 (a) TEM image, (b) HRTEM image, (c) electron diffraction pattern and (d) lattice spacing of TiO_2 nanocrystals

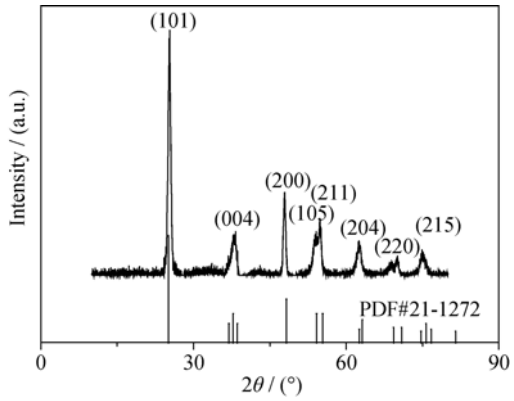

Fig. S2　X-ray diffraction (XRD) pattern of TiO_2 nanocrystals

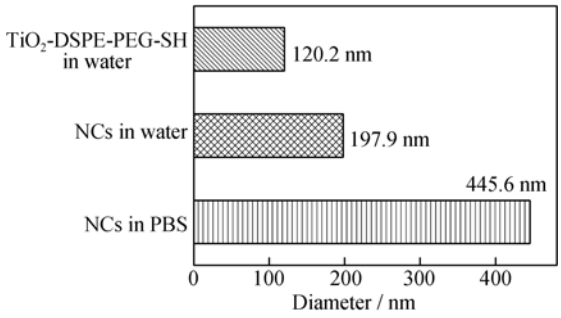

Fig. S3　Hydration diameters of DSPE-PEG_{2000}-SH modified TiO_2 in deionized water, NCs in deionized water and PBS observed by dynamic light scattering

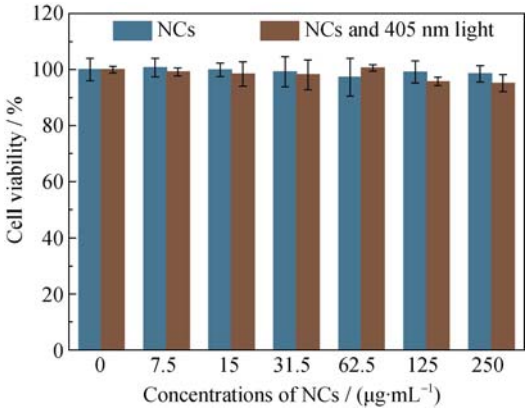

Fig. S4　Cell viability assessed by CCK-8 on NCs-treated PC12 cells as well as NCs and illumiantion-treated PC12 cells