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Peptide-fluorophore/AuNP conjugate-based two-photon excited fluorescent nanosensor for caspase-3 activity imaging assay in living cells and tissue†

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In the present study, a novel fluorescence resonance energy transfer (FRET) strategy-based two-photon excited nanosensor *via* the assembly of two-photon dye-labeled peptides on the gold nanoparticle's surface has been developed and successfully applied in the caspase-3 imaging assay in living cells and ischemia-reperfusion surgery-treated rat liver tissue slices.

Apoptosis is an important physiological process that occurs either during the course of development and normal maintenance of homeostasis or in pathological circumstances including cancer,¹ neurodegenerative diseases,² autoimmune disorders,³ and heart failure.⁴ Caspase-3 has been identified as the most important mediator among the caspase family for the initiation and propagation of apoptosis.⁵ Consequently, the development of intracellular protease sensors toward caspase-3 is essential for understanding the roles of this protease in physiological and pathological processes as well as for the therapeutic effect of cancer treatment or screening new drug candidates for dysregulated apoptosis. It is well known that caspase-3 can specifically cleave the N-terminus of the tetrapeptide Asp–Glu–Val–Asp (DEVD), which has been used to develop caspase-specific probes for the homogeneous or inhomogeneous analysis of caspase-3 activity. Usually, homogeneous assay methods include colorimetric assays,⁶ fluorescence polarization or fluorescence correlation spectroscopy-based assays,^{7,8} and luminescence-based detection.^{9–13} Meanwhile, inhomogeneous assay methods include Western blot assays,¹⁴ atomic force microscopy-based assays,¹⁵ and electrochemical detection.¹⁶ Among these developed technologies, fluorescence resonance energy transfer (FRET)-based homogeneous detection^{11,12} has taken a crucial place due to the excellent properties of fluorescence detection, such as high sensitivity, specificity, fast response, and technical simplicity. However, among the dyes acknowledged as energy donors, almost all were single-photon fluorescent

dyes, resulting in limitation of their application under complicated biological conditions.

Thus, considering the desirable properties of two-photon excitation (TPE) such as lower tissue autofluorescence and self-absorption, reduced photodamage and photobleaching, high spatial resolution and deeper penetration depth,¹⁷ we present herein the attempt to fabricate a novel AuNP/peptide-TP dye nanoconjugate-based two-photon nanosensor for the caspase-3 imaging assay in apoptotic cells and liver tissue slices obtained from a rat hepatic ischemia reperfusion model (Scheme 1). The nanosensor system is composed of ethyl-4-[3,6-bis (1-methyl-4-vinylpyridium iodine)-9H-carbazol-9-yl] (BMVC) molecules as the TPE fluorophore, the peptide

Scheme 1 (A) Schematic illustration of the AuNP/peptide-TP dye nanosensor for the caspase-3 activity assay. (B) Schematic diagram of the nanoconjugate sensor for caspase-3 activity monitoring and imaging in living cells. (1) Endocytosis of the sensing nanoconjugates; (2) cell apoptosis and generating activated caspase-3; (3) cleavage of the DEVD motif and recovery of the TPE fluorescence emission resulting from the release of BMVC molecules from the AuNP's surface, and further great enhancement when they were inserted into dsDNA.

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sequence CANLNDEVDK as the caspase-3 cleavable linker with a cleavage site between D and K, and AuNPs as the appropriate quencher and carrier vehicle for the delivery of the nanosensor to live cells through fast endocytosis. It is worth noting that the TPE fluorescence emission of the free BMVC molecules used in this work is very weak under the aqueous conditions, while the TPE fluorescence emission will be enhanced greatly when these TPdye molecules are inserted into double-stranded DNA sequences.¹⁸ This special characteristic of the BMVC molecules is very beneficial to the fabrication of desirable fluorescent imaging biosensors with excellent properties such as a low background and a high signal to noise ratio. The TPdye BMVC-labeled specific peptide sequence CANLNDEVDK-BMVC is anchored on a citrate-stabilized AuNP surface to form an AuNP/peptide-TPdye nanoconjugate *via* the Au-S strong interaction strategy. The AuNP/peptide-TPdye nanoconjugate initially emits negligible fluorescence due to the efficient quenching of AuNPs to the TPdye BMVC adjacent to the noble metal surface. In the apoptotic cells or injured rat liver tissue slices resulting from the hepatic ischemia reperfusion surgery, caspase-3 is processed to its active form, which can cleave the peptide sequence CANLNDEVDK-BMVC, and the released TPdye BMVC molecules will be inserted into the intracellular double-stranded DNA, thus triggering the TPE fluorescence emission recovery of the TPdye BMVC molecules and even great enhancement, and then realizing the caspase-3 activity imaging assay.

The formation and characterization of the CANLNDEVDK-BMVC functionalized AuNPs were first characterized by using transmission electron microscopy (TEM), UV-vis spectroscopy, dynamic light-scattering (DLS) and zeta potential analysis. One can see from Fig. 1A that the TEM image shows that the CANLNDEVDK-BMVC-coated AuNPs are regular and spherical in shape, indicating that the morphology of AuNPs was not changed obviously during the functional modifica-

tion process. The UV-vis absorption spectrum of AuNPs shows an approximately 4 nm red shift after the surface modification by CANLNDEVDK-BMVC (Fig. 1B), perhaps resulting from the influence of the peptide sequences modified. DLS (Fig. 1C) shows that the size of CANLNDEVDK-BMVC-coated AuNPs is about 16.5 nm, slightly larger than that of the AuNPs (~12.6 nm), and the value of PDI is 0.3 which means good dispersion of the sample. To further obtain the surface charge information of the AuNP/peptide-TPdye nanoconjugate, zeta-potential measurement was also carried out. The zeta-potential of the AuNP/CANLNDEVDK-BMVC nanoconjugate was determined to be about -38.0 mV, which showed a slightly negative shift compared with that of the AuNPs (-33.5 mV) because "CANLNDEVDK" has a PI of 3.7 and is negatively charged in neutral solution (Fig. 1D). All these results indicate that the conjugation between the activatable caspase-specific peptide and AuNPs and the successful formation of the AuNP/peptide-TPdye nanoprobe are clearly achieved during the self-assembly process.

The stability and biocompatibility of the AuNP/CANLNDEVDK-BMVC nanoconjugate under various environmental conditions were then investigated through testing its fluorescence intensity. One can see from Fig. S2 (ESI†) that no significant changes in fluorescence intensity were noticed with the addition of any metal ions or thiol-containing compounds, indicating that the synthesized AuNP/peptide-TPdye nanoconjugate is stable in the presence of various physiological relevant metal ions and thiol-containing amino acids. The stability of this AuNP/peptide-TPdye nanoconjugate in cell media such as DMEM or human cervical carcinoma (HeLa) cell lysate was also investigated (Fig. S3, ESI†). The result indicates that the AuNP/CANLNDEVDK-BMVC nanoconjugate can be stable for at least seven hours in these media solutions. The cytotoxicity tests were performed with HeLa cells as the model cell line using the standard cell viability assay—the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Here we compared the biological toxicity of the AuNP/CANLNDEVDK-BMVC nanoconjugate with that of the AuNPs. After culturing with the two nanostructures for 48 h, the cellular viability displays that both the AuNP/peptide-TPdye nanoconjugate and the AuNPs exhibit excellent cell viability. And no significant difference in the proliferation of the HeLa cells is observed in the absence or presence of the two nanostructures even when their concentrations are increased up to 0.6 nM (concentration of the nanostructure refers to the concentration of AuNPs) (Fig. S4, ESI†). The MTT results show that the obtained AuNP/peptide-TPdye nanoconjugate has no obvious cytotoxic effect on the living system.

We also explored the signaling mechanism. To verify the design scheme, we investigated the real-time records of OPE fluorescence intensity changes of the AuNP/CANLNDEVDK-BMVC nanosensing conjugate in buffer solution upon addition of the recombinant human caspase-3 (Fig. 2A). In an aqueous buffer solution, the fluorescence emission of the nanosensing conjugate was very weak, indicating the

Fig. 1 (A) TEM image of the AuNP/peptide-TPdye nanoconjugate. (B) The normalized UV-vis absorption spectrum, (C) DLS data and (D) zeta-potentials of AuNPs (black curve) and the AuNP/peptide-TPdye nanoconjugate (red curve) in aqueous solution.

Fig. 2 (A) Real-time fluorescence records of the AuNP/peptide-TPdye nanoconjugate upon addition of caspase-3 in the reaction buffer. (B) Fluorescence emission spectra of the AuNP/peptide-TPdye nanoconjugate (black curve), in the presence of caspase-3 (blue curve, the final concentration of caspase-3 is 468 ng mL⁻¹) and upon further addition of HPDNa (5'-AATCAAGCTTCCCCAAGCTTGAATTC-3', red curve). (C) Fluorescence emission spectra of the AuNP/peptide-TPdye nanoconjugate (1 nM, concentration of the nanoconjugate refers to the concentration of AuNPs) at various concentrations of caspase-3 (from bottom to top: 0, 0.63, 4.5, 36, 72, 144, 288, and 350 ng mL⁻¹, respectively). (D) Plot of the relative fluorescence emission intensity (F/F_0) at 560 nm of the nanoconjugate versus the concentration of caspase-3, which was obtained from (C). F and F_0 represent the fluorescence emission intensity of the reaction solution in the presence and absence of caspase-3, respectively. Data were presented as average $\pm$ SD from three independent measurements.

interaction of the AuNP-acceptor with the BMVC-donor, allowing the energy transfer process to occur. The continuous increase of the fluorescence emission after the addition of caspase-3 indicates that the fluorescence molecules of BMVC departed from the AuNP's surface due to the cleavage reaction catalyzed by caspase-3, resulting in the fluorescence emission recovery of the nanosensor. Moreover, further significant enhancement of the fluorescence emission intensity can be observed when dsDNA was added into the reaction solution (Fig. 2B), resulting from the formation of the BMVC/dsDNA complex. All these results verified the feasibility of this sensing scheme and that the synthesized AuNP/CANLNDEVDK-BMVC nanoconjugate could act as a sensor for caspase-3 with a desirable "signal-on" architecture.

We then investigated the capability of the AuNP/peptide-TPdye nanoconjugate as a protease sensor for the *in vitro* assay of caspase-3 activity. Mixtures of the AuNP/CANLNDEVDK-BMVC nanoconjugate and protease were prepared and incubated at 37 °C. The OPE fluorescence spectra were measured in the range from 480 to 700 nm after 60 min of incubation. One can see from Fig. 2C that the fluorescence peaks dynamically increase with increasing caspase-3 concentration and the plot of fluorescence intensity at the maximum emission wavelength of 560 nm versus caspase-3 concentration in the range from 4.5 to 144 ng mL⁻¹ reveals a linear response characteristic ($R^2 = 0.91$) of the protease sensor

(Fig. 2D), with a readily achieved detection limit of 0.63 ng mL⁻¹.

After investigating the response characteristics of the AuNP/peptide-TPdye nanosensor *in vitro*, we then explored its potential for live-cell imaging of caspase-3. Human cervical carcinoma (HeLa) cells were first incubated with the AuNP/CANLNDEVDK-BMVC nanoconjugate in the cell growth medium for 2 h at 37 °C. Then, the cells were treated with PEITC, an apoptosis inducer,¹⁹ for another 1 h at 37 °C, and finally the fluorescence emission images were obtained with two-photon confocal microscopy. As shown in Fig. 3, upon PEITC-mediated caspase-3 activation, all the cells showed strong TP fluorescence signals, while no appreciable contrast in the fluorescence images can be observed for the cell without PEITC treatment. To validate that the fluorescence signal resulted from the uptake of AuNP/peptide-TPdye nanoconjugate rather than the direct entrance of TPdye-labeled peptides into the cell, we further performed the experiment using the peptide "CANLNDEVDK-BMVC" to incubate with HeLa cells. As shown in Fig. S5 (ESI[†]), obvious fluorescence contrast cannot be observed for all the cells treated with/without PEITC, which further confirmed the excellent acceptor/carrier ability of the AuNPs. These results clearly demonstrate that the fluorescence activation in apoptotic cells was specific to caspase-3 activation. To further verify the internalization of the AuNP/peptide-TPdye sensing nanoconjugates in apoptotic HeLa cells, Z-scanning confocal imaging was performed (Fig. S6, ESI[†]). It was clear that bright fluorescence was present throughout the whole cells, which suggested efficient delivery of the nanosensing conjugate into the cytosol. This revealed that the nanosensing conjugate afforded a robust intracellular protease sensor for high-contrast TPE imaging of caspase-3 activation in live cells.

During liver surgery, surgeons may perform temporary inflow and/or outflow occlusion by vascular clamping to reduce intraoperative blood loss; this technique is referred to as hepatic ischemic reperfusion (HIR). However, this HIR can usually lead to the apoptosis of liver cells, thus resulting in injury of the liver tissue after surgery. Therefore, we finally applied the formed AuNP/peptide-TPdye nanosensor to

Fig. 3 TPM images of HeLa cells treated with (+)/without (-) PEITC. The experimental steps were described in the experimental section (ESI[†]).

Fig. 4 TPE images of the 1.0 mm-thick rat liver tissue slice at a depth of 100 μm and the corresponding 3D images accumulated along the Z-direction at a depth of 0–300 μm after incubation with the AuNP/peptide-TPdye nanoconjugate. IR: with hepatic ischemia–reperfusion surgery; normal: without hepatic ischemia–reperfusion surgery.

monitor caspase-3 activity in a 1.0 mm-thick liver tissue slice obtained from a normal rat model and an ischemia reperfusion-treated rat model by using a TP confocal microscope after the sensing nanoconjugate incubation. The TP fluorescence images (Fig. 4) show that the AuNP/peptide-TPdye nanosensor provided high TPE fluorescence emission signal for the hepatic ischemic reperfusion-treated rat model, while the TPE fluorescence emission signal from the normal rat model was substantially weaker, indicating the significantly increased activity of caspase-3 in liver cells for the ischemic–reperfusion-treated rat model due to the apoptosis of liver cells, and thus will result in the injury of liver tissue. Overall, our presented data show that the prepared AuNP/peptide-TPdye nanosensor can be used for high-contrast TPE caspase-3 imaging assay in apoptotic cells and deep tissues.

In conclusion, we have developed a TPE nanosensor for detecting caspase-3 in live cells and tissues. The nanosensor is formed *via* the assembly of TPdye-labeled peptide sequences onto the AuNP's surface. The AuNP/peptide-TPdye nanoconjugate exhibits good cell permeability, high stability and good biocompatibility. Compared with the nowadays prevailing strategy of one-photon-excitation-based fluorescent biosensor construction, the introduction of a two-photon dye as the signal reporter effectively eliminates problems of self-absorption and autofluorescence in biological matrices while using long excitation wavelength and offers high, larger depth penetration, as well as less photodamage. Moreover, the TPdye BMVC used in this work has very weak fluorescence emission in aqueous solution, while its fluorescence emission will be enhanced greatly when the molecules are inserted into dsDNA, thus resulting in a high signal-to-background ratio. *In vitro* and *in vivo* assays revealed that the AuNP/peptide-TPdye nanoconjugate provided a robust, sensitive, and selective sensor for the measurement of caspase-3 even under complex biological conditions. To the best of our knowledge, this is the first time that an AuNP/peptide-TPdye two-photon nanosensor has been successfully used for the caspase-3 assay in live cells and liver tissues treated by ischemic reperfusion surgery. This new AuNP/peptide-TPdye two-photon nanosensor is expected to have great potential for applications in anti-HIRI drug screening, medical research and clinical diagnostics.

Conflict of interest

The authors declare no competing interests.

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