The Development of a TNTs@MPDA@MnO_2/CG Composite Hydrogel: Synergistic Multi-Enzymatic Activities and Photothermal Effect for Enhanced Diabetic Wound Repair

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Experimental Section

1.1 Materials

Titanium oxide (TiO_2, AR, 99.5%), 1, 3, 5-trimethylbenzene (TMB, AR, 97%), tris (hydroxymethyl) aminomethane (Tris, 99.9%), dopamine hydrochloride (DOPA 98%), sodium chloride (AR), salicylic acid (SA, 99.5%) and riboflavine (98%) were purchased from Aladdin industrial Inc. Pluronic® F-127 were obtained from Sigma-Aldrich. Agar powder (BR), 3,3′,5,5′-tetramethyl benzidine (TMB, 98%) and titanic sufate ($Ti(SO_4)_2$, 96%) were purchased from Maeklin. Yeast extract was purchased from Solelybio mall. Ethanol (AR), Acetone (AR), potassium permanganate ($KMnO_4$), anhydrous ethanol (C_2H_5OH) was purchased from Sinopod Group Chemical Reagent Co., Ltd. Ferrous sulfate ($FeSO_4$) was purchased from Tianjin Baishi Chemical Co.,Ltd. , Methionine and cationic guar gum (CG) with a viscosity of ≥ 2000 mPa·s at 1 % concentration were purchased from Shanghai yuanye Bio-Technology Co., Ltd. Nitrotetrazolium Blue chloride (NBT, 98%) was obtained from Meryer (Shanghai)Chemical Technology Co., Ltd. *Escherichia coli* (CGMCC 1.2385) and *Staphylococcus aureus* (CGMCC 1.12409) were obtained from the China General Microbiological Culture Collection Center (CGMCC). Both strains were cultured in LB medium at 37 °C.

1.2 Characterization

The morphology of the samples was characterized by highresolution

transmission electron microscopy (HRTEM, FEI Crop TF 20, JEOL, Japan) and scanning electron microscopy (SEM, ZEISS Gemini SEM 300, Carl Zeiss AG, Germany). The elements of the nanoparticles were determined by X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo Scientific, USA). The specific surface area, pore volume, and pore diameter of the samples were characterized using a fully automatic specific surface and porosity analyzer (BET, ASAP 2460, McMuritik Instrument Co., Ltd). The functional groups of the nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR, VERTEX 70, Bruker, Germany). UltravioletVisible spectrometry (UV-Vis, NanoDrop 2000c, Tianmei Instrument Co., Ltd) was used to study the methods for Evaluating Enzyme-like Activities and Hydroxyl Radical Scavenging Ability of Samples The stability and particle size of the nanomaterials in the aqueous phase were determined using a nanoparticle size and zeta potential analyzer (Malvern Zetasizer Nano ZS90, Malvern Instruments Ltd, UK).

1.3Preparation of the TNTs@MPDA@MnO_2 nanoparticles

1.3.1 Preparation of TNTs nanoparticles. First, 2.5 g of TiO_2 powder was mixed with 35 mL of 10 M (mol/L) NaOH solution (prepared by dissolving 14 g of NaOH in 35 mL of deionized water) in a 50 mL polytetrafluoroethylene-lined autoclave. Subsequently, the autoclave was placed in a constant-temperature oven at 140 °C for 48 h, yielding a white gel after the reaction. The gel was then washed with deionized water until neutral, then soaked in 10 mL of 0.1 M (mol/L) HCl solution (prepared by diluting 1 mL of concentrated hydrochloric acid to 100 mL) for 1 h. Finally, it was dried at 105 °C for 5 h to get TNTs powder for further experiments.

1.3.2 Preparation of TNTs@MPDA nanoparticles. First, 60 mg of TNTs were dispersed in 2 mL of deionized water and ultrasonicated to achieve a homogeneous dispersion, which was then reserved for later use. Next, 65 mL of deionized water and 60 mL of anhydrous ethanol (EtOH) were measured, and 0.3632 g of F127 block copolymer was weighed. Also, 625 μL of TMB was measured. Both F127 and TMB were added almost simultaneously to the mixed solution of ethanol and water,

followed by magnetic stirring at room temperature for 30 min (without heating). Then, 90 mg of tris(hydroxymethyl)aminomethane (Tris) was dissolved in 5 mL of deionized water and added to the above mixture, with continued stirring for 2 min. Then, 40 mg of dopamine Hydrochloride (DOPA) was dissolved in 5 mL of deionized water and added to the mixture. The mixture was transferred to a shaker, and the pre-dispersed TNTs were quickly added to the mixture. The reaction was carried out in the shaker at 40 °C for 24 h. After the reaction, the resulting TNTs@MPDA nanocomposite was transferred to a beaker and dried in an oven at 50 °C for 6 h. Once dried, the product was scraped off and stored in centrifuge tubes for subsequent use.

1.3.3 Preparation of TNTs@MPDA@MnO_2 nanozymes. First, 0.1 g of TNTs@MPDA was dispersed in 10 mL of deionized water and ultrasonicated for 30 min to ensure achieve a homogeneous dispersion. Then, a potassium permanganate ($KMnO_4$) solution was added dropwise to the dispersion under continuous stirring. After a 1 h reaction, the crude product was collected by centrifugation and washed twice with anhydrous ethanol and deionized water to remove impurities. Finally, the product was dried in a vacuum oven at 50 °C for 12 h to obtain TNTs@MPDA@MnO_2 bioactive nanozymes.

1.4 Preparation of TNTs@MPDA@MnO_2/CG hydrogel. 1 g of CG powder was added to 20 mL of deionized water and vigorously stirred at 25 °C for 30 min to ensure complete dissolution. Then, TNTs@MPDA@MnO_2 (40 mg/mL) was added to the solution, and the total volume of the reaction mixture was adjusted to 40 mL using deionized water. After vigorous stirring for an additional 2 min at 25 °C, the mixed solution was allowed to stand at 25 °C to form the hydrogel.

1.5 POD-Like Activity. The POD enzyme facilitated the decomposition of H_2O_2 to generate ·OH. These ·OH radicals then oxidized 3,3',5,5'-TMB into its oxidized form (OxTMB). This oxidation reaction yielded a water-soluble blue-colored product with characteristic UV-vis absorption maxima at 370 nm and 652 nm. The POD-like catalytic activity of hydrogels can be quantitatively evaluated using UV-vis spectroscopy. Specifically, it is determined measuring the absorbance changes at these two characteristic wavelengths after the hydrogels react with H_2O_2.

1.6 CAT-Like Activity. Catalase catalyzed the decomposition of H_2O_2into H_2O and O_2. The catalase enzyme facilitates the catalytic. The remaining H_2O_2 then reacted with titanium (IV) sulfate ($Ti(SO_4)_2$) to form a yellow peroxo-titanium coordination complex. This complex exhibited a characteristic absorption peak at 405 nm. By accurately measuring the absorbance at 405 nm using UV-vis spectroscopy, the concentration of H_2O_2 could be quantitatively determined.

1.7 SOD-like activity. Riboflavin underwent reduction upon exposure to light and then re-oxidized under aerobic conditions to generate $O_2^{-}\cdot$. These superoxide anions could reduce NBT to form blue formazan, which had a characteristic absorption peak at 560 nm. SOD could scavenge the superoxide anion and inhibit this reaction. Therefore, the SOD-like activity of cryogels could be measured by detecting the absorption peak at 560 nm using UV - vis spectroscopy.

1.8 Swelling and Degradation Properties of Hydrogels.

1.8.1 Swelling Properties of Hydrogels. First, the lyophilized hydrogel samples (maintaining their original dimensions) were precisely weighed and the initial weight (W_0) was recorded. Then, the samples were immersed in PBS (pH 7.4) and the swelling experiment was conducted at a constant temperature of 37°C. At predetermined time intervals, the samples were removed, the surface was gently blotted with filter paper to remove excess solution, and their weight was immediately measured. The weight changes of the hydrogels over time (W_t) were continuously monitored and recorded until the swelling equilibrium was reached, and calculations were carried out using the following formula (1.1).

$$\text{Swelling ratio}(\%) = {}^{W_t - W_0}\!/_{W_0} \times 100\% \tag{1.1}$$

1.8.2 Degradation Properties of Hydrogels. To systematically assess the degradation characteristics of the designed hydrogel, the samples were first immersed in PBS (pH 7.4) at 37°C until they reached swelling equilibrium. Recorded the sample mass as W_s. Then, at set intervals, took out the samples, blotted them gently with filter paper to remove excess solution, and weighed them immediately to get W_t.

Calculated the degradation ratio (DR) at specific time points using the following formula (1.2).

$$\text{Degradation ratio}(\%) = {}^{Ws\ -\ Wt}\!/_{Ws} \times 100\% \tag{1.2}$$

1.9 Investigation of Photothermal Properties. To systematically explore the photothermal conversion efficiency of various nanocomposite hydrogels, this study selected pure CG, TNTs/CG, TNTs@MPDA/CG, and TNTs@MPDA@MnO_2/CG as research objects. An 808 nm near-infrared laser (power density: 2 W/cm²) was used to evaluate the photothermal performance. Each sample was continuousil irradiated for 600 seconds. After irradiation, the laser was switched off, and temperature monitoring continued for another 600 seconds. This heating-cooling cycle was repeated three times to assess the photothermal stability of hydrogels doped with different nanoparticles. The photothermal conversion efficiency of each hydrogel group was calculated and comparatively analyzed according to Equations (1.3, 1.4, and 1.5).

$$\eta = \frac{hs\left(T_{max,m} - T_{surr}\right) - hs(T_{max,water} - T_{surr})}{I(1 - 10^{-A_{808}})} \tag{1.3}$$

$$\tau_s = \frac{m_s C_s}{hs} \tag{1.4}$$

$$t = -\tau_s \ln({}^{\Delta T}\!/_{\Delta T_{max}}) \tag{1.5}$$

In the photothermal conversion efficiency calculation model, the parameters were defined as follows: h represented the heat transfer coefficient (W/m²·K), s denoted the specific surface area of the sample container (m²), m_s and C_s corresponded to the mass of water (g) and specific heat capacity (4.2 J/g·℃), respectively. I indicated the laser output power (2 W), A 808 represented the sample absorbance at a wavelength of 808 nm, $T_{max,\ m}$ was the maximum temperature increase of the sample (℃), T_{surr} referred to the ambient temperature (℃), ΔT and ΔT_{max} represented the instantaneous and maximum temperature differences between the hydrogel and ambient temperature at time t (℃), respectively. η denoted the photothermal conversion efficiency (%), and τ_s represented the thermal time constant of the hydrogel during the natural cooling

process.

1.10 Investigation of Mechanical Properties of Hydrogels. The mechanical properties of all cubic hydrogel samples (with dimensions of 15 mm × 11.5 mm × 9 mm) were evaluated using a mechanical testing system from Stable Micro Systems, UK. The hydrogel samples were compressed at a constant rate of 10 mm/s until a strain of 80% was reached Stress-strain curves were recorded during the entire compression process for subsequent analysis of mechanical properties.

1.11 In Vitro Antibacterial Activity Evaluation

1.11.1 Preparation of LB Medium and Bacterial Culture. The LB liquid medium was prepared by dissolving 10 g sodium chloride, 10 g tryptone, and 5 g yeast extract in 1 L of deionized water. The mixture was thoroughly stirred until complete dissolution. Then, 400 mL of the solution was aliquoted into four sterile conical flasks, with each flask containing 100 mL, and the flasks were sealed for later use. To the remaining 600 mL of the solution, 16 g of agar powder was added. The mixture was heated until the agar powder dissolved and then evenly distributed into four conical flasks to prepare solid medium.

1.11.2 Preparation of Bacterial Suspension. All experimental instruments were sterilized by autoclaving. 15-20 mL of LB solid medium was poured into each sterile Petri dish and left to solidify at room temperature. Single colonies of *E. coli* and *S. aureus* were separately inoculated into 100 mL LB liquid medium. The cultures were then cultured at 37 °C with shaking at 200 rpm for 16 h. Subsequently, the bacterial suspensions were diluted to a concentration of 1×10^6 CFU/mL using sterile PBS buffer.

1.11.3 Antibacterial Performance Evaluation. 10 μL of bacterial suspension was co-cultured with CG, TNT/CG, TNTs@MPDA/CG, and TNTs@MPDA@MnO_2/CG samples at 37 °C for 1 h. Subsequently, the samples were irradiated with an 808 nm near-infrared laser irradiation (power density: 2 W/cm²) to assess their photothermal antibacterial properties. After irradiation, 990 μL of sterile PBS was added to each sample, which was then vortexed for 2 min to thoroughly resuspend the surviving

bacteria. 20 μL of the bacterial suspension was spread onto LB agar plates and incubated at 37°C for 12 h for colony counting analysis.

1.12 Investigation of Cytocompatibility. L929 cells were seeded in 48-well plates at a density of 1×10^4 cells per well and cultured for 12 hours to ensure they adhered completely. Subsequently, the original culture medium was replaced with hydrogel extracts derived from CG, TNTs/CG, TNTs@MPDA/CG, and TNTs@MPDA@MnO_2/CG. These extracts were then co-incubated with the cells for 1 day. After the incubation period, the cells were washed three times with PBS. Then, 300 μL of CCK-8 working solution was added to each well. Finally, the OD at 450 nm was measured using a microplate reader. In contrast, the control group was treated with complete culture medium without hydrogel extract, serving as a reference for comparison.

1.13 Wound Healing Assay. L929 cells were seeded in 24-well plates at a density of 10×10^4 cells per well and cultured until they reached 100% confluency. A sterile 1 mL pipette tip was used to create a uniform scratch in the cell monolayer. The wells were gently washed twice with PBS to remove dislodged cells, and initial images of the scratched area were captured using an inverted fluorescence microscope. Subsequently, hydrogel extracts from CG, TNTs/CG, TNTs@MPDA/CG, and TNTs@MPDA@MnO_2/CG were added to their respective wells and co-cultured with the L929 cells. After 24 hours of incubation, the scratched area was re-imaged to assess the migration of cells from the periphery into the scratch region. The blank control group was treated with complete medium without any hydrogel.

1.14 Transwell Assay. L929 cells (1×10^5 cells per well) were seeded in the upper chamber of a 24-well Transwell system (4 μm pore filter; Corning, USA) and co-cultured with various hydrogels for 24 hours. After incubation, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with DAPI. The upper membrane surface was swabbed to remove non-migrated cells, and a laser microscope was used to quantify the migrated cells.

1.15 Scavenging of ROS in L929 Cells. L929 cells (2.0×10^4 cells per well) were seeded in 48-well plates and cultured overnight. Subsequently, these cells were

treated for 12 hours with hydrogel extracts from CG, TNTs/CG, TNTs@MPDA/CG, and TNTs@MPDA@MnO_2/CG, each containing 100 μM H_2O_2. Intracellular ROS were stained with DCFH-DA (Beyotime Biotechnology) for 30 minutes, and then the cells were washed three times with PBS.

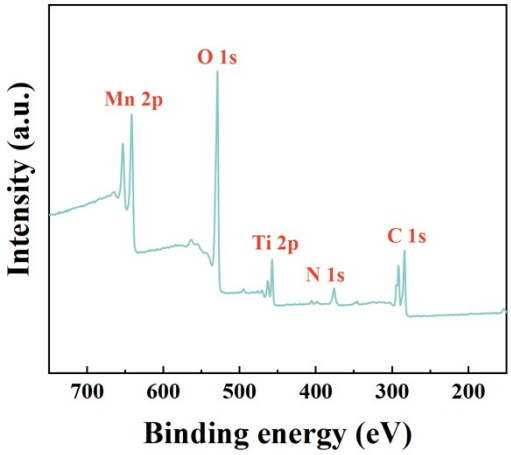

Figure S1 Full XPS spectra of TNTs@MPDA@MnO_2 nanoparticles

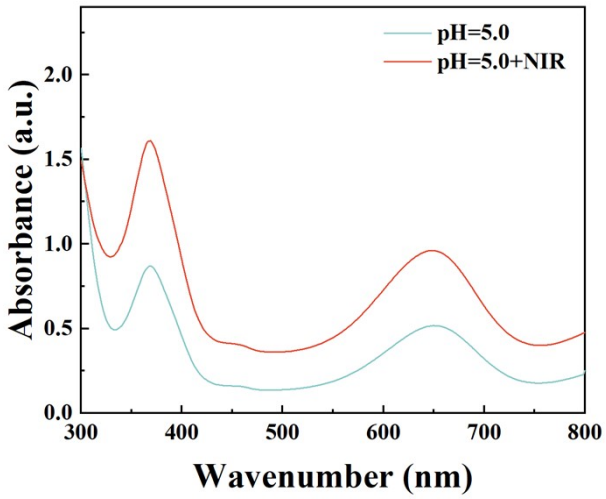

Figure S2 Enhanced POD-like activity of MnO_2 nanozymes under NIR irradiation.

Declaration of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled