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Modular Engineering of a Synthetic Biology-Based Platform for Sustainable Bioremediation of Residual Antibiotics in Aquatic Environments

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ABSTRACT

Tetracycline (TC) residues from anthropogenic activities undesirably present in nature as an emerging sustainability challenge and thereby require innovations in remediation technologies. Herein, as inspired by the microcompartment structure in living organisms, we adopt a synthetic biology approach to engineer the FerTiG, a modular enzyme assembly, to robustly scavenge TC residues with improved performance. The FerTiG consists of three functional modules, namely, a TC degradation module (Tet(X4)), a cofactor recycling module glucose dehydrogenase (GDH), and a protection module (ferritin), to organize diverse catalytic processes simultaneously as a biological circuit. The incorporation of GDH suitably fuels the FerTiG-dependent TC degradation by regenerating expensive nicotinamide adenine dinucleotide phosphate (NADPH) cofactor with glucose. The ferritin shields the catalytic core of FerTiG to resiliently decompose TC under unfavorable conditions. Due to collaboration among functional modules, FerTiG strongly catalyzes the residual TC removal from multiple environmental matrices. The degradation pathways and environmental/biological safety of FerTiG are then elaborated, indicating the promising readiness for the application of FerTiG. In summary, this work presents a synthetic biology-based strategy to spontaneously impose residual antibiotic biodegradation for better sustainability management. The FerTiG is engineered as a proof-of-principle for TC removal; however, this 'microcompartment-mimicking' concept is of great interest in mitigating other sustainability challenges where modular catalytic machinery is applied.

1. Introduction

As one of the fundamental discoveries of the 20th century, the introduction of antibiotics into clinical use revolutionized modern medicine and greatly extended the human lifespan [1]. In addition to their frequent application in clinical settings to eradicate infections, a large amount of antibiotics has also been consumed for agricultural purposes (mainly including pastoralism and aquaculture) to enhance health and productivity of animals [2,3]. Several recent surveys have predicted that the total consumption of antibiotics will exceed approximately 200 000 t in 2030, among which agricultural use accounts for the majority [4,5]. In this regard, the cumulative utilization of antibiotics in such quantities poses a global challenge for environmental management and public health, as antibiotic residues are an inevitable consequence of the intensified production and consumption of antibiotics [6–8]. Among the commonly utilized antibiotics, tetracycline (TC) class antibiotics (i.e., TC, chlortetracycline (CTE), oxytetracycline, and doxycycline (DOX)) have been employed to eradicate infections caused by an extensive panel of microbial pathogens [9]. This

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broad-spectrum nature, combined with the advantage of low cost, makes TCs extensively applicable in agriculture, especially in the livestock industry [10]. In this regard, TC has been one of the most extensively used antibiotics over the years in major continents, where China, as well as the European Union and the United States are both important producers and consumers of TC for veterinary purposes [11]. Given the considerable demand for TCs as a first-choice empiric treatment, even though TCs are no longer permitted for use as feed additives in most countries and regions, the application and production of TCs foster a market worth over 1.4 billion USD per year and is still growing [12].

In face of TCs produced and consumed in very large quantities, it is well established that TCs are always poorly absorbed and metabolized in the host body and are generally emanated into the surroundings via excreta in their prototype [13,14]. These TCs remaining in the host, along with the improper disposal of pharmaceutical effluents during TC production, collectively contribute to the massive TC residues discharged into both natural and anthropogenic environments [15]. As exemplified in a prior study, TCs were found to be the most dominant antibiotic residues in the soil in many sites in China, probably as a result of the use of animal manure for fertilization [16]. However, this scenario is even more worrisome because TC residues are reported to accumulate at an alarming rate in ecologies closely related to human life, such as sewage, lakes, rivers, and even drinking water [17,18]. On the one hand, the persistence of such unwanted TC residues has been shown to promote the evolution and transmission of antibiotic-resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) by elevating the selection pressure in a given niche [19]. On the other hand, residual TCs are able to directly cause nephrotoxic, neurotoxic, or reproductive toxicity to humans or animals after accumulating through the food chain or water supply to certain levels [20]. Considering their environmental and biological toxicity, the TCs are identified as an emerging environmental pollutant belonging to the pharmaceuticals and personal care products (PPCPs) that are capable of exerting harmful effects at low concentrations [21]. Given the harmful persistence of TCs, it is essential to take rapid and sustainable measures to reduce TC pollution, which accumulates in the environment at a very high rate. Although residual TCs could be degraded by photolysis or hydrolysis under natural conditions, the corresponding amount and rate are still very limited. The majority of these TC residues persist in the environment for up to 100 days if no action is taken [22]. To address this, a plenty of methodologies have been developed to degrade or inactivate residual TCs from their active form. Common techniques often decompose the TC by physicochemical destruction, including photocatalysis, electrochemical oxidation, photoelectrocatalytic degradation, and Fenton chemistry. Current physicochemical approaches [23], which are rapidly evolving with nano and catalytic technologies, are generally efficient. However, the technical factiousness and potential introduction of harmful intermediates for these methodologies are the biggest challenges that have not yet been overcome [24]. Emerging as an alternative, have gradually gained special attention due to their merits in eco-friendliness and easy-handling properties [25]. In the history of antibiotic biodegradation, a range of environmental organisms, including bacteria and fungi, have been identified to decompose the TCs under certain conditions [26]. Recently, modern biotechnology has enabled the bioengineering of bacteria to heterogeneously express specific enzymes to inactivate antibiotics [27]. In addition to the use of such organisms, the discovery and use of degradative antibiotics to remove residual antibiotics has also been a vital option. As demonstrated in our previous work, enzymes such as Tet(X4) are able to actively modify a wide range of TCs without introducing hazardous substances [28–30]. However, it is thought that there are advantages and disadvantages to using living microorganisms or degradative antibiotics (Fig. 1(a)). For instance, the self-replicating nature of degradative microorganisms allows them to spontaneously eradicate residual TCs efficiently and at a low cost. However, these applications are sometimes risky because those living organisms are likely to colonize the hosts and mediate the genetic drift of antibiotic resistance to commensal microbe [31]. In contrast, the use of pure TC destructases as non-replicable, DNA-free proteins is quite safe; however, their efficiency and cost are often suboptimal due to poor flexibility and the need for expensive cofactors [28]. As such, there is still a need for innovative approaches that balance cost, efficiency, and safety. In the search for novel biodegradation alternatives, it is always important to draw inspiration from living organisms because natural selection has evolved numerous structures, models, systems, and processes optimized for a wide panel of functionalities [32]. In contrast to enzymes, living degradative organisms incorporate multi-component catalytic machinery to constantly fuel the enzymatic eradication of residual TCs [33]. Additionally, the catalytic core enzymes are encapsulated by lipid membranes to offset the environmental stresses for efficient TC removal in the presence of various harsh conditions. In prokaryotes, many metabolic reactions take place in microcompartments defined as proteinaceous organelles to confine specific enzyme cores in a thin protein-based shell to organize chemical transformations in space (Fig. 1(b)) [34]. Inspired by this unique structure, we leverage the power of synthetic biology to design a microcompartment-like biodegradation system composed of functional protein modules to mimic living organisms for the robust removal of TC residues. For this purpose, a modular protein complex, consisting of a Tet(X4) for TC decomposition, a glucose dehydrogenase (GDH) for co-factor recycling, and a ferritin for core enzyme protection, was constructed as ferritin-caged Tet(X4) with co-factor recycling property that powered by GDH (FerTiG). This FerTiG platform was observed to degrade residual TCs with improved efficiency and cost-effectiveness under both lab and various environmental conditions, including tap water, lake water, livestock sewage, and pharmaceutical Wastewater. Compared with the exploitation of free enzymes, FerTiG was highly resilient to adverse environmental conditions and long-term storage. Further analysis confirmed the favorable biosafety and environmental safety of FerTiG, which was applied without potential risk to the environment and host. Overall, FerTiG was constructed as an enzymatic approach to remediate the residual TC contamination with benefits to living organisms. As a proof-of-principle, we engineered a minimal set of functional modules that were capable of spontaneous and robust TC removal from environments. However, this synthetic biology-based biodegradation platform possesses great potential for incorporating more modules to facilitate additional functionalities for engineering in sustainability management.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli (*E. coli*) strain DH5α was cultured at 37 °C in Luria broth (LB) medium with kanamycin at a concentration of 50 μg·mL^{-1} for plasmid DNA extraction. For protein expression, *E. coli* strain BL21 (DE3) was cultured at 37 °C in LB medium with 50 μg·mL^{-1} kanamycin.

2.2. Construction of FerTiG

The pET28a-Tet(X4)-GDH was first constructed by fusion of Tet(X4) fragment and GDH fragment using ClonExpress Ultra One Step Cloning Kit (catalog No.: C115-02; Vazyme China). It
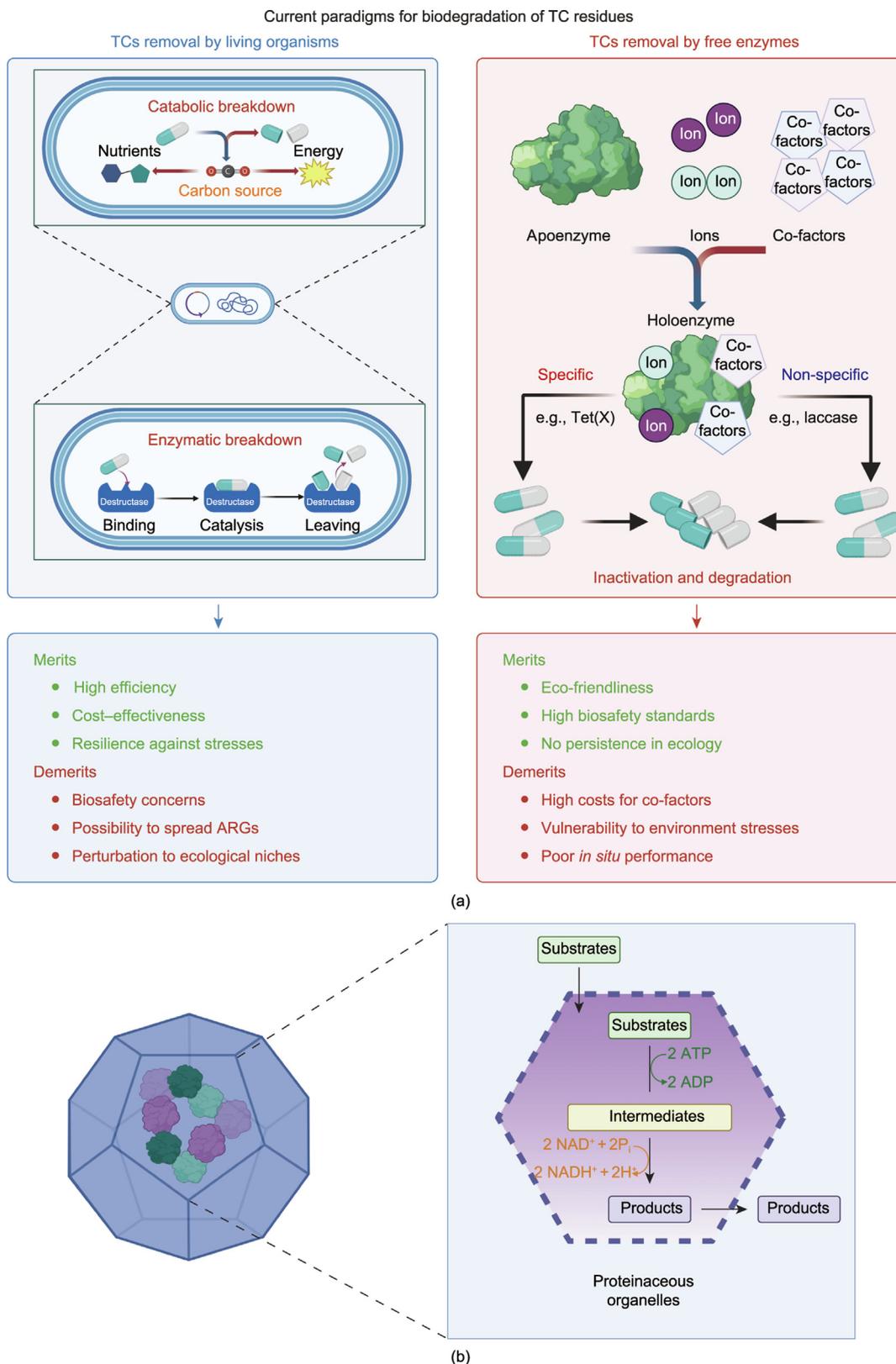
Fig. 1. Paradigms and perspectives in context of residual TC biodegradation. (a) The current paradigms for biodegradation of TC residues based on living organisms and enzymes; (b) model for the organized catalytic process in the microcompartments of living organisms.

was then used as a template to obtain pET28a-Tet(X4)-GDH-recombinant human heavy-chain ferritin (rHF) by polymerase chain reaction (PCR). The recombinant plasmid prepared according to the previous protocol with minor modifications was electroporated into BL21, and positive transformants were identified via primers pET28a-F/pET28a-R and confirmed by sequencing.

2.3. Expression and purification of FerTiG enzyme

The strain carrying pET28a-Tet(X4)-GDH-rHF was cultivated at the temperature of 37 °C and angular velocity of 180 r·min^{-1} to an optical density at 600 nm (OD$_{600}$) of 0.6, then isopropyl-β-D-thiogalactopyrandoside (IPTG) was added to a final concentration of 1 mmol·L^{-1}. The culture was next induced in the presence of temperature and angular velocity of 16 °C and 180 r·min^{-1}, respectively, in a shaking incubator overnight. The bacterial pellets were then harvested for lysis using sonication, and the lysates were subjected to a pre-equilibrated HisTrap HP column (GE Healthcare, USA) for subsequent purification. In continuing, the captured FerTiG protein was appropriately released by elution buffer (wash buffer with 500 mmol·L^{-1} imidazole) and then measured by bicinchoninic acid assay (BCA) protein quantification kit (Thermo Scientific, USA) followed by dialysis for 20 h at 4 °C.

2.4. Characterization of FerTiG

The as-prepared FerTiG was first characterized by immunoblotting via purified Tet(X4) as a control, which was visualized by a chemiluminescence multiplex blot imaging system (Tanon-5200SF; Tianneng Co., China). The size exclusion chromatography (SEC) analyses were then performed according to the previous protocol [35] with minor modifications via a Waters 515 solvent delivery system (Waters, USA). Morphological observations of FerTiG were performed by negative staining with phosphotungstic acid for transmission electron microscopy (TEM) observation, in which 30 μL (0.25 mg·mL^{-1}) of Tet(X4) and FerTiG were both adopted.

2.5. Enzymatic kinetics of FerTiG

FerTiG enzymatic kinetics were performed based on the reference work [28]. Briefly, the reaction was carried out in TC substrate mixture at various concentrations (0, 2.5, 5.0, 8.0, 10.0, 20.0, 30.0, 40.0 μg·mL^{-1}) supplemented with 2.5 mmol·L^{-1} nicotinamide adenine dinucleotide phosphate (NADPH) and 5 mmol·L^{-1} MgCl$_2$ in a final volume of 200 μmol·L^{-1} in 100 mmol·L^{-1} TAPS buffer (pH 7.4). The reaction was initiated by the addition of either Tet(X4) or FerTiG and then visualized by the dynamical change of absorbance at 360 nm (wavelength of maximum absorbance for the substrate) via a microtiterplate reader (TECAN, Germany). The curve was plotted against substrate concentration according to the Michaelis–Menten equation, and the kinetic was determined as K_{cat}/K_M (K_{cat}: first-order rate constant; K_M: Michaelis constant).

2.6. Comprehensive analysis of TC degradation by FerTiG

The microbiological degradation assay was performed per the previous research work [29]. In brief, a total of 10 parts per million (ppm, 1 ppm = 1 mg·L^{-1}) TC solution was subjected to a panel of treatments, including phosphate-buffered saline (PBS; as control), Tet(X4), and FerTiG with or without glucose. After incubation at 37 °C, samples were detected at 0.5, 1, 3, 6 h, and quenched with trifluoroacetic acid (TFA). Aliquots of each sample were then spotted on the Mueller–Hinton agar plates containing *Bacillus stearothermophilus* ATCC 7593. The plates were incubated for 5 h at 60 °C, and degradation was determined by the size of the inhibition zones.

The kinetics of FerTiG degradation were then investigated using high-performance liquid chromatography (HPLC), as previously reported, with minor modifications. Briefly, a total of 50 ppm TC solution was incubated with the above treatments continuously at 37 °C, and samples were harvested at 0.5, 1, 3, 6, and 9 h after incubation. Then, 500 μL aliquots were combined with 5 μL TFA to quench the reaction at each interval for HPLC analysis via

Athena C18-WP with a mobile phase of water containing 0.1% formic acid (A) and methanol (B). To reveal the broad spectrum nature of FerTiG, the degradation kinetics of CTE, DOX, tigecycline (TGC), and even eravacycline (ERA) at 50 ppm concentration were subjected to the same system.

2.7. Cofactor recycling property of FerTiG

The cofactor recycling of FerTiG was determined as the regeneration of the NADPH in the presence of glucose. The free Tet(X4) or FerTiG were incubated with various concentrations of NADP$^+$ with or without the glucose, and thereafter, the generation of NADPH was assayed by the colorimetric method based on the commercial kits (Solarbio, China). The reaction was monitored at 460 nm for 30 min via a microtiterplate reader (TECAN).

2.8. Resilience analysis of FerTiG

To evaluate the resilience against environmental stresses provided by the protection module in FerTiG, the degradation efficiency of FerTiG was evaluated against various adverse factors, including pH (2–11), temperature (4–40 °C), ionic strength (NaCl, 0–50 mmol·L^{-1}), ultraviolet (UV) irradiation (30–90 min). In addition, the storage stability of FerTiG was methodically examined by keeping FerTiG at three distinct temperatures, namely 4, 25 (room temperature), and 37 °C. Then, at 0, 1, 3, 5, and 7 days post storage, FerTiG was taken out for degradation analysis as mentioned above. The free Tet(X4) was then utilized as a control throughout the resilience analysis.

2.9. TC degradation by FerTiG in environmental matrices

To address the practical application of FerTiG for *in-situ* TC degradation, FertiG was utilized to eradicate the residual TC from tap water, lake water, livestock sewage, and pharmaceutic wastewater as representative aquatic environments reported to be contaminated with TC residues. The tap water, lake water, and livestock sewage were spiked with 50 μg·mL^{-1} TC, whereas the pharmaceutic wastewater was employed as such. The FerTiG was then applied to these environmental matrices, and the TC residues were detected after 3 and 6 h based on the approach described above.

2.10. Identification of the TC transformation products

The TC transformation products were elucidated by FerTiG-mediated degradation by a previously described protocol [29]. Briefly, TC (50 μg·mL^{-1}) was incubated with FerTiG in pH 6.0 with potassium phosphate buffer at 37 °C, and then the mixture containing the transformation intermediates was appropriately analyzed via an Agilent 1290–6540 Q-TOF equipped with a Zobrax Extend-C18 column (Agilent, USA). The spectrometer was operated in positive electrospray ionization mode with a full spectrum scan from 150 to 2000 m/z (m/z). The system was driven by a quaternary pump, and the mobile phase consisted of water containing acetonitrile as well as methanol with gradient elution. The flow rate was set as 0.3 mL·min^{-1}, and the injection volume was set equal to 5 μL for each analysis.

2.11. Total organic carbon measurement

In order to measure the mineralization of the TCs, the total organic carbon (TOC) was measured before and after treatment of FerTiG. In brief, the TC solution was incubated with FerTiG according to aforementioned protocol and the samples were spike at 9 and 24 h post the incubation. The samples were analyzed by

using a TOC analyzer (TOL-L; Shimadzu, Japan) and the data were presented in triplicates.

2.12. Aquatic toxicity prediction

The ecological risk associated with the aquatic toxicities of FerTiG was first predicted using the ecological structure–activity relationships (ECOSAR, v2.0) program developed by USEPA [36]. Based on the molecular structures of the intermediates, the aquatic toxicity was expressed by the predicted lethal concentration 50% (LC50) values.

Safety evaluation: To further assess the toxicity of FerTiG and the products, a biosafety test was first conducted on a zebrafish model in which 180 zebrafish embryos were randomly divided into three groups. All embryos were incubated in the culture medium containing FerTiG and the transformation products at 28.5 °C. The medium was refreshed every 24 h. The morphology and hatching rate of zebrafish were recorded continuously for 7 d. Meanwhile, acridine orange and hematoxylin and eosin (H&E) staining were performed on zebrafish juveniles to decipher the abnormality.

To further evaluate the *in vivo* biosafety in mammals, the BALB/c mice were treated with FerTiG (high/low) or PBS control, and the required observations were conducted. The body weights of mice were consecutively recorded for 9 consecutive days. After the 9th day, the mice were sacrificed to collect the kidney, liver, and ileum for pathological study with H&E staining. The animal trial was carefully reviewed and approved by the Institutional Animal Ethics Committee of South China Agricultural University (2023G012).

2.13. Statistical analysis

All experiments were performed in biological replicates, and results are presented as mean ± standard deviation (SD). To this end, the descriptive analysis of the standard deviation was performed via GraphPad Prism v8.0 for data visualization. The statistical significance of differences between groups was also examined using an unpaired t-test or ordinary one-way analysis of variance (ANOVA) based on IBM SPSS (v22; SPSS Inc., USA). In addition, the differences were considered significant at p-values of < 0.05.

3. Results and discussion

3.1. FerTiG is designed by the inspiration of microcompartment structure in prokaryotes

In our previous work, a broad-spectrum TC destructase, the Tet(X4), was developed for the removal of TC residue in various aqueous environments due to desirable degradation efficiency (Fig. S1 in Appendix A) [28]. However, the holoenzyme assembly of Tet(X4) calls for exogenous supplementation of costly NADPH as cofactors, thereby substantially increasing the costs and technical difficulty for scale-up. Furthermore, as an enzyme-based degradation approach, the suboptimal efficiency of such methodologies under adverse conditions (e.g., pH/temperature/ionic strength) undermined their further application in practical scenarios [29]. Inspired by the microcompartment structures in living organisms, we leverage the power of synthetic biology to establish a modular enzyme complex that combines multiple functional modules to enhance the catalytic performance of Tet(X4). As demonstrated in Fig. 2(a), the multienzyme complex FerTiG (ferritin-caged Tet(X4) with co-factor recycling property that powered by GDH) was designed to encompass three basic modules, namely a TC degradation module, a cofactor recycling module, and a protection module. In this complex, the Tet(X4) was genetically fused to a

Bacillus-derived GDH and a rHF, which together contribute to Tet(X4)-mediated TC removal efficiently and robustly. In the initial design, the GDH and rHF were genetically fused to the C- and N-termini of Tet(X4) spaced by a flexible linker (GGGGS)₃. However, the multienzyme assembly was derived as inclusion bodies in an insoluble form (data not shown). This finding was also consistent with those of previous reports that the proteinaceous cages fused by ferritin subunits are prone to be captured inside the inclusion bodies if not folded properly in an exogenous expression system [35,37,38]. To address this, the spacer peptide between Tet(X4) and rHF was expanded to five tandem repeats of GGGGS for a suitable spatial arrangement of various proteins [39]. As expected, optimization of the spacer peptide led to successful multienzyme assembly (Fig. 2(b)). Compared with unengineered Tet(X4), the molecular weight of FerTiG alters to 99 kDa due to the incorporation of GDH and rHF. Additionally, the SEC of FerTiG and free Tet(X4) revealed that the microcompartment mimic FerTiG exhibits a relatively larger hydrodynamic radius than the Tet(X4) apoenzyme (Fig. S2 in Appendix A). To further confirm the structural features of FerTiG, TEM was implemented. The obtained results were indicative of the fact that the FerTiG formed the globular-shaped scaffold, in which the three modular proteins resembled a microcompartment-like architecture (Fig. 2(c)). It is well established that, to some extent, the spatial proximity of a functional enzyme to other proteins may impair the catalytic activity by introducing steric hindrance at functional domain [40]. Thus, the logical next step was to validate whether genetic engineering affects the catalytic nature of Tet(X4). To this end, we first found that the Tet(X4) fused to FerTiG retained high activity for TC degradation as the peak for TC at 6.6 min was significantly reduced after 9 h treatment with FerTiG (Fig. 2(d)). Furthermore, the kinetic parameters for FerTiG were shown to be dynamically reduced by the dynamics in maximum absorption over time using spectrophotometry, and the results showed that the modular engineering gave Tet(X4) a slightly superior catalytic efficiency as the K_{cat}/K_M for FerTiG increased (Fig. 2(e)). This was consistent with the findings of a previous report, as it was observed that the enzymatic activity of methyl parathion hydrolase was improved when incorporated with the ferritin [39]. Although the exact mechanism of the improved enzymatic performance was unclear, we proposed that the interior cavity of the ferritin proteinaceous cage served as a reaction chamber to accelerate the catalysis by the proximity effect, which was expected to maximally overcome the diffusion-limited kinetics [41]. In summary, a modular enzyme assembly, FerTiG, was generated by the genetic integration of different protein modules, which was further employed to assess whether the modular assembly facilitates Tet(X4)-mediated TC eradication.

3.2. GDH in FerTiG fuels TC degradation via cofactor recycling

As mentioned above, the application of Tet(X4) in the reduction of unwanted TC residuals from the environment is mainly restricted by the constant consumption of the costly cofactor NADPH. Therefore, efforts to recycle such cofactors for maximally reducing the cost and tediousness are commonly taken as the only way to facilitate the application and feasibility of Tet(X4). It has been well-established that NADPH and its reduced form NADP⁺ are involved in a wide range of anabolic reactions in almost all organisms, where NADPH and NADP⁺ can be converted to each other by various metabolic pathways and enzymes [42]. Since the Tet(X4) mediates TC conversion at the expense of NADPH consumption to NADP⁺ as a flavin-dependent monooxygenase [43], we proposed that combination with enzymes for the reverse catalysis of NAPH⁺ to NADPH was able to power the TC removal and reduce
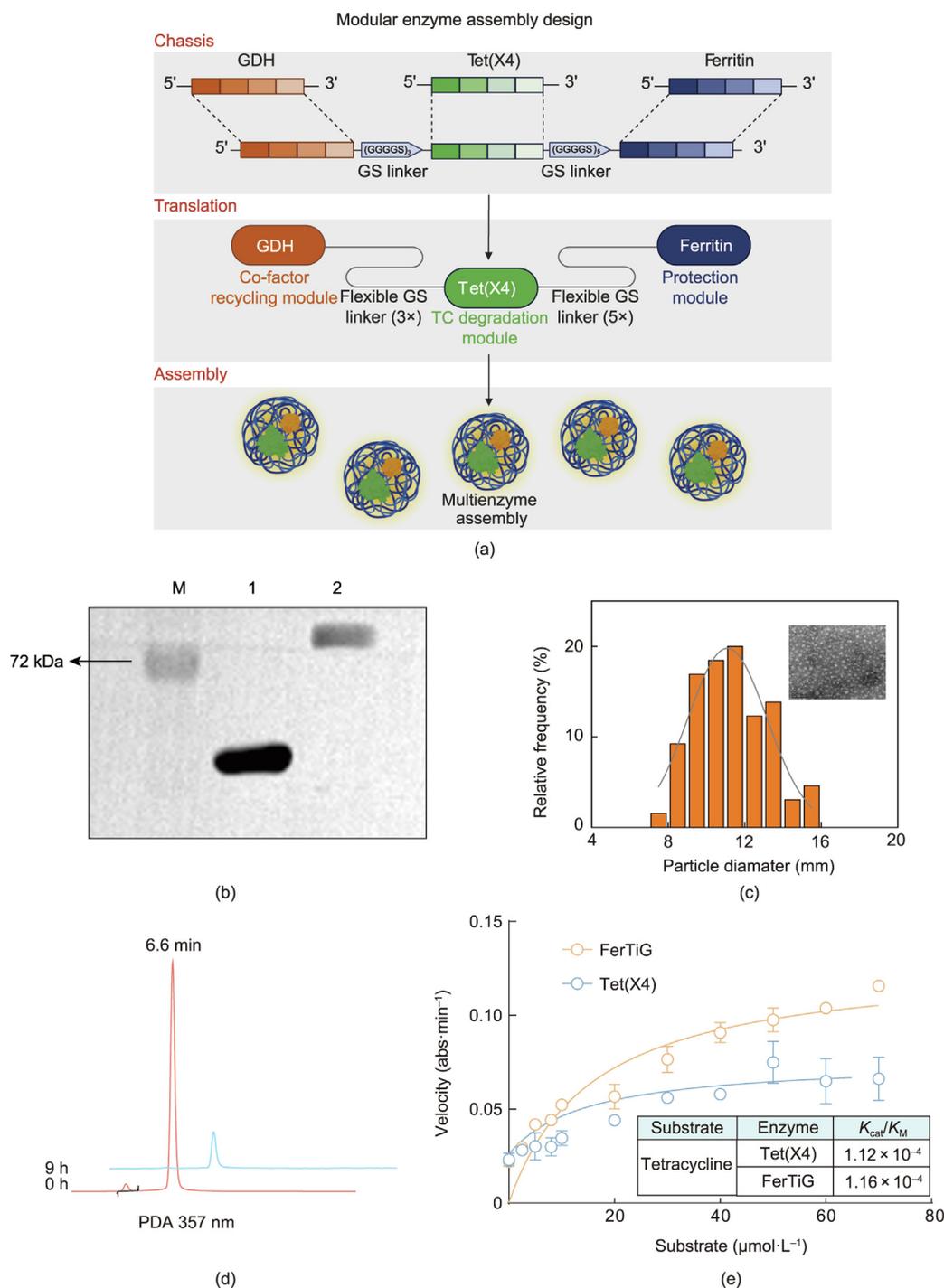
Fig. 2. Design and characterization of FerTiG. (a) Schematic illustration for design and assembly of FerTiG; (b) representative immune-blotting of free Tet(X4) and FerTiG (lane M: marker; lane 1: free Tet(X4); lane 2: FerTiG); (c) representative TEM images of negatively stained FerTiG; (d) HPLC analysis suggested FerTiG displayed efficient TC degradation; (e) determination of kinetic parameters of FerTiG-mediated TC degradation. PDA: photo diode array; abs: absolute velocity.

the cost. In this regard, a GDH from *Bacillus* was designated to recycle NADPH from NADP$^+$ using glucose as a cost-effective substrate (Fig. 3(a)). To investigate whether the incorporation of GDH facilitates the regeneration of NADPH, NADPH was measured based on a colorimetric methodology. As demonstrated in Fig. 3(b), it was observed that NADP$^+$ was efficiently converted to NADPH in a dose-dependent manner, but the absence of GDH or glucose slowed down the reaction as NADPH was not detected. To better understand the improved performance of FerTiG, the time-resolved degradation dynamics were plotted in Fig. 3(c). A more

pronounced degradation profile was observed in FerTiG compared to free Tet(X4), as both the rate and degradation were noticeably improved. Notably, FerTiG working with 10 μmol·L^{-1} NADPH showed even higher potency than free Tet(X4) combined with 100 μmol·L^{-1} NADPH, thereby fully confirming the enhancement of Tet(X4)-catalyzed TC removal by GDH. To strengthen the notion that GDH was able to facilitate TC removal by Tet(X4), the microbiological degradation assays were then performed with TC solution treated with free Tet(X4) or FerTiG supplemented with relatively low NADPH. The results demonstrated that the free
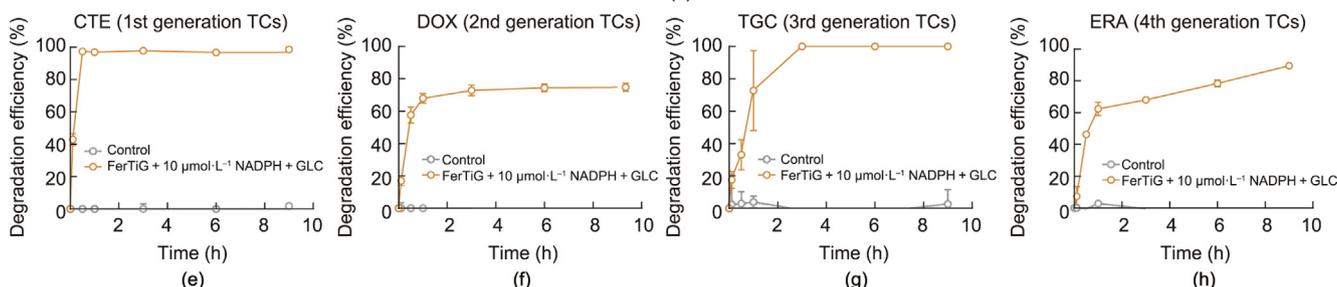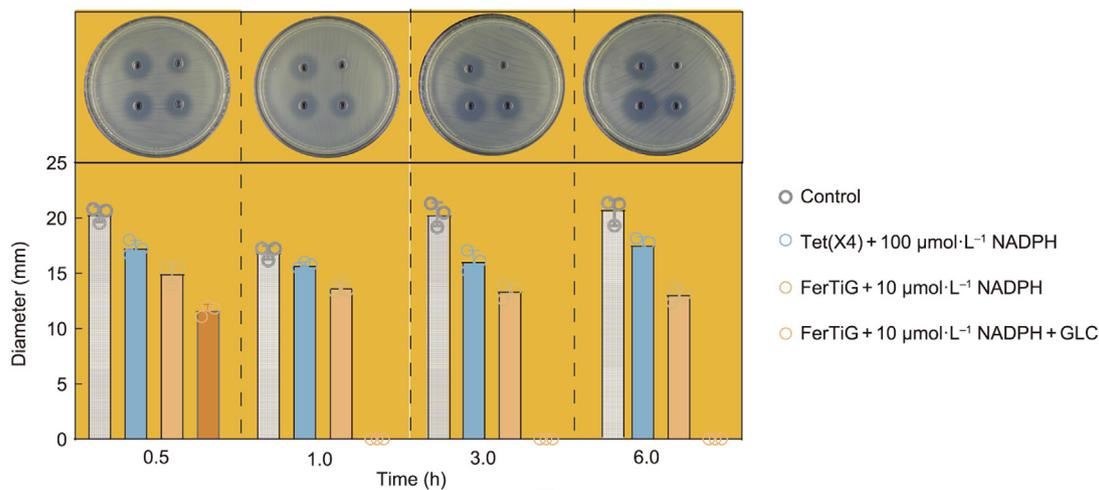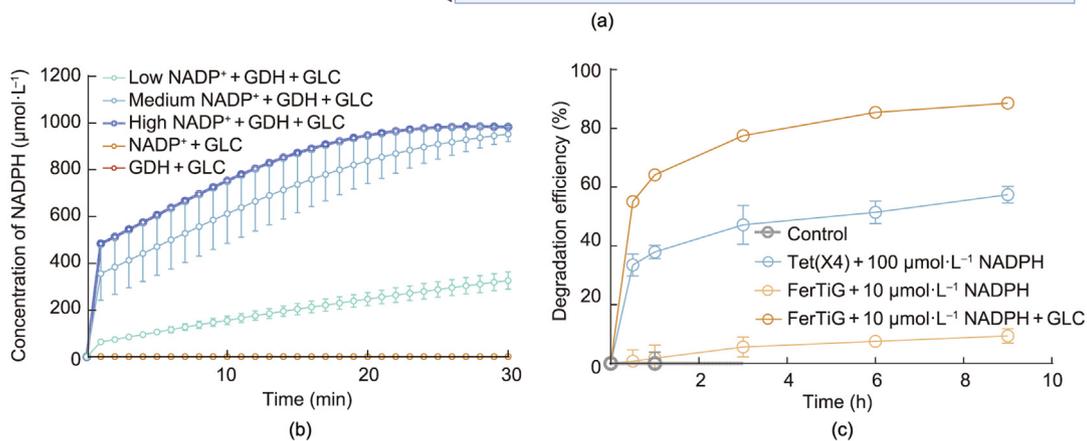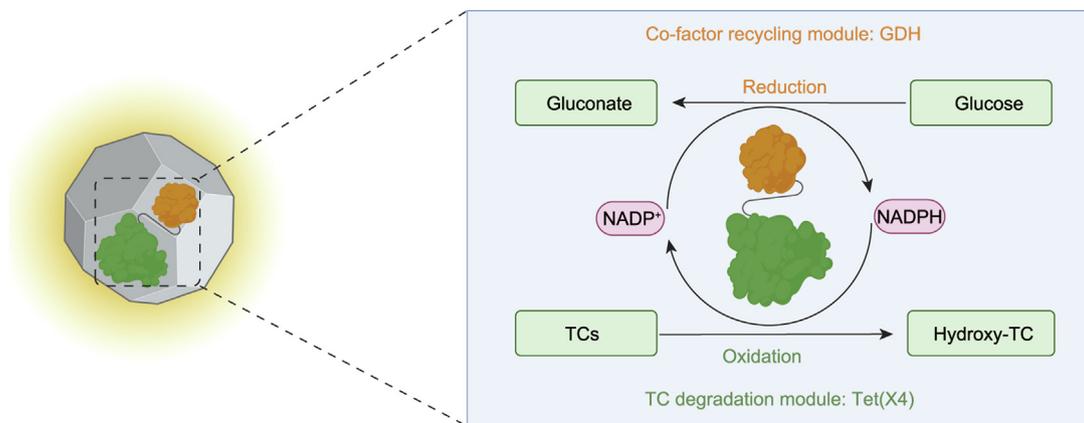
Fig. 3. Incorporation with GDH improved FerTiG efficiency via cofactor recycling. (a) Schematic working model for GDH to booster TC degradation via cofactor recycling; (b) GDH in FerTiG efficiently regenerated NADPH in presence of glucose; (c) GDH enhanced Tet(X4)-mediated TC degradation in FerTiG; (d) microbiological degradation assay endorsed the efficiency of FerTiG; (e–h) degradation efficiency of FerTiG on (e) CTE, (f) DOX, (g) TGC, and (h) ERA.

Tet(X4) only partially reduced the inhibition area, but FerTiG removed TC to a negligible level in a shorter time than the control group (Fig. 3(d)). These results were consistent with our previous findings that Tet(X4) suffered from limited TC scavenging activity under low NADPH conditions, which was well compensated by the incorporation of GDH as a cofactor recycling module. Thus, this module is necessary to enable better spontaneous TC removal by Tet(X4) under natural conditions where substrates such as glucose are expected to be more abundant than NAPDH [29,30]. In addition to TC, other antibiotics of the TC class have been reported to accumulate in environments [44], we next sought to investigate the functional versatility of FerTiG for the removal of other TC antibiotics. Encouragingly, the FerTiG exhibited broad applicability to efficiently degrade CTE, DOX, TGC, even ERA as the representatives as 1st, 2nd, 3rd, and 4th generation of TCs, as it rapidly decomposed such TCs in a larger extent within 1 h (Figs. 3(e)–(h)). Collectively, these results demonstrated that FerTiG possesses TC spontaneous degradation performance provided by cofactor recycling via GDH, significantly benefiting its efficiency and cost–effectiveness for practical application.

3.3. Ferritin confers stability to FerTiG upon exposure to adverse environmental factors and long-term storage

Previous investigations have shown that the biodegradation of residual antibiotics is affected by adverse environmental factors [45,46], which always recalcitrate the degradation by interfering with enzymatic activity [47,48]. The living organism generally exploits the lipid membranes to confine and protect the inner enzyme cores for robust enzymatic reactions. However, engineering a protection module with lipid-based materials such as liposomes is often expensive and technically challenging [49]. Hence, we considered ferritin as the protection module to form a proteinaceous cage for shielding the core enzyme. To evaluate the protection of this module against loss of activity after exposure to adverse environmental factors, the FerTiG was employed for TC degradation in presence of adverse pH, temperature, UV and ionic strength including monovalent ion, divalent ion as well as anions (Fig. 4(a)). As shown in Figs. 4(b)–(g) and Figs. S3 and S4 in Appendix A, the catalytic efficiency of free Tet(X4) was observed to be severely impaired in response to such adverse factors especially for the UV exposure, upon which the Tet(X4) was intermediately inactivated within 30 min. This observation was consistent with our previous findings that Tet(X4) was generally responsive to various environmental conditions [28–30]. It is also reasonable that its enzymatic activity was less active at sub-optimal pH, temperature, ionic conditions, or under detrimental UV radiation [50]. In contrast, incorporation with the protective module (ferritin) conferred significant resilience against such adverse environmental stimuli, as the FerTiG maintained high efficiency throughout all tested conditions even in certain extreme conditions (such as pH = 2 or high abundance of divalent ions (Figs. S3 and S4)). Therefore, it can be concluded that the enzyme assembly cloaking with ferritin confirms improved TC removal performance in the complex matrix of aquatic environments. It should also be mentioned that the protective module makes FerTiG more tolerant to UV damage, but its activity gradually lessens in a time-dependent manner. We reason that it is essentially related to ferritin forming the shielding cage as a protein scaffold, and the vulnerability to overexposure to UV is a general nature of all proteins [51]. As an antibiotic-destructase-based approach, another potential challenge for *in-situ* degradation of FerTiG is in storing sufficient amounts of active enzyme until the time of application [52]. For comprehensively defining the storage stability, the FerTiG was monitored for one week under various

storage conditions with free Tet(X4) as a comparison. The subsamples of FerTiG were taken at 1, 3, 5, and 7 d after storage under various conditions and subjected to TC degradation assays. Interestingly, the results demonstrated that FerTiG retained significant degradation activity at 4 °C and at room temperature such that there was no remarkable lessening in degradation efficiency observed. As both FerTiG and free Tet(X4) were subjected to a temperature of 37 °C, the loss of activity was observed on the first day after storage for free Tet(X4) yet the seventh day for FerTiG (Fig. 4 (h)). Taken together, these results reveal the favorable flexibility and stability of FerTiG to withstand a broad range of harsh conditions and suggest the general applicability of FerTiG for the removal of TC contaminants in well-defined adverse environments.

3.4. FerTiG robustly catalyzes in-situ *TC removal in environmental matrices*

It is noteworthy that *in vitro* antibiotic degradation hardly replicates its *in-situ* application since aquatic environments generally contain complex matrices that may alter the response of substrates to enzymes [53,54]. In this regard, the degradation performance of FerTiG was evaluated in tap water, lake water, livestock sewage, and pharmaceutical wastewater, which were previously reported to persist in concerning amounts [55,56]. In this case, we first measured the initial concentration of TC residues in the collected samples and found that no TC was detected in tap water, lake water, and livestock sewage, yet high levels of residual TC were found in pharmaceutical wastewater (Fig. 5(a)). For better evaluation, tap water, lake water, and livestock sewage were characterized by 10 ppm TC for subsequent experiments. The obtained results demonstrated that TC residues in the prototype remained stable in both tap water and pharmaceutical wastewater; however, a slight decrease was observed in samples in lake water and livestock sewage without the addition of FerTiG. This was conceivably because there were certain substances in such matrices that enhanced the spontaneous degradation of TC, although it was not fast enough and efficient under natural conditions [57]. However, the application of FerTiG rapidly reduced TC residues in the tested matrices, as shown in Fig. 5(b). To date, FerTiG has completely eradicated TC in the specified lake water and livestock sewage, with a removal efficiency of 100% within 3 h. In tap water, up to 81.27% of the residual TC was removed within 3 h. This result echoes the aforementioned findings that TC residues are difficult to naturally degrade in tap water and that there may be substances in lake water or livestock sewage that synergize with FerTiG by accelerating natural degradation. Notably, in pharmaceutical wastewater, where TC residues were present at high levels, more than half of residual TC (58.43%) was degraded in a rapid period (12.33 mg·(L·h)$^{-1}$). In our previous work, the efficiency of Tet(X4) was limited in certain environmental samples, indicating that the free enzyme without protection is subject to suboptimal activity due to potential recalcitrant in the environments [28]. To overcome the unfavorable performance of enzymes in environments, several investigations have utilized microorganisms such as *E. coli* as a chassis for the heterologous expression of the degrading antibiotic to recycle the cofactor and protect the enzyme. Such approaches have been established to remove residual TC with similar efficiency compared to FerTiG [25]. However, the aforementioned approaches were generally more time-consuming to achieve maximum degradation. On the other hand, such methodologies based on live bacteria are associated with risks of spreading antibiotic resistance genes carried in environmental compounds, and the potential spillover of antibiotic resistance throughout the ecology should be considered. In contrast, the FerTiG in the present
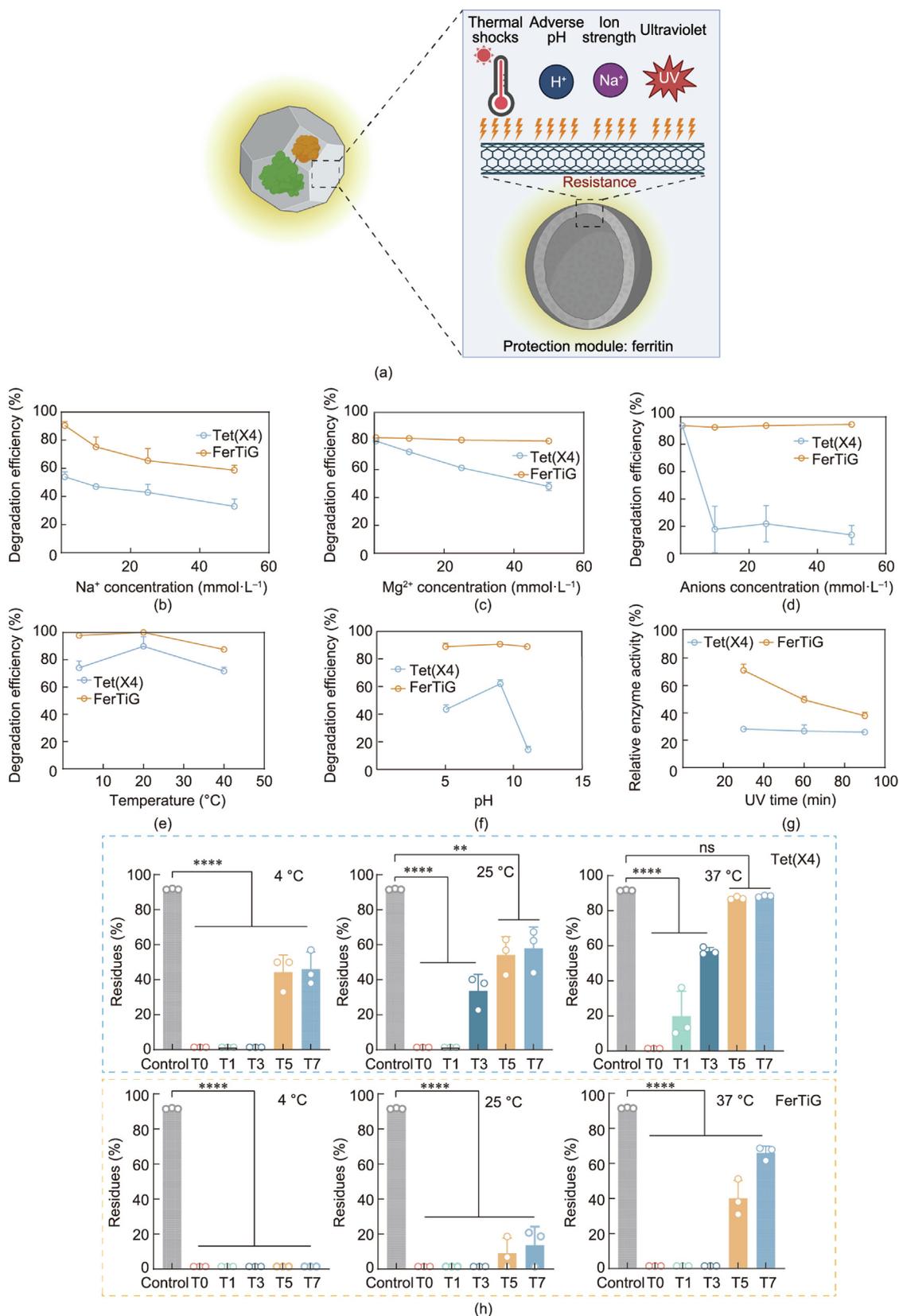
Fig. 4. Shielding by ferritin rendered FerTiG resilient to the adverse environmental stresses. (a) Schematic working model for ferritin to protect catalytic core of FerTiG against the adverse stresses; (b–g) the degradation performance of FerTiG under defined adverse conditions: (b) monovalent ion strength, (c) divalent ion strength, (d) anion strength, (e) temperatures, (f) suboptimal pH, and (g) UV exposure; (h) the storage stability of FerTiG in comparison with free Tet(X4). T0–T7: time-point 0–7 (0–7 days post the storage). $**p < 0.01$; $****p < 0.0001$; ns: not significant.
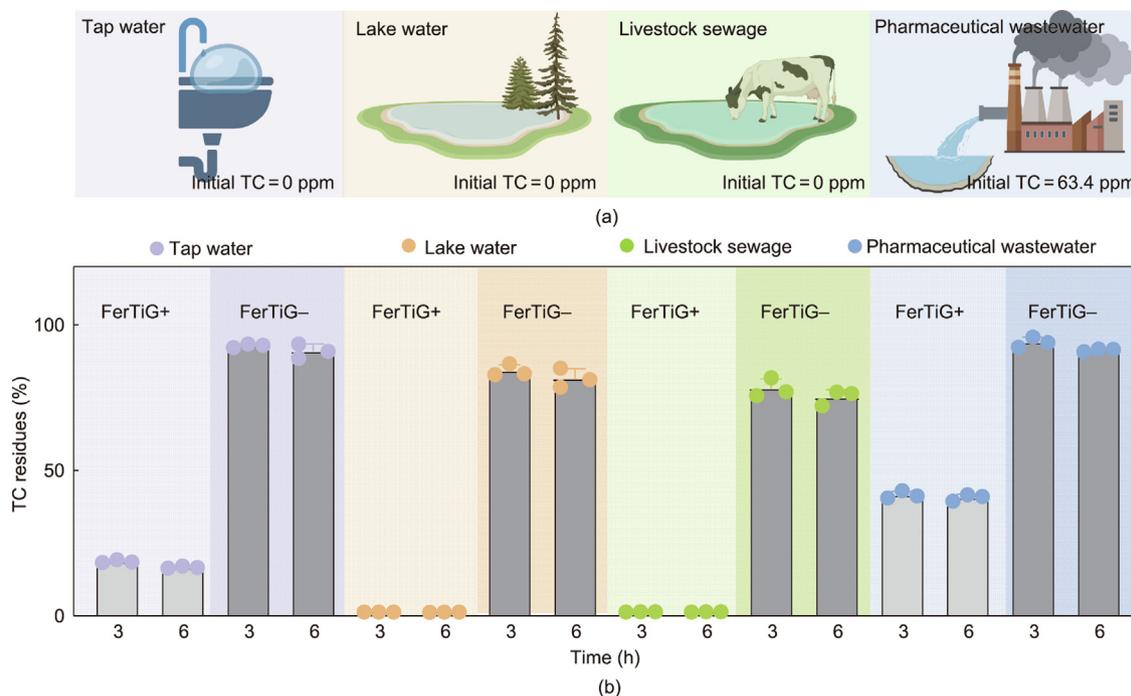
Fig. 5. *In situ* TC degradation from environmental matrices by FerTiG. (a) The representative aquatic environments and the initial TC concentration therein; (b) TC residue in environmental matrices with or without FerTiG application.

study was constructed in a DNA-free manner, thus circumventing the ecological risks of spreading antibiotic resistance. These results collectively demonstrated that the FerTiG versatilely eliminated the TC contaminants in various types of aqueous matrices with favorable performance.

3.5. Proposed degradation pathways of FerTiG to eradicate TC residues

To uncover plausible degradation pathways, the transformation intermediates were profiled by quadrupole time of flight mass spectrometer (QTOF-MS) analysis (Figs. S5–S8 in Appendix A) and summarized in Fig. 6. It has been well established in previous studies that TC degradation by Tet(X4) involves the first regional hydroxylation of TC at C11a and subsequent non-enzymatic breakdown of hydroxytetracycline to harmless micromolecules [58]. To elucidate the potential degradation pathway using FerTiG, intermediates during TC transformation were suitably profiled using high-resolution QTOF-MS. As expected, FerTiG first catalyzed the hydroxylation of TC to produce 11a-hydroxy-tetracycline (P1, m/z = 461), which is an unstable transformation product to convert to smaller molecules under natural conditions [29,30]. As revealed in a previous study, the dimethylamino group at the C4 position of this molecule was readily oxidized to form P2 (m/z = 477), which was suggested to be ecologically detoxified due to the loss of the dimethylamino group in its natural structure [59,60]. This modification destabilized the linear fused tetracyclic core backbone to lead to the collapse of the A ring and accelerated the decomposition of TC into micromolecules (P4) [61]. Aside from being partially oxidized, the demethylation was also reported at C4 to cleave the dimethylamino group under given conditions to convert P1 to P5 or P8 (m/z = 431/432), respectively. In addition, P5 was suggested to be converted to P6 (m/z = 415) via a dehydration reaction at C3. Through a ring-opening reaction on a ring of the tetracyclic backbone, it was converted to the product m/z = 301 (P7), which could eventually be converted to P4 as the final product (m/z = 149). In the case of P8, it has been reported that N4 was easily dealkylated

following the attack by the proton to generate an unstable intermediate whose cyclic structure readily collapsed to form P9 (m/z = 376). P9 was further oxidized to form P10 (m/z = 279) and then to form the final product P11 [62]. To date, P1 has been reported to form a molecule m/z = 459 (P12) via hydroxylation or oxidation, which naturally decomposes to finally produce P13 (m/z = 209) [63]. It is noteworthy that these transformation products in smaller molecular size conversion products are generally considered to be biodegradable materials, most of which can be further degraded to CO_2 and H_2O [64,65]. This was further proved by the TOC analysis as a total of 23.72% (9 h) and 28.19% (24 h) TOC were removed after FerTiG treatment (Fig. S9 in Appendix A).

3.6. FerTiG is free of biosafety concerns

Biosafety is a prerequisite for a generally applicable residue remediation system, as they are likely to persist in the aquatic system and eventually accumulate in the human body through the food chain or water supply [66,67]. In this regard, it is crucial to determine whether there were biosafety concerns for FerTiG. For this purpose, ECOSAR 2.0 was first utilized to predict the aquatic toxicity of the transformation products. The obtained results demonstrated that only four products with predictable acute LC50 were found; however, they were above 2040 $\mu g \cdot mL^{-1}$, which would rarely allow accumulation at such levels under natural conditions (Fig. 7(a)). The environmental and biological safety of FerTiG was then assessed in two common models. The zebrafish test was first conducted to answer the question of whether the application of FerTiG to the aquatic environment would have adverse ecological impacts on the aquaculture that resides there (Fig. 7(b)). The microscopic analysis revealed that no morphological abnormalities were found in the fish receiving FerTiG (Fig. 7(c)). Furthermore, acridine orange staining analysis on juvenile fish showed that FerTiG supplementation in the medium did not lead to apoptotic damage to zebrafish, indicating the ecologically harmless nature of FerTiG (Fig. 7(d)). Further analysis also revealed that neg-
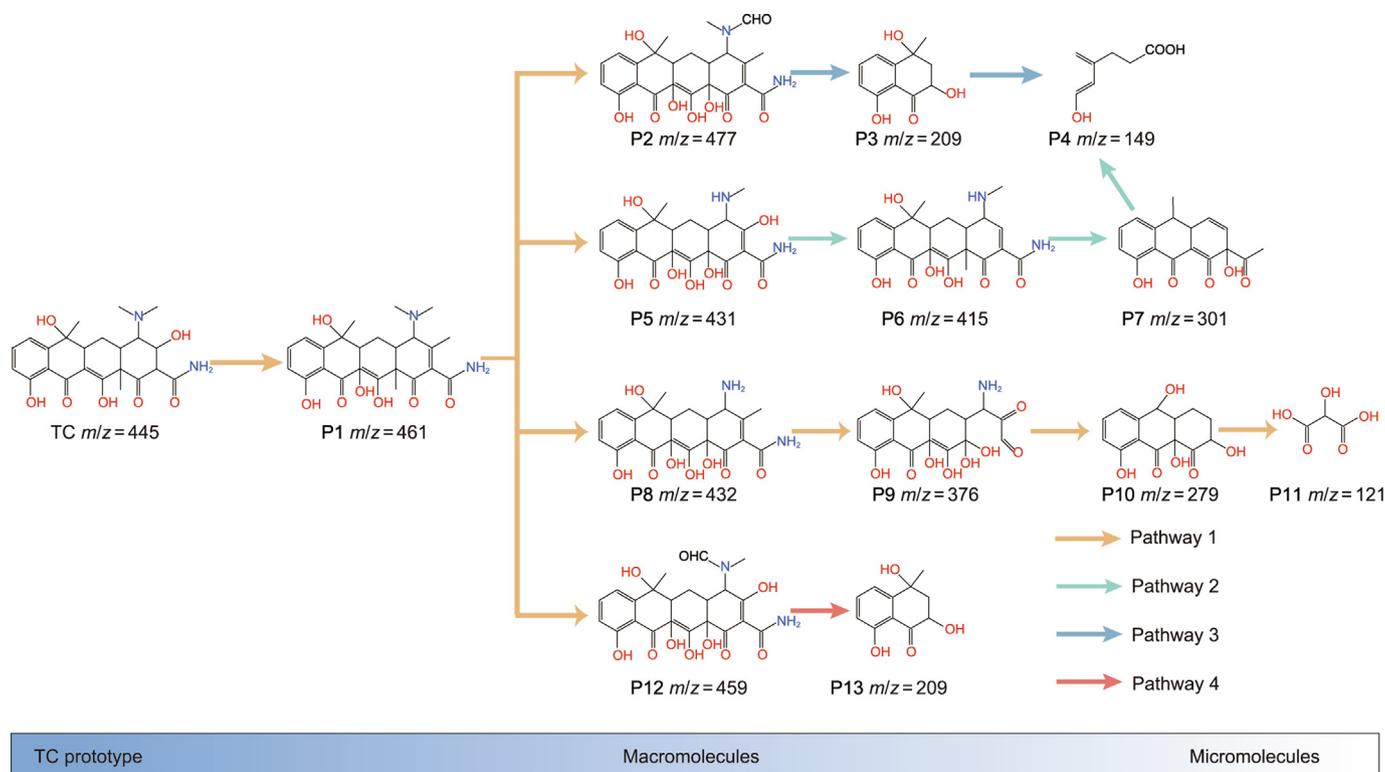
Fig. 6. Proposed degradation pathways and transformation intermediates for FerTiG-mediated TC degradation.

ligible deformity or delayed hatching rates were observed in zebrafish embryos incubated with high or low concentrations of FerTiG simultaneously Figs. 7(e) and (f). To confirm the biosafety of FerTiG in mammals, a mouse model was utilized to elucidate the host biological responses after mimicking the accumulation of FerTiG in the body (Fig. 7(g)). No deaths were observed during the experiment (Fig. S10 in Appendix A). It was important to elucidate the effect of FerTiG on host organs responsible for metabolic functions, as they are usually exposed to and bioaccumulate substances from the environment. For this purpose, the kidneys, livers, and intestines of mice from different treatments were collected for the H&E staining to examine whether FerTiG accumulation caused pathogenesis and histological changes in these organs. As illustrated in Fig. 7(h), neither damage nor inflammatory response was observed in the target organs of mice that received FerTiG. In addition to the tissue damage, the body weights of mice receiving FerTiG were observed to be insignificantly different compared with the control mice receiving saline buffer (Fig. 7(i)). A slight decrease in body weight was observed for the first day in both FerTiG and control mice immediately after treatment. This was consistent with observations in our previous studies, and this weight loss might have been caused by gavage stress rather than FerTiG accumulation [30]. These data from the two modeling investigations collectively confirmed the promising biosafety of FerTiG for its real-world application in aquatic environments without concerns for the introduction of hazardous substances or intermediates.

4. Conclusions

In the present study, an efficient modular enzyme assembly, FerTiG, was developed as a novel TC biodegradation platform inspired by modular catalytic machinery in the context of synthetic biology. In FerTiG, Tet(X4) efficiently catalyzed the TC removal as

the degradation module fed by GDH as the cofactor recycling module. Being shielded by a protective module composed of ferritin, FerTiG was able to eliminate TC contamination in defined harsh environments and then remained stable under various storage conditions. Contributed by the interactions between functional modules, FerTiG strongly reduced residual TCs in multiple environmental matrices, including tap water, lake water, livestock sewage, and pharmaceutical wastewater. The QTOF-MS analysis revealed potential transformation intermediates and demonstrated that the degradation of TCs was achieved by regioselective-hydroxylation subsequent non-enzymatic breakdown. Desirable eco- and biosafety were confirmed to FerTiG as no hazardous effects on environments and hosts were observed. Overall, this study presented a novel biodegradation system that structurally and functionally mimics the prokaryotic microenvironment. This system applied synthetic biology to combine the advantages of enzyme-based and living organism-based approaches for the mitigation of residual TC and highlighted the great potential of such modular enzyme assemblies for the sustainable management of environmental contaminants.

CRediT authorship contribution statement

Hao Ren: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Meilin Qin:** Methodology, Investigation, Formal analysis. **Lin Zhang:** Investigation, Formal analysis. **Zemiao Li:** Methodology, Investigation. **Yuze Li:** Investigation. **Qian He:** Investigation. **Jiahao Zhong:** Investigation. **Donghao Zhao:** Formal analysis. **Xinlei Lian:** Formal analysis. **Hongxia Jiang:** Formal analysis. **Xiaoping Liao:** Funding acquisition. **Jian Sun:** Writing – review & editing, Funding acquisition, Conceptualization.
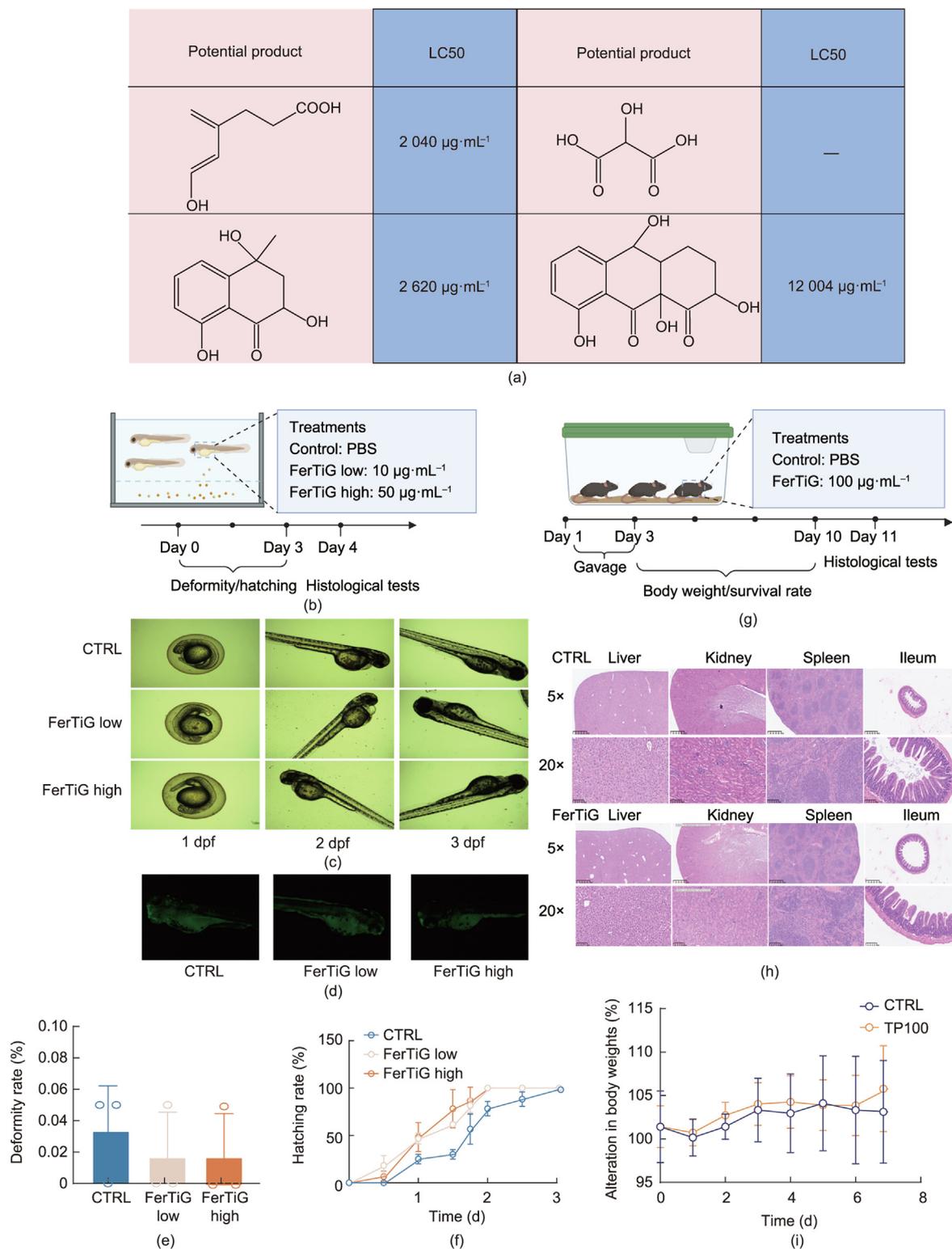
Fig. 7. Eco- and biosafety analysis of FerTiG application. (a) The LC50 of transformation intermediates based on ECOSAR prediction; (b) schematic illustration for animal trial based on zebrafish; (c) the development of zebrafish embryo under FerTiG exposure; (d) the acridine orange staining indicated no apoptosis caused by FerTiG exposure on zebrafish, the intensity of green fluorescence indicates the degree of apoptosis; (e) the embryo deformity rate of zebrafish under FerTiG exposure; (f) the embryo hatching rate of zebrafish under FerTiG exposure; (g) schematic illustration for animal trial based on mice; (h) histological examination of liver, kidney, spleen, and ileum of mice received FerTiG; (i) alteration in body weights of mice received the FerTiG. dpf: days post fertilization; CTRL: control; TP100: TC degradation products (initial TC concentration: 100 μg·mL^{-1}).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2025.03.033.

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