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L-Tyrosine-based biocompatible low-toxic substrate of peroxyoxalate chemiluminescent reaction

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1. Synthesis of bis(N-benzoyltyrosine ethyl ether) oxalate (BTE-oxalate)

N-Benzoyl-L-tyrosine ethyl ether ("BTEE") was acylated with oxalyl chloride in an aprotonic solvent in the presence of pyridine as a base.

Oxalyl chloride is an extremely active chlorohydride, so all solvents were carefully dried according to standard techniques and distilled on the day of the experiment. BTEE (1.0093 g, 3.22 mmol, Sigma-Aldrich) was placed in a 50 ml flask, benzene (15 ml) was added, and water was removed by azeotropic distillation of benzene. The operatopn was repeated three times. Then the flask was filled with argon and immediately plugged with a rubber septum. Pyridine (1.8 ml) and THF (10 ml) were syringed under an argon atmosphere, and the mixture was stirred till complete dissolution of BTEE. Then 0.64 M oxalyl chloride solution was prepared by mixing oxalyl chloride (0.34 ml, Sigma-Aldrich, 3.2 mmol, 2-fold molar excess of BTEE OH-groups) with THF (5 ml), and this was added slowly in 0.2 ml portions to the BTEE reaction mixture cooled in an ice bath. Between additions, 2-min breaks were taken. One hour later, the reaction mixture was removed from the ice bath and allowed to warm to room temperature. The pyridine hydrochloride precipitate was separated by filtration on a Schott filter and washed with THF (5 ml). Hexane (100 ml) was added to the filtrate containing the oxalate to precipitate the BTEE oxalate. The precipitate was filtered under vacuum on a Schott filter, transferred to a 100 ml flask and dissolved in chloroform (40 ml). The solution was washed in a separating funnel first with water, then with 1M HCl and again with water. Then the solution was transferred to a flask containing magnesium sulfate (10 g) and left overnight for dehydration. The next day, the magnesium sulfate was removed by filtration on a Schott filter. The BTEE-oxalate solution was evaporated to dryness and additionally kept under vacuum at a residual pressure of 0.01 mbar. The yield was 680 mg (60%).

Table S1. Signals assignment in the ^{1}H-NMR spectra of BTEE and BTEE-ox

BTEE	Chemical shift, ppm	Integral intensity	BTEE-ox	Chemical shift, ppm	Integral intensity
a	6.53 1H s	0.96	a'	–	–
b	6.75 2H d	2.01	b'	7.21 2H d	2.01
c	7.00 2H d	2.01	c'	7.25 2H d	1.99
d	3.21 2H ddd	2.02	d'	3.32 2H ddd	2.05
e	5.06 1H dd	0.99	e'	5.08 1H dd	1.00
f	4.24 2H q	2.00	f'	4.23 2H q	2.04
h	6.70 1H d	0.94	h'	6.67 1H d	0.99
g	1.30 3H t	3.02	g'	1.29 3H t	3.30
k	7.73 2H d	2.00	k'	7.75 2H d	2.00
o	7.42 2H t	1.99	o'	7.45 2H t	2.00
m	7.51 1H t	1.00	m'	7.53 1H t	0.98
$CHCl_3$	7.28 s	0.14	$CHCl_3$	7.28 1H s	0.28
H_2O	1.84 s	0.28	H_2O	1.63 2H s	0.26
			Benzene	7.37 6H s	0.02
			Hexane	0.90 8H t	0.03

2. POCL reaction

The exact concentration of hydrogen peroxide in the solution was determined by iodometric titration.

Chemiluminescence in THF/water mixture was evaluated as follows. A BTEE-ox solution in THF (0.02 M, 50 µl) was mixed with perylene solution in THF (0.01 M, 20 µl) and freshly distilled THF (930 µl), and this was placed in a fluorescence cuvette that was mounted in a fluorimeter cuvette holder thermostatted at 37 °C. Separately, hydrogen peroxide solution (80 µl, 0.106 M), sodium hydrogen carbonate solution (320 µl, 0.1 M, pH 7.5), and THF (600 µl) were mixed. This solution was also thermostatted at 37 °C. This solution was then injected into a cuvette using a syringe, and the chemiluminescence signal at 440 nm (emission slit 20 nm) was recorded using a Hitachi HR-3010 fluorimeter.

Chemiluminescence in the micelles of PLLA61-PEG87 was measured similarly. PLLA61-PEG87 block copolymer (15 mg) was dissolved in freshly distilled THF (80 µl) and mixed with perylene solution (20 µl, 0.01M) and BTEE-ox or CPPO solution in THF (46 µl, 20 mM), this was stirred thoroughly, and after temperature control added to 1 ml PBS (37 ºC) in a cuvette mounted in a fluorimeter holder. PBS (870 µl) mixed with hydrogen peroxide solution (80 µl, 0.1061 M) was injected into this solution, and the chemiluminescence signal was recorded at 37 °C.

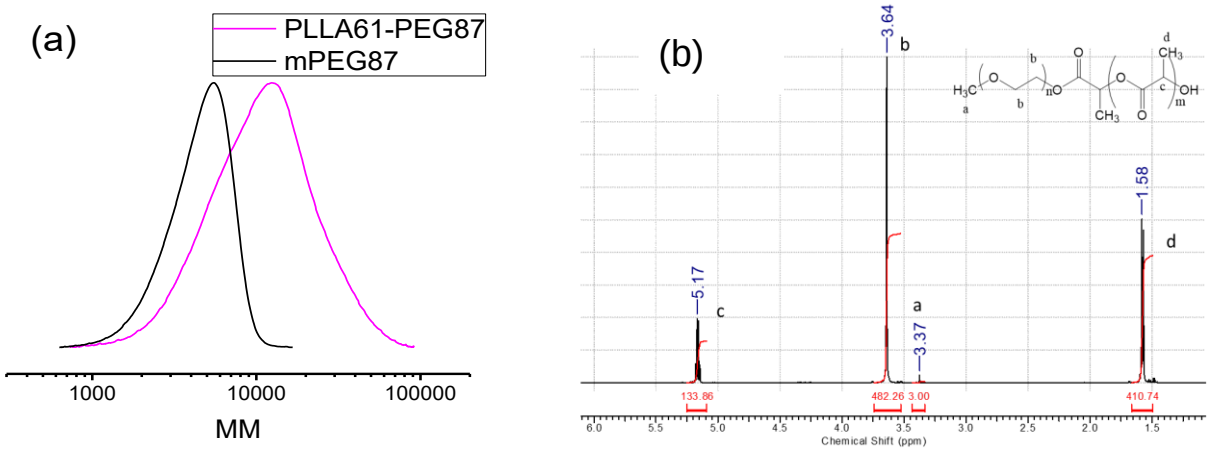

Figure S1 PLLA63-PEG87 analysis (*a*) Molecular mass distributions of the initial PEG87 (black) and PLLA61-PEG87 (magenta) calculated from GPC analysis using Waters HR-3 column with THF as eluent. (*b*) ^{1}H-NMR spectrum of PLLA61-PEG87 block copolymer in $CDCl_3$.

Table S2. Molecular mass characteristics of pTyr-PEG and PLLA-PEG copolymers

	M_n(NMR)	M_n(GPC)	M_w(GPC)	M_w/M_n
mPEG87		3817	4715	1.24
PLLA61-PEG87	10738	8226	14569	1.77

3. Characterization of PLLA61-PEG87 micelles

Samples for the dynamic light scattering analysis were prepared immediately before the measurement by adding a 10% polymer solution in THF (150 µl) to PBS (2 ml) heated to 37 ºC. The final concentration of the polymer in the sample was 7 g dm^{-3}. The experiment was performed at 37 ºC. Autocorrelation functions of scattered light intensity fluctuations (ACF) and mean intensities of scattered light were recorded using a Zetasizer NanoZS (Malvern Instruments Ltd., United Kingdom). A He-Ne laser (λ = 633 nm, 15 mW) was used as a light source. The scattering angle was 173º. Autocorrelation functions were measured using a 288-channel FotoCor-SP correlator with a logarithmic time scale from 2.5×10^{-8} to 6800 s. Data were collected for 3 minutes (10 repetitions) to obtain the ACF. Correlation function analysis was performed using Zetasizer DTS Nano software (Malvern Instruments Ltd, United Kingdom). The hydrodynamic diameter of micelles was calculated using the Stokes-Einstein equation in the spherical particle approximation.

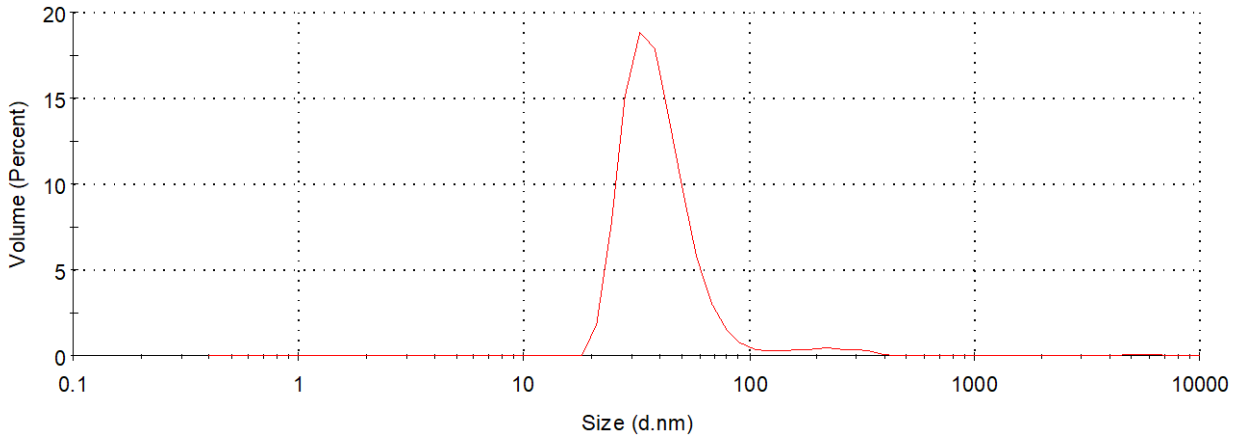

Figure S2. Volume distribution of PLLA61-PEG87 micelles at 37 ºC in PBS.

4. Cytotoxicity

Toxicity of oxalates was tested on adherent human ovarian adenocarcinoma cells of NCI/ADR-RES line (previously designed as MCF-7/ADR) resistant to different drugs. The cells were cultured in Nunc plastic vials (Nunclon) in DMEM medium supplemented with 4 mM glutamine, 100 μg ml^{-1} streptomycin, 100 units per ml penicillin and 10% bovine fetal serum (complete medium), in a CO_2 incubator (NAPCO) at 37 ºC in an atmosphere of 5% CO_2 and 95% humidity.

A day before the experiment, cells were seeded into a 96-well plate, 4 000 cells/well, in complete medium and kept overnight in the CO_2 incubator for cell adhesion. On the day of the experiment, test samples were dissolved in THF (0.15 ml) and thoroughly dispersed in serum-free DMEM (2 ml) using Vibrofix shaker. Series of dilutions of each sample in serum-free medium was prepared. The complete medium was then removed from the wells of the plate and 0.2 ml/well of test compounds of different concentrations were added. Each dilution of each compound was tested on cells in 3 to 4 wells. Medium (0.2 ml) without additives was added to control wells. The cells were incubated with test substances for 1-1.5 hours in the incubator, then they were removed and cells were cultured for additional three days in complete medium (0.2 ml/well) without additives. The number of living cells per well was assessed with MTT test at the end of the experiment. Cytotoxicity of the tested compounds was evaluated by their concentrations decreasing the number of survived cells to 50% (IC_{50}).

5. Evaluation of pKa of BTEE-oxalate by spectrophotometric titration.

The absorption spectrum of *N*-benzoyl-tyrosine ethyl ester (BTE) in alkaline medium has a characteristic shoulder at 275 nm. With increasing pH, the optical density of the solution at this wavelength increases, and at pH about 10.6 the shoulder at 275 nm shifts to the long-wavelength region, and a clearly distinguishable peak appears at 293 nm. When the pH is increased to 11, the peak increases. In the strongly alkaline region the degree of absorption change decreases. The dependence of optical density on pH has an S-shaped character (Figure S3) typical for chromophores containing ionizable groups. Processing of the pH dependence of the optical density at 293 nm using the Logistic function shows that the inflection corresponds to pK_a = 10.00 ± 0.03.

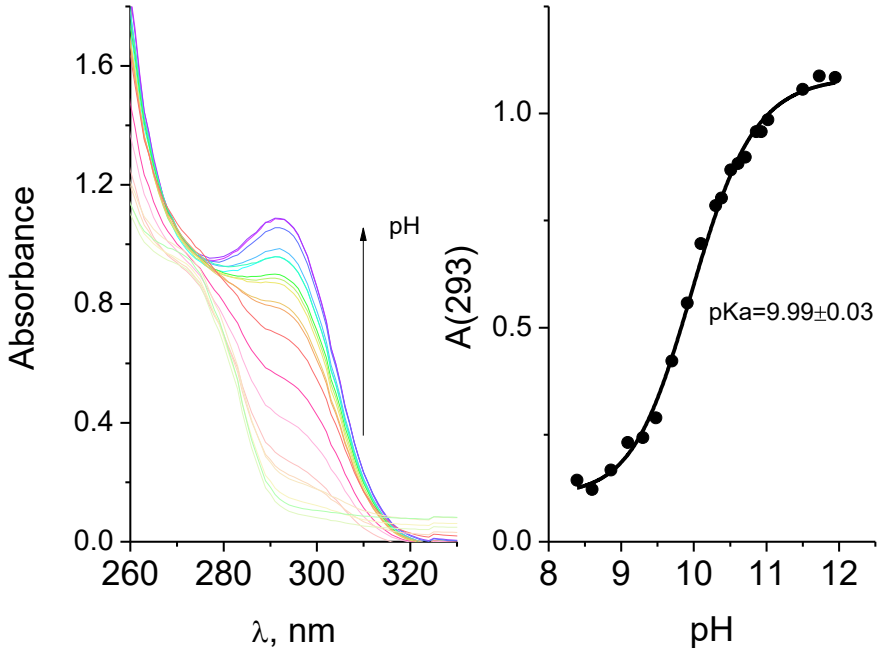

Figure S3. (*a*) Absorption spectrum of 0.5 mM BTEE in aqueous solution at pH from 8.39 to 11.95 (the arrow shows the increase in pH of the solution), (*b*) pH dependence of absorbance of BTEE solution at 293 nm.

Table S3. pK_a values of phenol oxalates calculated by ACDLabs 6.0, and their MTC and IC_{50} values in PLLA61-mPEG87 micelles

Oxalate	Cytotoxicity of Oxalates		pK_a of phenolic constituents in oxalate
	Maximum non-toxic concentration, mM	IC50, mM	
CPPO	0.016	0.055 ± 0.005	4.56±0.45
BTEE-ox	0.068	0.152 ± 0.032	9.75±0.15