Electronic Supplementary Information

Fabrication of gelatin hydrogels using pre-coordinated lanthanide complexes via imine crosslinking

Chen-Cheng Yu,[a] Yu Yun Hsu,[a] Yu-Chia Su,[a] Yi-Chen Yang,[a] Tzu-Ying Wang,[a] and Yi-Cheun Yeh[a]*

[a]Institute of Polymer Science and Engineering, National Taiwan University, Taipei 10617, Taiwan

* Corresponding author

E-mail: yicheun@ntu.edu.tw

Experimental section

Materials

Polyethyleneimine (PEI) (branched, 800 Da) and gelatin (from bovine skin) were purchased from Sigma-Aldrich. Sodium cyanoborohydride ($NaBH_3(CN)$), ninhydrin, and hydrindantin dihydrate were purchased from Fluorochem (UK). 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) was purchased from Acros Organics. Protocatechuic aldehyde (PA) was purchased from BLDpharm. Europium(III) nitrate hexahydrate ($Eu(NO_3)_3•6(H_2O)$), terbium(III) nitrate hexahydrate ($Tb(NO_3)_3•6(H_2O)$), samarium(III) nitrate hexahydrate ($Sm(NO_3)_3•6(H_2O)$), dysprosium(III) nitrate hexahydrate ($Dy(NO_3)_3•6(H_2O)$) and ytterbium(III) nitrate hexahydrate ($Yb(NO_3)_3•6(H_2O)$) were purchased from MorrChem. 3-[(Ethylimino)methylidene]amino-N,N-dimethylpropan-1-amine (EDC) was purchased from Fluorochem. N-Hydroxysuccinimide (NHS) was purchased from Alfa Aesar. Triethylamine (99%) (TEA) was purchased from Alfa Aesar. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and alamarBlue assay were purchased from ThermoFisher.

Instruments

Fourier-transform infrared spectroscopy (FT-IR) was performed by Spectrum Two (PerkinElmer). Scanning electron microscopy (SEM) was analyzed by TM-3000 (Hitachi). The rheology test was measured by AR2000EX (TA Instrument) with an 8 mm parallel plate. Hydrogels were lyophilized (-80 °C, 10 mTorr) using a freeze dryer (UNISS FDM-2). The porosity and pore size distribution of the hydrogels were quantified using a mercury porosimeter (AutoPore IV 9520). Luminescence spectra analysis of the hydrogel samples was carried out on a microplate reader (Hybrid Multi-mode reader, BioTek Synergy H1). The TA Affinity isothermal titration calorimeter (ITC) quantifies the thermodynamic properties of chemical and physical equilibria.

Synthesis of polyethyleneimine-modified gelatin (PG)

Polyethyleneimine-modified gelatin (PG) was synthesized through amide coupling. To synthesize PG, PEI (1 g) was added to 100 mL of MES buffer (0.976 g, 5 mmol MES powder in 100 mL of DI water) in a single-neck flask and stirred until dissolved. Subsequently, EDC (1.16 g, 7.5 mmol), NHS (0.345 g, 3 mmol), and gelatin (1 g) were sequentially added to the mixture. After complete dissolution, the mixture was stirred overnight at 37℃. The resulting solution was dialyzed for 3 days using a 3000 Da dialysis membrane (Cellu • Sep T1, MFPI, USA). Finally, the product was obtained by lyophilization, yielding polyethyleneimine-modified gelatin (PG).

Preparation of lanthanide complexes

The lanthanide metals and ligands were mixed according to the desired molar ratio for the experiment. Lanthanide compounds ($Eu(NO_3)_3 \cdot 6H_2O$, $Tb(NO_3)_3 \cdot 6H_2O$), and the ligand protocatechuic aldehyde (PA) were dissolved in DI water. The pH was adjusted to 10.0 using triethylamine to form pre-coordinated lanthanide complexes ($Eu(PA)_n$ and $Tb(PA)_n$).

Isothermal titration calorimetry (ITC) analysis

In this experiment, we aim to determine the stoichiometry of PA binding to $Eu(NO_3)_3$, and $Tb(NO_3)_3$) compounds in DI water through isothermal titration calorimetry (ITC). The experiment utilized an ITC instrument in DI water at a pH of 10.0 (adjusted using triethylamine) to determine the ligand binding to $Ln(NO_3)_3$ and the enthalpy and equilibrium binding constants of these interactions.

Preparation of $Ln(PA)_3$/PG hydrogels

PG was dissolved in DI water (10 wt%). The pre-coordinated lanthanide metal complexes were also dissolved in DI water. The pre-coordination solution [$Ln(PA)_x$] and the PG solution were mixed in equal volumes using a dual syringe and injected into 1 mL and 3 mL syringes, respectively. The resulting mixture was allowed to stand for 4 hours to ensure the formation of appropriate hydrogels before further use. In the polymer and pre-coordinated [$Ln(PA)_x$] complexes, the final concentration of PA was 5 wt% and 0.35-0.55 wt%, respectively.

Microstructural analysis of hydrogels

The hydrogels were characterized for pore size by freeze-drying. First, the hydrogels were frozen at -80°C for 12 hrs, and then lyophilized in a lyophilizer at -80°C under 10 mTorr for three days. The lyophilized hydrogels were examined using a scanning electron microscope (Hitachi TM-3000 tabletop) and mercury porosimeter (AutoPore IV 9520). Pore sizes of hydrogels determined from the SEM images were conducted with ImageJ software, and the average pore size for each hydrogel was calculated based on the analysis of 30 individual pores, with each pore being measured 30 times from different directions.

Rheological tests of hydrogels

The rheological properties of [$Ln(PA)_3$]/PG hydrogels were tested using a rheometer (AR 2000 EX and HR-2, TA Instruments) equipped with a plate (cone angle = 0°, diameter

= 20 mm). [Ln(PA)$_3$]/PG hydrogels were prepared in 3 mL syringes and allowed to stand for 24 hours before being placed in the center of the test platform. In oscillatory strain sweep tests, conducted at 1 Hz and 25℃, the strain was varied from 0.1% to 1000% with 10 points per cycle. Frequency sweep tests were performed at a strain of 10% and 25℃, with angular frequency ranging from 0.01 to 100 Hz and 10 points per cycle. Oscillation time sweep tests, conducted at low strain (1%) and high strain (1000%) for 1 minute at 1 Hz and 25℃, demonstrated the self-healing properties of the hydrogels. Shear thinning properties of the hydrogels were demonstrated through step flow tests with shear rates ranging from 0.1 s^{-1} to 100 s^{-1}.

The crosslinking density of hydrogels can be further calculated by the following formula:

$$G' = \nu RT$$

Where G' represents storage modulus in Pa, ν represents crosslinking density in mol/m^3, R represents gas constant (8.314 J K^{-1} mol^{-1}), and T represents the temperature in K.

Degradation behavior of hydrogels

The initial weights of the hydrogels were recorded after lyophilization (W_i). Subsequently, the hydrogels were immersed in the phosphate-buffered saline (PBS) solution. To facilitate uniform degradation, the hydrogels were placed in a shaking incubator with a flat platform at 37 ℃ and shaken at a speed of 100 rpm. At different time intervals (1, 3, 5, and 7 days), the hydrogels were frozen, lyophilized, and weighed again (W_f). The percentage of hydrogel weight remaining was calculated using the following equation.

$$\text{Remaining weight (\%)} = \frac{W_f}{W_i} \times 100\%$$

Cytocompatibility tests of hydrogels

Mouse embryonic fibroblasts (MEFs) isolated from C57BL/6N mouse embryos were generously provided by Prof. Richard Assoian of the University of Pennsylvania.

MEFs were cultured in a 5% CO_2 atmosphere at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% HEPES.

The cytocompatibility of the hydrogels was tested using an AlamarBlue assay. To prepare hydrogel extracts for cell culture, the hydrogels were sterilized by UV irradiation for 1 hr and then immersed in serum-containing DMEM at a volume ratio of 1:10 (hydrogel to medium) for 24 hrs. MEFs (1.5×10^4 cells/mL, 200 μL) were seeded in a 96-well plate containing hydrogel extract solutions and incubated in a 5% CO_2 atmosphere at 37°C. For quantification, 200 μL of AlamarBlue solution (10% in serum-containing DMEM) was added to each well, and the plates were reincubated for 4 hours. The solution was then transferred to a 96-well plate to detect cell viability. Fluorescence intensity was measured using a microplate reader (Biotek Synergy H1) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The results for each sample at different time points were normalized to day 1 values and each experiment was performed in triplicate.

Statistical analysis

All experiments were conducted with three replicates unless specified in the figure captions. Error bars in figures represent the standard deviation (s.d.) unless stated otherwise. The statistical significance of data differences was determined using a t-test, with significance defined at $p < 0.05$. Symbols *, **, or *** indicate p values less than 0.05, 0.01, or 0.001, respectively.

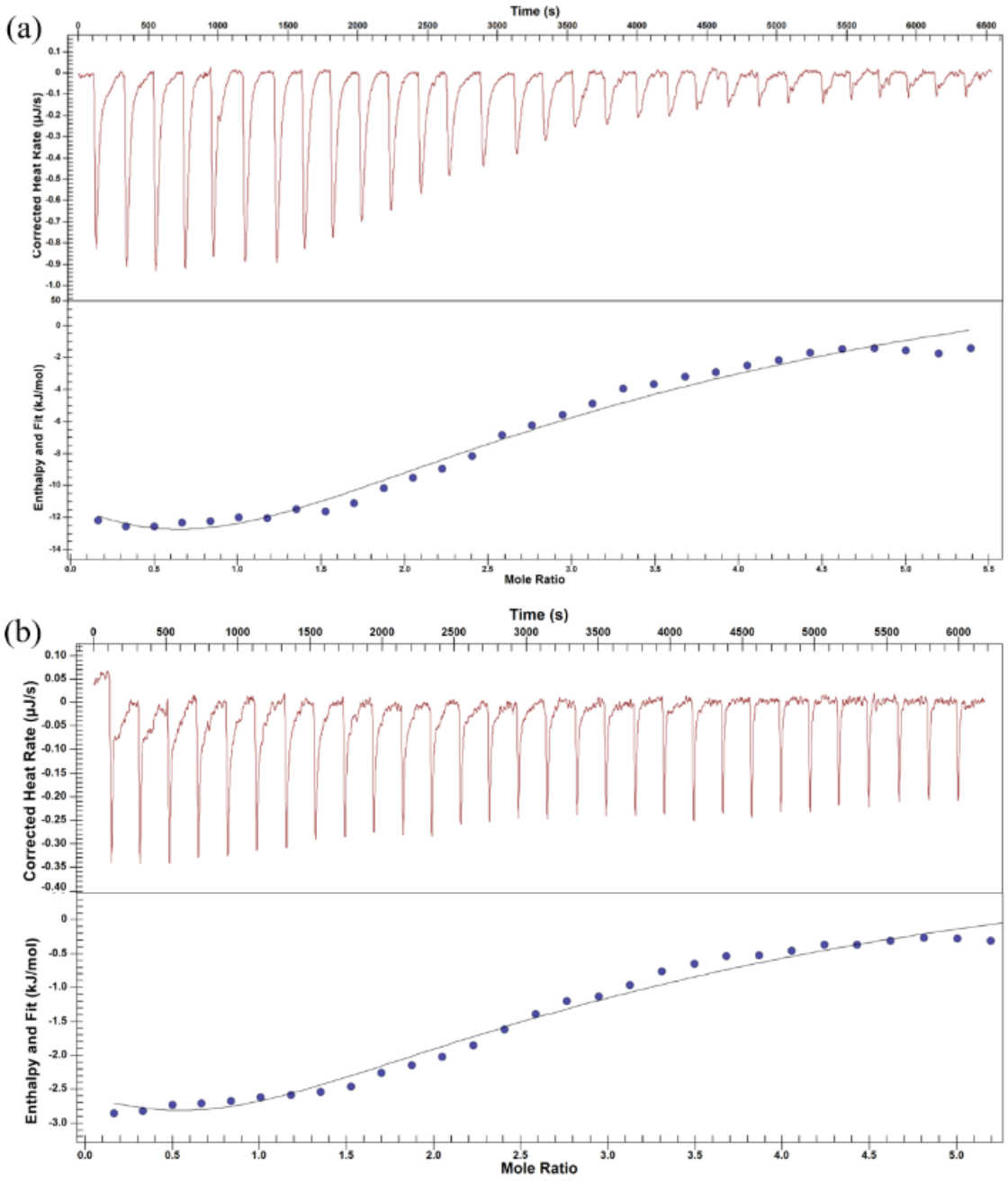

Figure S1. Exothermic diagram for PA mixed with (a) $Eu(NO_3)_3$ and (b) $Tb(NO_3)_3$ in DI water at pH 10.0 measured by ITC.

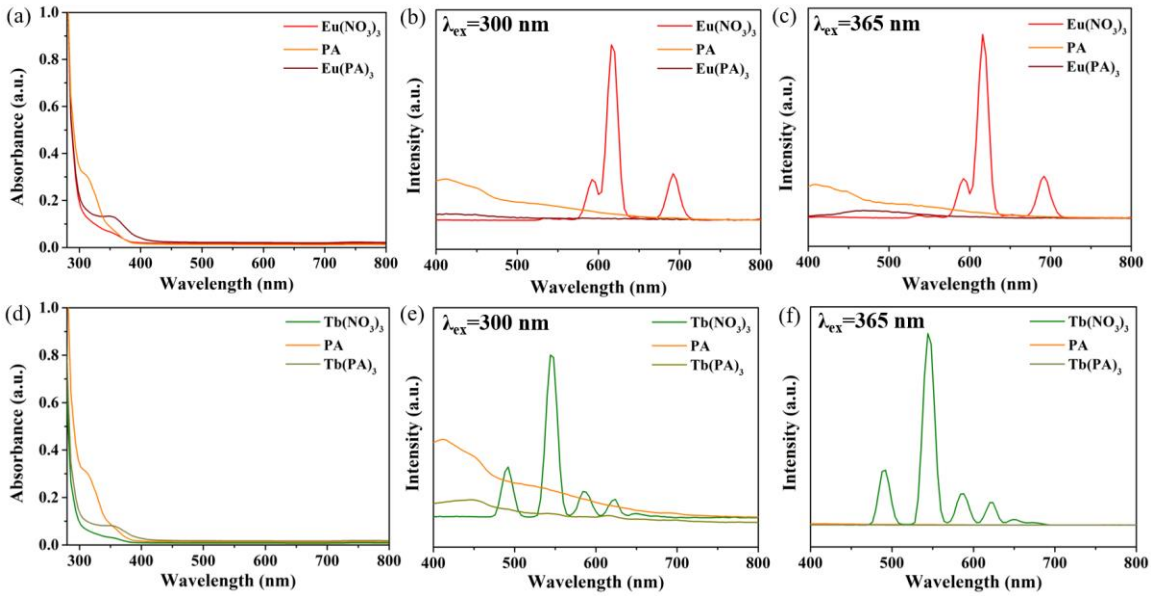

Figure S2. (a) Absorption and (b, c) luminescent spectra of $Eu(NO_3)_3$, PA, and $Eu(PA)_3$. (d) Absorption and (e, f) luminescent spectra of $Tb(NO_3)_3$, PA, and $Tb(PA)_3$. Excitation wavelength (λ_{ex})= 300 or 365 nm.

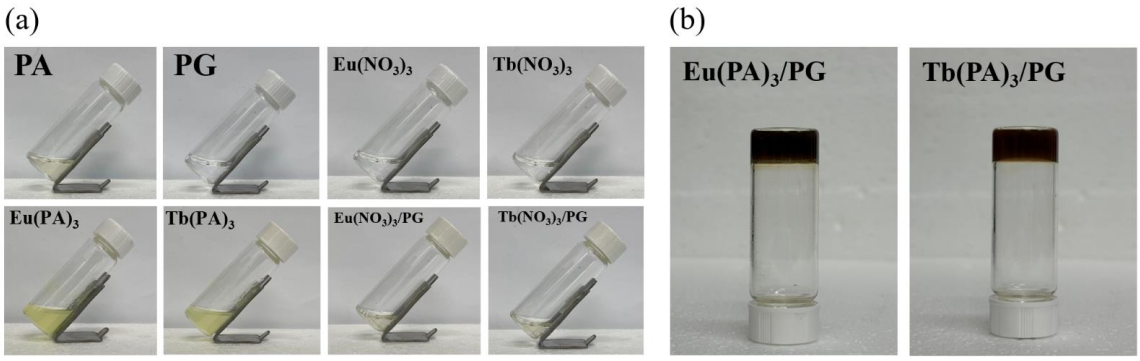

Figure S3. (a) Control groups and (b) $Ln(PA)_3$/PG hydrogels.

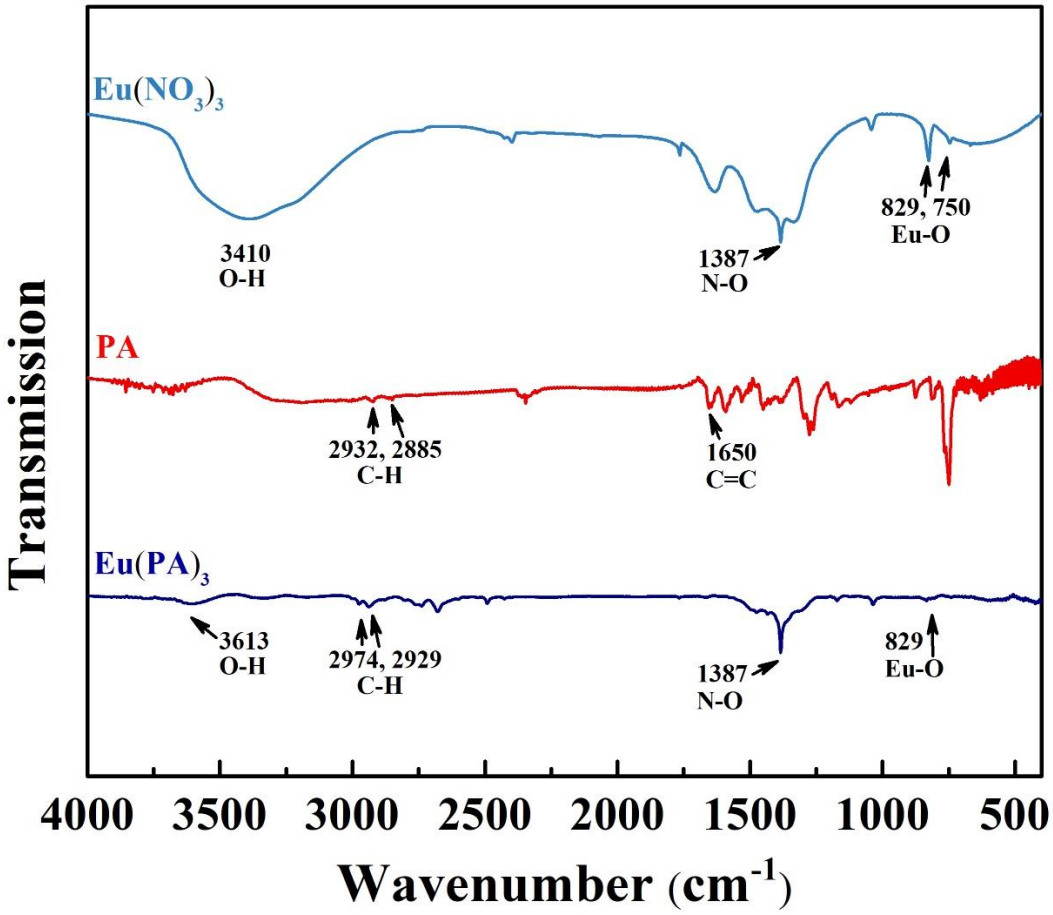

Figure S4. FTIR spectra of $Eu(NO_3)_3$, PA, and $Eu(PA)_3$.

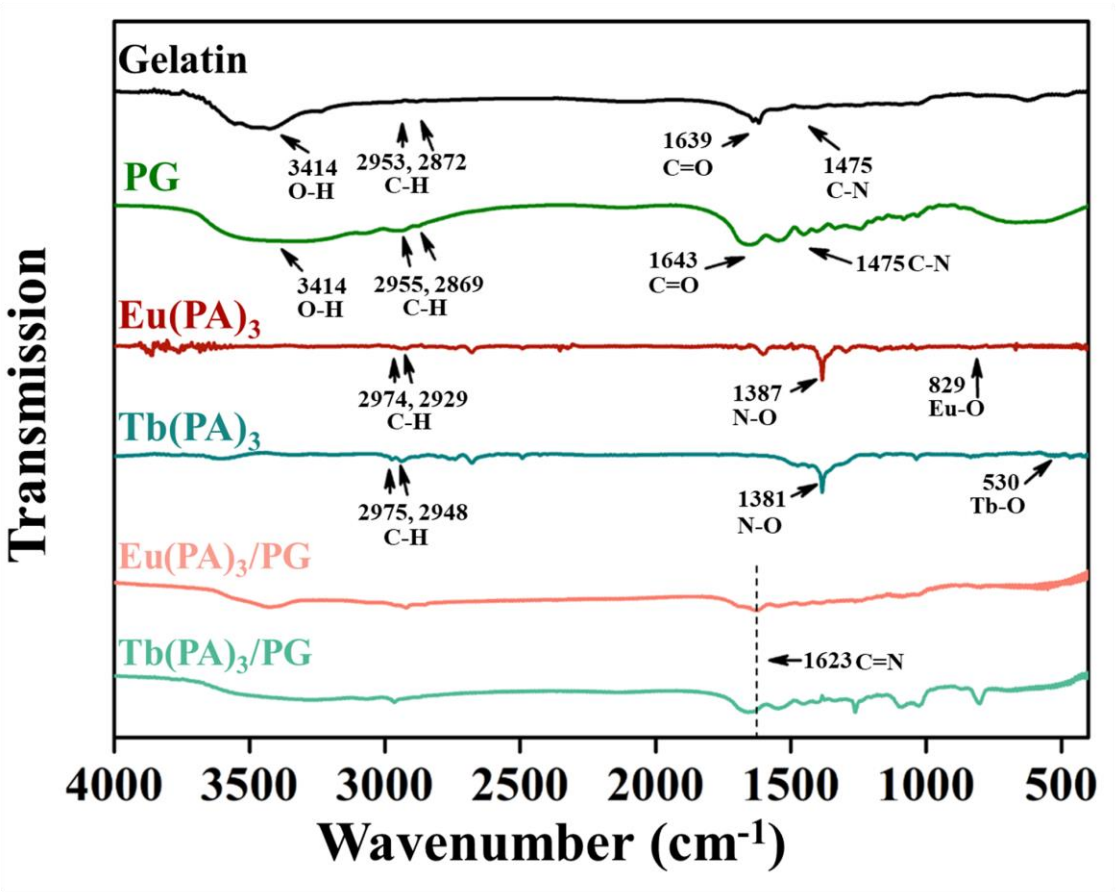

Figure S5. FTIR spectra of gelatin, PG, $Eu(PA)_3$, $Tb(PA)_3$, $Eu(PA)_3$/PG, and $Tb(PA)_3$/PG.

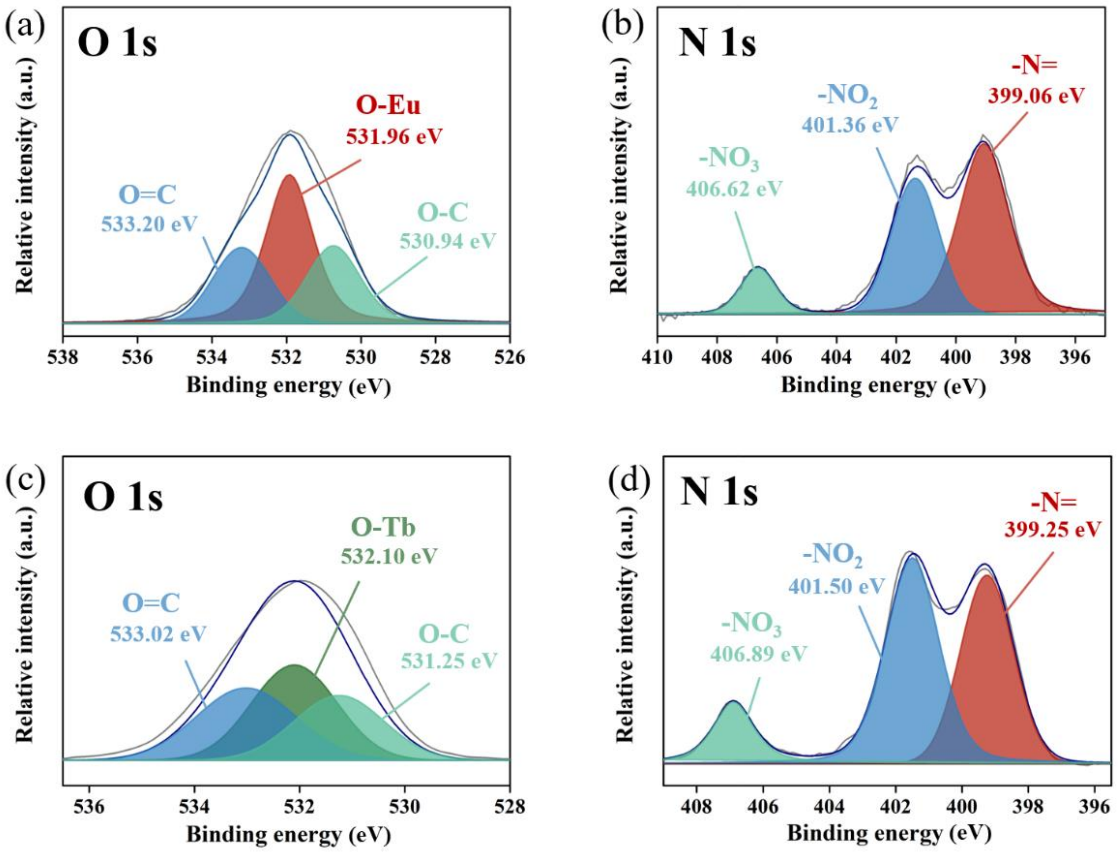

Figure S6. XPS spectra of (a) O 1s and (b) N 1s for $Eu(PA)_3$. XPS spectra of (c) O 1s and (d) N 1s for $Tb(PA)_3$.

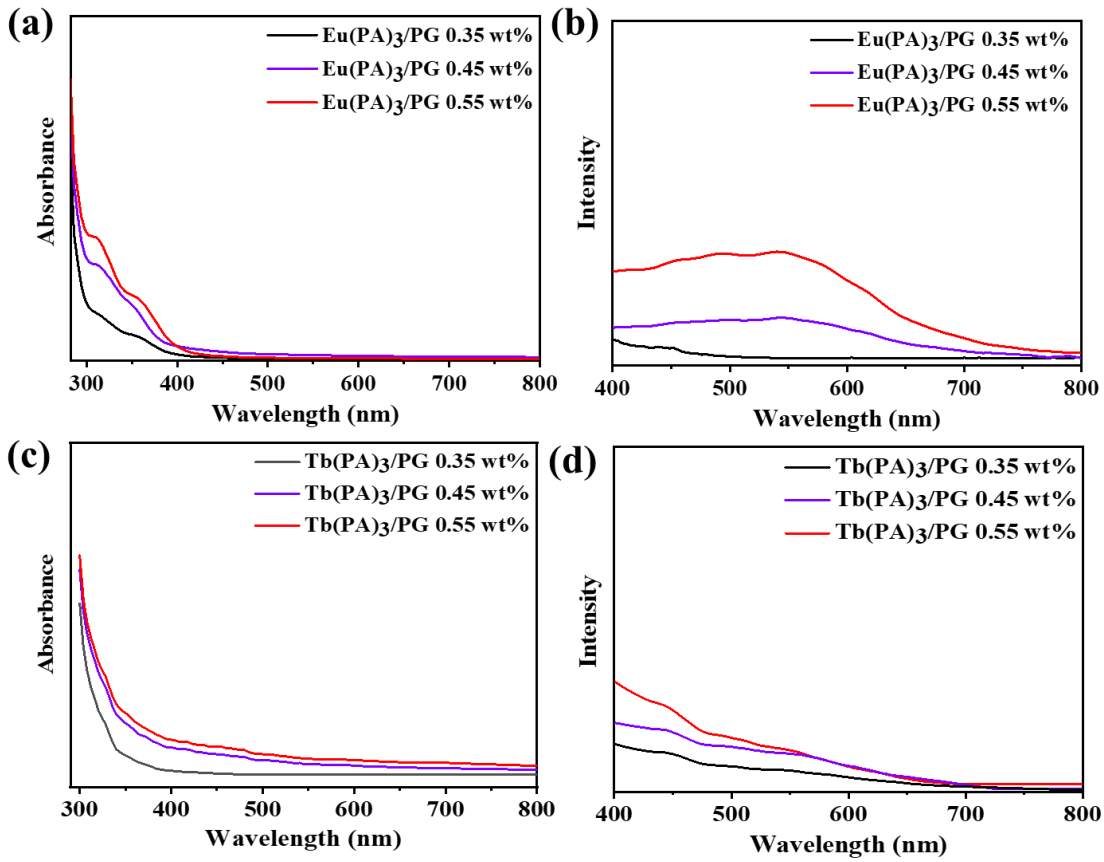

Figure S7. (a) UV-Vis and (b) luminescent spectra of $Eu(PA)_3$/PG hydrogels prepared with varying concentrations of $Eu(PA)_3$. (c) UV-Vis and (d) luminescent spectra of $Tb(PA)_3$/PG hydrogels prepared with varying concentrations of $Tb(PA)_3$.

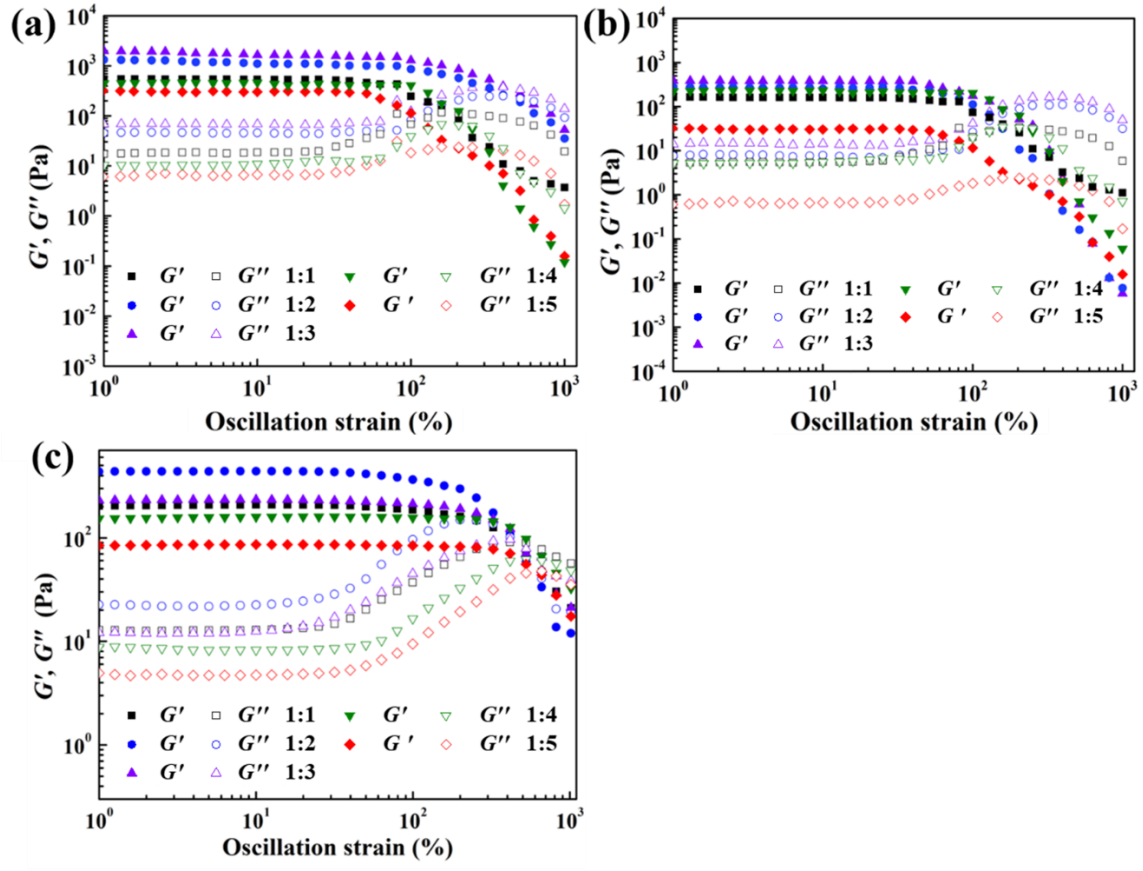

Figure S8. Oscillation strain sweep tests of $Ln(PA)_n$/PG hydrogels with different Ln:PA ratios: (a) Sm^{3+}, (b) Dy^{3+}, and (c) Yb^{3+} ions.

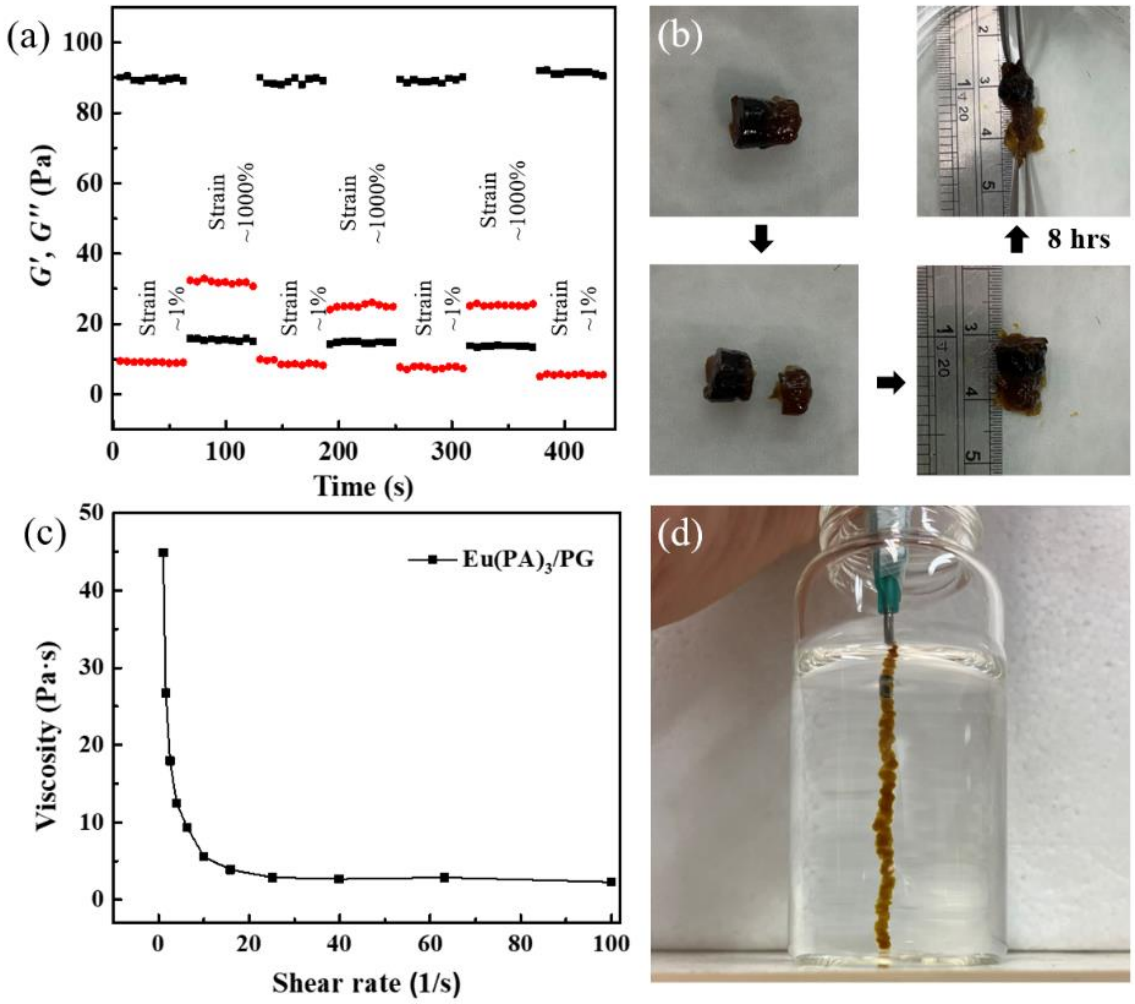

Figure S9. (a) *G'* and *G''* of $Eu(PA)_3$/PG hydrogel were recorded under the cyclic strain time sweep changes between strains of 1% and 1000%. (b) Demonstration of the self-healing behavior of $Eu(PA)_3$/PG hydrogel. (c) Continuous flow sweeps of $Eu(PA)_3$/PG hydrogel. (d) Demonstration of the injectability of $Eu(PA)_3$/PG hydrogel.

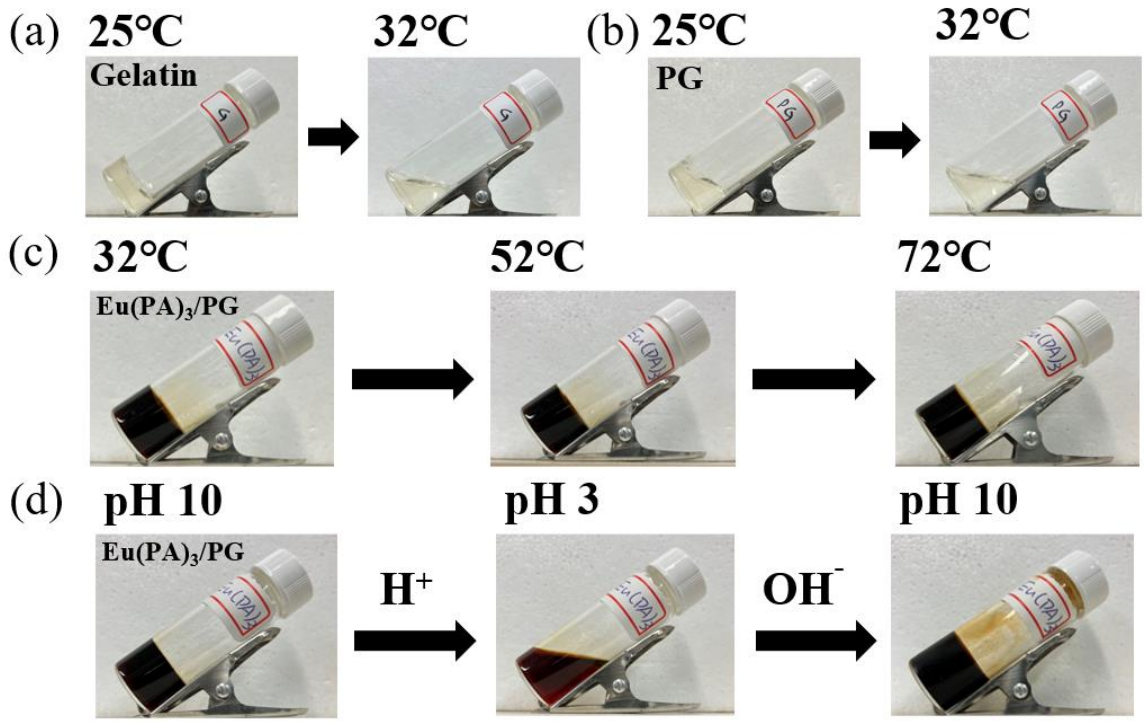

Figure S10. Thermal stability tests of (a) gelatin, (b) PG, and (c) $Eu(PA)_3$/PG hydrogel. (d) The pH-responsiveness of $Eu(PA)_3$/PG hydrogel.

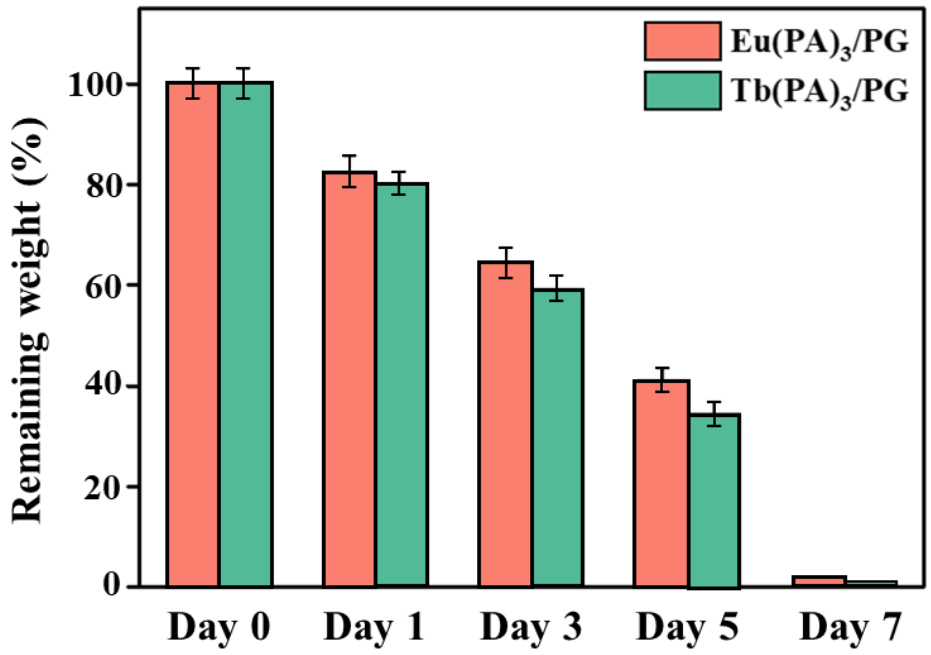

Figure S11. Degradation tests of hydrogels.

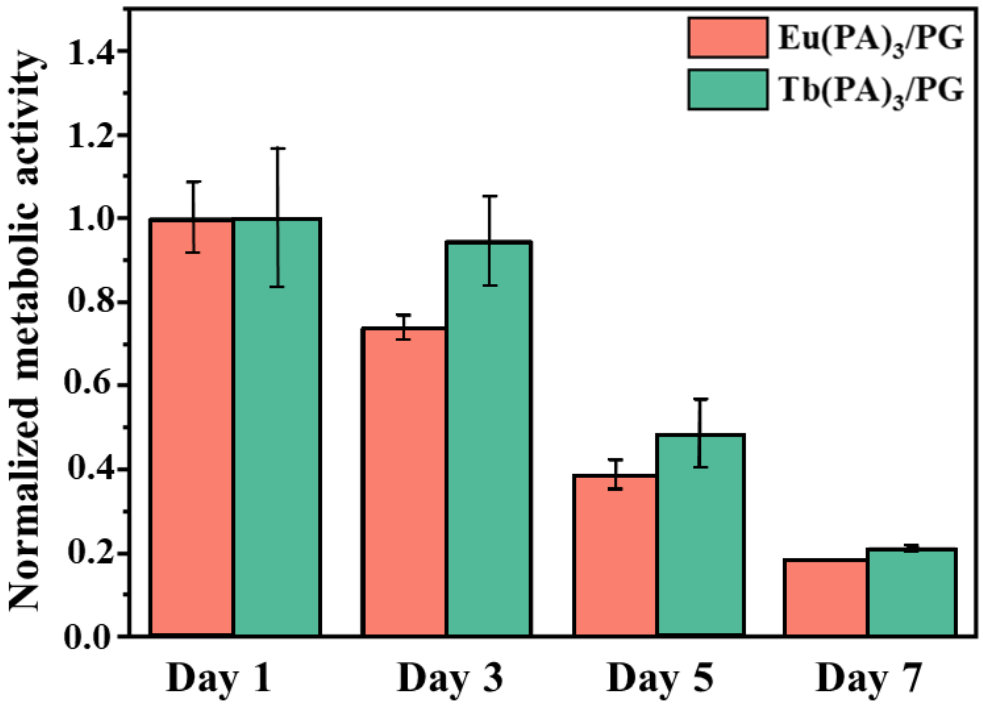

Figure S12. Normalized metabolic activity of MEFs cultured in the extract solutions of the hydrogels.

Table S1. ITC data of Ln-PA complexation.

$Ln(NO_3)_3$	Ligand (PA)	K_a (M^{-1})	ΔH (kcal/mol)	ΔS (cal/mol)	ΔG (kcal/mol)
$Eu(NO_3)_3$	First	1.93 ± 0.12E1	-5.59 ± 0.17	-1.87 ± 0.06	-5.03 ± 0.15
	Second	2.48 ± 1.83E3	3.18 ± 0.08	1.07 ± 0.03	2.86 ± 0.07
	Third	2.03 ± 0.07E5	2.41 ± 0.12	8.18 ± 0.39	-0.03 ± 0.00
$Tb(NO_3)_3$	First	5.80 ± 2.71E1	-0.56 ± 0.38	-1.84 ± 1.28	-0.01 ± 0.00
	Second	1.28 ± 0.29E3	-0.21 ± 0.13	-1.22 ± 0.57	0.15 ± 0.29
	Third	3.73 ± 1.33E5	0.77 ± 0.26	2.70 ± 0.87	-0.03 ± 0.00

Table S2. Strain sweep tests of $Eu(PA)_3$/PG hydrogels with different amount of $Eu(PA)_3$

$Eu(PA)_3$ amount (wt%)	0.35	0.40	0.45	0.50	0.55
Storage modulus (Pa)	234.04	376.79	441.95	389.59	242.29
Loss modulus (Pa)	13.97	16.47	20.04	16.97	13.49

Table S3. Strain sweep tests of $Tb(PA)_3$/PG hydrogels with different amount of $Tb(PA)_3$

$Tb(PA)_3$ amount (wt%)	**0.35**	**0.40**	**0.45**	**0.50**	**0.55**
Storage modulus (Pa)	204.75	253.93	313.75	303.06	222.92
Loss modulus (Pa)	11.20	14.88	17.75	15.37	12.04

Table S4. Strain sweep tests of $Eu(PA)_x$/PG hydrogels with different Eu:PA ratios:

Eu:PA	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
Storage modulus (Pa)	223.44	278.79	441.95	274.71	117.35
Loss modulus (Pa)	13.29	15.62	20.04	15.61	6.94
Crosslinking density (mol/m^3)	0.09	0.11	0.18	0.11	0.05

Table S5. Strain sweep tests of $Tb(PA)_x$/PG hydrogels with different Tb:PA ratios:

Tb:PA	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
Storage modulus (Pa)	183.38	244.22	313.76	236.02	103.18
Loss modulus (Pa)	11.46	14.94	17.75	14.04	7.26
Crosslinking density (mol/m^3)	0.07	0.10	0.13	0.10	0.04

Table S6. Strain sweep tests of $Sm(PA)_x$/PG hydrogels with different Sm:PA ratios:

Sm:PA	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
Storage modulus (Pa)	548.41	1326.52	1989.78	448.26	323.33
Loss modulus (Pa)	17.33	45.25	67.87	10.38	6.02
Crosslinking density (mol/m^3)	0.22	0.54	0.80	0.18	0.13

Table S7. Strain sweep tests of $Dy(PA)_x$/PG hydrogels with different Dy:PA ratios:

Dy:PA	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
Storage modulus (Pa)	164.52	274.89	384.89	224.13	32.33
Loss modulus (Pa)	5.20	7.62	13.93	5.19	0.60
Crosslinking density (mol/m^3)	0.06	0.11	0.16	0.09	0.01

Table S8. Strain sweep tests of $Yb(PA)_x$/PG hydrogels with different Yb:PA ratios:

Yb:PA	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
Storage modulus (Pa)	204.25	436.93	230.70	154.00	84.57
Loss modulus (Pa)	12.84	22.63	12.09	8.81	4.94
Crosslinking density (mol/m^3)	0.08	0.18	0.09	0.06	0.03

Table S9. MIP analysis of $Ln(PA)_3$/PG hydrogels.

Hydrogel	Total intrusion volume (mL/g)	Total pore area (m^2/g)	Median pore diameter (μm)	Porosity (%)
$Eu(PA)_3$/PG	9.43	3.26	6.58	93.36
$Tb(PA)_3$/PG	13.29	4.90	6.79	87.87