Supporting Information

A new insight into the Biginelli reaction: the dawn of multicomponent click chemistry?

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Experimental Section

1. Materials

2-(Acetoacetoxy)ethyl methacrylate (AEMA, Aladdin, 95%), benzaldehyde (Aladdin, > 99.5%), urea (Sinopharm Chemical Reagent Co.,Ltd, AR), $MgCl_2$ (Sinopharm Chemical Reagent Co.,Ltd, AR, 99%), methoxypolyethylene glycol (mPEG, M_n ~ 5000, Aldrich), methoxypolyethylene glycol amine (mPEG-NH_2, M_n ~2000, Aladdin, ≥ 0.4 *mmol/g* NH_2 loading), diketene (Beijing Ouhe tech Co., Ltd., 98%), 4-formylbenzoic acid (Aladdin, 98%), 4-hydroxybenzaldehyde (Aladdin, AR, 98%), N,N'-dicyclohexylcarbodiimide (DCC, Aldrich, ≥ 99.0%), 4-dimethylamiopryidine (DMAP, Aladdin, 99%), D-(+)-glucosamine hydrochloride (J&K Chemical, 99%), 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl, Aladdin, 98%), N,N-dimethylethylenediamine (Aladdin, 98%) were used as purchased.

2,2'-azobis(2-methylpropionitrile) (AIBN, J&K Chemical, 99%) was recrystallized from acetone prior to use. Dry dichloromethane (DCM) was distilled over calcium hydride while anhydrous tetrahydrofuran (THF) was distillation from sodium metal. Other solvents were used directly without further purification.

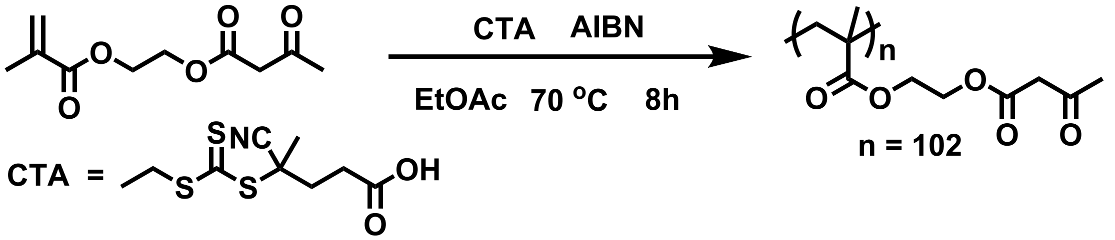
4-cyano-4-(ethylthiocarbonothioylthio) pentanoic acid[1], 4-(2-bromoethoxy) benzaldehyde[2], N,N-dimethyl-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-ethane -diamine[3], and N-acetoacetylglucosamine (dione-Glu)[4] were synthesized as previous literatures.

2. Instrumental Analysis

Gel permeation chromatography (GPC) analyses of polymers were performed using N, N-dimethyl formamide (DMF) containing 50 *mM* LiBr as the eluent. The GPC system was a Shimadzu LC-20AD pump system consisting of an auto injector, a MZ-Gel SDplus 10.0 *μm* guard column(50 × 8.0 *mm*, 10^2 *Å*) followed by a MZ-Gel SDplus 5.0 *μm* bead-size column (50 – 10^6 *Å*, linear) and a Shimadzu RID-10A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 to 10^6 $g\ mol^{-1}$. ^{1}H NMR and ^{13}C NMR spectra were obtained using a JEOL JNM-ECA400 (400 *MHz*) spectrometer for all samples. The ESI-MS data were collected using a Micro TOF-QII Bruker. The FT-IR spectra were made in a transmission mode on a Perkin-Elmer Spectrum 100 spectrometer (Waltham, MA, USA). UV-Visible absorption spectra were recorded on UV/Vis/NIR Perkin-Elmer lambda750 spectrometer (Waltham, MA, USA) using quartz cuvettes of 1 *cm* path length. The fluorescence measurements were obtained on a Perkin-Elmer LS-55 spectrometer equipped with quartz cuvettes of 1 *cm* path length.

3. Method

3.1. Synthesis of PAEMA:

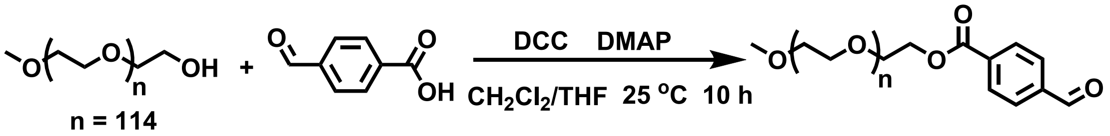


The synthesis of PAEMA is based on literature.[5] AEMA (4.28 *g*, 20 *mmol*), 4-cyano-4-(ethylthiocarbonothioylthio) pentanoic acid as CTA (35 *mg*, 0.13 *mmol*) and AIBN (6.6 *mg*, 0.04 *mmol*) were charged into a dry Schlenk tube along with ethyl acetate (EtOAc, 12 *mL*). The Schlenk tube was sealed with a rubber septum and purged by nitrogen flow for 20 *min*. The tube was then put into an oil bath maintained at 70 °*C* for 8 *h*. The crude was then precipitated from EtOAc to cold petroleum for 3 times, and then dried under vacuum to obtain the pure polymer for further use and characterizations.

^{1}H NMR (400 *MHz*, DMSO-d6)/*ppm*: 4.27 (s, $C\underline{H_2}OCOCH_2COCH_3$), 4.11 (s, $C(CH_3)COOC\underline{H_2}$), 3.62 (s, $COC\underline{H_2}COCH_3$), 2.20 (s, $C\underline{H_3}CO$), 2.00-0.60 (m, $C\underline{H_2}C\underline{H}(CH_3)CO$).
IR (*v*/*cm*$^{-1}$): 2990, 2958, 2893, 1714, 1448, 1411, 1361, 1315, 1239, 1141, 1054, 961, 858,802, 747.

The polymerization conversion (68%) was calculated by ^{1}H NMR of the crude, comparing the peaks of the vinyl protons and the methylene protons between the β-dione group, inferring the degree of polymerization (DP) and the polymer molecular weight (DP ~ 102, M_{nNMR} ~ 21800). The molecular weight was also obtained by measuring the UV absorbance of the trithiocarbonate group on the polymers compared to the one on CTA (ε_{EtOAc} = 6082 L· cm^{-1}· mol^{-1}, DP ~ 118, M_{nUV} ~ 25200), which fits reasonable to the ^{1}H NMR result.
GPC was also tested to evaluate the PDI and molecular weight of the final polymer (M_{nGPC} ~ 44900, *PDI* = 1.18, **Fig. S1**).

3.2 Synthesis of CHO-mPEG:



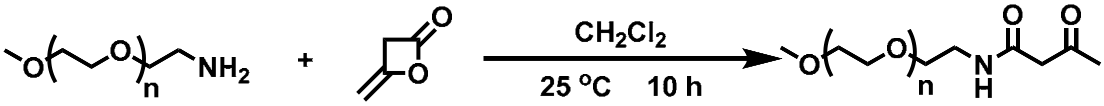
The synthesis of CHO-mPEG is based on literature[6]. Methoxypolyethylene glycol (M_n ~ 5000, 2.00 *g*, 0.4 *mmol*) was treated with toluene to remove the remaining water, then was dissolved with 4-formylbenzoic acid (0.12 *g*, 0.8 *mmol*) and DMAP (5.0 *mg*, 0.04 *mmol*) in 40 *mL* of dry CH_2Cl_2 and 10 *mL* of anhydrous THF. DCC (0.17 *g*, 0. 8 *mmol*) was added to the mixture under nitrogen atmosphere. The system was stirred at 25 °*C* for 10 *h*. After removing the solvents, 15 *mL* of toluene was added and the insoluble white solid was removed by filtration. The final polymer was obtained by precipitation from toluene to diethyl ether for three times.

^{1}H NMR (400 *MHz*, DMSO-d6, δ/*ppm*): 10.12 (s, C$\underline{H}$O), 8.16 (d, *J* = 8.3 *Hz*, C$\underline{H}$CCHO), 8.07 (d, *J* = 8.3 *Hz*, C$\underline{H}$CHCCHO), 4.46-4.41 (m, COOC$\underline{H_2}$), 3.80-3.73 (m, COOCH$_2$C$\underline{H_2}$), 3.71-3.45 (m, OC$\underline{H_2}$C$\underline{H_2}$O), 3.24 (s, OC$\underline{H_3}$).

IR (v/cm^{-1}): 2882, 1722, 1704, 1466, 1341, 1279, 1240, 1146, 1103, 1060, 959, 841.

M_{nGPC} ~24100, *PDI* = 1.03.

3.3 Synthesis of dione-mPEG:



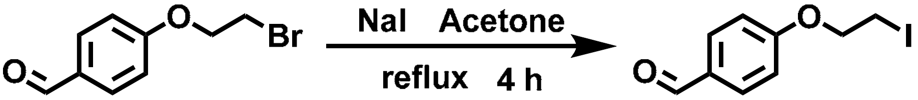
Methoxypolyethylene glycol amine (M_n ~ 2000, 0.80 *g*, 0.4 *mmol*) was dissolved in dry CH_2Cl_2, diketene (68 *mg*, 0.8 *mmol*) was added dropwise at room temperature. The solution was then stirred at 25 *°C* for 10 *h*. After removing the solvent, 5 *mL* of toluene was added to re-dissolve the polymer. The final polymer dione-mPEG was obtained by precipitation from toluene to petroleum ether for three times. NH_2 group conversion is around 95.8% calculated by ^{1}H NMR.

^{1}H NMR (400 *MHz*, DMSO-d6, δ/*ppm*): 8.11 (m, N$\underline{H}$CO), 3.68 (m, OC$\underline{H}$CCHNH), 3.70-3.35 (m, OC$\underline{H_2}$C$\underline{H_2}$O, OC$\underline{H_2}$C$\underline{H_2}$N, and COC$\underline{H_2}$CO), 3.29 (s, NHC$\underline{H_2}$), 3.24 (s, OC$\underline{H_3}$), 2.13 (s, C$\underline{H_3}$CO).

IR (v/cm^{-1}): 2882, 1719, 1651, 1466, 1341, 1279, 1240, 1146, 1105, 960, 841.

M_{nGPC} ~ 10900, *PDI* = 1.07.

3.4 Synthesis of 4-(2-iodoethoxy) benzaldehyde:



In a 25 *mL* round-bottom flask, 4-(2-bromoethoxy) benzaldehyde (0.46 *g*, 2 *mmol*), NaI (0.60 *g*, 4 *mmol*) were dissolved in 3 *mL* of acetone and refluxed for 4 *h*. The reaction mixture was cooled down to 25 °*C*, filtered to remove particulates, and concentrated under vacuo. The crude was then dissolved in CH_2Cl_2 and washed with aqueous $Na_2S_2O_3$ for three times. After drying over $MgSO_4$, the organic layer was concentrated under reduced pressure to obtain the final product. (0.49 *g*, 89.1% yield).

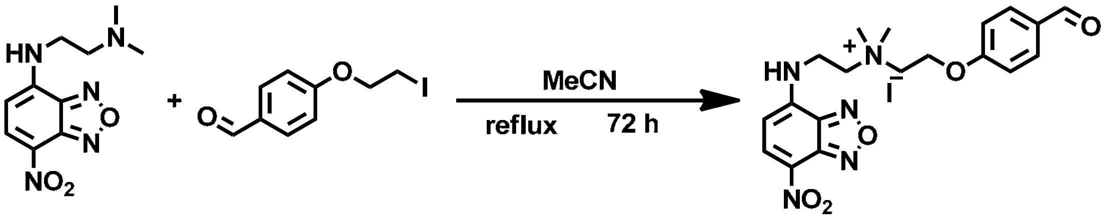
^{1}H NMR (400 *MHz*, DMSO-d6)/*ppm*: 9.88 (s, C<u>H</u>O), 7.88 (m, C<u>H</u>CCHO), 7.14 (m, C<u>H</u>CHCCHO), 4.38 (t, *J* = 6.1 *Hz*, $ICH_2C\underline{H_2}O$), 3.55 (t, *J* = 6.1 *Hz*, $IC\underline{H_2}CH_2O$).

^{13}C NMR (100 *MHz*, DMSO-d6)/*ppm*: 191.35, 162.71, 131.86, 129.97, 115.11, 68.56, 3.26.

IR (v/cm^{-1}): 2959, 2927, 2858, 2799, 2737, 1727, 1677, 1600, 1576, 1506, 1457, 1417, 1389, 1247, 1159, 1067, 988, 869, 825, 746.

ESI-MS: observed (expected): 298.9540 (298.9539) [M+Na$^+$].

3.5 Synthesis of NBD-CHO:



In a 25 *mL* round-bottom flask, N,N-dimethyl-N'-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-1, 2-ethanediamine (75 *mg*, 0.3 *mmol*), and 4-(2-iodoethoxy) benzaldehyde (0.17 *g*, 0.6 *mmol*) were dissolved into 2 *mL* of acetonitrile (MeCN) and refluxed for 48 *h*. The reaction mixture was cooled to 25 °*C*, filtered to get crude product. The product were then washed with diethyl ether and dried under vacuo to get pure product (84 *mg*, 53.1%).

^{1}H NMR (400 *MHz*, DMSO-d6)/*ppm*: 9.90 (s, C<u>H</u>O), 9.36 (s, ArNH), 8.59 (d, *J* = 8.8 *Hz*, $C\underline{H}CNO_2$), 7.90 (d, *J* = 8.6 *Hz*, C<u>H</u>CCHO), 7.18 (d, *J* = 8.6 *Hz*, C<u>H</u>CHCCHO), 6.59 (d, *J* = 8.9 *Hz*, $C\underline{H}CHCNO_2$), 4.62 (m, $NCH_2C\underline{H_2}O$), 4.07 (m, $C\underline{H_2}NHAr$), 3.96 (m, $NC\underline{H_2}CH_2O$), 3.83 (t, *J* = 6.1 *Hz*, $NC\underline{H_2}CH_2NHAr$), 3.28 (s, $NC\underline{H_3}$).

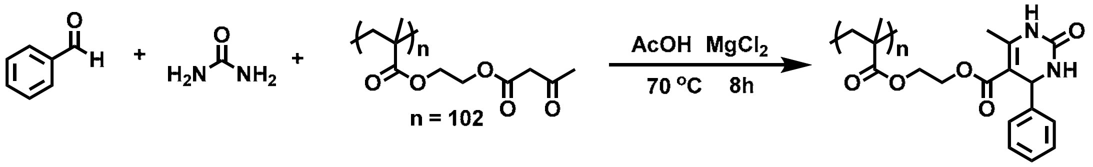
^{13}C NMR (100 *MHz*, DMSO-d6)/*ppm*: 191.38, 162.13, 144.47, 144.32, 144.01, 137.54, 131.72, 130.26, 122.09, 115.22, 100.32, 62.66, 61.89, 61.27, 51.36, 37.49.

IR (ν/cm^{-1}): 3162, 3128, 2983, 1683, 1576, 1492, 1331, 1248, 1165, 995, 899, 810.

ESI-MS: observed (expected): 400.1615 (400.1615) $[M\text{-}I]^+$

PL: λ_{max}^{ex} = 467 *nm*, λ_{max}^{em} = 534 *nm*.

3.6 Post-modification of PAEMA:



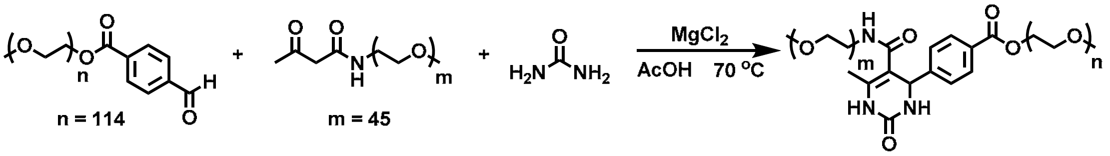
PAEMA (1.07 *g*, 0.049 *mmol*, containing ~5.0 *mmol* 1, 3-dione group), benzaldehyde (0.80 *g*, 7.5 *mmol*), urea (0.45 *g*, 7.5 *mmol*), and $MgCl_2$ (48 *mg*, 0.5 *mmol*) were charged into a dry Schlenk tube along with acetic acid (AcOH, 8.0 *mL*). The Schlenk tube was sealed with a rubber septum and put into an oil bath maintained at 70 *°C* for 8 *h* under the N_2 atmosphere. Samples were taken periodically for ^1H NMR analyses for Biginelli reaction conversion calculation (**Fig. S2**). At the end of the reaction, the mixture was firstly precipitated with water to remove the most urea and the catalyst, $MgCl_2$. Then, the precipitation was re-dissolved by AcOH and precipitated into diethyl ether for three times, and then dried under vacuum for further characterization.

^1H NMR (400 *MHz*, DMSO-d6)/*ppm*: 9.27 and 7.73 (s, $N\underline{H}CON\underline{H}$), 7.35-7,20 (m, $Ar\underline{H}$), 5.16 (s, $ArC\underline{H}$), 4.50-3.70 (m, $COOC\underline{H_2}C\underline{H_2}O$), 2.23 (s, $C\underline{H_3}C$), 2.00-1.30 (m, $CH_2C\underline{H}(CH_3)CO$), 1.30-0.60 (m, $C\underline{H_2}CH(CH_3)CO$). The peak at 3.70-3.40 (s, $COC\underline{H_2}CO$) is negligible.

IR (ν/cm^{-1}): 1702, 1644, 1456, 1228, 1088, 758, 699.

The functionality of final polymer was calculated using ^1H NMR by comparing the peaks between 4.50-3.70 (m, $COOC\underline{H_2}C\underline{H_2}O$) and 5.16 (s, $ArC\underline{H}$), leading to more than 99% of the dione groups were converted. The PDI and molecular weight of the final polymer were calculated through GPC analyses as M_{nGPC} ~ 55300, *PDI* = 1.15 **(Fig. S1)**.

3.7 The linkage of CHO-mPEG and dione-mPEG:

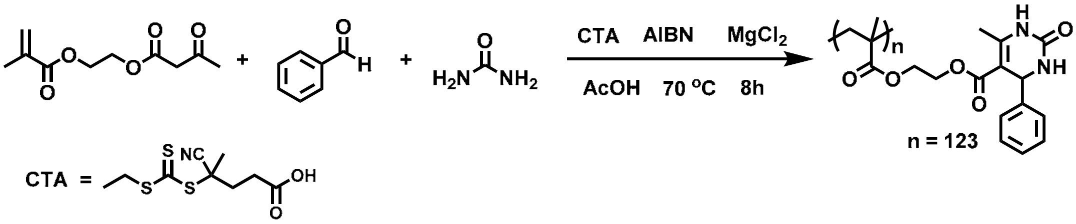


CHO-mPEG (0.10 *g*, 0.02 *mmol*), dione-mPEG (48 *mg*, 0.024 *mmol*), urea (24 *mg*, 0.4 *mmol*) and $MgCl_2$ (20 *mg*, 0.2 *mmol*) were charged into a dry Schlenk tube along with AcOH (1.0 *mL*). The Schlenk tube was sealed with a rubber septum and put into an oil bath maintained at 70 °*C* for 5 *h*. Samples were taken periodically for ^{1}H NMR and GPC analyses for conversion and molecular weight determination, respectively. The mixture was dialyzed against methanol using dialysis membrane (3500D) for 3 days. After removing the solvents, the final polymer was obtained by precipitation from toluene to petroleum ether.

^{1}H NMR (400 *MHz*, DMSO-d6, δ/*ppm*): 8.59 and 7.54 (s, N$\underline{H}$CON$\underline{H}$), 7.91 (d, *J* = 7.7 *Hz*, C$\underline{H}$CCHO) 7.62 (m, N$\underline{H}$CO), 7.37 (d, *J* = 7.7 *Hz*, C$\underline{H}$CHCCHO), 5.31 (s, ArC$\underline{H}$), 4.37 (m, COOC$\underline{H_2}$), 3.80-3.40 (m, OC$\underline{H_2}$C$\underline{H_2}$O, COOCH$_2$C$\underline{H_2}$, OC$\underline{H_2}$C$\underline{H_2}$N, and OC$\underline{H}$CCHNH), 3.24 (m, OC$\underline{H_3}$, NHC$\underline{H_2}$), 1.99 (s, C$\underline{H_3}$C).

M_{nGPC} ~30500, *PDI* = 1.04.

3.8 One-pot synthesis of Biginelli-type homopolymer:



AEMA (2.14 *g*, 10 *mmol*), benzaldehyde (1.59 *g*, 15 *mmol*), urea (0.90 *g*, 15 *mmol*), CTA (18 *mg*, 0.067 *mmol*), AIBN (3.3 *mg*, 0.02 *mmol*), $MgCl_2$ (95 *mg*, 1.0 *mmol*) were accurately weighed and charged into a dry Schlenk tube along with AcOH (8.0 *mL*). The Schlenk tube was sealed with a rubber septum and purged by nitrogen flow for 20 *min*. The tube was then put into a 70 °*C* oil bath for 8 *h*. Samples were taken periodically using degassed syringe for ^{1}H NMR and GPC analyses. At the end of the polymerization, the mixture was firstly precipitated with water to remove the most urea and the catalyst, $MgCl_2$. The precipitation was then re-dissolved by AcOH and precipitated by diethyl ether for three times, and then dried under vacuum to obtain the product.

^{1}H NMR (400 *MHz*, DMSO-d6)/*ppm*: 9.27 and 7.73 (s, $N\underline{H}CON\underline{H}$), 7.35-7,20 (m, $Ar\underline{H}$), 5.16 (s, $ArC\underline{H}$), 4.50-3.70 (m, $COOC\underline{H_2}C\underline{H_2}O$), 2.23 (s, $C\underline{H_3}C$), 2.00-1.30 (m, $CH_2C\underline{H}(CH_3)CO$), 1.30-0.60 (m, $C\underline{H_2}CH(CH_3)CO$). The peak at 3.70-3.40 (s, $COC\underline{H_2}CO$) is negligible.
IR (ν/cm^{-1}): 1706, 1645, 1455, 1229, 1089, 706, 699.

The functionality of final polymer was calculated by ^{1}H NMR by comparing the peaks between 4.50-3.70 (m, $COOC\underline{H_2}C\underline{H_2}O$) and 5.16 (s, $ArC\underline{H}$), leading to more than 99% conversion of the functional groups were obtained. GPC was tested to evaluate the PDI and molecular weight of the final polymer ($M_{nGPC} \sim 55800$, $PDI = 1.13$, **Fig. S5**).

3.9 Cell Culture:

A549 cells were cultured in Dubecco's modified eagle medium (DMEM) supplemented with 10% FBS, 5% penicillin and incubated at 37 °*C*, 5% CO_2. The medium was changed every two days. The cells were harvested with PBS containing 0.025% (w/v) trypsin and 0.01% EDTA, centrifuged and re-suspended in the DMEM medium.

3.10 Cytotoxicity Test:

The cell viability of dione-Glu on A549 cells was evaluated by WST assay. Cells were firstly seeded in 96-well microplates at a density of 5×10^4 cells mL^{-1} in 160 μL of respective media containing 10% FBS. After 24 *h* of cell attachment, the cells were incubated with various concentration of dione-Glu for 24 *h*. Dione-Glu was then washed out with PBS three times. 10 μL of CCK-8 dye and 100 μL of DMEM cell culture media was added to each well and incubated for 2h at 37 *°C*. Plates were then analyzed with a microplate reader (VictorШ, Perkin-Elmer). Measurments of formazan dye absorbance were carried out at 450 *nm*, with the reference wavelength at 620 *nm*. The values were proportional to the number of live cells. The percent reduction of WST was compared to controls (cells not exposed to dione-Glu), which represented 100% WST reduction. Three replicate wells were used for each control and test concentrations per microplate, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean ± standard deviation (SD).

The cytotoxicity tests of urea and NBD-CHO on A549 cells were conducted in similar procedures. After 24 *h* of cell attachment, the cells were incubated with various concentration of urea or NBD-CHO for 8 *h* instead 24 *h*.

3.11 Cell labeling and detection by fluorescence microscopy:

A549 cells were seeded in a glass bottom dish with a density of 1×10^5 cells per dish, culturing with medium containing dione-Glu (final concentration: 500 *μM*) for 3 days. The cells were then labeled with NBD-CHO (180 *μM*) in the presence of urea (180 *μM*) for 3 *h* at 37 *°C*. Afterwards, the cells were washed three times with PBS and then fixed with 4% paraformaldehyde for 10 *min* at room temperature. After three washes with PBS, the cells were stained with hoechst 33258 for 5 *min* and then washed with PBS for three times. Cell images were taken with a Confocal Laser Scanning Microscope (CLSM) Zesis 710, 3-channels (Zesis, Germany) with the excitation wavelengths of 405 *nm* and 488 *nm*.

The control cells were treated through similar process except using medium without dione-Glu (control 1) or incubation without urea (control 2).

Supporting Data

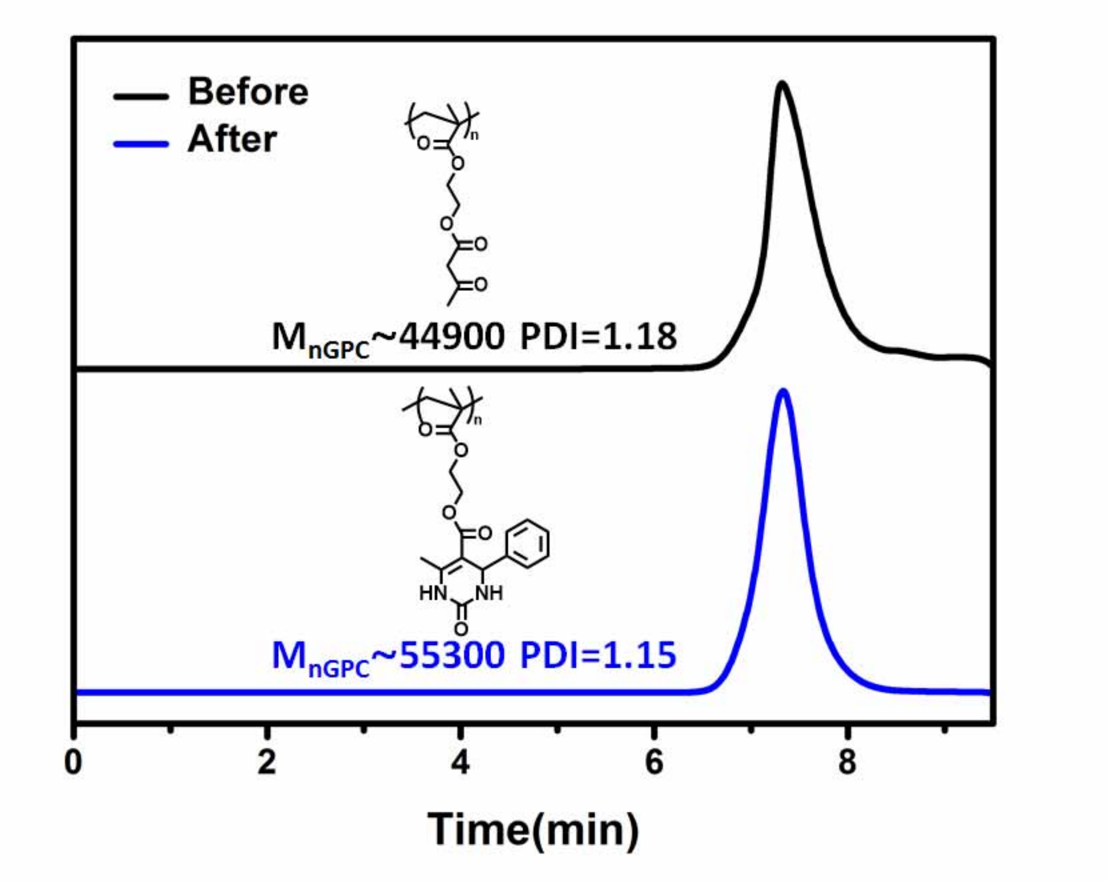


Figure S1. GPC analyses of the polymers before (black, PAEMA) and after (blue, Biginelli-type homopolymer) the Biginelli-modification.

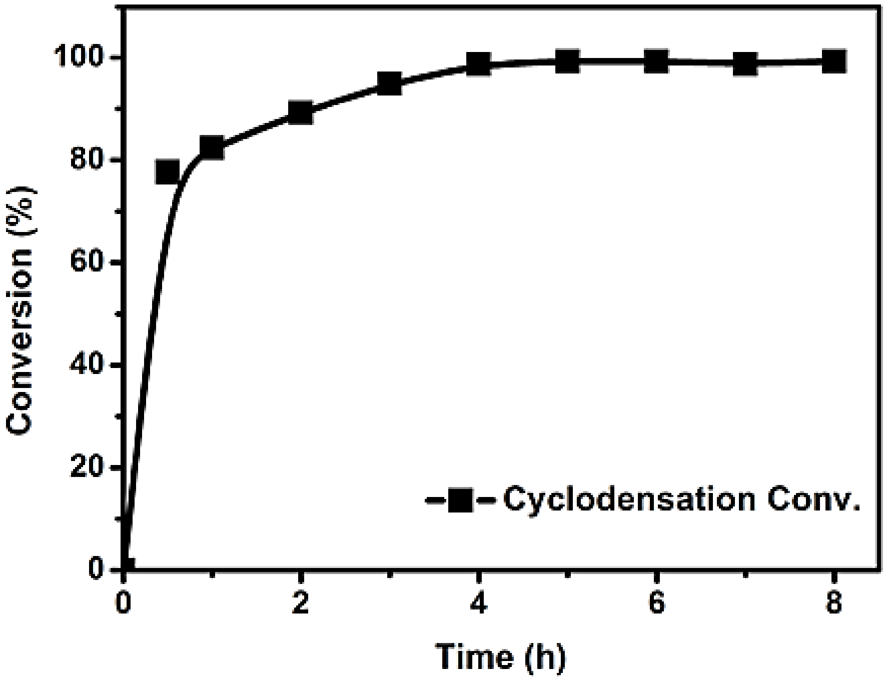


Figure S2. Biginelli reaction conversion of the PAEMA side chain versus reaction time (calculated by ^{1}H NMR analysis).

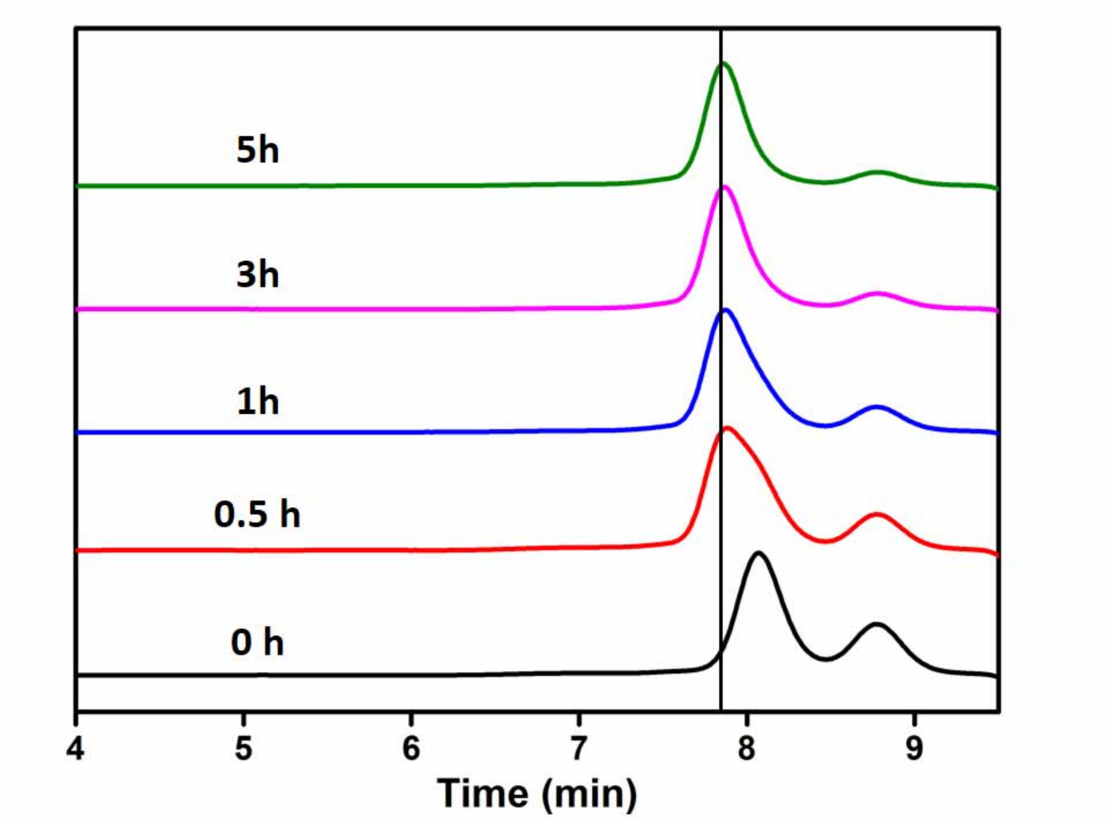


Figure S3. GPC tracking the generation of Biginelli-locked polymer (7.86 *min*) from CHO-mPEG (black, 8.07 *min*) and dione-mPEG (black, 8.77 *min*).

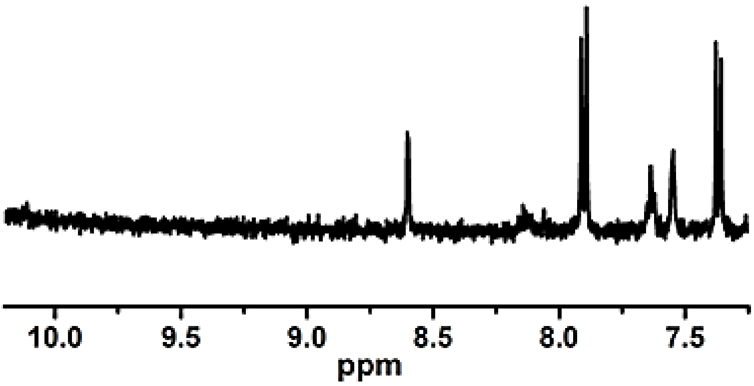


Figure S4. ^{1}H NMR analysis (DMSO-d6, 400 *MHz*, portion) of the crude of Biginelli-coupling reaction after 3 *h*.

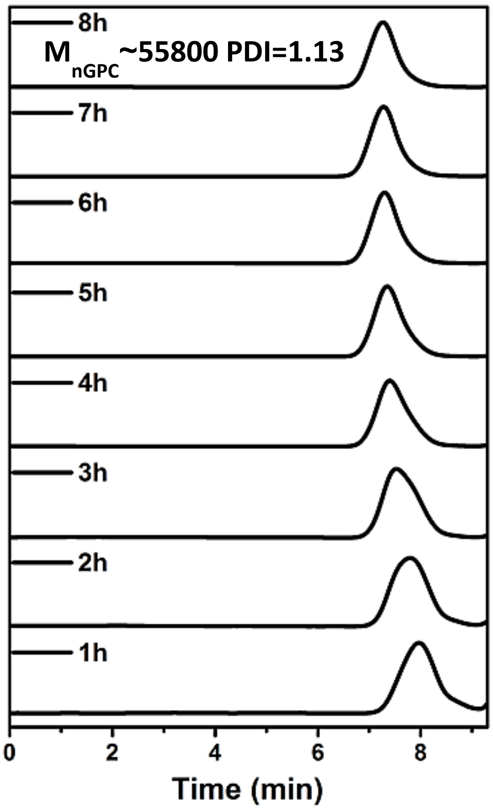


Figure S5. GPC analysis of the samples during one-pot Biginelli-RAFT synthesis of homopolymer.

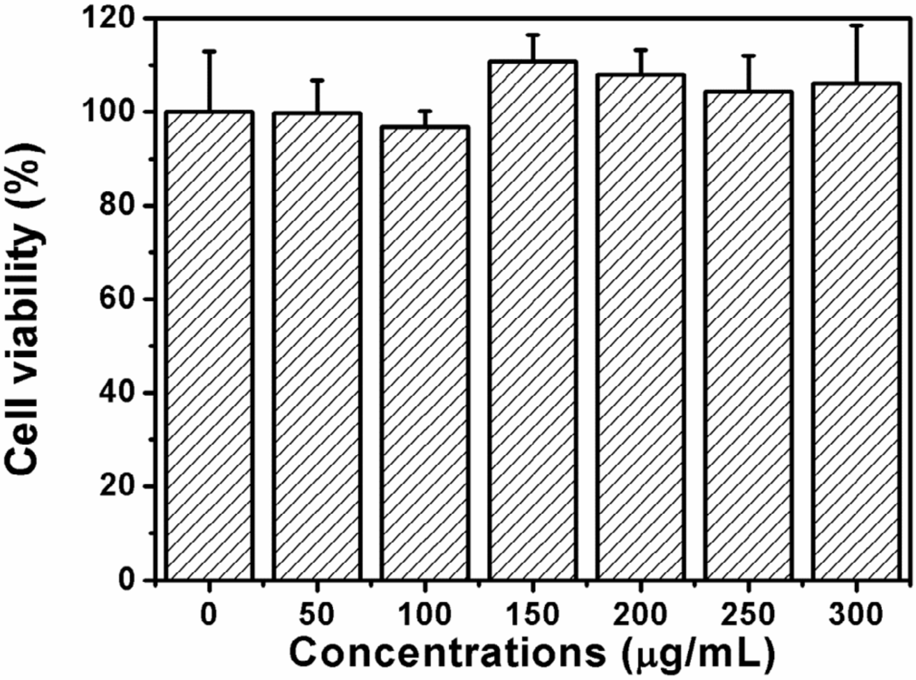


Figure S6. Dose-dependent cytotoxicity of dione-Glu, the cell viability was determined by WST assay.

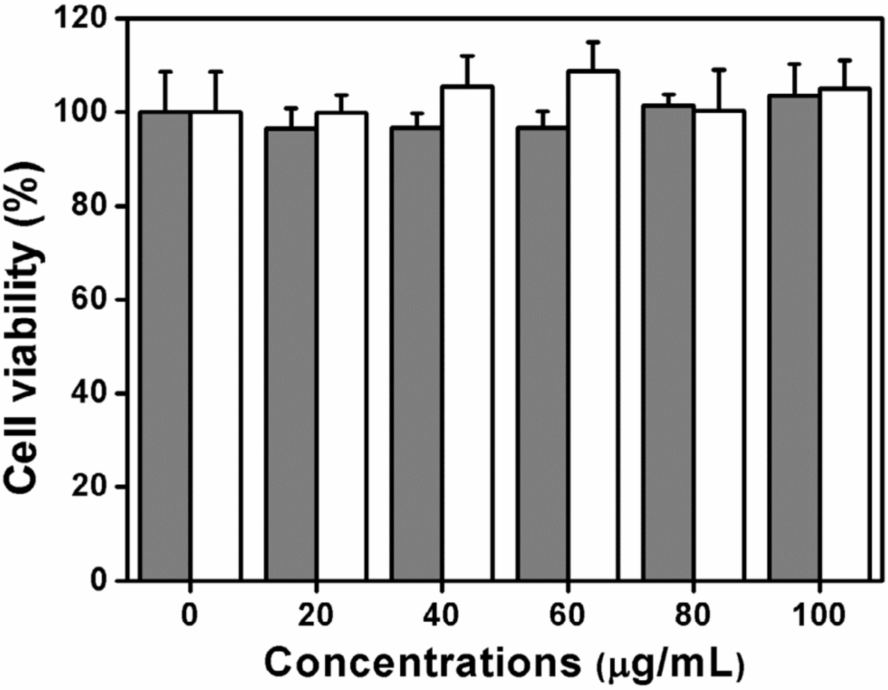


Figure S7. Dose-dependent cytotoxicity of urea (gray) and NBD-CHO (white), the cell viability was determined by WST assay.

Reference

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