

Electronic Supplementary Information

Integrated process for enzymatic catalysis allowing product recovery and enzyme reuse by applying thermoreversible aqueous biphasic systems

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1. Materials

The reagents used in the zwitterions (ZIs) synthesis were 1,3-propanesultone (≥ 99 wt % pure), triamylamine (≥ 98 wt % pure), tripropylamine (≥ 98 wt % pure) and trimethylamine (*ca.* 13 % v/v in acetonitrile) from Tokyo Chemical Industry Co. The following organic solvents, purchased from Kanto Chemical Co., Inc, were also used: acetonitrile (≥ 99.5 wt % pure), diethyl ether (≥ 99 wt % pure), ethyl acetate (≥ 99.5 wt % pure), hexane (≥ 96 wt % pure) and methanol (≥ 99.8 wt % pure). Deuterium oxide, D₂O (99.8 atom % D) from ACROS Organics was used in nuclear magnetic resonance (NMR) experiments.

To prepare aqueous biphasic systems (ABS), polyethylene glycol (PEG) with different molecular weights, namely 1540, 2000, 4000 and 6000 g/mol (abbreviated as PEG 1540, PEG 2000, PEG 4000 and PEG 6000, respectively) were used. They were obtained from Wako Pure Chemical Industries, Ltd, with the exception of PEG 2000 that was obtained from Kanto Chemical Co., Inc.

All studies were performed using commercial laccase from *Trametes versicolor* and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), acquired from Sigma. The salts used were C₆H₈O₇·H₂O (> 99%) from Fisher Scientific, and Na₂HPO₄ (> 99%) from Merck.

2. Experimental Procedure

Determination of the ABS phase diagrams and tie-line. The binodal curves were determined through the cloud point titration method, previously described by us,¹ at 25, 35, and 45 °C (± 1 °C) and atmospheric pressure. Aqueous solutions of polymer at 50-70 wt % and aqueous solutions of each ZI with concentrations ranging from 50 to 70 wt % were prepared and used. Repetitive drop wise addition of the aqueous polymer solution to the aqueous solution of ZI was carried out under constant stirring until the detection of a cloudy solution, followed by the drop wise addition of ultrapure water until the finding of a clear and limpid solution. Whenever necessary, and to better characterize the solubility curves, the addition of the ZI aqueous solution to the polymer aqueous solution also was carried out. The ternary system compositions were determined by weight quantification within $\pm 10^{-3}$ g.

Tie-line (TLs) were determined by a gravimetric method originally described by Merchuk *et al.*². A mixture at the biphasic region was gravimetrically prepared (PEG + ZI+ water) within $\pm 10^{-4}$ g, vigorously stirred, and left for at least 12 h at the desired temperature to reach the complete separation and equilibration of the coexisting phases. After the separation step, both top and bottom phases were weighted.

The experimental binodal curves were fitted using Equation (1)²:

$$[PEG] = A \exp[(B[ZI]^{0.5}) - (C[ZI]^3)] \quad (1)$$

where $[PEG]$ and $[ZI]$ are the PEG and ZI weight percentages, respectively, and A , B and C are constants obtained by the regression.

Oxidation reaction and separation of laccase. The following mixtures compositions were used to carry out the enzymatic catalysis: 39 wt% of N₅₅₅C3S + 13 wt% of PEG 6000 and 20 wt% of N₁₁₁C3S + 20 wt% of PEG 6000. The oxidation reaction in aqueous PEG/ZI monophasic systems was investigated using laccase solutions prepared with a concentration circa 10 g·L⁻¹ dissolved in PBS (phosphate buffered saline at 0.01M and pH ≈ 7.4 at 25 °C), as well as aqueous solutions of ABTS with a concentration of 0.8 g·L⁻¹ dissolved in PBS. In each system, a small amount of the solution containing laccase (≈ 250 μL) and a small amount of the solution containing ABTS (≈ 50 μL) were added to the phase-forming components to reach a total weight of 1 g. The oxidative reactions were carried out at 45 °C and 25 °C in N₅₅₅C3S- and N₁₁₁C3S-based ABS, respectively. Each mixture was carefully mixed during 1 min. Then, the reaction systems were cooled down to 25 °C or heated up to 45 °C in N₅₅₅C3S- and N₁₁₁C3S-based ABS, respectively. A careful separation of both phases was then performed. The quantification of oxidized ABTS was carried by UV-spectroscopy, using a UV-Vis spectrophotometer, at a wavelength of 420 nm, in each phase. Laccase was quantified by its activity, for which 10 μL of each phase was mixed with 50 μL of ABTS, and 700 μL of citrate/phosphate buffer (0.1M) at pH 4.5. The increase in absorbance/min was recorded in a UV-Vis spectrophotometer at 420 nm. Each procedure has been repeated at least three times to determine the average in the compounds extraction efficiencies, as well as the respective standard deviations. Possible interferences of the ZI and polymer with the analytical method were taken into account, and control samples were always prepared using PBS solutions instead of laccase and ABTS aqueous solutions. The percentage extraction efficiency of oxidase ABTS ($EE_{ABTS}\%$) and laccase ($EE_{laccase}\%$) are defined as the percentage ratio between the amount of oxidase ABTS/active laccase in the ZI-rich aqueous phase or PEG-rich phase and that in the total mixture. The ZI interference or the bottom-phase interference in the laccase activity was also evaluated by preparing 5 mixtures, all of them with 50 μL of a laccase solution, and 37 μL of PBS, ZI or bottom-rich phase. The laccase activity was determined in each mixture.

Recovery and activity of laccase. To investigate the recovery and stability of laccase, five consecutive cycles of oxidative reaction of ABTS were performed. Each cycle (reaction + separation) was carried out in a maximum of 1 h. The laccase activity was assayed as previously described. After the reaction, the formation of ABS was induced by changes in temperature. The upper phase was removed (substrate-rich phase), and a fresh substrate solution was added (same volume of solution removed with a composition determined by the TL), and a new cycle started. The 5 cycles were carried out in a maximum of 5 h. The operational stability along the cycles was evaluated by the relative enzyme activity. The relative laccase activity (%) is defined as the ratio of the enzyme activity at the end of each cycle in respect to the enzyme activity in the first cycle.

Recovery of PEG. An ultrafiltration step was applied to the PEG-rich phase using amicons with a cut-off 3 kDa. 2 cycles at 10000 rpm, 20 min each, were applied. The phases obtained were analysed by FTIR and UV-Vis spectroscopies to address the presence of polymer, ZI and ABTS.

3. Results

Zwitterions

N₅₅₅C3S: δ_H (400 MHz; D₂O; δ /ppm relative to TMS): 0.77 (9H, t, $J=7.00$), 1.22 (12H), 1.52 (6H), 2.01 (2H), 2.83 (2H, t, $J=7.00$), 3.10 (6H), 3.25 (2H). Elemental analysis (%) for C₁₈H₃₉NO₃S: Found: C, 61.37; H, 11.67; N, 3.76; C/N, 16.32. Calculated: C, 61.85; H, 11.25; N, 4.01; C/N, 15.42.

N₃₃₃C3S: δ_H (400 MHz; D₂O; δ /ppm relative to TMS): 0.83 (9H, t, $J=7.30$), 1.55 (6H), 1.98 (2H), 2.83 (2H t, $J=7.00$), 3.06 (6H), 3.26 (2H). Elemental analysis (%) for C₁₂H₂₇NO₃S: Found: C, 53.22; H, 10.50; N, 44.89; C/N, 10.89. Calculated: C, 54.31; H, 10.25; N, 45.28; C/N, 10.29.

N₁₁₁C3S: δ_H (400 MHz; D₂O; δ /ppm relative to TMS): 2.12 (2H), 2.86 (2H, t, $J=7.30$), 3.03 (9H), 3.37 (2H). Elemental analysis (%) for C₆H₁₅NO₃S: Found: C, 39.60; H, 8.51; N, 7.38; C/N, 5.36. Calculated: C, 39.76; H, 8.34; N, 7.73; C/N, 5.14.

Determination of the ABS phase diagrams and tie-line

Table S1. Experimental weight fraction data for the systems composed of PEG (1) + N₁₁₁C3S (2) + H₂O (3) at 25 °C and atmospheric pressure.

PEG 1540		PEG 2000		PEG 4000		PEG 6000	
100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂
61.7421	6.0918	34.0487	18.7480	4.5019	39.9471	36.5480	13.8659
55.4399	8.5957	31.8726	20.3877	38.5272	14.1616	31.6993	16.3996
48.1452	12.2903	30.3512	21.6191	33.8958	17.2392	28.1107	17.9649
42.1343	15.3750	28.3233	22.1655	30.0458	19.7265	26.1921	18.7581
34.7956	20.4897	25.5365	24.1730	28.0121	20.6579	23.8267	20.0611
28.1009	25.5694	23.2277	25.7972	25.4302	22.5751	20.3927	21.5968
23.3294	29.1927	21.4455	26.7825	23.1789	24.0596	18.6645	22.8338
19.9088	31.9590	19.3712	28.6400	20.7934	25.9579	17.0672	24.0653
16.9317	33.9948	18.0385	29.5860			15.8596	24.9044
3.6458	51.5846	16.7994	30.6240			15.3595	25.2720
13.7774	37.1920	16.1899	30.8026			14.4565	25.8687
20.6973	31.1082	15.1847	31.4162			13.6024	26.3826
25.9894	26.6601	14.4760	32.2904			12.9157	26.7802
32.2135	21.7758	13.4289	33.2900			12.1127	27.4744
38.6463	17.1432	13.0566	33.4685			11.5231	27.9370
45.5854	12.8555	12.7045	33.6969			10.9030	28.6900
		11.8409	34.5139			10.2791	29.3006
		11.5821	34.7636			9.8782	29.5406
		11.2841	34.9267			9.4401	29.9541
		11.0058	34.9162			8.9751	30.3724
		10.7818	35.0899			8.6531	30.5118
		10.5888	35.2309			8.3425	30.7703
		10.2064	35.7282			7.9672	31.1481
		9.8278	36.1299			7.6797	31.3330
		9.4236	36.3663			7.3563	31.5805
		9.1261	36.6079			7.0926	31.7867
		8.7886	36.8812			6.8398	32.0830
		8.5036	37.1197			6.6460	32.1985
		8.2073	37.3645			6.5345	32.1756
		7.7852	38.0516			5.1742	33.3822
		7.4631	38.4183			5.0224	33.4928
		7.2257	38.5334			4.8670	33.6513
		6.9512	38.9100			4.7271	33.8350
		6.7573	39.0754			4.5967	34.0021
		6.6478	39.0029			4.4796	34.0722
						4.3459	34.2717

Table S2. Experimental weight fraction data for the systems composed of PEG (1) + N₅₅₅C3S (2) + H₂O (3) at 25 °C and atmospheric pressure.

PEG 2000		PEG 4000		PEG 6000	
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
22.2531	38.6759	31.8241	25.8809	36.5072	18.5734
17.8769	42.2702	29.4176	26.9142	33.7211	19.3230
13.2438	46.1743	27.4069	27.9340	30.8400	20.6449
		24.9894	29.1468	28.0395	22.3740
		22.5272	31.2166	26.0183	23.1997
		20.8345	32.6053	24.3382	23.9173
		18.2265	35.5452	22.0932	25.7930
				20.5848	26.9534
				18.9978	28.2837
				17.6259	29.4506
				16.3132	30.7081
				15.2276	31.8042
				14.1827	33.0016
				13.3341	34.1333
				12.3330	35.5667
				11.5755	36.5125
				10.7007	37.9364
				10.0149	38.8572
				9.3636	39.7651
				8.9328	40.3753
				8.3697	41.2818
				7.8662	42.0420
				7.4306	42.8574
				7.0137	43.5142
				6.6308	44.1926
				6.2891	44.8622

Table S3. Experimental weight fraction data for the systems composed of PEG (1) + N₁₁₁C3S (2) + H₂O (3) at 35 °C and 45 °C and atmospheric pressure.

PEG 1540				PEG 2000			
35 °C		45 °C		35 °C		45 °C	
100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂
2.5799	52.4192	12.3438	30.7170	37.8487	14.1892	2.7565	35.8610
4.6128	42.3991	20.0573	25.4501	29.3328	19.5811	6.2559	31.7276
6.1163	39.3528	24.8130	21.0591	22.8144	24.0034	41.1505	10.2282
9.1282	35.5950	28.1497	18.7405	20.3801	25.3364	37.0615	12.4077
14.0020	31.4270	29.1972	17.3232	18.1173	27.6502	34.0536	13.8191
20.6191	26.1840	33.3398	15.2324	14.7896	29.5112	31.2460	15.2157
21.4814	25.1298	36.0052	13.7126	12.8064	31.7178	29.1164	16.1726
29.4090	20.0588	38.0713	12.6729	11.3756	32.8514	26.9461	17.1540
34.6321	16.5671	38.9525	11.4103	10.0079	33.8398	25.2384	17.9872
40.6361	13.0729	39.8117	10.1940	9.2179	34.6020	18.9339	21.7057
		45.3637	8.1480	7.2448	36.4122	17.5744	22.7771
				6.3520	37.7940	16.6291	23.4499
				5.8848	38.2306	15.0332	24.8597
				5.6474	38.2865	14.2061	25.8340
				5.4368	38.5683	13.7490	25.9092
				4.9457	39.7139		
				4.7770	39.7568		

Table S4. Experimental weight fraction data for the systems composed of PEG (1) + N₁₁₁C3S (2) + H₂O (3) at 35 °C and 45 °C and atmospheric pressure.

PEG 4000				PEG 6000			
35 °C		45 °C		35 °C		45 °C	
100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂	100 w ₁	100 w ₂
40.5840	11.8056	2.5259	34.7317	40.5129	11.4816	45.2757	5.7506
36.8467	13.8912	3.6572	31.4290	25.4078	16.5983	40.2826	8.0785
33.2146	15.9229	4.7655	29.7844	21.4521	18.0844	32.8029	10.6718
30.2343	17.6603	8.1131	26.8450	20.1425	18.8188	30.4045	11.9240
25.7113	20.0444	41.6749	9.5992	19.0414	19.3449	25.0506	13.9550
24.0681	21.2838	36.2602	11.0490	17.8234	19.9910	22.6949	14.7161
20.5196	23.8284	33.2506	12.7646	16.8645	20.6571	19.8310	15.9964
18.7608	24.7111	30.9704	14.1185	14.5630	22.1402	16.4714	17.8744
16.7048	25.7350	28.7147	15.0193	12.3231	23.2632	14.6027	19.3607
14.9147	27.0727	25.2678	16.8539	10.6804	24.5741	12.7138	20.6243
13.2412	28.2875	22.5188	18.7483	9.7350	25.2282	11.2953	21.4441
12.1310	29.1904					10.1615	22.1220
10.7508	29.8224					9.6225	22.6210
9.9066	31.2383					8.7220	23.1279
8.5305	32.7156					6.8447	23.9302
7.6329	33.4654						

Table S5. Experimental weight fraction data for the systems composed of PEG 6000 (1) + N₅₅₅C3S (2) + H₂O (3) at 35 °C and atmospheric pressure.

35 °C	
100 w ₁	100 w ₂
30.8761	26.2935
28.1694	27.4792
16.4114	36.4320
12.4479	41.5483
10.2233	43.5569
7.8140	47.1157

Table S6. Identification of the ZI-PEG systems able (✓) or not able (✗) to form ABS at 25 °C.

Polymer	N ₅₅₅ C3S	N ₃₃₃ C3S	N ₁₁₁ C3S
PEG 1540	✗	✗	✓
PEG 2000	✓	✗	✓
PEG 4000	✓	✗	✓
PEG 6000	✓	✗	✓

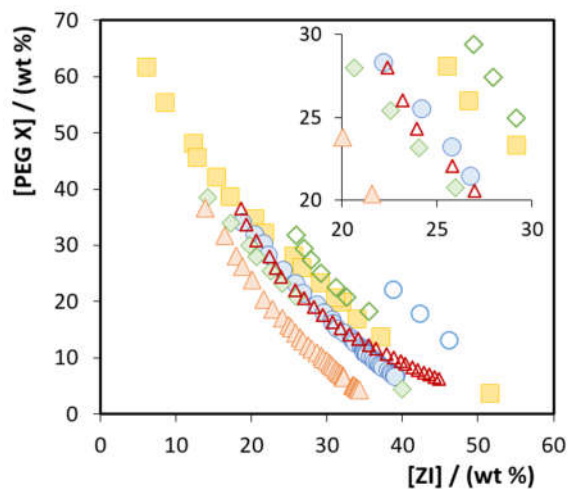


Figure S1. Ternary phase diagrams of ZI-PEG-based ABS at 25 °C. N₁₁₁C3S (full symbols); N₅₅₅C3S (open symbols): PEG 1540 (■), PEG 2000 (●), PEG 4000 (◆) and PEG 6000 (▲).

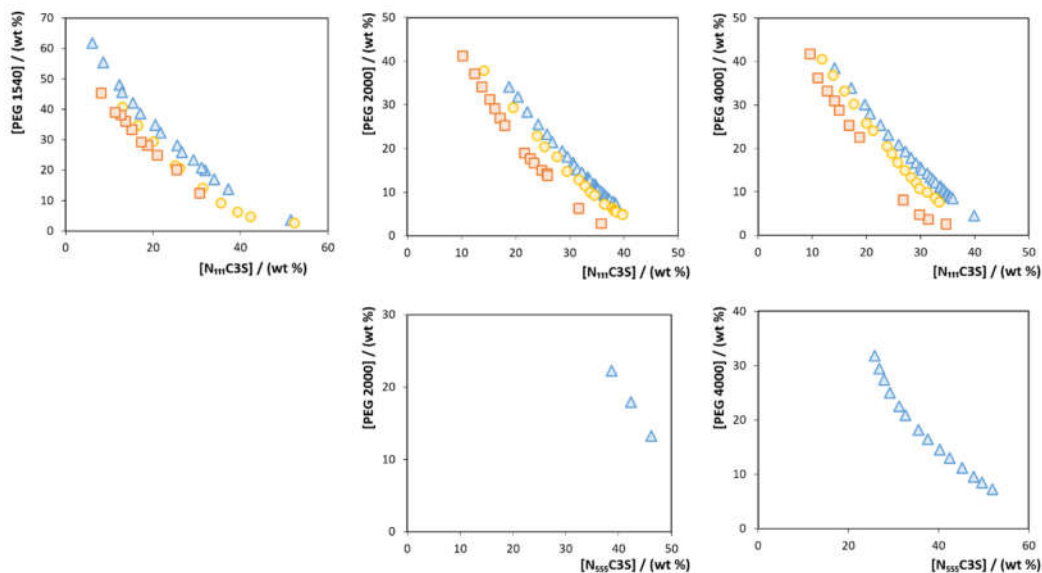


Figure S2. Temperature effect in the phase diagrams of ternary systems composed of ZI + water + PEG at 25 °C (▲), 35 °C (●), and 45 °C (■).

Table S7. Values for the constants A , B and C , obtained by the regression of the experimental binodal data as described by Equation (1)². Respective standard deviations, σ , and correlation coefficients, R^2 , are also given. ^aNot determined due to insufficient experimental data to describe the binodal curve; ^bestimated.

ZI	PEG	T (°C)	$A \pm \sigma$	$B \pm \sigma$	$10^6(C \pm \sigma)$
N ₅₅₅ C3S	2000	25 ^a			
	4000	25 ^a			
	6000	25	333.8 ± 10.4	-0.524 ± 0.004	0.48 ± 0.08
		35	413.4 ± 13.5	-0.488 ± 0.009	0.57 ± 0.13
	1540	25	110.5 ± 4.9	-0.234 ± 0.014	1.23 ± 0.13
		35	108.6 ± 32.4	-0.259 ± 0.078	1.95 ± 0.48
45		85.1 ± 16.7	-0.220 ± 0.060	2.32 ± 0.81	
N ₁₁₁ C3S	2000	25	78.0 ± 1.3	-0.154 ± 0.002	2.44 ± 0.05
		35	84.0 ± 11.0	-0.192 ± 0.033	2.61 ± 0.18
		45	106.0 ± 19.6	-0.281 ± 0.054	3.75 ± 0.62
	4000	25	81.3 ± 16.3	-0.178 ± 0.053	2.72 ± 0.43
		35	92.8 ± 12.0	-0.225 ± 0.036	3.27 ± 0.30
		45	94.2 ± 27.2	-0.255 ± 0.088	5.67 ± 1.22
	6000	25	103.8 ± 1.2	-0.269 ± 0.009	3.76 ± 0.14
		35	253.3 ± 2.0	-0.524 ± 0.011	3.85 ± 0.72
		45	96.5 ± 2.0	-0.302 ± 0.011	7.90 ± 0.72
40 ^b		168.2 ± 0.6	-0.434 ± 0.001	5.49 ± 0.04	

Table S8. Weight fraction percentage (wt %) composition of the initial mixture and of the coexisting phases of the ZI-based ABS used in the enzyme recovery, and pH value.

ZI	PEG	T (°C)	Weight fraction composition / wt %						
			[PEG] _{PEG}	[ZI] _{PEG}	[PEG] _M	[ZI] _M	[PEG] _{ZI}	[ZI] _{ZI}	pH
N ₁₁₁ C3S	6000	40	30.4	13.2	20.0	20.03	2.9	30.9	4.6

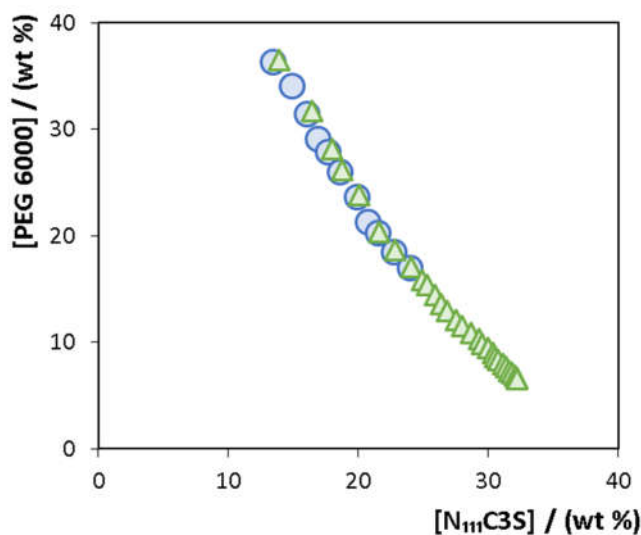


Figure S2. Ternary phase diagram of the N₁₁₁C3S-PEG6000-based ABS at 25 °C: (▲) ABS composed of N₁₁₁C3S, PEG 6000 and water; (●) ABS composed of N₁₁₁C3S, PEG6000 and water, in presence of laccase, ABTS and PBS, at concentrations of 2.50 g/L, 4.00x10⁻² g/L and 4.83x10⁻⁴ g/L, respectively.

Oxidation reaction and separation of Laccase

Table S9. Extraction efficiencies (*EE* %) of the studied systems for oxidized ABTS and laccase for opposite phases in ZI-PEG-ABS.

	<i>EE</i> _{ABTS} (PEG-rich phase) %	<i>EE</i> _{laccase} (ZI-rich phase) %
N ₁₁₁ C3S	100	100
N ₅₅₅ C3S	80.9 ± 4.0	85.0 ± 3.6

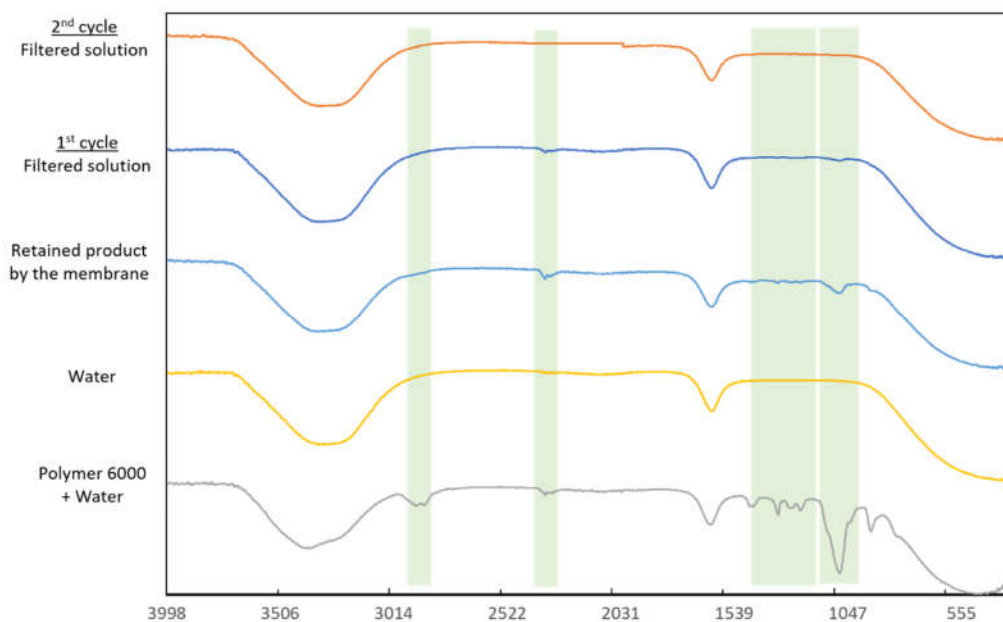


Figure S3. FT-IR (Fourier-transform infrared) spectroscopy spectra corresponding to the PEG-recovery assays.

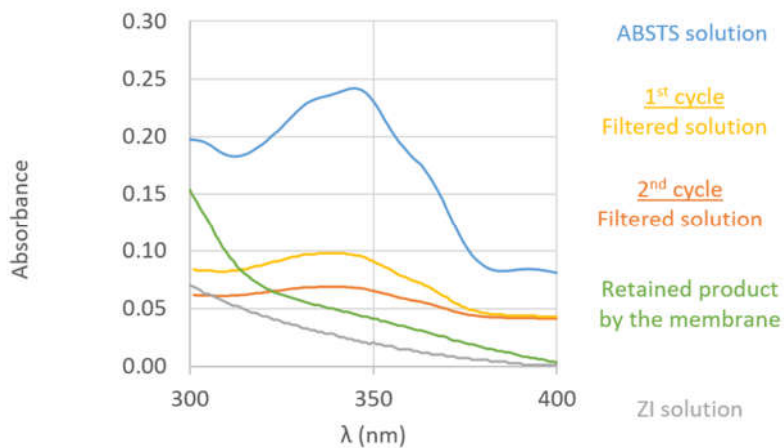


Figure S4. UV-Vis spectroscopy spectra corresponding to the PEG-recovery assays.

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