Supporting Information for:

Soluble enzyme cross-linking via multi-component reactions; a new generation of cross-linked enzymes

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Materials and instrumentation

The organic solvents used in this research were purchased from Merck. *Thermomyces lanuginosus* lipase (Lipozyme® TL 100 L) and the lipase from *Rhizomucor miehei* (Palatase® 20000 L) were supplied by Novozymes (Bagsvaerd Denmark). Bovine serum albumin, *para*-nitrophenyl butyrate, cyclohexyl isocyanide, glutaraldehyde bisulfite were from Sigma. Dynamic laser scattering (DLS) was performed on Malvern ZEN 3600. Scanning Electron Microscopy (SEM) was performed on a TESCAN, VEGA3 (Tescan, Czech Republic) operating at 20 kV accelerating voltage at ambient temperature. UV analyses were conducted by a PG instruments-T 80^{+} UV-Visible spectrometer.

Methods

Experiments were performed in triplicate and the presented results are as their mean value.

CLEs production by multi-component reaction

For the cross-linking of RML and TLL, 500 µL of each enzyme (5 and 20 mg/mL, respectively) were added in a 5 mL glass tube. Then, 30-290 mM of GA and 18-330 mM of cyclohexyl isocyanide were added to the solution. The reaction mixture was stirred on a magnetic stirrer for 16-18h for RML and 3-18h for TLL at 25 °C. The obtained CLEs were then centrifuged at 10 000 rpm for 5 min and washed five times with distilled water to remove unreacted components. Finally, CLEs were suspended in 25 mM phosphate buffer (pH 7) and stored at 4 °C. Two control experiments for both lipases were conducted one of them in the absence of cyclohexyl isocyanide and the other in the absence of glutaraldehyde.

CLEs production in the presence of BSA

To a total volume of 600 µL RML and TLL (3 and 12.5 mg/mL, respectively) in distilled water, 100 mM GA and 2 µL of cyclohexyl isocyanide (36 mM) were added. The solutions were stirred on a magnetic stirrer at 25 °C up to 24 h. The resulting CLEs were then centrifuged at 10 000 rpm for 5 min, washed five times and suspended in 25 mM phosphate buffer (pH 7) and stored at 4 °C.

Enzyme activity assay

The activity of free lipases and immobilized preparations was determined by the hydrolysis of *p*-NPB by adding 20 µL of 50 mM *p*-NPB in acetonitrile to 2 mL of 10 mM phosphate buffer (pH 7). The product concentration (*p*-nitrophenol) was determined from the increase in absorbance at 348 nm (ε =5150 $M^{-1}cm^{-1}$). Specific activity (U/mg lipase) is expressed as micromole of substrate hydrolyzed per minute per mg of each enzyme.

Protein Quantification

Protein Quantification was performed by Bradford's method. Briefly, 20 µL of enzyme solution was added to 1 mL of Bradford reagent and incubated at room temperature for 10 min. The absorbance of the solution was then measured at 595 nm. The protein content of each solution was calculated by using a calibration curve of BSA as a standard. The ratio of the amount of remaining enzyme in the supernatant to the initial amount of offered enzyme for immobilization was calculated and reported as a percentage of immobilization.

Thermal inactivation of different RML and TLL immobilized preparations

To investigate the thermal stability of immobilized enzymes, the free and immobilized enzymes were incubated in 10 mM sodium phosphate buffer (pH 7) under different temperatures for 2 h in a water bath. The activity of each sample was determined using *p*-NPB assay as described above.

Co-solvent stability of the free and immobilized preparations of the lipases

Free enzyme and immobilized preparations were incubated in a solution containing 10 mM sodium phosphate buffer (pH 7) and 20% of dioxane, 1-propanol, 2-propanol, methanol and ethanol at room temperature. The activity of each sample was measured using the *p*-NPB assay.

Determination of the optimum pH activity

Free enzyme and the immobilized preparations were added in 2 mL of a solution containing 10 mM sodium phosphate buffer with different pH from 4 to 10 at 25 °C. The activity of each sample was measured using the *p*-NPB assay and the highest activity was considered as 100 %.

Table S1. General parameters of RML and TLL immobilization:

Enzyme	Immobilization yield (%)[a]	Immobilization time (h)	Specific activity (U/mg Enzyme)[b]
RML	57-97	16-18	0.87-21.3
TLL	57-97	3-18	6.5-15.9

Immobilizations were performed as described in the experimental section.[a]Yield is defined as the percentage of the soluble enzyme that becomes cross-linked. [b]Specific activity is expressed as micromole of substrate hydrolyzed per minute per mg of protein. Specific activities of free RML and TLL is 1.73 U/mg and 8.9 U/mg, respectively

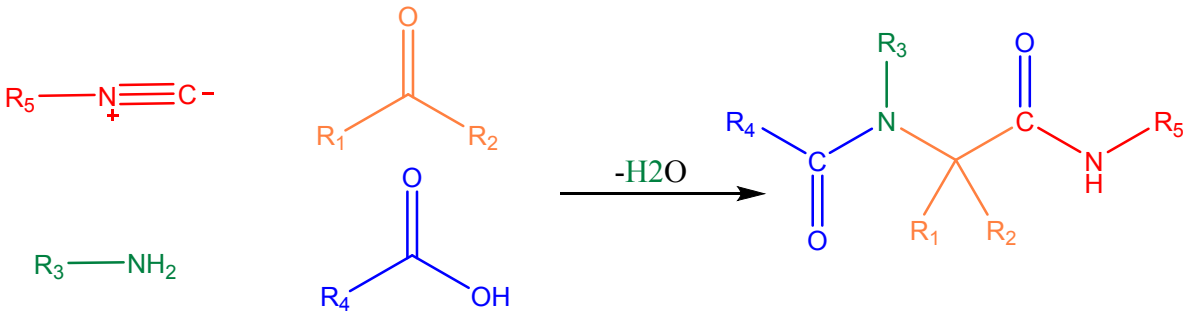
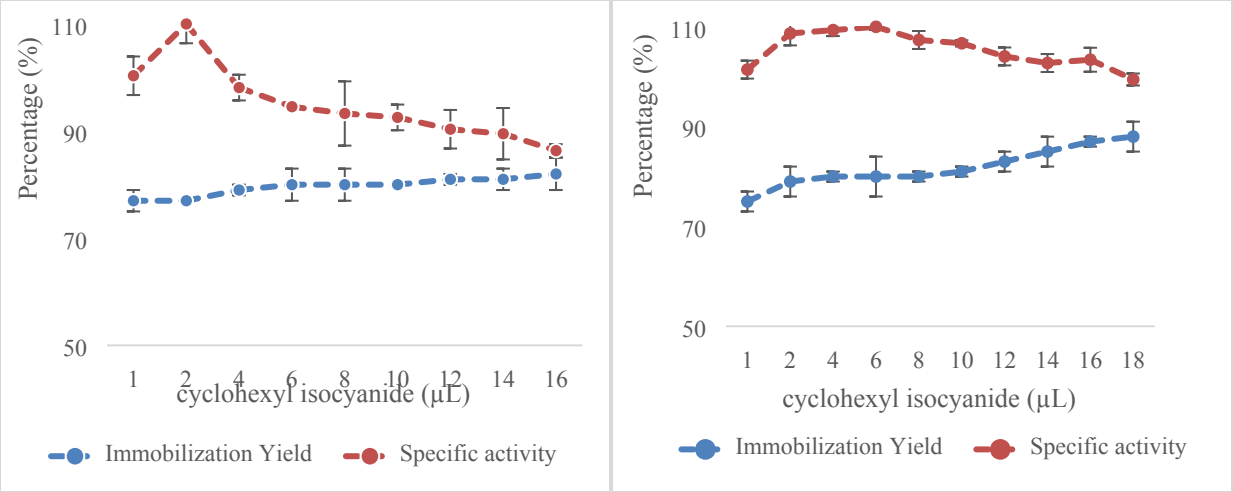


Figure S1. Components of the Ugi reaction



a b

Figure S2. The effect of cyclohexyl isocyanide on the specific activity and immobilization yield of a) RML b) TLL in the presence of constant amount of GA (65 mM) at 25 °C.

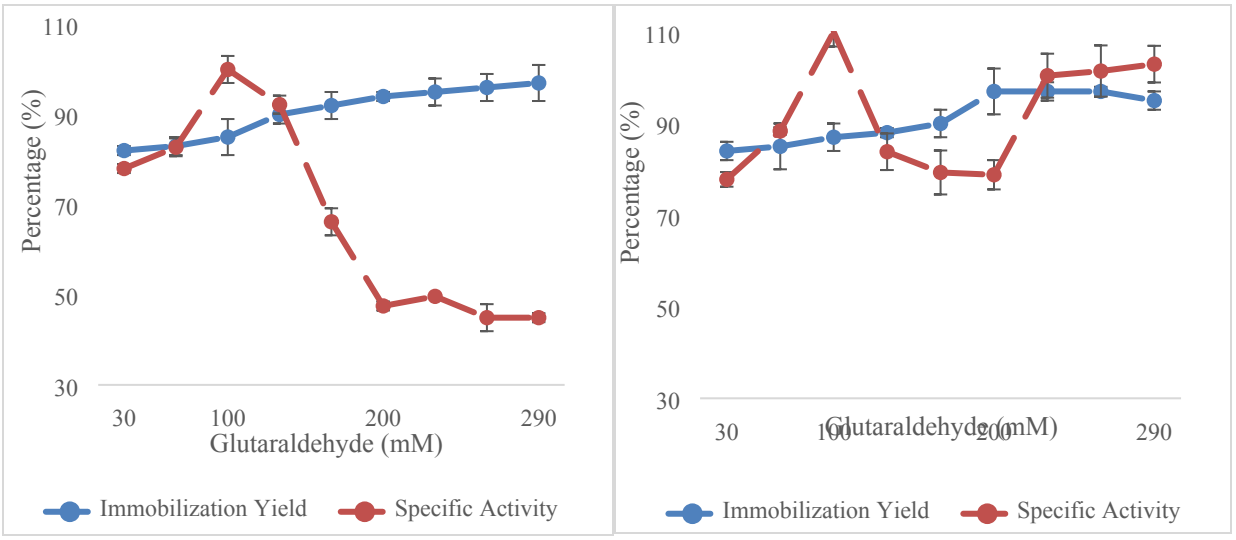


Figure S3. The effect of GA on the specific activity and immobilization yield of a) RML b) TLL in the presence of cyclohexyl isocyanide (36 mM) at 25 °C.

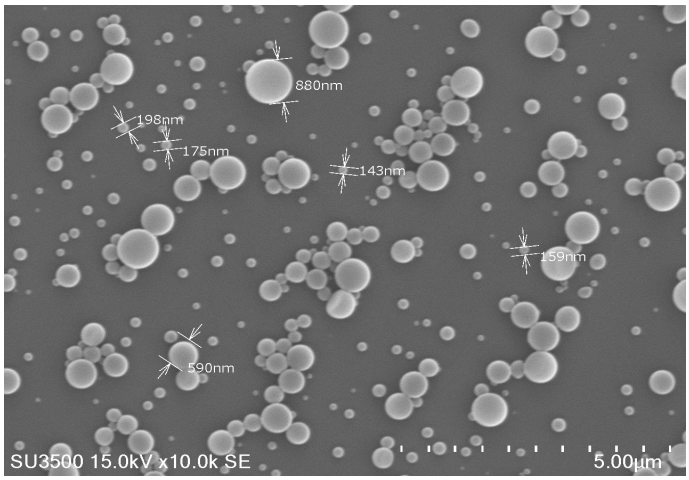


Figure S4. SEM image of immobilized RML (Immobilization was performed in the presence of 20 mg BSA)

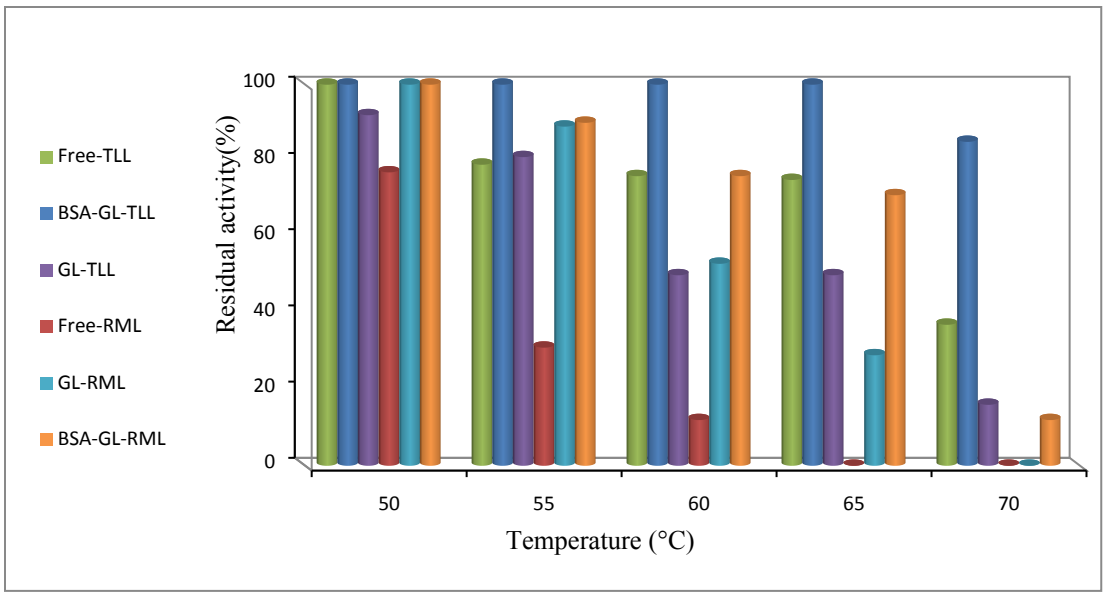


Figure S5. Thermal stability of the immobilized derivatives of RML and TLL. Experimental condition: Increasing a certain amount of each biocatalyst in 1mL of sodium phosphate buffer 25 mM (pH 7.0) and incubation at different temperatures for 2h. The initial activity of the free enzymes and each immobilized derivative was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

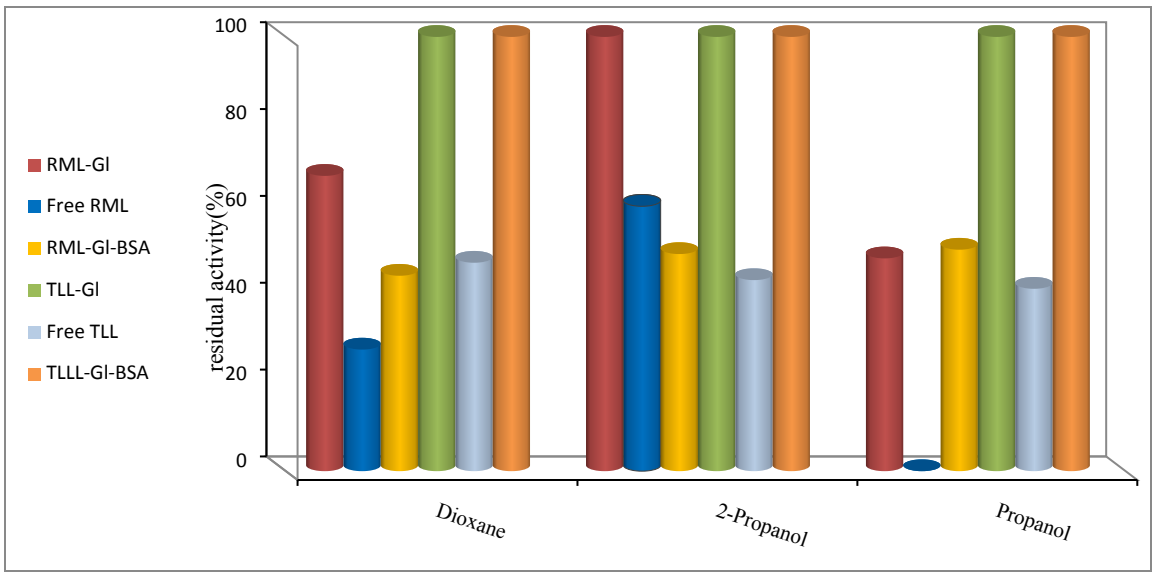


Figure S6. Co-solvent stability of the immobilized derivatives of RML and TLL in 20% of organic solvents. Experimental condition: Incubation of each biocatalyst in 1 mL solution containing 25 mM sodium phosphate buffer (pH 7.0) and 20% of three organic solvents at 25°C. The initial activity of RML, TLL and each immobilized derivative was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

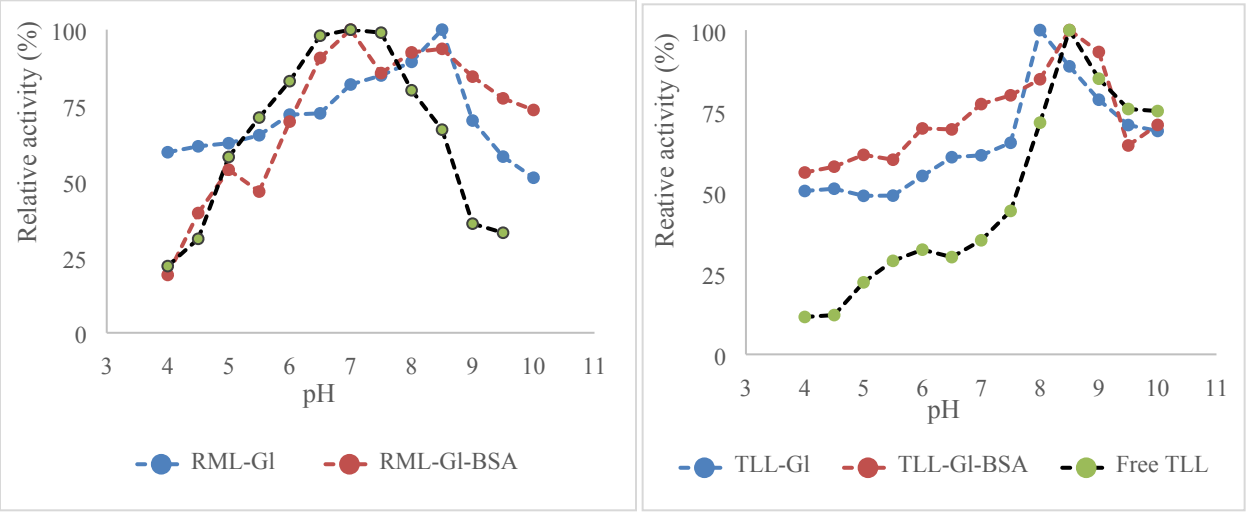


Figure S7. Optimum pH activity of the immobilized derivatives of RML and TLL

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	118.9	Peak 1:	86.15	100.0	10.91
PdI:	1.000	Peak 2:	0.000	0.0	0.000
Intercept:	7.57	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

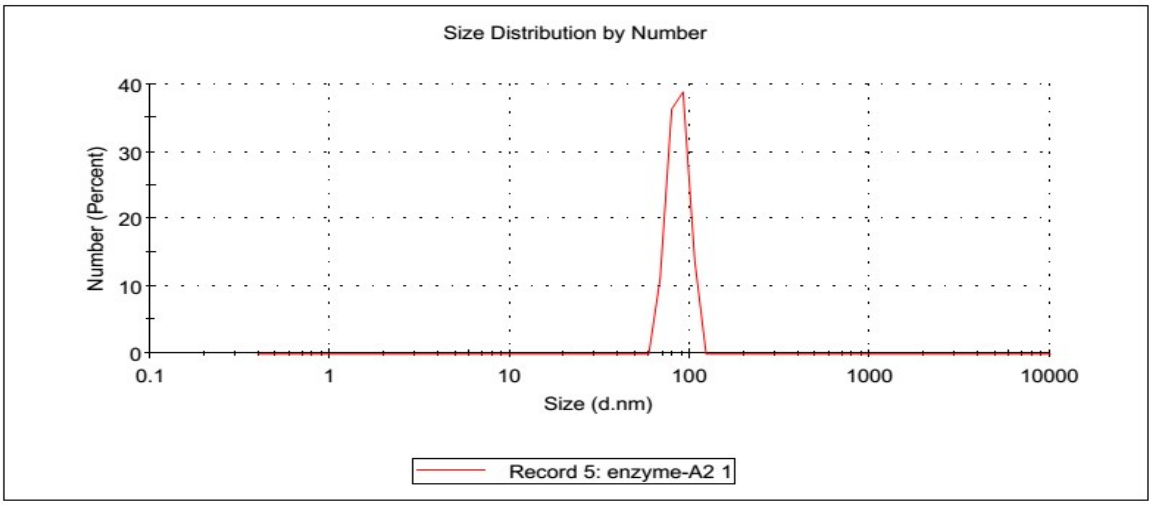


Figure S8-1. DLS analysis for the cross-linked TLL prepared in the presence of 0.5 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	119.6	Peak 1:	84.59	100.0	10.76
PdI:	1.000	Peak 2:	0.000	0.0	0.000
Intercept:	7.29	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

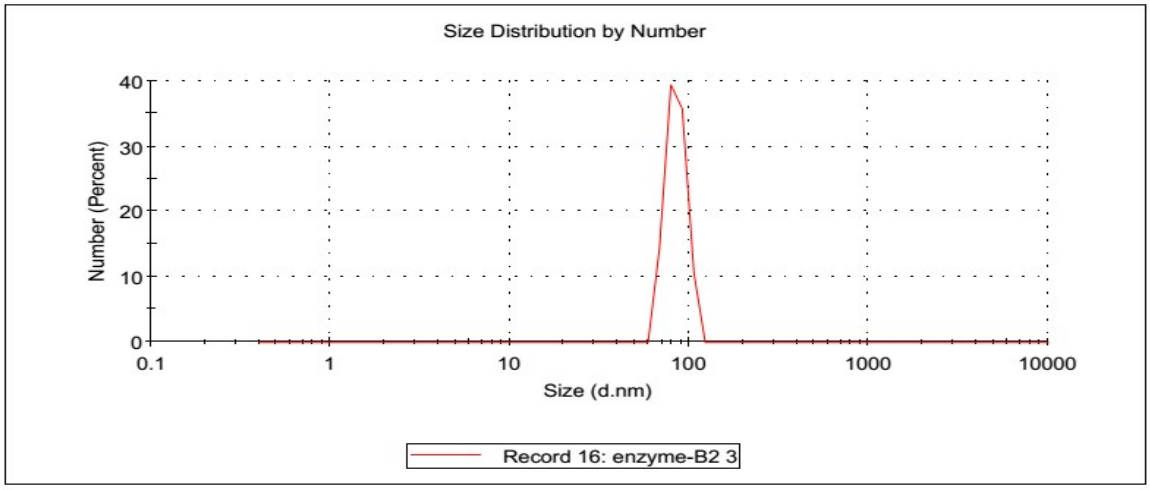


Figure S8-2. DLS analysis for the cross-linked TLL prepared in the presence of 6 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	121.3	Peak 1:	85.17	100.0	10.84
PdI:	1.000	Peak 2:	0.000	0.0	0.000
Intercept:	7.30	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

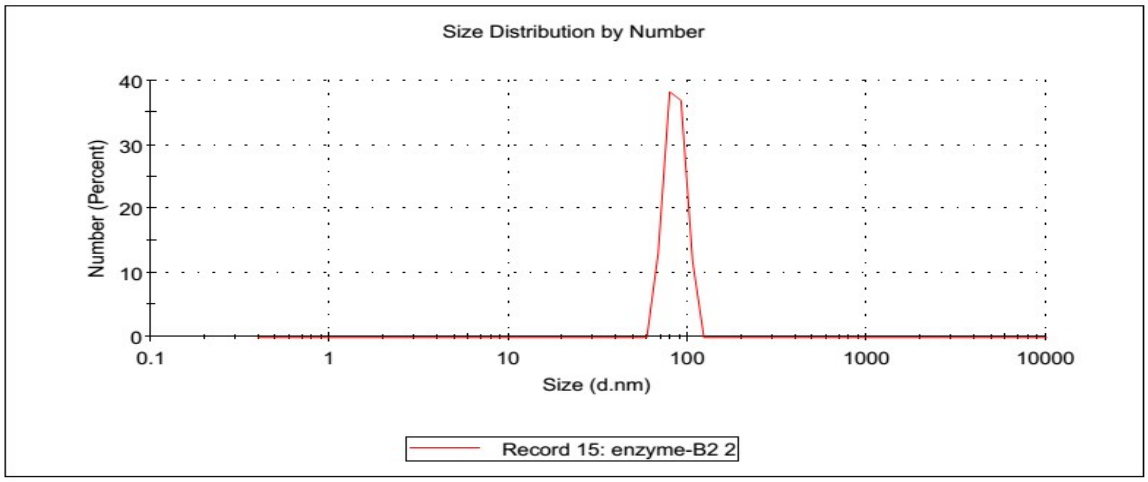


Figure S8-3. DLS analysis for the cross-linked TLL prepared in the presence of 14 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	174.5	Peak 1:	11.00	100.0	2.367
PdI:	0.448	Peak 2:	44.77	0.0	15.36
Intercept:	0.927	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

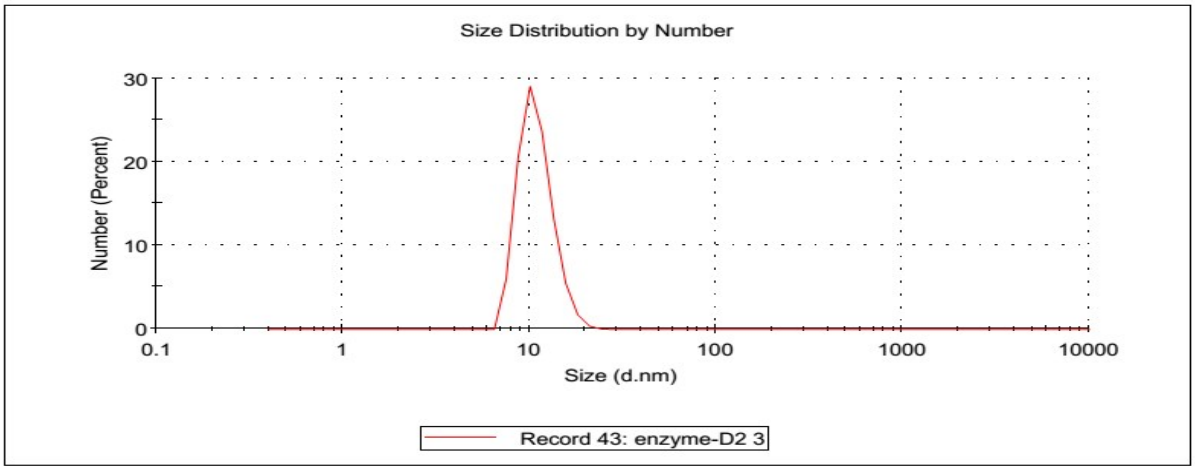


Figure S8-4. DLS analysis for the cross-linked TLL prepared in the presence of 20 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	1823	Peak 1:	809.4	33.4	169.7
PdI:	0.949	Peak 2:	231.5	66.6	41.80
Intercept:	0.948	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

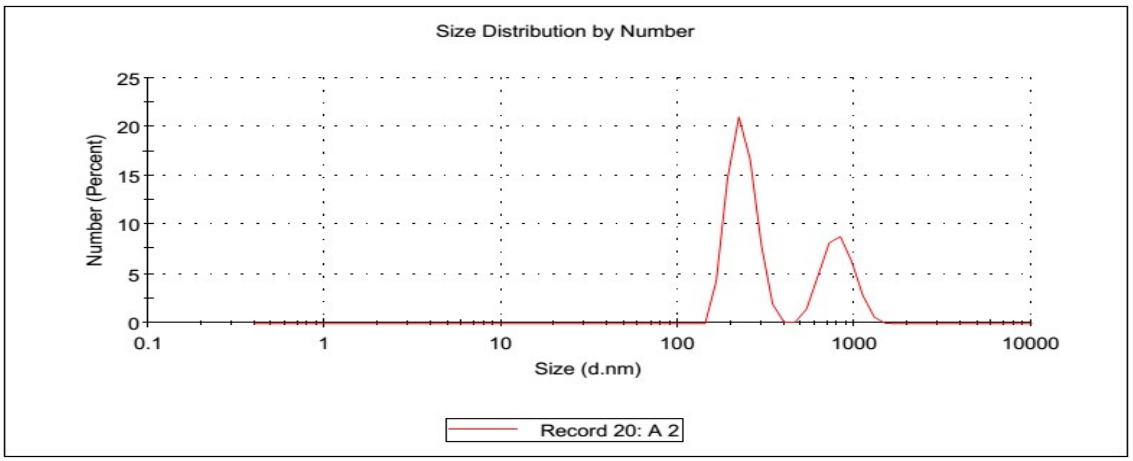


Figure S8-5. DLS analysis for the cross-linked RML prepared in the presence of 0.5 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	1090	Peak 1:	554.1	17.4	144.2
PdI:	0.715	Peak 2:	108.4	82.6	19.20
Intercept:	0.930	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

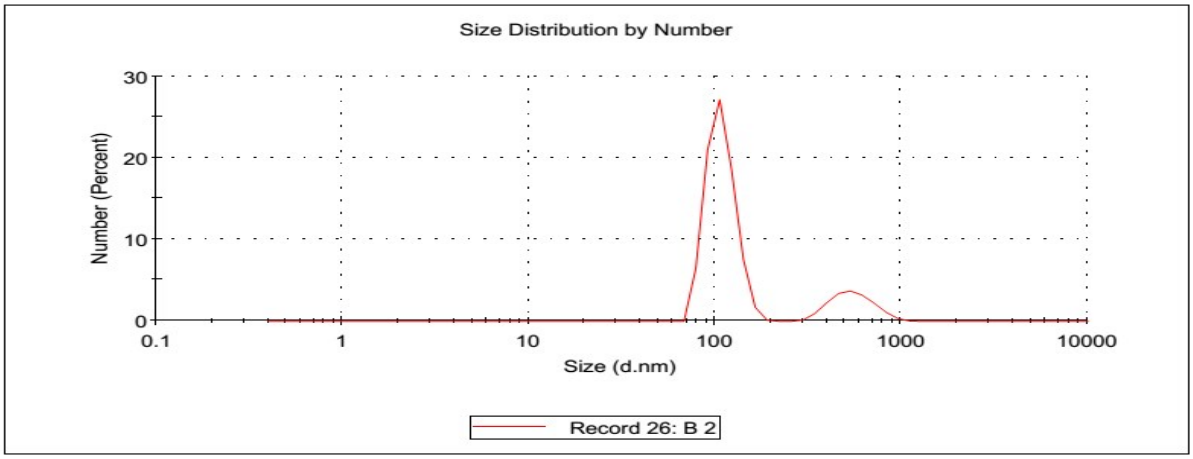


Figure S8-6. DLS analysis for the cross-linked RML prepared in the presence of 6 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	950.1	Peak 1:	25.58	84.2	4.343
PdI:	0.960	Peak 2:	57.41	15.8	44.33
Intercept:	0.764	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

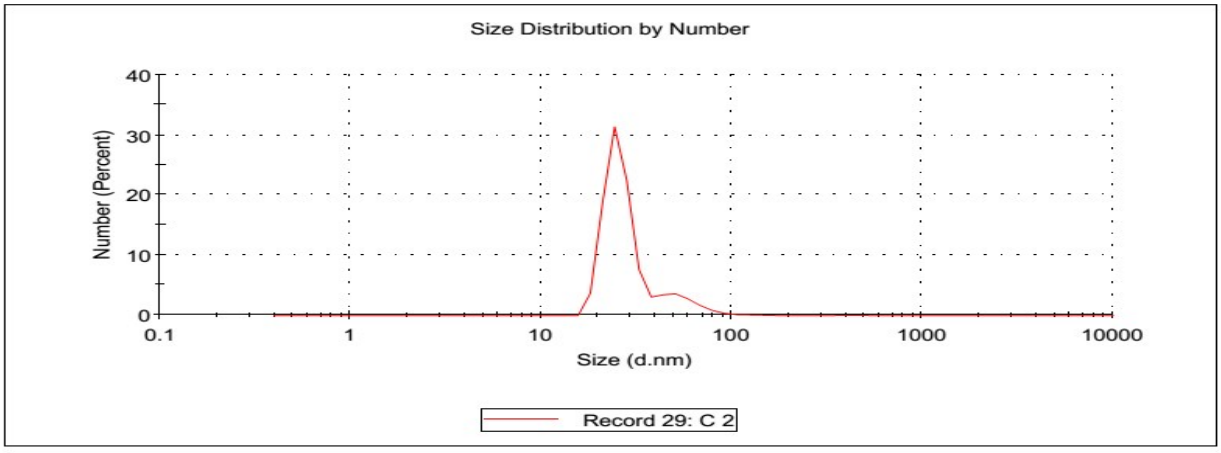


Figure S8-7. DLS analysis for the cross-linked RML prepared in the presence of 14 mg of BSA

			Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm):	837.5	Peak 1:	173.6	89.9	39.72
PdI:	1.000	Peak 2:	835.0	10.1	218.2
Intercept:	0.888	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

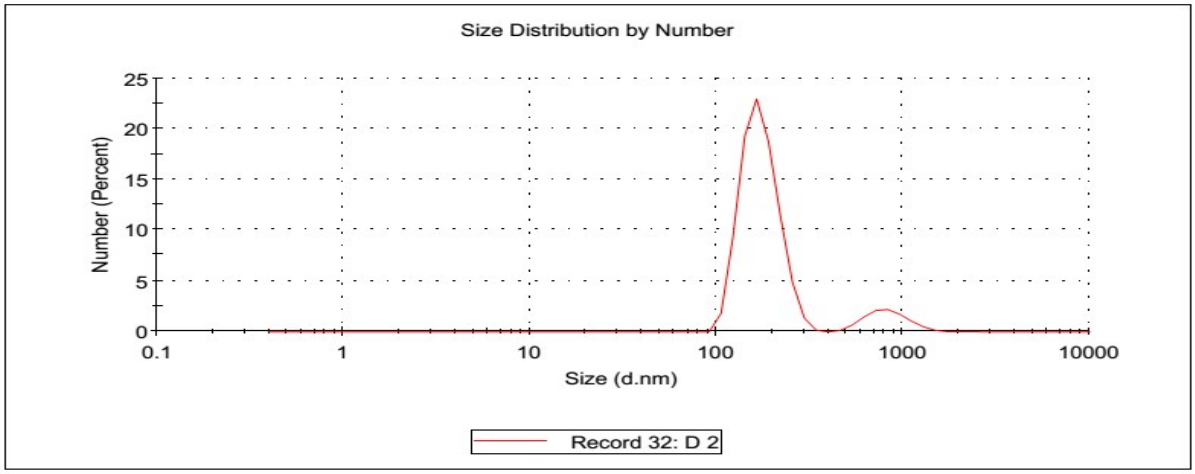


Figure S8-8. DLS analysis for the cross-linked RML prepared in the presence of 20 mg of BSA