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Supplementary Materials

Synthesis and Characterization of Amine and Aldehyde-Containing Copolymers for Enzymatic Crosslinking of Gelatine

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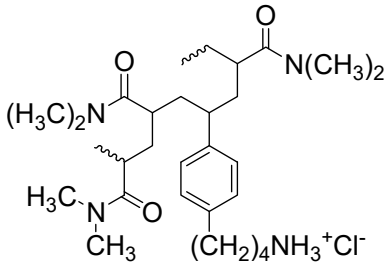


Figure S1. Linear LO substrate copolymer (P1c) previously prepared by us of interest for this new study.

(a)

11a (n=2), **11b** (n=4), **11c** (n=6)

(b)

Figure S2. Chemical structure of monomer **5** (a); chemical structure of amidoamine monomers **11a-c** (b).

Table S1. Copolymerization of **5** with DMAA at 60 °C.

5 (mg, mmol)	DMAA (g, mmol)	M_5	AIBN (mg, %)	DMF (mL)	Time (h)	Isolation solvent	Copolymer (g, %)
50.4, 0.238	2.5, 25.23	0.009	25.9, 1.02	11.0	3 h 30′	DIPE	CP5/DMAA-0.9 * 2.07, 80.9
75.4, 0.356	2.31, 23.29	0.015	24.7, 1.04	11.0	3 h 30′	DIPE	CP5/DMAA-1.5 * 1.74, 72.9
155.4, 0.734	1.38, 13.95	0.052	15.8, 1.03	7.1	24	Et_2O	CP5/DMAA-5.2 * 0.53, 34.4
151.2, 0.714	0.637, 6.43	0.111	7.9, 1.00	3.6	24	Et_2O	CP5/DMAA-11.1 * 0.504, 63.9
101.4, 0.479	0.421, 4.25	0.113	5.99, 1.15	3.0	24	Et_2O	CP5/DMAA-11.3 * 0.117, 22.3
698.0, 3.295	0.766, 7.73	0.426	15.3, 1.05	7.5	7	Et_2O	CP5/DMAA-42.6 * 0.678, 46.3
953.0, 4.50	1.04, 10.48	0.429	21.3, 1.07	9.5	24	Et_2O	CP5/DMAA-42.9 * 0.860, 81.4
811.0, 3.83	0.88, 8.88	0.431	17, 1.01	8.0	21	Et_2O	CP5/DMAA-43.1 * 0.599, 35.4

DMAA = *N, N′*-dimethylacrylamide; M_5 = molar fraction of **5** in the feed; AIBN= 2,2'-azo*bis*-(2-methyl-proprionitrile); DIPE= di-isopropyl ether; * the copolymer code number indicates the percentage of **5** in the polymerization feed.

Table S2. Results from characterization analyses performed on CP5/DMAA-42.9.

Entry	μequivNH_2/g *	Mn	Z AVE (nm)	ζ-p(mV)	PDI	mmolNH_2/g **	β	β AVE
CP5/DMAA-42.9	894±12	5100	334 ± 27	+57.6 ± 1.7	1.012 ± 0.007	12.5 ± 0.03	0.667	0.2305 ± 0.1354

* Volumetric titration; ** potentiometric titrations; PDI = polydispersion index. AVE = average; β = buffer capacity (dV (HCl)/d (pH); β AVE = average buffer capacity. Defined as the volume of HCl necessary to cause a variation of pH equal to one unit in the pH range 4.5–7.5.

Table S3. Solubility of DMAA homopolymer (HP-DMAA) and of prepared copolymers using **5**, DMAA and AA.

Solvent	CP5/DMMA-1.5 *	CP5/DMAA-42.9 *	CP5/DMMA/AA	HP-DMAA
Petrol	-	-	-	-
Et_2O	-	-	-	-
Toluene	Swells on heating	Swells on cooling	-	Partially soluble
THF	Partially soluble	Partially soluble	-	Soluble on heating
Dioxane	Swells on cooling	Swells on cooling	-	Soluble on heating
Acetone	Partially soluble	-	-	+
$CHCl_3$	+	-	Partially soluble	+
DCM	+	-	Swells on cooling	+
MeOH	+	+	+	+
DMF	+	+	+	+
DMSO	+	+	+	Soluble on heating
H_2O	+	+	+	+

Petrol = petroleum ether 40–60 °C; DMAA = *N, N′*-dimethylacrylamide; AA = acrylic acid; THF = tetrahydrofuran; DCM = dichloromethane; DMF = *N, N*-dimethylformamide; DMSO = dimethyl sulfoxide; * the copolymer code number indicates the percentage of **5** in the feed.

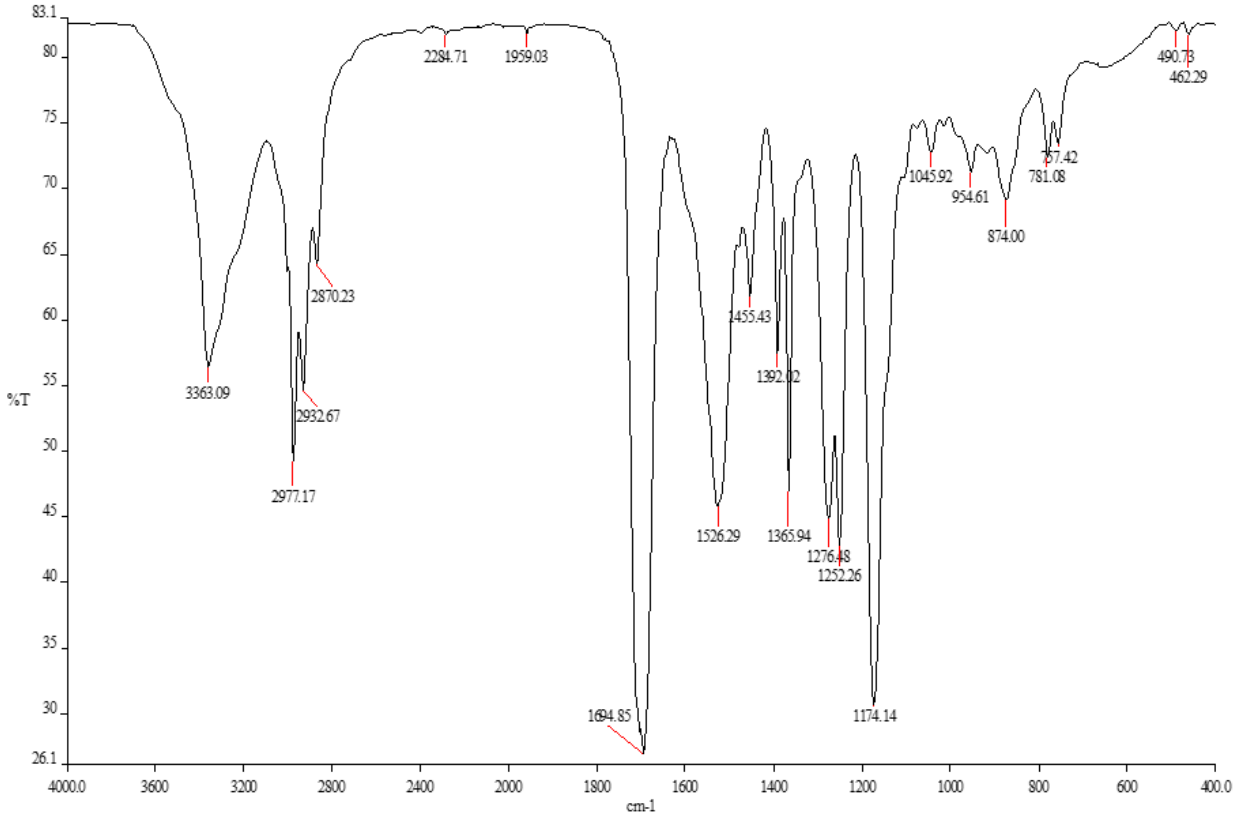


Figure S3. FTIR (film) spectrum of **6a**.

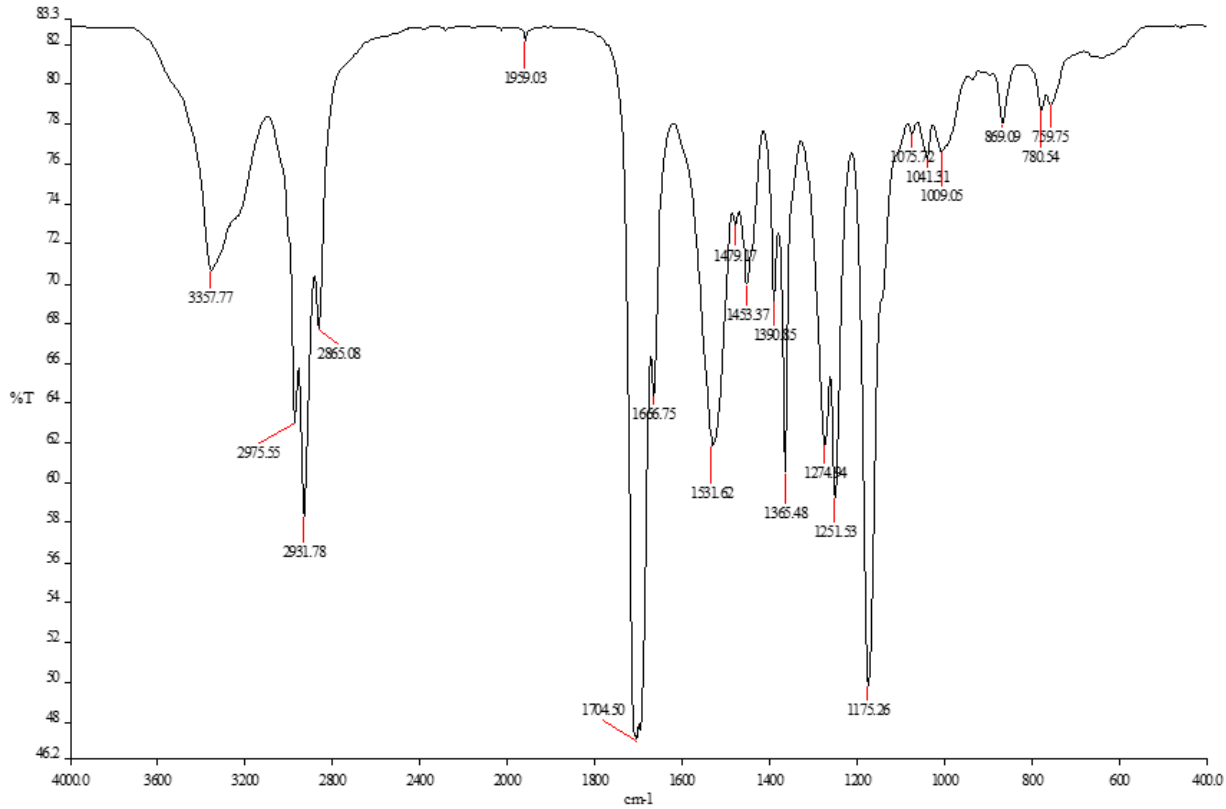


Figure S4. FTIR (film) spectrum of **6b**.

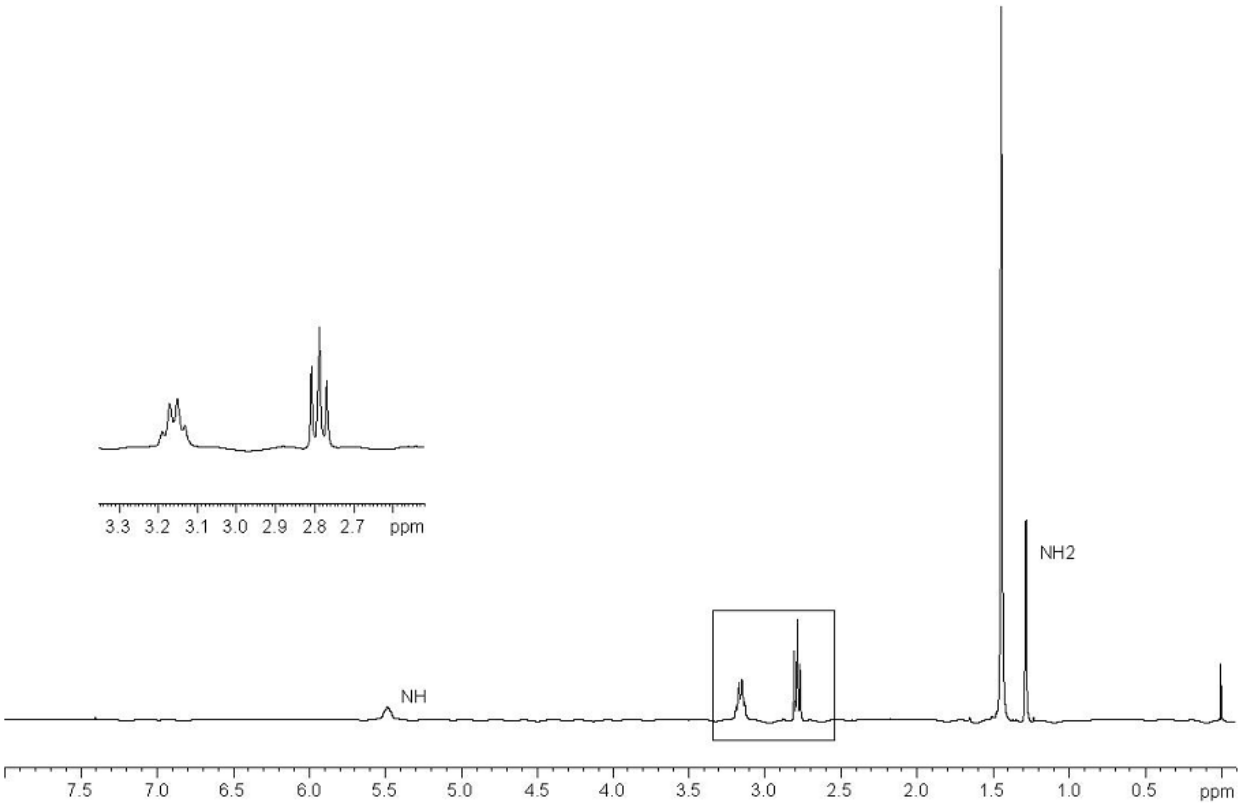


Figure S5. ^{1}H NMR ($CDCl_3$, 300 MHz) spectrum of **6a**.

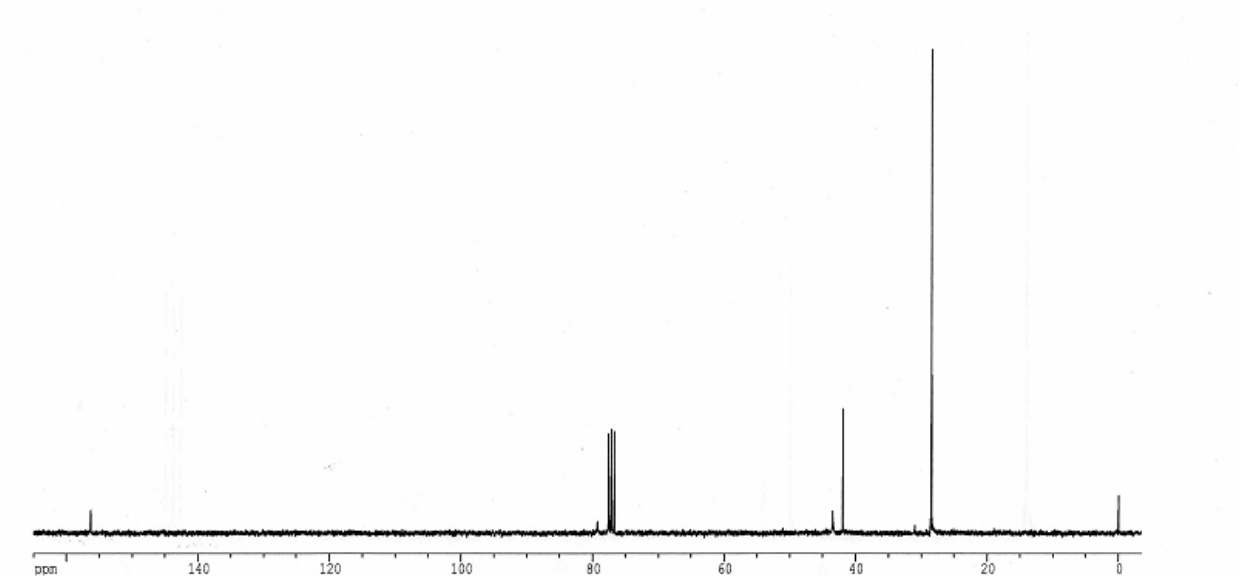


Figure S6. ^{13}C NMR ($CDCl_3$, 75.5 MHz) spectrum of **6a**.

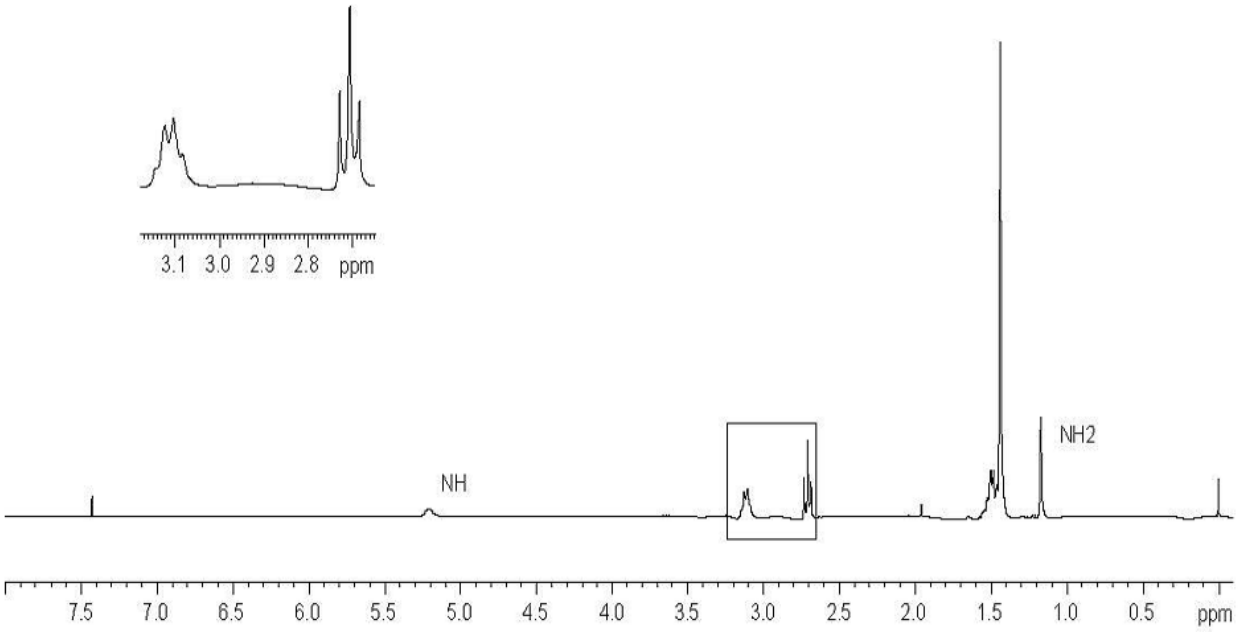


Figure S7. ^{1}H NMR ($CDCl_3$, 300 MHz) spectrum of **6b**.

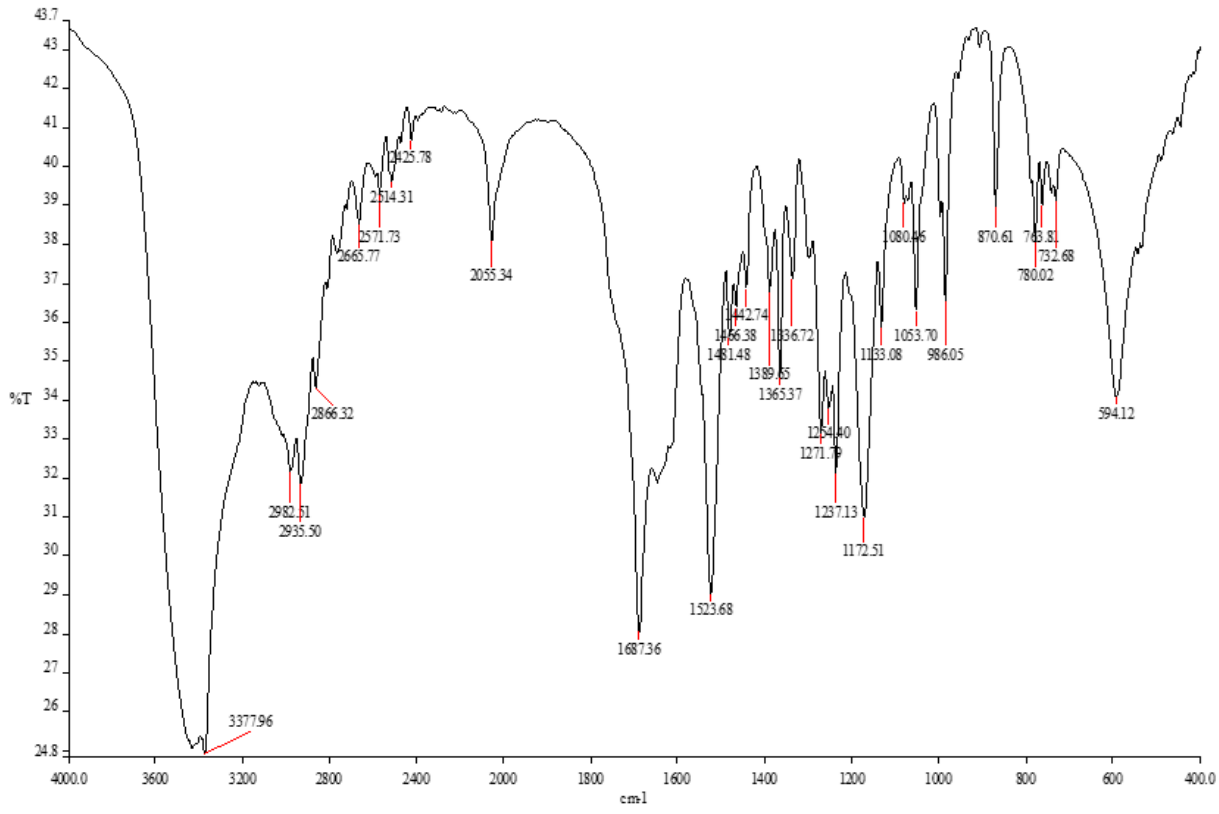


Figure S8. FTIR (KBr) spectrum of the hydrochloride salt of **6c**.

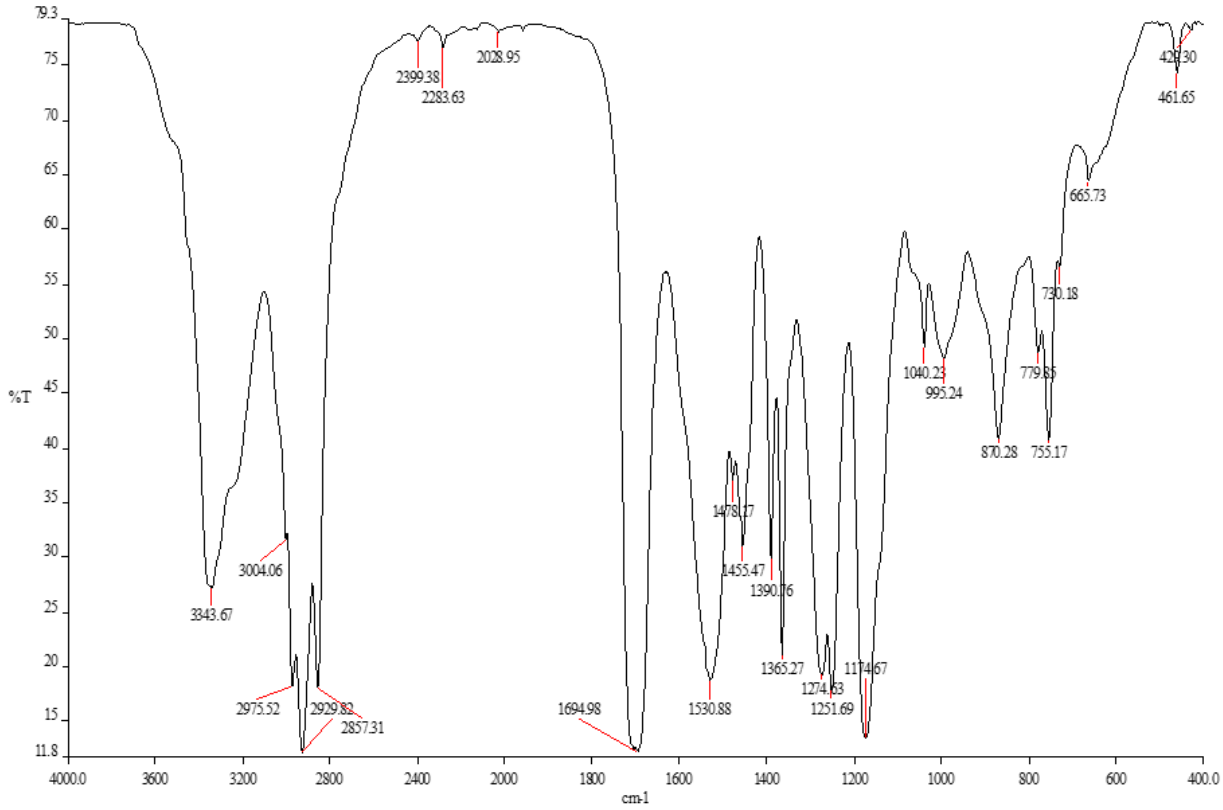


Figure S9. FTIR (film) spectrum of **6c**.

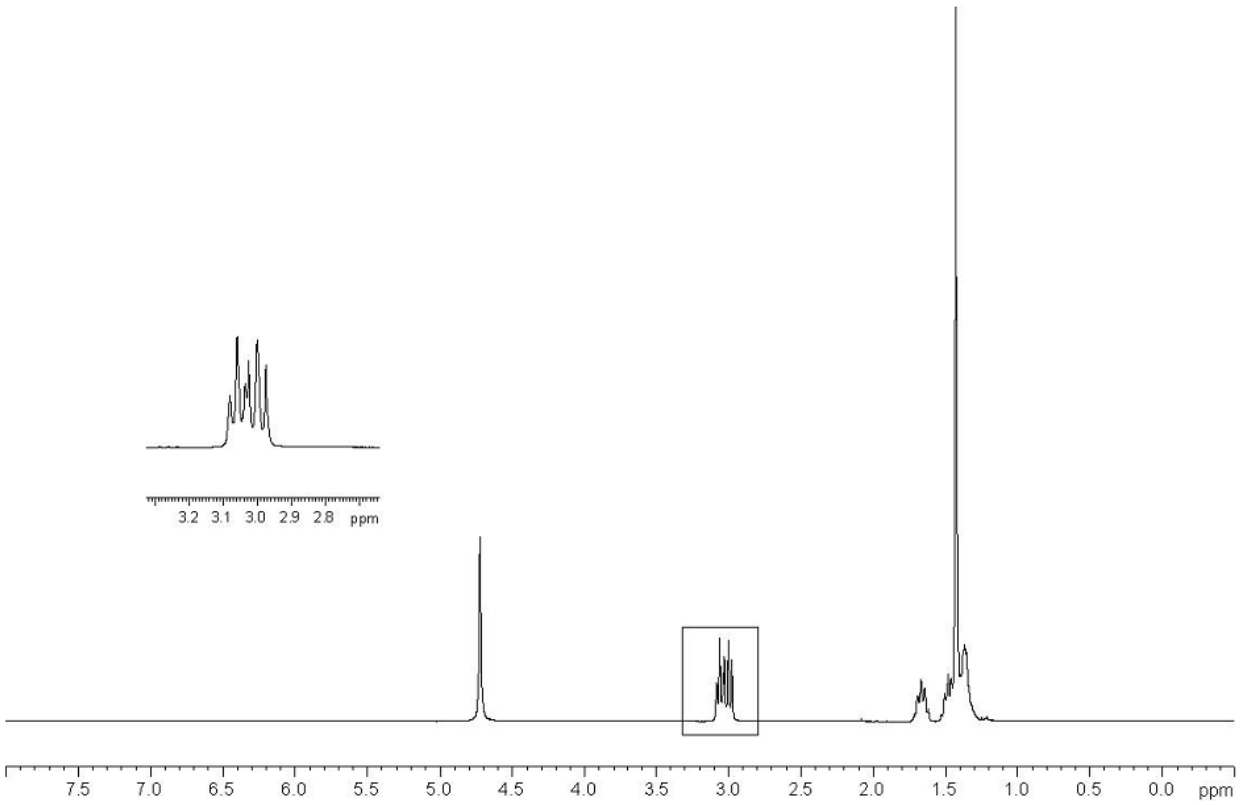


Figure S10. ^{1}H NMR (D_2O, 300 MHz) spectrum of the hydrochloride salt of **6c**.

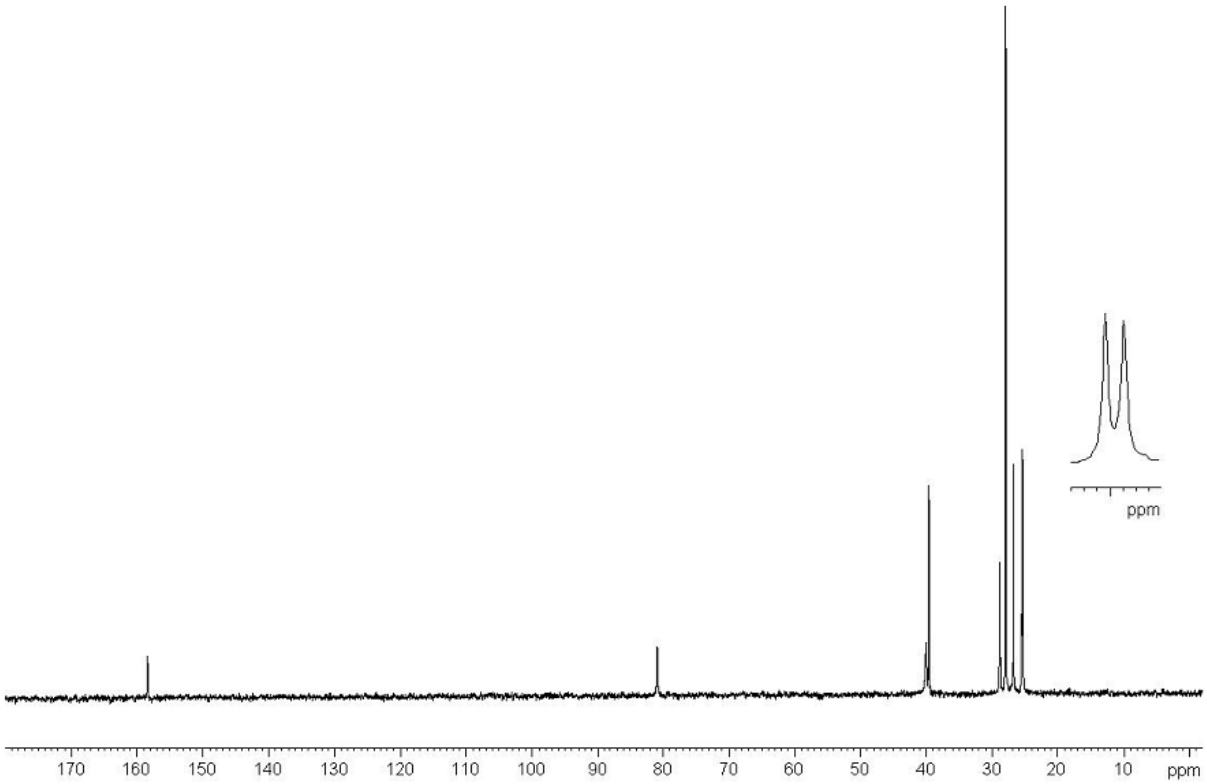


Figure S11. ^{13}C NMR (D_2O, 75.5 MHz) spectrum of the hydrochloride salt of **6c**.

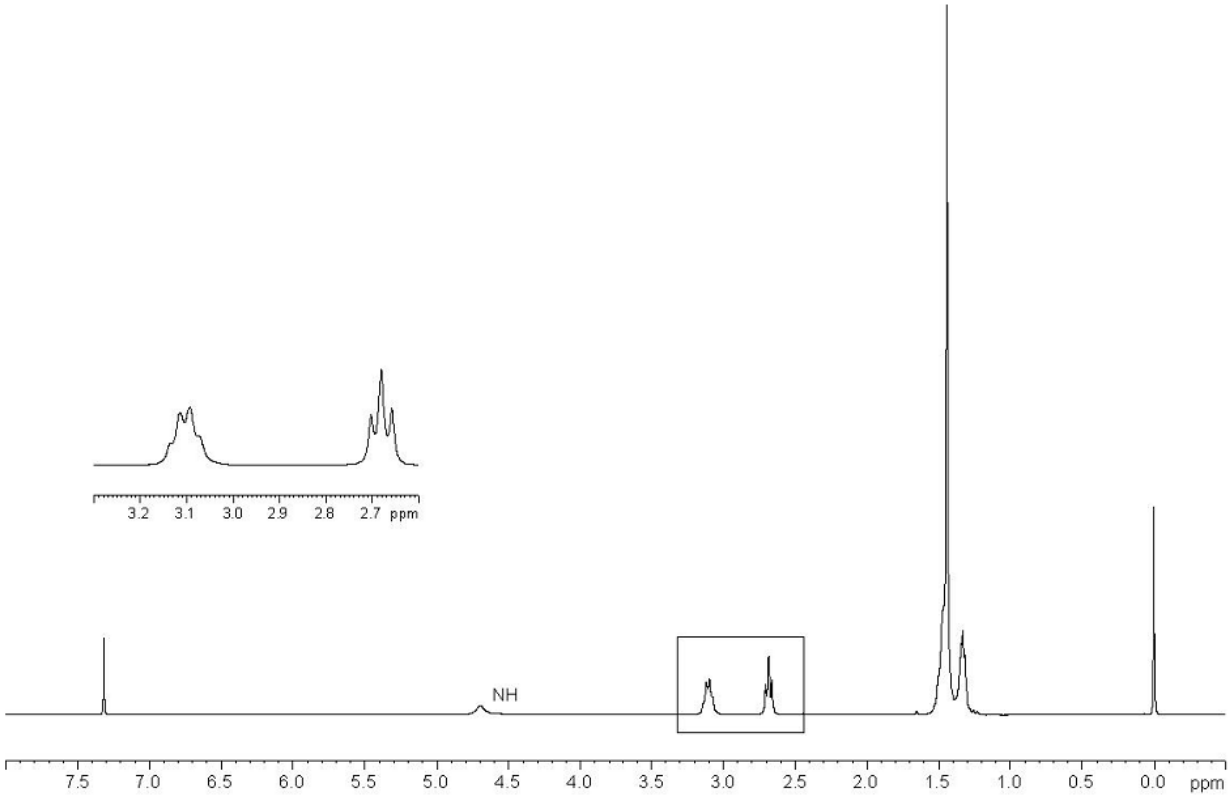


Figure S12. ^{1}H NMR ($CHCl_3$, 300 MHz) spectrum of **6c**.

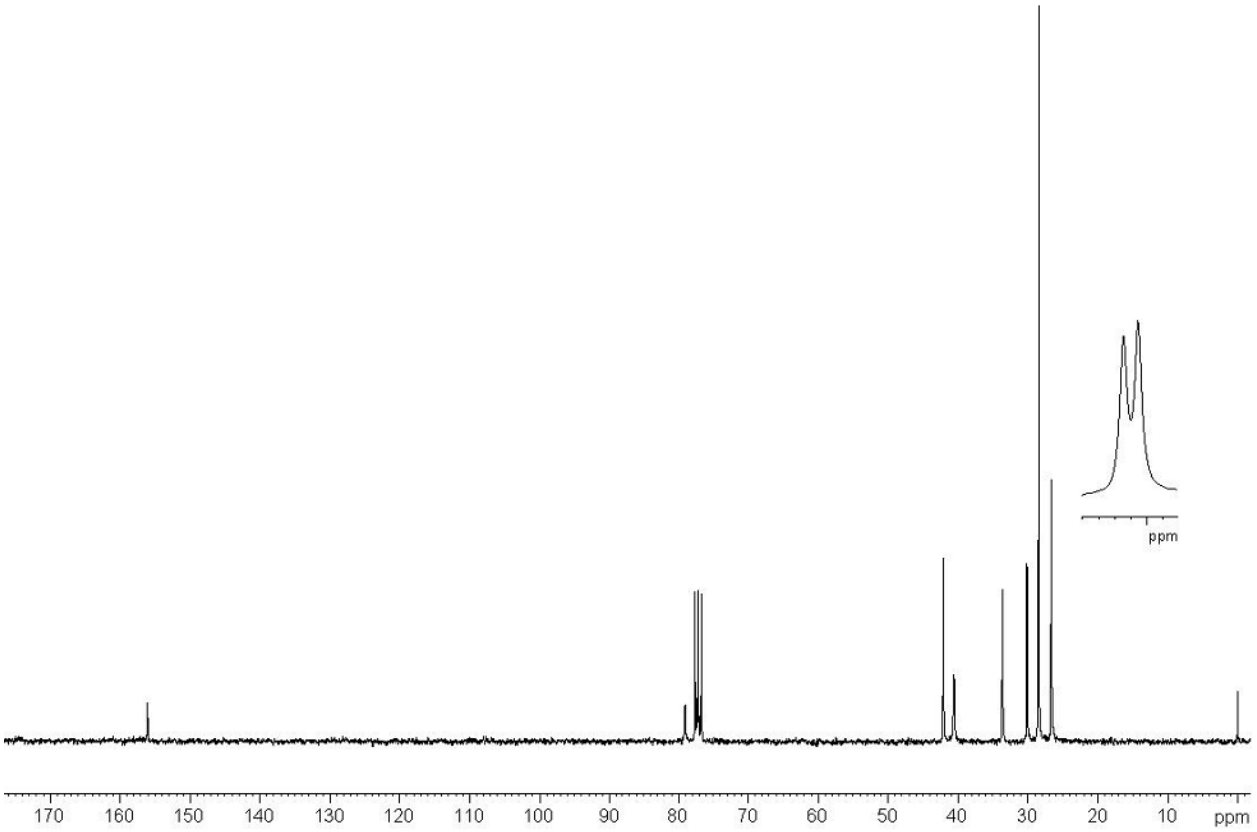


Figure S13. ^{13}C NMR ($CHCl_3$, 300 MHz) spectrum of **6c**.

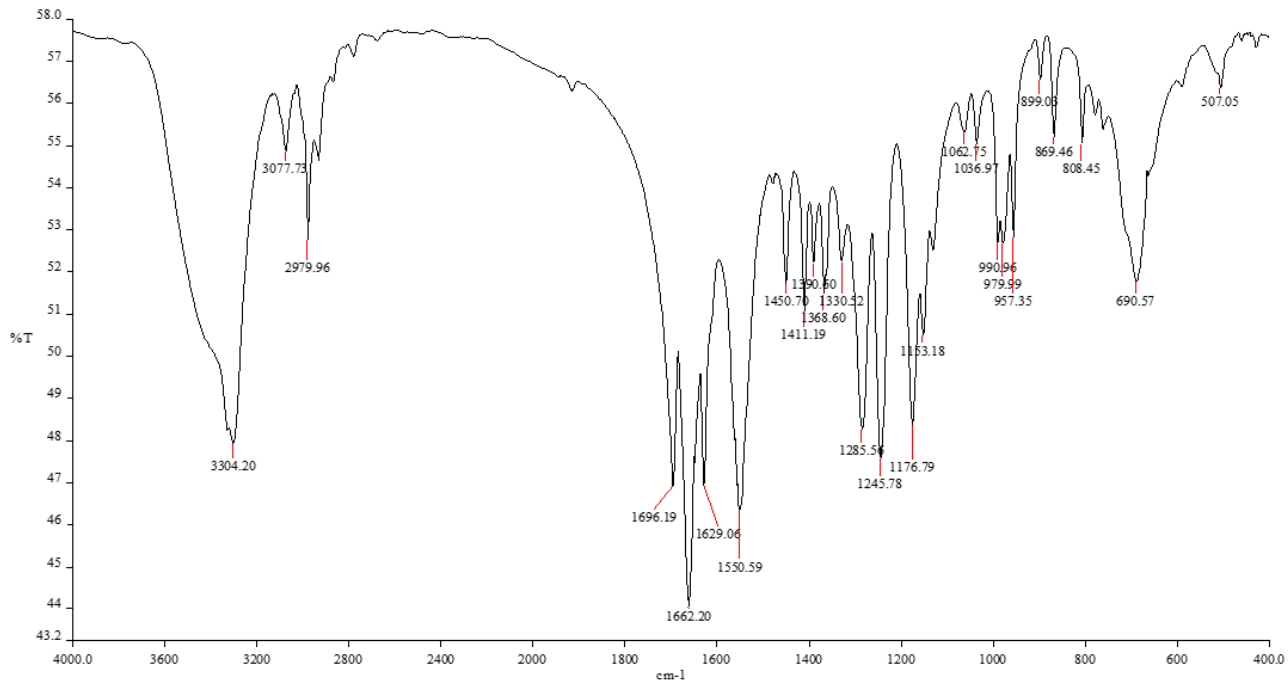


Figure S14. FTIR spectrum (KBr) of compound **10a**.

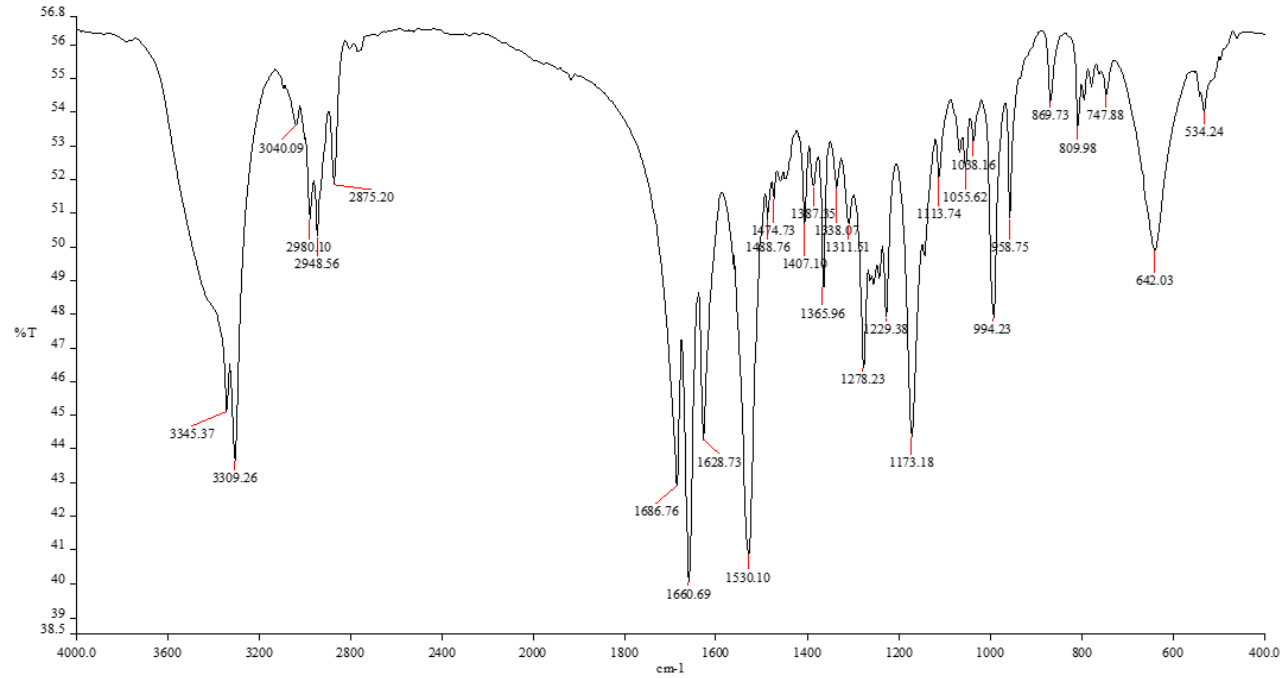


Figure S15. FTIR spectrum (KBr) of compound **10b**.

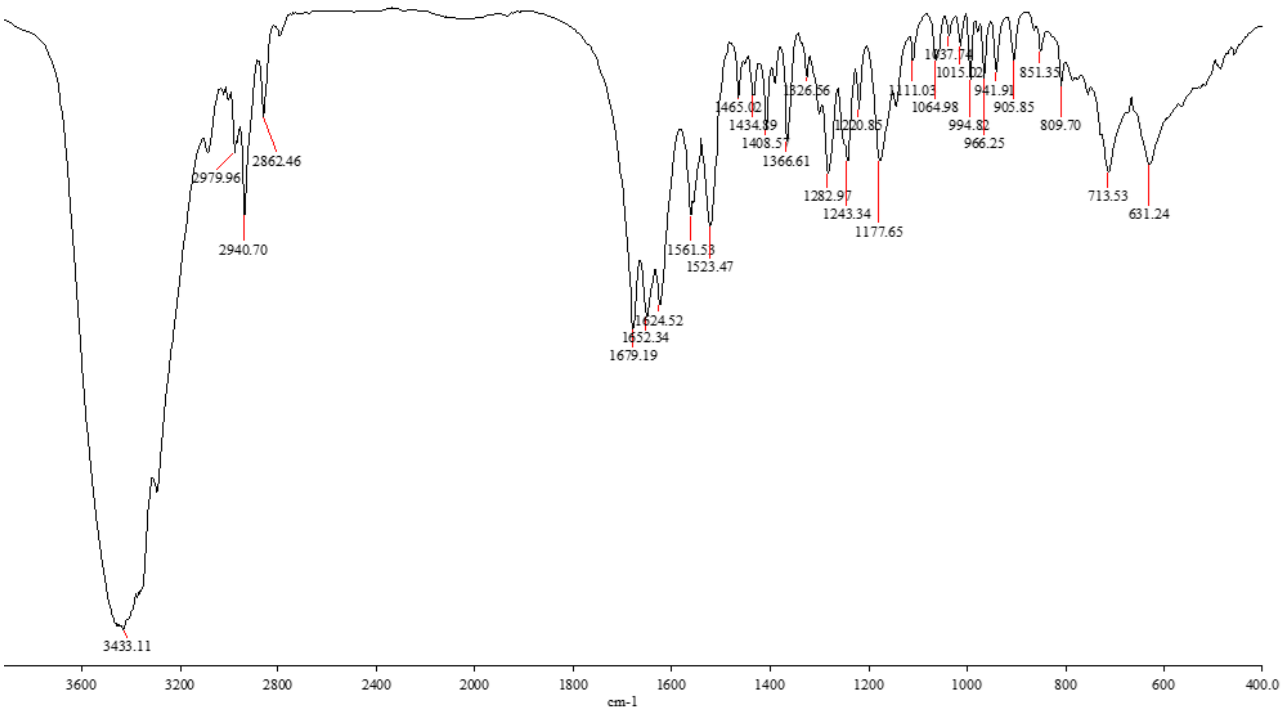


Figure S16. FTIR spectrum (KBr) of compound **10c**.

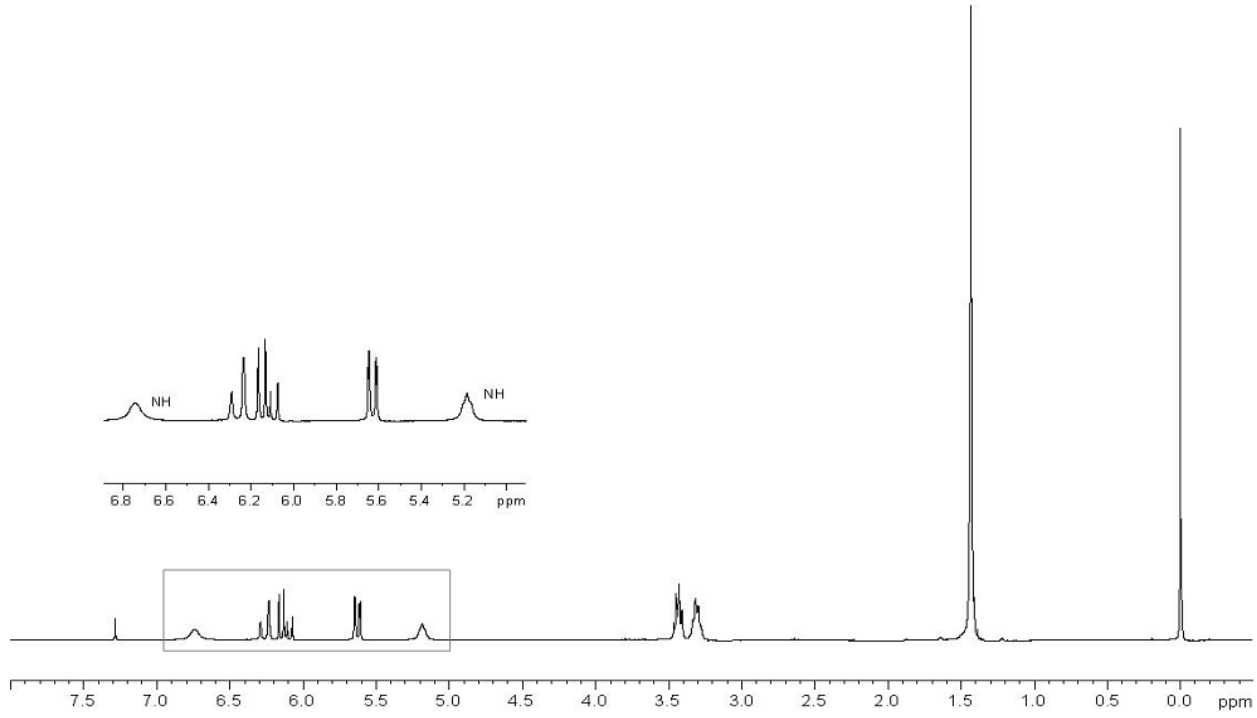


Figure S17. ^{1}H NMR spectrum ($CHCl_3$, 300 MHz) of compound **10a**.

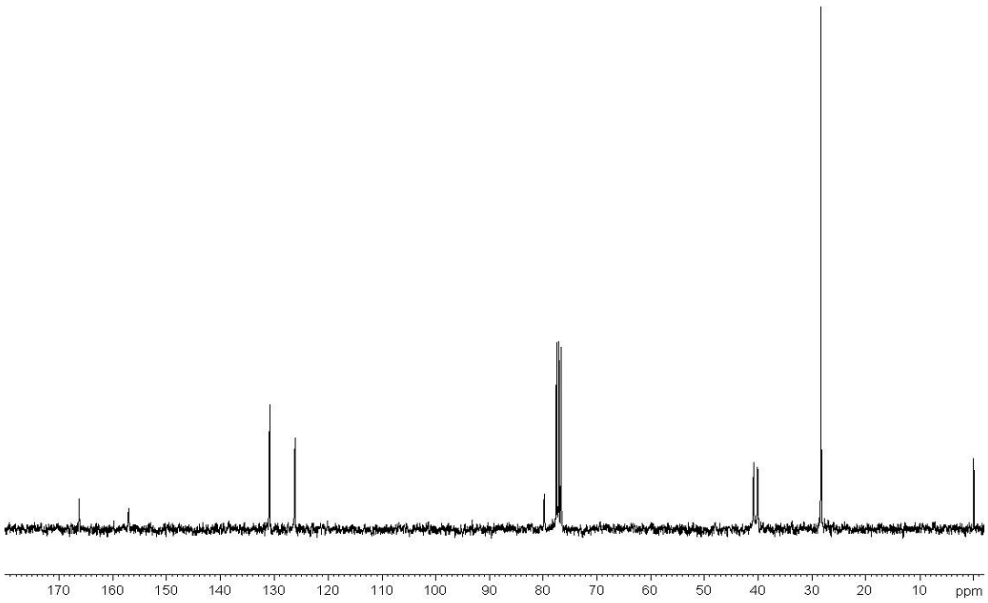


Figure S18. ^{13}C NMR spectrum ($CHCl_3$, 75.5 MHz) of compound **10a**.

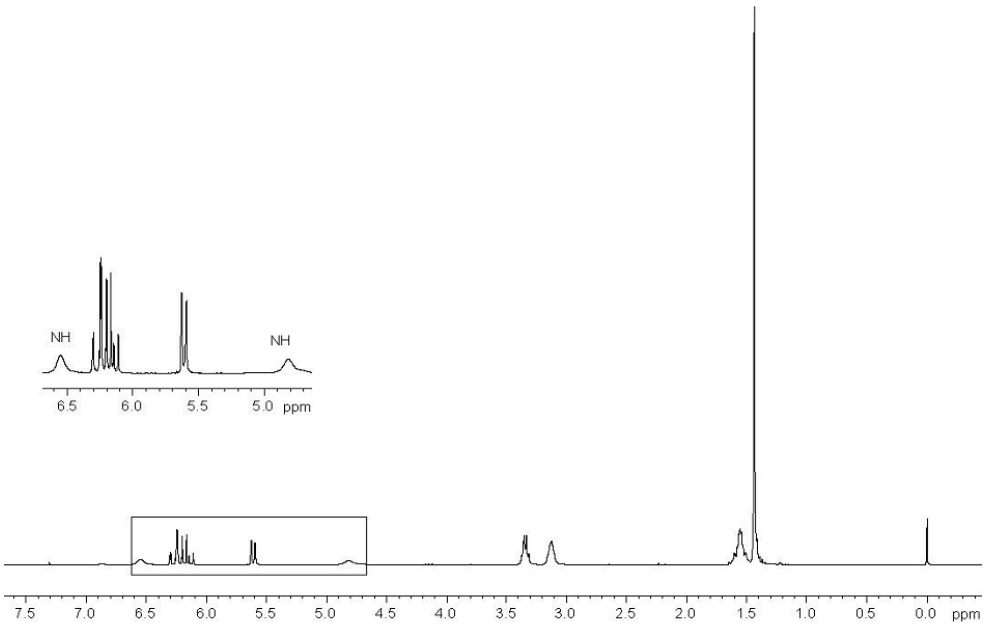


Figure S19. ^{1}H NMR spectrum ($CHCl_3$, 300 MHz) of compound **10b**.

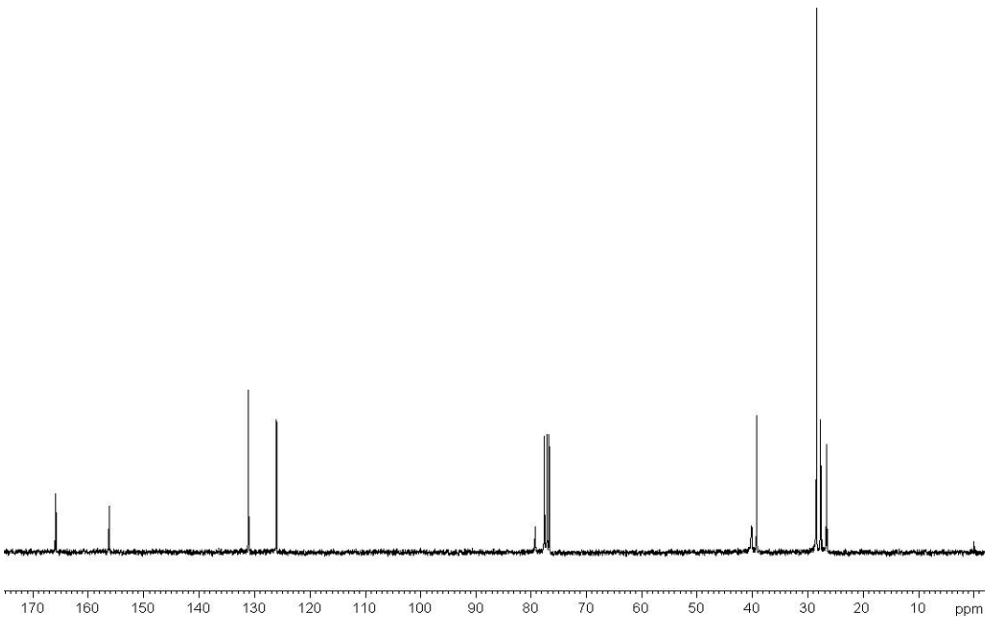


Figure S20. ^{13}C NMR spectrum ($CHCl_3$, 75.5 MHz) of compound **10b**.

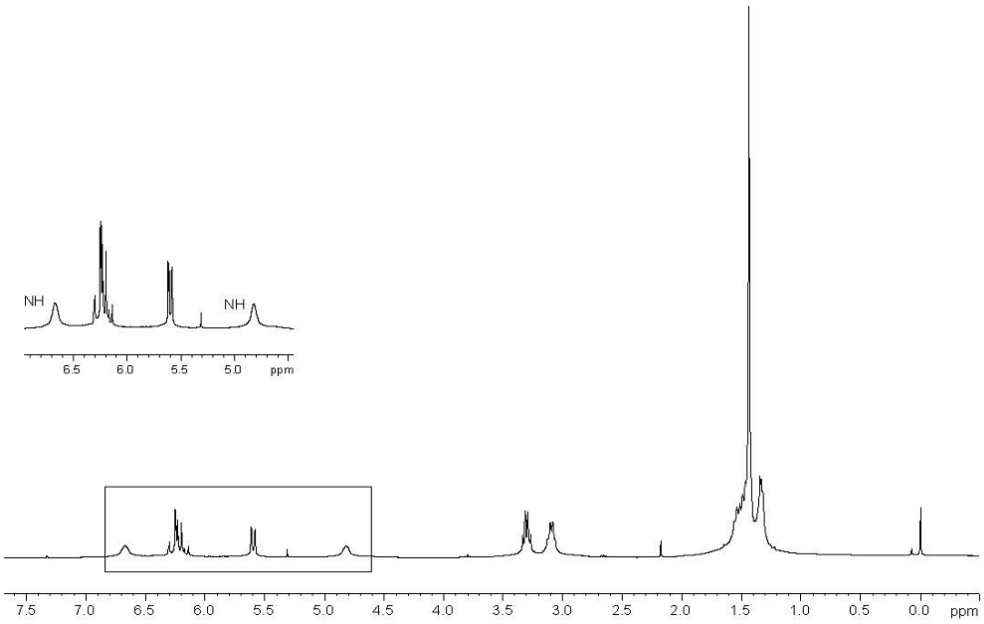


Figure S21. ^{1}H NMR spectrum ($CHCl_3$, 300 MHz) of compound **10c**.

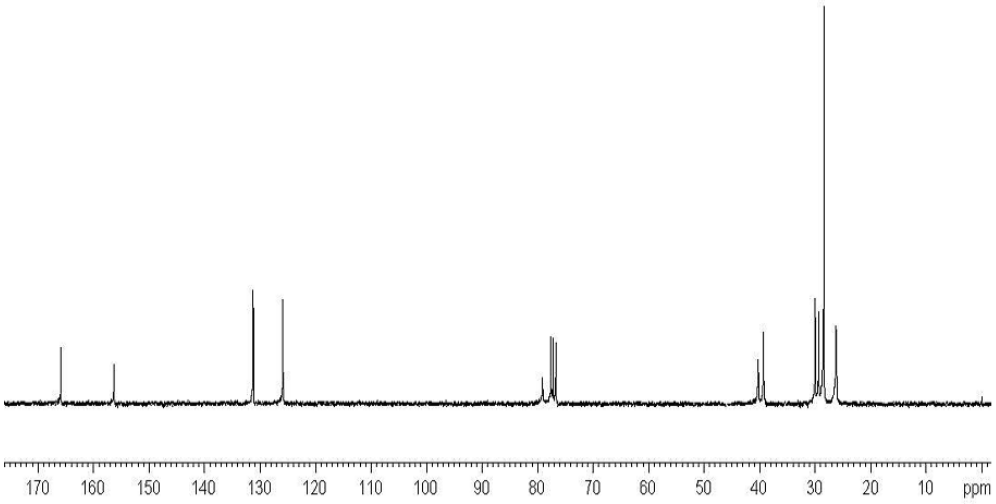


Figure S22. ^{13}C NMR spectrum ($CHCl_3$, 75.5 MHz) of compound **10c**.

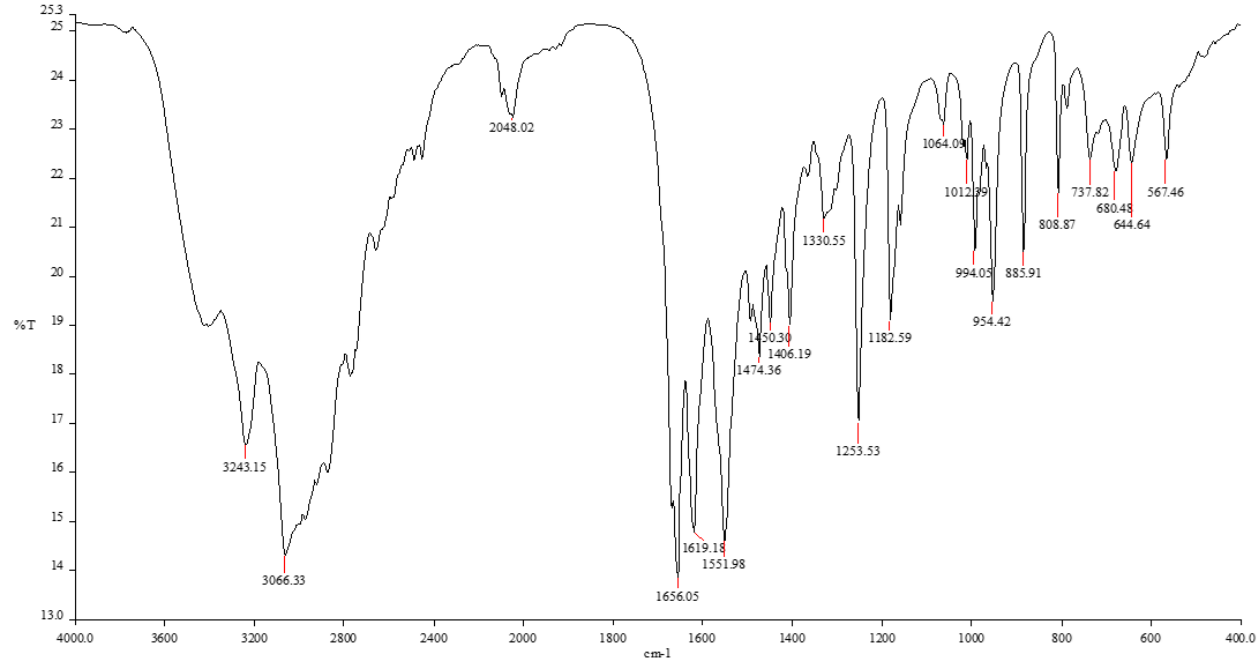


Figure S23. FTIR spectrum (KBr) of compound **11a**.

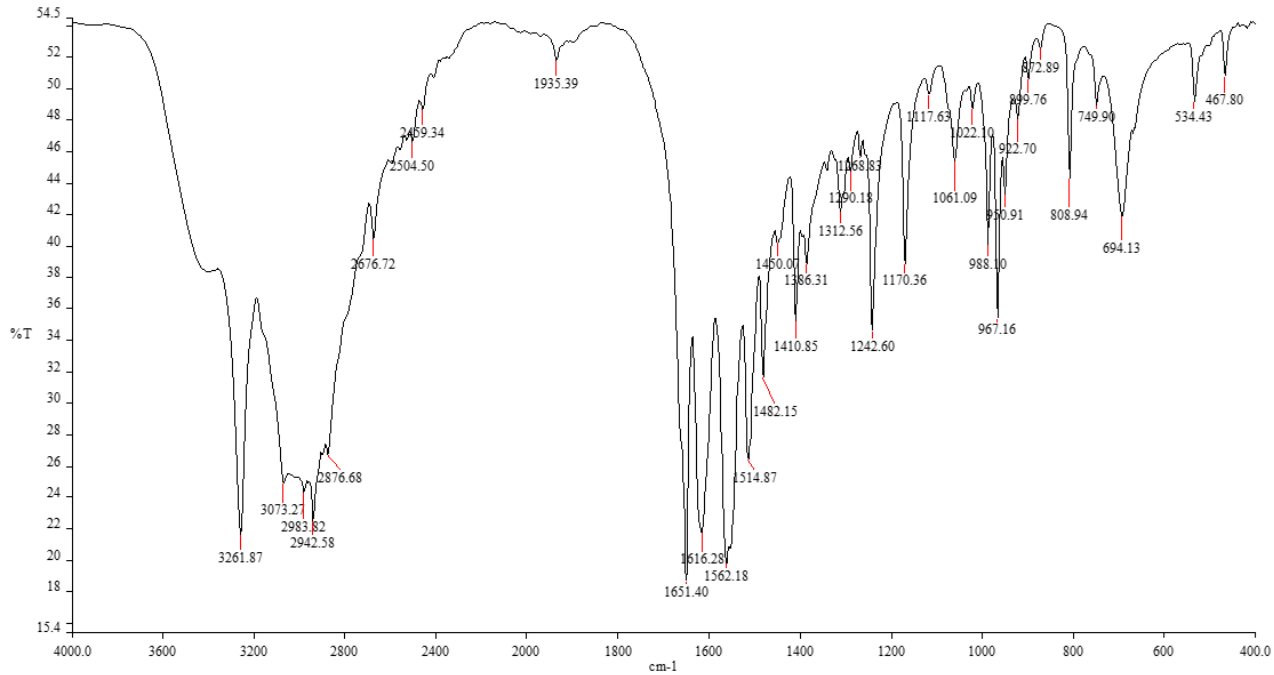


Figure S24. FTIR spectrum (KBr) of compound **11b**.

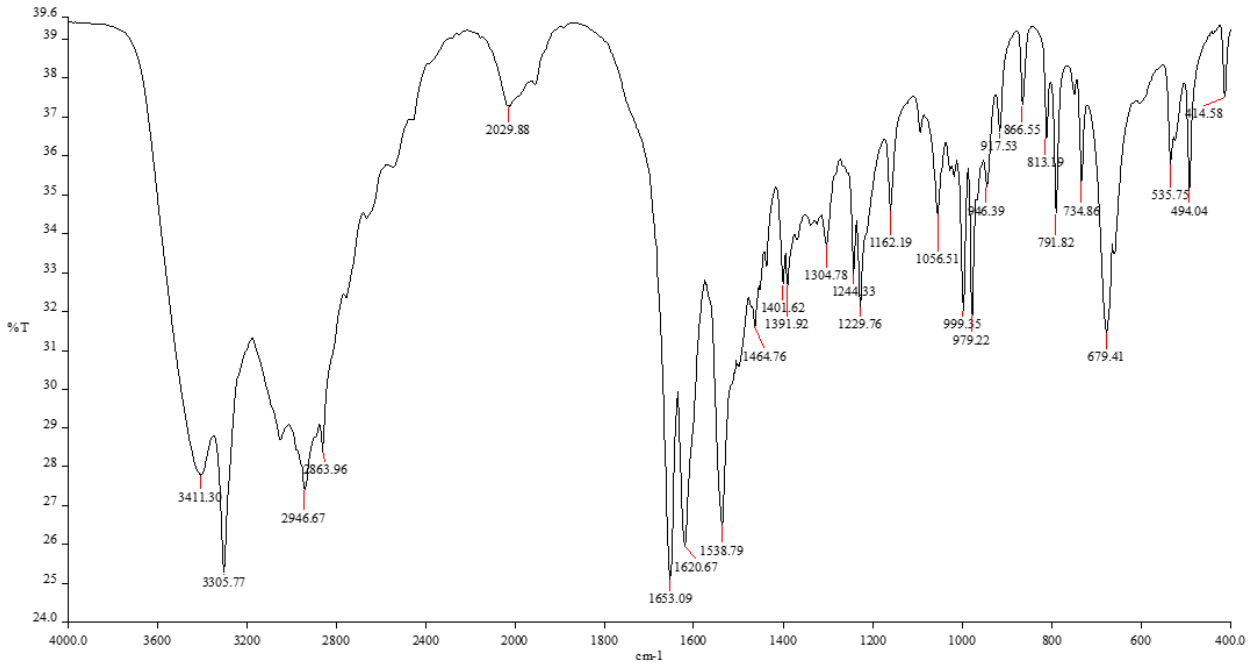


Figure S25. FTIR spectrum (KBr) of compound **11c**.

Table S4. Solubility of the prepared copolymers and of the DMAA homopolymer prepared for comparison purposes.

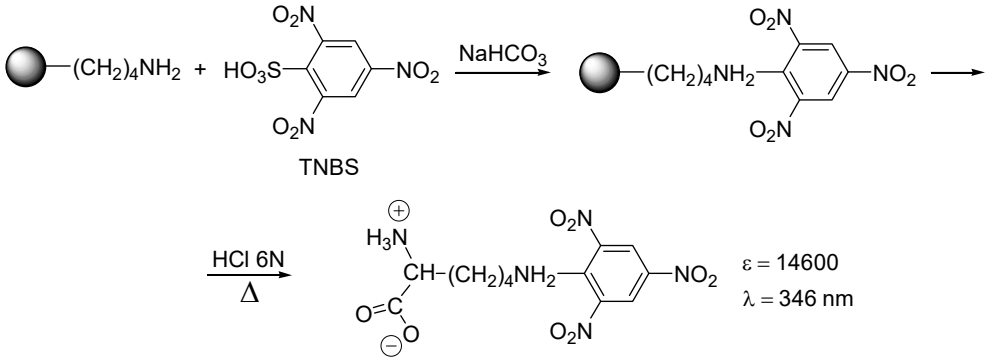
Solvent	CP10a/DMAA	CP10b/DMAA	CP11a/DMAA	CP11b/DMAA	CP11c/DMAA	HP-DMAA
Petrol	-	-	-	-	-	-
Et_2O	-	-	-	-	-	-
Toluene	Partially soluble *	Partially soluble *	Swells *	Swells *	Swells *	Partially soluble
THF	+	+	Swells *	Swells *	Swells *	Soluble *
Dioxane	+	+	Swells *	Swells **	Swells **	Soluble *
Acetone	+	+	Swells *	Swells *	Swells **	+
$CHCl_3$	+	+	+	+	Soluble *	+
DCM	+	+	+	+	+	+
MeOH	+	+	+	+	+	+
DMF	+	+	+	+	+	+
DMSO	+	+	+	+	+	+
H_2O	+	+	+	+	+	+

* Under heating; ** under cooling; petrol = petrol ether boiling at 40–60 °C; DMAA = *N*,*N*′-dimethylacrylamide; THF = tetrahydrofuran; DCM = dichloromethane; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; HP-DMAA = DMAA homopolymer.

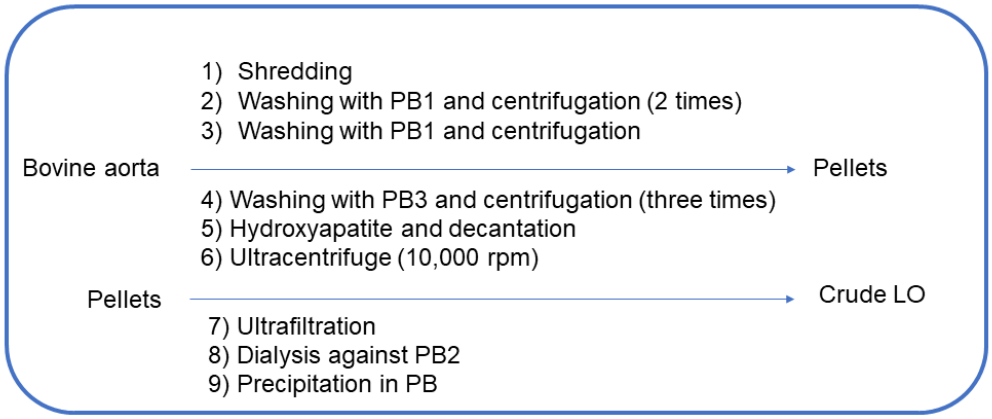
Table S5. Main features of commercially available Gel B used in this study.

Bloom grade (Bloom)	FTIR (cm^{-1})	NH_2 (mmol/g)	
250	1645, 1540 *	0.391± 0.02 **	0.219± 0.03 ***

* C=O amides; ** acid-base titration; *** UV determination; Bloom grade = unit of measurement of the solidity of a gel.

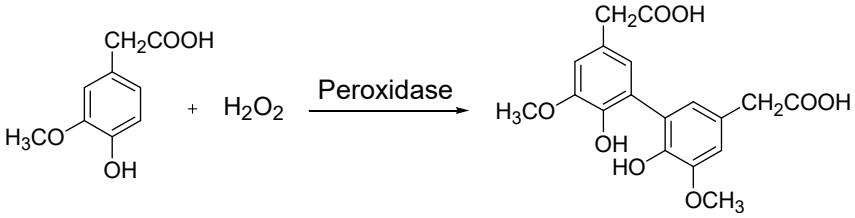


Scheme S1. Reaction between gelatine and TNBS leading to the formation of chromophore measurable by UV-Vis analyses.



Legend: LO = Lysyl oxidase; PB = 1M potassium phosphate buffer;
PB1= buffer 0.4M NaCl, 16mM potassium phosphate (pH=7.8);
PB2= 16mM potassium phosphate buffer (pH=7.8);
PB3= 4M urea 16mM potassium phosphate buffer (pH=7.8).

Chart S1. Main steps of procedure performed to extract and purify LO from bovine aorta.



Scheme S2. Reaction between homo vanillic acid and hydrogen peroxide in presence of peroxidase leading to the formation of the fluorescent dimer measurable by fluorimetry.

Table S6. Oxidation assays of selected copolymers with copper-containing amino oxidases (CAO).

Entry	Copolymer (mg)	Solvent (μL)	CAO (μL)	CAO stock solution (mg/mL)	Schiff test
1	HP-DMAA 7.1	H_2O mQ 250	-	-	-
2	CP5/DMAA-43.1 * 4.7	BU6M 500	LO 150	152.5/1.50 BU6M	+
3	CP5/DMAA-43.1 * 10.2	BU6M 500	PAO 400	4.8/1.00 BU6M	+
4	CP5/DMAA-11.3 * 28.3	BU6M 250	LO 450	159.0/1.08 BU6M	+ **
5	CP5/DMAA-11.3 * 24.3	BU6M 250	PAO 250	4.6/0.75 BU6M	-
6	CP5/DMAA-5.0 * 23.5	BU6M 250	LO 450	159.0/1.08 BU6M	+ **
7	CP5/DMAA-5.0 * 23.5	BU6M 250	PAO 250	4.6/0.75 BU6M	-
8	TP5/DMAA/AA=1/8/1 10.0	BU6M 250	LO 250	152.5/1.50 BU6M	-
9	TP5/DMAA/AA=1/8/1 9.2	BU6M 250	PAO 250	4.8/0.75 BU6M	-
10	CP11a/DMAA-10.0 * 17.1	BU6M 250	LO 450	152.5/1.50 BU6M	-
11	CP11a/DMAA-10.0 * 8.8	BU6M 250	PAO 200	4.8/1.25 BU6M	-
12	CP11b/DMAA-10.0 * 15.3	BU6M 250	LO 600	46.0/0.60 BU6M	+
13	CP11b/DMAA-10.0 * 14.7	BU6M 250	PAO 200	1.2/0.20 BU6M	+
14	CP11c/DMAA-9.9 * 13.3	BU6M 250	LO 450	152.5/1.50 BU6M	+/-
15	CP11c/DMAA-9.9 * 16.8	BU6M 250	PAO 200	4.8/1.25 BU6M	+

* The code number indicates the percentage of monomers in the reaction feed; ** heating; CAO = copper-containing amino oxidases; **5** = 4-aminobutylstyrene hydrochloride; DMAA = *N*,*N*-dimethylacrylamide; BU6M= borate buffer 0.05 M, Urea 6M; LO = lysyl oxidase; PAO = plasma amine oxidase; **11a** = *N*-acryloyl-1,2-diaminoethane hydrochloride; **11b** = *N*-acryloyl-1,4-diaminobutane hydrochloride; **11c** = *N*-acryloyl-1,6-diaminohexane hydrochloride.

Table S7. Size, PDI and ζ-p of CP5/DMAA, CP11b/DMAA, CP11c/DMAA and CPMA/DMAA.

Entry	Z AVE (nm)	PDI	ζ-p (mV)
CP5/DMAA	334.0 ± 27.0	1.012	+57.6 ± 1.7
CP11b/DMAA	2590 ± 56.7	0.281	+6.5 ± 3.7
CP11c/DMAA	372.8 ± 196.8	0.326	+24.8 ± 5.1
CPMA/DMAA	112.2 ± 82.8	0.590	+18.3 ± 9.9

Table S8. Crosslinking of gelatine with copolymers.

Exp	Gel B (mg)	Solvent (mL)	Copolymer (mg)	Wt %	Solvent mL	CAO mL	Gel B Crosslinked (mg, %)
1	500.1	BU6M 5.0	CP5/DMAA-43.1 50.5	10.1	-	LO§ 2.0	M21 76.6, 13.9
2	503.1	BU6M 4.0	CP11b/DMAA-10.0 51.5	10.2	BU6M 1.0	LO† 5.0	M33 1.9, 0.38
3	502.7	BU6M 4.0	CP11c/DMAA-10.0 52.4	10.4	BU6M 1.0	PAO‡ 0.600	M31 37.3, 7.4
4	500.8	PB 4.0	CPMA/DMAA-20.0 51	10.2	PB 2.0	-	M23 498.7, 99.5
5	500.6	PB 4.0	CPMA/DMAA-20.0 25.4	5.1	PB 1.0	-	M26 345.5, 69.0
6	500.2*	PB 6.5	CPMA/DMAA-20.0 25.5	5.1	H_2O mQ 1.0	-	M32 310.7, 62.1
7	650.1	BU6M 5.2	CPMA/DMAA-20.0 65.0	10.0	BU6M 1.5	LO♯ 5.2	M34 399.8, 57.2

5 = 4-aminobutylstyrene hydrochloride; DMAA = *N*,*N*-dimethylacrylamide; MA = methacrolein; **11c** = *N*-acryloyl-1,6-diaminohexane hydrochloride; **11b** = *N*-acryloyl-1,4-diaminobutane hydrochloride; LO = lysyl oxidase; PAO = plasma amine oxidase; BU6M = 0.05 M borate buffer, 6M Urea (pH = 8.1 - 8.3); PB (phosphate, pH = 7.8); * gelatine was added to a hyaluronic acid (HA) solution obtained by dissolving 10.2 mg of HA in 5 mL of phosphate buffer. § 0.5029g /5.0 mL BU6M; ‡ 0.0037g/0.6 mL BU6M; † 0.4092/5.5 mL BU6M; ♯ 0.5254/5.2 mL BU6M.

Table S9. UV and NaOH titration of pristine Gel B and of some selected crosslinked derivatives.

Sample (mg)	CL agent (% *)	NH_2/g (UV) (mmol)	NH_2/g (NaOH) (mmol)	Crosslinking (%)
Gel B				
11.2	-	0.219±0.02	-	-
251.3	-	-	0.391±0.03	-
M21	CP5/DMAA-43.1			
11.4	10.1	0.065±0.001	-	70.5
M31	CP11c/DMAA-10.0			
11.9	10.4	0.208±0.04	-	5.0
220.1	10.4	-	0.372±0.03	4.9
M23	CPMA/DMAA-20.0			
11.4	10.2	0.207±0.03	-	5.5
222.9	10.2	-	0.157±0.02	59.8
M26	CPMA/DMAA-20.0			
11.6	5.1	0.225±0.02	-	≈ 0
221.9	5.1	-	0.190±0.01	51.4
M32	CPMA/DMAA-20.0			
12.9	5.1	0.174±0.01	-	20.5
220.5	5.1	-	0.164±0.01	58.1
M34	CPMA/DMAA-20.0			
11.5	10.0	0.118±0.015	-	46.1

CL = Crosslinking; * % wt/wt with Gel B.

Table S10. Experimental data of the synthesis of compounds **6a, b**.

Entry (g, mmol, mL)	DCM (mL)	Boc_2O (g, mmol)	Time (h)	Side product (g, mmol, %)	Product (g, mmol, %)	Physical state
1,2-ethylenediamine 5.39, 89.68, 6.0	50 * 25 **	4.90, 22.45	21	**7a** 0.6067, 2.33, 10.4	**6a** 2.64, 16.45, 73.3	Light-yellow oil
1,4-diaminobutane 6.14, 69.65, 7.0	70 * 30 **	3.80, 17.39	21	**7b** 0.3896, 1.35, 7.8	**6b** 3.13, 16.64, 95.7	Yellow oil

Boc_2O = *t*-butyl-di-carbonate; * for dissolving diamines; ** for dissolving Boc_2O.

Table S11. Experimental data of the synthesis of compounds **10a-c**.

Entry (g, mmol)	Solvent (mL)	Acryloyl chloride (g, mmol, mL)	TEA (g, mmol, mL)	Time (min)	Product (g, mmol, %)	Physical state
6a 2.17, 13.57	32.5 * 16.5 **	1.43, 16.29, 1.35	1.37, 13.57, 1.9	90 ***/135 °	**10a** 2.51, 11.71, 86.3	Solid
6b 3.01, 16.01	38.0 * 19.5 **	1.7, 19.21, 1.6	1.6, 16.01, 2.2	45 ***/120 °	**10b** 3.26, 13.45, 84.1	Solid
6c 3.04, 14.05	34.0 * 17.0 **	1.53, 16.86, 1.4	1.42, 14.05, 1.95	90 ***/overnight °	**10c** 3.03, 11.21, 79.8	Solid

TEA = triethylamine; * for dissolving acryloyl chloride; ** for dissolving compounds **6a-c**; *** for the dropwise adding of TEA and **6a-c** solution; ° reaction time.

Table S12. Experimental data of the synthesis of monomers **11a-c**.

Entry (g, mmol)	EtOAc (mL)	HCl 3N (mL)	HCl 37% (mL)	Time (min)	Product (g, mmol, %)	Physical state
10a 2.90, 15.50	---	7.5	---	80 °	**11a** 1.77, 11.80, 76.1	Solid
10b 1.60, 6.61	20	---	Needed to pH = 1	N.R. °	**11b** 0.97, 5.50, 83.2	Solid *
10c 2.98, 11.02	---	12	---	60 °	**11c** 2.25, 10.87, 98.6	Solid

° Reaction time; * for characterization the dark solid was recrystallized by acetonitrile (CH_3CN).

Table S13. Copolymerization reactions of **10a**, **10b** and **11c** with DMAA a 60 °C.

Entry	mg (mmol)	DMAA (g, mmol)	M_m	AIBN (mg, %)	MeOH (mL)	Time (min)	Copolymer	(g, %)
10a	201.1 (0.94)	0.84, 8.47	0.0997	10.4, 1.00	7.0	290	CP10a/DMAA	0.658, 63.2
10a	200.0 (0.93)	0.88, 8.40	0.0997	10.5, 0.97	7.0	210	CP10a/DMAA	0.749, 69.4
10b	200.3 (0.83)	0.74, 7.47	0.0997	9.68, 1.03	6.5	270	CP10b/DMAA	0.435, 46.3
10b	400.6 (1.65)	1.47, 14.85	0.1000	18.7, 1.00	13.0	270	CP10b/DMAA	1.458, 79.8
11c	100.9 (0.49)	0.44, 4.44	0.0994	5.46, 1.01	3.0	150	CP11c/DMAA	0.478, 94.7
11c	201.0 (0.97)	0.88, 8.75	0.0998	11.0, 1.02	4.0	210	CP11c/DMAA	0.819, 76.1

DMAA= *N, N′*-di-methylacrylamide; M_m= molar fractions of the monomers into the feed; AIBN = 2,2'-azo-*bis*-(2-methylpropane nitrile).

Table S14. Boc deprotection of copolymers CP10a/DMAA e CP10b/DMAA.

Copolymer	g	HCl 3N* (mL)	Time(h)	Product	g, %
CP10a/DMAA	0.337	3.3	4	CP11a/DMAA	0.277, 82.2
CP10a/DMAA	0.406	4.0	4	CP11a/DMAA	0.261, 64.3
CP10b/DMAA	0.267	2.0	4	CP11b/DMAA	0.180, 67.4
CP10b/DMAA	0.396	4.0	4	CP11b/DMAA	0.376, 94.9

* in EtOAc; DMAA = *N, N′*-di-methylacrylamide.