Electronic Supplementary Material (ESI) for Materials Advances.

SUPPORTING INFORMATION

Development of a tissue construct with spatially controllable stiffness via one-step 3D bioprinting and dual-crosslinking process

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Content:

1. Scheme of the alginate methacrylation reaction

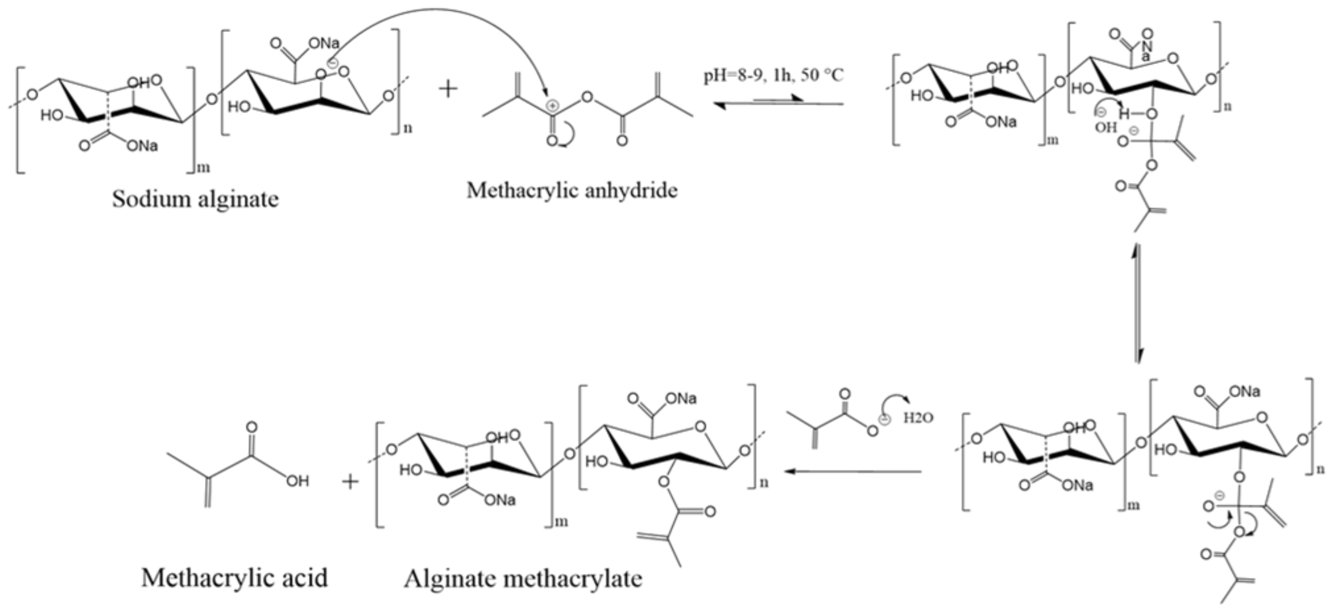

Figure S1. Schematic steps of alginate methacrylation by grafting methacrylate groups into sodium alginate chain.

2. Detailed interpretation of FTIR spectra

As observed from Figure 1(d and e), both spectra display wide bands in the region ranging from 3600 cm^{-1} to 3000 cm^{-1} and from 2980 cm^{-1} to 2850 cm^{-1}, ascribable to the stretching of –OH and –CH groups, respectively, typically of the polysaccharides [1]. The others bands might be more representative of the alginate chains since the stretching of carboxylate anions $–COO^{-}$ falls around 1595 cm^{-1} [1][2], the C–OH deformation along with the symmetric stretching of O–C–O of the carboxylate groups [2][3] falls around 1403 cm^{-1} , while the vibrations of C–O and C–C of the pyranose rings produce the bands at ca. 1083 cm^{-1} and at ca. 1026 cm^{-1}, respectively [1]. Furthermore, all the bands at low frequency (946 cm^{-1}, 883 cm^{-1} and 812 cm^{-1}) might be assigned to the C–O stretching of uronic acid [1], to the C1–H deformation of β-mannuronic acid [1], and to the C–H bending vibrations of mannuronic acid residues [4]. In addition to these bands, the spectrum related to the AlgMa product exhibits more intense peaks at 2980 cm^{-1}-2850 cm^{-1} and the presence of a "shoulder" at 1711 cm^{-1} (both highlighted with the red arrows in Figure 2e), not observed in the pristine alginate spectrum (Figure 2 d). These marked bands along with the "shoulder" can be respectively assigned to the stretching of the –CH of the alkane and alkene bonds contained in the methacrylate functionalization and to the stretching of the C=O group of the esters.

3. Mass spectrometry data of AlgMa products after hydrolysis

Table S1. Mass spectrometry data of AlgMa products after hydrolysis, reported as observed m/z values, error in ppm and formula assignments; (Δ) -COC_3H_5 (methacrylation); Hexuronic acid: Glucuronic acid/Mannuronic acid

Observed mass (m/z)	Error (ppm)	Formula	Structure
371.08	**5.0**	**$[C_{12}H_{19}O_{13}]^+$**	**Hexuronic acid$_2$**
339.09	5.0	$[C_{12}H_{19}O_{11}]^+$	Hexuronic acid$_2$
353.07	7.0	$[C_{12}H_{17}O_{12}]^+$	Hexuronic acid$_2$
355.09	6.0	$[C_{12}H_{19}O_{12}]^+$	Hexuronic acid$_2$
407.12	4.5	$[C_{16}H_{23}O_{12}]^+$	Hexuronic acid$_2$ +Δ
441.12	5.5	$[C_{16}H_{25}O_{14}]^+$	Hexuronic acid$_2$ +Δ
493.16	7.0	$[C_{20}H_{29}O_{14}]^+$	Hexuronic acid$_2$ +2Δ
509.15	6.0	$[C_{20}H_{29}O_{15}]^+$	Hexuronic acid$_2$ +2Δ
547.12	**5.8**	**$[C_{18}H_{27}O_{19}]^+$**	**Hexuronic acid$_3$**
603.18	8.0	$[C_{22}H_{35}O_{19}]^+$	Hexuronic acid$_3$ +Δ
617.16	5.7	$[C_{22}H_{33}O_{20}]^+$	Hexuronic acid$_3$ +Δ
685.18	5.0	$[C_{26}H_{37}O_{21}]^+$	Hexuronic acid$_3$ +2Δ
703.21	6.3	$[C_{30}H_{39}O_{19}]^+$	Hexuronic acid$_3$ +3Δ
719.20	5.7	$[C_{30}H_{39}O_{20}]^+$	Hexuronic acid$_3$ +3Δ
753.22	7.0	$[C_{30}H_{41}O_{22}]^+$	Hexuronic acid$_3$ +3Δ
723.14	6.1	**$[C_{24}H_{35}O_{25}]^+$**	**Hexuronic acid$_4$**
756.16	6.0	$[C_{28}H_{36}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
758.17	6.0	$[C_{28}H_{38}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
760.19	5.5	$[C_{28}H_{40}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
770.14	5.5	$[C_{28}H_{34}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
772.15	4.9	$[C_{28}H_{36}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
774.18	7.2	$[C_{28}H_{38}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
776.20	7.1	$[C_{28}H_{40}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
784.12	6.0	$[C_{28}H_{32}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
786.13	6.0	$[C_{28}H_{34}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
788.15	5.0	$[C_{28}H_{36}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
790.16	5.6	$[C_{28}H_{38}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +Δ
796.20	7.1	$[C_{31}H_{40}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
798.21	5.0	$[C_{31}H_{42}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
800.22	6.0	$[C_{31}H_{44}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
802.24	5.3	$[C_{31}H_{46}O_{24}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
812.19	5.0	$[C_{31}H_{40}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
814.20	4.5	$[C_{31}H_{42}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
816.22	5.5	$[C_{31}H_{44}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
826.20	6.0	$[C_{32}H_{42}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
828.23	7.0	$[C_{32}H_{44}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
830.23	5.0	$[C_{32}H_{46}O_{25}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
838.16	5.7	$[C_{32}H_{38}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
840.19	6.0	$[C_{32}H_{40}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
842.21	7.1	$[C_{32}H_{42}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
844.22	8.0	$[C_{32}H_{44}O_{26}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
852.14	5.7	$[C_{32}H_{36}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
854.17	6.3	$[C_{32}H_{38}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
856.19	6.1	$[C_{32}H_{40}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ
858.20	6.0	$[C_{32}H_{42}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +2Δ

906.20	6.0	$[C_{36}H_{42}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
908.21	5.5	$[C_{36}H_{44}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
910.23	5.6	$[C_{36}H_{46}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
912.24	4.9	$[C_{36}H_{48}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
914.26	7.2	$[C_{36}H_{50}O_{27}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
924.20	5.0	$[C_{36}H_{44}O_{28}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
926.22	5.1	$[C_{36}H_{46}O_{28}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
928.24	6.0	$[C_{36}H_{48}O_{28}]^{\bullet+}$	Hexuronic acid$_4$ +3Δ
899.17	**4.3**	$[C_{30}H_{43}O_{31}]^{+}$	**Hexuronic acid$_5$**
864.16	5.6	$[C_{30}H_{40}O_{29}]^{\bullet+}$	Hexuronic acid$_5$
866.19	7.1	$[C_{30}H_{42}O_{29}]^{\bullet+}$	Hexuronic acid$_5$
868.21	5.9	$[C_{30}H_{44}O_{29}]^{\bullet+}$	Hexuronic acid$_5$
870.22	7.2	$[C_{30}H_{46}O_{29}]^{\bullet+}$	Hexuronic acid$_5$
872.24	6.0	$[C_{30}H_{48}O_{29}]^{\bullet+}$	Hexuronic acid$_5$
878.14	5.1	$[C_{30}H_{38}O_{30}]^{\bullet+}$	Hexuronic acid$_5$
880.17	6.0	$[C_{30}H_{40}O_{30}]^{\bullet+}$	Hexuronic acid$_5$
882.18	4.3	$[C_{30}H_{42}O_{30}]^{\bullet+}$	Hexuronic acid$_5$
884.19	5.6	$[C_{30}H_{44}O_{30}]^{\bullet+}$	Hexuronic acid$_5$
886.22	7.1	$[C_{30}H_{46}O_{30}]^{\bullet+}$	Hexuronic acid$_5$
1075.21	**8.0**	$[C_{36}H_{51}O_{37}]^{+}$	**Hexuronic acid$_6$**
1209.26	8.2	$[C_{44}H_{57}O_{39}]^{+}$	Hexuronic acid$_6$ +2Δ
1284.34	7.5	$[C_{48}H_{68}O_{40}]^{\bullet+}$	Hexuronic acid$_6$ +3Δ
1359.42	7.2	$[C_{52}H_{79}O_{41}]^{+}$	Hexuronic acid$_6$+4Δ
1427.27	**6.0**	$[C_{48}H_{67}O_{49}]^{+}$	**Hexuronic acid$_8$**

Table S2. Isotope cluster area (ICA) of all identified species is reported. Three sets of experiments (**1**, **2**, **3**) were performed. The listed ICA values are calculated as media from three consecutive acquisitions to obtain a total of 9 subset data. In black are reported the oligosaccharides chains with no derivatization sites; in red are reported methacrylate species.

	1	**2**	**3**
Observed (m/z)	**ICA**	**ICA**	**ICA**
339.09	100663.7	189102.5	226460.7
353.07	113900.4	106517.5	141293.1
355.09	252417.8	169726.3	206883.1
371.08	233331.1	165196.7	233408.6
407.12	93049.0	107333.8	91277.5
441.12	208892.6	170514.3	393901.9
493.16	44751.0	42209.8	87516.0
509.15	12283.0	13228.4	29695.9
547.12	65984.4	43626.6	50261.5
603.18	37072.6	32687.0	46770.4

617.16	15552.1	6474.2	26693.8
685.18	21694.3	21486.8	38624.6
703.21	72892.7	48356.2	86437.2
719.20	29531.0	16025.6	32745.9
723.14	4821.6	3449.0	3528.0
753.22	10883.0	4031.4	4213.5
756.16	13470.5	9399.8	15550.4
758.17	23191.7	11464.4	26539.4
760.19	25181.2	14430.2	23780.2
770.14	19105.0	12010.6	19778.9
772.15	38201.5	25629.0	43505.2
774.18	28048.2	20738.0	33132.2
776.20	9166.3	5072.1	5351.9
784.12	19005.4	12605.7	22092.7
786.13	39395.7	26348.5	41325.6
788.15	34343.9	22897.5	42779.1
790.16	15481.3	11303.4	9516.7
796.20	15567.2	8677.0	17946.5
798.21	39132.4	33379.9	47264.3
800.22	66395.1	47332.3	76686.6
802.24	40989.8	27666.7	49429.7
812.19	35587.5	22448.1	43324.2
814.20	57817.3	43087.9	66495.1
816.22	42240.2	28973.0	52247.2
826.20	64070.3	46933.7	83361.4
828.23	70726.1	54696.5	84982.5
830.23	39595.3	28972.3	48588.3
838.16	11631.8	10461.0	16842.9
840.19	38195.3	25191.9	40558.9
842.21	50207.0	33149.4	49625.6
844.22	28572.5	20474.1	31849.2
852.14	27912.9	16100.6	29160.1
854.17	51601.1	33108.4	58315.9
856.19	37175.3	27464.8	45381.6
858.20	18045.7	10290.9	17363.3
864.16	3557.4	1705.7	2783.3
866.19	12136.6	7233.6	11353.5
868.21	16930.3	14585.7	22188.2
870.22	22118.8	14651.3	21759.8
872.24	11291.5	8303.2	9653.6
878.14	8669.2	6093.8	7717.9
880.17	17297.4	9036.9	18124.8
882.18	28955.5	17305.7	30367.3
884.19	17388.3	11255.4	18712.5

886.22	10599.5	5365.2	9911.5
899.17	7141.0	1607.0	2309.4
906.20	4836.3	2867.8	5360.1
908.21	8385.3	5470.8	7984.2
910.23	6526.8	4863.0	9536.1
912.24	10817.6	6828.9	10106.4
914.26	9350.9	4712.0	12423.4
924.20	5411.9	2689.9	6073.1
926.22	9824.6	4994.3	8697.4
928.24	7242.2	2893.5	7944.9
1075.21	519.3	746.0	2826.2
1209.26	2024.2	1433.5	7225.0
1284.34	2462.6	1739.8	9076.7
1359.42	1137.4	410.5	1022.9
1427.27	376.9	262.6	670.6
	Σ (ICA 1) 2542776.0	**Σ (ICA 2)** 1967331.7	**Σ (ICA 3)** 3086318.7
	Σ (ICA_M 1) 1603791.4	**Σ (ICA_M 2)** 1187527.7	**Σ (ICA_M 3)** 2061888.6
	$\frac{\sum ICA_M 1}{\sum \text{ICA1}} * 100$ **63.07**	$\frac{\sum ICA_M 2}{\sum \text{ICA2}} * 100$ **60.36**	$\frac{\sum ICA_M 3}{\sum \text{ICA3}} * 100$ **66.81**
		AVERAGE	***63.41***
		Standard Deviation	***3.24***

4. Stability tests and cell viability results

Table S3. Summary of the stability tests and cell viability results of different AlgMa ink formulations.

Sample	I2959 concentration	UV time	Stability test[1]	Cell viability[2]
AlgMa (4% w/v)	0.05 % (w/v)	1 min	Solubilized after 5 min	/
	0.05 % (w/v)	5 min	Solubilized after 15 min	/
	0.05 % (w/v)	10 min	Solubilized after 20 min	/
	0.1 % (w/v)	1 min	Solubilized after 20 min	/
	0.1 % (w/v)	5 min	Solubilized after 2 h	/
	0.1 % (w/v)	10 min	Solubilized after 1 day	/
	0.2 % (w/v)	1 min	Stable until 3 days but circular shape is slightly lost	79%
	0.2 % (w/v)	1,5 min		62%
	0.2 % (w/v)	2 min		42%
	0.2 % (w/v)	5 min	Stable until 3 days	0%
	0.25 % (w/v)	1 min	Stable until 3 days	58%
	0.25 % (w/v)	1,5 min		52%
	0.25 % (w/v)	2 min		43%
	0.4 % (w/v)	2 min		33%

[1]stability tests were performed in PBS at 37°C by observing structural modification and the dissolution of the material during time.
[2]cell viability was assessed by live-dead analysis with Calcein-AM and Propidium Iodide. HeLa were used as cell type.

5. Rheological results

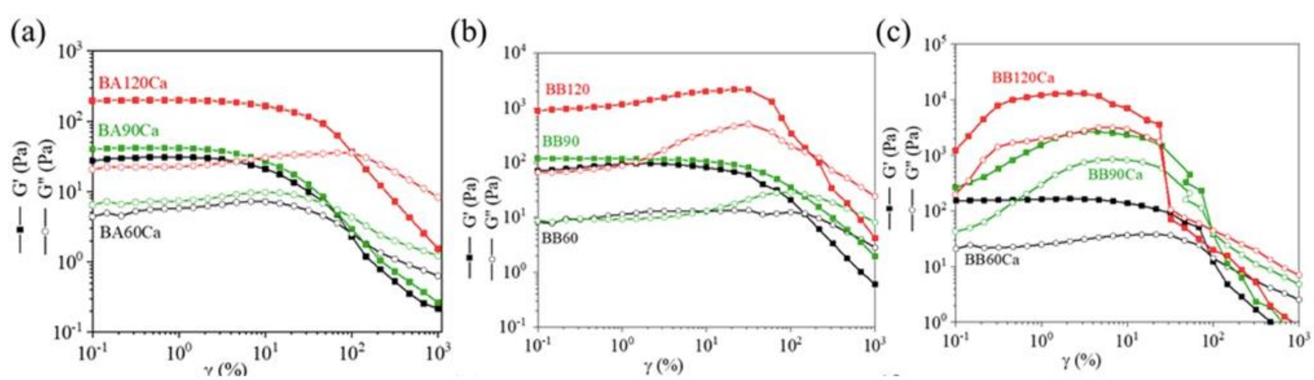

Figure S2. Rheological evaluation of 3D bioprinted (a) BA after dual cross-linking process with different UV irradiation time: 60 sec (BA60Ca, black curve), 90 sec (BA90Ca, green curve), 120 sec (BA120Ca, red curve); (b) BB after photo-crosslinking at 60 sec (BB60, black curve), 90 sec (BB90, green curve) and 120 sec (BB120, red curve) of UV light; (c) BB after dual cross-linking process with different UV irradiation time: 60 sec (BA60Ca, black curve), 90 sec (BA90Ca, green curve), 120 sec (BA120Ca, red curve).

Table S4. Summary of the storage (G') and loss (G") moduli values of the 3D bioprinting scaffolds produced by using bioink A and bioink B (colored in yellow), and the corresponding scaffolds not containing cells (colored in green), after dual crosslinking processes.

3D bioprinted scaffold	UV irradiation time (s)	Time of immersion in $CaCl_2$ (min)	G' (kPa)	G" (kPa)
IB120Ca	120	5	99	16
IB90Ca	90	5	23	5
IB60Ca	60	5	14	2.8
BB120Ca	120	5	2	0.20
BB90Ca	90	5	0.310	0.040
BB60Ca	60	5	0.120	0.020
IA120Ca	120	5	21	7
IA90Ca	90	5	17	7
IA60Ca	60	5	5	2
BA120Ca	120	5	0.200	0.020
BA90Ca	90	5	0.041	0.007
BA60Ca	60	5	0.030	0.002

References

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[3] Mathlouthi M and Koenig J L 1987 Vibrational Spectra of Carbohydrates *Advances in Carbohydrate Chemistry and Biochemistry, Academic* Press **44** 7-89

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