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Controlled *in situ* acidification enables the 3D printability of GelMA-Dextran aqueous two-phase hydrogel with unique interconnected porosity

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Supplementary Information

Identification of the IEP of GelMA

The IEP of GelMA is defined as the pH corresponding to a zero electrophoretic mobility, which can vary from one synthetized batch to another. The preparation of GelMA aqueous solutions ($1 mg/mL$) with gradually increasing HCl or NaOH concentrations is followed by pH measurements (Metrohm 744 pH-Meter) to target a pH range (3.0−4.5) or (5.0−9.5), respectively. Each solution is added to a disposable folded capillary cell (DTS1070) and their electrophoretic mobility is measured by Malvern's Nano-Zetasizer, after equilibration at T=37 °C for 180s. For each pH value, five measurements with max. 100 runs per measurement and no delay time between them are taken.

For the batches used in this work, pI≈ 4.00, as shown in **Figure S1A**. The IEP always shifts to a lower value than that of native gelatin (pI≈ 7.7), because some of the amino- and carboxylic groups in gelatin have been substituted by methacrylic groups. At a pH range of $(3.5 - 4.5)$, the global charge of GelMA is close to zero, hence this is the demixing range for GelMA-dextran aqueous solutions. If this pH range is shifted, then so are the relative concentrations to trigger nucleation and growth or spinodal decomposition. Variation of the isoelectric point after changing the synthesis protocol of GelMA is also shown in **Figure S1B**. Although the methacrylic anhydride is halved or quartered, there is no significant impact in the determined isoelectric points.

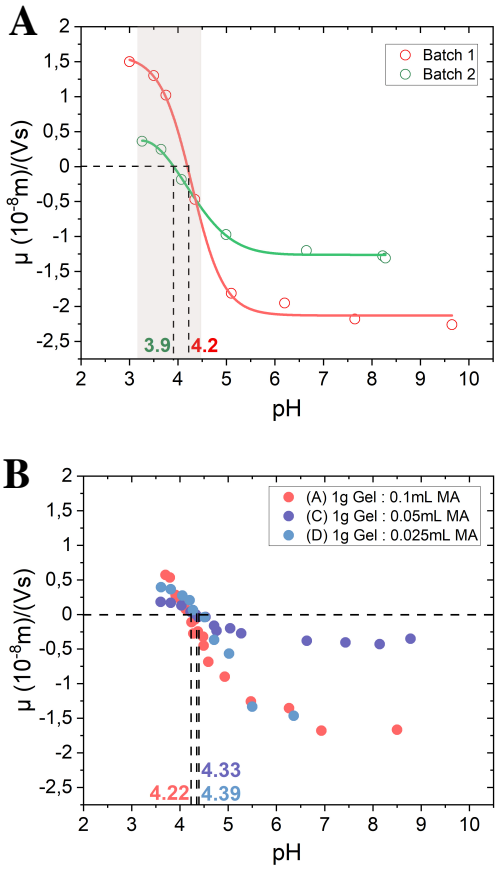

Fig. S1 A) Variation of isoelectric points of different GelMA batches with the same methacrylic anhydride used during the synthesis and the same starting gelatin batch. B) Variation of isoelectric points of three GelMA batches with varying methacrylic anhydride proportions per 1 g of gelatin: 0.1 mL MA (red), 0.05 mL MA (purple) and 0.025 mL MA (blue).

Phase diagram of GelMA-dextran

The creation of a GelMA-Dextran phase diagram requires the preparation of many mixtures of the two polymers in different relative concentrations. Each sample is allowed to equilibrate at 37 °C in a Cultura-M incubator for 24 h, after which it may evolve from a homogeneous solution to one that is macroscopically phase separated, with two distinct phases, the GelMA- and the dextran-rich. The binodal line in a phase diagram separates the biphasic from the monophasic region, where samples phase separate or not (**Figure S2A**), respectively. In some cases, turbidity assessment of the solutions or optical microscopy is employed to confirm phase separation. In this work, the phase diagram of **Figure S2B** is created from mixtures acidified by HCl to target a

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pH of 4.7 close to the IEP of GelMA. For a [GelMA]= 50mg/mL, [dextran]= 50mg/mL composition, no macroscopic phase separation is observed. The solution remains monophasic and it is concluded that in neutral pH conditions the binodal line would have to be shifted to higher concentrations of the two polymers. This would make the hydrogels stiffer, as the polymer percentage would be higher, and this would be an undesirable attribute for bio-applications with cells[1].

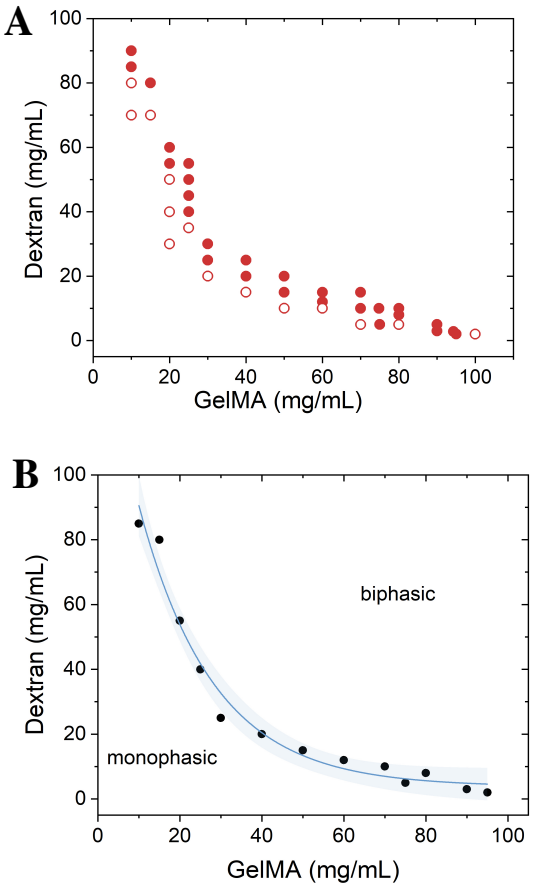

Fig. S2 A) Determination of the binodal line for the phase diagram of GelMA and dextran, after adjusting the pH≈ 4.7 with HCl. Closed circles are for the compositions that phase separate and open circles for the ones that do not. B) Phase Diagram of GelMA-dextran at pH≈ 4.7. The rectangular symbol represents the solution of GelMA and dextran in milli-Q water. The binodal line is determined and depicted with a 95% confidence band.

Comparison of hydrochloric to gluconic acid

It is known that hydrochloric acid requires careful handling due to its corrosive nature and potential to produce harmful fumes. On the other hand, gluconic acid is milder and non-toxic, hence it is generally handled in food and pharmaceutical applications. The nature of the two acidifiers can be further compared, within the scope of this study, in terms of their intrinsic properties that should be taken into account for triggering segregation of GelMA and dextran in water. A mixture of [GelMA]= 52mg/mL, [dextran]= 26mg/mL (for bicontinuous microstructure) is prepared and its turbidity is measured as function of pH. The starting point of the GelMA-dextran solution in milli-Q water is pH≈ 5.5 and gradually HCl (black curve) or gluconic acid (orange curve) is added to it, measuring pH and turbidity at 37°C. It is important to emphasize that in this experiment gluconic acid –and not GDL– is added to the GelMA-dextran solution, in order to avoid any other kinetics effects. For this, a high GDL concentration of 10% w/v is diluted in milli-Q water and left overnight until hydrolysis is complete.

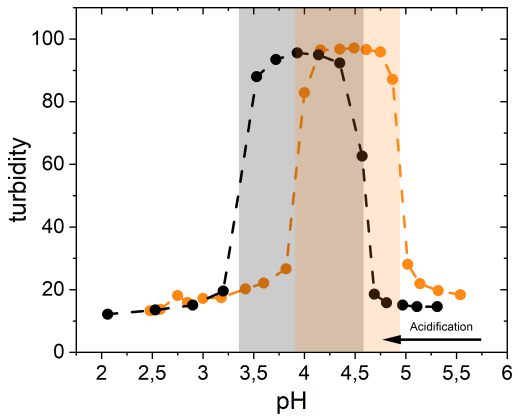

Fig. S3 Turbidity as function of pH for [GelMA]= 52mg/mL, [dextran]= 26mg/mL to target the bicontinuous microstructure, following acidification by hydrochloric (black) or gluconic acid (orange), at 37 °C.

In **Figure S3** the pH range (3.3−4.6) for HCl and, slightly higher, pH (3.9−4.9) for gluconic acid is highlighted. For these pH values, the GelMA-dextran solutions turn from transparent to turbid, to transparent again when pH < 3.3 (HCl) or pH < 3.9 (gluconic acid). GelMA and dextran do not phase separate at such low pH values because GelMA is sufficiently charged. The acidification of GelMA using gluconic acid instead of HCl demonstrates a shift of approximately +0.5 pH units, as determined by electrophoretic mobility and turbidity measurements. This discrepancy is likely related to the fundamental differences in the acid dissociation behavior of the two acids. HCl is a strong acid that dissociates completely in aqueous solution, releasing protons and acidifying the medium. In contrast, gluconic acid is a weak acid that only partially dissociates. Therefore, when weak acids or their conjugate salts are used, the measured IEP should be interpreted as a relative isoelectric point rather than an absolute one[2]. Furthermore it is known that ion-specific effects influence the binding to biomolecules[3]. Thus, the additional presence of ionized species such as gluconate can influence the local charge environment around GelMA, effectively shifting the balance point at which the net charge of the polymer becomes zero. As a result, the isoelectric point region observed with gluconic acid differs from that measured in the case of acidification with a strong acid like HCl.

The turbidity results from UV-Vis spectroscopy can be also coupled with analyzing the induced microstructures in the different stages of acidification of the GelMA-dextran system. For this, a

small quantity of GelMA-dextran with a specific amount of gluconic acid to target different pH values is UV-crosslinked and taken to the confocal microscope. In this experiment the aim is to examine to what extent the induced porosity in the hydrogels is symmetrical before/ after the pH range of high turbidity. In **Figure S4(c1-c2)**, and for the [GelMA]= 52mg/mL and [dextran]= 12mg/mL (regular disconnected droplets) system, one can observe that before (c1) and after (c4) turbidity increases, the hydrogel is homogeneous and non porous because GelMA was charged in solution state (before UV-crosslinking). Near the IEP of GelMA (c2, c3) nucleation and growth takes place and disconnected pores in the labeled continuous GelMA hydrogel can be distinguished.

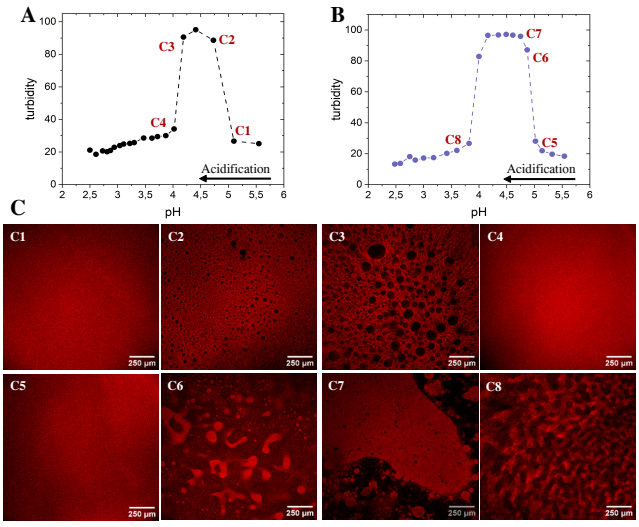

Fig. S4 A) Turbidity as function of pH for [GelMA]=52mg/mL, [dextran]=12mg/mL that phase separated by nucleation and growth, following acidification by gluconic acid at 37 °C. B) Turbidity as function of pH for [GelMA]=52mg/mL, [dextran]=26mg/mL that phase separated by spinodal decomposition, following acidification by gluconic acid at 37 °C. C) CLSM images of the casted hydrogels with regular disconnected (first row) and interconnected (second row) pores, for different pH conditions, following the turbidity curves for each system. GelMA is labelled red. Images (c1-c4) come from the pH-evolution of nucleation and growth (black curve) and images (c5-c8) from that of spinodal decomposition (purple curve).

For the [GelMA]= 52mg/mL, [dextran]= 26mg/mL (bicontinuous) system in **Figure S4(c5-c8)**, a homogenous non-porous hydrogel (c5) is obtained after UV-crosslinking the GelMA-dextran solution at pH≈ 5, where there is no segregation and GelMA is still charged. Spinodal decomposition is triggered at a pH< 5 (c6). As soon as turbidity reaches the maximum value (c7), a multiple emulsion formation is observed, that is, GelMA-rich droplets dispersed in dextran continuous phase (G/D) and dextran-rich droplets dispersed in GelMA continuous phase (D/G). Continuing to lower pH values, where the turbidity of the mixture has decreased to ≈ 40%, the bicontinuous microstructure is again obtained (c8). This experiment proves that the sensitivity and changes of the microstructure can be indicated by the turbidity of the GelMA-dextran solution.

Reproducibility studies for triggering phase separation

Turbidity is an indicator of the triggering point of phase separation. In **Figure S5** the same experiment of measuring the temporal evolution of turbidity of a [GelMA]=54mg/mL, [dextran]=22mg/mL and [GDL]=10mg/mL solution is performed in quantiplicate. This is to test if the spike of the value of turbidity can be a trustworthy indication that spinodal decomposition started and then there are ≈ 5 min for the interwoven channels to fully grow, i.e. the time window in which photocrosslinking should take place to obtain the hydrogel. As it can be seen, the onset time of phase separation for the same composition of GelMA-dextran-GDL is very reproducible, with an average of $t_{ON} = (8.3 \pm 0.3)$ min.

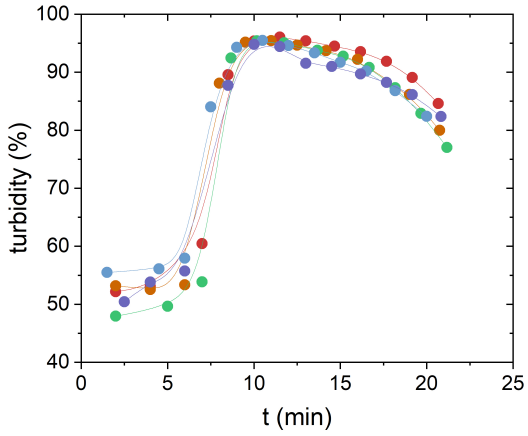

Fig. S5 Reproducibility of onset times of phase separation for a [GelMA]=54mg/mL, [dextran]=22mg/mL and [GDL]=10mg/mL solution at 37 °C. The onset time of phase separation is indicated by the sudden increase of the turbidity value.

Macroscopic images of hydrogels after inkjet 3D printing

In this work it is demonstrated that a macroscopic single-layered structure can be 3D printed on a petri dish, using inkjet printing of the GelMA-dextran ATPS at 37°C. In **Figure S6A** this macroscopic structure is depicted, along with what the microstructure looks like for different parts of the 3D printed sample (**Figure S6C, D**). The high resolution allows for 3D printing more complex shapes of the hydrogels. After performing tile scans with the confocal microscope, it is confirmed that the microstructure is homogeneous and bicontinuous everywhere along the sample. Lastly, in **Figure S6B** the 3D representation of the GelMA-dextran hydrogel is portrayed, after performing a zeta-stacking with the confocal microscope. Bicontinuity is also confirmed along the third axis.

References

1 S. R. Caliari and J. A. Burdick, *Nature methods*, 2016, **13**, 405–414.

2 G. Scatchard and E. S. Black, *The Journal of Physical Chemistry*, 1949, **53**, 88–100.

3 W. Kunz, *Specific ion effects*, World Scientific, 2010.

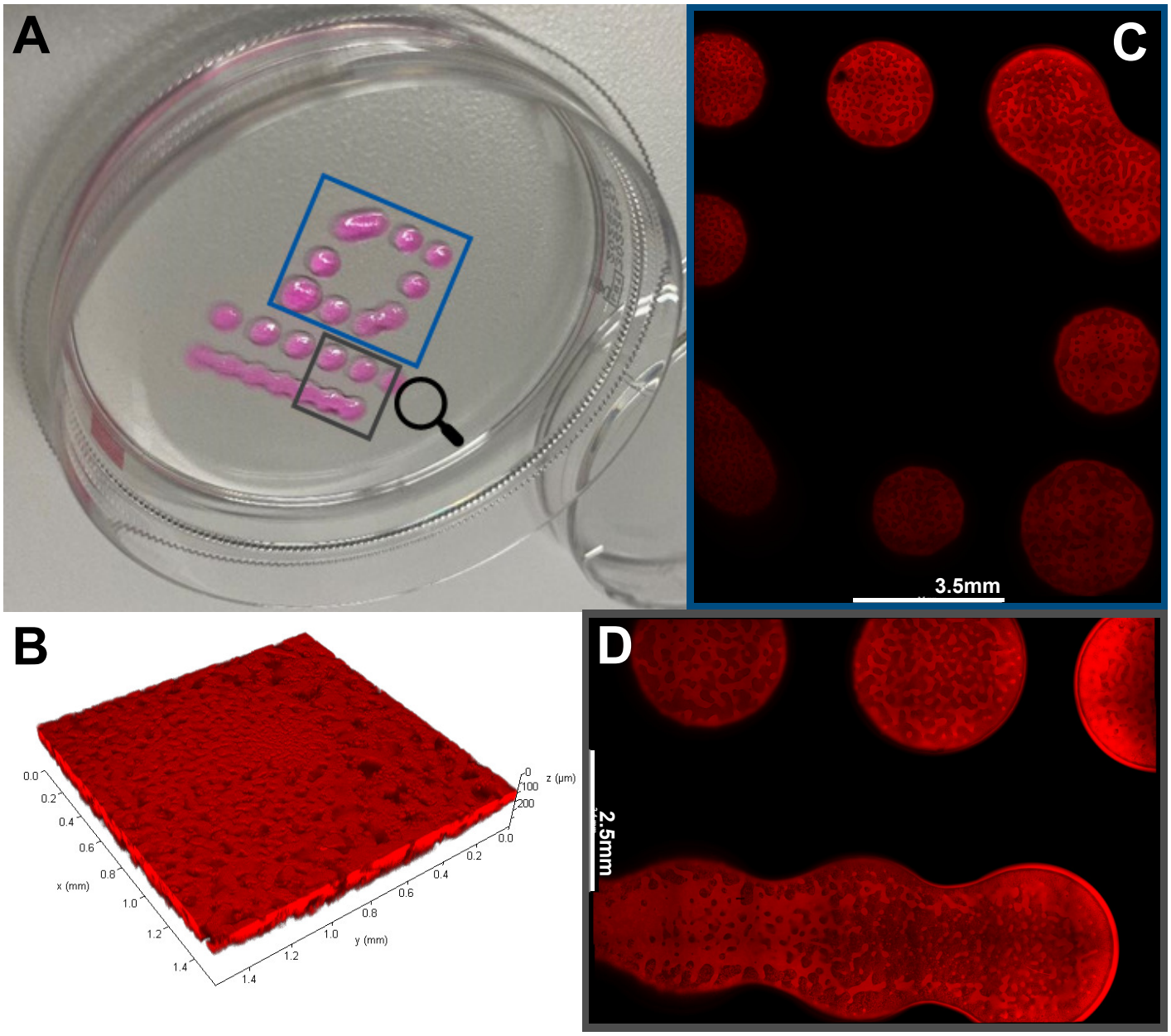

Fig. S6 A) Macroscopic image of the inkjet 3D printed hydrogel. B) Zeta stacking to create a 3D representation of the printed hydrogel. C) and D) Tile scan imaging of the blue- and grey-colored areas of image (A) where the distinct bicontinuous microstructure is captured after UV-crosslinking at $t = 8{:}45$ min.