Supporting Information

Continuous hydrogen production by immobilized cultures of *Thermotoga neapolitana* on a novel hydrogel with pH-buffering properties

Maria Assunta Basile,[a] *Cosimo Carfagna,*[b] *Pierfrancesco Cerruti,*[*b] *Giovanna Gomez d'Ayala,*[b] *Angelo Fontana,*[c] *Agata Gambacorta,*[c] *Mario Malinconico*[b] *and Laura Dipasquale*[*c]

[a]Department of Biochemistry and Biophysics, Second University of Naples, Via L. De Crecchio, 7 - 80100 Naples, Italy
[b]Institute of Polymer Chemistry and Technology (CNR) - Via Campi Flegrei, 34 - 80078 Pozzuoli (Na), Italy
[c]Institute of Biomolecular Chemistry (CNR) - Via Campi Flegrei, 34 - 80078 Pozzuoli (Na), Italy

Experimental section

Preparation of the immobilizing hydrogel

The acrylic monomers 2-hydroxyethyl methacrylate (HEMA), [2-(methacryloyloxy)ethyl]trimethylammonium chloride (METAC), 2-(dimethylamino)ethyl acrylate (DMAEA), ethylene glycol dimethacrylate (EGDMA) and the free radical initiator 2,2'-azoisobutyronitrile (AIBN) were purchased from Aldrich and used as received.

HMD copolymer was prepared via radical chain polymerisation using AIBN 0.1% (w/w) as initiator and EGDMA 0.4% (w/w) as cross-linking agent.[S1] The molar ratio of monomers in the polymerization mixture was HEMA/METAC/DMAEA (HMD) = 5:3:3. Polymerization was carried out by loading the mixture on sealed glass chambers separated by a silicon frame, and curing in a nitrogen-purged oven as follows: 2 h at 60 °C, 4 h at 70 °C and 1 h at 85 °C. After polymerization, the gel was extensively washed with sterile distilled water, dried and ground into particles which were passed through a 1-mm sieve.

Solid-state ^{13}C MAS-NMR

For the solid-state ^{13}C MAS-NMR measurements, HEMA-based hydrogels were powdered using a freezer mill and then dried under vacuum at 60 °C for 24 h. Measurements were carried out using a Bruker Avance II 400 spectrometer operating at 100.47 MHz. Samples were spun at 10 kHz in 4 mm zirconium oxide rotors. Spectra were collected using a single pulse excitation sequence with a ^{13}C 90° pulse width of 3.8 μs, and a recycle delay of 20 s, by averaging from 4000 to 10000 scans.

Swelling properties

The supporting material was immersed in distilled water and allowed to reach the equilibrium swelling degree. Then 5 g of the swollen hydrogel were dehydrated to constant weight at 90 °C. The dried material was immersed in distilled water and weighed at different times until constant weight, to calculate water absorption. Water desorption and absorption ratio is expressed as percent weight change with respect to the initial weight.

Titration of HMD 5:3:3

The buffering activity of the synthesized hydrogel was evaluated by titrating 50 mL of the standard culture medium adjusted at pH 8.5 containing 2 g hydrogel with a 0.1 M acetic acid solution using a Crison TitroMatic 1S.

Fourier transform infrared spectroscopy (ATR-FTIR)

A diamond crystal Perkin Elmer Universal ATR sampling accessory mounted on a Perkin Elmer Spectrum 100 FTIR spectrometer was used.

Spectra were recorded on powdered samples as an average of 32 scans in the range 4000–600 cm^{-1}, with a resolution of 4 cm^{-1}. For the quantitative measurements, the area of the carbonyl stretching (1800–1650 cm^{-1}) was used as a reference.[S2]

Microrganism

Thermotoga neapolitana (DSM 4359^{T}) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Medium and culture conditions

Routine growth was performed using a standard culture medium containing (g/L): NaCl 10.0; NH_4Cl 1.0; $MgCl_2 \cdot 6H_2O$ 0.2; KCl 0.1; $CaCl_2 \cdot 2H_2O$ 0.1; K_2HPO_4 0.3; KH_2PO_4 0.3; cysteine-HCl 1.0; yeast extract 2.0; tryptone 2.0; glucose 5.0; resazurin 0.001; before sterilization, the pH was adjusted at 8.5 with 1 M NaOH at room temperature. After sterilization, the medium was supplemented with 10 mL/L of both filter-sterilized vitamins and trace element solutions (from DSM medium 141).

Aliquots of this medium were splitted into 120 mL-glass serum bottles. Oxygen was removed by heating batch reactor while sparging its content with a stream of O_2-free N_2 gas until the solution was colourless. Then the bottles were sealed, capped and sterilized by autoclaving for 10 min at 110

°C. After the inoculum (6% v/v) under sterile and anaerobic conditions, the serum bottles were incubated at 80 °C. All transfers and sampling of cultures were performed with sterile syringes and needles; for the use as inoculum, the bacteria were stored in liquid culture under anaerobic conditions at 4 °C.

Suspended and immobilized culture tests in bioreactor

After 16 h of incubation at 80 °C, the *batch* cultures were used as 6% (v/v) inoculum for a 3.8 L-glass reactor with working and headspace volumes of 1 L and 2.8 L, respectively. The cultures were kept at 80 °C under continuous stirring at 250 rpm.

Prior to bacterial immobilization, the polymeric hydrogel was swollen and acclimated in the standard medium lacking of cysteine–HCl, yeast extract, tryptone and glucose. For the immobilization tests, acclimated supporting material and standard culture medium were mixed in a 1:3.3 volume ratio; control tests without supporting material were performed by using standard culture medium: in all cases the final volume was 1 L.

N_2 sparging was applied at regular intervals and, in the suspended cultures, the pH was maintained constant at 7.5 (measured at room temperature) by addition of 1 M NaOH.

After the first *batch* fermentation step of the immobilized system, the exhaust medium was withdrawn and replaced with fresh medium while sparging the culture with O_2-free N_2 gas; then another *batch* step was run under the same conditions as described above. This procedure was repeated three times. All the tests were performed in duplicate.

Analytical determinations of culture parameters

At regular intervals, samples were taken for the determination of organic acids and glucose contents in the supernatant after cell centrifugation at 10000 rpm for 30 min at 5 °C.

The amounts of lactic and acetic acids produced during growth were quantified by ^{1}H-Nuclear Magnetic Resonance (NMR, 400 MHz) by using a Bruker Avance 400 spectrometer and 10–20% (v/v) of a standard solution of D_2O containing 0.05% (w/w) 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (Sigma–Aldrich) as standard.[S3]

Glucose concentration was determined by the decrease of reducing groups with the dinitrosalicylic acid method.[S4] The samples were boiled at 100 °C for 5 min and then immediately cooled on ice. The absorbance was measured at 546 nm. The dinitrosalicylic acid colour reaction was calibrated under the assay conditions by using a standard solution of glucose.

The bioreactor headspace was sampled using a gas-tight syringe and gaseous end products (H_2 and CO_2) were analyzed by using a Gas Chromatograph Thermo Scientific, equipped with a 3 m Hayesep Q column. The temperatures of the thermal conductivity detector, injector and analytic column were 200 °C, 50 °C and 50 °C, respectively. The carrier gas was nitrogen at a flow rate of 20 mL/min. H_2 and CO_2 were quantified by using calibration curves made by means of pure gases.

SEM observations

The interaction between *T. neapolitana* cells and the hydrogel support was confirmed by Scanning Electron Microscopy (SEM) observations of the matrix surface after cell growth.[S1,S5] Bacterial cells immobilized on the hydrogel were fixed with 2.5% (v/v) glutaraldehyde in saline solution (0.9% (w/v) NaCl in distilled H_2O) overnight at 4 °C, rinsed with phosphate buffer (PBS 0.05 M, pH 7.5) and then dehydrated in water-acetone mixtures from 20% (v/v) to absolute acetone for 15 minutes.

Supplementary figures

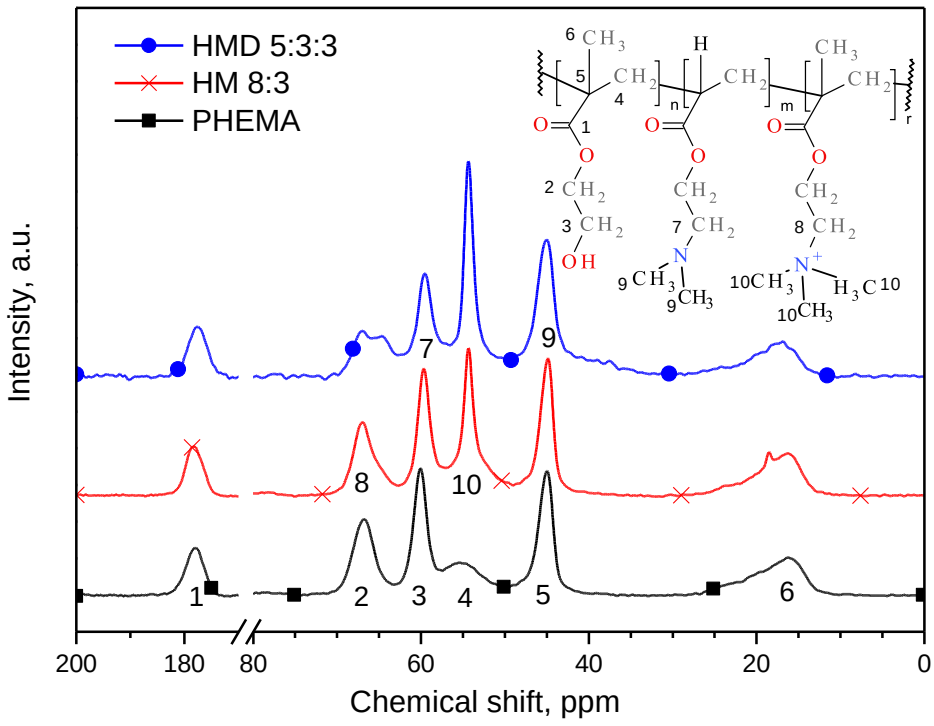

Figure S1. ^{13}C MAS-NMR spectra of PHEMA, HM 8:3 and HMD 5:3:3 hydrogels measured at room temperature, with the identification of the relative carbon atoms.

The solid state ^{13}C-NMR spectrum of HMD 5:3:3 showed several overlapping peaks, in particular the broad band at 65-70 ppm, and the resonance at 55 ppm. Therefore, in order to identify the respective carbon atoms and to quantify the relative amount of species present in the copolymer, NMR spectra of the HEMA homopolymer (PHEMA), as well as of the 8:3 molar ratio HEMA:METAC copolymer (HM 8:3) were recorded (Fig. S1). PHEMA NMR spectrum displayed the characteristic chemical shifts of the carbon atoms of the constituting repeat unit of the polymer: the signals of carbonyl groups at 178 ppm, the peaks of the pendant -CH_2- groups at 67 and 60 ppm, the weak signal of the chain -CH_2- at 55 ppm, the quaternary carbon resonance at 45 ppm, and the broad methyl peak at 16 ppm.[S6]

In the spectrum of HM 8:3, the peak of the nitrogen-bound methyl groups (C10) resonates at 54.5 ppm, hence it overlaps the signal of the -CH_2- on the backbone, while the signal of the methylene carbon vicinal to nitrogen at about 65 ppm, is convoluted with that of C2 of PHEMA.[S7]

In the case of HMD 5:3:3, further complications arise from the overlapping of DMAEA methyl (C10) and methylene (C7) groups at 45 and 60 ppm, respectively.[S8] Moreover, the backbone ternary carbon is not visible, as it is likely masked by the intense peak at 55 ppm.

Therefore, quantification of the comonomer relative amount in the ternary hydrogel was not trivial. Mathematical peak deconvolution was inconclusive as it gave no reliable results, hence a quantitative, though approximate estimation of the composition was obtained by comparing the values of the ratio of the area of saturated carbons (80 to 40 ppm) to that of methyls bound to the polymer backbone (30 to 10 ppm) for all the spectra (Fig. S2).

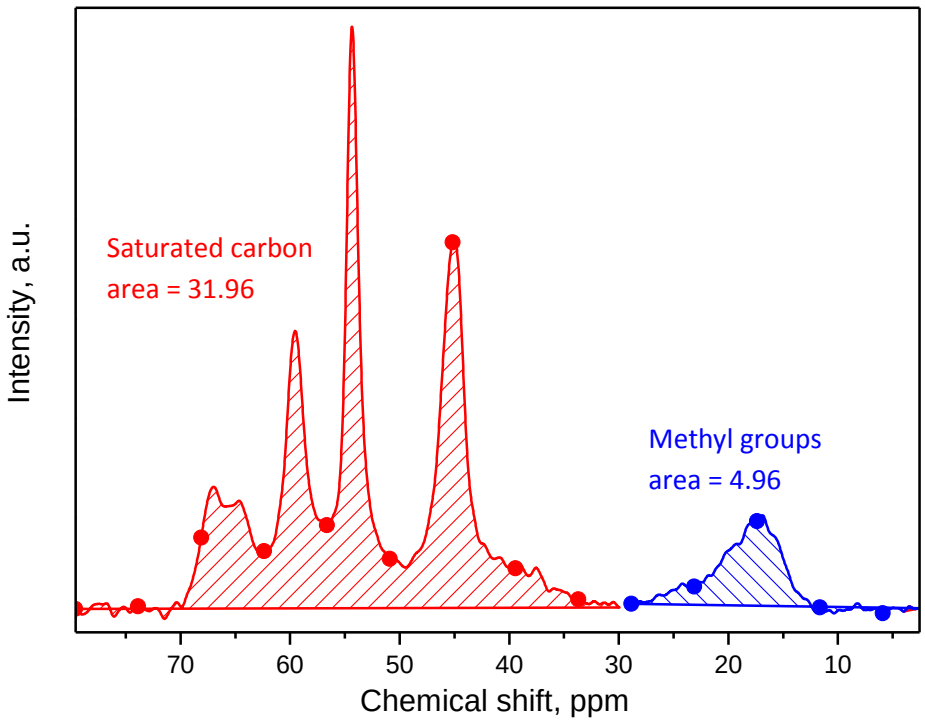

Figure S2. 80 to 10 ppm range of the ^{13}C MAS-NMR spectrum of HMD 5:3:3, with the identification of the integration regions and their relative values.

The obtained values were 3.94, 4.74, 6.44 for PHEMA, HM 8:3, and HMD 5:3:3, respectively.

HEMA and METAC mole fractions in HM 8:3 were determined using the following equations:

$1 = x_H + x_M$

$4.74 = 3.94\ x_H + 7\ x_M$

where x_H and x_M are the mole fractions of HEMA and METAC, respectively. Solution of the above equations yields a x_M value of 0.26, thus very close to the feed mole ratio.

Similar reasoning was made for HMD 5:3:3. In this case, the equations were:

$1 = x_H + x_M + x_D$

$6.44 = 3.94\ x_H + 7\ x_M + 6\ x_D$

where x_D is the mole fraction of DMAEA. Replacing x_M with 0.26 enabled to calculate the DMAEA mole fraction, which was found to be 0.17.

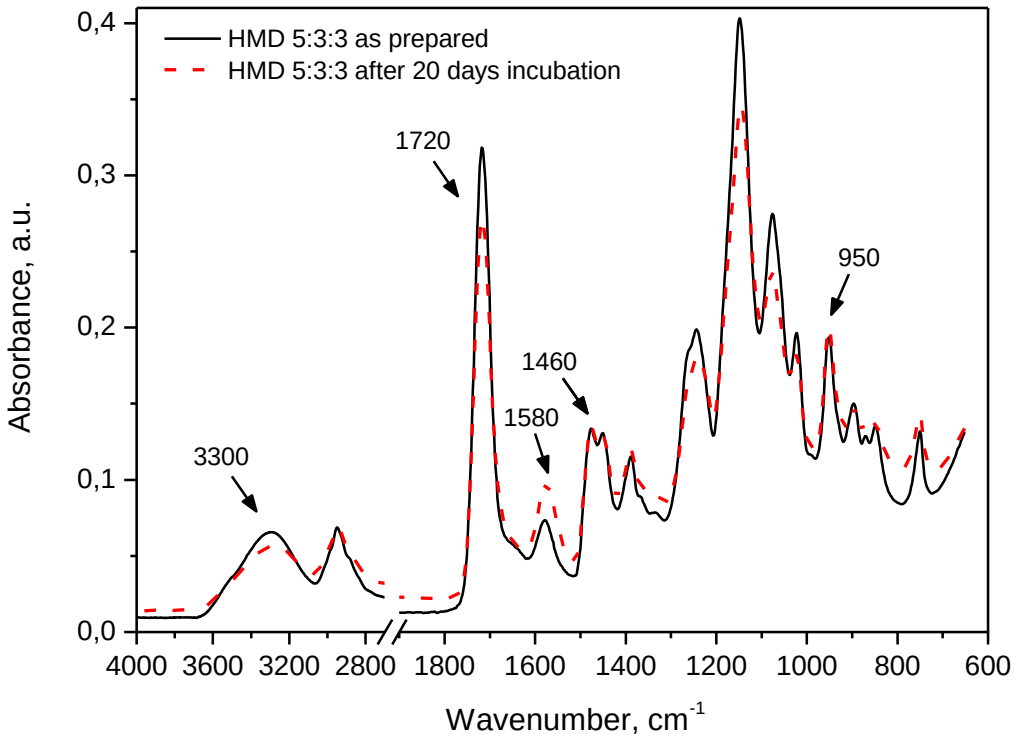

Figure S3. ATR-FTIR spectra of HMD 5:3:3 hydrogel before and after 20-day incubation in properly modified culture medium at 80 °C.

In the region of functional groups, the FTIR spectrum of HMD 5:3:3 (Fig. S3) shows the broad absorption band at 3300 cm^{-1} corresponding to O-H stretching of HEMA, and the convoluted peak at 2950 cm^{-1} corresponding to C–H stretching. The carbonyl absorption is peaked at 1720 cm^{-1}, while it was peaked at 1708 cm^{-1} for PHEMA. The carboxylate groups absorption is visible at 1580 cm^{-1}, while the presence of the amine-bearing comonomers is confirmed by the peaks at 1460 and 950 cm^{-1}.

References

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