SUPPORTING INFORMATION

Soft Micron-Sized Polypeptide Microgels: Preparation, Crosslink Density, Topography and Nanomechanics in Swollen State

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Characterization of microgel precursor

^{1}H NMR

^{1}H NMR spectra were recorded on a Bruker Avance III 600 spectrometer (Rheinstetten, Germany) equipped with a 5 mm diffusion probe-head. Measurement conditions were as follows: 90° pulse width 10 μs, acquisition time 5.45 s, spectral width 6002 Hz, relaxation delay 10 s, and 128 scans.
The amount of the functional groups (mmol/g) in the P2HPG-Tyr structure was calculated using Equation S1.

$$C = \frac{I_s / N_H}{I_{CH}} \times \frac{1}{M_{copolymer}} \qquad \text{(S1)}$$

where I_s is the area under the specific signal (2H of tyramine at 7.1 ppm or 2H of propargyl at 3.97 ppm), N_H is the number of hydrogen atoms related to the specific signal, I_{CH} is the area under the hydrogen signal assigned to the CH group in the backbone of the polymer and $M_{copolymer}$ is the molecular weight of the copolymer repeat unit was calculated using Equation S2:

$$M_{copolymer} = \sum \frac{I_i / N_{H,i}}{I_{CH}} \times M_i \qquad \text{(S2)},$$

where M_i is the molecular weight of the comonomers unit.

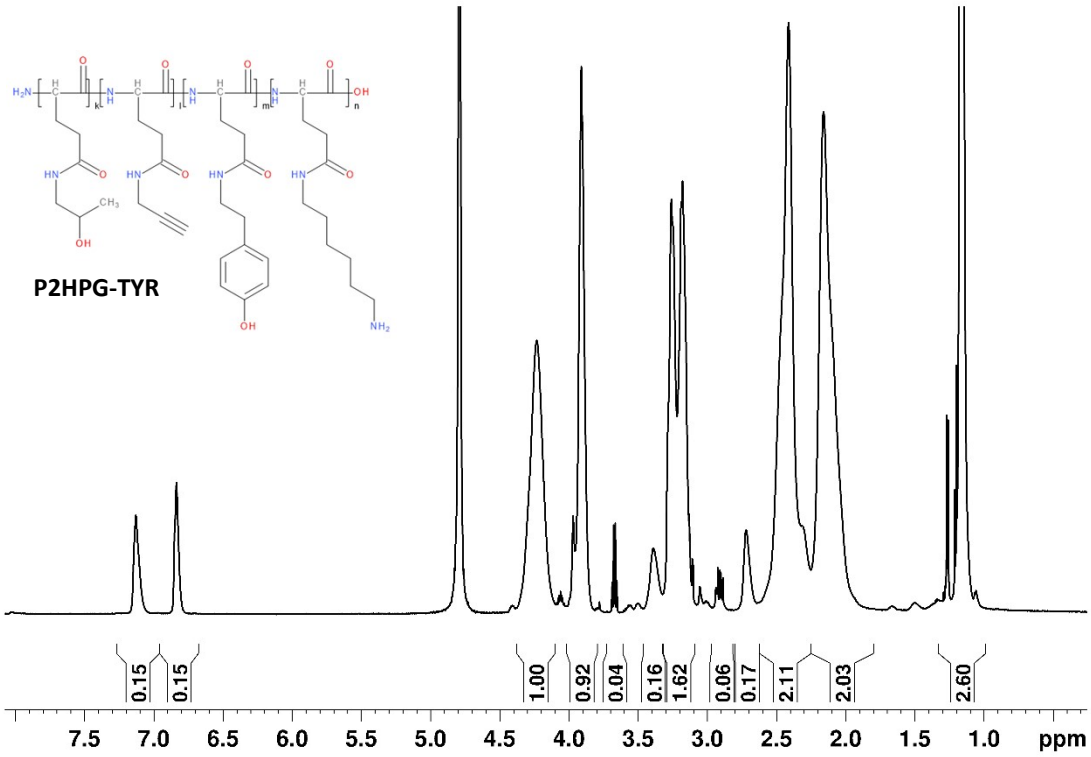

Figure S1: The structure of the copolymer P2HPG-Tyr was confirmed by high-resolution ^{1}H NMR Spectroscopy (600 MHz, 25 °C, D_2O) with the following assignment of hydrogen signals: 7.0 ppm (aromatic 2H), 6.7 ppm (aromatic 2H), 4,2 (1H, C<u>H</u>CH_2CH_2), 3.9 ppm (2H, C<u>H</u>$_2$C≡CH), 3.6 ppm (2H, OHCH_2C<u>H</u>$_2$), 3.3 ppm (2H, OHC<u>H</u>$_2$CH_2), 2.8-3.0 ppm (2H, CH_2 next to aromatic ring), 2.6 ppm (2H, CH_2C<u>H</u>$_2$NH), 2.4 ppm (2H, CHCH_2C<u>H</u>$_2$), and 1.9-2.2 ppm (2H, CHC<u>H</u>$_2$CH_2) (Figure S1). Note that even though most resonances have a multiplicity of a doublet or higher, due to the high molecular weight of the polymer, the signals are rather represented as broad singlets or doublets. Therefore, the multiplicity was not determined.

The concentration of tyramine and propargyl units was found using Equation S1 and was 0.69 and 0.14 mmol/g, respectively. The tyramine concentration was also confirmed using UV–Vis spectroscopy on a SPECORD 250 spectrophotometer (Jena Analytics, Germany) as a complementary method. The absorbance was measured in acetate buffer-MeOH (3:1) at λ_{max} = 275 nm using tyramine as the standard for calibration curve. The concentration of tyramine on the P2HPG-Tyr precursor obtained from the UV-Vis spectroscopy was 0.71 mmol/g.

Size exclusion chromatography (SEC)

The number-average molecular weights (M_n), weight-average molecular weights (M_w) and dispersities ($Đ$) of the polymer PBLG were measured using size exclusion chromatography (SEC) on a Watters system equipped with a Waters 410 differential refractometer and light scattering detector PD2020 (Precision detectors, USA). The PL-gel 1000 Å column and % formamide in THF as eluent at a flow rate of 1 ml/min were used.
The number-average molecular weights (M_n), weight-average molecular weights (M_w) and dispersities ($Đ$) of the water soluble PHEG-Tyr were measured using size exclusion chromatography (SEC) on a Shimadzu HPLC-SEC system equipped with UV detector

(Shimadzu), differential refractive index detector (Wyatt Optilab T-rEX), and multi-angle light scattering detector DAWN EOS (Wyatt Technology). The Superose 12 column and sodium acetate buffer eluent (pH 6.5) at a flow rate of 0.5 ml/min were used.

Determination of Microgels Crosslinks Density

For calculating the concentration of elastically active network chains noted as ν_e [moles of EANCs/cm^3 of a dry hydrogel network], we assume that each average chain of molecular weight $\bar{M}_n$ = 22,100 g/mol bears 12 tyramine units, i.e. the average length of chain between the two neighbour tyramines is approximately 22,100/12 ≅ 1842 g/mol, so this is a sufficiently long segment to form connecting elastic chain. When such chains connect branched nodes which are constituted by units with at least three bonds with connections to the infinite structure, they are called "elastically active". Further, we consider that the bond between the two tyramines formed by the peroxy-mediated reaction is short and provides rather a point-like connection of two aromatic rings, so the link between two tyramines do not contribute to the elasticity of the network and thus it is not counted within the number of EANCs. The tyramine dimer is considered a unit called "crosslink" with maximally four bonds connected with the hydrogel network. Indeed, the tyramine dimers are continuously formed during the crosslinking process and their number and functionality gradually increase. Each of the bonds issuing from the crosslinks represents ½ of an elastically active network chain: so, the concentration of EANC is equal to the expression: ½ × i × N_i, where i is the functionality of a crosslink > 2 (in our case i = 3 and 4), and N_i is the number of i-functional crosslinks in the chosen volume element. Then, the number of EANCs is calculated from hydrogel composition using the formula expressed by Eq. 6 in the main text reprinted for reader's convenience here:

$$\nu_e = \frac{1}{2}\frac{\sum_{i=1}^{i} i\,N_i}{\sum_{i=1}^{i} n_i M_i}\varrho \qquad (3)$$ – reprinted Eq. 6 of main text,

where n_i is a molar fraction of units of functionality i, M_i is the molar mass of the i-functional unit and Q is the specific gravity of the dry system [g/cm^3] that is considered 1.3.

Our other approach employed the statistical theory of branching processes (TBP) that offers the statistical description of topological properties of a system formed by the cross-linking of the multifunctional chains[1] such as our example. The explanation of both ways and comparison of all the resulting values are included in the main text.

To summarize, the agreement of the values of ν_e obtained from both calculations was satisfactory and supports the relevancy of the chosen approaches. It is also worth mentioning that the value of ν_e determined herein for the hydrogels corresponds well to crosslink density of moderately crosslinked synthetic hydrogels based on poly(2-hydroxyl methylmethacrylate) investigated previously.

The TBP is in general capable of providing other parameters of the crosslinking systems such as conversion in the gel point (critical conversion), fraction of structures in the elastically inactive part of the network (average size of dangling chains), development of the gel fraction during the crosslinking process (gel fraction vs. sol fraction as a unique functions of conversion) etc., however, here it serves as an example of an advanced treatment of the gelled system.

To answer the question whether all connections between the crosslinks are long enough to be elastically active, we have calculated the distrinbution of chain end-to-end distances for the case

of freely jointed chain model with varying number of freely rotating segments. The outcome is plotted in Figure S2. The Figure shows that in this model, already 3 jointed segments exert the maxima on the distribution function. Then, the chains composed of 4 and more segments behave all similarly displaying the maxima and their behavior is very close to that of chains composed of an infinite number of segments. The details of approach to this calculation can be found in the book [2].

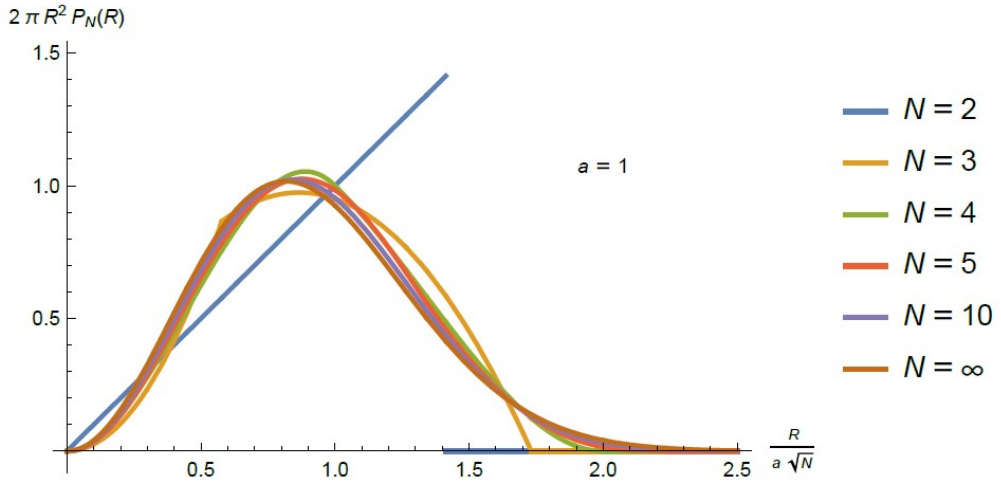

Figure S2. Chain end-to-end distance distribution function calculated for freely jointed chain for varying number of segments N constituting the chain. The details of calculation in [2] y-axis: the end-to-end distance intensity parameter, x-axis: the end-to-end distance.

Table SI shows that the molar fraction of chains up to 2 segments is 0.012 (i.e. the portion of "short, likely elastically inactive" chains is less than 1.2 molar %. Thus, the chains since their $N \geq 3$, if one copolymerized unit is considered a sufficiently long single (stiff) segment, constitute more that 98 molar % of all network chains. In other words, the 98% of network chains are "entropically" elastically active.

Table SI. The molar fraction of segments between crosslinks for the average length of a connecting chain 8 as it corresponds to the P2HPG-Tyr networks.

number of segments in chains between crosslinks, N	molar fraction of chains of length N in network
0	0.000250829
1	0.00216505
2	0.0092536
3	0.0261106
4	0.0547119
5	0.0908026
6	0.124323
7	0.14442
8	0.145291
9	0.128581
10	0.101342

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