## Supplementary Materials

# The Role of Crosslinker Content of Positively Charged NIPAM Nanogels on the In Vivo Toxicity in Zebrafish

**Roberta Bilardo [1], Federico Traldi [1], Caroline H. Brennan [2] and Marina Resmini [1,*]**

[1] Department of Chemistry, School of Physical and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK; r.bilardo@qmul.ac.uk (R.B.); f.traldi@qmul.ac.uk (F.T.)
[2] School of Biological and Behavioural Sciences, Queen Mary University of London, London E1 4NS, UK; c.h.brennan@qmul.ac.uk
* Correspondence: m.resmini@qmul.ac.uk

**Table S1.** Chemical composition, monomer conversions ([1]H NMR) and chemical yields of positively charged NIPAM-based nanogels, containing 10 mol% *t*-BAEMA as functional monomer, before the optimization of the polymerization conditions. All nanogels were synthesized with MBA as the crosslinker in relative concentrations ranging between 5 – 20 mol%. For all the formulations, the initiator (AIBN) was 1% of the total moles of double bonds in the mixture, while the total monomer concentration ($C_M$) used was kept equal to 1 or 2%.

| Nanogel | Composition (mol%) | | | $C_M$ (%) | Monomer Conversion (%) | | | | Yield (%) |
|---|---|---|---|---|---|---|---|---|---|
| | NIPAM | MBA | t-BAEMA | | NIPAM | MBA | *t*-BAEMA | Overall | |
| $NG_{20}$-tBAEMA-1 | 70 | 20 | 10 | 1 | 67 | 88 | 97 | 75 | 64 |
| $NG_{10}$-tBAEMA-1 | 80 | 10 | 10 | 1 | 60 | 75 | 98 | 65 | 48 |
| $NG_5$-tBAEMA-1 | 85 | 5 | 10 | 1 | 55 | 68 | 97 | 59 | 29 |
| $NG_{20}$-tBAEMA-2 | 70 | 20 | 10 | 2 | 79 | 95 | 99 | 84 | 79 |
| $NG_{10}$-tBAEMA-2 | 80 | 10 | 10 | 2 | 74 | 91 | 99 | 79 | 62 |
| $NG_5$-tBAEMA-2 | 85 | 5 | 10 | 2 | 70 | 83 | 99 | 74 | 50 |

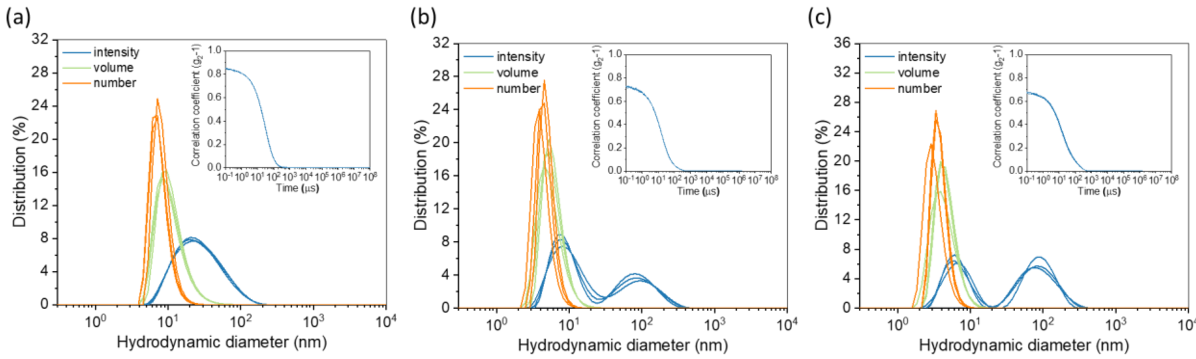



**Figure S1.** Triplicate DLS measurements of neutral nanogels, with crosslinker content between 5 and 20 mol%: **(a)** $NG_{20}$-1; **(b)** $NG_{10}$-1; and **(c)** $NG_{5}$-1 in deionized water (1 mg/mL, 25°C) by intensity (blue), volume (green) and number (orange) distributions. Inserts show correlogram of each triplicate measurement. The presence of a single peak by volume and number distributions suggests that the second population, observed by intensity for $NG_{10}$-1and $NG_{5}$-1, represents a negligible fraction of the samples.

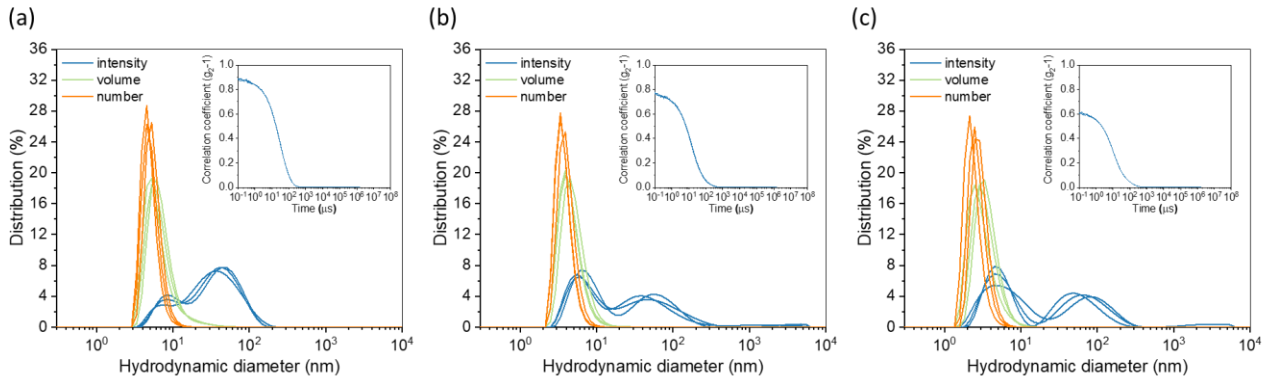



**Figure S2.** Triplicate DLS measurements of *t*-BAEMA nanogels, with crosslinker content between 5 and 20 mol%: **(a)** $NG_{20}$-tBAEMA-3; **(b)** $NG_{10}$- tBAEMA-3; and **(c)** $NG_{5}$- tBAEMA-3 in deionized water (1 mg/mL, 25°C) by intensity (blue), volume (green) and number (orange) distributions. Inserts show correlogram of each triplicate measurement. The presence of a single peak by volume and number distributions suggests that the second population, observed by intensity, represents a negligible fraction of the samples.

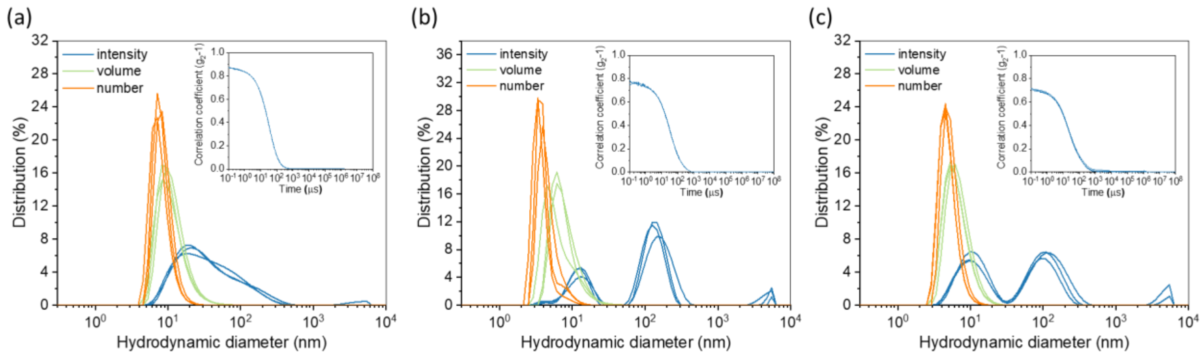



**Figure S3.** Triplicate DLS measurements of neutral nanogels, with crosslinker content between 5 and 20 mol%: **(a)** $NG_{20}$-1; **(b)** $NG_{10}$-1; and **(c)** $NG_{5}$-1 in fish water with pH = 7 (1 mg/mL, 25°C) by intensity (blue), volume (green) and number (orange) distributions. Inserts show correlogram of each triplicate measurement. The presence of a single peak by volume and number distributions suggests that the second population, observed by intensity, represents a negligible fraction of the samples.

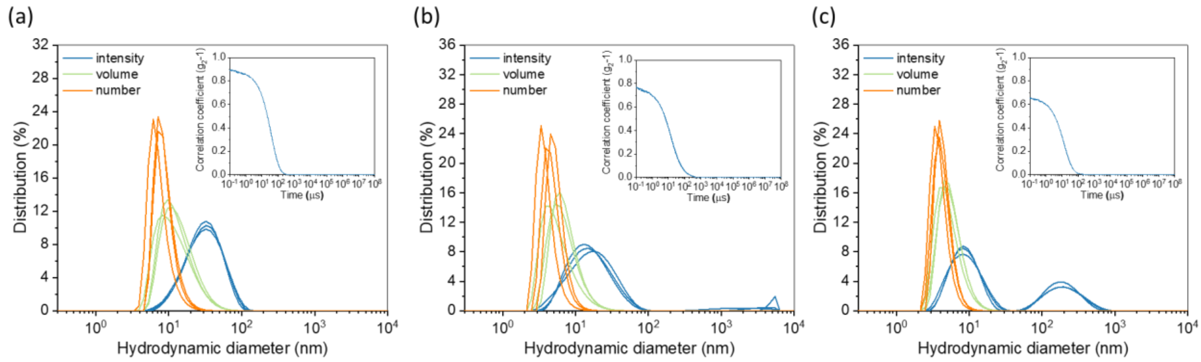
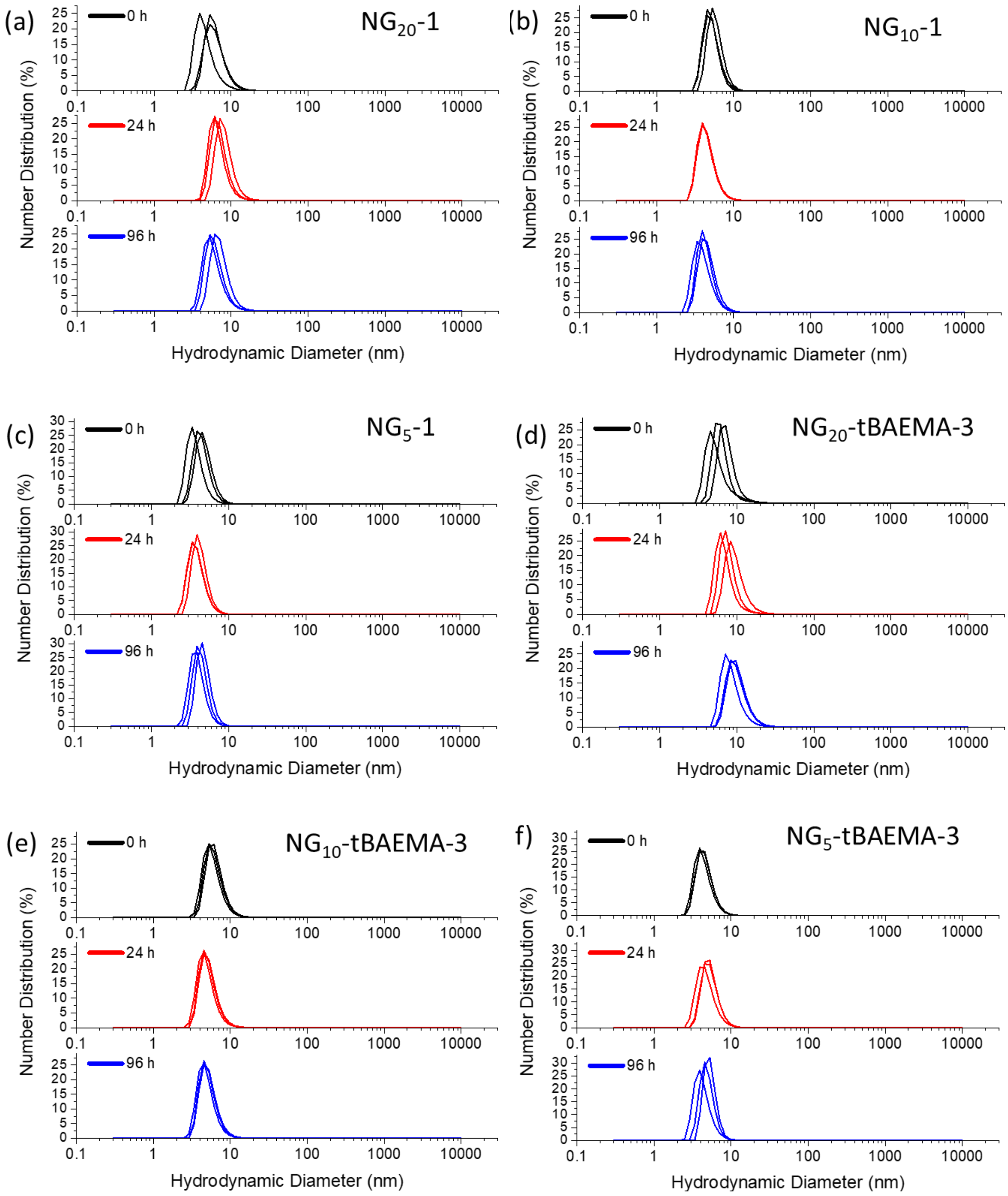



**Figure S4.** Triplicate DLS measurements of *t*-BAEMA nanogels, with crosslinker content between 5 and 20 mol%: **(a)** $NG_{20}$-tBAEMA-3; **(b)** $NG_{10}$- tBAEMA-3; and **(c)** $NG_{5}$- tBAEMA-3 in fish water with pH = 7 (1 mg/mL, 25°C) by intensity (blue), volume (green) and number (orange) distributions. Inserts show correlogram of each triplicate measurement. The presence of a single peak by volume and number distributions suggests that the second population, observed by intensity for $NG_{5}$-tBAEMA-3, represents a negligible fraction of the samples.



(a)
NG$_{20}$-1
(b)
NG$_{10}$-1
(c)
NG$_{5}$-1
(d)
NG$_{20}$-tBAEMA-3
(e)
NG$_{10}$-tBAEMA-3
f)
NG$_{5}$-tBAEMA-3
0 h
24 h
96 h
Number Distribution (%)
Hydrodynamic Diameter (nm)
0.1
1
10
100
1000
10000

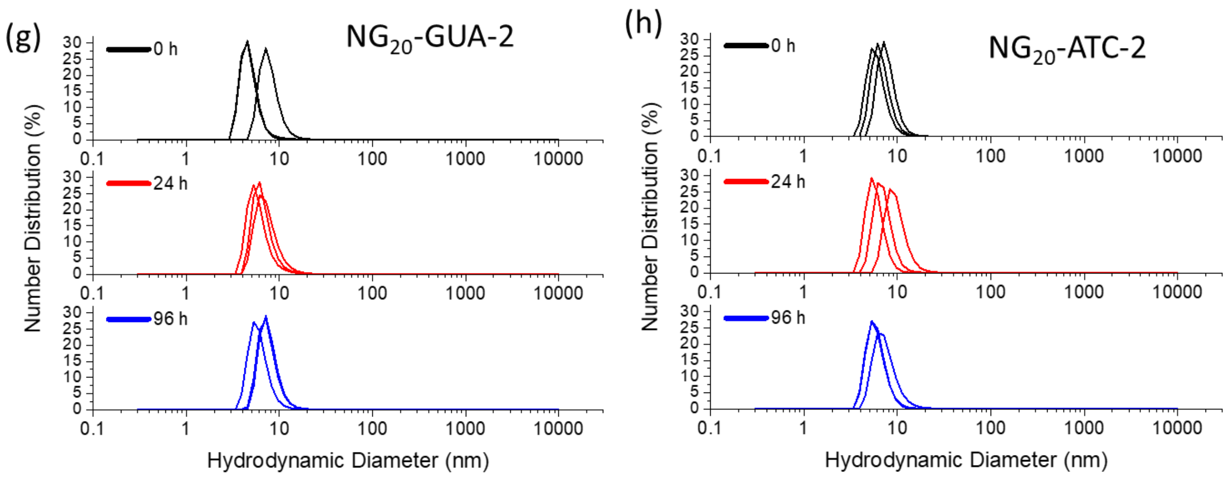



**Figure S5.** Colloidal stability studies of nanogels in fish water (pH = 7) at 28 °C (0.5 mg/mL). Triplicate DLS measurements (number distribution) of **(a)** $NG_{20}$-1; **(b)** $NG_{10}$-1; and **(c)** $NG_{5}$-1 **(d)** $NG_{20}$-tBAEMA-3; **(e)** $NG_{10}$- tBAEMA-3; and **(f)** $NG_{5}$- tBAEMA-3; **(g)** $NG_{20}$-GUA-2; **(h)** $NG_{10}$- ATC-2 were obtained after 0 (black), 24 (red), and 96 (blue) hours after reconstitution of the dry nanogel powders. Data show that nanogels are colloidal stable within the time frame of the *in vivo* toxicity studies.

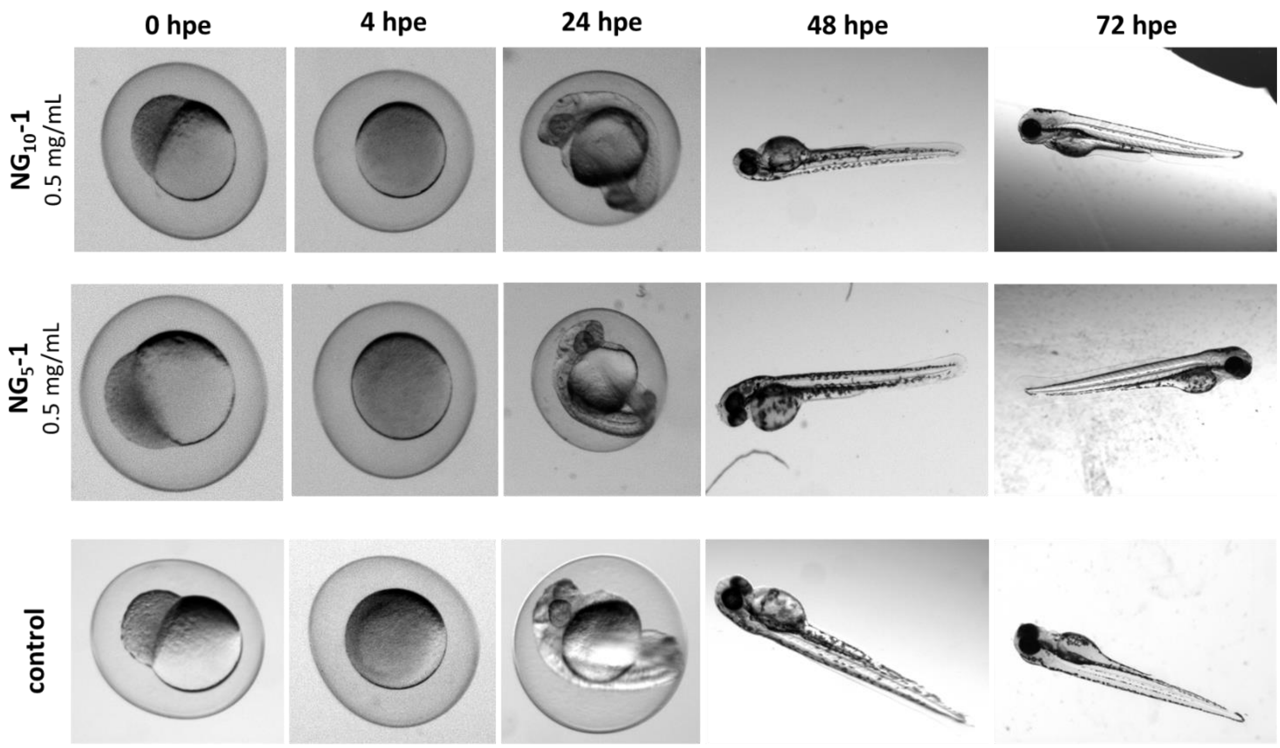



**Figure S6.** Stereomicroscopy assessment of the biocompatibility of neutral nanogels $NG_{10}$-1 (10 mol% crosslinker, first row) and $NG_5$-1 (5 mol% crosslinker, second row), in zebrafish up to 72 hours post exposure (hpe). The morphology and development of the embryos/larvae, exposed by immersion in nanogel solutions in fish water (concentration 0.5 mg/mL), was monitored at 0, 4 24, 48 and 72 hours after the administration and compared to non-exposed embryos/larvae used as negative control. These data are representative of the biocompatibility of neutral nanogels also at lower concentrations, *i.e.* 0.3 mg/mL and 0.1 mg/mL.

**Table S2.** Chemical composition, monomer conversions ([1]H NMR) and chemical yields of positively charged NIPAM-based nanogels, containing 10 mol% of either *t*-BAEMA, or GUA, or ATC as functional monomer. All nanogels were synthesized keeping the crosslinker (MBA) content equal to 20 mol%. For each formulation, the optimization of the polymerizations led to a concentration of the initiator (AIBN) and a total monomer concentration ($C_M$) between 1 and 2 mol% (of the total moles of double bonds in the mixture) and between 2 and 3%, respectively. The individual monomer conversion of ATC could not be determined because of the overlapping of its peaks with the ones of MBA.

| Nanogel | Composition (mol%) | | | AIBN (mol%) | $C_M$ (%) | Monomer Conversion (%) | | | | Yield (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | NIPAM | MBA | Co-monomer | | | NIPAM | MBA | Co-monomer | Overall | |
| | | | t-BAEMA | | | | | t-BAEMA | | |
| $NG_{20}$-tBAEMA-3 | 70 | 20 | 10 | 1 | 3 | 90 | 98 | 99 | 92 | 77 |
| | | | GUA | | | | | GUA | | |
| $NG_{20}$-GUA-2 | 70 | 20 | 10 | 2 | 2 | 89 | 97 | 94 | 91 | 60 |
| | | | ATC | | | | | ATC | | |
| $NG_{20}$-ATC-2 | 70 | 20 | 10 | 2 | 2 | 86 | 97 | - | 89 | 54 |

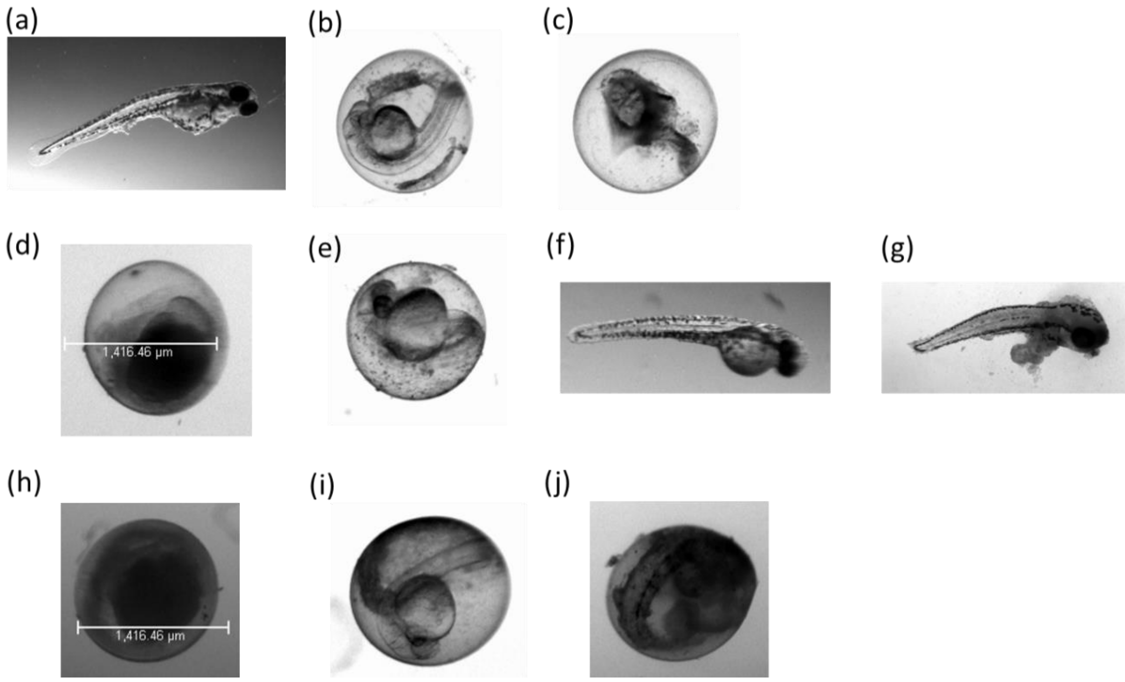



**Figure S7.** Examples of embryos/larvae dead after the immersion into nanogels solutions, for different times. In particular, the reported pictures show embryos/larvae exposed to : **(a)** $NG_{20}$-tBAEMA-3 0.1 mg/mL for 96 h; **(b)** $NG_{10}$-tBAEMA-3 0.1 mg/mL for 24 h; **(c)** $NG_{5}$-tBAEMA-3 0.1 mg/mL for 24 h; **(d)** $NG_{20}$-ATC-2 0.1 mg/mL for 4 h; **(e)** $NG_{20}$-ATC-2 0.1 mg/mL for 24 h; **(f)** $NG_{20}$-ATC-2 0.05 mg/mL for 48 h; **(g)** $NG_{20}$-ATC-2 0.03 mg/mL for 72 h; **(h)** $NG_{20}$-GUA-2 0.1 mg/mL for 4 h; **(i)** $NG_{20}$-GUA-2 0.1 mg/mL for 24 h; **(j)** $NG_{20}$-GUA-2 0.05 mg/mL for 48 h. Panels **(d)**, **(g)**, **(h)** and **(j)** were found to be necrotic, while the other panels show embryos/larvae with normal morphology but appearing to be disintegrating, as common after the death.

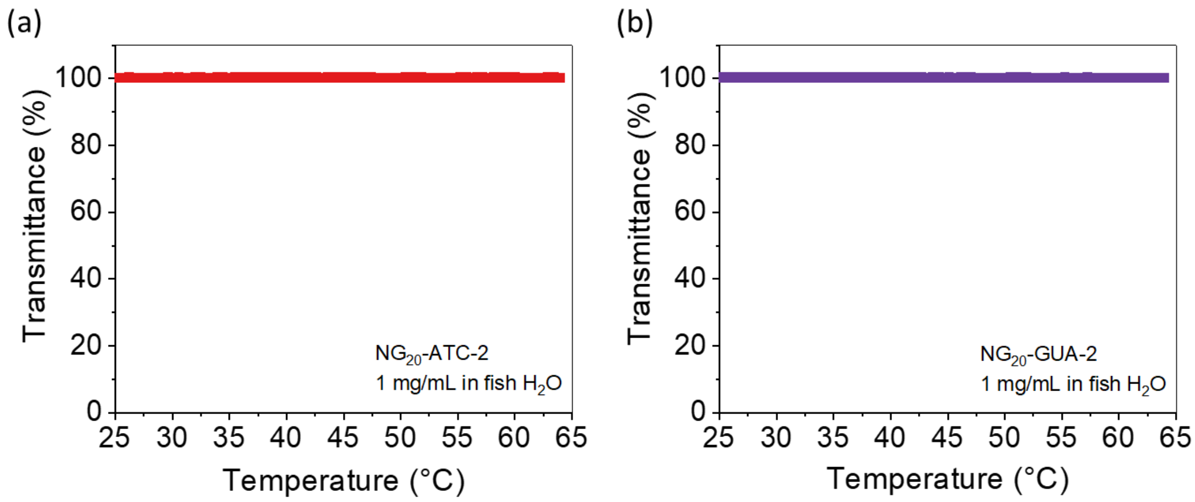



**Figure S8.** UV-visible spectroscopy study of the thermo responsive properties of the positively charged nanogels, containing 20 mol% of crosslinker, **(a)** $NG_{20}$-ATC-2 and **(b)** $NG_{20}$-GUA-2 in fish water (concentration 1 mg/mL), in a temperature range between 25 and 65°C.

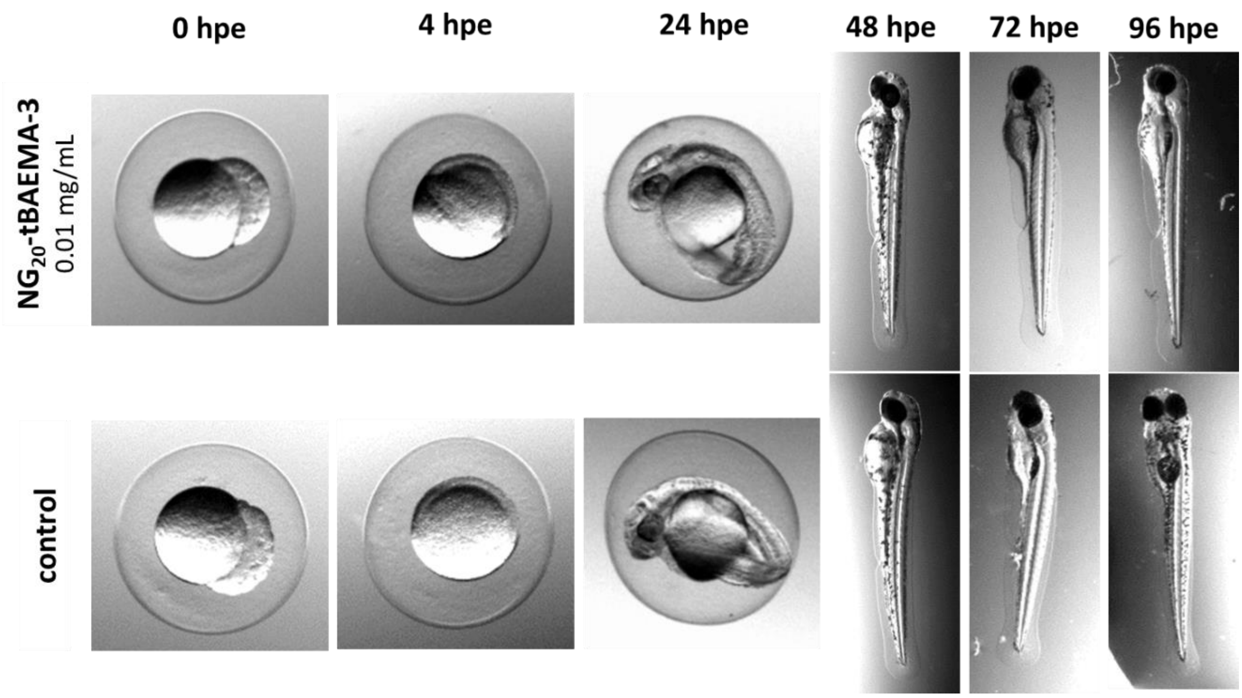



**Figure S9.** Stereomicroscopy assessment of the biocompatibility of the positively charged nanogel $NG_{20}$-tBAEMA-3 (20 mol% crosslinker, and 10 mol% of *t*-BAEMA), in zebrafish up to 96 hours post exposure (hpe). The morphology and development of the embryos/larvae, exposed by immersion in nanogel solutions in fish water (concentration 0.01 mg/mL), was monitored at 0, 4 24, 48, 72 and 96 hours after the administration and compared to non-exposed embryos/larvae used as negative control.