The Potential Health and Environmental Risks of 3D-engineered Polymers

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Supplementary Method 1: Collection of leachates from the plastics for toxicological assays. Cell culture medium and ultrapure water at (23^{o}C and 18.2MΩ.cm; Milli-Q water; Millipore, Darmstadt, Germany) were conditioned for 24 h with routinely detoxified PIC100, E-Shell200, E-Shell300, PDMS, and PS structures in a humidified atmosphere of 5% CO_2 and 5% O_2 at 38.5^{o}C. A ratio of 2.5 mL of media/water per cm^2 of polymer structure was used and the dimensions of the polymers were 1 cm in diameter and 0.2 cm in thickness. As a control, 2.5 mL milliQ water or cell culture medium was subjected to the same procedure to act as a negative control. The resulting polymer conditioned MilliQ water or media and their negative controls were kept at -20^{o}C until use in one of the assays.

Reference mass spectrum of diethyl-phthalate. Diethyl-phthalate (1 µM end concentration) was first solubilized 1:50 in distilled ethanol and subsequently diluted 1:1 in MilliQ water and further diluted to the end concentration in MilliQ water. This solution was injected directly into the ESI mass spectrometer as described in the materials and methods (Figure S1). The exclusive presence of the $[M+H]^+$ peak (223.10 m/z) and the fragment ion (177.06 m/z) as well as the relative abundance of both correspond to those measured in Figure 2. The satellite peaks on the right are C^{13} isotope peaks (224.10 and 178.06 m/z respectively). Note that we have made a series of dilutions of diethyl-phthalate in MilliQ water (10^{-3} to 10^{-13} M) and with this titration curve we have quantified the amount of released diethyl-phthalate from the diverse plastics as is indicated in Table SI.

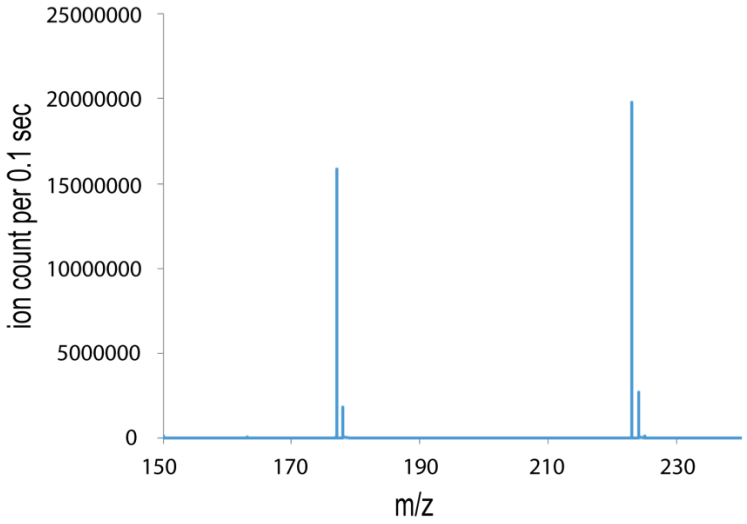

Figure S1. ESI output of Diethyl-phthalate. Note the exclusive presence of the $[M+H]^+$ peak (223.10 m/z) and the fragment ion (177.06 m/z) as well as the satellite peaks on the right, which correspond to C^{13} isotope peaks (224.10 and 178.06 m/z respectively).

Table SI. Effect of conditioning of IVM medium with PIC100 structures on oocyte maturation. Data is present as percentage of total oocytes put in maturation.

Oocyte stage	Control (%)	PIC100 conditioned medium (%)
Degenerated	4.2*	30.8**
Not mature	19.1[1]	21.6[2]
Mature	76.6[#]	47.6[##]

Different symbols and number signs indicate statistically significant differences ($p<0.05$).

Isolation of oviduct cells and long-term oviduct cell culture

Cow oviducts were collected from a local abattoir immediately after slaughter and transported to the laboratory on ice. The oviducts were dissected free of surrounding tissue and washed three times in cold PBS supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin. BOECs were isolated by squeezing the total oviduct content out of the ampullary end of the oviducts, and collected in HEPES buffered Medium 199 supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were washed twice by centrifuging at 500 x g for 10 min at 25^{o}C in HEPES buffered Medium 199 supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin. Next, cells were cultured for 24 h in HEPES buffered Medium 199 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal calf serum (FCS; Bovogen Biologicals, Melbourne, Australia). During these 24 h, the cells arranged themselves into floating vesicles with outward facing actively beating cilia; these vesicles were collected, centrifuged at 500 x g for 10 min at 25^{o}C, resuspended in DMEM/Ham's F12 medium (DMEM/F-12 Glutamax I, Gibco BRL, Paisley, U.K.) supplemented with 1.4 mM hydrocortisone, 5 mg/mL insulin, 10 mg/mL transferrin, 2.7 mM epinephrine, 9.7 nM tri-iodothyronine, 0.5 ng/mL epidermal growth factor, 50 nM trans-retinoic acid, 2% bovine pituitary extract (containing 14 mg/mL protein), 1.5 mg/mL fatty acid free bovine serum albumin (BSA, a6003, Sigma Chemical Co., St. Louis, MO), 100 mg/mL gentamycin, and 2.5 mg/mL amphotericin B (3D culture medium), and pipetted up and down several times to mechanically separate the cells. Next, cells were seeded into a transwell device (Corning Inc, NY, USA; 3D culture; 0.6 x 10^6 cells/cm^2). Cells were cultured in 3D culture medium in a humidified atmosphere of 5% CO_2-in-air at 38.5^{o}C until they reached confluence (5-7 days). Thereafter, an air-liquid interface was established by removing the medium in the apical compartment for up to 42 days in a humidified atmosphere of 5% CO_2-in-air at 38.5^{o}C. The basolateral medium was

completely refreshed with 3D culture medium twice a week. A total of six animals and 8 transwells for each animal were used for 3D culture.

In vitro fertilization (IVF) and culture (IVC) using 3D cultured BOECs

Fertilization medium and culture media (synthetic oviductal medium; SOF medium) were conditioned for 24 h with routinely detoxified PIC100 structures, and compared to control media that have not been exposed to PIC100 structures. At day 28 of BOEC culture the basolateral 3D medium was replaced by fertilization medium (conditioned and not conditioned) and a total of 50 in vitro matured COCs were added to the apical compartment (with 500 µL of fertilization medium not conditioned, but supplemented with 10 µg/ml heparin, 20 µM d-penicillamine, 10 µM hypotaurine, and 1 µM epinephrine) of each of the 3D culture transwells (n=8 transwells; 35-50 COCs per insert). Sperm was also added to a final concentration of 10^6 sperm cells/mL.

After 20-22 h of co-incubation under a humidified atmosphere of 5% CO_2-in-air at 38.5^{o}C, cumulus cells were removed by pipetting, the presumptive zygotes were cultured in the apical compartment (with 500 µL of SOF medium not conditioned; 35-50 zygotes per insert) and the basolateral fertilization medium (conditioned and not conditioned) was replaced by SOF medium (conditioned and not conditioned). The embryos were cultured for eight days under a humidified atmosphere of 5% CO_2 and 5% O_2 at 38.5^{o}C. At day 5 post-fertilization all embryos were transferred to fresh SOF medium.

Similar IVF and IVC procedures were performed in transwell without BOECs as a control group. Three replicates for both control and 3D culture groups (with conditioned and not conditioned medium) were performed. A total of 380 COCs in conditioned medium group and 396 COCs in control non-conditioned medium group were fertilized and further cultured in the same conditioned versus non-conditioned media in the 3D culture transwell, respectively. In parallel a

total of 376 COCs were fertilized in conditioned medium group and 380 COCs in non-conditioned medium group and their further embryo development was followed in the absence of 3D cultured BOECs. Results are presented in Figure S2.

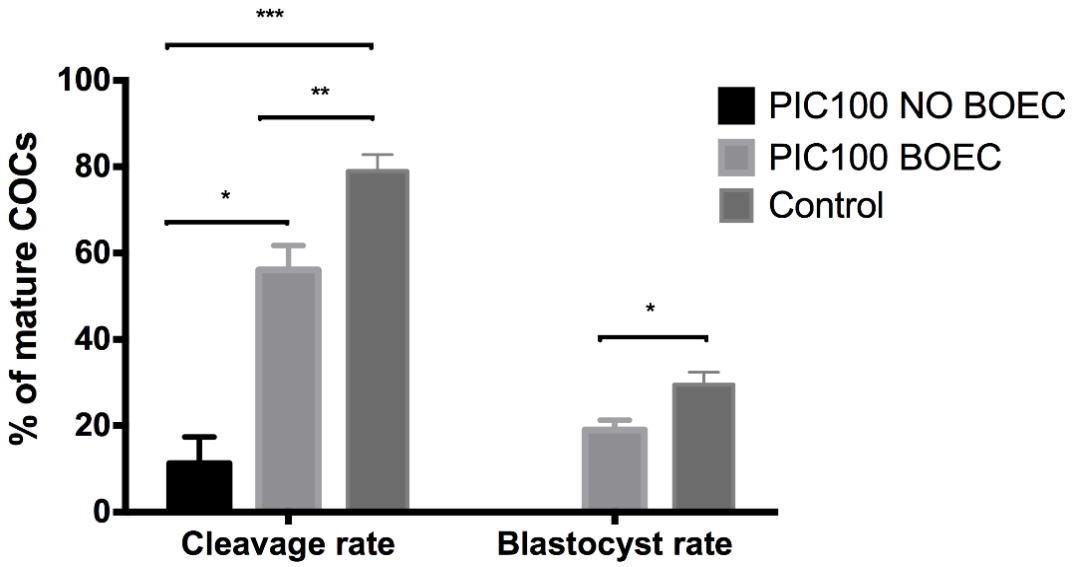

Figure S2. Cleavage and blastocyst rates are shown as percentage of cleaved embryos and blastocysts, respectively, of the total mature cumulus oocyte complexes (COCs) used for *in vitro* fertilization (IVF). IVF and *in vitro* culture (IVC) were performed using medium conditioned with PIC100 material in absence or presence of a bovine oviductal epithelial cell culture (PIC100 NO BOEC and PIC100 BOEC, respectively). Standard IVF and IVC were also performed without conditioned medium (Control). The cleavage rate was reduced for both PIC100 BOEC and PIC100 NO BOEC compared to the control group ($p<0.05$). A reduction in blastocyst rate was observed for PIC100 BOEC, compared to the control group ($p<0.05$) and blastocyst formation was completely blocked in PIC100 NO BOEC. Different symbols indicate statistically significant differences ($p<0.05$).

MS analysis for identification of compounds leached from the plastics. MilliQ water (negative control) and MilliQ water exposed to plastic materials were processed for MALDI-MS detection of any compounds leached from the plastics. To this end, the samples were first dried in a vacuum and the resulting dried powders were then solubilized in 20 µL of a 1:1 methanol:tetrahydrofuran mixture. For each analysis, 0.5 µL of this solution was mixed with 0.5 µL of dihydrobenzoic acid matrix in solution at 10 mg/mL in methanol with 0.1% formic acid, and this mixture was deposited on a MALDI polished steel target, and left to dry and crystallize. MALDI-MS analysis was performed using a Voyager-DE™ PRO Biospectrometry Workstation (Applied Biosystems) controlled by Voyager Control Panel Software (Foster City, CA, USA). Analyses were conducted in the positive linear mode using a nitrogen laser (337 nm) for ionization and Data Explorer 4.0 software from Applied Biosystems. Results are presented in Figure S3.

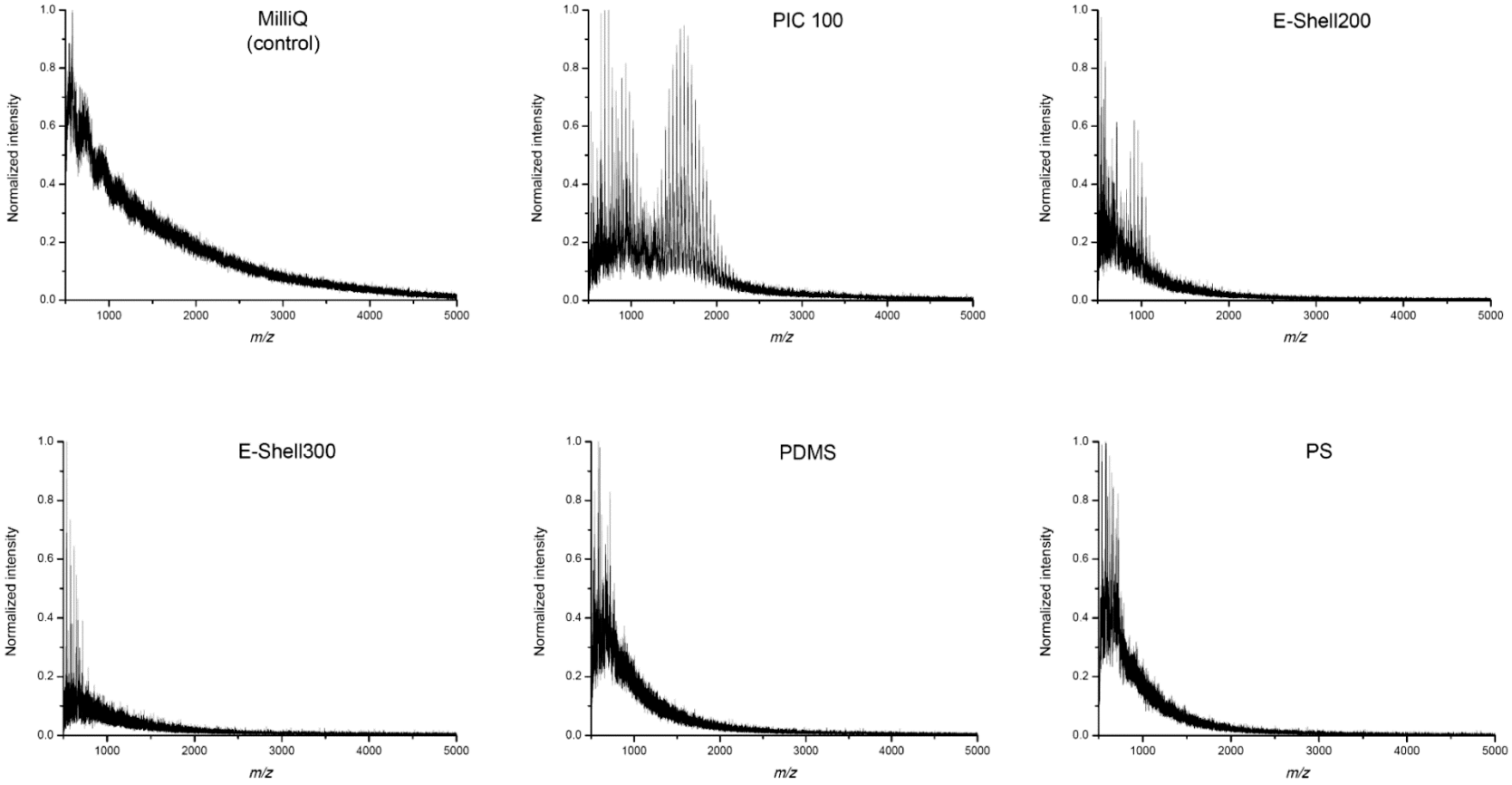

Figure S3. MALDI analysis of the leachate samples: MilliQ water only, and milliQ water conditioned by PIC100, E-Shell200, E-Shell 300, PDMS and PS. Clusters of peaks separated by 44 *m/z* were indicative of the release of diverse polyethylene glycols into MilliQ water.

***In vitro* fertilization (IVF) and culture (IVC) in the presence of DEP.** A >99% pure fluid of diethyl-phthalate was dissolved into DMSO (stock solution of 200 µM) and diluted 1:2000 to IVF and IVC media as described above (final concentration of diethyl-phthalate was 100 nM). The developmental progress of IVC was compared to 1:2000 DMSO additions with 0 nM diethyl-phthalate as negative control). Three independent experiments were performed with in 144, 155 and 299 COCs for the DMSO control and 146, 153 and 289 COCs for the 100 nM in DMSO condition, respectively. Results are presented in Figure S4.

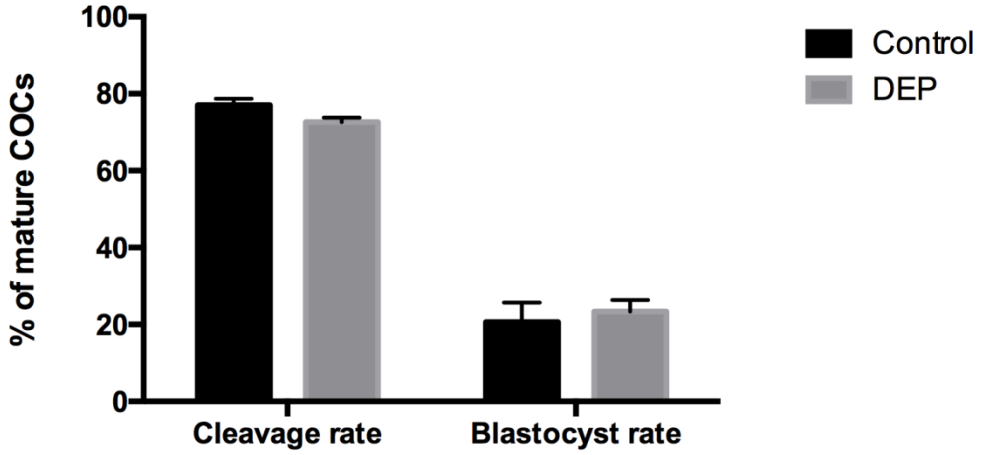

Figure S4. Bovine cleaved embryo and blastocyst production efficiency are displayed as percentages of the total number of mature cumulus oocyte complexes (COCs) submitted for *in vitro* fertilization (IVF). IVF and *in vitro* culture (IVC) were performed in the presence of 100 nM of diethyl-phthalate (DEP). Standard IVF and IVC were also performed without DEP (Control).