Functional Nanoparticles

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Biocompatible Functionalized Polyglycerol Microgels with Cell Penetrating Properties**

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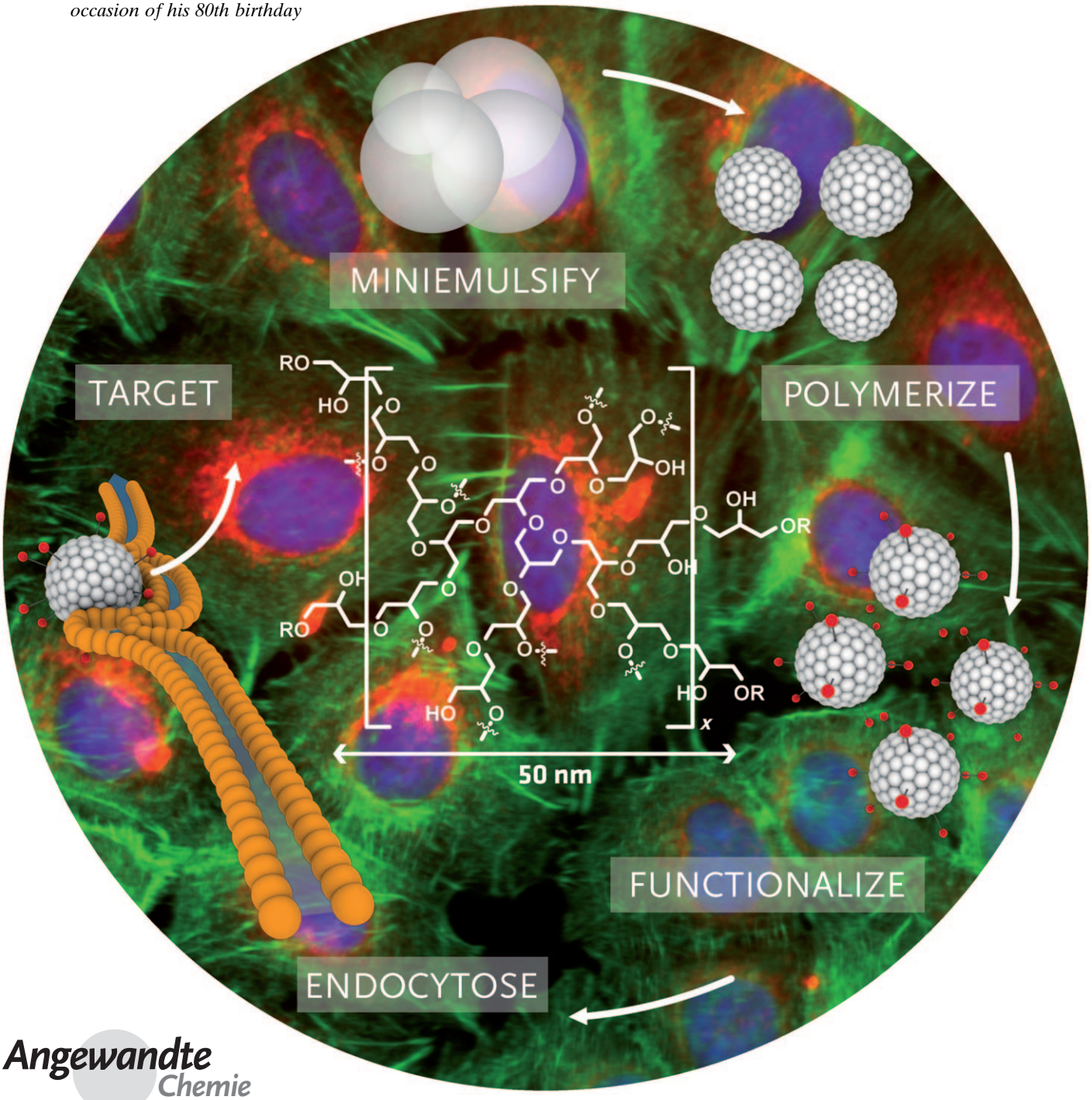
Dedicated to Professor Helmut Ringsdorf on the occasion of his 80th birthday

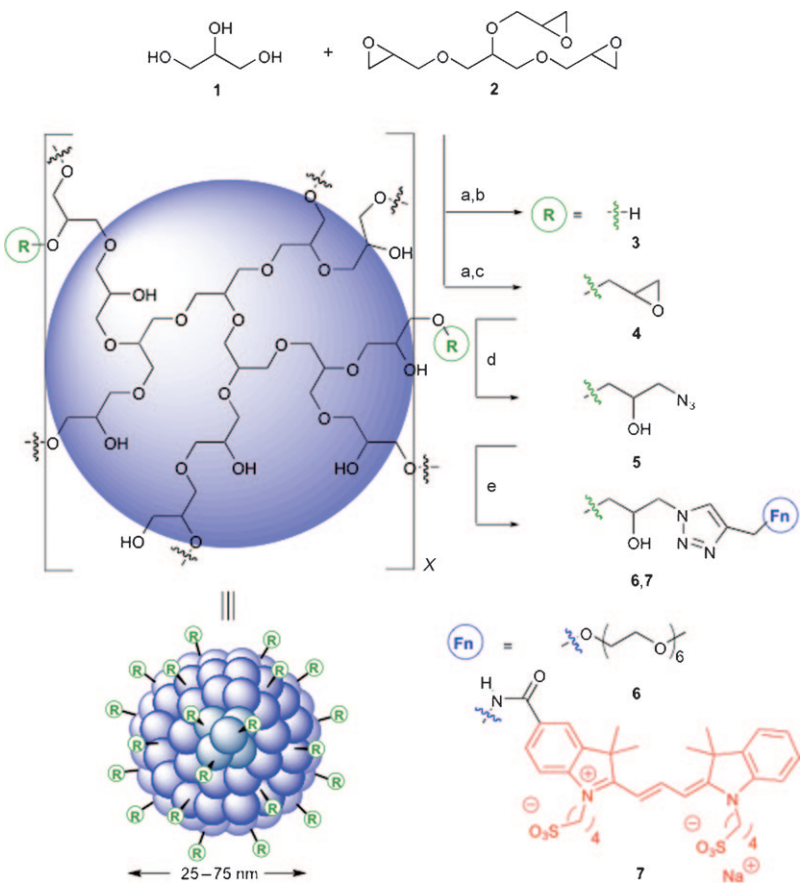


Dendritic architectures, being globular structures with low viscosity and dense surface functionality, have become well established in chemical and biomedical applications.[1] This description encompasses not only the monodisperse yet tediously synthesized perfect dendrimers, but also hyperbranched polymers which are typically obtained in one step and retain the favorable characteristics of perfect dendrimers.[2] Hyperbranched polyglycerols (HPG),[3] make up one such class of "dendrimer-like" polymers, traditionally synthesized by ring-opening polymerization of glycidol (3-hydroxypropylene oxide).[4] HPGs exhibit good biocompatibility and have been synthesized up to $M_n \approx 1$ MDa (diameter ≈ 10 nm) with narrow polydispersity and good solubility in polar solvents.[5] Macrogels based around HPGs have been developed for drug-delivery and tissue-engineering purposes.[6] However, efficient routes to produce biocompatible microgels in the "nano" size range (10–200 nm) are currently underdeveloped.[7,8] Microgels are a special case of high molecular weight polymers with characteristics of both hyperbranched and macroscopically cross-linked materials, and are formed when polymerization is carried out in an enclosed volume (emulsion, micelle etc.).[8a] Microgels can be dissolved somewhat analogously to linear polymers but because of sporadic cross-linking the conformation is almost fixed. Cross-linked poly *N*-isopropylacrylamides (pNIPAMs) which form microgels have been intensely studied for stimuli-responsive physical behavior, as novel materials, and as drug-delivery agents.

Based upon insights into the mechanisms of nanoparticle endocytosis,[9] we hypothesized that polyglycerol microgels (PG-μ-gels) of optimum size may be efficiently and non-disruptively taken up into cells, making them attractive scaffolds for drug-delivery agents and for probing biological phenomena. Particles with diameters in the range of 25–50 nm would be most desirable according to previous studies. Furthermore, particles in this size range passively accumulate in disease-distressed tissues by the so-called 'enhanced permeation and retention' effect.[10]

Among the methods available to prepare particles in the required size range, polymerization within miniemulsion was considered most attractive for our purpose. Miniemulsions are special heterophase dispersions of relatively stable nanodroplets in a continuous nonsolvent phase.[11] As a result of the high shearing of the biphasic system in the presence of an appropriate surfactant, droplets with a narrow size-distribution can be prepared in the 20–200 nm range. Each droplet behaves as a discrete nanoreactor and a whole range of polymerization reactions can be initiated within, leading to size-defined products.[12] Linear products of diols and bivalent electrophiles with high molar mass have thus been synthesized for example, polyesters,[13] polyurethanes,[14] and polyethers (diol/bisepoxide).[15] Extension of such methodology to branched monomers leads naturally to the formation of highly branched and cross-linked products. In this way sophisticated nanoparticles may be prepared that incorporate further features, such as biodegradability or surface functionalization.[16]

Herein we present a route to high molecular weight PG-μ-gels based upon acid-catalyzed polyaddition of cheap commercially available starting materials: the triol glycerol (**1**) and the trisepoxide, glycidyl glycerolether (**2**; Scheme 1). By utilizing miniemulsion conditions it is possible to obtain discrete PG-μ-gels with diameters up to 80 nm that show full solubility in polar solvents including water.[17] Our system is based upon inverse miniemulsion in which the polar reactants



Scheme 1. Synthetic pathways towards pure PG-μ-gel and surface functionalized PG-μ-gel particles: a) cyclohexane/DMSO/block copolymer, sonication-initiated miniemulsion formation 4×1 min; b) *p*-TSA (cat.), 115 °C, 16 h; c) *p*-TSA (cat.), 115 °C, varied reaction time (Table 2); d) NaN_3, DMF, 60 °C, 24 h; e) propargyl derivative, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O, 24 h.

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are dispersed in nonpolar cyclohexane. A poly(ethylene-*co*-butylene)-*block*-poly(ethyleneoxide) surfactant, was found to act as an excellent stabilizer of glycerol/trisepoxide nanodroplets,[18] particularly if a small amount of dimethysulfoxide (DMSO) is mixed in as an ultralipophobe (suppresses destabilization of the droplet by Ostwald ripening).[19] Miniemulsions were formed by ultrasonication with a sonic tip prior to addition of *para*-toluenesulfonic acid (*p*-TSA) catalyst. Our cationic ring-opening polymerizations in miniemulsion proceeded to give nanoparticles of defined size as the continuous nonsolvent phase prevents macroscopic aggregation. Products were obtained quantitatively and free from surfactant, after purification by solid/liquid extraction with hexane, and subsequent dialysis in water.

To control the size of the nanoparticles various parameters were altered (see Table 1). Maintaining a standard 0.2 mM concentration of surfactant in cyclohexane, it was found that

Table 1: Parameter variation allows particle size control.[a]

Product	Monomer ratio[b]	Monomer [mg][c]	DMSO [mL]	Diameter [nm][d]
3a	3:2	135	0.05	23.7 ± 2.8[d]
3b	3:2	270	0.10	22.4 ± 2.2[e]
3b	3:2	270	0.10	24.1 ± 2.5[d]
3c	3:2	1080	0.20	49.0 ± 7.6[d]
3d	3:2	2160	0.40	67.3 ± 5.9[d]
3e	3:3	2160	0.40	54.3 ± 6.0[d]
3f	3:1	2160	0.40	80.6 ± 6.9[d]

[a] Constant parameters: cyclohexane = 15 mL; surfactant = 20 mg; *p*-TSA = 20 mg. [b] Ratio is alcohol:epoxide. [c] Summation of **1** and **2** quantity with the aforementioned ratio. [d] Measured by DLS. [e] Measured by TEM.

varying the volume ratio of disperse phase (monomers + DMSO) relative to the continuous phase resulted in PG-μ-gels **3a–d** with sizes controllable between 25 and 65 nm diameter. A lower limit diameter was reached at 25 nm as shown by comparison of products **3a** and **3b**. A further factor influencing diameter is the initial monomer ratio **1**:**2**. By comparison of products **3d–f** it is seen that a relative increase of **2** in the reaction mixture leads to smaller products, a phenomenon relating to a higher degree of branching and more compact globular nature of the microgels formed as the number of cross-linking units is increased. Inverse gated ^{13}C NMR spectroscopy (see Supporting Information) indicates a degree of branching (fraction of ether oxygen atoms relative to hydroxy oxygen atoms) of approximately 60% in the typical case of **3d**. Further studies on internal structures are currently being undertaken. Particle sizes were determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM; see Figure 1).

Under the conditions employed, complete reaction, as evidenced by full consumption of epoxide units (^{1}H NMR spectroscopy), was achieved after heating for 16 h. Advantageously, nanoparticles of comparable size and quality may be formed that still bear a significant quantity of residual epoxide groups by heating the reaction for reduced time to give products **4** as shown in Table 2. These epoxide groups can

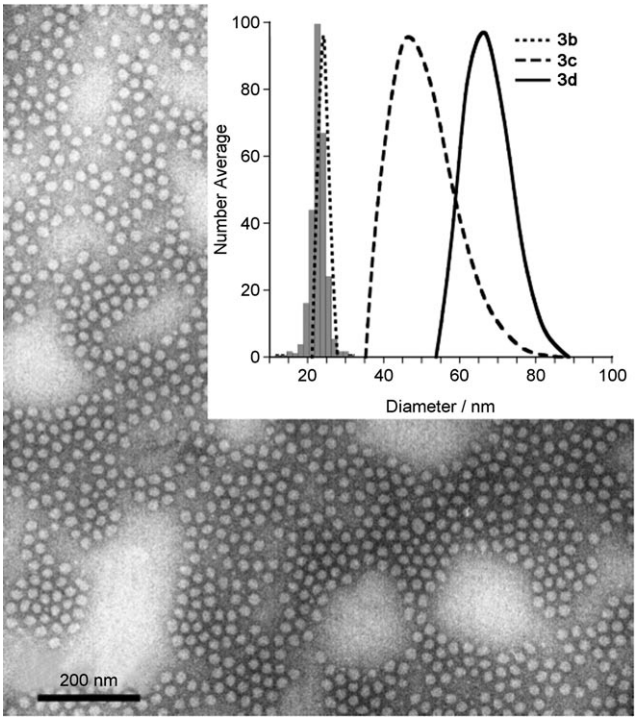

Figure 1. TEM image of PG-μ-gel **3b** negatively stained with phosphotungstic acid. Inset: DLS curves of PG-μ-gels **3b–d** and a histogram of PG-μ-gel **3b** particle sizes counted from the corresponding TEM image.

Table 2: Varying reaction time leads to epoxide-functionalized nanoparticles by incomplete reaction.[a] Particle sizes of **5b** and **6** are also given.

Product	*t* [h]	Epoxide units/glycerol repeat unit[b]	Size [nm][c]
4a	3.0	0.19	44.3 ± 7.5
4b	5.5	0.15	45.7 ± 6.4
4c	7.5	0.12	48.1 ± 6.3
4d	12.0	0.05	47.2 ± 5.9
4e	16.0	ca. 0	45.2 ± 4.8
5b	–	0.15 (azide)	44.2 ± 4.9
6	–	0.15 (mPEG)	47.1 ± 5.6

[a] Constant parameters: cyclohexane = 15 mL; surfactant = 20 mg; *p*-TSA = 20 mg; monomer ratio = 3:3, monomer quantity = 1080 mg, DMSO = 0.2 mL. [b] Measured by ^{1}H NMR spectroscopy. [c] Average (mean), measured by DLS.

undergo a standard ring-opening reaction with sodium azide to provide a facile route to azide functionalized PG-μ-gels **5**. These gels with up to 0.19 azide units per glycerol repeat unit in the final product can be used as substrates for further reactions. For example, propargylated polyethyleneglycol monomethyl ether (mPEG 350) was treated with **5b** (0.15 azides/glycerol repeat) in a copper-catalyzed Sharpless/Huisgen click reaction.[20] IR spectrocopy (Supporting Information) indicated that complete reaction occurred showing that the azide groups are highly accessible in the final polymer. This result suggests either a very exposed series of networks in

the final microgel or that, as a result of the polymerization mechanism, all the residual epoxide groups were located on the outer surface of the particle. DLS and TEM analyses of mPEG shell-functionalized PG-μ-gels **6** are shown in Figure 2.

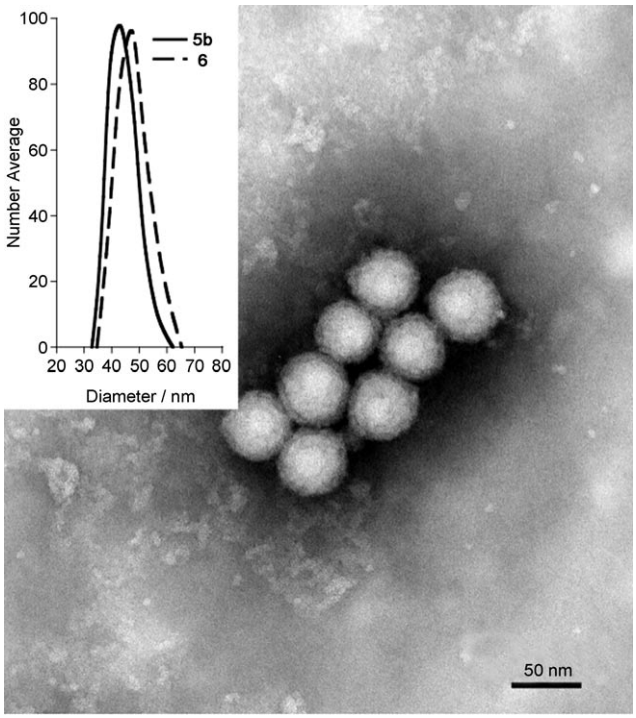

Figure 2. TEM image of PG-μ-gel **6** negatively stained with phosphotungstic acid. Inset: DLS curves of PG-μ-gels **5b** and **6**.

To probe cellular uptake of such PG-μ-gels, **5d** (0.05 azide units per glycerol repeat) was conjugated to a fluorescent dye as shown in Scheme 1. The fluorophore is based on an indocarbocyanine dye (ICC; absorption max. 550 nm, fluorescence max. 580 nm) containing a monocarboxy functionality, which was modified to a propargyl amide to permit click coupling to the PG-μ-gel **5d**.[21] Initial tests on cellular uptake show that these nanoparticles **7** localize in the perinuclear region of human lung cancer cells at 37 °C; there is significantly lower uptake at 4 °C. In comparison, the low molecular weight control dye **8** with only one triglycerol unit did not lead to cellular fluorescence signals. These data (Figure 3), may be evidence for a size-dependent endocytotic mechanism of cell entry and future work will be devoted to studying this phenomenon in more detail.

We anticipated that our polyglycerol materials would show high biocompatibility, as is the case for lower molecular weight analogues.[5] We selected microgel **3d** as a test compound and investigated effects on human hematopoietic U-937 cell proliferation (cell number), viability, and metabolic activity (MTT test; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). Experiments were carried out in 10% fetal calf serum and conducted at varying concentrations of **3d**, against pure buffer as a control and against apoptosis-inducing dexamethasone (Dex) as a positive control (Figure 4). Only a very high concentration of **3d**

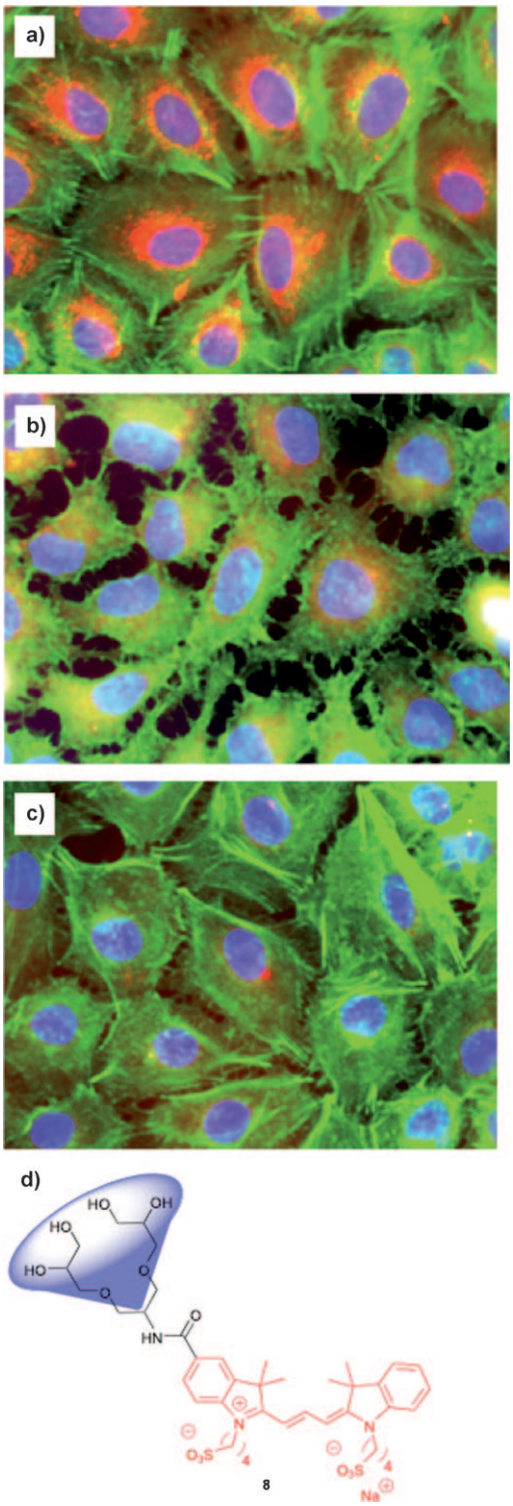

Figure 3. Culture of the human lung cancer cell line A549 incubated with ICC-labeled PG-μ-gel **7** for a) 4 h at 37 °C and b) 4 °C, or c) incubated with control dye **8** at 37 °C. Actin cytoskeleton was stained with Alexa Fluor 488 phalloidin (green), nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). d) Structure of **8**; red: ICC dye fragment, blue cone: low-molecular-weight polyglycerol dendron.

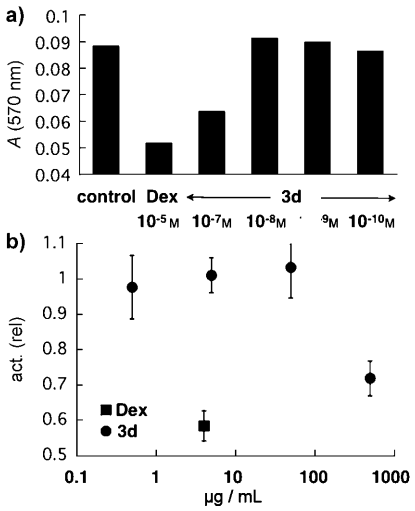

Figure 4. Human hematopoietic U-937 metabolic activity (MTT) tests on microgel **3 d** with negative (pure buffer) and positive (Dex) controls: a) Metabolic activity (in $mol\,L^{-1}$) as measured by monitoring formazan UV/Vis absorption at $\lambda = 570$ nm. b) Metabolic activity (in $\mu g\,mL^{-1}$) relative to negative control. Procedures and more details (cell proliferation, viability) are given in the Supporting Information.

(10^{-7} M = 0.5 $mg\,mL^{-1}$) had a negative influence on the cells, comparable with effects on cell cultures of using 10^{-5} M Dex (0.004 $mg\,mL^{-1}$). Thus, these microgels are considered to be highly biocompatible materials exhibiting minor interaction with biological structures.

In conclusion, we have designed a facile and versatile approach to the synthesis of polyglycerol microgels of dimensions previously unobtainable, providing simple access to materials in the nanoscale. Beneficially such nanoparticles have a narrow size distribution and can be readily functionalized with a wide range of groups by click chemistry. The biocompatible nature of polyglycerol materials is very promising for future applications as delivery vehicles for drugs and dyes. Owing to the size of these PG-µ-gels they rapidly and harmlessly internalize into cells by an endocytotic pathway. Future research is oriented towards incorporating specific cell-targeting moieties and the loading of active molecules onto these highly functional nanoparticles.

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[17] We observed only a small dependence of solvent (e.g. methanol, pure water, phosphate buffered saline) on particle sizes. Furthermore, solution diameters (TEM) were closely matched by solid particle sizes (TEM). We infer that these microgels are not particularly swellable but have a relatively rigid structure.

[18] The precise batch of surfactant used is a poly(ethylene-*co*-butylene)-*block*-poly(ethylene oxide), $M_n = 8100$, 41 wt% ethylene oxide, 39 wt% ethylene, 20 wt% butylene as used in; J. K. Oh, H. Dong, R. Zhang, K. Matyjaszewski, H. Schlaad, *J. Polym. Sci. Part A* **2007**, *45*, 4764–4772; Synthetic details given in A. Thomas, H. Schlaad, B. Smarsly, M. Antonietti, *Langmuir* **2003**, *19*, 4455–4459.

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