Supporting Information

A tunable scaffold of microtubular graphite for 3D cell growth

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MATERIALS AND METHODS

Biofunctionalization: A 4:1 mixture of 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amine(PEG)2000] (DSPE-PEG2000-NH₂; Avanti Polar Lipids) and DSPE-PEG2000-cRGD was prepared in ultrapure water with a PEG-lipid concentration of 1 mg/ml. AG samples were placed into a 12-well plate and 500 µl of PEG-lipid mixture were carefully pipetted into each well. The well-plate was covered with a lid and placed into a desiccator, which was evacuated and purged with nitrogen twice. Then samples remained under nitrogen atmosphere for 24 hours. Afterwards, the samples were washed twice with ultrapure water and left to soak in ultrapure water for another 24 h to remove unbound PEG-lipids. Finally, the solution was exchanged for cell culture medium and AG samples were directly used for cell seeding.

Covalent coupling of cRGD to amine terminated PEG-lipids: Cyclo(Arg-Gly-Asp-D-Tyr-Lys) (c(RGDyK); EUROGENTEC) was coupled via the amine terminus to DSPE-PEG2000-NH₂ (M=2,790 g/mol) according to following procedure. 10 mg of PEG-lipids were dissolved in 3 ml standard PBS containing 8% glutaraldehyde (Sigma) assisted by brief (4 sec) sonication and 10 min treatment in a thermoshaker at 1500 rpm and 40°C. The solution was allowed to incubate overnight at room temperature. Activated PEG-lipids were concentrated and washed 3 times with 200 µl ultrapure water using centrifugal filters (Vivacon 500, 2,000 MWCO; Sartorius) at 10,000 g for 90 min. For reductive amination of activated PEG-lipids (DSPE-PEG2000-glutaraldehyde), 2.5 mg c(RGDyK) (1.1 eq; 4 µmol) were dissolved in 500 µl carbonate buffer pH 8.3. Then 236 mg of sodium cyanoborohydride NaCNBH₃ (M=62.8 g/mol) were dissolved in 200 µl carbonate buffer pH 8.3, of which 20 µl were added to the recovered DSPE-PEG2000-glutaraldehyde solution together with the dissolved c(RGDyK). The solution was briefly sonicated (4 sec), then placed in a thermoshaker for 15 min at 40°C and 1500 rpm, and left for incubation over night at room temperature. The solution was then filtered using vivacon 500
centrifugal filters at 10,000 g for 120 min to remove carbonate buffer followed by 3 washing steps with ultrapure water (200 µl) at 10,000 g for 40 min for purification of the resulting DSPE-PEG2000-cRGD. The recovered concentrated cRGD-PEG-lipids were stored at -20°C until use.

**Cell culture and cell seeding:** Rat embryonic fibroblasts (REF52) and fluorescent rat embryonic fibroblasts (REF52 YFP Pax), were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Laboratories) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 2 mM L-glutamine (all from Gibco Laboratories) in an incubator at 37°C and 5% CO₂ level. About 20,000 cells were seeded on functionalized AG and 10,000 cells were seeded into empty control wells. Growth was visually inspected until control cells reached confluence after day 4. Then the cells were fixed with 4% paraformaldehyde (PFA) in Hank’s buffered salt solution (both Sigma) for 20 min and washed with PBS.

**Actin fiber and nuclear staining with Phalloidin and Hoechst:** PFA fixed control cells and AG/cell samples were washed with PBS three times and 0.1% Triton X-100 in PBS was added for 5 min followed by three times washing with PBS. Next 20 µl PBS and 5 µl Phalloidin and 0.2 µl Hoechst (10 µg/ml) were mixed. Phalloidin stains actin fibers, and Hoechst stains the nucleus of a cell. Four µl of staining solution were added to each sample and incubated for 20 min at 37 °C. Afterward, the solution was removed and the wells were washed with PBS three times and transferred to an IX81 microscope to examine them.

**Fluorescence microscopy:** Fluorescence imaging was done on an Olympus IX81 inverted microscope equipped with a metal halide lamp, and a monochrome CCD camera (Hamamatsu C9300). In order to observe the different fluorescently labeled cell features the following filters were used: YFP to observe YFP conjugated paxillin that marks adhesion complexes, DAPI for nuclear staining with Hoechst, and RFP to visualize phalloidin stained actin fibers. Setup control
and image recording were assisted by Olympus CellSense Dimension software. Control cells were recorded in epi-fluorescence configuration at 0.1 sec illumination. Images of AG bulk samples were taken as optical image stacks of 20 to 80 sections in epi-fluorescence by motor-stage assisted change of the focal plane in 5 µm-steps. Illumination was set to 5 sec per image to provide enough fluorescence signals at reasonable signal to noise ratio. Image processing of monochrom images was done using CellSense Dimension software. Composite images of all three channels were done in Image J with adjustment of contrast and brightness.

**MTT based viability assay:** In accordance with protocols in part 12 of ISO 10993 (ISO 10993-12:2004) ‘Sample preparation and reference materials’, we have prepared extracts from three PEG-lipid functionalized and three pristine AG samples in cell culture medium with a mass/volume ratio of sample and medium of 0.1 g/ml taking into consideration the ‘standard surface areas and extract liquid volumes’ outlined in Table 1 of the ISO 10993:12:2004. The immersed samples were gently agitated at 37 ºC over a period of 72 h. Following protocols in part 5 of ISO 10993 (ISO 10993-5:2009) ‘Tests for in vitro cytotoxicity’ the viability of REF 52 fibroblasts was tested with an MTT metabolic activity assay after 24 hours of exposure of seeded cells to the extracts. REF cells were cultured for 24 h at 37 ºC with the undiluted extracted medium. Cells treated with 15% DMSO served as positive control, and cells incubated in untreated culture medium were used as negative control. After 24 h incubation the medium was exchanged to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Germany) solution in Eagle minimum essential medium (Biochrom, Germany) without supplements and phenol red (at concentration of 1mg/ml) followed by 4 h incubation at 37 ºC. The MTT converted to formazan was extracted by adding isopropanol alcohol, and the optical density of formazan was measured in a plate reader (Bio-Tek instruments, µQuant) (at 570 nm).
**Super critical point drying and scanning electron microscopy:** PFA fixed AG/cell samples were dehydrated stepwise in graded ethanol (EtOH) of increasing alcohol concentrations starting at 60% EtOH and incubation period of 30 min followed by 80% (30 min), 90% (30 min), 95% (1 h) and 100% (>1 h). Thereafter, EtOH was substituted against acetone and the samples were transferred into the chamber of the critical point dryer (CPD, BAL-Tec CPD030). For SEM imaging a 7.5 nm layer of gold was applied directly after CPD. Imaging was done in Supra 55VP field emission SEM (Zeiss) applying an acceleration voltage of 2 kV. SEM images have been cropped but are otherwise displayed as recorded. Scale bars have been included using CorelDraw X7.

**Preparation of AG sections:** PFA fixed AG/cell samples were dehydrated in graded EtOH. Concentrated EtOH was then substituted against xylene, which was changed 3 times with 1 h settling time for each step. Afterwards samples were soaked in paraffin of 56-58 °C for 1.5 hours, drained and immersed again for embedding into blocks of solidified paraffin. After complete hardening, paraffin was trimmed down to the sample surface and sections of 9 µm thickness were prepared with a microtome. Sections were placed onto glass slides and dried at 37 °C. Prior to hematoxylin/eosin (HE) staining with Mayer’s Haemalaun and 0.1 % Eosin-B solution (both from Merck) paraffin was removed and sections were rehydrated by reversal of the dehydration procedure.

**Dewaxing of AG/cell sections and haematoxylin/eosin staining:** Dewaxing was done by placing glass slides with paraffin sections in xylene three times for 3 min. Then the glass slides were passed through ethanol solutions of decreasing concentrations (100% EtOH 3 x 3 min, 90% 2 x 3 min, 80% 2 x 3 min, 70% 1 x 3 min, 50% 1 x 3) and finally into deionized water for two
times 3 min. After rehydration glass slides were incubated in Mayer’s Haemalaun solution (Merck) for 3 min, rinsed with 0.1% HCl for 2 sec and washed under running water for 1 min. Next, glass slides were placed in 0.1% Eosin-B solution (Merck) for 3 min followed by 30 sec rinsing with water. Lastly, samples were dehydrated again in an ascending graded ethanol series, soaked in xylene two times, and then covered with Neo-Mount (Merck) and a cover slip.

*Optical bright field microscopy* was done on an Olympus BX43 equipped with a color CCD camera (ImagingSource). Final image contrast and brightness of presented micrographs were adjusted using CorelDraw X7.

SI APPENDIX I: Surfactants for immersion of AG in aqueous media – test series

A vast number of molecules have been investigated for dispersion of carbon based materials.\(^1\) We have chosen amphiphilic agents that were successfully tested for large diameter CNTs, as AG filaments range from several 100 nm up to a few micrometers. We have tested the following agents at different concentrations in aqueous solution:

1. Bovine serum albumin (BSA), a serum albumin protein derived from cows, which is a standard low cost protein in lab experiments and cell culture, and shows no adverse effects.

2. Carboxymethyl cellulose (CBMC) is used in detergents and in purified form for food and pharmaceutical applications.

3. Amine terminated poly(ethylene glycol) conjugated phospholipids (e.g. DSPE-PEG2000-NH\(_2\)), are engineered biopolymers that have been proven to successfully disperse CNTs and graphene. The hydrophobic tails of the phospholipid DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine) are assumed to attach non-covalently to the AG surface via hydrophobic interactions, whereas PEG extends into the aqueous phase imparting
hydrophilicity to AG. Applications of PEG in medicine are manifold. It is used to increase blood circulation times of therapeutics and prevent rapid renal clearance, serves as coating and lubricant for pharmaceutical products and in eye drops.

4. Cell culture medium supplemented with 10% fetal bovine serum (FBS) is used for eukaryotic in vitro cell culture. Apart from BSA as the major component, FBS contains a rich variety of proteins including growth factors. The nutrients and proteins of FBS may adsorb onto the AG surface and facilitate AG immersion in aqueous solutions as well as afford a suitable matrix for cell adhesion.

Figure S1 illustrates the results of the series of experiments. BSA and CBMC at different molecular weight were not successful in improving immersion of AG. Cell culture medium supplemented with 10% FBS showed improved immersion of AG after a 2 days incubation period. DSPE-PEG2000-NH₂ has shown to readily immerse AG in aqueous solutions and thus proven to be the most promising candidate for AG surface functionalization.
Figure S1. Transfer of hydrophobic AG into aqueous media employing amphiphilic molecules as immersing agents. AG pellets of 8 mm diameter are fixed to silicon substrates and placed in wells containing aqueous suspensions of different agents. A) Bovine serum albumin (serum protein) The aqueous solution is strongly repelled by AG and collects around the Si-chip, whereas AG remains dry. B) Carboxymethyl cellulose with a molecular weight of 90 kD. The liquid is strongly repelled and collects in the top part of the well, and away from the Si-chip. C) Carboxymethyl cellulose with a molecular weight of 250 kD is repelled as demonstrated by the drop forming on the AG disk, similarly to plain water. D) 2-distearoyl-sn-glycero-3-phosphoethanolamine- N-[amine(PEG2000)] (DSPE-PEG2000-NH₂) readily wets AG and the Si-chip is entirely submerged in the liquid. E) Cell culture medium supplemented with 10% fetal bovine serum (proteins). F. Water as control sample to illustrate super-hydrophobicity of unmodified AG.

SI APPENDIX II: Additional SEM images of DSPE-PEG2000-NH₂ functionalized AG

We employed SEM imaging to investigate the surface of biofunctionalized AG scaffolds at high resolution. Figure S2 shows a representative surface scan with different magnifications of DSPE-PEG2000-NH₂ functionalized AG. The sample was dehydrated by drying at ambient conditions and imaged without further surface treatment. The adsorbed molecules become visible at high magnification as fine hairy structures extending from the side walls (Figure S2 B) and as faint bright dots (Figure S2C). Pristine AG was subjected to Au sputtering as a control for scanning electron micrographs of biofunctionalized AG (Figure S3).
**Figure S2.** Scanning electron micrographs of AG non-covalently functionalized with DSPE-PEG2000-NH₂ without Au coating.

**Figure S3.** Scanning electron micrographs of pristine AG with Au coating.
SI APPENDIX III: Paraffin thin section of AG of different densities

Three different AG structures C111, C107 and C116 of varying specific weight ranging from 0.2 mg/ccm to 1.5 mg/ccm were investigated. The CVD parameters influence the wall thickness of the graphitic shells and thus the density of the resulting AG samples. By varying the toluene injection rate various AG densities were obtained and estimated to be 0.2 – 0.4 mg/ccm for the most lightweight batch C111 (injection rate 5 ml/h per g(ZnO)), 0.5-0.7 mg/ccm for batch C107 (injection rate 5 ml/h per g(ZnO)), and 1.0-1.2 mg/ccm for batch C116 (injection rate 6 ml/h per g(ZnO)). Figure S4 shows representative cross sections and a photograph of all 3 types in dry state. It is important to mention that all three types were produced based on a cylindrical ZnO template of 6 mm diameter and 3 mm height. In C107 and C106 the original size of the template is preserved with a minor gain in size of 1-2%. The ultra-lightweight structure of C111 - on the other hand - exhibits much reduced dimensions, as a consequence of compression under its own weight.

The samples were transferred into bi-distilled water through non-covalent surface modification by hydrophobic adsorption of PEG-lipids. For paraffin embedding dehydration was carried out by sequentially exchanging water for ethanol, subsequent transfer into xylene and soaking into liquid paraffin. During this procedure the samples were constantly kept in liquid to prevent collapse of the AG structure due to surface tension during evaporation. After solidification the paraffin blocks were trimmed down and either horizontal thin sections or vertical sections of 9µm thickness were prepared for assessment of the network within the bulk material (Figure S4).
Figure S4. Cartoons illustrating the preparation of horizontal and vertical sections from different paraffin embedded AG samples. Embedded AG disks were 6 mm in diameter and 3 mm in height. (A) Side view cartoon depicting the preparation of 9 µm thick horizontal cross sections to about the equatorial line of the AG disk at 1.5 mm. The photograph to the right shows what remains from the paraffin embedded AG disks after sectioning. (B) The top view cartoon on the left shows the preparation of vertical sections of 9 µm thickness and 3 mm height, which were were cut from the first 2 mm of the 6 mm AG sample. The top view photograph of the paraffin block shows the remaining sample.

The sections were investigated under a light microscope to assess filament thickness, pore size, matrix connectivity, and homogeneity of these parameters (Figure S5A-C). Sections of the ultra-lightweight AG C111 generally revealed that the filament lengths and diameters, as well as the pore sizes ranged below typical structural parameters presented by natural extracellular matrix. Sections of AG C107 showed reduced connectivity of filaments, which might be an artificial result of the harsh treatment during paraffin embedding where paraffin was repeatedly filled and drained into and from the bulk sample, or may be related to structural problems of the ZnO template. Regions with disrupted network connectivity were much fewer in C116 with the
highest specific weight of > 1mg/ccm. Thus we assumed that interfilament connections were more robust, suggesting that this type of AG might be more suitable for future experiments.

**Figure S5.** (A) Photograph showing AG samples of different specific weight before paraffin embedding; C111 ultra-lightweight AG with average specific weight of ~ 0.25 mg/cm³, C107 AG with average specific weight of 0.6 mg/cm³, C116 AG with average specific weight of 1.1 mg/cm³. (B-C) Light microscopy images of paraffin sections revealing the network structure of carbon microfilaments within the bulk samples of C111, C107 and C116. Scale bars 100 µm.
SI APPENDIX IV: Additional light microscopy images of haematoxylin and eosin stained AG sections

**Figure S6.** (A) HE-staining makes REF52 YFP Pax cells visible by coloring the nuclei blue and the cytosol pink. Due to the vigorous de-waxing and staining treatment, the original section (Figure S5D) is disrupted and in part has been washed off the glass slide. B)-G) Higher magnification reveals cells that are well interfaced with AG filaments and illustrate morphologies typical for fibroblasts. Scale bars 10 µm.

REFERENCES

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