The next generation of low adhesion – increasing flexibility and performance

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Introduction

Cell based *in vitro* assays are becoming more important in the basic research but also in applied research in pharmacology and toxicology. All these methods are generating important information and finally help to understand the human (patho-) physiology. In order to improve the predictivity of these assays, traditional two-dimensional cell culture models evolve into three-dimensional models (spheroids and organoids) and microfluidic systems (e.g. organ on a chip)[1]. Both trends require a robust and reproducible coating of the used hardware to generate valid data[2].

Low attachment U-bottom shaped plates for spheroid generation are very convenient for the generation and usage of such spheroids and most of the major laboratory suppliers provide such plates in a variety of formats. However, this first wave of “low-binding plates” differs strongly in quality and a range of flaws. For example, multiple spheroids or irregular aggregates can be observed leading to reduced uniformity and reproducibility of assay results. Furthermore, plates are available in a limited number of formats, restricting applications severely to the standard microtiter surfaces and dishes. “Self-made systems” to coat other surfaces, e.g. based on agarose, come with increased variability and challenges in handling. More standardized 3D cell culture procedures could therefore further reduce data variability and enhance biological relevance of *in vitro* assays[3].

To address these shortcomings, we have developed a polymeric coating solution. The BIOFLOAT™ FLEX coating solution generates a surface with excellent protein and cell-repellent properties, leading to a biologically inert surface on different kinds of lab consumables. Owing to its highly anti-adhesive properties, cell-to-cell interactions are favored, leading to the formation of highly uniform spheroids which float in the medium without interaction with the surface. The coating solution allows to treat different formats of plates or microfluidic devices, which are amenable for 3D spheroid screening approaches or organoid models in cancer research or toxicology.

Results

The BIOFLOAT™ FLEX coating solution passivates plastic and glass surfaces

The aim was to develop a polymer solution that i) rapidly and strongly binds to the surface without any complex pretreatment of the surface, that (ii) is highly protein and cell-repellent, better than previously available systems and that iii) is stable under cell culture conditions.

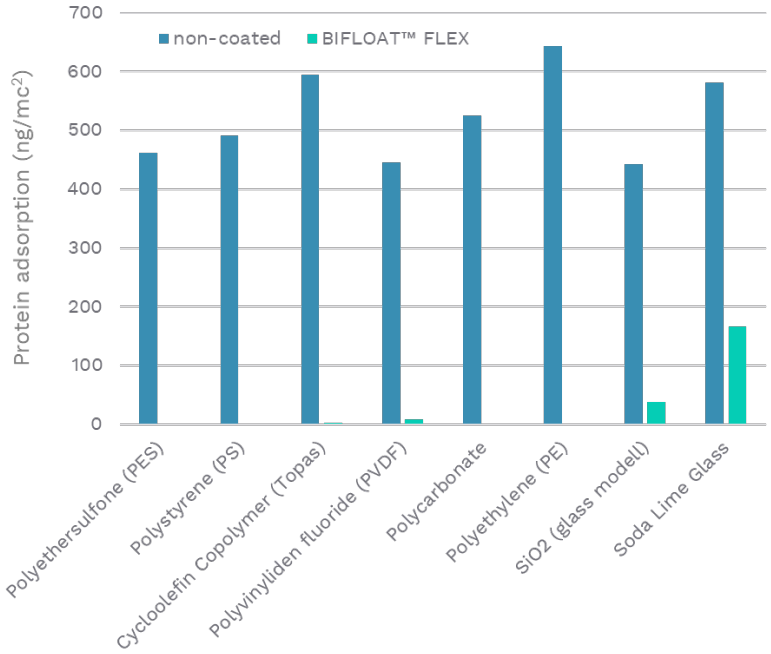


Fig. 1 Reduction of unspecific protein binding. The QCM detects mass changes of a sensor surface due to the binding of protein. Such protein adsorption is found in all materials tested including plastic and glass materials. In contrast, after coating the surface using BIOFLOAT FLEX coating solution, protein binding is significantly reduced.

After optimizing the polymer chemistry and composition, the physical properties of the coated surfaces were initially characterized using a Quartz-Crystal Microbalance (QCM), which detects the kinetics of mass adsorption and allows to precisely monitor the coating process. Hereby, a strong adsorption of the BIOFLOAT™ FLEX coating solution to polystyrene was detected already seconds after passing the solution through the flow cell. The adsorbed polymer layer of about 600 ng / cm^2 corresponds to a monolayer of polymer molecule. Accordingly, the polymer-coated surface will not affect the geometry of the device. This is of importance, in particular for microfluidic devices since the diameter and flow rate of the microfluidic channels will not be changed by the deposition of such a layer.

The protein repellency of the BIOFLOAT™ FLEX coating solution was analyzed on different commonly used substrate materials such as polystyrene, quartz glass, polycarbonate and polyethylene. To this end, a casein solution was introduced into the QCM flow cell as a model protein to monitor the protein loading on the surfaces. The control experiments clearly showed that casein adsorbs to all materials tested in the absence of any other coating. However, once the surfaces were passivated with the BIOFLOAT™ FLEX coating solution, protein adsorption was fully prevented as can be seen from the minimal mass loadings in the QCM study.

Using BIOFLOAT™ surfaces for spheroid generation

Motivated by this result, the BIOFLOAT™ FLEX coating solution was evaluated on U-shaped polystyrene 96 well plates for the generation of spheroids. Since fibroblast cells are known to be strongly adherent to cell culture consumables, they were used as model cell line to evaluate the cell-repellent nature of the BIOFLOAT™ FLEX coating solution.

The plates were passivated simply by adding the BIOFLOAT™ FLEX coating solution to the well for three minutes, followed by aspiration of the liquid. After a short drying step of 30 min, cells were seeded into each well. Immediately, the cells started to settle and to form round aggregates. After a few hours, robust and compact spheroids had formed, displaying a very regular and round shape with a size correlating to the seeding density of the cell (Fig. 2). While only one well is shown for illustration, identical spheroids formed in all treated wells (n=84). In contrast, cells grown in the remaining not-coated control wells strongly adhered to the bottom to form a confluent layer as expected.

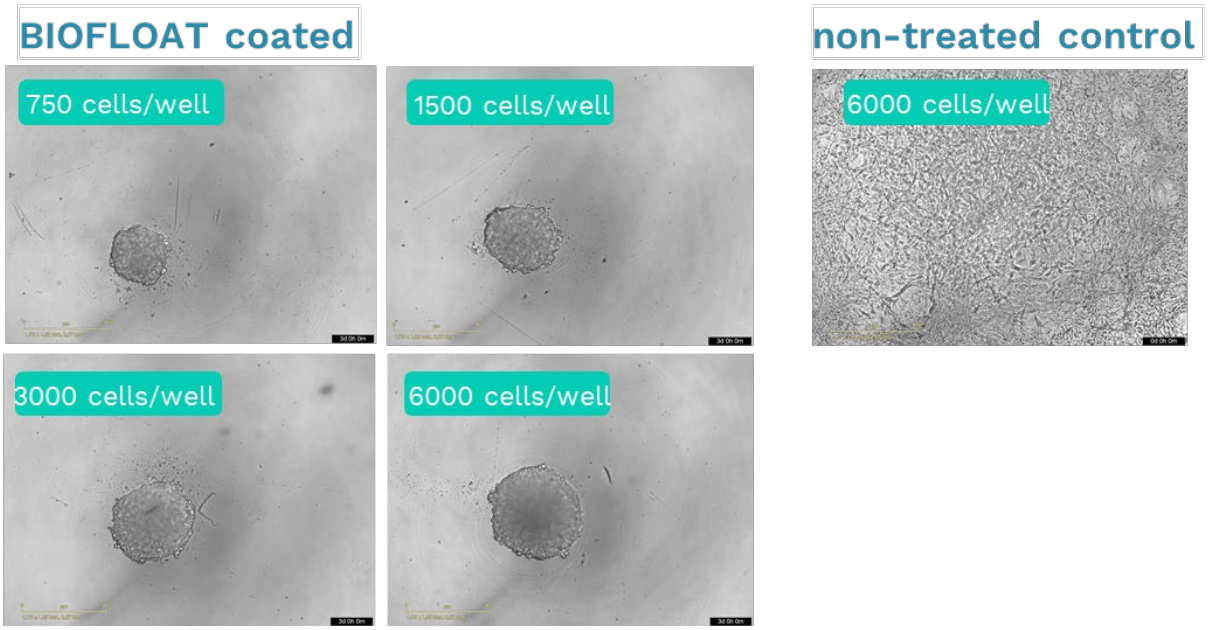


Fig 2 **Formation of spheroids on BIOFLOAT™ coated wells.** Perfectly round shaped spheroids are formed already hours after seeding between 700 to 6000 cells. Microscopic evaluation was performed 3 days post seeding. The size of the spheroids correlates with seeding density.

The BIOFLOAT™ FLEX coating solution passivates within a few seconds, avoiding long incubation steps

The QCM data showed an extremely fast adsorption of the polymer (not shown), which prompted us to test also short incubation time points for the absorption to the polystyrene plate. The coating and incubation time was investigated by applying the coating solution to non-treated polystyrene surfaces from 1 second to a few minutes.

A coating contact between the BIOFLOAT™ FLEX coating solution and the surface for one second is sufficient to generate a fully functional ultra-low adhesion surface.

These results demonstrate the ease and stability of the surface treatment. Any substrate or device can be conveniently passivated by simple and short rising or pipetting (“zip-spit”). Finally, when coating multiwell plates, an incubation period of 3 minutes is recommended, which corresponds to the duration in which the coating can be pipetted conveniently into each well.

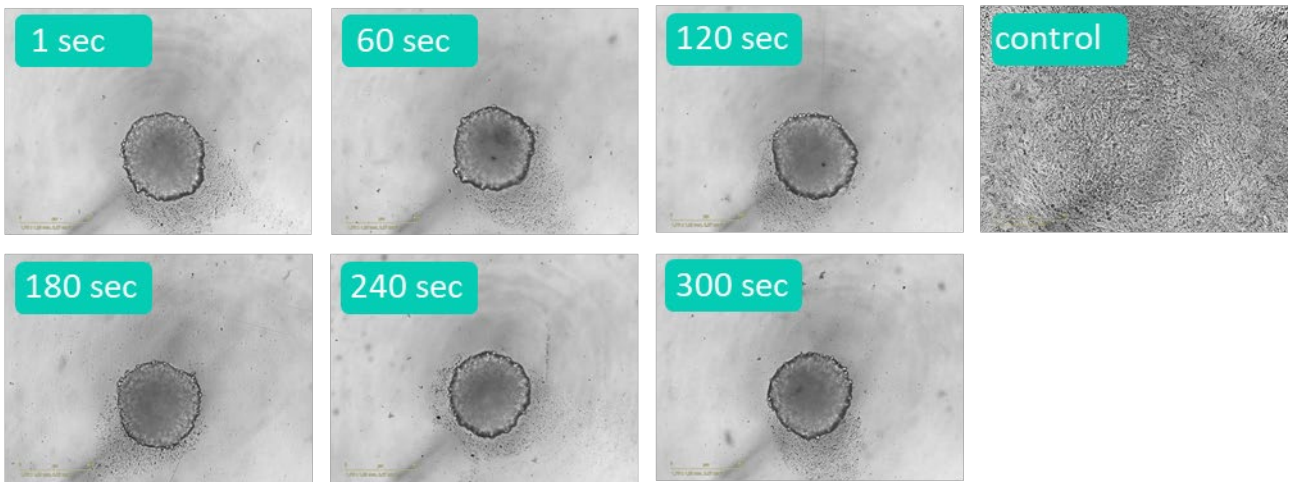


Fig 3 **Coating kinetics of** U-bottom polystyrene plates which had been coated for different time periods with BIOFLOAT™ FLEX coating solution before seeding cells for spheroid generation.

BIOFLOAT™ FLEX coated plates generate faster and more regular spheroids than benchmark products

After successful validation of the layer formation on plastics and glass, the BIOFLOAT™ FLEX coated plates were benchmarked against common low adhesion plates for spheroid and organoid generation. Fibroblasts were cultivated on BIOFLOAT™ FLEX coated 96 well plates in comparison to benchmark low-adhesion plates. Morphological characterization of the spheroids over a period of 3 days displayed a rapid and reliable spheroid formation in each well for the BIOFLOAT™ treated surface. In contrast, irregular aggregate formation was observed on some of the benchmark surfaces, which could not be washed away, indicating a high number of adhesion points (Fig. 4). The morphological evolution over time was then further characterized including the early stages of spheroid formation.

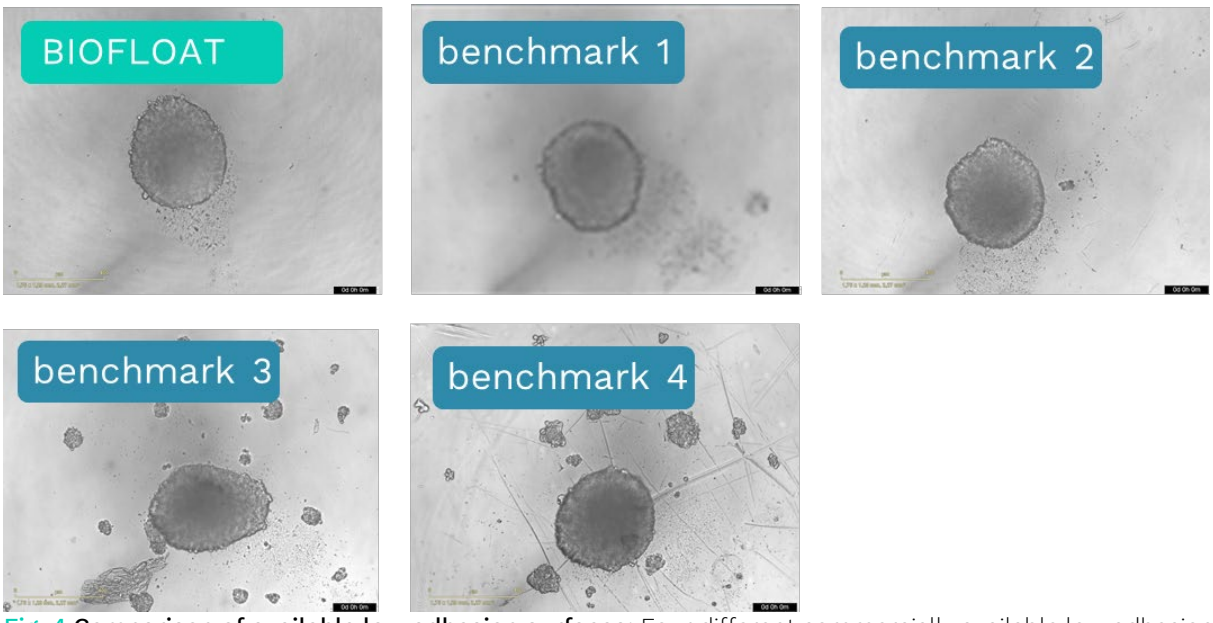


Fig. 4 **Comparison of available low adhesion surfaces:** Four different commercially available low adhesion plates were compared to BIOFLOAT™ FLEX coated plates using fibroblast cells. Two of the plates generated additional cell aggregates around the main spheroid (benchmark 3 and 4). The remaining plates generated a single spheroid in most of the wells.

Cell aggregation quickly started after seeding and completed within 8 hours to deliver one single, compact spheroid on the BIOFLOAT™ FLEX coated plates, similar to benchmark 1. In contrast, the cell aggregation on benchmark 2 was significantly slower and the evolution over time yielded less defined aggregates (Fig. 5). Finally, the circularity analysis of cell aggregates grown on BIOFLOAT™ FLEX coated surfaces revealed an extremely regular growth with increasing circularity over time to deliver highly uniform spheroids for perfect data consistency.

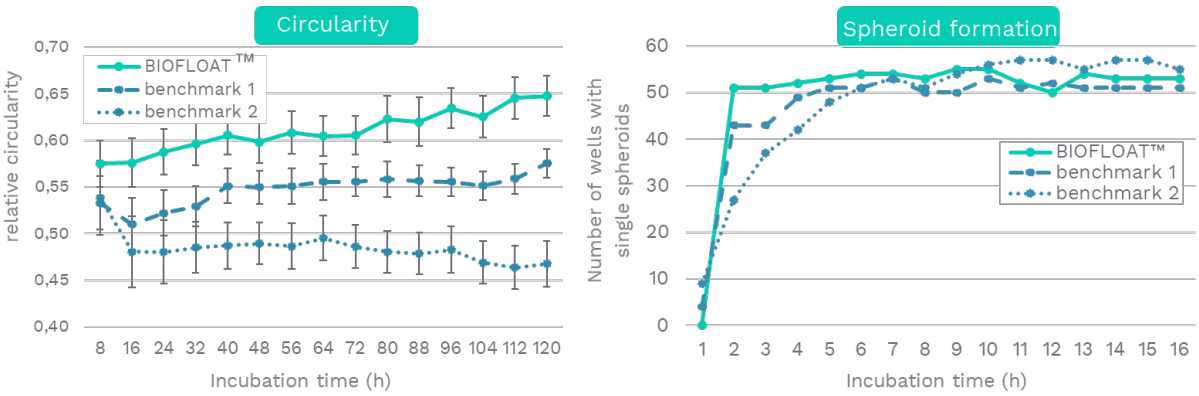


Fig. 5 Generation of spheroids over time: continuous monitoring of spheroid generation, including shape of spheroids and number of wells with single spheroids, reveals that cell spheroids grown on BIOFLOAT™ FLEX coated plates are generated faster and have a very regular shape.

BIOFLOAT™ FLEX coated plates are more robust than benchmark products

Besides the low adhesion performance we wanted to ensure that the coating also performs in different assay setups, including multiple washing steps or long-term incubation. In order to simulate repetitive media exchange, the influence of multiple washing steps on spheroid formation was evaluated with PBS buffer solution and compared with product benchmark 1. While BIOFLOAT™ FLEX coated plates could sustain a total of 12 washing cycles and still generate compact round spheroids, a performance loss was observed for the competitor benchmark 1 (Fig. 6)

Further, the scratch resistance of the BIOFLOAT™ FLEX coated plates was investigated in comparison to benchmark 1. Surprisingly, the performance of the competitor benchmark 1 heavily dropped when scratched with a standard pipette tip (Fig. 7). Vulnerability to physical damage was particularly visible when increasing the physical stress to the surface. Interestingly, the BIOFLOAT™ FLEX coating stayed intact even under these harsh conditions. (Fig. 7).

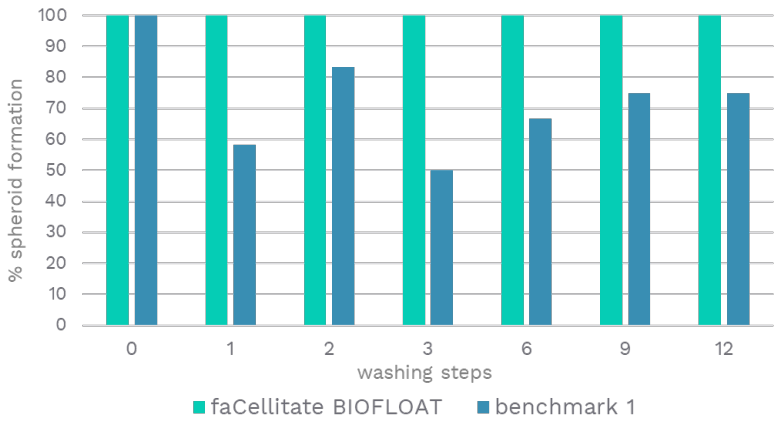


Fig. 6 Washing stability of BIOFLAT™ FLEX coated surfaces: In contrast to benchmark products, the BIOFLOAT surface generated singular spheroids even after 12 washing cycles with PBS buffer

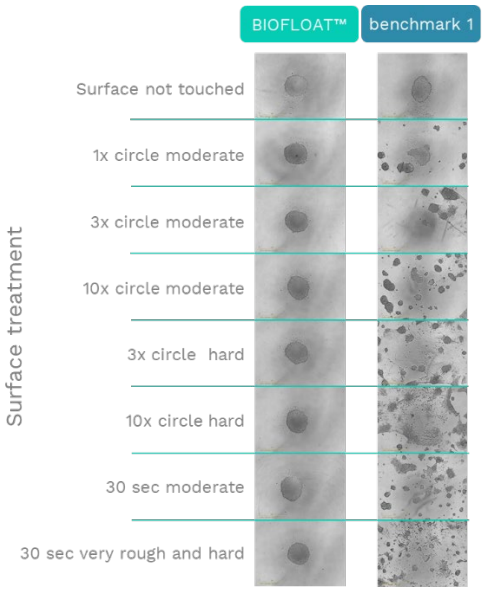


Fig. 7 **Scratch resistance of BIOFLAT™ surfaces:** In contrast to benchmark products, the BIOFLOAT™ surface can be scratched with a pipette tip or even exposed to stronger physical stress and still generate singular spheroids in all wells.

Spheroids grown on BIOFLOAT™ surfaces are well compatible with a broad range of assays

The fibroblast model is well suited to demonstrate the biologically inert character of the on BIOFLOAT™ coating. However, the compatibility of BIOFLOAT™ surfaces with histological methods and high content imaging was demonstrated with HegG2 liver cells, which are relevant models *in vitro* toxicology.

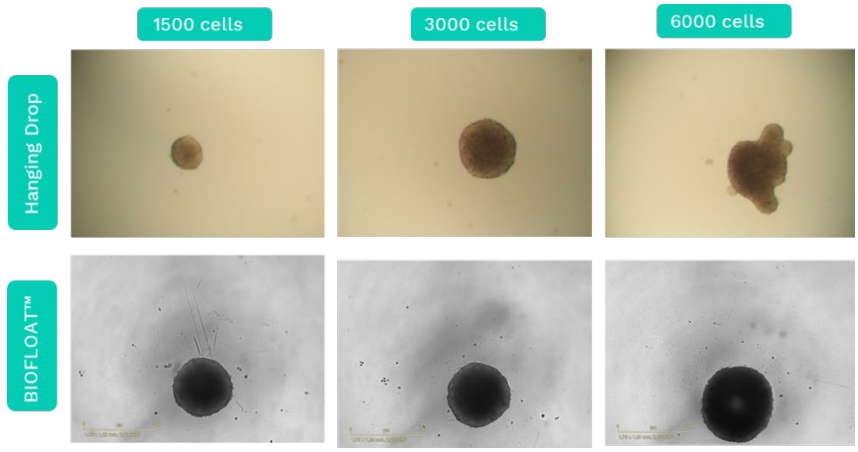


Fig. 8 **Comparison of hanging-drop system and BIOFLOAT coated plates.** In both systems spheroids can be formed. On BIOFLOAT™ surfaces the shape of the spheroids are more regular compared to the classical hanging drop system.

In a first step the spheroid formation of HepG2 spheroids in BIOFLOAT™ coated plates was compared to a classical hanging drop system (Fig. 8). As expected, round and regularly shaped spheroids formed on BIOFLOAT™ coated surfaces. In contrast, irregularly shaped aggregates were observed in the hanging drop system.

The characterization of the spheroids was completed by using high content imaging for different ranges of different markers. Changes in mitochondrial mass indicate the loss of total mitochondria or mitochondrial membrane potential, which imply mitochondrial swellings or adaptive responses to cellular energy demands. As expected, no differences in mitochondrial activities were observed compared to other low adhesion systems (Fig. 10). This holds true also for markers which indicate imbalances in the oxidative state such as

oxidative stress and glutathione content. Finally, the ATP levels, which are an indicator for metabolic activity, were comparable to existing systems.

Taken together, this panel of tests demonstrates a comparable behavior for cell spheroids cultivated on BIOFLOAT™ surfaces compared to other spheroid cultivation systems. It is important to note that the BIOFLOAT™ system provides improved reproducibility and reliability but the absolute values do not differ from historical data. Ensuring the consistency of old and new data sets enables an easy change between culture systems.

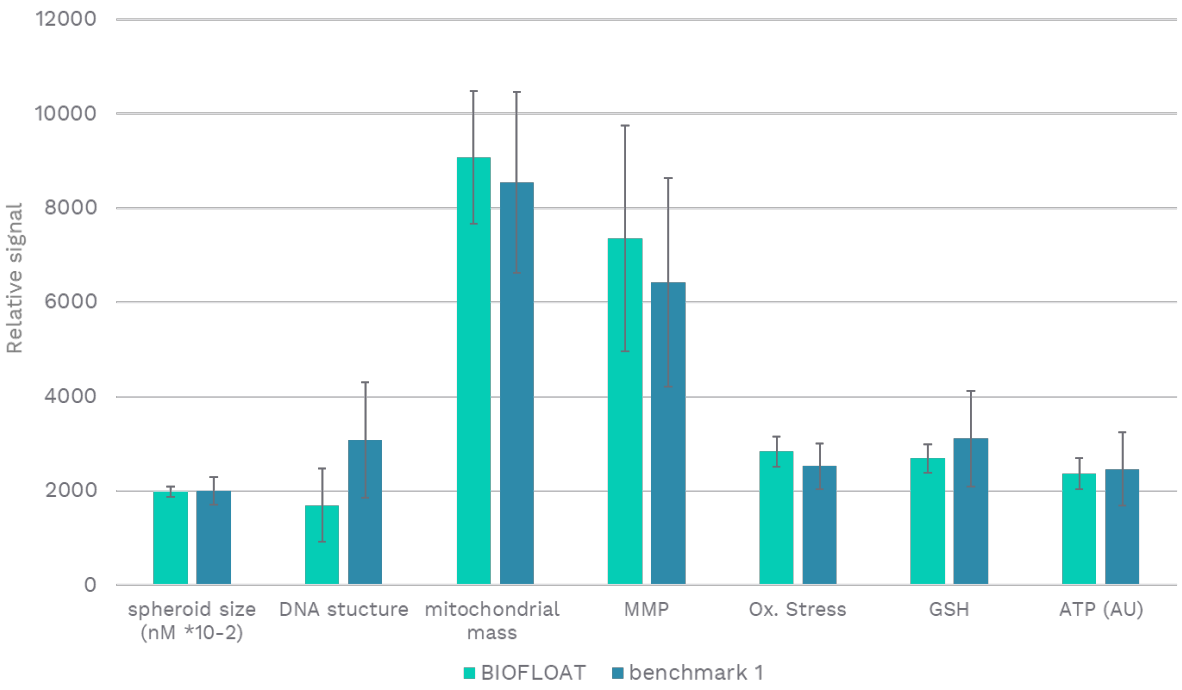


Fig. 9 **Comparing historical data for benchmark products** regarding spheroid size, DNA structure, mitochondrial mass, mitochondrial membrane potential (MMP), glutathione (GSH) and ATP levels show similar readouts on BIOFLOAT™ surfaces and benchmark 1. Changing the spheroid system allows for more consistency and stable handling but had no influence on the absolute readout in this assay panel. All parameters were evaluated by microscopy and life stain imaging.

Conclusion

The goal of this development was to generate a coating solution, which reproducibly passivates plastic and glass surfaces, independently of the geometry. The BIOFLOAT™ FLEX coating solution provides a high flexibility since it stably adsorbs to a broad range of materials without any special coating procedure. The instant adsorption process accelerates the handling and allows for applications, which only require short pipetting steps or passing by the liquid in a flow as given for microfluidic devices or flow cells. At the same time, the coating does not influence the geometry of the device. Although the coating consists of a physically adsorbed monomolecular layer, it is highly robust and resistant to stress factors such as scratching and media changes.

Surfaces coated with BIOFLOAT™ FLEX coating solution are highly cell-repellent and therefore ideally suited for the 3D cell culture of spheroids. In contrast to other low-adhesion

surfaces, single, round and compact spheroids are reliably formed on BIOFLOAT™ FLEX coated surfaces without any remaining interaction points on the surface. Knowing that every well generates such spheroids in each experiment reduces the variably of results and provides reliable data. Most importantly, the rapid generation of spheroids saves time and demonstrates the homogeneous passivation of the surface.

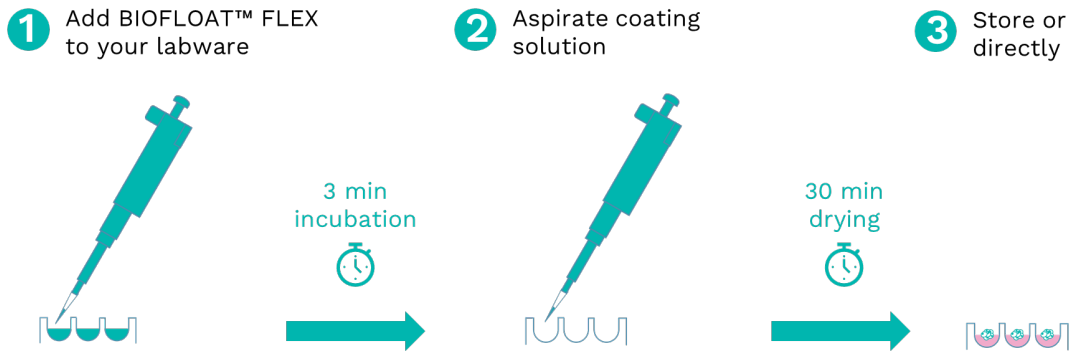
Methods

Quartz-Crystal Microbalance

The surface adsorption (SA) was determined by a Quartz-Crystal Microbalance with dissipation monitoring (QCM-D, a special embodiment of the QCM method) which measures the resonance frequency of a freely oscillating quartz crystal after excitation. The shift in resonance frequency scales inversely proportionally with mass changes at the quartz surface. The SA was calculated from the shift of the 7th overtone of the resonance frequency according to the method of Sauerbrey. The Q-Sense E4 (Biolin Scientific Holding AB) operating system has a mass sensitivity of about 2 ng/cm2. QCM measurements were performed using standard flow-through methods with a flow rate of 50 µL/min at 23°C.

Coating procedure

BIOFLOAT™ FLEX coating solution was pipetted into each well (100µl per well) of a U-shaped 96 well plate and incubated at room temperature for 3 min. After incubation, the solution was removed using a standard pipette. After air-drying for 30 minutes for at room temperature, within the laminar flow hood, the plate was immediately used or stored at room temperature for later use.



Spheroid analysis

The adherence and spreading behavior of the cells was evaluated using a murine fibroblast cell line, namely BALB- 3T3 (3T3) cells clone A31 (ECACC; Lot No. 03L010, passage 82). Cells were maintained in Dulbecco's Minimal Essential Medium (sourced from Biochrom), supplemented with Newborn Bovine Calf Serum (10 v/v%), 4 mM Glutamine and 1% Penicillin/Streptomycin (PAN Biotech) in T75 flasks with 20 mL medium at 37°C in a 95% humidified atmosphere containing 5% CO_2 to a confluence of 80-90 %. For the analysis of adherence on different polystyrene surfaces, semi-confluent 3T3 cells from passage 6-15 were trypsinized using Trypsin/EDTA (0,05%/0,02%) in PBS (PAN Biotech), incubated at 37°C and 5% CO_2 and seeded at approximately 30.000 cells/mL using 200µl per well into polystyrene-based 96 multi well plates.

The morphology was characterized using an automated light microscope (IncuCyte System, Sartorius) with a 10x objective, phase contrast and photo documentation taking strictly defined 16 pictures per well every 8 hours. Quantitative analysis of cell adherence was done by means of automatic microscopy and image analysis using corresponding software of the automated microscope (IncuCyte S3 2018B, confluence mask) three days after seeding.

Life stain

Analysis was done in collaboration with a service provider specialized for high content imaging. Protocol in short: for characterization DNA structure, mitochondrial mass, mitochondrial membrane potential (MMP), glutathione (GSH) and ATP levels cells were seeded into ultra-low adhesion 96-well black walled clear bottomed spheroid microplates. At the end of the incubation period, the spheroids were loaded with the relevant dye/antibody for each cell health marker. The plates were then scanned using an automated fluorescent cellular imager, ArrayScan® (Thermo Scientific Cellomics).

References

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