A novel polymer solution to generate ultra-low cell attachment surfaces and highly uniform spheroids in 3D primary cell cultures

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Introduction

It is well known that primary cell cultures provide physiologically more relevant data compared to immortalized cell lines, primary cell cultures maintain many of the important cell markers and phenotypes observed *in vivo* (1). However, once isolated, primary cells are usually cultivated in an artificial environment on plastic or glass substrates where they grow as a 2D layer of cell sheets. Limitations of 2D cultures, such as altered morphology and behavior, are increasingly becoming recognized. Therefore, different systems are being studied and established to culture primary cells, for example as 3D spheroids (2).

While there are different strategies to form cell spheroids, low attachment surfaces have the advantage of being chemically defined and can be conveniently handled and scaled (3). However, the residual cell-material interaction of the cells with the plastic or glass surfaces influences the morphology and behavior of the spheroids.

In this approach, we have cultured primary hepatocyte cell spheroids on 'faCellitate BIOFLOAT™' coated U-bottom plates and compared it to other state-of-the-art, ultra low attachment plates (ULA). Interestingly, the excellent cell-repellent nature of the 'BIOFLOAT™' surface enabled the rapid formation of primary cell spheroids with well-defined geometry and consistency. Additionally, the BIOFLOAT™ system made it possible to form spheroids on difficult primary cell types that did not form on any other low attachment surface.

Materials and Methods

Spheroid culture of primary hepatocytes

Cryopreserved Cynomolgus hepatocytes (Primacyt, Lot CH140707) were seeded into different benchmark surfaces and faCellitate BIOFLOAT™ 96-well U-bottom plates at a density of 2500 viable cells per well in 3D-HMM (3D-Hepatocyte Maintenance Medium, PRIMACYT).

Directly after seeding, the plates were centrifuged at 500 x g for 2 min. The spheroid cultures were monitored daily using phase contrast microscopy.

Cryopreserved primary human hepatocytes (Lot CH140707) were seeded into benchmark ultra low attachment surfaces and faCellitate BIOFLOAT™ 96-well U-bottom plates at a density of 2500 viable cells per well. Subsequently after seeding, cells were centrifuged at 92 x g for 5 min. Cells were monitored daily using phase contrast microscopy. The cells were cultivated in 100 µL/well Williams E medium until the spheroids were formed. After spheroid formation, 50 µL of the medium was replaced every 48-72 h by 50 µL FBS-free medium.

CYP induction and quantification of metabolites

Monooxygenase activity (Cytochrome P450) was induced for 72 h from day 6 till day 9 in culture with a cocktail of the common inducers, Phenobarbital (CYP2B17 Cynomolgus isoform for human CYP2B6), ß-Naphthoflavone (CYP1A1/2) and Rifampicin (CYP3A8 Cynomolgus isoform for human CYP3A4). After induction, reference CYP substrates (Acetaminophen, Bupropion, Midazolam) were applied for 4 h. The supernatants were harvested for metabolite analysis by LC-MS/MS. Spheroids were lysed for cell harvesting and protein quantification. The metabolites were normalized to protein content.

Cell viability test

The cell viability was assessed by using the luminescence assay CytoTox-Glo ® Cytotoxicity Assay (Promega) according to the manufacturer's protocol for 2D monolayers and optimized for the 3D cell culture. The assay was performed at day 8 and day 11 after spheroid formation by removing 50 µL medium from each well and adding 50µL CytoTox-Glo ®-Reagent to achieve a 1:1 dilution. The contents were mixed for 2 minutes on an orbital shaker to lyse the cells, followed by an incubation at room temperature for 10 minutes. The luminescent signal was detected using the luminometer LUMIstar Galaxy.

Results

For the characterization of spheroids, primary Cynomolgus hepatocytes were cultured in BIOFLOAT™ coated plates and compared to two other benchmark surfaces.

Already during seeding and during the first 24 h in culture, differences between benchmark 2 and BIOFLOAT™ surfaces were observed. Whereas in the BIOFLOAT™ system the cells grouped into the central part of the wells, benchmark 2 showed significantly less central accumulation and multiple aggregations of cells. (Fig. 1)

While a compact cell spheroid was formed within three days on BIOFLOAT™ surfaces and benchmark 1, a compact aggregation of cells leading to an irregularly shaped spheroid was only observed after nine days for benchmark 2.

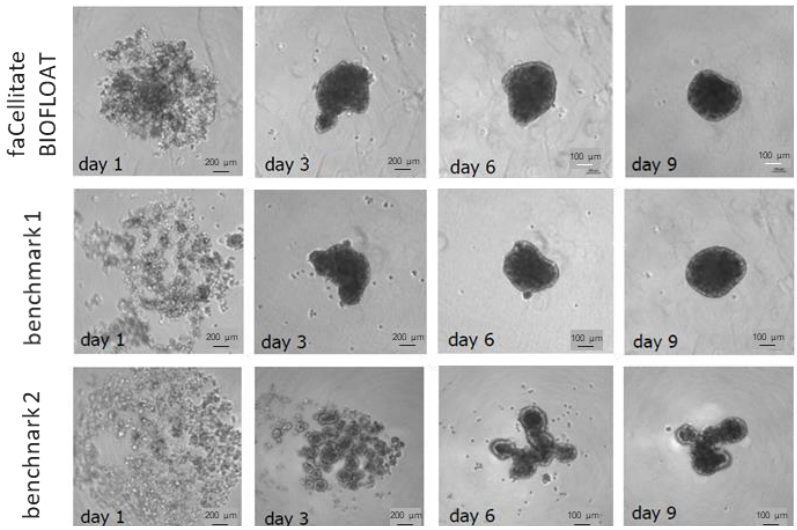

Fig 1 Spheroid formation of primary Cynomolgus hepatocytes

Functional data of the hepatocyte spheroids were collected by testing their accessibility to compounds and their metabolic activity using a range of different P450 monooxygenases, such as CYP1A1/2, CYP2B17 and CYP3A8. In the absence of inducer cocktail, all spheroids demonstrated a similar robust basal enzyme activity, independent of the culture surface. However, after compound application, a 1.5 - 2.5-fold induction of all CYP activities was found for spheroids grown on the BIOFLOAT™ surface. (Fig 2)

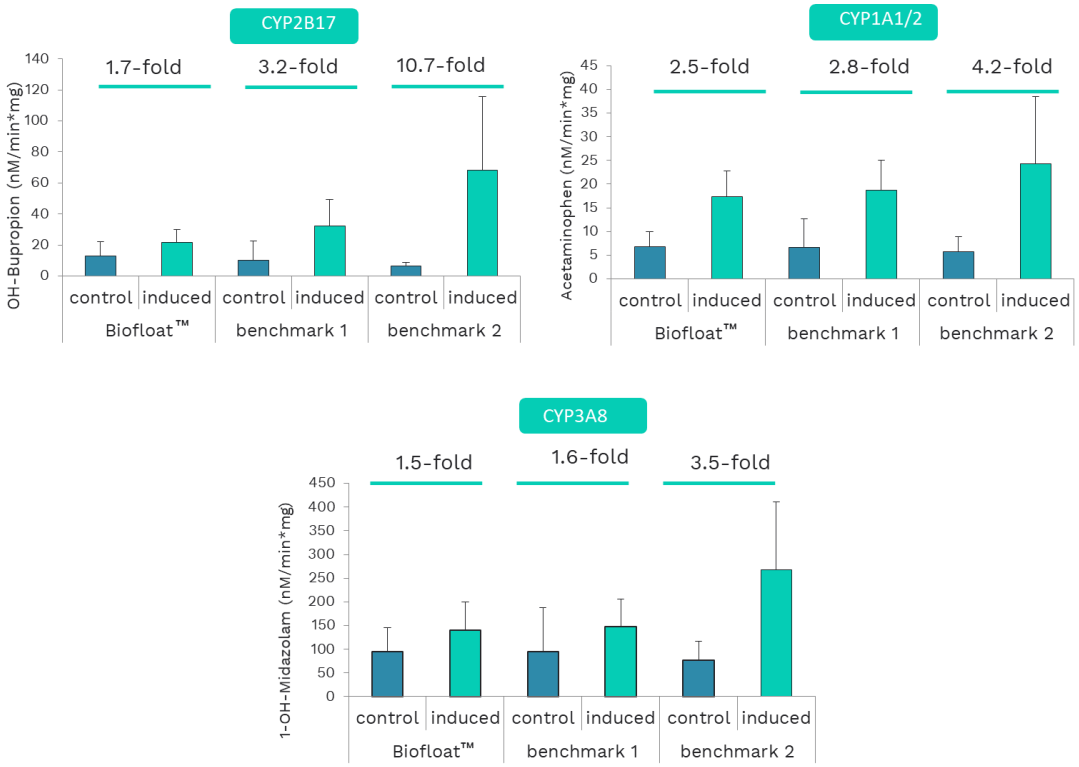

Fig. 2 Characterization of hepatocytes based on basal and induced CYP activity

The range of induction activity was similar to spheroids grown on benchmark 1. Interestingly, a significantly stronger induction was observed for the unshaped aggregates grown on benchmark 2, ranging from 3.5-fold for CYP3A8 to 10.7-fold for CYB2B17.

The observed differences in CYP activity obviously reflect the difference in surface and accessibility for the cells towards the inducer. On a fully inert surface, such as BIOFLOAT™-coated substrates, cells grow fully round and compact, with limited accessibility towards drugs. In contrast, interaction and surface adhesion on plates with lower quality may lead to irregularities in shape, resulting in increased surface area of the spheroid.

The system was further challenged by using primary human cells which had not formed spheroids in earlier experiments on benchmark ULA plates (benchmark 1). The primary human hepatocytes were seeded on plates coated with BIOFLOAT™ and on benchmark 1 plates, which had performed similarly in the previous experiments with Cynomolgus hepatocytes. However, while cells on benchmark surfaces formed either irregular shaped spheroids or multiple independent aggregates, highly defined circular cell spheroids were generated on BIOFLOAT™ substrates (Fig. 3).

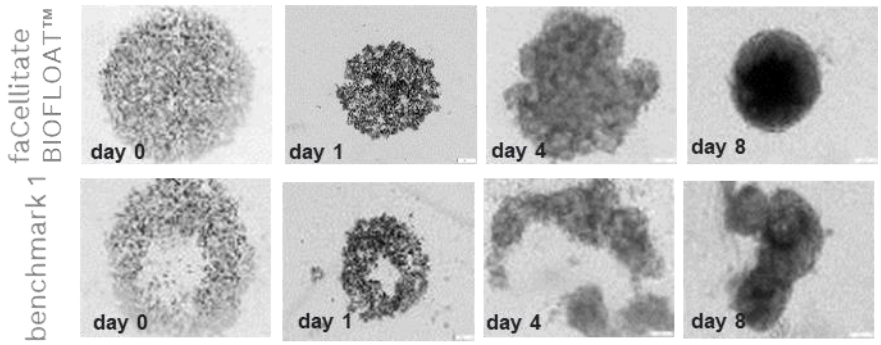

Fig 3 Spheroid formation of primary human hepatocytes.

A luminescent assay was used to assess cellular viability and cytotoxicity, which can result from a loss of membrane integrity. While clear differences in the cell morphology were observed earlier, no significant differences could be detected in the ATP level, indicating a comparable fitness of the cells in both plates (Fig. 4).

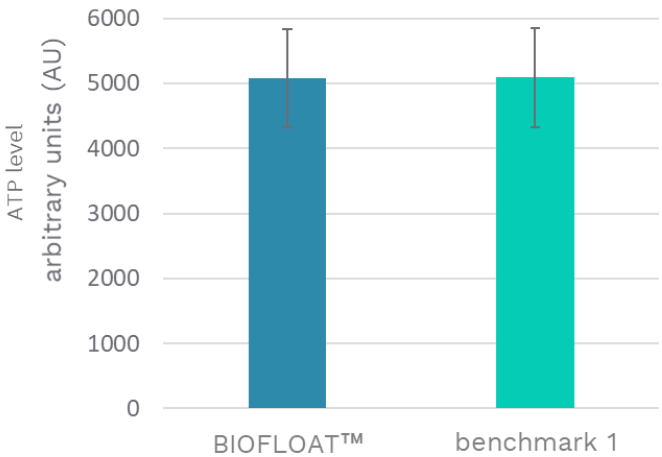

Fig 4 Characterization of the hepatocytes based on CYP activity

Summary

There is a range of cell culture products available on the market with low binding properties, which are promoted for growing spheroids from primary cells. In this study, we compared the state-of-the-art products against a novel self-coating formulation – 'faCellitate BIOFLOAT™´, which generates highly defined and cell-repelling surfaces. All products enabled the cultivation of spheroids, however, clear differences were observed in the morphology and kinetics of spheroid formation on the different surfaces. In contrast to BIOFLOAT™ surfaces, pre-coated benchmarks did form spheroids, which were less regular in shape and needed more time to form a compact aggregate. This is likely due to remaining adhesion points and interactions of the primary cells with areas on the plastic surface that were not passivated. Furthermore, the observed variability of spheroid quality within different experiments or wells on common state-of-the-art products is a critical variable when performing routine testing using spheroids. Hence, one should carefully choose the growth substrate as this has a major impact on the quality of the cell aggregates and thereby may influence its physiological properties. In contrast, a continuous quality of size and shape was observed for spheroids on BIOFLOAT™ surfaces for all tested primary cells, which makes them premium candidates for spheroid culture of primary cells.

References

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