RESEARCH ARTICLE

Insulin-dependent glucose consumption dynamics in 3D primary human liver cultures measured by a sensitive and specific glucose sensor with nanoliter input volume

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Abstract
The liver plays a central role in glucose homeostasis and hepatic insulin resistance constitutes a key feature of type 2 diabetes. However, platforms that accurately mimic human hepatic glucose disposition and allow for rapid and scalable quantification of glucose consumption dynamics are lacking. Here, we developed and optimized a colorimetric glucose assay based on the glucose oxidase-peroxidase system and demonstrate that the system can monitor glucose consumption in 3D primary human liver cell cultures over multiple days. The system was highly sensitive (limit of detection of 3.5 µM) and exceptionally accurate (R² = 0.999) while requiring only nanoliter input volumes (250 nL), enabling longitudinal profiling of individual liver microtissues. By utilizing a novel polymer, off-stoichiometric thiol-ene (OSTE), and click-chemistry based on thiol-Michael additions, we furthermore show that the assay can be covalently bound to custom-build chips, facilitating the integration of the sensor into microfluidic devices. Using this system, we find that glucose uptake of our 3D human liver cultures closely resembles human hepatic glucose uptake in vivo as measured by euglycemic-hyperinsulinemic clamp. By comparing isogenic insulin-resistant and insulin-sensitive liver cultures we furthermore show that insulin and extracellular glucose levels account for 55% and 45% of hepatic glucose consumption, respectively. In conclusion, the presented data show that the integration of accurate and scalable nanoliter glucose sensors with physiologically relevant organotypic human liver models enables longitudinal profiling of hepatic glucose consumption dynamics that will facilitate studies into the biology and pathobiology of glycemic control, as well as antidiabetic drug screening.

KEYWORDS
glucose disposition, insulin resistance, off-stoichiometric thiol-ene, organotypic cell culture, thiol-Michael addition reaction, type 2 diabetes

Abbreviations: BSA, bovine serum albumin; FBS, fetal bovine serum; GOx, glucose oxidase; HRP, horseradish peroxidase; HTS, high-throughput screening; LOD, limit of detection; LOQ, limit of quantification; NAFLD, nonalcoholic fatty liver disease; OSTE, off stoichiometric thiol-ene; PBS, phosphate buffered saline; PHH, primary human hepatocytes; qPCR, quantitative polymerase chain reaction; RIM, reaction injection molding; SD, standard deviation; SEM, standard error of the mean.

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1 | INTRODUCTION

The liver plays a central role in the maintenance of glycemic homeostasis. As soon as plasma glucose level rises, for example, postprandially, the increasing insulin levels stimulate hepatic glucose uptake. In contrast, during times of fasting and starvation, the liver releases glucose to maintain blood glucose levels and critical energy supply to other organs. While insulin normally stimulates glucose consumption, glycogen synthesis and lipogenesis, in insulin resistant livers gluconeogenesis is only insufficiently repressed, whereas at the same time lipid synthesis is retained.\textsuperscript{1,2} This pathway selective insulin resistance results in hyperglycemia and hypertriglyceridemia and constitutes a central defect in type 2 diabetes.\textsuperscript{3,4}

To obtain a deeper understanding of the biology and pathobiology of glucose homeostasis and glycemic control, mice constitute most widely used model. However, rodents and humans differ drastically in glycemia, lipidemia, and insulin resistance, as defined by a subnormal biological response to normal insulin concentrations,\textsuperscript{5} resulting in notable limitations and caveats.\textsuperscript{6,7} These inter-species differences impair result translatability and necessitate the use of alternative or complimentary studies in human systems. Yet, measurement of insulin sensitivity and insulin-stimulated glucose utilization in humans requires hyperinsulinemic euglycemic clamps, which are highly labor- and time-intensive,\textsuperscript{8} or the use of surrogate markers based on the quantitative insulin sensitivity check index or homeostasis model assessment, which are simple but require robust input data and careful interpretation.\textsuperscript{9,10} The ability to measure insulin resistance and glucose homeostasis in a more accessible and, importantly, physiologically relevant tissue model of human liver is thus essential to facilitate mechanistic studies of human glucose disposition.

Three-dimensional (3D) culture systems have become increasingly accepted in recent years as the new in vitro model standard, as scientists gradually moved away from traditional monolayer culture platforms and towards organotypic culture paradigms.\textsuperscript{11,12} Particularly for human liver models, 3D culture methods have dramatically extended the functional live span. Whereas primary human hepatocytes (PHH) in conventional monolayers rapidly lose their molecular phenotype\textsuperscript{13} and exhibit metabolic aberrations, including mitochondrial dysfunction, perturbed AMPK signaling and a shift toward glycolysis,\textsuperscript{14} in 3D culture they retain their transcriptomic, proteomic and metabolic configuration, as well as their functionality for at least 5 weeks in 3D culture.\textsuperscript{14-17} However, whether these 3D liver models can accurately recapitulate glucose utilization and serve as useful models for human liver glucose disposition remained unclear.

While a multitude of electrochemical, fluorescent and colorimetric assays for glucose measurements have been developed,\textsuperscript{18-20} the vast majority of these are intended for blood glucose quantifications and require sample preparation, instrumentation or input volumes that are incompatible with the time-lapse monitoring of scalable tissue models for which only very limited material is available. In addition, most in vitro sensors require the utilization of fluorescent or radio-labeled glucose analogues, which require cell lysis for uptake quantification, rendering them inapplicable for longitudinal measurements of the same cell population.\textsuperscript{21} Other methods, such as electrochemical-based glucose sensors (such as those utilized in finger-prick test strips) and high-performance liquid chromatography are not amenable for rapid turnover and high-throughput analysis.

By contrast, glucose quantification based on enzymatic assays with a colorimetric output coupled to optical high-throughput absorbance measurements constitutes a rapid and facile means for glucose quantification. To be useful for longitudinal measurements of glucose metabolism in 3D human microtissues, the assay of choice needs to be accurate and precise in cell culture media, and have a high sensitivity to detect small fluctuations in glucose concentrations despite low input volumes. Furthermore, the system should be sufficiently stable and allow for repeated and dynamic measurements for multiple days.

Here, we developed and calibrated a customized glucose sensor based on the enzymatic Saifer-Gerstenfeld method that allows the measurement of glucose consumption in 3D primary human liver cell cultures over multiple days at physiologically relevant concentrations. The assay can detect glucose uptake from minute quantities of cells and retains high specificity and sensitivity in culture medium using as little as 250 nL input volume. Using surface functionalization and thiol-Michael addition reactions, we covalently bound the assay enzymes to custom-build devices fabricated using reaction injection molding of the novel polymer OSTE (off-stoichiometric thiol-ene), allowing for longitudinal on-chip glucose measurements. Using this sensor, we quantify glucose uptake dynamics in 3D liver cultures and find that glucose consumption closely resembles hepatic glucose uptake in humans in vivo measured by euglycemic-hyperinsulinemic clamp. By comparing isogenic insulin-sensitive liver spheroids with spheroids conditioned in high insulin or our previously developed insulin-resistant NAFLD model,\textsuperscript{22} we quantify the importance of hormonal regulation and show that insulin controls approximately 55% of hepatic glucose consumption while 45% are driven by extracellular glucose levels. We conclude that the system presented here provides a facile, accessible, and scalable platform for the long-term monitoring of glucose consumption in primary human 3D tissue cultures,
which opens new avenues for the study of glucose homeostasis and the screening for antidiabetic medications.

2 | MATERIALS AND METHODS

2.1 | Enzymatic glucose quantification

Glucose concentrations were quantified based on the enzymatic Saifer-Gerstenfeld method, which utilizes glucose oxidase (GOx) and horseradish peroxidase (HRP) to oxidize a chromogen. Instead of o-dianisidine, 4-aminocinapyrine, or o-Phenylenediamine as electron donor, we used 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as chromogen due to its higher extinction coefficient and favorable toxicity profile. GOx and HRP were obtained from myBioSource (USA; cat #: MBS537543) and Sigma-Aldrich (Sweden; cat #: S5512), respectively. Unless otherwise indicated, ratios of enzymes in the reaction were 1:100 of GOx:HRP. Both GOx and HRP were conjugated to streptavidin to allow for surface immobilization. ABTS+ formation was monitored by measuring 2 minutes interval absorbance at 420 nm (A420) on a Synergy2 microplate reader (BioTek Instruments). Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as average of enzymatic reaction rate for the first t minutes from A420 data as follows:

\[
\text{Activity (AU*min}^{-1}} = \frac{\sum_{i=1}^{n}(A_{420nm,t_i} - A_{420nm,s_0})}{2n} \times 100.
\]

where n is the number of data points, excluding the measurement at t0 and A420nm,t is the absorbance at 420 nm at time point t (in minutes). Absorbance readings were performed on a Synergy™2 microplate reader (BioTek Instruments). Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as LOD = \( \frac{3\sigma_0}{m} \) and LOQ = \( \frac{10\sigma_0}{m} \) with m as the slope of the standard curve, obtained using linear regression and \( \sigma_0 \) as the standard deviation of blank measurements with 0 mM D-glucose (n = 8).

2.2 | Analysis of enzyme kinetics

Activity was calculated as average of enzymatic reaction rate for the first t minutes from A420 data as follows:

\[
\text{Activity (AU*min}^{-1}} = \frac{\sum_{i=1}^{n}(A_{420nm,t_i} - A_{420nm,t_{i-1}})}{2n} \times 100.
\]

where n is the number of data points, excluding the measurement at t0 and A420nm,t is the absorbance at 420 nm at time point t (in minutes). Absorbance readings were performed on a Synergy™2 microplate reader (BioTek Instruments). Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as LOD = \( \frac{3\sigma_0}{m} \) and LOQ = \( \frac{10\sigma_0}{m} \) with m as the slope of the standard curve, obtained using linear regression and \( \sigma_0 \) as the standard deviation of blank measurements with 0 mM D-glucose (n = 8).

2.3 | On-chip glucose sensing

An aluminum mold was micromachined using a micromilling machine (Minitech Machinery Corp., USA). The mold featured nine pillars with a diameter of 7 mm, depth of 5 mm, and interspacing of 18 mm inside a rectangular cavity (61 × 59 cm²). OSTE polymer (OSTEMER 322) was purchased from Mercene labs, Sweden. The polymer precursor was then mixed, degassed and prepared for the reaction injection molding (RIM) process, which was performed according to the established OSTE-RIM process. Following the injection and UV curing of the polymer precursor inside the aluminum mold, the replica was demolded and the polymerized OSTE chip with replicated wells were then subject to surface modification using 10 mM maleimide-PEG2-biotin (ThermoFisher, Sweden) solution in PBS.

2.4 | Cell culture

Primary human hepatocytes from two donors were obtained commercially from BioIVT with available informed written consent for all donors. 3D liver spheroids were cultured as previously described. In short, primary human hepatocytes were thawed according to the supplier’s instructions and seeded into ultra-low attachment 96-well plates (Corning) at 1500 viable cells per well unless otherwise stated. Cells were seeded in 100 µl culture medium (Williams E medium with 11 mM glucose supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5.5 µg/mL transferrin, 6.7 ng/mL sodium selenite, 100 nM dexamethasone, and 10% FBS) containing either (a) 0.1 nM (LI), (b) 1.7 µM insulin (HI; conventional insulin cell culture concentration) or (c) 1.7 µM insulin, as well as 480 µM free fatty acids (1:1 mixture of oleic and palmitic acid) and 5 mM fructose, as indicated. Spontaneous self-aggregation of the hepatocytes occurred and after few days a single spheroid could be observed per well. When the spheroids were sufficiently compact, serum was phased out.

2.5 | Cell viability assay

Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Sweden) according to manufacturer’s instructions. Briefly, individual spheroids were disrupted in 25 µL of the supplied buffer followed by incubation at 37°C in 5% CO₂ for 20 minutes. Subsequently, luminescence was read using a MicroBeta LumiJET 2460 Microplate Counter (Perkin Elmer, USA).

2.6 | Intracellular lipid quantitation

Intracellular lipid accumulation was quantified using the AdipoRed Adipogenesis Assay Kit (Lonza, Switzerland) following the manufacturer’s instruction. Briefly, 3.5 µL of AdipoRed Assay Reagent was added to trypsinized
single spheroids. After 10–15 minutes incubation in the dark, spheroids were disrupted by vigorous pipetting followed by fluorescence measurement (Ex/Em: 485/572) in a whole-plate fluorometer (Spectra Max Gemini, Göteborgs Termometerfabrik, Sweden).

### 2.7 Glucose consumption assays in organotypic 3D liver cultures

For glucose consumption experiments, culture medium was changed to medium containing glucose and hormone concentrations based in part on our previous work as follows: (a) 11 mM glucose + 1.7 μM insulin (HI + HG), (b) 11 mM glucose + 0.1 nM insulin + 100 nM glucagon (LI + HG), (c) 5.5 mM glucose + 1.7 μM insulin (HI + LG) and (d) 5.5 mM glucose + 0.1 nM insulin + 100 nM glucagon (LI + LG). The concentration of 0.1 nM insulin resembles physiological fasting insulin levels, while 1.7 μM corresponds to concentrations in conventional cell culture media. At the indicated time points, 10 μL of medium supernatant and 80 μL of HRP-ABTS mix were mixed and the reaction was initiated by addition of 10 μL GOx solution to a final stoichiometry of 1:100 GOx:HRP and 2mM ABTS in duplicates. The glucose uptake per cell was calculated as $C_t \cdot V_t \cdot F/V_c$, where $C_t$ is the initial glucose concentration in the culture media, $V_t$ is the remaining volume of culture media at time point $t$, $V_c$ is the initial volume of culture media, $n$ is the number of cells per spheroid and $V_F$ is the volumetric factor of the considered spheroids.

### 2.8 Human in vivo liver glucose uptake

Human liver glucose uptake was measured from 326 non-diabetic individuals as 22.4 ± 9.2 μmol × kg$^{-1}$ × m in$^{-1}$ during the euglycemic-hyperinsulinemic clamp. For human liver, we assumed a range of cellularity from 0.6 × 10$^{11}$ to 1.9 × 10$^{11}$ cells × kg$^{-1}$ × m in$^{-1}$, resulting in an uptake of 0.2 fmol × cell$^{-1}$ × min$^{-1}$ (range: 0.07 to 0.53 fmol × cell$^{-1}$ × h$^{-1}$) or 12 fmol × cell$^{-1}$ × h$^{-1}$ (range: 4.2 to 31.8 fmol × cell$^{-1}$ × h$^{-1}$).

### 2.9 Gene and protein expression analysis

Total RNA was extracted from pooled (n = 32-48) spheroids. Following cDNA synthesis from at least 100 ng of RNA, expression levels were analyzed by qPCR using Taqman probes against PCK1, G6PC, FASN, FOXO1, MLXIPL (ChREBP), SREBF1 (SREBP), and GAPDH (Table S1). Gene expression was calculated using the ΔΔC_T method using GAPDH as endogenous reference. Insulin response on protein level was evaluated by exposing insulin sensitive or insulin resistant 3D liver cultures to high insulin levels (1.7 μM) for 7 minutes. Subsequently, 32-48 spheroids per sample were pooled and lysed in RIPA buffer supplemented with protease inhibitor (complete Tablets EASYpack, Roche) and phosphatase inhibitor cocktail (PhosSTOP, Sigma). The protein blots were blocked with 5% (w/v) bovine serum albumin (BSA) in TBS with 0.1% Tween-20 for 1 hour at RT, then, incubated either overnight at 4°C or 1 hour at RT with antibodies against pAKT$^{Ser473}$ (4060T, Cell Signaling Technology) and vinculin (129002, Abcam).

### 2.10 Statistical analysis

Statistical analyses were conducted in Prism version 6.01 (GraphPad Software, USA). Details about the respective statistical tests used for each experiment are provided in the corresponding Figure captions. In all tests, $P < .05$ was considered statistically significant.

### 3 RESULTS

#### 3.1 Calibration of a sensitive and specific enzymatic glucose sensor based on the Saifer-Gerstenfeld method

We quantified glucose levels based on a two-step enzymatic reaction using GOx and HRP. In presence of atmospheric oxygen, D-glucose is oxidized by GOx into D-gluconolactone and hydrogen peroxide. HRP couples the reduction of H$_2$O$_2$ to the oxidation of the chromogen ABTS, resulting in the production of two chromogenic ABTS•+ radicals with maximum absorbance ($λ_{max}$) at 420 nm per molecule of hydrogen peroxide (Figure 1A).

To optimize reaction efficiency, we first titrated the stoichiometry of GOx and HRP. We thus initially tested a GOx:HRP ratio of 1:20 based on available reaction parameters. However, our titration experiments showed that at this enzymatic ratio, signals from 10 to 20 mM glucose samples were not adequately resolved (Figure 1B). Based on these findings, we increased the ratio to 1:100 of GOx to HRP, resulting in markedly improved signal resolution (Figure 1C). Since our aim was to develop an assay, which yields linear signals across the physiologically relevant glucose range, that is, up to 20 mM of glucose, this enzyme stoichiometry was used in further experiments.

To evaluate the specificity of the reaction, we compared enzymatic activity of D-glucose to various structurally similar monosaccharides with identical molar mass, including the L-enantiomer of glucose, D-galactose, and D-fructose.
Notably, even at 10-fold higher concentrations of these sugars, assay activity was <5% compared to D-glucose. Activity was similarly low for sucrose, a disaccharide of D-glucose and D-fructose. These data suggest that the assay is highly selective for D-glucose and results in only negligible activity using other nutritionally relevant carbohydrates under the condition presented used in this study.
3.2 Reaction kinetics allow for accurate long-term monitoring and high-throughput screening of glucose levels

Next, we quantified the reaction kinetics. Responses were highly linear at lower glucose concentrations (0-2.5 mM; R² ≥ 0.96), whereas signals saturated at higher glucose levels (>5 mM) irrespective of assay time (Figure 1E). Based on these results we concluded that media with glucose levels in the physiological or pathophysiological range (0.5-20 mM) can be diluted 10-fold to achieve linear response kinetics, which allowed to drastically reduce the required input volumes. To evaluate the suitability of the assay for long-term monitoring of glucose levels, we tested enzymatic stability. Importantly, GOx and HRP maintained enzyme activity at 25°C and their metabolic rates did not significantly differ from enzyme stored at 4°C (P = .58), suggesting that enzymatic stability supports longitudinal profiling of glucose concentrations for at least 1 week (Figure 1F).

A common problem when performing enzyme-based colorimetric assays in a high-throughput screening (HTS) format is the “lagging sample effect” where reactions in the last plate of a stack of sample plates or the last column on a multiwell plate (ie, “the lagging samples”) are initiated later than in the first plates or columns (ie, “the leading samples”) due to the time required for plate handling and pipetting. When the enzymes employed have high catalytic activity, this poses a significant problem that can affect the accuracy of the analyses. To determine whether the lagging sample effect might affect our glucose sensor, we compared the measured activity of lagging and leading samples with identical predefined glucose concentrations. Importantly, end-point absorbance readings underestimated the concentration of the lagging samples by 50% (slope = 0.5; R² = 0.96), whereas the activity of leading and lagging samples had a strikingly linear correlation (slope = 0.99; R² = 0.993; Figure 1G). These data demonstrate that under optimized conditions in the linear activity range, the use of kinetic parameters (activity) of the enzymatic cascade instead of static end-point absorbance measurements eliminates lagging sample effects for the presented glucose assay, thus, enabling robust HTS applications.

Importantly, when testing the optimized and calibrated assay using 10 µL of media with defined glucose concentrations, the measured glucose values were highly correlated with glucose inputs, explaining 99.7% of experimental glucose variability (Figure 1H-i). Furthermore, even when reducing input volumes 40-fold to 250 nL excellent accuracy was retained (R² = 0.999) with a LOD and LOQ of the assay of 3.5 and 12 µM glucose, respectively (Figure 1J). We conclude that our modified Saifer-Gerstenfeld method provides highly accurate readouts across the physiological and pathophysiological range of glucose concentrations (1-20 mM) with nanoliter amounts of sample input.

3.3 Design of a high-density on-chip glucose sensor module using thiol-Michael addition reactions

We sought to use the established assay to develop an on-chip glucose sensor module that would allow its integration into microfluidic devices. To achieve this aim, we fabricated polymer devices featuring reactive surfaces using reaction injection molding of OSTE. After the UV curing process, the reactive thiol surface of the polymer enables a wide range of applications for device integration and surface modification. Here, we used maleimide tags to covalently bind biotin moieties to the OSTE surface via thiol-Michael addition reactions.

This, in turn, enabled tight binding of streptavidin-conjugated GOx and HRP using biotin-streptavidin affinity chemistry, for quantitative on-chip glucose sensing (Figure 2A).

First, we assessed surface occupancy. To this end, we incubated the biotinylated OSTE surface with 10 nM (1 pmol enzyme), 100 nM (10 pmol enzyme), and 1 µM (100 pmol enzyme) of streptavidin-conjugated enzymes while maintaining the ratio of GOx to HRP ratio at 1:100. After 30 minutes incubation, the unbound enzyme was
removed and its activity was measured. Notably, unbound activity was below the detection limit when 10 or 100 nM of enzyme (GOx + HRP) was added, whereas addition of 1 µM resulted in a residual activity of 64.7% ± 6.3% (n = 6; Figure 2B). These data suggest that approximately 35%, that is, 35 pmol of enzyme were surface-bound, corresponding to 0.7 pmol of biotin-conjugated enzyme per mm² of OSTE surface.

On-chip, the bienzymatic reaction cascade had a $V_{\text{max,chip}}$ of 28 nM × sec⁻¹, which is close to previously suggested $V_{\text{max}}$ values for HRP of 32.7 nM × sec⁻¹ when analyzed in solution. Combined, these data suggest that thiol-Michael addition chemistry can be used to develop an on-chip glucose sensor on functionalized OSTE surfaces without impacting assay performance.

### 3.4 Scaling of glucose consumption with spheroid size

Following the establishment and calibration of the assay, we aimed to use the glucose sensor to profile long-term glucose consumption with spheroid size.
uptake in organotypic 3D cultures of primary human liver cells. We first evaluated whether limited mass transport of oxygen and nutrients within the spheroid might impact glucose consumption. To this end, we seeded hepatocyte spheroids of different cell numbers (500, 1500, and 3000 cells) and spheroid formation was observed in all groups after 5 days (Figure 3A). Notably, size distribution analysis showed significantly larger spheroid diameters with increasing cell number at seeding with coefficients of variance <10% for each group (Figure 3B). Once spheroids were formed, we evaluated glucose consumption upon insulin stimulation over the course of 6 hours. Our data demonstrate that total glucose consumption per spheroid scaled linearly with spheroid size for spheroids of up to 1500 cells. However, for larger spheroids (3000 cells) glucose consumption increased only under-proportionally and, consequently, the glucose consumption rate per cell decreased (Figure 3C), in agreement with previous reports. We conclude that 1500 cells constitutes a suitable spheroid size to further investigate glucose consumption dynamics.

3.5 | Modulation of insulin sensitivity in hepatocyte spheroids

Importantly, the use of a scalable and long-term stable culture model allows to modulate insulin sensitivity in vitro, which enables comparisons of isogenic insulin-sensitive and insulin-resistant liver microtissues. We induced insulin resistance by culturing spheroids for 7-14 days in medium containing high insulin concentrations (“insulin resistant group”) or in medium containing high insulin as well as elevated levels of FFA and fructose (“NAFLD group”), and subsequently compared insulin response as well as glucose consumption dynamics with liver microtissues from the same donor cultured in media with physiological insulin

**FIGURE 3** Glucose consumption scales with cell number in spheroids consisting of up to 1500 cells. A, Brightfield micrographs of spheroids formed from 500, 1500, and 3000 cells at the time of seeding (upper row) and after 5 days of aggregation (lower row). Scale bar = 100 µm. B, Spheroid size distribution as a function of cell number is shown for n = 12-24 spheroids per group. C, Total glucose consumption per spheroid increases with cell number. Cell number and glucose consumption scales linearly for 500- and 1500-cell spheroids but not for 3000-cell spheroids. D, Consequently, glucose consumption per cell remains constant in spheroids of 500 and 1500 cells, whereas consumption per cell is reduced in larger spheroids, likely due to limited mass transport. * and **** corresponds to $P < .05$ and .0001 in an unpaired two-tailed heteroscedastic $t$ test
levels (“insulin sensitive group”; Figure 4A). First, we assessed spheroid viability and found no significant difference between treatment groups (Figure 4B). Next, we evaluated intracellular triglyceride accumulation and detected a moderate increase in the insulin resistance group (2.2-fold; \( P < .05 \)), whereas steatosis in the NAFLD model was substantially higher (17.8-fold; \( P < .0001 \); Figure 4C).

In agreement with elevated intracellular lipids, we observed a characteristic hypertrophy and darkening of the spheroid in light microscopic images, characteristic of steatotic cells. In agreement with elevated intracellular lipids, we observed a characteristic hypertrophy and darkening of the spheroid in light microscopic images, characteristic of steatotic cells. 

Notably, conditioning of 3D liver cultures in high insulin alone resulted, if at all, only in a mild induction of genes encoding key enzymes of gluconeogenesis and de novo lipogenesis. The induction of pathway-selective insulin resistance in organotypic 3D human liver cultures (Figure 4A). Alternatively, cells were cultured in high-insulin media with further supplementation of free fatty acids and fructose (“NAFLD”). After conditioning, cells were exposed to different combinations of glucose (HG = high glucose = 11 mM; LG = low glucose = 5.5 mM) and insulin concentrations (HI = high insulin = 1.7 µM; LI = low insulin = 0.1 nM) and insulin response and glucose uptake were measured at the indicated time points. B, Column plot showing cellular ATP levels as proxy for viability. Note that spheroid viability was not affected by the different conditioning protocols. n = 8-16. C, Spheroids conditioned in high insulin show a significant increase in intra-cellular lipids. This steatosis is further exacerbated by exposure to elevated levels of free fatty acids and fructose (NAFLD), as previously reported. n = 20-24. D, Chronic expression changes of candidate genes implicated in insulin resistance after 1-2 weeks of conditioning. n = 3-6. E, Representative Western blot images and densitometry analysis of the corresponding pAKT signals (60kDa) before and 7 min after exposure to insulin are shown normalized to vinculin (124 kDa). Note that pAKT signals in insulin resistant liver cultures did not respond to insulin stimulation in contrast to cells cultured at physiological insulin levels that showed a marked 3.5-fold increase (\( P < .05 \) using a one-tailed unpaired t test). n = 3 F and G, Response in gene expression upon acute insulin exposure (6h). F, In response to acute insulin exposure, expression of the key gluconeogenetic genes \( G6PC \) and \( PCK1 \) is significantly repressed in insulin-sensitive but not in insulin-resistant liver cultures (neither in IR nor NAFLD) compared to baseline expression (dashed line). Statistical tests refer to comparison to IS using Tukey’s multiple comparison t test. n = 3-6 g, the rate-limiting lipogenesis gene \( FASN \) is induced in both insulin-sensitive cells. Notably, levels after stimulation are indistinguishable from insulin resistant cells (both IR and NAFLD), demonstrating pathway-selective insulin resistance in these models. n = 3-6. Data are represented as mean ± SEM. * * * * and **** corresponds to \( P < .05, .01, .001 \), and .0001 in an unpaired two-tailed heteroscedastic t test. n.s. = not significant, as defined by \( P > .05 \).
lipogenesis (<2-fold; \( P > .05 \)), respectively (Figure 4D). In contrast, spheroids in the NAFLD group featured highly significant increases in expression of \( G6PC \) (6.2-fold; \( P < .0001 \)), \( PCK1 \) (6.1-fold; \( P < .0001 \)), and \( FASN \) (13.3-fold; \( P < .0001 \)). Furthermore, we observed significant induction of ChREBP (encoded by the \( MLXIPL \) gene; 7.9-fold, \( P < .0001 \)), the key transcription factor regulating the glucose-dependent induction of genes involved in de novo lipogenesis, including \( FASN \), whereas expression of \( FOXO1 \) and \( SREBF1 \) (SREBP) remained unaffected.

To profile insulin sensitivity directly, we measured acute responses to insulin on the protein and transcript level. Notably upon insulin challenge, insulin sensitive 3D liver cultures exhibited a marked 3.5-fold increase in phosphorylation of AKT, a key step in intracellular insulin signal transduction (\( P < .05 \); \( n = 4 \); Figure 4E). In contrast, insulin-resistant spheroids showed only minor responses to insulin challenge (1.1-fold increase in the IR group; \( P = .66 \); \( n = 4 \)).

On the gene expression level, insulin strongly repressed the gluconeogenetic genes \( PCK1 \) (4.1-fold downregulation) and \( G6PC \) (3.9-fold downregulation) in insulin sensitive spheroids, but its response was significantly blunted in both insulin resistant and NAFLD spheroids (Figure 4F), in the latter despite higher basal expression (compare Figure 4D). By contrast, \( FASN \) expression increased upon insulin challenge.

**FIGURE 5** Long-term profiling of glucose consumption dynamics in insulin-sensitive and insulin-resistant 3D cultures of primary human liver cells. A, Glucose consumption over time under different glucose and hormone levels (see Methods section for details). \( n = 3-6 \). B, Net glucose consumption per cell is shown over the course of 6h. \( n = 3-6 \). For glucose consumption rates at 24 and 72 h, we refer to Figure S3. Two-sided unpaired \( t \) tests were performed pairwise between groups as indicated. C, Differential glucose consumption kinetics in insulin-sensitive versus insulin-resistant groups (IR and NAFLD) at different glucose and hormone levels. Note that the extent of glucose uptake is both glucose and insulin dependent and is overall higher in insulin sensitive 3D liver cultures. \( n = 3-6 \). \( P \)-values refer to one-way ANOVA with post hoc multiple comparison tests vs. IS. D, Pie chart showing the insulin-sensitive and insulin-independent fractions of glucose uptake in primary human hepatocytes. The relative contributions were estimated based on differences in glucose uptake between insulin resistant and insulin sensitive cells. Data are shown as mean ± SEM. *\( P \leq .05 \), **\( P \leq .01 \). HG, high glucose; HI, high insulin; LI, low insulin; LG, low glucose (we refer to the method section for details).
in insulin sensitive cultures whereas expression plateaued in NAFLD spheroids with substantially higher FASN baseline expression (Figure 4G). These results demonstrate that culture of primary human hepatocytes in media with conventional insulin and glucose concentrations (1.7 µM insulin + 11 mM glucose) rapidly desensitize spheroids to insulin, which hampers their utility for metabolic studies. By contrast, insulin sensitivity was retained in culture conditions with physiological insulin and glucose levels (0.1 nM insulin + 5.5 mM glucose).

3.6 The role of insulin in controlling human hepatic glucose consumption

Previous reports strongly suggested that glucose uptake in the liver is to certain degree insulin-independent; however, disentangling the contribution of insulin-dependent and insulin-independent contributions to liver glucose uptake has remained challenging. To establish an association between hepatic insulin sensitivity and glucose uptake, we measured glucose consumption dynamics in isogenic insulin sensitive, insulin resistant and NAFLD spheroids using our calibrated glucose sensor (Figure 5A). In all groups, we detected considerable glucose uptake with a declining slope, indicating that glucose uptake rates decreased over time. Notably, the declining rate of glucose consumption was not caused by reduced cell viability (Figure S1). Moreover, glucose concentrations decreased by <1 mM over the 72 h of measurements, indicating that declining uptake rates were not due to depleted glucose level in the media per se.

Glucose consumption was most sensitive to extracellular glucose levels across all groups. However, also insulin signaling had considerable effects on glucose fluxes. In insulin sensitive cells, physiological insulin concentrations (0.1 nM) were already sufficient to elicit maximal glucose uptake and a further 17 000-fold increase in insulin concentrations to 1.7 µM only resulted in an additional 1% increase in glucose consumption (from 9.9 to 10 fmol × cell⁻¹ × h⁻¹; Figure 5B). At low glucose levels, however, insulin sensitive cells strongly increased glucose consumption in response to insulin stimulation and glucose uptake more than doubled resulting in insulin sensitive cells (from 3 to 6.1 fmol × cell⁻¹ × h⁻¹; +102%).

By contrast, in spheroids conditioned in high insulin, high insulin levels were necessary to cause a significant increase in glucose uptake and insulin increased glucose uptake in both high (11 mM) and low (5.5 mM) glucose conditions by 2.7 and 1.3 fmol × cell⁻¹ × h⁻¹ (38% and 37%), respectively (Figure 5B). Furthermore, insulin effects on net glucose uptake were almost completely abolished in NAFLD spheroids across tested glucose levels. These findings corroborate the observed lack of insulin response at that transcriptional level, which combined demonstrates the severe insulin resistance in these cells. Notably, glucose handling differed quantitatively between donors with insulin resistant liver microtissues of some donors showing net glucose production during early time points (Figure S2).

When analyzing glucose consumption as a function of insulin sensitivity, we found that glucose uptake of insulin sensitive cells was consistently higher with the exception of insulin-resistant microtissues cultured in low insulin and low glucose conditions for which no difference was observed (Figure 5C, Table 1 and Figure S3). These findings are in agreement with the insulin response dynamics measured at the molecular level, which indicate that basal levels of insulin signaling cells were increased in insulin resistant cells (compare Figure 4). The most significant impacts of insulin resistance are seen in high insulin conditions. In normoglycemic media (5.5 mM glucose), average net glucose consumption was reduced from 6.1 fmol × cell⁻¹ × h⁻¹ in insulin sensitive cells to 2.6 fmol × cell⁻¹ × h⁻¹ in NAFLD spheroids (57% reduction; Figure 5B,C). Similarly, insulin-mediated glucose uptake in hyperglycemic conditions was reduced from 10 fmol × cell⁻¹ × h⁻¹ in insulin sensitive cells to 4.7 fmol × cell⁻¹ × h⁻¹ in NAFLD spheroids (53% reduction). Based on these data combined, we estimate that insulin contributes approximately 55% to hepatic glucose uptake while the remaining 45% are insulin-independent and driven by external glucose supply (Figure 5D).

4 DISCUSSION

The liver plays a key role in the disposition of glucose. During fasting, the liver produces glucose by gluconeogenesis (short-term fasting) and glycogenolysis (long-term fasting), whereas the liver has a positive net hepatic glucose uptake postprandially. In insulin resistance, hepatic glucose uptake is significantly decreased. Notably, while glucose transporters in other tissues, such as GLUT4 in skeletal muscle, are insulin sensitive, activity of the predominant hepatic glucose transporters GLUT1 and GLUT2, is not regulated by insulin. However, despite the lack of glucose transporter regulation, overall hepatic glucose uptake is clearly insulin sensitive in humans in vivo, at least in part due to the role of insulin in promoting intracellular glucose utilization and regulating hepatic glucose output through suppression of gluconeogenesis and glycogenolysis, which contributes to the maintenance of a steep glucose gradient and, in turn, facilitates influx. However, the detailed mechanisms underlying this insulin-dependent control of hepatic glucose uptake remain incompletely understood.

To evaluate the relationship between insulin sensitivity and tissue-specific glucose uptake, in vivo studies are considered the gold standard. However, they are technically challenging,
invasive or require the use of positron emission tomography combined with euglycemic-hyperinsulinemic clamps. Moreover, dissecting the contribution of insulin and glucose concentrations in liver glucose uptake in vivo is complicated by the positive feedback loop between glucose level and insulin secretion. To circumvent these problems, there has been increasing interest in modeling glucose disposition and metabolism in cell culture models. Yet, metabolism in cancer cell lines is highly perturbed with proliferation being closely linked to a transition from respiration toward glycolysis. Furthermore, hepatic cell lines exhibit drastically increased basal glucose uptake, changes in lipogenesis and altered insulin response and, consequently, these cell lines cannot serve as relevant models for physiological energy homeostasis. In addition to the choice of cell model, the selection of culture paradigm has major impacts on hepatic metabolism. In the last years, it has become increasingly clear that 3D culture methods support the maintenance of in vivo metabolic features, whereas metabolism in conventional 2D monolayer cultures is highly perturbed. The development of a platform that integrates physiologically relevant human tissue models with sensitive and specific glucose sensing in an accessible and high-throughput compatible format thus constitutes an important tool to study tissue-specific glucose disposition.

A plethora of electrochemical, radiometric and optical glucose detection methods has been developed to quantify glucose concentrations. Electrochemical glucose sensors are commonly used for point-of-care applications and generally require ≥30 µL of sample, which renders them unsuitable for long-term monitoring of glucose homeostasis in microtissue cultures where volumes are low. Similarly, measurement of glucose uptake using radiolabeled-glucose requires cell lysis, which makes this assay incompatible with longitudinal glucose consumption profiling.

Optical assays offer versatility in that they can be tailored to high-content glucose quantifications with relatively lower marginal cost. Here, we optimized an enzymatic colorimetric assay to be highly accurate for measurements of glucose concentrations with an error rate <0.5% across the entire range of physiologically and pathophysiologically relevant glucose concentrations. By titrating enzyme stoichiometries, we calibrated the system so that glucose oxidase would be rate-limiting, which enabled direct correlation between the analyte and the products of the second-step reaction. Using kinetic (activity) instead of static reaction parameters (endpoint absorbance) further eliminated the lagging sample effect, thereby facilitating assay scalability. Moreover, the calibrated assay retained high accuracy (R² = 0.999) with as little as 250 nL of input volume. This accuracy compares well to accuracy recommendations of <5% error rates by the American Diabetes Association, whereas point-of-care blood glucose sensors can exhibit error rates up to 20%, particularly for samples in the at the hypo- and hyperglycemic extremes of the physiological range.

Notably, multiple commercial systems based on the GOx-HRP system are available. However, the LODs of these systems are higher (13-40 µM compared to 4 µM in our assay) and data on assay calibration and performance, such as kinetic parameters and lagging sample effects are not readily available. Furthermore, these systems are not compatible with on-chip

### TABLE 1 Glucose consumption rates of 3D primary human liver cultures in response to glucose and insulin

| Time frame (h) | Spheroid group | HI-HG | LI-HG | HI-LG | LI-LG |
|---------------|----------------|-------|-------|-------|-------|
| 0-6 h         | Insulin sensitive | 10.0 ± 2.4 | 9.9 ± 2.5 | 6.1 ± 1.7 | 3.0 ± 0.8 |
|               | Insulin resistant | 9.7 ± 2.4 | 7.0 ± 2.2 | 4.9 ± 1.2 | 3.5 ± 1.3 |
|               | NAFLD           | 4.7 ± 0.5 | 5.6 ± 0.6 | 2.6 ± 0.5 | 2.9 ± 0.7 |
| 6-24 h        | Insulin sensitive | 3.5 ± 0.7 | 3.5 ± 0.8 | 2.4 ± 1.1 | 1.2 ± 0.3 |
|               | Insulin resistant | 2.9 ± 0.9 | 1.9 ± 0.5 | 1.7 ± 0.3 | 1.5 ± 0.5 |
|               | NAFLD           | 2.3 ± 0.3 | 1.5 ± 1.2 | 0.5 ± 0.8 | 0.9 ± 0.3 |
| 24-72 h       | Insulin sensitive | 1.4 ± 0.4 | 1.3 ± 0.3 | 0.9 ± 0.4 | 0.4 ± 0.1 |
|               | Insulin resistant | 1.0 ± 0.2 | 0.9 ± 0.1 | 0.6 ± 0.1 | 0.5 ± 0.1 |
|               | NAFLD           | 0.8 ± 0.0 | 0.5 ± 0.5 | 0.3 ± 0.2 | 0.3 ± 0.1 |
| 0-72 h        | Insulin sensitive | 2.6 ± 0.6 | 2.6 ± 0.6 | 1.7 ± 0.6 | 0.8 ± 0.2 |
|               | Insulin resistant | 2.2 ± 0.5 | 1.7 ± 0.2 | 1.2 ± 0.2 | 1.0 ± 0.3 |
|               | NAFLD           | 1.1 ± 0.0 | 0.9 ± 0.5 | 0.4 ± 0.2 | 0.7 ± 0.2 |

*Note: Data are represented as mean ± SD (n = 6 for insulin sensitive and insulin resistant groups; n = 3 for NAFLD group).*

Abbreviations: HG, high glucose (11 mM); HI, high insulin (1.7 µM); LI, low insulin (0.1 nM); LG, low glucose (5.5 mM).

*aAnalogous to hyperinsulinemic-euglycemic clamp.*
solutions using thiol-Michael additions and have consider-
ably higher costs (1.14-3.22 USD/test for commercial assays
compared to 0.09-1.02 USD for the system presented here),
which can be relevant for high-throughput screening appli-
cations that analyze effects on glucose consumption.

The chemistry of OSTE polymer allows for selective and
robust biofunctionalization. Thiol-click reactions not only
allow for thiol-maleimide Michael addition as demonstrated
here, but can also be applied to a wide range of other thiol-cou-
pling-based surface chemistries. Notably, device fabrication
by reaction injection molding (RIM) enables to integrate the
on-chip sensor module with microfluidic components, such
as additional tissue compartments, micro-channels, fluidic
actuators, and tube connectors. In addition, OSTE-RIM pro-
vides a scalable platform for batch fabrication that can pave
the way for the commercialization of such devices.54

Only few studies have evaluated glucose dynamics in or-
ganotypic cultures of primary hepatocytes. In rat hepatocyte
spheroids significant glucose consumption only occurred at
concentrations ≥20 mM, in substantial excess of physiolog-
ical serum glucose levels in rat (<7 mM), suggesting that
the culture conditions might disrupt physiological glucose
control.55,56 Glucose handling in primary human hepatocy-
tes was only analyzed in micropatterned co-cultures.57,58
The studies showed that hepatocytes produced considerable
amounts of glucose during the first 3 days but sharply de-
creased by >95% thereafter. However, PCK1 expression in-
creased in both hyperglycemic and hypoglycemic conditions,
suggesting perturbed regulation of gluconeogenesis. In our
model, we could observe net glucose production in selected
donors and conditions. However, further vectorial decompo-
sition requires the use of labeled probe substrates, such as
isotope-labeled glucose or glucose analogues.

We substantially extended these findings and reveal glucose
flux dynamics of primary human hepatocytes in organotypic 3D cultures. Using our optimized highly sensi-
tive glucose sensor, we can detect glucose uptake of single
microtissues and quantified the net glucose consumption
for insulin sensitive hepatocytes in normoglycemic hyper-
insulinemic conditions to be 6.1 ± 1.7 fmol × cell⁻¹ × h⁻¹ which closely resembles hepatic glucose uptake in humans in vivo measured by euglycemic-hyperinsulinemic clamp (12
fmol × cell⁻¹ × h⁻¹ with a range of inferred inter-individual
variability from 4.2 to 31.8 fmol × cell⁻¹ × h⁻¹; see methods
for calculation). These results indicate that our model accu-
rately recapitulates human liver glucose uptake both qualita-
tively and quantitatively. Furthermore, we demonstrate that
we can model hepatic pathway-specific insulin resistance,
manifested as the paradox concomitant increase of both lipo-
genesis and gluconeogenesis, thus, linking insulin resistance
to nonalcoholic fatty liver disease (NAFLD).

By studying effects of insulin resistance on glucose con-
sumption dynamics, we experimentally quantified, for the
first time, the contribution of insulin signaling to hepatocytic
glucose uptake. Importantly, we find that glucose uptake
under physiological glucose conditions is significantly insu-
lin-dependent, whereas in insulin resistant, steatotic hepato-
cyes insulin control of glucose uptake is lost and glucose
uptake is controlled almost exclusively by extracellular glu-
cose concentrations. Overall, 55% of glucose consumption
is insulin-dependent, thus supporting and refining previous
modeling-based estimates.40 Combined, these data show
that the presented platform enables studies of glucose dis-
position of primary human cells in organotypic cultures with
unprecedented accuracy and incentivizes its integration into
microfluidic systems as a module for longitudinal glucose
monitoring.

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CONFLICT OF INTEREST
VML is co-founder, CEO and shareholder of HepaPredict
AB, a contract research organization offering pharmacologi-
cal and toxicological services based on 3D spheroid cultures
of primary human hepatocytes. In addition, VML discloses
consultancy work for EnginZyme AB. The other authors de-
clare no conflict of interest.

AUTHOR CONTRIBUTIONS
A.M. Kemas and S. Youhanna performed the research and analyzed the data; R. Zandi Shafagh fabricated OSTE
devices; V.M. Lauschke designed the research and ana-
yzed data; all authors contributed to the writing of the
manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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