Incorporation of Gelatin Microspheres into HepG2 Human Hepatocyte Spheroids for Functional Improvement through Improved Oxygen Supply to Spheroid Core

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The multicellular spheroid three-dimensional cell culture system can be used as a formulation for cell-based therapy. However, the viability and functions of the cells in the core region of the spheroid tend to decrease because of limited oxygen supply. In this study, we incorporated gelatin microspheres (GMS) into HepG2 human hepatocyte spheroids to allow oxygen to reach the spheroid core. GMS with an approximate diameter of 37 µm were fabricated by water-in-oil emulsification followed by freeze drying. GMS-containing HepG2 spheroids (GMS/HepG2 spheroids) were prepared by incubation of the cells with GMS at various mixing ratios in agarose gel-based microwells. Increasing the GMS ratio increased the diameter of the spheroids, and few spheroids formed with excess GMS. HepG2 cells in the GMS/HepG2 spheroids were more oxygenated than those in the GMS-free spheroids. GMS incorporation increased the viability of HepG2 cells in the spheroids and increased the CYP1A1 activity of the cells to metabolize 7-ethoxyresorufin, although mRNA expression of the CYP1A1 gene was hardly affected by GMS incorporation. These results indicate that incorporating GMS into HepG2 spheroids improves the hypoxic microenvironment in the spheroids and increases cell viability and CYP1A1 metabolic activity.

Key words sphere; gelatin microsphere; CYP1A1; oxygen supply

INTRODUCTION

Multicellular spheroids are three-dimensional cell aggregates, in which cells have more extensive interaction with other cells and extracellular matrix components than two-dimensional monolayered cells. Upregulation of albumin secretion and metabolic activity of rat hepatocytes and HepG2 human hepatocytes has been described in multicellular spheroids. Therefore, multicellular spheroids of hepatocytes are considered a useful in vitro drug screening system for drug metabolism and toxicity. Multicellular spheroids may also have greater therapeutic potential for cell-based therapy. It was reported that multicellular spheroid formation increased the viability of the constituent cells.

Multicellular spheroids are densely packed with cells and have no vasculature. This can lead to a condition where oxygen is consumed by the cells on the surface of the spheroids. This limits the level of oxygen that reaches the core region, resulting in hypoxia at the core region of the spheroids. In general, hypoxia is linked with disadvantageous biochemical events, such as apoptotic cell death, low cell proliferation rate, and anaerobic glycolysis. Especially, hepatocytes demand more oxygen than other types of cells to maintain viability and metabolic activity. Since CYP1A1 subtype requires oxygen for its metabolic activity, hepatocytes in a hypoxic environment in spheroids may not be fully functionally active.

A variety of biomaterials have been incorporated into multicellular spheroids or other three-dimensional artificial tissues to maintain the viability and functions of the cells. Microspheres are often used to apply scaffolds and empty spaces, to control the stiffness, or to improve tissue functions. Gelatin is a superior biomaterial that has been incorporated into several types of spheroids because of its suitable characteristics that include cell attachment, biocompatibility, ease of application, and thermosensitive gel-sol transition. Gelatin microspheres (GMS) have nanopores through which oxygen can pass. The observation that GMS incorporation increased the aerobic respiration of bone marrow-derived mesenchymal stem cell aggregates supports the expectation that GMS can prevent the hypoxic conditions of multicellular spheroids.

In this study, we incorporated GMS into multicellular spheroids of HepG2 human hepatocytes to improve their functions. GMS were fabricated by water-in-oil (W/O) emulsification method and stabilized by cross-linking with glutaraldehyde. GMS-containing multicellular spheroids of HepG2 cells (GMS/HepG2 spheroids) were prepared using agarose gel-based microwells. We evaluated the size, cell viability, oxygen supply, and CYP1A1 metabolic activity of the spheroids.

MATERIALS AND METHODS

Chemicals Dulbecco’s modified Eagle’s medium (DMEM, low glucose type) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS), 5-(and-6)
carboxyfluorescein diacetate succinimidyl ester (CFSE), and NHS-rhodamine were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Trypan blue stain solution, Span 60, and Tween 20 were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Trypsin was obtained from Becton Dickinson and Co. (Mansfield, MA, U.S.A.). Ethylenediaminetetraacetic acid and disodium salt, dihydrate (EDTA-2Na) were obtained from Dojindo Laboratories (Kumamoto, Japan). Mono azo rhodamine (MAR) was obtained from Goryo Chemical (Hokkaido, Japan). Gelatin (type-b, bovine bone derived) and agarose were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Preparation of GMS GMS were prepared by chemical cross-linking of gelatin in the W/O emulsion state, as previously described with some modification. An aqueous solution of 10 wt% gelatin was preheated at 60°C. Four milliliters of gelatin solution were added to 120 mL of olive oil containing 0.1 wt% Span 60, followed by stirring at 1000 rpm for 1 h at 60°C to prepare the W/O emulsion. As the temperature gradually decreased to 4°C on ice, emulsions containing gelatin formed microspheres at hydrogel state. GMS were retrieved by washing three times with cold acetone and centrifugation (3300 \( \times g \), 4°C, 5 min), followed by drying to remove residual acetone. Dried GMS were suspended in 40 mL of water containing 0.05 wt% glutaraldehyde and 0.1 wt% Tween 20, followed by stirring at 1000 rpm for 24 h at 4°C to chemically cross-link gelatin. The remaining aldehyde groups of gelatin were blocked by a 3 h incubation with 40 mg glycine. GMS were washed three times with cold water and collected by centrifugation (3300 \( \times g \), 4°C, 5 min). Finally, GMS suspended in water were frozen at –80°C, lyophilized, and stored. The size of GMS in phosphate-buffered saline (PBS) was measured using a model BZ-X710 Biozero microscope (Keyence, Osaka, Japan). Prior to their incorporation into spheroids, GMS were sterilized by UV irradiation (253.7 nm) for 1 h.

Cell Culture HepG2 human hepatocyte cancer cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FBS, 0.2% NaHCO\(_3\), 100 units/mL penicillin, 100 µg/mL streptomycin, and 293 µg/mL L-glutamine (PSG; Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO\(_2\).

Preparation of Agarose Gel-Based Microwells Agarose gel-based microwells were prepared using a micro-molding technique. Micropillar arrays were fabricated as previously described. Heated agarose solution (3%) in PBS was poured over the micropillar array placed in a cell culture dish. The agarose solution was gelled by 30 min incubation at 20–25°C. The agarose gel-based microwell sheet was peeled off from the micropillar array and trimmed to fit a well in a 6-well culture plate. The structure of the microwells was observed using the aforementioned Biozero microscope. The height, intermediate diameter, and maximum diameter were 523 ± 15, 539 ± 42, and 910 ± 55 \( \mu m \), respectively.

Preparation of GMS/HepG2 Spheroids HepG2 hepatocytes were harvested using a trypsin (0.25% (w/v))-EDTA (0.68 mM) solution, resuspended in a culture medium, and mixed with UV-sterilized GMS. The concentrations of the cells and GMS in 3 mL volumes of culture medium were set to 2.5 \( \times 10^6 \) cells and 5.0 \( \times 10^6 \), 1.25 \( \times 10^7 \), or 2.5 \( \times 10^7 \) particles, respectively. Next, the mixed cells and GMS suspended in each 3 mL of culture medium were added to agarose gel-based microwells placed in six-well culture plates. After a 1 h incubation at 37°C in a humidified 5% CO\(_2\) incubator, the microwells containing the cells and GMS were transferred to a 15-cm dish to prevent lack of nutrients. After an additional 72 h incubation at 37°C in a humidified 5% CO\(_2\) incubator, the multicellular spheroids that formed were manually picked using a micropipette. The diameter of the spheroids was measured using a microscope. To count the number of live cells in spheroids, the spheroids were dispersed by using trypsin-EDTA in PBS. Then, the dispersed cells were stained with trypan blue and non-stained cells were counted as live cells. The toxicity of GMS to HepG2 hepatocytes was estimated by the WST-8 assay using Cell Count Reagent SF (Nacalai Tesque Inc.) 24 h after culturing 1 \( \times 10^4 \) HepG2 cells with 1, 2, 5, or 10 \( \times 10^3 \) GMS particles in a 96-well plate.

Scanning Electron Microscopy (SEM) GMS and spheroids were fixed with 4% paraformaldehyde and 1% glutaraldehyde solution at 20–25°C overnight. Samples were dehydrated with increasing concentrations of ethanol, which was replaced with 1-butanol, followed by lyophilization. The dried samples were observed by SEM using a model S-4700 microscope (Hitachi, Tokyo, Japan).

Confocal Fluorescence Microscopy Suspended HepG2 hepatocytes were fluorescently labeled by incubation in Opti-MEM containing 1 \( \mu M \) CFSE for 5 min at 37°C. GMS were fluorescently labeled by incubation in 20 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid solution containing 10 µg/mL NHS-rhodamine for 15 min at 37°C. Fluorescently labeled GMS/HepG2 spheroids were prepared using the CSFE-labeled HepG2 cells and rhodamine-labeled GMS as described above. Next, the spheroids were optically cleared by using the ScaleS method. Briefly, the spheroids in microwells were washed three times with PBS and fixed with 4% paraformaldehyde in phosphate buffer (Nacalai Tesque, Inc.) for 15 min at 20–25°C. After washing three times with PBS, the spheroids were optically cleared using ScaleS\(_4\)(0) (22.5% (w/v)-sorbitol and 9.1 M urea [pH 8.4]) for 2 h at 37°C and then with ScaleS\(_4\)(0) (40% (w/v)-sorbitol, 10% (w/v) glycerol, 4 M urea, and 20% (v/v) dimethyl sulfoxide) for 2 h at 20–25°C. The optically cleared spheroids were observed by confocal fluorescence microscopy using a model A1R MP microscope (Nikon, Tokyo, Japan).

Estimation of Hypoxic Microenvironment in Spheroids HepG2 spheroids with or without GMS were prepared as described above. One hundred spheroids in each well of a six-well plate received 1 \( \mu M \) of the MAR hypoxia-sensitive fluorescent probe. After a 6 h incubation at 37°C in a humidified 5% CO\(_2\) incubator, spheroids were dispersed using trypsin-EDTA (0.25% (w/v)) in PBS. Mean fluorescence intensity (MFI) of the cells was measured by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter, Miami, FL, U.S.A.). Data were analyzed using Kaluza software (version 1.0; Beckman Coulter).

Live/Dead Cell Staining HepG2 spheroids with or without GMS were prepared as described above. One hundred spheroids per well received Opti-MEM containing 1 \( \mu M \) CFSE and 5 \( \mu g/mL \) propidium iodide. After 30 min incubation,
spheroids were optically cleared using the ScaleS method described above, and were observed using confocal fluorescence microscopy.

**Quantitative PCR** Total RNA was extracted using Sepasol RNA-I Super G (Nacalai Tesque), according to the manufacturer’s instructions. cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO Co., Ltd., Osaka, Japan), according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using StepOnePlus (Applied Biosystems Inc., Foster City, CA, U.S.A.) and the following primers: β-actin forward (5'-CAA CTC CAT CAT GAA GTG TGAC-3’), β-actin reverse (5’-GCC ATG CCA ATC TCA TCT TG-3’), CYP1A1 forward (5’-TCC AGA GAC AAC AGG TAA AAC A-3’), CYP1A1 reverse (5’-AGG AAG GGC AGA GGA ATG TGA T-3’); PCR was performed using the following amplification conditions: 40 cycles of 95°C for 3 s and 60°C for 30 s. Quantification of the mRNA was performed by normalizing the levels of target cDNAs to the expression levels of the endogenous reference gene β-actin.

**Ethoxyresorufin-O-deethylase Assay** CYP1A1 activity of HepG2 hepatocytes was measured using the 7-ethoxyresorufin-O-deethylase (EROD) assay as previously reported. In brief, 1 × 10^5 suspended HepG2 hepatocytes or 20 HepG2 spheroids (approximately 1 × 10^5 cells) were added to each well in 24-well plates with 2 µM of 7-ethoxyresorufin in EROD reaction buffer (50 mM Na_2HPO_4 with pH adjusted to 8.0 with 50 mM NaH_2PO_4). After 1 h incubation at 37°C in a humidified 5% CO_2 incubator, the fluorescence of the 7-ethoxyresorufin metabolite was measured using a FluoroMax-4 spectrophotometer (HORIBA, Ltd., Kyoto, Japan).

**Statistical Analysis** Differences between groups were statistically evaluated using the Student’s t-test. p-Values <0.05 were considered statistically significant.

**RESULTS**

**Multiporous GMS Fabricated by W/O Emulsion Technique** GMS were prepared by the W/O emulsion technique. SEM of GMS revealed angular and microporous nature of the surface (Figs. 1A, B). A microscopic image of GMS suspended in water showed that they were spherical (Fig. 1C). The diameter of GMS was 10 to approximately 100 µm, with a mean diameter of 37.4 µm (Fig. 1D). No significant toxicity was observed when GMS were added to HepG2 hepatocytes, irrespective of the amount of GMS (Fig. 1E).

**Generation of GMS/HepG2 Spheroids Using Agarose Gel-Based Microwells** GMS/HepG2 spheroids were prepared by culturing HepG2 with GMS at different mixing ratios of GMS and HepG2 hepatocytes (0.02, 0.05, 0.1, and 0.2). GMS/HepG2 spheroids were successfully formed at all except the highest mixing ratio of 0.2 (Fig. 2A). The diameters of the spheroids increased as the GMS/HepG2 ratio increased (Figs. 2A, B). Figure 2C shows a representative confocal microscopy image of GMS/HepG2 spheroids prepared using CFSE-labeled HepG2 and rhodamine-labeled GMS. Rhodamine-derived fluorescence was observed in the GMS/HepG2 spheroids, indicating that GMS were incorporated in the HepG2 spheroids. SEM images revealed that the surface of the GMS/HepG2 spheroids was uneven with gaps (Fig. 2D). Taken together, these results indicate that GMS were successfully incorporated in HepG2 spheroids.

**GMS Incorporation Reduces Hypoxic Region in HepG2 Spheroids** The MAR probe is hypoxia-sensitive and fluoresces under hypoxic conditions. The probe was used to check whether the cells in the HepG2 spheroids were in a hypoxic environment. The fluorescence intensity of MAR-treated GMS/HepG2 spheroids was lower than that of spheroids without GMS (Fig. 3). In addition, the intensity tended to decrease with increasing GMS/HepG2 ratio, and was hardly changed.

![Fig. 1](image-url)  
(A, B) SEM images of multiporous freeze-dried GMS fabricated by the W/O emulsion technique. Scale bar = 10 µm in (A) and 1 µm in (B). (C) Representative image of GMS in water. Scale bar = 100 µm. (D) Histograms of the diameter of 300 GMS. (E) The number of live HepG2 cells cultured with GMS, as estimated by the WST-8 assay. Results are expressed relative to control groups as the mean ± standard deviation (S.D.) of four experiments. There were no significant differences.
when the GMS/HepG2 ratio was 0.05 or higher. The results suggested that GMS incorporation into HepG2 spheroids reduced the hypoxic region in the spheroids.

**GMS Incorporation Increases Viability of HepG2 Hepatocytes in Spheroids**

Confocal observation of optically cleared GMS/HepG2 spheroids after live/dead staining of the cells was performed. GMS in HepG2/GMS spheroids were seen as yellow dots in the images because of the autofluorescence of glutaraldehyde used for GMS fabrication. In the HepG2 spheroids without GMS, almost all the whole cells, except those close to the surface, were dead. In GMS/HepG2 spheroids, more live cells were observed in the spheroids, particularly around GMS (Fig. 4A). The number of live cells in the spheroids also increased by incorporation of GMS when the GMS/HepG2 ratio was 0.02 and 0.05 (Fig. 4B). The findings indicated that incorporating GMS into HepG2 spheroids improved the viability of the enclosed HepG2 cells.

**GMS Incorporation Increases CYP1A1 Metabolic Activity of HepG2 Hepatocytes in HepG2 Spheroids**

The EROD assay showed that CYP1A1 metabolic activity of GMS/HepG2 spheroids was significantly higher than that of HepG2 spheroids without GMS (Fig. 5A). By contrast, the mRNA expression of CYP1A1 of HepG2 cells in GMS/HepG2 spheroids decreased with increasing GMS/HepG2 ratio (Fig. 5B). These results suggested that HepG2 cells in GMS-containing spheroids efficiently activated CYP1A1 metabolization without increasing mRNA expression of CYP1A1.

**DISCUSSION**

Incorporating GMS into HepG2 spheroids improved the hypoxic conditions (Fig. 3), cellular viability (Fig. 4), and metabolic activity (Fig. 5) in the spheroids. These results suggest that GMS improve oxygenation within the spheroids. HepG2 spheroids incorporating GMS are functionally superior compared to HepG2 spheroids with no GMS.

GMS were incorporated by self-assembly into spheroids by simply adding them to the microwells along with HepG2 cells. A prior study reported that gelatin microspheres, which attached to cells, were more efficiently incorporated into embryonic stem cell spheroids than agarose and poly(lactic-co-glycolic acid), which do not attach to cells. We assume that the cell–GMS interaction caused an automatic incorporation of GMS into the spheroids. However, spheroids were not formed when the GMS-to-cell ratio exceeded 0.1, since the excess GMS reduced cell–cell and cell–GMS contacts.

Incorporating GMS into HepG2 spheroids prevented hypoxic conditions and improved cell viability in the spheroids. Direct observation of the hypoxic regions could indicate that the hypoxic core regions of the spheroids are rescued by incorporating GMS. However, MAR used for the staining of the hypoxic regions was released from cells during the optical clearing process, which was indispensable for the direct observation.
of the spheroid cores. Moreover, the relationship between the improved hypoxic conditions and cellular viability was not examined. Incorporated GMS could contribute to cellular viability by a mechanism unrelated to the improvement of hypoxia, such as a cell-matrix interaction that could reduce cell apoptosis.23)

CYP1A1 contributes to the metabolism of xenobiotics and several endogenous chemicals with the use of oxygen.24) Presently, EROD activity of HepG2 hepatocytes in the spheroids was increased by the incorporation of GMS (Fig. 5A). EROD activity requires oxygen, and the improved hypoxic conditions resulting from the incorporation of GMS into spheroids could lead to high CYP1A1 activity. In addition to oxygen supply, cell–extracellular matrix interaction would also be important for CYP1A1 activity. Incorporation of GMS into spheroids is considered to increase cell–extracellular matrix interaction. However, incorporation of an excess amount of GMS resulted in the reduced CYP1A1 metabolic activity in the spheroids.3 In addition, expression of the CYP1A1 of HepG2 hepatocytes was increased in larger spheroids.3) In addition, exposure of male Sprague Dawley rats to hypoxic conditions was reported to increase EROD activity in the liver.25) Therefore, the cell–cell interaction and the hypoxic condition would upregulate the mRNA expression of CYP1A1 of HepG2 hepatocytes, which could be the reason for the low mRNA expression of CYP1A1 of HepG2 hepatocytes in GMS/HepG2 spheroids. In addition, we think that O₂ supply and the balance between the cell–cell interaction and cell–extracellular matrix interaction in spheroid are important for the construction of a liver tissue model with suitable properties. Based on the results of the present study, it can be said that the highest CYP1A1 activity of the HepG2/GMS spheroids with GMS/HepG2 ratio of 0.05 is owing to the increased expression of CYP1A1 mRNA by hypoxia and cell–cell interaction, and the increased CYP1A1 activity by O₂ supply and cell–extracellular matrix interaction.

Incorporation of GMS into hepatocyte spheroids could also induce in vivo-like cell–extracellular matrix interaction. The liver consists of cells as well as extracellular matrix derived from non-hepatocyte cells, such as hepatic stellate cells.26) Drug efficacy and resistance have been related to the presence of extracellular matrix. Therefore, hepatocyte spheroids mixed with stromal cells have been used as a more realistic liver tissue model.27) However, stromal cells reportedly cannot efficiently produce extracellular matrix in spheroids without any extracellular matrix, such as gelatin.28) Therefore, the construction of highly-functional liver tissue models requires the incorporation of some extracellular matrix in the hepatocyte spheroids. In our study, GMS was incorporated into hepatocyte spheroids as an element of the extracellular matrix. This proved to be a better approach for constructing a liver tissue model.

GMS are used as drug carriers.29) Therefore, incorporat-
ing drug-containing GMS into spheroids would bestow additional functions. GMS loaded with fibroblast growth factor 2 (FGF-2) can accelerate fibroblast proliferation and capillary formation in artificial dermis transplanted into skin defects.\(^6\) Therefore, incorporating GMS-FGF-2 into spheroids could induce early angiogenesis after transplantation and increase cellular viability and the therapeutic effect. Spheroids incorporating GMS-drugs will be explored in future studies.

In conclusion, HepG2 spheroids incorporating GMS were fabricated by culturing HepG2 in microwell with GMS. GMS incorporation improved hypoxic conditions and cellular viability, and increased CYP1A1 metabolic activity of HepG2 cells.

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**Conflict of Interest**  The authors declare no conflict of interest.

**REFERENCES**

1. Ong CS, Zhou X, Han J, Huang CY, Nashed A, Khatri S, Mattson G, Fukumitsu T, Zhang H, Hibino N. *In vivo* therapeutic applications of cell spheroids. *Biotecnol. Adv.*, 36, 494–505 (2018).
2. Sakai Y, Yamagami S, Nakazawa K. Comparative analysis of gene expression in rat liver tissue and monolayer- and spheroid-cultured hepatocytes. *Cells Tissues Organs*, 191, 281–288 (2010).
3. Nishikawa T, Tanaka Y, Nishikawa M, Ogino Y, Kusamori K, Mizuno N, Mizukami Y, Shimizu K, Konishi S, Takahashi Y, Takakura Y. Optimization of albumin secretion and metabolic activity of cytochrome P450 1A1 of human hepatoblastoma HepG2 cells in multicellular spheroids by controlling spheroid size. *Biol. Pharm. Bull.*, 40, 334–338 (2017).
4. Kyllin JA, Sharma P, Leedale J, Colley HE, Murdoch C, Harding AL, Mistry P, Webb SD. Characterisation of a functional rat hepatocyte spheroid model. *Toxicol. In Vitro*, 55, 160–172 (2019).
5. Underhill GH, Khetani SR. Bioengineered liver models for drug testing and cell differentiation studies. *Cell. Mol. Gastroenterol. Hepatol.*, 5, 426–439 (2018).
6. Uchida S, Itaka K, Nomoto T, Endo T, Matsumoto Y, Ishii T, Kataoka K. An injectable spheroid system with genetic modification for cell transplantation therapy. *Biomaterials*, 35, 2499–2506 (2014).
7. Brancato V, Garziano A, Gioiella F, Urciuolo F, Imparato G. Gelatin as biodegradable biomaterials—oxygen releasing scaffolds. *Acta Biomater.*, 115, 695–699 (2013).
8. Echave MC, Saenz del Burgo L, Pedraz JL, Orive G. Gelatin as biomaterial for tissue engineering. *Curr. Pharm. Des.*, 23, 3567–3584 (2017).
9. Hayashi K, Tabata Y. Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. *Acta Biomater.*, 7, 2797–2803 (2011).
10. Gunji S, Obama K, Matsui M, Tabata Y, Sakai Y. A novel drug delivery system of intraperitoneal chemotherapy for peritoneal carcinomatosis using gelatin microspheres incorporating cisplatin. *Surgery*, 154, 991–999 (2013).
11. Jeong OL, Jeung SH, Syed IHA, Sing MN, James JY. Functionalized biomaterials—oxygen releasing scaffolds. *J. Biotechnol. Biomater.*, 5, 1000182 (2015).
12. Ghollipournakabadi M, Zhao S, Harrison BS, Mozafari M, Seifalian AM. Oxygen-generating biomaterials: a new, viable paradigm for tissue engineering? *Trends Biotechnol.*, 34, 1010–1021 (2016).
13. Munro AW. Cytochrome P450 1A1 opens up to new substrates.